RAT HEPATIC PHOSPHATIDYLETHANOLAMINE *N*-METHYLTRANSFERASE: ENZYME PURIFICATION AND CHARACTERIZATION

by

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Thesis Abstract

Phosphatidylethanolamine (PE) *N*-methyltransferase catalyzes the stepwise transfer of methyl groups from *S*-adenosyl-L-methionine (AdoMet) to the amino headgroup of PE. Successive methylation results in the formation of the two intermediates, phosphatidyl-*N*-monomethylethanolamine (PMME) and phosphatidyl-*N*,*N*-dimethylethanolamine (PDME), and the final product phosphatidylcholine (PC). PE *N*-methyltransferase is an integral membrane protein localized primarily in the endoplasmic reticulum (microsomal fraction) of liver.

PE-, PMME- and PDME-dependent PE *N*-methyltransferase activities were purified from Triton X-100 solubilized microsomes 429-, 1542- and 832-fold, respectively. The purified enzyme was composed of a single 18.3 kDal protein as determined by SDS-PAGE. Molecular mass analysis of purified PE *N*-methyltransferase (in Triton X-100 micelles) by gel filtration on Sephacryl S-300 indicated the enzyme existed as a 24.7 kDal monomer. PE *N*-methyltransferase catalyzed the complete conversion of PE to PC and had a pH optimum of 10 for all three steps. A Triton X-100 mixed micelle assay was developed to assay PE-, PMME- and PDME-dependent activities of both pure and microsomal PE *N*-methyltransferase. The *N*-terminal amino acid sequence of rat liver PE *N*-methyltransferase and the recently cloned 23.1 kDal *S. cerevisiae* PEM 2 were found to be 35% homologous.

Double reciprocal plots for PE *N*-methyltransferase at fixed Triton X-100 concentrations and increasing PE, PMME or PDME were highly cooperative. Similar cooperative effects were noted when phospholipid was fixed and Triton X-100 increased. The cooperativity could be partially abolished if a fixed mol% of nonsubstrate phospholipid such as PC was included in the assay. This would

indicate that PE *N*-methyltransferase has specific binding requirements for a site(s) in contact with the micellar substrate. The occupation of this boundary layer by phospholipid is essential for full expression of enzyme activity. Kinetic analysis revealed that PMME and PDME methylation followed an ordered Bi-Bi mechanism. The overall mechanism involves initial binding of PE to a common site and successive methylation steps involving the binding and release of AdoMet and *S*-adenosyl-L-homocysteine, respectively. Cysteine residue(s) (which are rapidly oxidized in the absence of reduced thiols) are involved in the catalytic mechanism.

Reverse-phase HPLC was used to fractionate the phospholipid products of PE *N*-methyltransferase into individual molecular species. Substrate specificity experiments on PE *N*-methyltransferase *in vitro* and *in vivo* revealed no selectivity for any molecular species of diacyl PE, PMME or PDME. The PE-derived PC, which is rich in 16:0-22:6, is rapidly remodeled to conform to the molecular species compositon of total hepatocyte PC *in vivo* .

The 18.3 kDal PE *N*-methyltransferase was found to be a substrate for cAMP-dependent protein kinase *in vitro*. However, only 0.25 mol phosphorus/mol of PE *N*-methyltransferase was incorporated, with no observed effect on activity. Studies on PE *N*-methyltransferase regulation in choline-deficient rat liver indicated that activity changes were due to elevated levels of cellular PE. Immunoblotting of choline-deficient liver microsomes or hepatocyte membranes with a anti-PE *N*-methyltransferase antibody revealed no alteration in enzyme mass. While more work is needed, initial indications are that hepatic PE *N*-methyltransferase is a constitutive enzyme regulated primarily by substrate and product levels.

Table of Contents

Legend to Tables

Legends to Figures

List of Abbreviations

AdoEt	*S*-adenosyl-L-ethionine
AdoHcy	*S*-adenosyl-L-homocysteine
AdoMet	*S*-adenosyl-L-methionine
A	ampere
ATP	adenosine triphosphate
BSA	bovine serum albumin
cAMP	adenosine 3', 5'-monophosphate
CDP	cytidine diphosphate
CHO	chinese hamster ovary
Ci	curie
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfate
CMC	critical micellar concentration
CoA	coenzyme A
cpm	counts per minute
CTP	cytidine triphosphate
kDal	kilodalton
DEAE	diethylaminoethyl
DME	*N,N*-dimethylethanolamine
dpm	disintegrations per minute
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
DTT	dithiothreitol
DZA	deazaadenosine
EDTA	ethylenediaminetetraacetate
EGTA	ethyleneglycol bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
Fig.	figure
g	gram
g	gravity
h	hour
H_{II}	hexagonal phase structure of phospholipid
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonate
HPLC	high performance liquid chromatography
IAA	iodoacetic acid
K_m	Michealis constant of an enzyme reaction
l	litre
lysoPC	lysophosphatidylcholine
lysoPE	lysophosphatidylethanolamine
m	meter
M	molar
MEM	modified Eagle's medium
min	minute
MME	*N*-monomethylethanolamine
NEPHGE	non-equilibrium pH gradient gel electrophoresis
Nonidet P40	same compound as Triton X-100-contains average of 9 moles of ethylene oxide per molecule
PAGE	polyacrylamide gel electrophoresis
PC	phosphatidylcholine
PDME	phosphatidyl-*N,N*-dimethylethanolamine
Ref.	reference

PI	phosphatidylinositol
PMME	phosphatidyl-*N*-monomethylethanolamine
rpm	revolutions per minute
PS	phosphatidylserine
SDS	sodium dodecyl sulfate
S.E.	standard error
SM	sphingomyelin
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
THF	tetrahydrofolate
TLC	thin-layer chromatography
TLE	thin-layer electrophoresis
TP-egg PE	PE prepared by transphosphatidylation of egg PC in the presence of ethanolamine
T_m	gel to liquid-crystaline phase transition temperature of phospholipids
Tris	tris (hydroxymethyl) aminomethane
Triton X-100	octyl phenoxy polyethoxyethanol
UV	ultraviolet
v	volume
V	volt
V*max*	maximum velocity of an enzyme reaction
w	weight

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INTRODUCTION

1.1 The Centrality of Phospholipids to Cell Function

The phospholipid bilayer affords eukaryotic cells a barrier from the ionic, pH and physical stresses of the external environment. Within this controled environment the multivalent activities that describe the cell phenotype are orchestrated. Phospholipid bilayers also house cellular organelles, within which specific biochemical reactions occur. The phospholipid bilayer is not, however, an inert 'bag' in which cellular components are conveniently stored, but is more accurately described as a "fluid-mosaic" of various phospholipids and proteins (1). Components of the bilayer itself are constantly being degraded and replenished by synthesis. In this regard, many of the synthetic functions involved in the maintenance of membrane structure are in or on the membrane itself. Also embedded in cellular membranes are a variety of enzymatic functions with no bearing on membrane synthesis or degradation. An example are the specialized receptors in the phospholipid bilayer which couple binding of an external ligand with an intracellular event or internalize essential molecules (2).

It is becoming increasingly apparent that phospholipids are not just structural elements. Receptor mediated hydrolysis of PI, an acid phospholipid, is a ubiquitous event in transduction of hormonal signals across cell membranes (3). Diglyceride, one of the products of PI hydrolysis, activates protein kinase C, which in turn has an important role in regulating cell functions (4). TPA, a potent tumor promoter, usurps the protein kinase C pathway by mimicking diglyceride (4). Other lipids (sphingosine and sphinganine) have been reported to inhibit protein kinase C-mediated cell responses (5).

Structural proteins, receptors and enzymes of the lipid bilayer have amphiphilic character, much the same as the phospholipid molecules with which they are associated. This infers unique solubility, kinetic and structural properties on the proteins, which in turn makes them extremely refractory to biochemical analysis.

Pathways for the synthesis of PC, the major phospholipid of eukaryotic cell membranes (6), are localized almost exclusively in membranes. As a result, the elucidation of molecular and regulatory properties of the PC biosynthetic pathways has been sluggish. In recent years, the development of enzyme purification techniques, membrane solubilizing surfactants and kinetic models for enzymes using lipophilic substrates has made study of PC biosynthesis more amenable.

1.2 Phosphatidylcholine Structure

PC, or lecithin (1,2-diacyl-*sn*-glycero-3-phosphocholine), the structure of which is shown in Fig. 1, was first isolated from egg yolk and brain in 1846-47 by Gobley (7) and chemically synthesized by Baer and Kates (8) in 1950. The amphiphilic nature of PC is conferred by a negatively charged phosphodiester and positively charged choline moiety (hydrophilic), and two *n*-alkyl chains (hydrophobic) in ester linkage. In solution, PC will spontanously assume an energetically favourable bilayer structure with the *n*-alkyl chains shielded from the aqueous environment (9).

1.3 Phosphatidylcholine Synthesis

There are 4 major routes for the synthesis of PC. The majority of research has been devoted to the liver and lung systems which possess great synthetic capacity owing to export requirements. Where applicable, references will be made to other tissues.

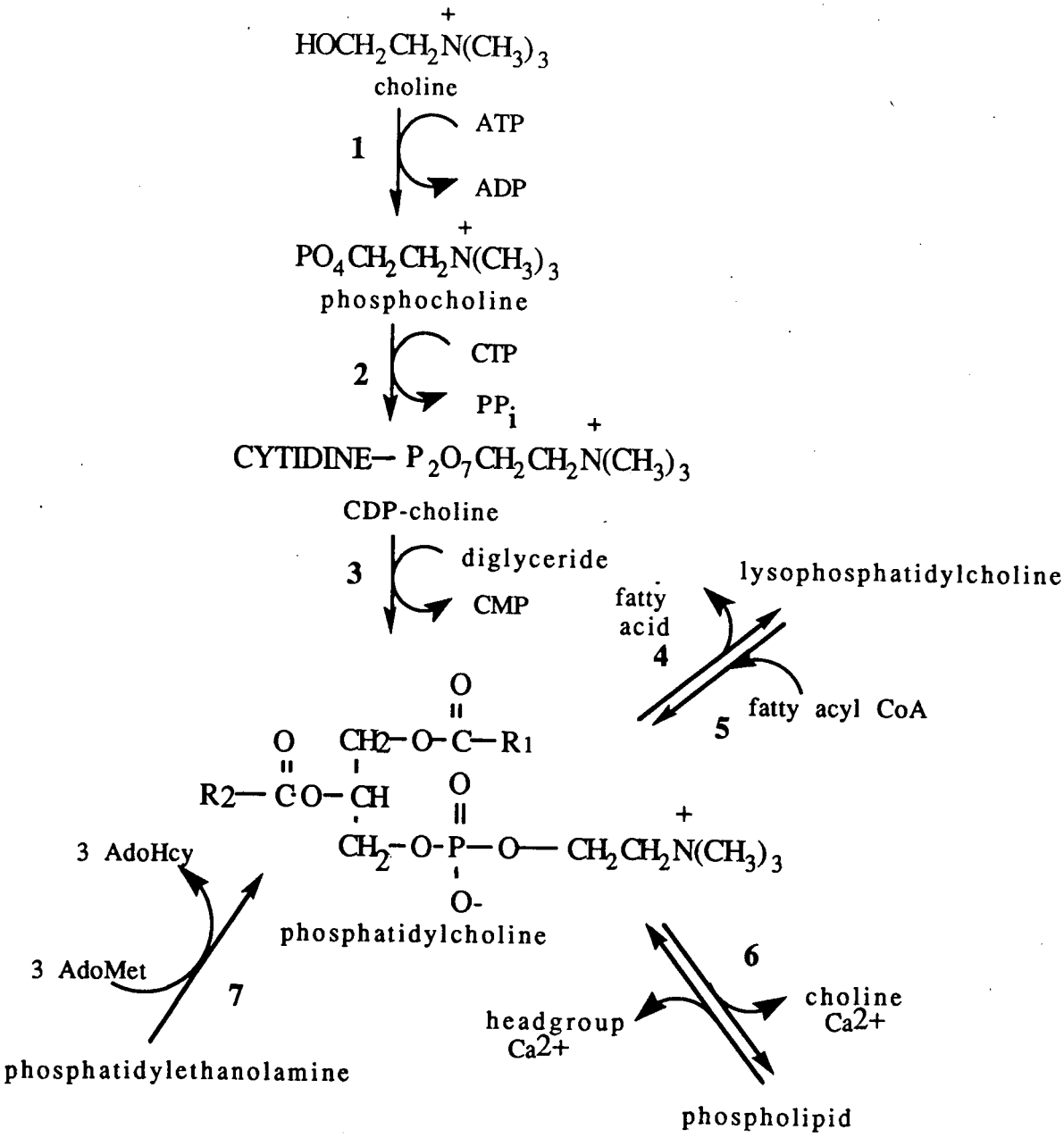

Figure 1. **PC biosynthetic pathways.** The numbers in bold indicate the enzymes involved; 1, choline kinase; 2, CTP:phosphocholine cytidylyltransferase; 3, CDP-choline:1,2-diglyceride phosphocholinetransferase; 4, phospholipase A_2 or A_1; 5, acyl-CoA:lysophosphatidylcholine acyltransferase; 6, base-exchange enzyme; 7, phosphatidylethanolamine *N*-methyltransferase.

1.3.1 CDP-Choline Pathway.

Synthesis via CDP-choline and diglyceride (Fig. 1) is quantitatively the most important route for PC synthesis. The pathway was elucidated through the work of Wittenburg and Kornberg (10), who discovered ATP:choline phosphotransferase (choline kinase, EC 2.7.1.32), and by Kennedy and Weiss (11) who described CTP:phosphocholine cytidylyltransferase (EC 2.7.7.15) and CDP-choline:1,2-diacylglycerol cholinephosphotransferase (choline phosphotransferase, EC 2.7.8.2).

Choline kinase synthesizes phosphocholine by the transfer of the ATP γ-phosphate to choline (10). Unlike the latter steps in the pathway, choline kinase activity is cytosolic. A 80 kDal (native) enzyme that phosphorylates both choline and ethanolamine was partially purified from monkey lung (12). More recently, Ishidate *et al.* (13) reported the complete purification of a 42 kDal ethanolamine/choline kinase from rat kidney. Native molecular weight was estimated at 75-80 kDal. Immunoprecipation experiments established identity between the kidney enzyme and ethanolamine/choline kinase activity in other rat tissues (14). Multiple ethanolamine/choline kinase isoforms have been demonstrated in rat liver, kidney, lung and intestine (15). While the function of the multiple isoforms are unknown, it is interesting to note that CCl_4 and certain polycyclic aromatic hydrocarbons will induce formation in liver of kinase isoproteins (16,17) immunologially distinct from the 42 kDal kinase (15). In light of these reports on the duality of choline- and ethanolamine-kinase activities it seems that reports of separate enzymes (18) should be re-examined.

In some transformed cell lines, choline phosphorylation is the rate-limiting step in PC formation (19,20). However, the general rule that the first step in a biosynthetic pathway is rate-limiting does not hold true for PC

synthesis in the majority of cases. It is now generally recognized that CDP-choline synthesis by the cytidylyltransferase is rate-limiting in liver, lung and a variety of cell lines (21). Cytidylyltransferase is unique in that it can be isolated in soluble and membrane-bound forms: thus it is termed an *ambiquitous* enzyme (22). Only the membrane bound form is active in the absence of exogenous phospholipid (23). It is the dual localization of cytidylyltransferase, and the translocation of enzyme between these two compartments, that governs CDP-choline (and PC) production (23). Translocation is stimulated by long-chain fatty acids, fatty alcohols and mono- and diglycerides *in vivo* (24-26). It should be cautioned that the effects of oleate are produced at non-physiological concentrations (1 mM in cell culture medium or 10 mol% in PC vesicles, Ref. 27). Pelech and Vance (28) have provided evidence that phosphorylation-dephosphorylation is important in regulation of cytidylyltransferase. To date no direct incorporation of phosphate into the enzyme has been recorded. Cytidylyltransferase was recently purified from rat liver cytosol (29). The enzyme consists of a single 45 kDal protein by SDS-PAGE, and a tetramer when analyzed by gel filtration in the presence of Triton X-100 (29).

The final step in PC synthesis is the condensation of CDP-choline with diglyceride. This reaction is catalyzed by an integral membrane protein of the endoplasmic reticulum (30). No complete purification has yet been reported. *In vitro,* cholinephosphotransferase shows a preference for 1-palmitoyl-2-linoleoyl- and 1-palmitoyl-2-docosahexaenoyl-diglyceride (31). 1-Palmitoyl-2-oleoyl and 1-palmitoyl-2-linoleoyl diglyceride seem to be the preferred substrates *in vivo* (32).

1.3.2 Base-Exchange.

The exchange of choline into PC was first described in 1959 (33). The microsomal activity had a K_m for choline of 8 mM, a requirement for Ca^{2+} (2-3 mM, Ref. 34) and was competitively inhibited by both L-serine and ethanolamine (35). [^{3}H]Choline labeling in rats indicated that base-exchange was a negligible route for PC synthesis (36,37). Contrary to this, Treble *et al.* (38) reported that the rate of choline base-exchange activity was four times that of phosphocholine incorporation into PC.

Since there exist exchange activities for choline, serine and ethanolamine, it is still an open question as to the number of enzymes involved and the requirements for lipid acceptor. Recently, Suzuki and Kanfer (39) purified to apparent homogeneity a serine- and ethanolamine-exchange enzyme activity from rat brain microsomes. The 100 kDal protein used exclusively PE or asolectin as a phospholipid acceptor. PS auxotrophs, with a 33% reduction in serine-exchange enzyme, have been isolated from mutagenized CHO cells (40,41). Concomitant with deletion of the structural gene or promoter for the serine exchange enzyme was a loss of 99% and 50% of choline- and ethanolamine-exchange activities, respectively (41). Comparison of remaining activities with those deleted prompted assignment of exchange activities to a choline-, serine- and ethanolamine-exchange function (serine-exchange enzyme I) and a serine- and ethanolamine-exchange activity (serine exchange enzyme II, Ref. 42). Serine-exchange enzyme I uses PC as an acceptor, while exchange enzyme II uses PE (42). Lack of decarboxylation of PS to PE, due to PS insufficiency, meant that serine exchange enzyme II could not fulfill the cell's requirement for PS, owing to its own requirement for PE as an acceptor. These auxotrophs were deficient in cellular PS but not PC, which again suggests PC synthesis by exchange is of minimal importance. It

is also interesting to note that choline-exchange in CHO cells requires PC as a lipid acceptor, indicating that exchange is a futile process (42).

1.3.3 Reacylation of Lysophosphatidylcholine

An elevated fatty acid/glycerol [^{14}C] labeling ratio in the PC (relative to TG) of lung slices suggested to Lands (43) that fatty acids in PC were turning over in preference to complete degradation. Lands (44,45) latter identified a rat liver microsomal acyl-CoA:1-acyl-*sn*-glycerol-3-phosphocholine acyltransferase (lysoPC acyltransferase, EC 2.3.1.23) as well as an enzyme that esterifies a fatty acid at the *sn*-2 position (EC 2.3.1.62). The two activities that acylate the *sn*-1 and *sn*-2 positions of lysoPC are specific for stearoyl- and linoleoyl-CoA, respectively (46). Thus, the acyl composition of the lysoPC acceptor was of little importance compared to the position of the free hydroxyl to be acylated (47,48). Evidence suggests that two separate microsomal enzymes exist for the esterification of arachidonoyl-CoA and oleoyl-CoA to 1-acyl lysoPC (49). The arachidonoyl-CoA-specific acyltransferase was recently purified from rat brain (50).

It is apparent that the reacylation pathway is more important in the remodeling of PC molecular species through the concerted action of a phospholipase A_2 or A_1. However, it should be noted that rat liver is capable of taking up and esterifing lysoPC, predominantly with arachidonate (51). LysoPC was also utilized as a precursor for PC in CHO cells cultured in choline-deficient medium (52).

In addition to the acylation pathways just described, there also exists a microsomal activity that catalyzes the transfer of fatty acid from the *sn*-2 position of intact PC or PI to the *sn*-2 postion of lysoPC (53) in a CoA-dependent fashion (independent of phospholipase A_2 activity). A microsomal

transacylase activity that forms PC and glycerophosphocholine from two lysoPC molecules has also been reported (54).

1.4 Synthesis of Phosphatidylcholine by Phosphatidylethanolamine Methylation

The main focus of this work was investigation of the molecular and regulatory properties of the PE methylation pathway in rat liver. As illustrated in Fig. 2, PE *N*-methyltransferase catalyzes the stepwise transfer of methyl groups from AdoMet to the amino headgroup of PE, thus generating the two partially methylated intermediates, PMME and PDME, and PC. Before starting a detailed discussion of PE *N*-methyltransferase, the properties and synthesis of it's lipid substrate will be examined.

1.4.1 Structure of Phosphatidylethanolamine.

PE (Fig. 3) was first isolated from brain by Thudichum (55) in 1884. It was not until 1930 that ethanolamine was unequivocally identified as a component of this phospholipid (56). PE, similar to PC, self-associates in aqueous environments such that contact of the hydrocarbon chains with one another is maximized and exposure to water is minimized. Unlike PC, PE exists in both bilayer and a unique hexagonal (H_{II}) structure (9). In this regard PE exhibits 'lipid polymorphism'. Based on ^{31}P-NMR studies, H_{II} structure exists as inverted cylinders of PE, which contain water in their interior in association with the ethanolamine headgroup (57,58). The transition between bilayer and H_{II} structure was shown to be temperature-dependent and reversible (59). The temperature dependence is related to the fatty acid composition of the PE and the transition to H_{II} structure usually occurs above the T_m of the PE in question (59). PE makes up 15-35% of phospholipid in biological membranes (6), so the potential for nonbilayer structure in these membranes does exist.

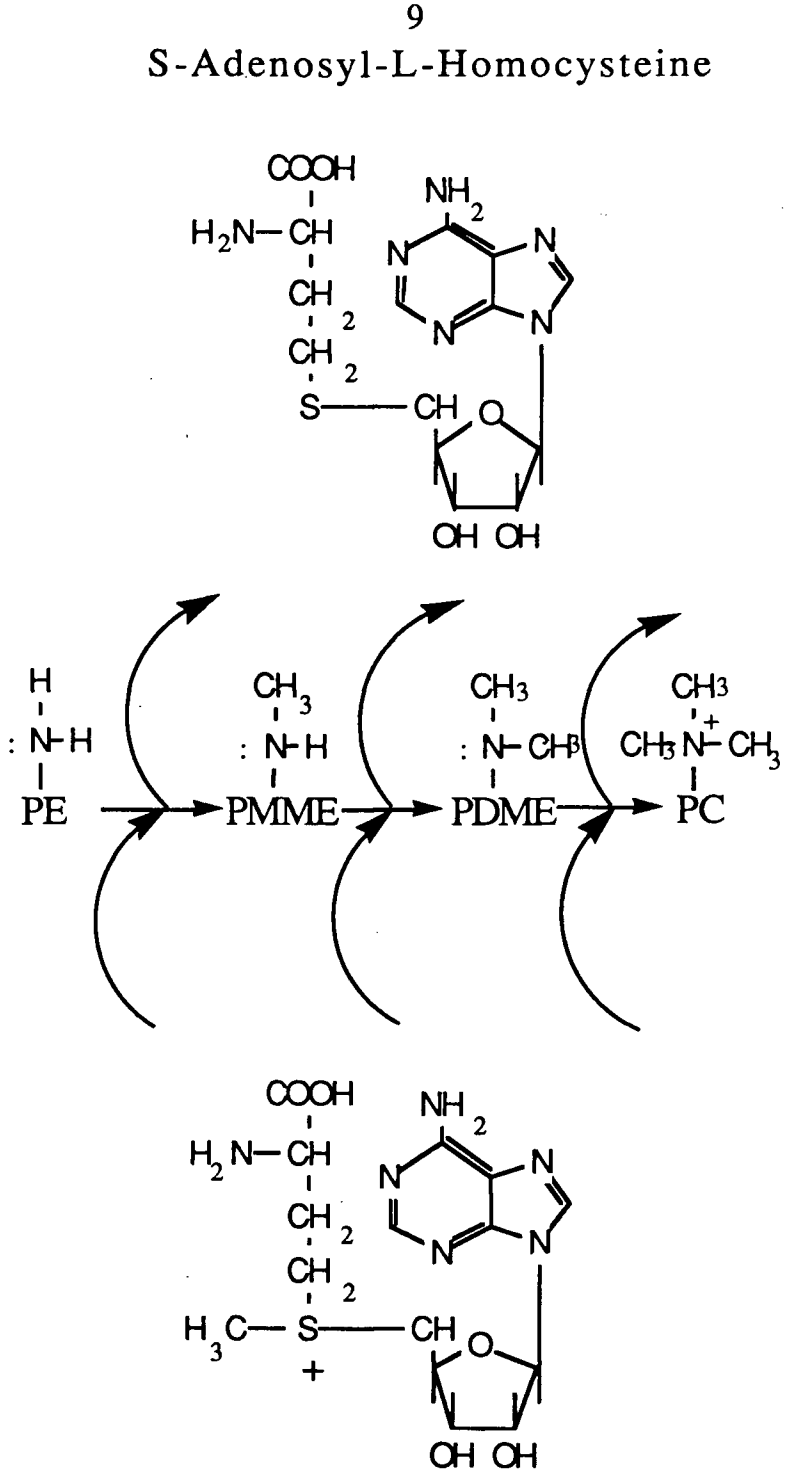

Figure 2. **Synthesis of PC by PE methylation.**

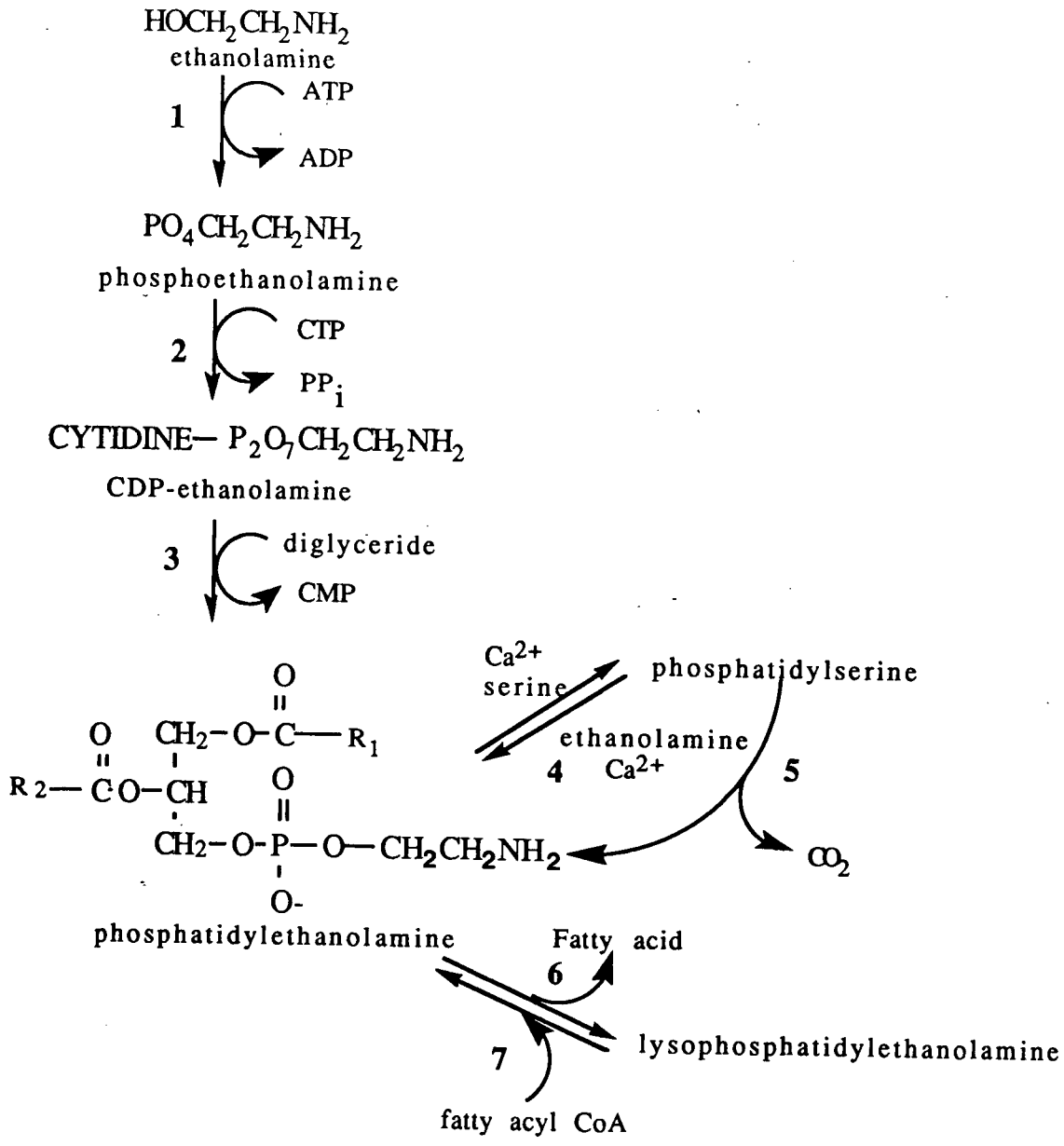

Figure 3. **PE biosynthetic pathways.** The bold numbers indicate the enzymes involved in the pathway; 1, ethanolamine kinase; 2, CTP:phosphoethanolamine cytidylyltransferase; 3, CDP-choline:1,2 diglyceride phosphoethanolaminetransferase; 4, base-exchange enzyme; 5, phosphatidyl-serine decarboxylase; 6, phospholipase A_2 or A_1 ; 7, acyl-CoA:lysophosphatidyl-ethanolamine acyltransferase.

1.5 Origins of Phosphatidylethanolamine Utilized for Methylation

Biosynthesis of PE occurs by four distinct routes (Fig.3). Except for PS decarboxylation, the pathways are similar to those for PC synthesis, but knowledge of regulation and enzymology is quite rudimentary.

1.5.1 The CDP-Ethanolamine Pathway.

This route for PE synthesis is enzymologically similar, though distinct from the CDP-choline pathway for PC synthesis. As discussed in Section 1.3.1, there appears to be good evidence that in rat tissues the same cytosolic enzyme catalyzes both choline and ethanolamine phosphorylation.

In 1956 (11) Kennedy and Weiss discovered that PE was synthesized in a manner analogous to PC; through a CDP-ethanolamine intermediate. It was recognized that CDP-ethanolamine and -choline synthesis was by separate enzymes, reversible upon addition of pyrophosphate and ubiquitous in rat tissues (11). Phosphoethanolamine cytidylyltransferase (EC 2.7.7.14) has since been purified (60) and reported to have a molecular mass of 49-50 kDal by SDS-PAGE and 100-120 kDal by gel filtration. The enzyme activity is totally dependent on reducing agents and $Mg^{2+}$for activity, and has a pH optimum of 8.0 (60). Phosphoethanolamine cytidylyltransferase seems to be localized primarily in the cytosolic fraction following liver homogenization in isotonic saline (61). Whether translocation between cytosol and membrane compartments occurs is unknown.

Ethanolaminephosphotransferase (EC 2.7.8.1) is an integral membrane protein of the endoplasmic reticulum (62), and catalyzes the final step in the pathway. Apparently, this enzyme is separate from the one catalyzing CDP-choline condensation with diglyceride (62,63). Unlike choline-phosphotransferase, which shows little specificity *in vitro* (31) and synthesizes saturated species of PC, ethanolaminephosphotransferase shows a

preference for 1-palmitoyl-2-docosahexaenoyl diglycerides *in vitro* and *in vivo* (31,64,67). The enzyme is also known to utilize 1-alkyl- and 1-alkenyl-2-acyl diglycerides (63,66,67).

Analogous to PC synthesis, the rate-limiting step in synthesis of PE occurs at the production of CDP-ethanolamine (68). PE synthesis is stimulated in an unknown fashion by oleic acid (68).

1.5.2 Phosphatidylserine Decarboxylation

PE can also be synthesized in mitochondria by phosphatidylserine decarboxylase (EC 4.1.1.65, Ref. 69,70). Due to the location of this enzyme, PS synthesized in endoplasmic reticulum by base-exchange (34,35) must be transported to the mitochondria for decarboxylation. Methylation of PE to PC or base-exchange with serine then necessitates movement back to the endoplasmic reticulum. The mammalian PS decarboxylase has yet to be purified and characterized extensively.

The major route for PE synthesis is not as well defined as that for PC. It appears that both the CDP-ethanolamine pathway and decarboxylation of PS are the major routes of synthesis, however, the net contribution of each pathway is in question. Yeung and Kuksis (71) reported that 50% of PS was decarboxylated to PE in rat liver, while Wise and Elwyn (72) found 100% conversion of PS to PE. These values are believed to be overestimates owing to an oversimplified one-compartment model for PS metabolism (73). The reported rates of PE synthesis in rat liver from ethanolamine are 3- to 4-times greater then those from PS (74). However, cells in culture do not require ethanolamine for growth and seem to be able to synthesize PE almost entirely from PS (75,76).

1.5.3 Acylation of Lysophosphatidylethanolamine.

Merkl and Lands (77) have reported the acylation of 1- and 2-acyl lysoPE in rat liver microsomes. This pathway is probably only important in tailoring the fatty acid composition of PE molecules (78). However, lysoPE can be used as a precursor for PE (without catabolism to ethanolamine) as indicated by its ability to rescue ethanolamine requiring CHO cell mutants with a defect in PS base-exchange activity (75).

1.6 Characterization of Phosphatidylethanolamine *N*-Methyltransferase

1.6.1 Elucidation of the Phosphatidylethanolamine Methylation Pathway

As early as 1941 (79) it was recognized that the methyl group of methionine was utilized for the synthesis of choline. Stetten (80) demonstrated that [^{15}N]ethanolamine feeding to rats resulted in production of labeled 'choline phosphatide'. The result was interpreted to indicate that ethanolamine was methylated to choline prior to incorporation into PC. In 1959, Bremer and Greenberg (81) demonstrated that the primary acceptor of methyl groups was phosphatidylethanolamine and not the water soluble precursors of the CDP-ethanolamine pathway. Acid hydrolysis of the liver lipid fraction of [^{3}H-*methyl*]methionine-treated rats, and separation of the labeled bases, showed >95% was in choline and the remainder in mono- and dimethylethanolamine (81,81a). These finding were later expanded to show that the majority of methylation activity was associated with the 100,000 x *g* fraction of rat liver homogenate (82,83). In addition, AdoMet was demonstrated to be the immediate donor of methyl groups to PE (82,83). It was further demonstrated, on the basis of product distribution, that the addition of the first methyl group to PE was rate-limiting (82). Rehbinder and Greenberg (84) found that methylation could be stimulated 2- to 3-fold by the addition of PMME and PDME to deoxycholate solubilized microsomes. Oddly, PE did not stimulate methylation (82,84,85). In retrospect, this was due to improper delivery of

substrate to the enzyme since it has been reported that the Triton X-100 solubilized enzyme is stimulated 8.9-fold by PE (86).

The enzyme is tightly associated with microsomal membranes and requires detergents for its release in a soluble form (ie. does not sediment when subjected to 100,000 x *g* x 1 h). Enzyme activity is dependent on a free sulfhydryl(s) for activity (84,85). Another interesting feature of PE *N*-methyltransferase is its alkaline pH optimum (10-10.5, Ref. 81a,84). This optimum could be a reflection of the ionization state of the ethanolamine headgroup of PE (pKa approximately 9.5). This infers that methyl transfer occurs predominately to the deprotonated form of PE. Whether this alkaline pH optimum has a physiological significance is unknown, but methylation at pH 7.5 occurs at about one-tenth the rate of that at pH 10 (84,87,88). The microsomal enzyme is insensitive to EDTA (85) and does not require Mg^{2+} for activity (89).

1.6.2 Tissue- and Subcellular-Localization

Bremer and Greenberg (85) were the first to show that liver had the highest methyltransferase specific activity. Kidney, heart, lung and testis microsomal fractions all contained measurable, but low (2-6% of liver), activity. Brain, spleen and intestine contained no measurable activity (85). Since this original observation, numerous studies on extrahepatic PE *N*-methyltransferase have been published. In all instances the specific activity is low compared to liver. The localization, specific activity and co-factor requirements of these extrahepatic activities are summarized in Table 1. Why such low activity is expressed in extrahepatic tissues is unknown, but a good explanation is differential gene expression. No reports exist on enzyme mass in these tissues so tissue-specific inhibitors cannot be ruled out. It is clear,

Table 1. Cell and Tissue Distribution of PE *N*-Methyltransferase

Tissue or Cell	Subcellular fraction	Specific Activity[1]	pH[2]	AdoMet[2] (μM)	Additional cofactors	Ref.
Rat liver	microsomes	450	9.2	200	---	89
		580	9.2	200	0.28 mg/ml PMME	89
		550	9.2	200	0.85 mg/ml PDME	89
Mouse liver	microsomes	209	8.8	24	---	86
		226	8.8	24	0.6 mM PE	86
		673	8.8	24	0.6 mM PMME	86
		463	8.8	24	0.6 mM PDME	86
Rat liver	microsomes	31	8.0	200	---	96
Rat heart	homogenate	4.3-9.6	9.0	200	10 mM $MgCl_2$	242
Rat heart	sarcolemma	4.3	10	150	---	236
	microsomes	4.6	10	150	---	236
	mitochondria	4.4	10	150	---	236
Hamster heart	sarcolemma	0.01	6	0.055	1 mM $MgCl_2$	237
	sarcolemma	0.05	7	10	1 mM $MgCl_2$	237
	sarcolemma	3.60	10	150	1 mM $MgCl_2$	237
Rat colonic epithelial cells	plasma membranes	3.66	8.0	200	---	238
Rat adipocytes	plasma membranes	65.0	7.4	20	0.4 mM PMME	173
Rat adipocytes	plasma membranes	4.8	8.5	10	0.1 mM PMME	171
Rat erythrocytes	plasma membranes	0.49	8.0	200	5 mM $MgCl_2$	98
Human neutrophils	microsomes	0.6	8.0	50	---	239
Human lymphocytes	microsomes	0.84	8.0	50	---	239
Rat aorta	microsomes	0.42	8.2	100	5 mM $MgCl_2$	240
Mouse thymus	microsomes	0.01	7.4	20	0.4 mg/ml PE	113
		0.02	7.4	20	1.0 mg/ml PMME	113
		0.06	7.4	20	1.0 mg/ml PDME	113
Bovine adrenal medulla	microsomes	3.87	7.0	4.0	5 mM $MgCl_2$	99
Whole rat brain	microsomes	0.91	8	200	1 mM $MgCl_2$	103
	synaptsomal plasma membrane	0.93	8	200	1 mM $MgCl_2$	103
Rat pituitary	homogenate	0.21	9.5	200	10 mM $MgCl_2$	241
	homogenate	0.06	6.5	200	10 mM $MgCl_2$	105
		0.11	9.5	200	10 mM $MgCl_2$	105
Dog lung	microsomes	0.78	8.2	200	6.6 mM $MgCl_2$, 0.3 mM dog lung PE	243

[1] pmol/min/mg

[2] indicate the pH and AdoMet concentration at which the assay was performed

however, that PE methylation is not a significant route for PC synthesis in these tissues.

In extrahepatic tissues, PE *N*-methyltransferase is localized primarily in microsomes or plasma membrane fractions (Table 1). Subcellular analysis of PE *N*-methyltransferase activity in rat liver has often produced conflicting results. Similar to earlier findings (84,85), Van Golde *et al.* (90) reported that the majority of PDME methylation activity was confined to endoplasmic reticulum. Unfortunately, no information was provided on endogenous PE methylation. Contrary to this report, Higgins and Fielding (91) found twice the PE *N*-methyltransferase activity in 'cis-enriched' Golgi compared to endoplasmic reticulum or 'trans-enriched' Golgi (assayed at pH 8.5). A systematic evaluation of PE *N*-methyltransferase activity in Golgi revealed that results varied depending on the fractionation techniques used (92). Thus, all fractionation procedures enriched for trans-Golgi, but methyltransferase specific activities ranged from 0 to 0.8 nmol methyl groups transferred/min/mg. It was estimated that Golgi contained 2.3% of the total cellular methyltransferase activity (92). In this same report, mitochondria and plasma membranes had PE *N*-methyltransferase above that due to contamination from endoplasmic reticulum. Localization of PE *N*-methyltransferase in subcellular organelles by immunocytochemistry would be the method of choice in such studies.

1.6.3 Topology in Membranes.

The enzymes of glycerol lipid synthesis are asymmetrically distributed to the cytosolic surface of the microsomal membrane (93,94). PE-, PMME- and PDME-dependent PE *N*-methyltransferase activities were all degraded by trypsin treatment (95). Latency of mannose-6-phosphate phosphohydrolase (an enzyme of the microsomal lumen) was maintained above 90% during the

course of digestion. Contrary to this, it was reported that PE was converted to PMME on the microsomal lumen and subsequently converted to PC by a second methyltransferase on the cytosolic surface (96). This conclusion relied on the use of phospholipase C as a probe for localizing PC, PMME and PDME, but the authors gave no evidence that PMME or PDME were hydrolyzed by the phospholipase C (96). The use of phospholipases as probes for membrane asymmetry has drawbacks which include perturbing effects of products and differential hydroysis due to the heterogenous nature of membranes (97). The fact that PMME and PDME are 'sequestered' from phospholipase C (96), even when the microsome is disrupted, suggests that these intermediates remain bound to PE *N*-methyltransferase until completely methylated. Because of the technical problems associated with the use of phospholipases, conclusions on asymmetric distribution of two methyltransferases in liver is unfounded. Similar observations have been made for methylation in erythrocyte membranes (98) and will be discussed in Section 1.6.4.

1.6.4 Molecular Structure and Kinetics

The fact that PE methylation occurs via a three step process immediately raises the question as to the number of enzymes involved. Those researchers that originally characterized liver PE methylation inferred that a single enzyme was responsible for all methylation events (84,85). As more tissues were examined and kinetic models were formulated, a body of literature developed in support of the concept of two enzymes. Axelrod and coworkers provided evidence for a two enzyme model in bovine adrenal medulla (99), erythrocyte membranes (98), rat liver microsomes (100), rat basophilic leukemia cells (102,103), rat brain synaptosomes (103) and reticulocyte ghosts (104). The model proposed that a unique methyltransferase catalyzes the synthesis of PMME from PE (methyltransferase I) and that a second enzyme

added the last two methyl groups to form PC (methyltransferase II). The majority of the evidence was derived from kinetic data, pH optima and divalent cation requirements. Methyltransferase I had a reported pH optimum of 6.5-8, a K_m for AdoMet of 0.6-4 μM and a requirement for Mg^{2+}. Methyltransferase II showed a pH optimum of 10-10.5, a K_m for AdoMet of 67-110 μM and no cation requirement (98-100,103,105-107). Lineweaver-Burke plots of initial velocity data at increasing AdoMet concentrations (at pH 8) were typically biphasic with both a high- and low-K_m component (100,103,105,106). The Mg^{2+} requirement was often variable and, depending on the tissue, absolute requirements (99) or only 15% stimulation in activity (103) were reported. It was reported by Prasad and Edwards (105) that sonication of rat pituitary extracts partially solubilized methyltransferase I. Contrary to this, Crews *et al.* (103) reported that sonication of rat brain synaptosomes released methyltransferase II into a 100,000 x *g* supernatant fraction.

The majority of studies indicating two methyltransferases based conclusions on the accumulation of PMME and PDME, both of which are intermediates in PE to PC conversion. Audubert and Vance (88) pointed out that PE methylation is a steady-state process. This implies that intermediates in the pathway (PMME and PDME) rapidly reach constant levels in 1 min, after which time only the amount of the end product (PC) increases. Thus, measurements of radioactivity in PMME and PDME are only an assessment of their steady-state levels. Based on this concept, formulae were derived that estimate the actual amounts of PMME, PDME and PC formed during methylation of microsomal PE. K_m values for AdoMet for the methylation of PE, PMME and PDME were reported to be 50-100 μM, and the pH optimum for all three methylations was 10.5 (88). This was taken to indicate that one enzyme catalyzed all three methylations. A similar conclusion was reached in calf

brain (108). Reconcilation of these conflicting reports awaited purification of the enzyme(s) for PE methylation.

Kinetic analysis of a multistep pathway, whether catalyzed by one or more enzymes, is very complex. While the formulae of Audubert and Vance (88) will determine the K_m for AdoMet, elucidation of the kinetic mechanism will require a different approach. The best approach would involve assaying the individual steps with PMME and PDME, since in both cases only one methyl addition occurs to yield PDME and PC, respectively (88,89,108,109). Assaying the methylation of PE to PMME poses a problem, for no discrete assay is yet available. Since this step is rate-limiting and no intermediates accumulate, the production of PC should reflect the rate of PE methylation. The presence of endogenous substrates and various inhibitors and activators in crude membrane fractions necessitates kinetic analysis of the pure enzyme. Earlier attempts at purification are discussed in the following section.

1.6.5 Purification

PE *N*-methyltransferase is tightly associated with membranes, no doubt due to one or more hydrophobic membrane spanning domains. Because of its integral nature, purification requires the use of amphiphiles or detergents to solubilize PE *N*-methyltransferase from membranes in a form free of lipids and other proteins. The enzyme could then be purified by various conventional techniques. The use of detergents in protein purification has been extensively reviewed (110-112). Any purification of a bioactive protein requires that a balance be struck between optimal solubilization with detergent and maintenance of biological activity. Selection of the proper detergent to achieve this end is largely empirical.

A variety of detergents have been used to solubilize PE *N*-methyltransferase. Most purifications have utilized liver microsomes as

starting material for the logical reason that it contains the highest methyltransferase specific activity. Schneider and Vance (89) reported that sonication of rat liver microsomes in the presence of 0.2% Triton X-100 released 44% of methyltransferase activity. Attempts at purifying the soluble enzyme were partially successful (28-fold purification from homogenate, Ref. 89). A similar solubilization of PE *N*-methyltransferase from mouse liver microsomes, using 0.2% Triton X-100, was reported at the same time (86). Percy, Moore and Waechter (108) also showed that the calf brain enzyme could be solubilized with Triton X-100. Partial purification of PE *N*-methyltransferase from mouse thymocytes using Triton X-100 has been reported (113). The activity in thymocytes is so low that the 1500-fold purified (PE-dependent activity) enzyme had a specific activity similar to that in rat liver microsomes.

Pajares *et al.* (114) reported the 200-fold purification of CHAPS solubilized rat liver microsomal PE *N*-methyltransferase. The alleged methyltransferase had a molecular mass of 25 kDal, and was shown to be photoaffinity labeled with [*methyl*-^{3}H]8-azido-AdoMet. Oddly, the authors showed that the enzyme also methylated fatty acids (114). Shortly after, a paper was published by the same group (115) which now claimed a 50 kDal protein was PE *N*-methyltransferase. By a series of elaborate and confusing experiments they concluded that the 50 kDal protein was actually a dimer of the 25 kDal protein described in the earlier report (114). In both of these publications (114,115) little direct evidence (such as co-chromatography of protein and activity) is provided that either a 25 or 50 kDal protein is PE *N*-methyltransferase. Another disturbing feature of these purifications (114,115) was that CHAPS was removed after the first DEAE chromatography step. Thus, no effort was made to remove endogenous lipids (or proteins) and

the result was purification of a large lipid-protein aggregate or 'membrane fragment'. Although all the aforementioned purifications were unsuccessful, they were unanimous on one point: one enzyme seemed to be responsible for all three methylation events.

1.6.6 Phosphatidylethanolamine Molecular Species Specificity of PE N-Methyltransferase

Specificity can imply both selectivity for different molecular species of the primary substrates PE, PMME and PDME (varying in fatty acid composition), or for compounds with widely divergent structural properties, but with a primary-, secondary- or tertiary-amino group. Molecular species specificity will be discussed first.

All reports to date have utilized argentation TLC (116) as a method for separating molecular species of phospholipids. Separation is on the basis of degree of unsaturation, and thus 'fractions' with the same number of double bonds, and not individual species, are resolved. Most reports on specificity have involved *in vivo* or *in vitro* labeling with [*methyl*-^{3}H]-methionine or -AdoMet, respectively, and analysis of the labeled PC molecular species by argentation TLC. *In vivo,* di- and tetra-enoic (117) or tetra- and hexa-enoic (118-120) fractions of PC were the chief products of PE methylation in rat liver. Arvidson (64) reported that [1,2-^{14}C]ethanolamine labeled hexaenate-rich PE was the primary substrate for methylation. This conclusion has since been shown to be incorrect, and the general consensus is that methylation of any particular PE species is dependent on its concentration (117,120) and not fatty acid composition. This same report (117) showed that the hexaenoic fraction of PC synthesized by methylation had the highest initial specific activity and appeared to turnover rapidly.

Rat brain, the only other organ in which PE *N*-methyltransferase specificty was examined, showed polyunsaturated-rich species of PC to be the

major methylation products. *In vitro* labeling of synaptosomal PC (121) and *in vivo* labeling of whole rat brain PC (122) showed tetra-, penta- and hexa-enoic species of PC to be the primary products of methylation. Similar to liver studies, there was a pronounced turnover of hexaenoic PC species and a concomitant increase in % label in more saturated PC fractions (122). The recent development of HPLC techniques for the separation of *individual* phospholipid molecular species (123,124) made analysis of methylated products of PE *N*-methyltransferase more amenable.

1.6.7 Substrate Specificity

There is a paucity of information indicating substrates other then PE, PMME and PDME are methylated by PE *N*-methyltransferase. The problem has been the unavailability of pure enzyme, thus activities in crude membranes cannot be ascribed to PE *N*-methyltransferase with complete certainty. It has been reported that rat liver microsomes possess an activity that methylates ceramide-phosphoethanolamine (125). A similar activity exists in rat brain microsomes (125). Contrary to this report, LeKim *et al.* (126) reported no hepatic methylation in rats of intravenously injected ceramide-phospho-*N,N*-dimethylethanolamine. Lyso-PMME and -PDME, but not lysoPE, are methylated by liver microsomes (127). These two lyso lipids compete with their diacyl analogues suggesting that methylation is catalyzed by the same enzyme. Clearly, more work is required (preferably with purified enzyme) to assign these activities to PE *N*-methyltransferase.

Modification of the base moiety of PE has given some insight into PE *N*-methyltransferase specificity. Incubation of hepatocytes with monoethylethanolamine, 2-aminopropanol and 2-aminobutanol resulted in their incorporation into phosphatides and the resultant addition of a single methyl group (128). Phosphatidyl-diethanolamine, -diethylethanolamine and

-dimethylaminopropan-2-ol were not methylated by microsomes (128). It was reported that *N*-isopropylethanolamine is converted to phosphatidyl-*N*-isopropylethanolamine *in vivo* and subsequently undergoes a single methylation (129). PE *N*-methyltransferase is sensitive to alterations in the ethanolamine moiety and to *N*-alkyl substitutions of the amino headgroup.

The structural features of AdoMet essential for methyl transfer are not well defined. An early study indicated that the ethyl sulfonium analogue of AdoMet, *S*-adenosyl-L-ethionine (AdoEt), was a competitive inhibitor of PE methylation (85). Incorporation of ethyl groups into PE was not assessed. When [*ethyl*-^{14}C]ethionine was injected into rats, label was recovered in liver PC, but only 1.5% compared to that recovered in PC following [*methyl*-^{14}C]methionine injection (130). Apparently AdoEt can occupy the AdoMet active site on PE *N*-methyltransferase, but ethyl transfer occurs only slowly.

1.7 Functions of Phosphatidylethanolamine *N*-Methyltransferase in Liver

1.7.1 Contribution to Phosphatidylcholine Synthesis

The widely quoted value is that PC synthesis via methylation contributes 20-40% of that in the total liver (68). Earlier reports that monitored PE methylation from [^{3}H]ethanolamine-labeled PE estimated that 3-10% of PC was derived by methylation (131).

1.7.2 As A Source of Phosphatidylcholine in Lipoproteins

Since methylation of PE contributes significantly to the total cellular pool, it is feasible that this PC has some preordained function. Both VLDL and HDL are actively secreted by hepatocytes (132). The surface monolayer of these lipoproteins is composed primarily of the choline-containing lipids, PC and SM (133), which function to solubilize the apolar core (triglyceride and cholesterol ester) components of these particles. The PC required for assembly and secretion of VLDL and HDL from hepatocytes in monolayer culture seems

to be synthesized by both the CDP-choline- and methylation-pathway (134). PC made via methylation of ethanolamine-derived PE is secreted in lipoproteins. However, cellular specific activity of this PC was found to be 2-fold greater than hepatocyte culture medium (134). The relative lack of input into lipoprotein PC by PE methylation was further demonstrated by treating hepatocytes with DZA. This adenosine analogue raises cellular AdoHcy pools and inhibits methylation of PE, but does not affect lipoprotein secretion (135). Oddly, DZA did not inhibit the conversion of [3-^{3}H]serine-labeled PE into PC (134,136), and in fact this serine-derived PC was preferentially and rapidly (30 min) secreted in lipoproteins. These results would seem to indicate that methylation of PS-derived PE is performed by a AdoHcy-insensitive enzyme or the methyltransferase is sequestered away from inhibitor in some manner. This rapid secretion is even more peculiar since methylation and decarboxylation occur in separate organelles and transit from endoplasmic reticulum to mitochondria is estimated at 6 h (75). This is not the only interpretation because the labeled serine used in these studies (134,135) will label the AdoMet pool via THF. A large proportion of the label in PC would be in the methyl group and not the ethanolamine moiety (71).

While it appears that PE methylation is not required for lipoprotein secretion, it is worth noting that VLDL secretion is impaired in choline- and methionine-deficient hepatocytes and supplementation of methionine alone is sufficient to resume VLDL secretion (137).

1.7.3 Does Phosphatidylethanolamine N-Methyltransferase Supply the Hepatocyte With Polyunsaturated Phosphatidylcholine?

Saturates, monoenes, dienes and trienes are the major molecular species of PC formed by the CDP-choline pathway (74,138). However, as discussed in Section 1.6.6, PC formed via methylation of PE is rich in tetra- and hexa-enoic species. It is tempting to speculate that PE *N*-methyltransferase supplies the

cell with PC enriched in unsaturated species by virtue of enzyme specificity or due to the highly unsatuated nature of PE fatty acids (138). There is still little direct proof for this hypothesis apart from specificity data on PE *N*-methyltransferase and cholinephosphotransferase. The deacylation-reacylation pathway in microsomes may be the primary route for incorporation of long-chain polyunsaturated fatty acids into PC (Section 1.3.3).

1.8 Regulation of Phosphatidylethanolamine *N*-Methyltransferase in Liver

1.8.1 Regulation by Substrate Levels.

Incubation of hepatocytes with 0.5 mM ethanolamine produces a 75% increase in methylation of PE (139) and an increase in cellular PE from 20 to 30% of total phospholipid. The effect of ethanolamine supplementation on PE *N*-methyltransferase activity assayed *in vitro* was less striking, but some stimulation in activity was observed (139). Experiments showed that reduction of the endogenous PE concentration (by treatment with the amino group-blocking reagent methylacetimidate) in microsomes caused a simultaneous reduction in methylation rates (128). The effect of PC (the end product of the methylation pathway) on enzyme activity has not been reported.

Supplementation of cultured cells with MME and DME resulted in the synthesis of the PE *N*-methyltransferase substrates PMME and PDME (68,140-142). In LM cells cultured with MME or DME up to 60% of cellular lipid was PMME or PDME (140). Because LM cells are deficient in PE methylation, little of the PMME and PDME is converted to PC. In hepatocytes, MME and DME supplementation raised the phosphatides of these bases to 10-20% of the total phospholipid and stimulated PE methylation by 40% (68). Since PMME and PDME are trace components in hepatic phospholipids (143), these studies have little physiological relevance.

In addition to PE, AdoMet and AdoHcy (or more importantly the ratio of the two) modulate PE methylation. Incubation of hepatocytes with 0.1 mM methionine stimulated PE to PC conversion by 2-fold (68). Higher concentrations had no further affect. Normal perfused rat liver has an AdoMet concentration of 32 nmol/g of tissue (144). This level is elevated to 120 and 300 nmol/g tissue when 0.05 and 2.25 mM methionine, respectively, are included in the perfusate (144). This increase in AdoMet levels is not accompanied by increased AdoHcy. However, perfusion of livers with 3.4 mM homocysteine and 4.0 mM adenosine elevates AdoHcy levels from 8 to 4000 nmol/g tissue and AdoMet to 1250 nmol/g tissue (144). This change in AdoMet/AdoHcy ratios from 5.6 to 0.3 results in a complete abolition of PE methylation.

Further insights on the influence of AdoMet/AdoHcy on PE methylation have been made using a variety of AdoHcy analogues or compounds that raise intracellular AdoHcy levels. The most widely used of these compounds is DZA, a compound that potently inhibits AdoHcy hydrolase (I_{50}=0.008 mM) and elevates cellular AdoHcy (145,146). As well, DZA is a good substrate for the reverse reaction of AdoHcy hydrolase (147). Intraperitoneal injection of DZA into rats decreased the AdoMet/AdoHcy ratio from 4.5 to 1.6 in 4 h (146). 3-DZA-AdoHcy levels were found to be similar to AdoHcy (151 compared to 125 nmol/g liver, respectively). Administration of DZA to rats caused a 90% decrease in [*methyl*-^{3}H]methionine labeling of PC (146). Thus, inhibition of PE methylation is due to appearance of two competitive inhibitors (AdoHcy and DZA-AdoHcy) of PE *N*-methyltransferase (146,148). Activity of PE *N*-methyltransferase in isolated microsomes was not affected (148). The AdoHcy analogues *S*-7-deazaadenosyl-homocysteine and 5'-deoxy-5'(1,4-diamino-pentanoic acid)adenosine (Sinefungin) also inhibit PE methylation (the latter

only *in vitro*, Ref. 149). Other adenosine analogues, 9-β-D-arabinofuranosyladenine, 5'-deoxy-5'-isobutylthioadenosine (SIBA) and A[(-)-9-[trans-2,trans-3-dihydroxy-4-(hydroxymethyl)cyclopent-4-enyl]adenine (Neplanocin A), are potent inhibitors of PE methylation *in vivo* (135,149) and appear to raise cellular AdoHcy levels in a manner analogous to DZA (149,150).

1.8.2 Hormonal Effects in Liver

The reported effects of various hormones on PE methylation in hepatocytes vary greatly. The majority of the studies have centered on the effects of glucagon, insulin, vasopressin and angiotensin on microsomal PE *N*-methyltransferase activity and *in vivo* conversion of PE to PC measured by [*methyl*-^{3}H]methionine labeling. Glucagon appears to act via the second messenger cAMP, while vasopressin and angiotensin exert their influence by a Ca^{2+}-mediated pathway (151).

Geelen *et al.* (152) were the first to demonstrate that pretreatment of hepatocytes with glucagon for 3 h caused a small (20%) increase in PE to PC conversion. Since glucagon also enhanced PE synthesis, the effect on methylation may have been due to an expanded substrate pool (152). Experiments performed in a similar manner reported a 33% reduction in PE to PC conversion after 2.5 hours in the presence of 100 nM glucagon (153). Incubation of hepatocytes with the cAMP analogue chlorophenylthio-cAMP reduced by 50% the incorporation of [1-^{3}H]ethanolamine-labeled PE into PC (154). cAMP analogues (155) and glucagon (153) also inhibit PC production from choline. In the two reports that showed *in vivo* inhibition of PE methylation by glucagon (153) and cAMP analogues (154), PE *N*-methyltransferase activity in a microsomal fraction was unchanged or stimulated 2-fold, respectively. Castano *et al.* (156) reported a 2-fold stimulation of PE *N*-methyltransferase activity in homogenates from

hepatocytes treated with glucagon. In accord with a role for cAMP-dependent phosphorylation, treatment of rat liver microsomes with cAMP and ATP caused a 2-fold stimulation in enzyme activity (157). In opposition to this result, Pelech *et al.* (158) reported no effect of cAMP-dependent protein kinase, protein phosphatase 1 or 2A, casein kinase II and calmodulin-dependent protein kinase on PE *N*-methyltransferase activity in microsomes. However, ATP or GTP and cytosol caused a 50% increase in activity (158).

Results from various laboratories, though quite contradictory, suggested that cAMP-dependent phosphorylation was activating PE *N*-methyltransferase. Various experiments were initiated to demonstrate that the putative 50 kDal PE *N*-methyltransferase (Section 1.6.5) was phosphorylated by cAMP-dependent protein kinase in response to glucagon. A 50 kDal protein was phosphorylated on a serine residue by cAMP-dependent kinase with a resultant 4-fold increase in PE methylation activity (159). AdoMet seemed to activate phosphorylation or inhibit dephosphorylation of partially pure methyltransferase (160). Also, the 50 kDal protein was immunoprecipitated from glucagon treated hepatocytes and shown to have incorporated ^{32}P (161). Protein kinase C also activated PE *N*-methyltransferase and phosphorylated the 50 kDal protein (162). However, evidence reported in this thesis will show that the 50 kDal protein is not PE *N*-methyltransferase.

Vasopressin, angiotensin and the Ca^{2+}ionophore A23187 inhibited choline labeling of PC (163), but activated by 2-fold PE *N*-methyltransferase in hepatocyte homogenates (164). Ca^{2+} and ATP also stimulated microsomal methyltransferase activity 2-fold, presumably through the mediation of calmodulin (165). Apparently, the β-adrenergic receptor agonist isoprenaline also stimulates PE *N*-methyltransferase activity in hepatocytes from

adrenalectomized rats and, to a lesser extent, control animals (166,167). Again, this activation appeared to be mediated by cAMP.

Insulin has no effect on PC synthesis from choline (153) or via methylation of PE (168). However, insulin was reported to inhibit the glucagon-dependent stimulation of methyltransferase activity and phosphorylation of a 50 kDal protein in hepatocytes (168).

1.8.3 Hormonal Regulation in the Adipocyte

PE *N*-methyltransferase of adipocytes seems to be activated by cAMP-dependent mechanisms in a manner similar to that of hepatocytes. Accordingly, adrenocorticotropin (169), epinephrine (170), isoproterenol (170) and forskolin (170,171) were all shown to stimulate methylation activity in intact adipocytes. Administration of oxytocin, a hormone that does not act via cAMP (172), to adipocytes resulted in a time- and dose-dependent 1.5-fold activation of PE methylation (170). Phorbol 12-myristate-12-acetate was reported to stimulate methylation activity 2-fold in rat adipocytes, presumably via protein kinase C (171).

Insulin was reported to abolish the effects of various hormones acting through cAMP-dependent mechanisms (169,170) and, by itself, to inhibit methylation in intact adipoctyes by 40% (169). Strangely, the same group reported that insulin stimulated PE *N*-methyltransferase activity in isolated adipocyte plasma membranes (179). Isoproterenol, as well as stimulating activity, was recently shown to enhance phosphorylation of the putative 50 kDal methyltransferase (174) in a manner analogous to hepatocytes (Section 1.8.2). Insulin and a phospho-oligosaccharide (prepared from rat liver membranes by PI-specific phospholipase C digestion) both inhibited isoproterenol-dependent phosphorylation (174). The phospho-oligosaccharide, presumed to be cleaved from its lipid moiety by an insulin-

responsive phospholipase C, is proposed to serve as a second messenger of insulin action (175). Considering that the 50 kDal protein is not PE *N*-methyltransferase, one should be wary of reports on enzyme phosphorylation and effects on activity.

1.8.4 Other Effectors

There appear to be cytosolic factors that influence PE *N*-methyltransferase activity (158), but they remain unidentified. A heat-stable, low molecular weight inhibitor has been identified in rat liver cytosol (158,176). This inhibitor may be similar to a 25 amino acid peptide isolated from rabbit liver cytosol that is proposed to inhibit various methyltransferases (177).

Unsaturated fatty acids are potent inhibitors of PE methylation in intact hepatocytes and microsomes (178). Long-chain fatty acyl-CoA esters were also effective inhibitors and inhibition was reversible upon addition of BSA.

1.8.5 Developmental Regulation

Two reports on PE *N*-methyltransferase in pre- and post-natal rat liver demonstrated a steady rise in activity from -5 to +15 or +20 days and a slow decline to adult values thereafter (179,180). Activities in prenatal rabbit livers were about 33% lower than values at birth and reached a maximum at +14 days (181). Similarly, rat brain PE *N*-methyltransferase activity was demonstrated to be the highest between day 5 and 30 (182). Since enzyme mass was not correlated with activity, it is feasible that activity changes noted after birth could be due to altered PE or AdoMet/AdoHcy levels.

1.8.6 Coordinate Regulation with the CDP-Choline Pathway

Several examples of coordinate regulation of the two major pathways for PC synthesis have been demonstrated. Unsaturated fatty acids, while potently inhibiting PE methylation (178), were found to stimulate PC

production from choline in cultured rat hepatocytes (183). This enhanced synthesis via the CDP-choline pathway was correlated with translocation of phosphocholine cytidylyltransferase activity from cytosol to microsomes.

The elevated cellular levels of AdoHcy caused by DZA treatment inhibited PE methylation (146), but caused a 2- to 3-fold increase in PC synthesis via the CDP-choline pathway and a 3-fold increase in microsomal cytidylyltransferase activity (148).

Maintenance of rats on a choline-deficient diet caused various metabolic perturbations including fatty liver, decreased circulating lipoprotein levels, increased hepatic PE and decreased hepatic PC (184,185). Accompanying these changes is a 2-fold elevation in PE *N*-methyltransferase activity, a 2-fold reduction in cytosolic cytidylyltransferase activity and no change in microsomal cytidylyltransferase activity (186,187). However, a recent report by Yao, Jamil and Vance (188) showed that microsomal cytidylyltransferase activity was elevated in choline- and methionine-deficient hepatocytes, and that a shift of activity to the cytosol occurred upon supplementation with choline of methionine. The mechanism of this redistribution in enzyme activity is as yet unknown.

1.9 Phosphatidylethanolamine *N*-Methyltransferase in Eukaryotic Microorganisms

1.9.1 Phosphatidylethanolamine Methylation in Yeast.

PE *N*-methyltransferase has yet to be purified from yeast, however, genetic evidence points to the involvement of two enzymes in conversion of PE to PC. In yeast, unlike mammalian cells, the majority of PC can be synthesized by PE methylation (189,190). Methionine seemed to be utilized about 4-times more effectively for PC synthesis than choline (190). Two mutants in PE methylation have been isolated from *S. cerevisiae*. The *opi 3* mutant (191) appeared to be defective in methylation of PMME to PC and

accumulated PMME and, to a lesser extent, PDME. Incorporation of [*methyl*-^{3}H]methionine into PC was about 10-20% of that in wild type strains (191). The *cho 2* mutant is defective in the conversion of PE to PMME (192). Growth of this strain on MME restores PC levels to normal (192). It is unclear whether these two mutations are the result of lesions in regulatory or structural genes. Work with several other choline auxotrophs of *S. cerevisiae* also indicated that two enzymes were required for the formation of PC (193,194). More recently, Kodaki and Yamashita (195) have successfully cloned the structural genes for two *S. cerevisiae* methyltransferases by means of genetic complementation. A double mutant carrying the *pem 1* gene accumulated PMME and was defective in synthesis of PDME and PC. Transfection of the double mutant with *pem 2* resulted in an intact PE methylation pathway, but PC levels were about one-half that of wild type values (195). Thus, one enzyme converts PE to PC (*PEM 2*) and the other catalyzes PMME synthesis only (*PEM 1*), but both are necessary for the maintenance of normal PC levels. Internal homology was observed in the predicted amino acid sequence of the *PEM 1* gene product, as well as between *PEM 1* and *PEM 2* (195).

Yeast PE *N*-methyltransferases were reported to be inhibited by choline in the growth medium (196). *Myo*-inositol was also observed to inhibit reversibly PE methylation in *S. cerevisiae* (193), an effect expressed only in the presence of choline (194). A detailed survey of all growth conditions showed that choline alone does not repress PE *N*-methyltransferase, and inositol alone is required for full expression of enzyme activity. Inositol and choline together led to 6- to 10-fold repression of activity (192). Repression of activity by inositol and choline is due to reduced enzyme synthesis since restoration of enzyme activities (by removal of inositol) is abolished by cycloheximide (193).

The *S. cerevisiae* sterol and fatty acid auxotroph GL-7, which is deficient in squalene epoxide cyclase (197), displays unusual sterol synergism. Addition of limiting quantities of cholesterol or ergosterol did not support growth, but addition of both sterols produced a marked increase in growth rates (198,199). PE methylation, measured by [*methyl*-^{3}H]methionine and [*methyl*-^{3}H]AdoMet incorporation *in vivo* and into microsomal PC, respectively, was found to be one of the processes stimulated by ergosterol/cholesterol supplementation (199). Since the total amount of sterol was the same in cholesterol versus cholesterol plus ergosterol supplemented cells, the results indicated a specific effect of ergosterol on PE *N*-methyltransferase (199). It was also observed that the GL-7 mutant (supplemented with ergosterol) had phosphocholine cytidylyltransferase activities 4-fold lower then wild type, and as such PE methylation was the major source of PC in these cells (190). Evidently, selective inhibition of methylation by ethionine resulted in enhanced PC synthesis from choline in both mutant and wild type cells (190).

Two mutant strains of the mold *Neurospora crassa* were shown to have phospholipid compositions consistent with a requirement for two enzymes in PE to PC conversion (200,201). Microsomes from one of these mutant strains appeared to be defective in PE methylation to PMME, while the second strain accumulated PMME, and thus may have a defect in the conversion of PMME to PC (202). Partial purification of the PMME- and PDME-dependent activities was also consistent with the notion of two enzymes for PE to PC conversion in *N. crassa.* (203). It is possible that, like *S. cerevisiae*, *N. crassa* has a PE methyltransferase that converts PE to PC and not just PMME to PC.

1.9.2 Phosphatidylethanolamine Methylation in Bacteria

As a general rule, bacteria do not contain PC or the partially methylated intermediates of the PE methylation pathway (204). There are some notable

exceptions to this rule. Twelve strains of hydrogen-oxidizing bacteria were found to contain 23 to 47% PC (205), 6 strains of the phototrophic purple bacteria (family Chromatiaceae) contained 10-17% of their total phospholipid as PC (205) and several strains of methane- and methanol-utilizing bacteria also contain PC as well as PMME and PDME (207,208). Phospholipids of thiobacilli were shown to be composed of PC, PMME and PDME (209). Eight strains of *Clostridium beijerinckii*, a butyric acid-producing bacteria, contained PMME but no other methylated phospholipids (209b). The source of the methyl groups in bacterial PC is methionine and AdoMet appears to be the direct methyl donor (209,209a). It has been proposed that the occurrence of PC among these bacteria is correlated with the presence of intracytoplasmic membrane systems or photosynthetic pigments (205). Such structure-function relationships are tenuous at best, since within a particular bacterial genus some species contain PC while others do not. Goldfine (210) noted that PC and PDME were found in bacteria rich in unsaturated fatty acids; a combination that would enhance membrane bilayer stability.

Characterization of the PE methylating system of *Agrobacterium tumefaciens* revealed the presence of a soluble enzyme catalyzing the synthesis of only PMME and a particulate enzyme that produced primarily PC from endogenous PE (211). The PE *N*-methyltransferase of *Rhodopseudomonas sphaeroides* (212) was found to be exclusively (>90%) associated with a soluble cell fraction. A single enzyme catalyzing all three methylations to PC was purified from Triton X-100 solubilized membranes of *Zymomonas mobilis* (213). The putative enzyme is composed of a single subunit of 42 kDal (213).

As yet, no choline nucleotide pathway has been identified for the synthesis of bacterial PC. This would indicate that of the two pathways for PC synthesis, PE methylation was the first to evolve. The prevalence of PMME and

PDME (often in the absence of PC) in some bacterial strains suggests that the pathway evolved in a stepwise manner (205,210).

1.9.3 Other Organisms

The ciliate protozoan *Tetrahymena* is peculiar owing to the presence of 20% 2-aminoethylphosphonolipid (an analogue of PE with 2-aminoethylphosphonate as the base) in its phospholipid (214). *Tetrahymena* contains about 29% PC, of which 60% is synthesized by PE methylation (214). Interestingly, 2-aminoethylphosphonolipid (AEP-lipid) and *N,N*-dimethylaminoethylphosphonolipid are not substrates for methylation (215,216) and *N,N,N*-trimethylaminoethylphosphonolipid (TMAEP-lipid) is not a natural component of *Tetrahymena* lipids (214). *Tetrahymena* grown on medium containing TMAEP incorporate this base into TMAEP-lipid such that it represents 20% of the total phospholipid (217). At this proportion of the total lipid, cell growth is seriously inhibited (217). The toxicity of TMAEP-lipid makes it imperative that AEP-lipid is not methylated. Whether the mammalian PE *N*-methyltransferase requires a phosphoester bond between the headgroup and phosphate is yet unknown. AEP injected into rats was found in AEP-diglyceride, but the trimethyl derivative was not observed indicating the O-P bond is necessary for methylation (218).

The slime mold *Dictyostelium discoideum* has been found to possess enzyme systems for the methylation of both neutral lipids and PE (219). PE *N*-methyltransferase of *D. discoideum* appears to be composed of two enzymes, a conclusion based on the biphasic nature of reciprocal plots of AdoMet initial velocity data (220). The major product of PE *N*-methyltransferase was reported to be PMME (219,220). It has been suggested that cell aggregation (a stage in mold development), mediated by cellular cGMP levels that are elevated in response to occupation of cAMP receptors on the cell surface, is accompanied

by a parellel stimulation in PE methylation (221). PE *N*-methyltransferase activity in cell homogenates was enhanced about 2-fold upon treatment with cGMP and calmodulin (220,221). However, PE methylation activity did not correlate with developmental changes mediated by cAMP, and instead seemed to be involved in membrane synthesis (219).

1.10 Phosphatidylethanolamine Methylation and Transmembrane Signaling

Bacteria respond to environmental stimuli (chemotactic effectors such as sugars and amino acids) by a series of receptor binding and covalent modification episodes that culminate in specific flagellar movements (222). Genetic manipulation techniques supported the role of protein methylation and demethylation being central to the chemotactic response. As is often the case, this bacterial model for chemotactic response was tested in eukaryotic cells, but with less spectacular results. Evidence suggested that increased protein methylation coincided with the chemotactic response in motile cells such as leukocytes, neutrophils and spermatids (223). Protein carboxymethylation may also play a role in secretion of hormones from the pituitary, parotid and adrenal glands (223). It was during the course of these aforementioned studies that methyl label from methionine or AdoMet was observed in phospholipids, presumably by PE methylation, and the association between PE methylation and signal transmission across membranes was formulated (99,224). Instances in which PE methylation was correlated with the response of a tissue or cell to a particular agonist or chemotactic effector are summarized in Table 2. In summary, these reports based the second-messenger function of PE methylation on the observation that synthesis of methyl labeled lipids preceeded the appearance of cAMP, arachidonate, Ca^{2+} or prostaglandins. Also, DZA, 3-deaza-SIBA and homocysteine-thiolactone, all inhibitors of PE methylation, appeared to attenuate the response

Table 2. Compilation of Reports For and Against PE Methylation in Cell Signaling Events

In Favour	Tissue and Effect	In Disagreement
Axelrod *et al.* (101) Axelrod *et al.* (102) Axelrod *et al.* (244) Axelrod *et al.* (245)	Rat basophilic leukemia cell, mast cell and thymocyte histamine and Ca^{2+}release	Moore *et al.* (229) Boam *et al.* (254)
Axelrod *et al.* (246)	Lymphocyte mitogenesis	Moore *et al.* (255)
Axelrod *et al.* (247)	Leukocyte chemotaxis	---
Axelrod *et al.* (225)	Reticulocyte cAMP levels	---
Kannagi *et al.* (248)	Platelet activition	Randon *et al.* (256)
Pike *et al.* (249)	Macrophage chemotaxis	Sung and Silverstein (257) Aksamit *et al.* (258)
Axelrod *et al.* (250)	cAMP and Ca^{2+} levels in fibroblasts	---
Wiesman *et al.* (251)	Na^{+} transport in A6 epithelia	---
Ganguly *et al.* (252)	Stimulation of SR Ca^{2+}-ATPase	---
Zelenka *et al.* (253)	Differentation of lens fiber epithelia	---
---	Myogenic cell cAMP	Koch *et al.*(259)
---	Paroid gland cAMP	Padel *et al.* (260)
---	Hepatocyte cAMP	Colard, *et al.* (261) Schanche *et al*(262)

of cells to various agonists (224). It was suggested that PE *N*-methyltransferase was in close association with the β-adrenergic receptor and PE methylation occurred in response to ligand binding. Generation of PMME (and PC) increased membrane fluidity, the result of which was coupling of receptor with adenylate cyclase and production of cAMP (225). Membrane fluidity changes were observed upon incubation of erythrocyte ghosts with AdoMet (226). As noted in Table 2, the second messenger role of PE methylation was largely advocated by Axelrod and coworkers. Results from other labs tend to refute most of the conclusions reached by these workers (specific examples are noted in Table 2). The evidence against PE methylation in signal transduction was based either on no observed stimulation of methyl labeled lipid synthesis or a lack of effect of methyltransferase inhibitors on response to various stimuli (Table 2).

The low PE methylation activity in most extrahepatic tissues led Vance and de Kruijff (227) to speculate that the 0.00033% of total PC derived via PE methylation in erythrocyte membranes could not account for the large changes in microviscosity (226). This observation was supported by work in mouse fibroblasts (LM cells, Ref. 140) and liposomes (228), which showed alteration of the proportion of PMME and PDME in membranes did not influence fluidity. An interesting report by Moore *et al.* (229) demonstrated that stimulation of rat basophilic leukemia cells, mast cells and mouse thymocytes, and subsequent release of histamine by a Ca^{2+}-dependent signal, was not coincident with PE methylation. Instead, the initial response to agonists was PI hydrolysis to inositol polyphosphates and diglyceride, which are known to mediate intracellular release of Ca^{2+} (3,229) or activate protein kinase C (230), respectively.

1.11 Rationale and Objectives of the Present Study

A multitude of studies on PE *N*-methyltransferase have appeared in the 27 years since this enzyme was first identified in liver microsomes. Work toward characterization of PE methylation at the molecular level has often lagged, primarily because the enzyme is an integral membrane protein and utilizes lipid substrates. As a direct result of the paucity of information, incorrect modes of regulation and questionable functions have been ascribed to PE *N*-methyltransferase. This statement may seem rather strong in light of the last 40 pages of literature review. However the remainder of this text, which is devoted to characterization of the liver methyltransferase, should substantiate this view.

Purification of PE *N*-methyltransferase from rat liver microsomes was undertaken. These efforts were quite fruitful and subsequent molecular and kinetic characterization of the purified enzyme revealed properties very dissimilar from those previously reported in the literature. Results suggested that previous reports of purification were erronous, and in fact these researchers had only isolated a partially pure enzyme and characterized a contaminating protein in their preparation.

Regulation of PE *N*-methyltransferase by phosphorylation and substrate levels (using the choline-deficient rat as a model) was investigated and again indicated a serious re-evaluation of published reports was required. A portion of the following results have appeared in publication (231-235).

EXPERIMENTAL PROCEDURES

2.1 Materials

PMME, PDME, dipalmitoyl-PE, distearoyl-PE, dimyristoyl-PE, 1-palmitoyl-2-oleoyl-PE, dioleoyl-PE, dilinoleoyl-PE, dilinolenoyl-PE and TP-egg-PE (prepared by phospholipase D action on egg PC in the presence of ethanolamine) were purchased from Avanti Polar Lipids, Birmingham, AB. AdoMet and ATP was purchased from Boehringer Mannheim, Canada. [*methyl*-^{3}H]AdoMet, [*methyl*-^{3}H]methionine, γ^{32}P-ATP, ^{125}I-protein A and [1-^{3}H]ethanolamine were from Amersham Corp., UK. DTT, collagenase, insulin, phosphoserine, phosphothreonine, phosphotyrosine, choline chloride, methionine, Triton X-100, catalytic subunit of cAMP-dependent protein kinase and BSA were from Sigma Chemical Co., St. Louis, MO. Molecular mass standards for gel filtration and SDS-PAGE, octyl Sepharose CL-4B, Sephacryl S-300, DEAE Sepharose CL-4B and PBE 94 polybuffer exchange resin for chromatofocusing were purchased from Pharmacia-LKB Biotechnology Inc. Preparative (2.0 mm) silica gel 60, analytical (0.2 mm) silica gel 60 and cellulose thin-layer plates were from Merck. HPLC grade methanol, chloroform and acetonitrile were from Fisher. Dulbecco's MEM (arginine-, choline-, and methionine-free) and Hanks' balanced salt solutions were obtained from Gibco Laboratory, Grand Island, NY. Primaria culture dishes were from Becton Dickinson and Co., Oxnard, CA. Gelatin and nitrocellulose were purchased from Biorad Laboratory, Richmond, CA. All other materials were of reagent grade.

2.2 Phosphatidylethanolamine *N*-Methyltransferase Purification

2.2.1 Isolation of Microsomes

Microsomes were isolated from the livers of female Wistar rats (175-225 g) in the following manner. Rats were sacrificed by cervical dislocation and the livers immediately removed and placed in ice cold 10 mM Tris HCl (pH 7.2)

buffer containing 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride and 1 mM DTT. The liver was cut into small pieces, suspended at a final concentration of 25% (w/v) in the Tris-saline buffer and homogenized using a motor driven Potter-Elvehjem apparatus. The homogenate was centrifuged at 12,000 x *g* for 10 min. The supernatant fraction was then subjected to centrifugation at 120,000 x *g* for 1 h. The cytosol was immediately decanted and the microsomal pellet resuspended in a 20 mM potassium phosphate buffer (pH 7.9) containing 1 mM EDTA, 1 mM DTT and 250 mM sucrose using a hand held glass dounce. Microsomes were also isolated from livers that had been perfused with 150 mM NaCl and 0.5 mM EGTA. Following perfusion the livers were treated in an identical manner as that described above. Microsomes prepared from perfused livers had a higher initial PE *N*-methyltransferase specific activity.

2.2.2 Preparation of Microsomal Membranes

Microsomes (20-30 mg/ml protein) were suspended in 100 mM Na_2CO_3 and 5 mM DTT, at a final protein concentration of 4 mg/ml, and stirred at 4°C for 30 min. The suspension was centrifuged at 120,000 x *g* for 1 h and the membrane pellet collected and resuspended (using a glass dounce) in 20 mM potassium phosphate buffer (pH 7.9) containing 10% (v/v) glycerol and 5 mM DTT. This buffer was used in all subsequent purification steps and will be referred to as buffer A. All purification steps were performed at 4°C.

2.2.3 Solubilization of Microsomal Membranes

Microsomal membranes were suspended in buffer A (containing 0.7% (w/v) Triton X-100) to a final protein concentration of 4 mg/ml and stirred for 1 h. The mixture was centrifuged at 120,000 x *g* for 1 h and the supernatant collected and used as a source of soluble enzyme.

2.2.4 Chromatography on Whatman DE-52 Cellulose

Soluble PE *N*-methyltransferase was passed through a column of DE-52 cellulose (30 x 2.5 cm), previously equilibrated in buffer A plus 0.7% Triton X-100, at a flow rate of 1.0 ml/min. PE *N*-methyltransferase activity was recovered in the unbound fractions.

2.2.5 Chromatography on Whatman P-11 Phosphocellulose

A column of Whatman P-11 phosphocellulose (16 x 1.6 cm) was equilibrated in buffer A containing 0.7% (w/v) Triton X-100. The pooled fractions from the previous step were applied to the column at a flow rate of 0.25 ml/min. When loading was complete the column was flushed in succession with 100 ml of buffer A containing 0.7% (w/v) Triton X-100 and 100 ml containing 0.25% (w/v) Triton X-100. PE *N*-methyltransferase activity was eluted from the column with a 350 ml linear gradient of NaCl from 0 to 0.8 M in 0.25% (w/v) Triton X-100. PE *N*-methyltransferase activity eluted in a broad peak from 0.2 to 0.6 M. We found it necessary to use P-11 phosphocellulose that had been used for several purifications (and not regenerated with HCl and NaOH according to the manufacturers specifications) in order to achieve good recoveries. The phosphocellulose could also be pretreated with 0.2% (w/v) BSA in Buffer A containing 0.7% Triton X-100 followed by elution with 2.0 M NaCl in the same buffer. This procedure seemed to block high affinity sites on the resin that would otherwise reduce recovery of PE *N*-methyltransferase and change its chromatographic characteristics on octyl Sepharose.

2.2.6 Chromatography on Octyl Sepharose CL-4B

Active fractions from the phosphocellulose column were pooled and diluted with Buffer A (no Triton X-100) to a final Triton X-100 concentration of 0.05% (w/v). The enzyme solution was pumped (0.5 ml/min) onto a column of octyl Sepharose (17 x 1.6 cm) equilibrated in buffer A containing 0.05% (w/v) Triton X-100. The column was flushed with 100 ml of buffer A containing

0.05% Triton X-100. It is critical to the final purity of the enzyme that this column be eluted so that the main PE *N*-methyltransferase peak is well separated from the major protein peak that precedes it (Figure 4). The best results were obtained by eluting the column with 100 ml of Buffer A containing 0.15% (w/v) Triton X-100, followed by a 250 ml linear gradient of Triton X-100 from 0.15 to 0.5% (w/v) in the same buffer.

2.2.7 Chromatography on PBE 94

The final step in the purification takes advantage of the very basic nature of PE *N*-methyltransferase. The pooled fractions from the previous step, adjusted to pH 9.4 with 250 mM ethanolamine, were applied to a column of PBE 94 (15 x 1.6 cm) equilibrated in 25 mM ethanolamine (pH 9.4), 5 mM DTT, 10% (v/v) glycerol and 0.1% (w/v) Triton X-100. PE *N*-methyltransferase was loaded at a flow rate of 0.2 ml/min. Similar to the DE-52 cellulose step, PE *N*-methyltransferase activity was not bound to this anion exchange resin. Concentration of the dilute, purified enzyme can be achieved by reapplying the enzyme solution to a Mono S column (cation exchange resin, Pharmacia-LKB Biotechnology Inc.). Briefly, the Mono S column was equilibrated in buffer A containing 0.1% (w/v) Triton X-100. Purified PE *N*-methyltransferase was adjusted to pH 7.9 with 0.5 M KH_2PO_4 and applied to the column at a flow rate of 0.2 ml/min. Enzyme was subsequently eluted at a flow rate of 0.6 ml/min with a linear gradient of NaCl from 0 to 1 M in buffer A. Total volume of the gradient was 15 ml. Concentration by Mono S chromatography did not alter the specific activity of PE *N*-methyltransferase using PE, PMME or PDME as substrates and recoveries averaged 75-80%. Enzyme was stored in the high salt eluate at 4°C in 0.1% Triton X-100. There was no loss of activity after at least two months.

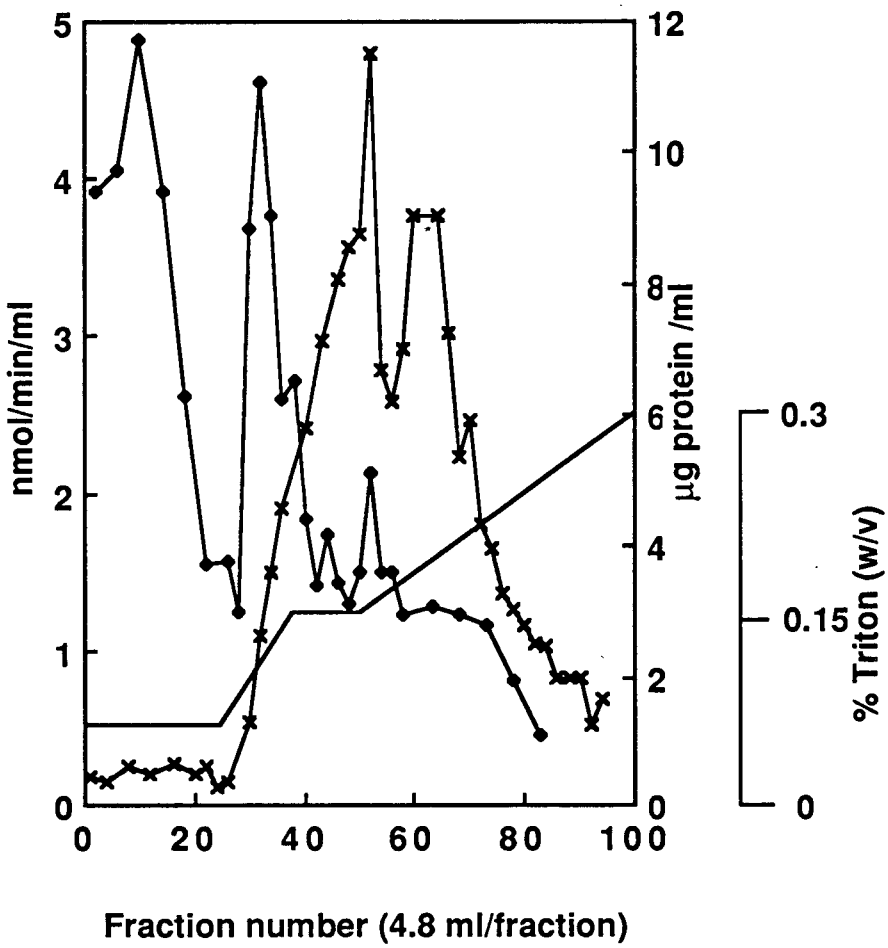

Figure 4. **Octyl Sepharose CL-4B chromatography of PE *N*-methyltransferase.** Pooled fractions from the P-11 phosphocellulose step were applied to an octyl Sepharose column as described in Section 2.2.6. Protein concentrations (◆) in the column fractions were measured by the silver binding method (267). PE *N*-methyltransferase activity was determined in the presence of 0.25 mM PMME and 0.5 mM Triton X-100 (✖). Triton X-100 concentrations (—) were determined by relating the absorbance of unknown samples to those of standards at 275 nm.

2.3 Assay of Phosphatidylethanolamine *N*-Methyltransferase

2.3.1 Assay using [methyl-^{3}H]S-Adenosyl-L-Methionine

The presence of microsomal phospholipids in partially purified fractions (step 1 to 4, refer to Table 3) necessitated the use of higher concentrations of Triton X-100 and exogenous phospholipid substrates in order to achieve maximal expression of PE *N*-methyltransferase activity. The complete removal of endogenous phospholipids after phosphocellulose chromatography (Table 3) allowed the use of lower Triton X-100 and exogenous lipid substrate concentrations. All assays were in 125 mM Tris HCl (pH 9.2) and 5 mM DTT, with a final assay volume of 150 µl. Samples from steps 1 to 4 contained 1.0 mM Triton X-100 and either no addition, 2.0 mM PE, 0.4 mM PMME or 0.4 mM PDME. No more than 25 µg of protein were assayed in these first 4 steps. Purification steps 5 to 7 (Table 3) were assayed in the presence of 0.5 mM Triton X-100 and 2.0 mM PE, 0.25 mM PMME or 0.45 mM PDME. Dilution of the enzyme source to 0.5 mM Triton X-100 was the major factor in deciding the volume of enzyme to be assayed in the final 3 purification steps. PE, PMME and PDME were added to the assay as vesicles prepared in the following manner. Lipids were dried under a stream of nitrogen and further dried under high vacuum (10 microns of Hg) for 30 min. The dry lipids were resuspended in 20 mM Tris HCl (pH 9.2), 0.01% (w/v) EDTA and 0.02% (w/v) Triton X-100 by vortexing for 1 min and immediately sonicated at 37°C for 3 min using a Son-IM-1 Ultrasonic Processor equiped with a Microtip (Heat Systems-Ultrasonics, Farmingdale, NY) at a 100 watt setting. Synthetic PEs were prepared in an identical manner and sonicated above their T_m. Following the addition of these assay components, the mixture was placed on ice and the enzyme source was added and allowed to equilibrate for 10 min. [*methyl*-^{3}H]AdoMet (21 µCi/µmol) was added to a final concentration of 200 µM and the mixture was incubated at

37°C for 10 min to assay PMME- and PDME-dependent activity or 30 min to assay PE-dependent activity, unless otherwise specified.

The assay was stopped by the addition of 2 ml of chloroform:methanol (2:1, v/v). Methylated phospholipids were extracted by addition of 2 ml 0.5% (w/v) NaCl, vortexed and centrifuged (2000 rpm for 5 min) to separate organic and aqueous phases. The organic phase was washed 3 times with 2 ml of 0.5% NaCl:methanol:chloroform (50:50:4, v/v/v) and dried under a stream of nitrogen. The dried residues were redissolved in 0.2 ml of chloroform/methanol (2:1, v/v) and the radioactivity was determined in a 0.1 ml aliquot. Chloroform:methanol was evaporated from the sample prior to liquid scintillation counting. Total chloroform:methanol (2:1, v/v) soluble counts were determined directly using a Beckman LS 3801 Liquid Scintillation Counter (Beckman Instruments, Fullerton, CA) and expressed as amount of methyl groups transferred to phospholipid/min/mg protein. PE, PMME and PDME methylation by purified PE *N*-methyltransferase was linear for 30 to 40 min (Fig. 5).

2.3.2 Assay using [^{3}H]Phosphatidylethanolamine

Methylation activity assayed with [^{3}H]PE was determined in a similar manner as described in Section 2.3.2, but in the presence of 200 μM unlabeled AdoMet and 2 mM [^{3}H]PE (2500 cpm/nmol). Following incubation for 30 min, PC, PMME and PDME were separated by TLC and radioactivity was determined as described in Section 2.3.2.

2.3.3 Analysis of Products by Thin-Layer Chromatography

Products of PE *N*-methyltransferase were separated by TLC in a solvent system of chloroform:methanol:acetic acid:water (50:30:5:2, v/v/v/v). Carrier PE, PC, PMME and PDME were added to the extracts prior to separation.

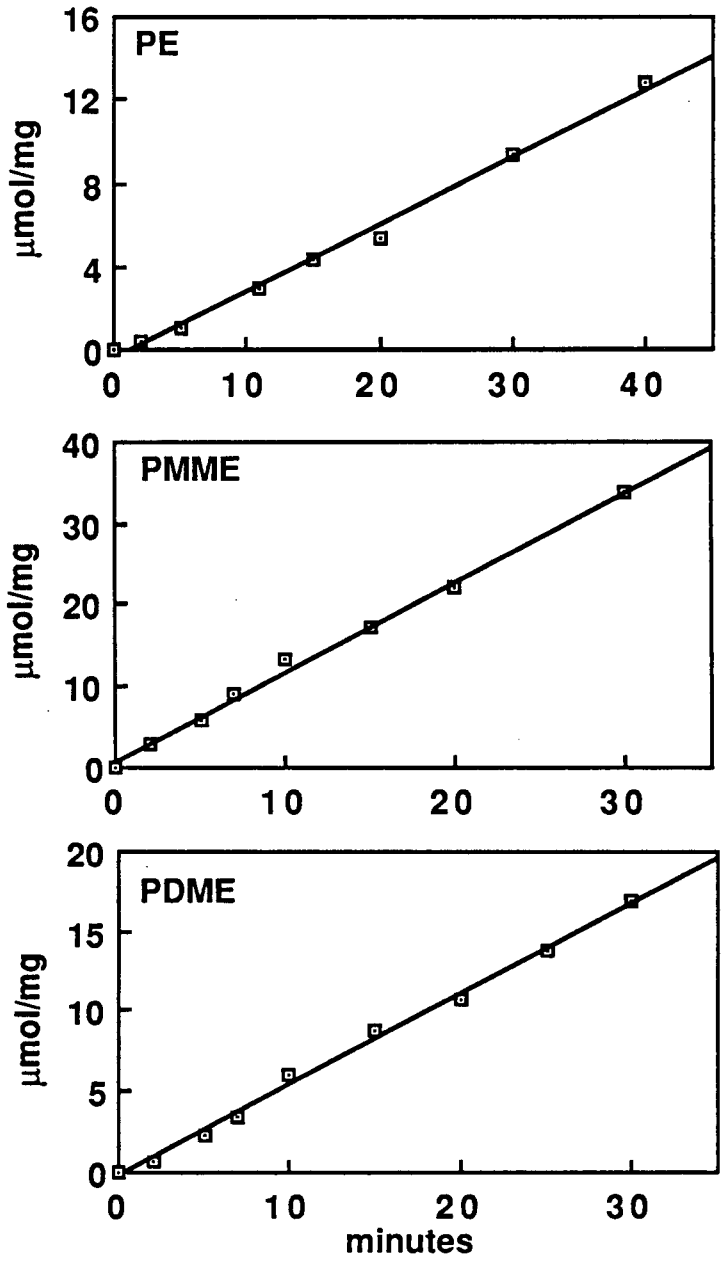

Figure 5. **Time course for methylation of PE, PMME and PDME.** 57, 60 and 24 ng of purified PE *N*-methyltransferase was assayed in the presence of 0.5 mM Triton X-100 and 2.0 mM PE, 0.25 mM PMME and 0.5 mM PDME, respectively, for the indicated periods of time.

Phospholipids were visualized by exposure to iodine vapours, scraped into scintillation vials containing 250 µl of water, 5 ml of Aqueous Counting Scintillant (Amersham Canada Ltd., Oakville, Ont.) was added and radioactivity was measured after 24 h.

2.3.4 Preparation of [1-^{3}H]Ethanolamine-Labeled Phosphatidylethanolamine

[^{3}H]PE was prepared by a 3 h continuous pulse of freshly prepared rat hepatocyte monolayers with 15 µCi of [1-^{3}H]ethanolamine. At the end of this time, medium was aspirated, cells were scraped from dishes and PE was purified from the extracted phospholipids by the method of Arvidson (64) as described in Section 2.4.1.

2.3.5 Repurification of S-Adenosyl-L-Methionine

AdoMet and [*methyl*-^{3}H]AdoMet were repurified on Dowex 1-X8 and Cellex-P, respectively, as previously described (269,270). AdoMet concentrations were determined by absorbance measurements at 257 nm (molar absorption coefficient = 15,000 M^{-1} cm^{-1}). AdoHcy used in kinetic experiments was not repurified, and its concentration was determined spectrophotometrically at 257 nm (molar absorption coefficient = 15,000 M^{-1} cm^{-1}).

2.4 High Performance Liquid Chromatographic Analysis of Phospholipids

2.4.1 Preparation of Radiolabeled Phosphatidylethanolamine N-Methyltransferase Products

Methyl labeled PDME and PC were prepared under conditions described previously for the assay of PE-, PMME-, and PDME-dependent methyltransferase activities (Section 2.3.1). Methylation of 0.25 mM PMME, 0.4 mM PDME, and 2.0 mM TP-egg PE or microsomal PE by purified PE *N*-methyltransferase was in 0.5 mM Triton X-100, 125 mM Tris-HCl (pH 9.2) and 5

mM DTT. The enzyme was preincubated on ice with the aforementioned components for 10 min. The reaction was initiated by the addition of [*methyl*-^{3}H]AdoMet (20 μCi/μmol) to a final concentration of 200 μM and incubated at 37°C for 20 min. PE in whole microsomes was methylated in a similar fashion except exogenous lipid substrates and Triton X-100 were absent.

Incubations were stopped by the addition of 2 ml of chloroform:methanol (2:1, v/v) and the methylated phospholipids extracted as described in Section 2.3.1. The chloroform:methanol extracts were evaporated under a stream of nitrogen, redissolved in chloroform and phospholipid classes were separated by TLC in a solvent system of chloroform:methanol:acetic acid:water (50:30:5:2, v/v/v/v). The plates were sprayed with 0.2% (w/v) dichlorofluorescein in 95% ethanol and phospholipids were visualized under UV light. Bands were scraped and extracted according to the of method of Arvidson (64). Phospholipids were stored in chloroform:methanol (2:1, v/v) at -20°C prior to separation by HPLC.

2.4.2 High Performance Liquid Chromatographic Analysis of Phospholipid Molecular Species

Separation of PC, PE and PDME molecular species was performed essentially as described by Patton *et al.* (123). Separations were performed on a 4.6 x 150 mm Pelosphere C18 cartridge column (Perkin-Elmer Corp., Norwalk, CT) using a Perkin-Elmer Series 4 solvent delivery system. Elution was monitored at 205 nm using a Perkin-Elmer Bio LC 90 spectrophotometer. PC, PE or PDME were applied to the reverse-phase column in 10-25 μl of absolute ethanol. Phospholipid molecular species were eluted in an isocratic mode using a solvent system of methanol:acetonitrile:water (90.5:2.5:7, v/v/v), containing 20 mM choline chloride, at a flow rate of 2 ml/min. Peaks corresponding to individual molecular species were collected using the peak cutting program on a Pharmacia Frac 100 fraction collector.

Individual peaks were pooled, dried under a stream of nitrogen and dissolved in 2 ml of chloroform:methanol (2:1, v/v). This was followed by extraction with 2 ml of 0.5% (w/v) NaCl and 2 ml of 0.5% (w/v) NaCl:methanol:chloroform (50:50:4, v/v/v). The organic phase was evaporated and dissolved in 2 ml of chloroform:methanol and stored at -20°C. Molecular species were identified by transmethylation of individual peaks with 14 % BF_3 in absolute methanol (Pierce Chemical Company, Rockford, IL). Fatty acid methyl esters were analyzed by gas-liquid chromatography on a 6' x 1/8" column of 100-120 mesh Supelcoport (Supelco Inc., Bellefonte, PA), coated with 5% diethyleneglycol succinate, using temperature programming.

2.5 SDS and Two-Dimensional Gel Electrophoresis

Purified PE *N*-methyltransferase and partially purified microsomal fractions were precipitated with 10% trichloroacetic acid in 0.15% Triton X-100. Precipitates were set on ice for 30 min and subsequently centrifuged at 10,000 rpm for 5 min in a bench top Eppendorf microcentrifuge. Precipitates were washed twice with one volume of acetone at -20°C, air dried and dissolved in a 2% SDS, 5% 2-mercaptoethanol, 20% glycerol and 0.25 M Tris HCl buffer (pH 6.8). Samples that did not require concentration were diluted 1:1 with this same SDS buffer. Electrophoresis was done in 10% acrylamide gels containing 0.1% SDS as described by Laemmli (263), or in 5-15% acrylamide gradient gels. The gradient gels were prepared in the following manner. A 5% acrylamide solution (w/v) was prepared by combining 2.8 ml of a 20% glycerol (v/v)-30% acrylamide-0.8% bis-acrylamide solution with 4.23 ml of 1.5 M Tris HCl (pH 8.8) and 9.8 ml of distilled water. The 15 % acrylamide solution (w/v) contained 8.5 ml of 60% glycerol (v/v)-30% acrylamide-0.8% bis-acrylamide solution, 4.23 ml of Tris HCl and 4.14 ml of distilled water. Both solutions were brought to 0.1% SDS (w/v) in a final volume of 17 ml. TEMED (6 µl) was added and the solutions

were degassed. Ammonium persulfate was added to a final concentration of 0.02% (w/v) and the solutions were placed in an acrylic gradient former. A linear gradient was poured over a 5 min period. The stacking gel solution consisted of 3% acrylamide (w/v), 0.125 M Tris HCl (pH 6.6) and 0.1% SDS (w/v), with ammonium persulfate and TEMED concentrations the same as the running gel. All SDS-PAGE was run at 25 mA constant current.

Purified PE *N*-methyltransferase was resolved in a two-dimensional electrophoresis system described by O'Farrell *et. al.* (264). Briefly, NEPHGE gels (first dimension) contained 9.2 M urea, 2% (w/v) Triton X-100 and 5% (v/v) pH 3-10 Ampholytes. PE *N*-methyltransferase was loaded at the anode and electrophoresed for 2000 V/h. The first dimensional gel was extruded, soaked in 60 mM Tris HCl (pH 6.8), 2-mercaptoethanol, 2% SDS and 10% glycerol for 10 minutes, applied to a 1% agarose cushion on a 5-15% gradient gel and electrophoresised at 25 mA. Gels were fixed in water:ethanol:acetic acid (50:40:10, v/v/v) and silver stained (279).

2.6 Purification of Microsomal Phosphatidylethanolamine and Phosphatidylcholine

Microsomes (400 mg of microsomal protein, 25 mg/ml) were mixed with 8 volumes of chloroform:methanol (1:1, v/v) and stirred at 20°C for 30 min. The mixture was filtered through a plug of glass wool, total lipids extracted by the method of Folch (265) and the organic phase flash evaporated. The lipids were dissolved in a small volume of chloroform and applied to preparative thin-layer plates (silica gel 60, 2.0 mm). Plates were developed in a solvent system of chloroform:methanol:water:acetic acid (50:30:2:5, v/v/v/v) and the PE and PC zones identified and eluted according to the method of Arvidson (64). Purified microsomal PE was stored in chloroform:methanol (2:1, v/v) under nitrogen at -20°C.

2.7 Protein and Phosphorus Assays

Protein was determined by the method of Lowry *et. al.* (266), modified to contain deoxycholate at a final concentration of 0.04% (w/v), or by a sensitive silver binding assay (267). Both protein assays used BSA as a standard. The very dilute protein concentrations in the PE *N*-methyltransferase purification scheme (particularly the final 3 steps) required the use of a sensitive protein assay to avoid using most of the pure sample to obtain accurate protein determinations. In regions of the purification scheme where protein concentrations could be determined accurately by both methods, the silver binding assay gave values that were 10-20% lower than Lowry determinations.

Lipid phosphorus was determined by the method of Rouser *et al.* (268). Sterile solutions of 1 mM KH_2PO_4 were used to generate standard curves.

2.8 Animal Maintenance and Hepatocyte Culturing

2.8.1 Animals and Diets

Male or female Wistar rats (150-175 gm) were maintained on standard Purina rat chow and water *ad libitum*. In the choline deficiency studies, male Sprague-Dawley rats (45-50 gm) were fed a diet consisting of 10% vitamin-free casein, 10% α protein, 56% sucrose, a 4% salt mixture and vitamins, but with no choline (ICN Biochemicals, Canada). Choline-supplemented rats had 0.4% (w/w) choline included in this ICN diet. In the molecular species studies, hepatocytes were isolated from the livers of female Wistar rats (150-175 gm).

New Zealand white rabbits were maintained on Purina rabbit chow (and an occasional lettuce leaf).

2.8.2 Preparation and Culturing of Primary Monolayers of Rat Hepatocytes

Rats were lightly anesthetized with diethylether followed by intraperitoneal injection of 5 mg pentobarbitone/100 g body weight. Hepatocytes were isolated as previously described (271,272), but with some modification (137). Briefly, livers were perfused through the portal vein with

40-50 ml of Hanks' solution (Ca^{2+} and Mg^{2+} free) supplemented with 20 mM glucose, 25 mM Hepes, 0.5 mM EGTA and 10 μg/ml insulin. This was followed by 50-60 ml of collagenase (6 mg/ml) dissolved in Hanks' buffer. The liver was excised and further digested in 10 ml of collagenase solution by gentle agitation at 37°C. The cell suspension was filtered, centrifuged (50 x *g* for 2 min) and resuspended in Dulbecco's MEM containing 17% fetal bovine serum. Cells were plated at a density of 3 x 10^6 cells/dish in 3 ml of Dulbecco's MEM containing 17% fetal bovine serum. Cells were allowed to plate for at least 5 hours at 37°C in a 5% CO_2 atmosphere prior to the start of experiments.

For experiments in which PC molecular species were probed, 30 min prior to the start of the experiment medium was exchanged for Dulbecco's MEM without serum or methionine. Experiments were initated by the addition of 15 μCi [*methyl*-^{3}H]methionine/dish. Following a 1 h incubation, the medium was replaced with Dulbecco's MEM containing 200 μM methionine, and cells and medium were sampled for a period up to 24 h. Phospholipid from cells and medium was extracted with chloroform:methanol (2:1, v/v), separated by TLC and the mol% and methyl label distribution of molecular species in PC was determined following fractionation by HPLC.

The choline-deficient rat model was developed by Zemin Yao, a graduate student in our laboratory. In our collaborative effort, Zemin was responsible for maintenance of choline-deficient rats and culturing of hepaotcytes. Enzyme assays and immunoblots were performed by myself. PE *N*-methyltransferase activity in choline- and methionine-deficient hepatocytes was probed in the following manner. Hepatocytes, isolated from the livers of choline-deficient rats, were plated for 6 h in choline- and methionine-free Dulbecco's MEM (containing 17% delipidated bovine fetal serum) and then supplemented with 200 μM methionine or 100 μM choline. At various times,

cells were rinsed with PBS (pH 7.4) and homogenized in 20 mM potassium phosphate (pH 7.9), 1 mM EDTA, 1 mM DTT and 250 mM sucrose by 40 strokes of a motor driven Potter-Elvehjem apparatus. A membrane fraction was prepared by centrifugation at 120,000 x *g* for 1 h and PE *N*-methyltransferase activity was assayed as in Section 2.3.1.

2.9 Immunochemistry

2.9.1 Immunoblotting

Proteins separated by SDS-PAGE were transferred to nitrocellulose according to the method of Tobin *et al.* (273), with several modifications. Briefly, 5-15% acrylamide gradient gels were soaked in transfer buffer (25 mM Tris HCl, 192 mM glycine, 20% methanol (v/v) and 0.03% SDS at pH 8.3) for 15 min. The transfer cassette was placed horizontally and a Scotch-Brite pad and filter paper (Whatman 4Chr), both soaked in transfer buffer, were placed on top. The SDS-gel was placed on the filter paper and any bubbles removed. Meantime, a nitrocellulose sheet was soaked in transfer buffer for 15 min. The surface of the SDS-gel was wetted with 2 ml of transfer buffer and the nitrocellulose placed on top. Contact was assured by gently smoothing the surface with a finger. Finally, filter paper and a Scotch-Brite pad were placed on the nitrocellulose, the cassette was closed, placed in a transfer chamber (Biorad Transblot Cell, Biorad, Richmond, CA) with the nitrocellulose facing the anode, immersed in transfer buffer and subjected to a field strength of 150 V (0.55 A) for 4 h.

2.9.2 Immunodetection of Phosphatidylethanolamine N-Methyltransferase on Nitrocellulose Membranes

Nitrocellulose blots were incubated in 25-50 ml of buffer B (150 mM NaCl, 5 mM EDTA, 50 mM Tris HCl, 0.05% (v/v) Nonidet P40 and 0.25% (w/v) gelatin at pH 7.4) containing 150 μg/ml of rabbit anti-PE *N*-methyltransferase IgG for 4 h at 20°C. The antibody solution was replaced with 100 ml of buffer B

and incubated for 1 h at 20°C. This wash solution was replaced with 20 ml buffer B containing 0.1 μCi/ml ^{125}I-protein A and incubated for 2 h at 37°C. The nitrocellulose was then washed with buffer C (1 M NaCl, 5 mM EDTA, 50 mM Tris HCl, 0.25% (w/v) gelatin and 0.4% (w/v) sodium lauryl sarcosine at pH 7.4) for 2 h at 37°C. Finally, the blot was rinsed in distilled water, dried and exposed to Kodak XAR-5 film at -70°C. Film cassettes were equiped with intensifying screens.

2.9.3 Production of Anti-Phosphatidylethanolamine N-Methyltransferase IgG.

PE *N*-methyltransferase antibodies were raised in female New Zealand white rabbits (1.5 kg) using TCA-precipitated enzyme or native enzyme. The TCA-precipitated enzyme was prepared by bringing unconcentrated purified enzyme solutions to 10% (w/v) TCA, stirring at 4°C for 1 h and centrifuging at 12,000 x *g* for 10 min. Supernatant was decanted and the pellet washed twice with 20 ml of -20°C acetone and each time centrifuged at 12,000 x *g* for 10 min. Residues were dried under nitrogen and dissolved in 1 ml of 20 mM Tris HCl (pH 7.0), 150 mM NaCl and 0.5% SDS. Native antigen was prepared by concentrating enzyme against solid polyetheneglycol 8000 to a volume of 1-2 ml and dialyzing versus 20 mM potassium phosphate (pH 7.4), 150 mM NaCl, and 0.1 mM DTT for 24 h. Both antigens (75-100 μg) were emulsified in equal volumes of Freund's complete adjuvent and injected subcutaneously at 5-6 sites on the rabbit's neck and back. After 4 weeks, a booster of 25 μg PE *N*-methyltransferase in Fruend's incomplete adjuvent was given in a manner identical to primary injections. After 2 weeks, a blood sample was drawn from an ear vein and the presence of PE *N*-methyltransferase antibody was tested by immunoblotting. When titre was detected, the rabbit was bled by cardiac puncture, blood clotted at room temperature for 2 h and serum separated by centrifugation for 10 min at

12,000 x *g*. Antibodies against the native and denatured enzyme could be used to detect enzyme protein on nitrocellulose membranes. Unfortunately, neither of the antibodies would immunoprecipitate enzyme activity or the 18.3 kDal protein under a variety of detergent and buffer conditions. Anti-PE *N*-methyltransferase raised against the denatured enzyme was used in all experiments described herein.

2.9.4 Purification of Rabbit Plasma IgG Fraction.

The IgG fraction of rabbit serum was purified by the method of Mayer and Walker (274). Briefly, serum was adjusted to 50% saturation with $(NH_4)_2SO_4$, centrifuged at 10,000 x *g* for 10 min and the pellet washed twice with 1.75 M $(NH_4)_2SO_4$. The pellet was redissolved in 10 mM potassium phosphate, 1 mM EDTA, 1 mM NaN_3 (pH 8.0) and dialyzed against the same buffer (2 litres) for 24-34 h. The dialyzed sample was applied to a 1.6 x 20 cm column of DEAE Sepharose CL-4B, equilibrated in the aforementioned phosphate buffer, and unbound fractions were collected, pooled and stored at -70°C.

2.10 Phosphorylation of Phosphatidylethanolamine *N*-Methyltransferase.

2.10.1 Enzyme Phosphorylation In Vitro

Phosphorylation of pure PE *N*-methyltransferase was tested using the catalytic subunit of bovine heart cAMP-dependent protein kinase. Phosphorylation was done in 1.5 ml plastic Eppendorf tubes using a 50 mM potassium phosphate (pH 7.0), 10 mM $MgCl_2$ and 10 mM DTT buffer system. Following the addition of these components, 1.5 µg of cAMP-dependent kinase, 0.1 mM ^{32}P-ATP (200-500 cpm/pmol) and pure PE *N*-methyltransferase (100-500 ng) were added. The final volume of the cocktail was 150 µl. Incubations were at 30°C for the times indicated in the pertinent Figures and Tables. Reactions were terminated by the addition of 150 µl of 20% (w/v) TCA, set on ice

for 20 min and centrifuged in an Eppendorf microcentrifuge (14,000 rpm for 10 min). Supernatant was removed, the pellet was washed once with ice cold acetone and dried at room temperature for 20 min. The phosphorylated enzyme was then subjected to SDS-PAGE, stained with Coomassie Blue R and exposed to Kodak XAR-5 film at -70°C. If the amount of phosphate incorporated was to be determined, the PE *N*-methyltransferase band was excised from the dried gel and subjected to liquid scintillation counting.

2.10.2 Analysis of Phosphoamino Acids by Thin-Layer Electrophoresis.

Phosphorylated amino acids of PE *N*-methyltransferse were identified by acid hydrolysis and TLE (275). Phosphorylated and TCA-precipitated PE *N*-methyltransferase (Section 2.10.1) was washed successively with 1 ml ice cold 20% (w/v) TCA, 5% TCA and ether:acetone (1:1, v/v). The sample was dissolved in 0.2 ml 6 N HCl, transferred to a glass screw cap vial with a teflon cap liner, flushed with nitrogen and heated at 110°C for 2 h. Following hydrolysis, the HCl was removed under a stream of nitrogen and the residues were dissolved in 10 µl of TLE electrode buffer, the composition of which was acetic acid:88% formic acid:distilled water (78:25:897, v/v/v). Phosphoserine, phosphothreonine and phosphotyrosine standards (16 µg) were added to the sample and it was spotted on a cellulose thin-layer plate. The plate was lightly sprayed with electrode buffer, placed horizontally in an electrophoresis chamber (Shandon 600 X 100 model, Cheshire, UK), Cambrelle wicks placed at both ends and a clean glass plate placed on top to prevent evaporation and ensure good contact between the TLE plate and wicks. The samples were subjected to electrophoresis toward the anode for 3 h at 0.5 kV. When electrophoresis was complete, the plate was air dried at room temperature for 30 min, sprayed with 0.2% (w/v) ninhydrin in 95% ethanol and developed in a 160°C oven. The plate was exposed to Kodak XAR-5 film at -70°C and the

mobility of the ^{32}P-labeled amino acids compared to the ninhydrin stained standards. Phosphorylated and hydrolyzed histone was also run as a phosphoserine standard.

2.10.3 Effect of Phosphorylation on Enzyme Activity In Vitro.

Phosphorylation was performed as in Section 2.10.1, except unlabeled ATP was used. At various time points during phosphorylation a sample was removed from the phosphorylation cocktail and assayed for PE- or PMME-dependent activities of the purified enzyme as described in Section 2.3.1.

2.11 Photolabeling of Phosphatidylethanolamine *N*-Methyltransferase in Microsomes

Before purification of PE *N*-methyltransferase was achieved, attempts at identifying the enzyme in microsomes by photoaffinity labeling were made. Azido-derivatives of AdoMet are expensive or difficult to synthesize. Fortunately, AdoMet is photosensitive and has been used to photolabel cytosolic methyltransferases (277,278). AdoMet was utilized to photolabel methyltransferases in microsomes in the following manner. Microsomes or microsomal membranes (100-200 μg) were suspended in 0.15 ml of 50 mM Tris-HCl (pH 9.5), 5 mM DTT, 4 μCi (0.35 nmol) of [*methyl*-^{3}H]AdoMet and irradiated for 20 min at 4°C with 254 nm UV light (hand-held Spectroline model ENF-24 lamp, Spectronics Corp., Westbury, NY) at a pathlength of 1 cm. Irradiation was performed in 0.5 ml Falcon microtitre wells. Following irradiation, 50 μl of 4% SDS disruption buffer (refer to Section 2.5) was added to the cocktail and the samples were subjected to SDS-PAGE (samples were not boiled), fixed in ethanol:water:acetic acid (40:50:10, v/v/v), stained with 0.04% (w/v) Coomassie Blue R in 10% (v/v) acetic acid and impregnated with fluor (En^{3}Hance, DuPont, Boston, MA). After drying, the gel was exposed to Kodak XAR-5 film at -70°C for 7-14 days.

2.12 *N*-Terminal Amino Acid Sequence Analysis of PE *N*-Methyltransferase

N-terminal amino acid sequencing was performed by the Northern Alberta Peptide Institute, University of Alberta, for a cost covering the use of reagents. Purified enzyme was precipitated with TCA as described in Section 2.10.1 and subsequently dissolved in 0.01% (w/v) DTT and 0.2% (w/v) SDS. This material was used directly for amino acid sequence analysis.

2.13 Data Analysis

Assays of PE *N*-methyltransferase activity were performed in duplicate and expressed as an average of the two values. Linear regression analysis (performed on a Macintosh Plus computer using the Cricket graph program) was used to determine the best fit of lines through data points on all Hill and double reciprocal plots as well as for replots of kinetic data. In cases where double reciprocal plots were nonlinear, curves were drawn by hand.

Values in tables were expressed as the average of the indicated number of determinations with either the range or standard deviation given. Where appropriate, significance of difference between the means of two sets of data was determined by the students T-test

RESULTS

3.1 PE *N*-Methyltransferase Purification and Molecular Properties

3.1.1 Purification

Table 3 shows a typical purification of PE *N*-methyltransferase from a crude microsomal fraction of rat liver. Activities for methylation of PE, PMME and PDME copurified, but not to the same degree. The activities for the methylation of PMME and PDME purified to the same degree to the phosphocellulose step, after which the PMME-dependent activity showed a substantially higher fold purification (1542- versus 832-fold). Microsomal PE-dependent methylation activity was found to purify 429-fold. These results can be rationalized by considering that the individual lipid substrates are also providing the enzyme with an unique phospholipid environment in each case. Differences in phospholipid head group and fatty acid composition would affect the properties of the mixed micelle substrate, thereby influencing PE *N*-methyltransferase activity. Unlike previous purification schemes that relied on endogenous PE in order to assay PE *N*-methyltransferase (114), it was found that PE *N*-methyltransferase activity was completely dependent on exogenous substrate following the phosphocellulose step. This is consistent with the observation that all measurable lipid phosphorus was removed following this step (Table 3).

The hydrophobic character of PE *N*-methyltransferase is illustrated by the high Triton X-100 concentration necessary to release it from microsomal membranes and by its high affinity for octyl Sepharose CL-4B (Fig. 4). This step offered the most substantial purification (100-fold) and was critical with regard to final enzyme purity. As illustrated in Fig. 4, PE *N*-methyltransferase elutes in a broad peak following single step elution with

TABLE 3. PURIFICATION OF PHOSPHATIDYLETHANOLAMINE *N*-METHYLTRANSFERASE

Fraction	Volume (ml)	Protein (mg)	Lipid phosphorus (μmol)	Specific activities[1]				Total Activity[2,3]	% Recovery[3]	Fold Purification
				NA[4]	PE	PMME	PDME			
Microsomes	35	1227.8	742	1.05	1.47	5.57	4.51	6838	100	—
Microsomal Membranes	41	743.7	623	1.49	1.73	8.96	5.29	6663	97	1.6
Soluble Membranes	180	478.8	577	0.25	0.44	6.18	7.55	2959	43	1.1
DE52 Cellulose	206	350.2	585	0.40	0.64	12.8	10.8	4486	65	2.3
P-11 Cellulose	183	40.3	N.D.[5]	N.D.	5.96	34.25	44.5	1380	20	6.1
Octyl Sepharose CL-4B	140	0.25	N.D.	N.D.	156	3560	1575	871	12	639
PBE 94	138	0.041	N.D.	N.D.	631	8590	3750	352	5	1540

[1]Specific activities expressed as nanomoles of methyl groups transferred/minute/mg of protein.

[2]Expressed as nmol/minute.

[3]Total activity, recovery and fold purification were calculated for PMME-dependent methylation.

[4]No addition of exogenous lipid substrates.

[5]Not detected

0.15% Triton X-100. The gradient was necessary to keep the volume of the pooled fractions to a minimum.

3.1.2 Analysis of Methylated Products

The distribution of methylated products at each step of the purification and for all three lipid substrates is shown in Table 4. As previously reported (88), PMME and PDME methylation resulted in the formation of predominately PDME and PC, respectively. Methylation of these two substrates using the Triton X-100 mixed micelle assay showed the same trend with 95-99% of the radioactivity found in these same two products. This trend was observed throughout the entire purification. PC (the major product of PE methylation) showed a 10-15% increase in content, with a concomitant decrease in PMME and PDME, as the enzyme was purified.

Methylation of PE, PMME and PDME was found to be linear for up to 30 min (Fig. 5). As previously reported (88), the major product of PDME methylation is PC. When PMME methylation was examined, the major product formed was PDME (Fig. 6). However, with increasing time there was a proportional increase in PC counts and decrease in PDME counts. This result would indicate that as the proportion of PDME in the mixed micelle increases it can compete effectively for methylation with PMME. Methylation of PE resulted in the linear accumulation of PC for 40 min (Fig. 6). PMME and PDME reached steady state levels in 2 to 5 min. As expected, PDME methylation (Fig. 6) produced PC linearly for 30 min. The linearity of the methylation reactions is a good indication that the rate of exchange of phospholipid substrate between micelles is not rate-limiting.

3.1.3 pH Optima for Methylation.

As illustrated in Fig. 7, all three methylation activities of the purified enzyme have a pH optimum at 10. The alkaline pH optima of the three

Table 4. DISTRIBUTION OF METHYLATED PHOSPHOLIPIDS DURING PE *N*-METHYLTRANSFERASE PURIFICATION

Distribution of the individial phospholipids are expressed as the percentage of the total counts in PC, PMME and PDME. The recovery of applied radioactivity was 60-70% with > 95% of the recovered counts in the three methylated products. Results are expressed as the average ± S.D for 3 determinations unless indicated by numbers in parentheses.

Fraction	Substrate	% Product distribution		
		PC	PDME	PMME
Microsomes	NA[1]	77.1±17.7	10.5±3.4	12.5±14.9
	PE	76.7±14.8	11.7±3.9	11.5±12.3
	PMME	3.2±1.8	95.7±2.3	1.1±0.5
	PDME	96.7±1.6	2.0±0.9	1.4±0.6
Membranes	NA	75.1±25.2	12.3±8.3	12.5±17.2
	PE	81.3±14.8	10.3±3.8	8.4±11.1
	PMME	6.8±2.4	92.8±2.6	0.5±0.2
	PDME	95.5±1.6	3.5±0.8	1.0±0.8
Soluble Membranes	NA	46.6(2)	32.4(2)	21.1(2)
	PE	82.0±9.7	9.1±4.1	8.8±6.7
	PMME	1.9±1.2	96.9±1.6	0.5±0.6
	PDME	98.1±1.0	1.6±0.6	0.3±0
DE 52 Cellulose	NA	39.6(2)	32.4(2)	21.1(2)
	PE	71.8±15.9	20.6±16.3	7.6±4.8
	PMME	3.0±1.7	96.5±2.0	0.2±0.3
	PDME	97.5±1.1	2.3±0.9	1.2±1.3
P-11 Cellulose	PE	78.1±4.7	16.5±2.5	5.4±3.4
	PMME	2.9±1.6	97.7±2.1	0.5±0.3
	PDME	98.8±0.8	1.1±0.9	0.2±0.1
Octyl Sepharose CL-4B	PE	81.2±12.9	14.7±8.3	3.8±4.8
	PMME	3.9±4.1	96.1±4.4	0.6±0.4
	PDME	99.0±0.4	0.6±0.4	0.4±0.2
PBE 94	PE	92.3±6.7	6.6±5.9	1.1±0.3
	PMME	2.5±1.1	96.5±1.2	1.0±0.8
	PDME	95.8±4.3	2.5±1.7	1.7±2.0

[1] no addition

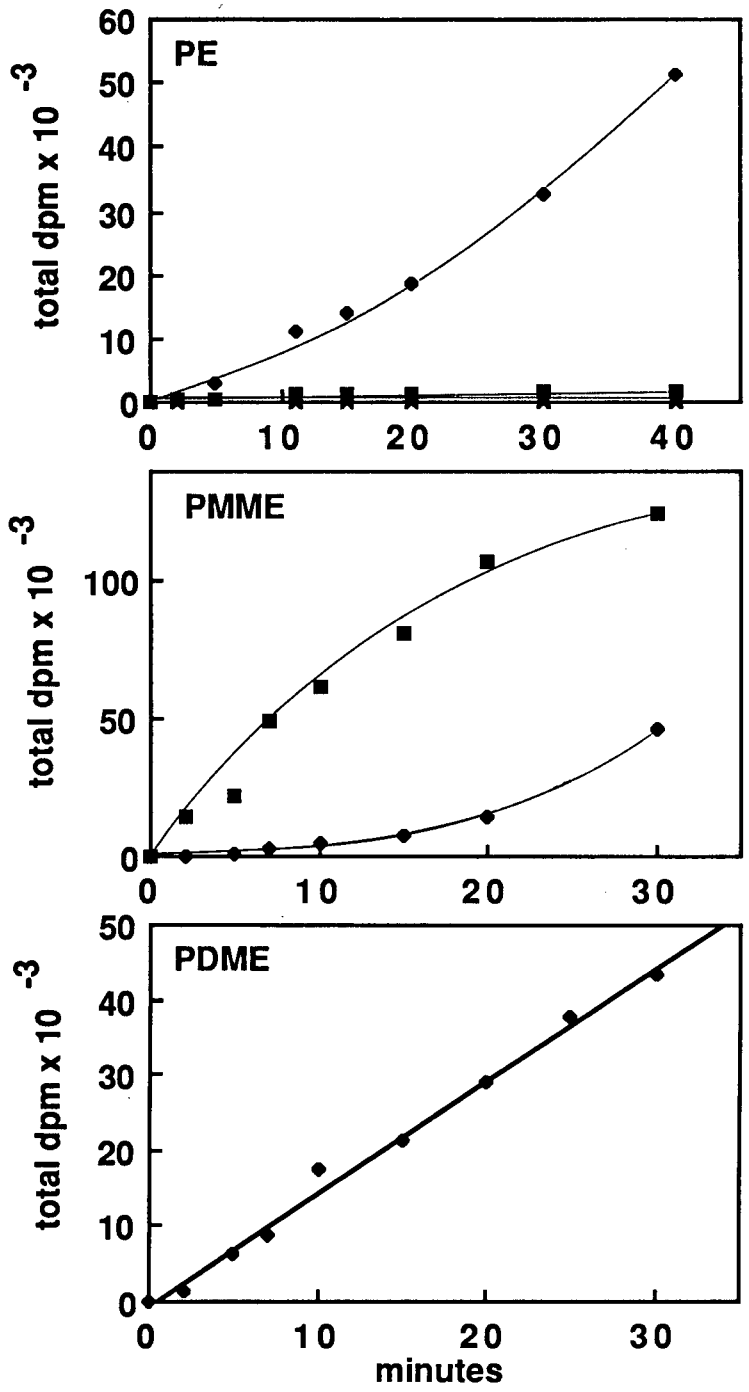

Figure 6. **Analysis of products formed during the time course of PE, PMME and PDME methylation.** Following methylation, radioactive PC (◆), PMME (✖) and PDME (■) were separated by TLC, counted and expressed as total dpm recovered. 65-70% of the applied counts were recovered for all time points. Purified PE *N*-methyltransferase was used in all experiments.

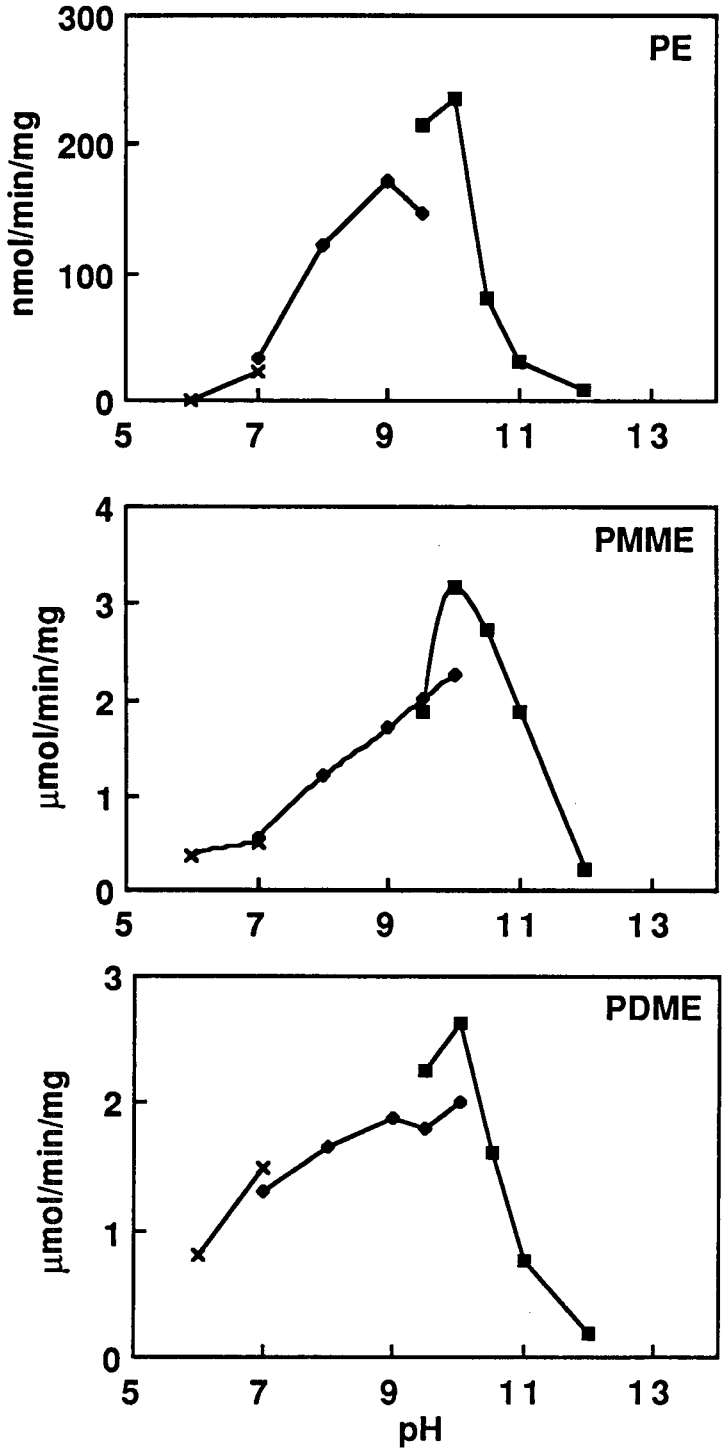

Figure 7. **pH curves for the methylation of PE, PMME and PDME by purified PE *N*-methyltransferase.** PE *N*-methyltransferase was assayed as described in Section 2.3.1. Assays contained 125 mM potassium phosphate (✖), Tris HCl (◆) or glycine KOH (■).

methylation activities probably reflects the pKa of the substrate's amino group. It is feasible that protonation of the substrate's amino group results in poor binding to the active site of PE *N*-methyltransferase. These pH optima are similar to that reported for microsomes (84,87,88).

3.1.4 Molecular Mass Determination by SDS-PAGE

Analysis of the purified enzyme by SDS-PAGE in 10% acrylamide gels indicated that PE *N*-methyltransferase was composed of a single 18.3± 0.7 (n=3) kDal subunit (Fig. 8, lane 1). It was apparent that during the purification of PE *N*-methyltransferase from microsomes there was an increase in the amount of a 18.3 kDal protein. The 50 kDal protein previously thought to be PE *N*-methyltransferase (114,115,159,162) steadily decreased in content (lane 7 to 2) and was absent from the pure enzyme.

3.1.5 Molecular Mass Determination by Gel Filtration.

Molecular mass analysis of the native enzyme in Triton X-100, by gel filtration on Sephacryl S-300, showed that PE-, PMME- and PDME-dependent activities co-chromatographed with a Stokes radius of 55.2 Å (n=2, Fig. 9A). Pure Triton X-100 micelles were found to have a Stokes radius of 53.1 Å when chromatographed on the same column. The apparent molecular mass difference between PE *N*-methyltransferase and pure micelles was determined to be 24.7 kDal. These results indicated that there was a single subunit per Triton micelle. Indeed, attempts to cross link the enzyme subunits with dimethylsuberimidate were negative. Analysis of the elution profile of the 18.3 kDal protein on Sephacryl S-300 is shown in Fig. 9B. SDS-PAGE and silver staining of concentrated column fractions revealed that the putative methyltransferase protein (insert) co-chromatographed with all three methylation activities. This is strong evidence to suggest that the 18.3 kDal

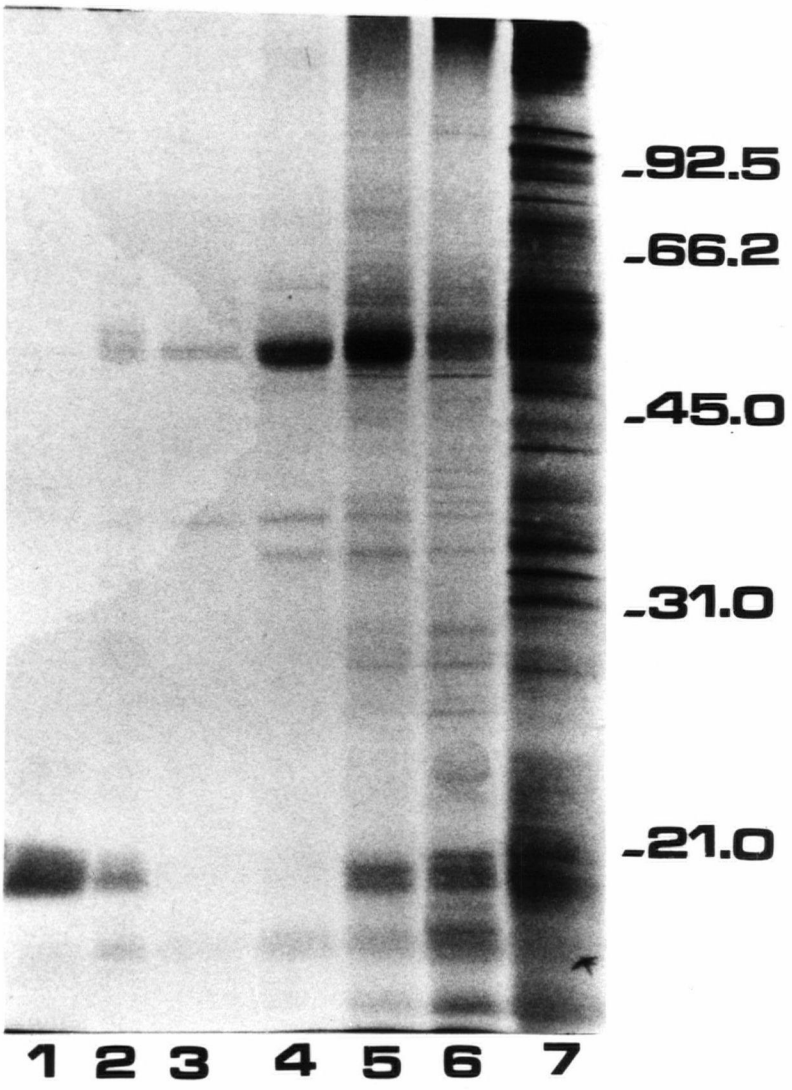

Figure 8. **Electrophoresis of partially purified and purified PE *N*-methyltransferase.** 1 μg of purified PE *N*-methyltransferase (lane 1) was subjected to SDS-PAGE in a 10% acrylamide gel and silver stained. Lanes 2-7 are protein profiles of the various steps from the purification scheme. Lane 2, octyl Sepharose (1 μg); lane 3, P-11 cellulose (1 μg); lane 4, DE 52 cellulose (25 μg); lane 5, soluble microsomal membranes (25 μg); lane 6, microsomal membranes (25 μg); lane 7, microsomes (50 μg).

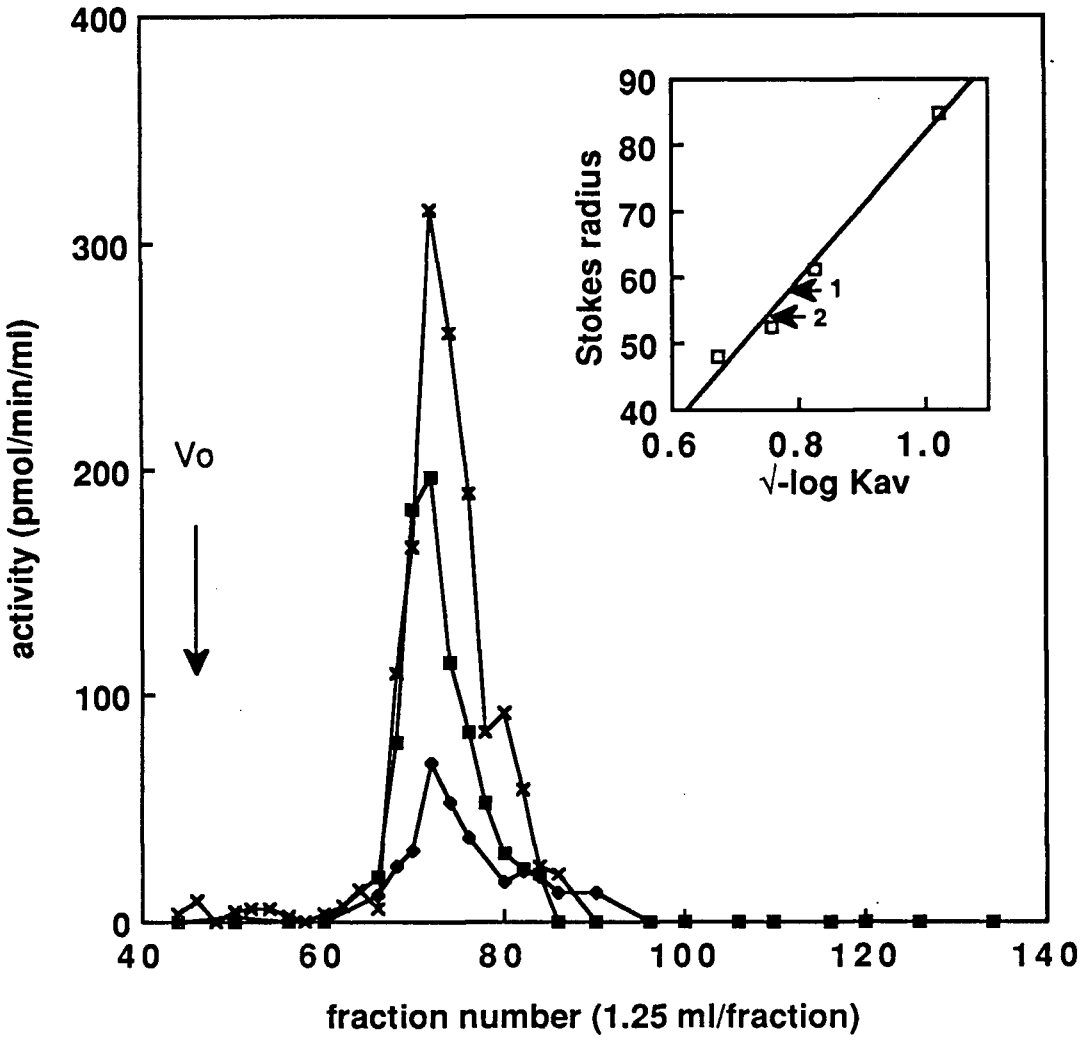

Figure 9A. **Sephacryl S-300 chromatography of purified PE *N*-methyltransferase.** A 96 x 1.6 cm column of Sephacryl S-300 was equilibrated in buffer A containing 0.1% Triton X-100. Panel A, 3.68 µg of PE *N*-methyltransferase (1.5 ml) was chromatographed at a flow rate of 16 ml/h. Fractions were assayed for PE-(◆), PMME-(✖) and PDME-(■) dependent activities as described in Section 2.3.1, except that incubation times were 20 min. Triton X-100 micelle size was determined by equilibrating the column in 0.35 mM Triton X-100 and chromatographing 1 ml of 8 mM Triton X-100 at a flow rate of 16 ml/h. The elution position of the Triton X-100 micelles was determined by absorbance at 275 nm. The column was calibrated using aldolase (158 kDa), catalase (232 kDa), ferritin (440 kDa) and thyroglobulin (669 kDa). The void volume was determined using Dextran Blue 2000. A plot of Stokes radii versus √-log Kav is shown in the insert. Arrows 1 and 2 show the positions of PE *N*-methyltransferase and pure Triton X-100 micelles, respectively.

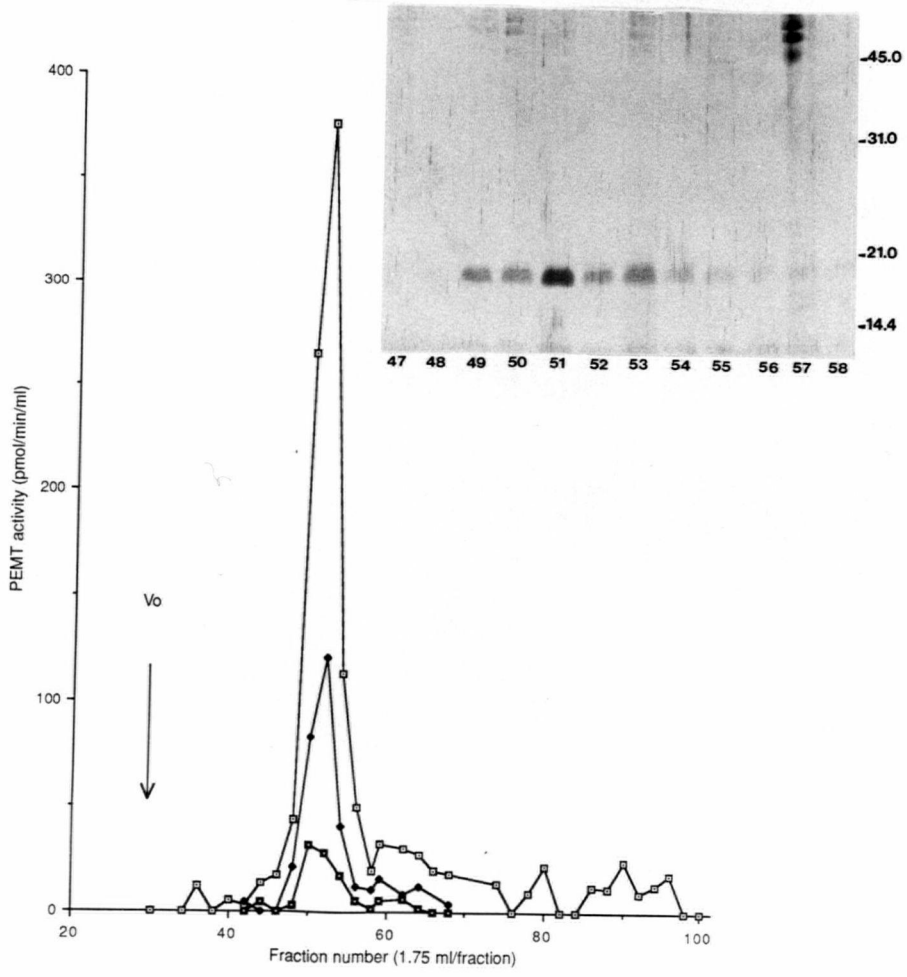

Figure 9B. 3.68 µg of enzyme was chromatographed at a flow rate of 16 ml/h and fractions were assayed for PE-(■), PMME-(❑) and PDME-(◆) dependent methyltransferase activity. Fractions around the activity peak were precipitated with 10% TCA and analyzed by SDS-PAGE in a 10% acrylamide gel (insert).

protein is indeed PE *N*-methyltransferase and that a single enzyme performs all three methylation reactions.

3.1.6 Two-Dimensional Gel Analysis

During purification it became apparent that PE *N*-methyltransferase possessed an extremely basic pI. This observation was corroborated by the finding that PE *N*-methyltransferase would not enter conventional isoelectric focusing gels (pH 3-10). A two dimensional electrophoresis system, employing NEPHGE (the method of choice for resolving basic proteins) in the first dimension and SDS-PAGE in the second, revealed that PE *N*-methyltransferase is composed of two (or possibly three) isoproteins (Fig. 10). The reason for the microheterogeneity is unknown.

3.1.7 Immunoblotting of Microsomes and Purified Enzyme

A rabbit polyclonal antibody was raised against the purified enzyme and used to analyze microsomes by immunoblotting. The concern was whether the purified enzyme was a proteolysis product and not the native microsomal enzyme. This concern proved to be unfounded since, as shown in Fig. 11, both the purified and microsomal enzyme were of identical molecular mass.

3.2. Kinetics of Phosphatidylethanolamine *N*-Methyltransferase.

3.2.1 Analysis of Micellar Substrates

We have developed a simple Triton X-100 mixed micelle assay to determine individually PE-, PMME- and PDME-dependent PE *N*-methyltransferase activities. The purified enzyme was found to be maximally active when assayed in the presence of 0.5 mM Triton X-100 and 2.0 mM (88 mol%) PE or 0.25 mM (49 mol%) PMME and 0.4 mM (61 mol%) PDME in 1.0 mM Triton X-100. The mol% of Triton X-100 at which maximum PMME and PDME methylation occurred is in the region where a homogenous population of micelles exists (279). The 88 mol% of PE required for maximal activity is well

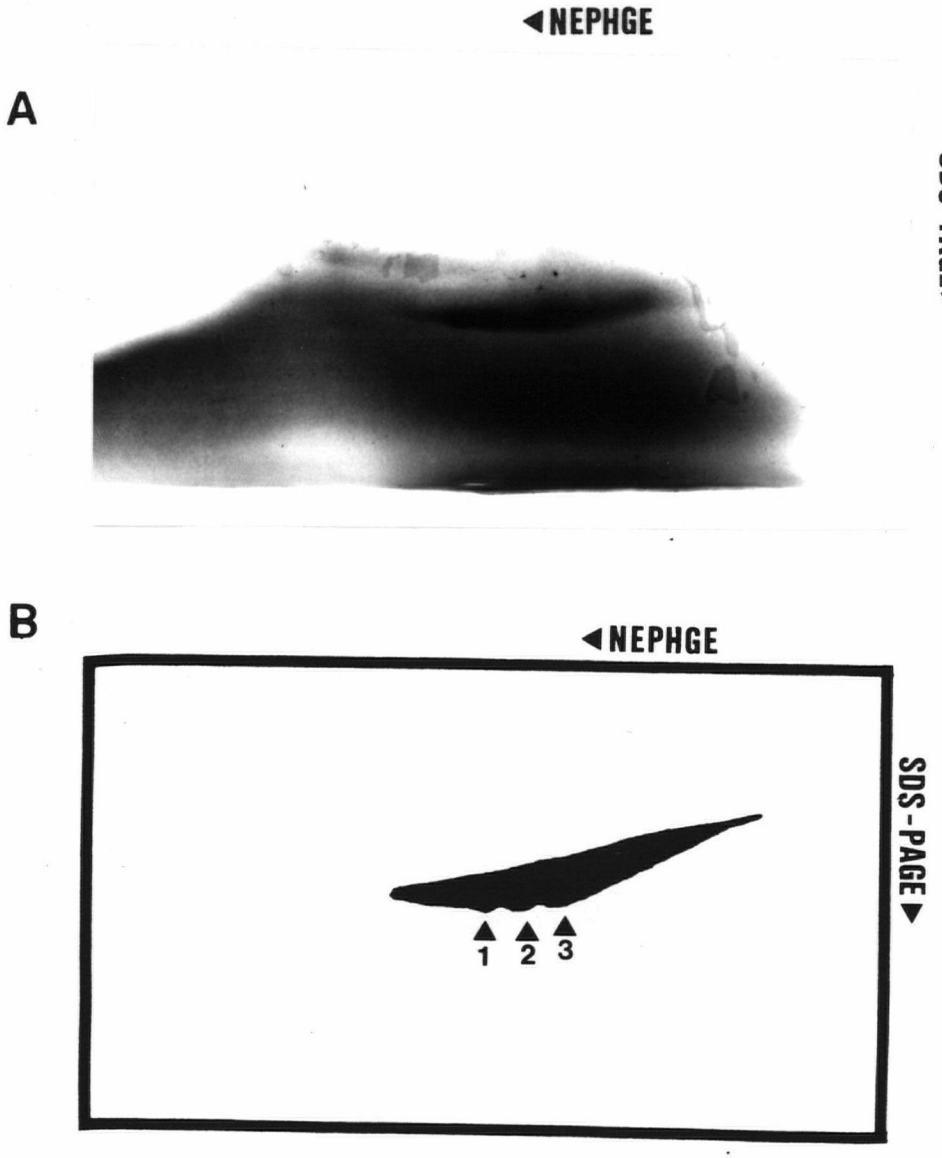

Figure 10. **Two-dimensional gel electrophoresis of PE *N*-methyltransferase.** A, two μg of purified enzyme was resolved in a NEPHGE system containing pH 3-10 ampholytes, followed by SDS-PAGE in the second dimension. The gel was fixed in 30% (v/v) methanol/10% (w/v) trichloroacetic acid/3.5% (w/v) sulfosalicyclic acid for 2 h prior to silver staining. B, a schematic drawing of the gel in A with background removed for clarity. The 3 isoforms are shown.

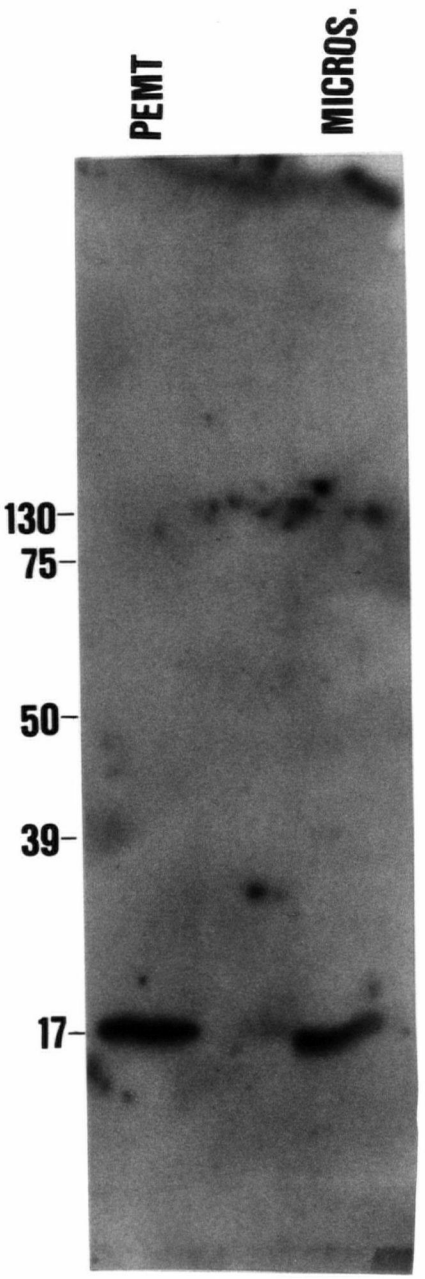

Figure 11. **Immunoblot of PE *N*-methyltransferase in microsomes.** 200 μg of microsomes and 150 ng purified enzyme were separated by 5-15% SDS-PAGE, transferred to nitrocellulose and probed with an anti-PE *N*-methyltransferase antibody. The autoradiogram shown was exposed for 7 h.

above 68 mol% (Triton X-100 mole fraction of 0.32) sphingomyelin, which is the effective limit for a monodisperse population of mixed micelles (279). Also, freeze fracture analysis of 91 mol% PE revealed the presence of large multilamellar vesicles (280).

A study was made of the structure of PE, PMME and PDME micellar substrates by gel filtration on a 1.6 x 96 cm column of Sephacryl S-300 equilibrated in 125 mM Tris HCl (pH 9.2), 5 mM DTT, 0.3 mM Triton X-100 and 3.3 mM potassium phosphate. Phospholipid substrates were prepared as if for an enzyme assay, but 0.75 ml of the mixture was instead loaded on the gel filtration column. Separations were performed at a flow rate of 20 ml/hr at room temperature. It is evident from the elution profiles shown in Fig. 12 that the 88 mol% PE mixture eluted at the void volume and thus consists of large (>1.5 x 10^6 dal) multilamellar structures. On the other hand, 49 mol% PMME and 61 mol% PDME mixed micelles are retained on the column and elute with Stokes radii of 52 and 63 Å, respectively. Pure Triton X-100 micelles had a Stokes radius of 55 Å. The similarity of PMME and PDME mixed micelle Stokes radii to micelles of pure Triton X-100 indicated that these two PE *N*-methyltransferase substrates are indeed homogenous and monodisperse. Methylation of PE is maximal in large non-micellar aggregates, the structure of which is presently undefined.

3.2.2 Methylation Adheres to the 'Surface Dilution' Model

The role of Triton X-100 in the mixed micelle assay was investigated to determine if PE *N*-methyltransferase had kinetic properties similar to those described for other membrane bound enzymes. Fig. 13 illustrates that all three methylation activities are subject to 'surface dilution' inhibition, a result often seen for enzymes that act on mixed micelle substrates (281,282). All three methylation activities showed a definite peak of activity between 0.5 and 2.5

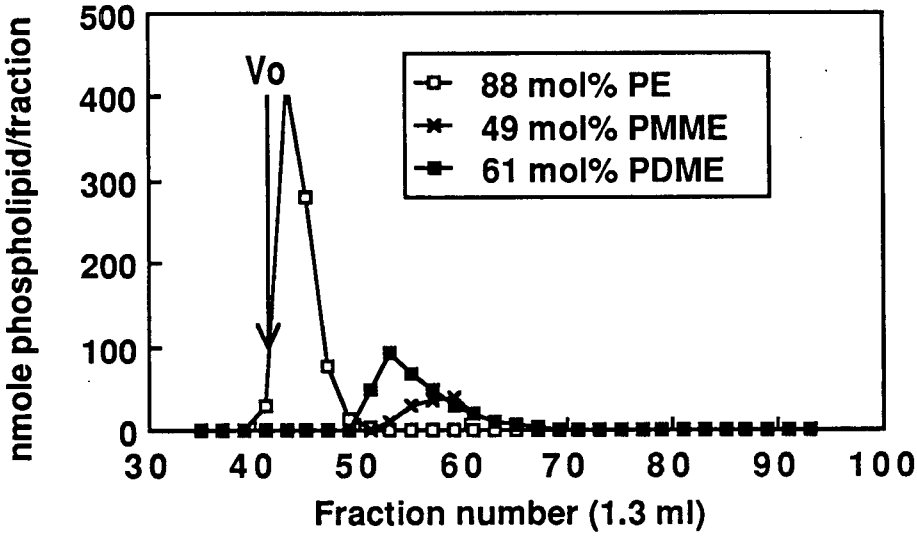

Figure 12. **Analysis of micellar substrates by gel filtration.** PE (88 mol%), PMME (49 mol%) and PDME (61 mol%) were separated by gel filtration as described in Section 3.2.1. Fractions were analyzed for lipid phosphorous following extraction by the method of Folch *et al.* (265).

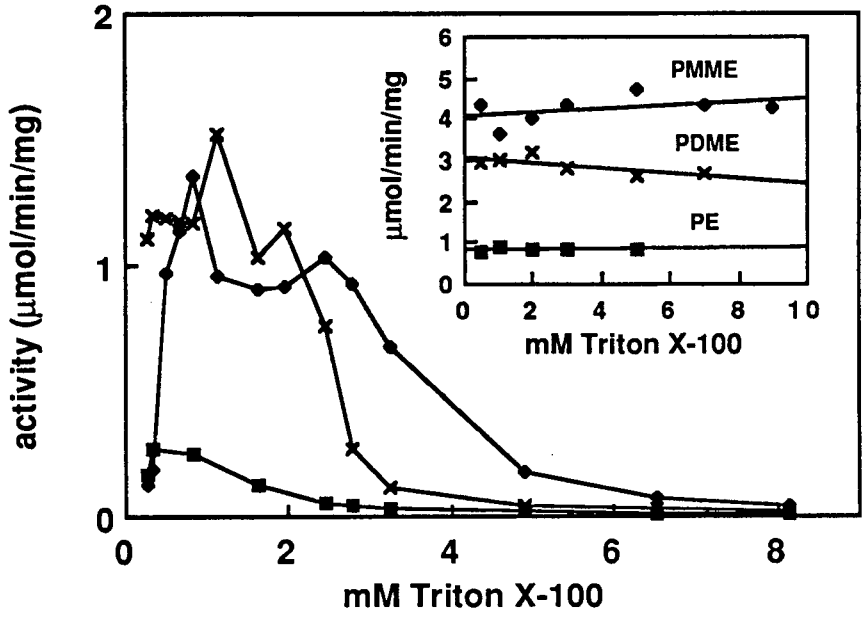

Figure 13. **Surface dilution of PE, PMME and PDME methylation activities.** Assay conditions were as described in Section 2.3.1, except 2 mM PE (■), 0.5 mM PMME (◆) and 0.5 mM PDME (✖) were assayed at various Triton X-100 concentrations. The insert shows PE-, PMME- and PDME-dependent activities assayed at 88, 49 and 61 mol%, respectively, over a range of Triton X-100 concentrations.

mM Triton X-100. However, PE and PDME methylation activities were not inhibited as much as PMME methylation by low surfactant concentrations. The inhibition of PE, PMME and PDME methylation was not the result of increasing micelle concentration (insert). Optimal enzyme activity was essentially linear over a range of Triton X-100 concentrations and at fixed PE, PMME and PDME mol%s of 88, 49 and 61, respectively. These results are taken to suggest that Triton X-100 reversibly inhibits activity by diluting phospholipid substrate in the mixed micellar phase.

3.2.3 Effects of Mixed Micelle Composition and Concentration

The enzymology of catalysis in mixed micelle substrates has some notable deviations from classical Michaelis-Menten kinetics. Instead of catalysis in the aqueous phase, enzymes that act on mixed micellar substrates catalyze reactions at the interface of a large detergent-amphiphile particle. In order to analyze these systems the concentration of micellar substrate and lipid concentration in the micelle must be varied independently of one another. This can be achieved in three ways: (i) micelle concentration, at fixed mol% phospholipid substrate, was varied, (ii) bulk substrate concentration was fixed and Triton X-100 concentration was varied, and (iii) Triton X-100 concentration was fixed and the bulk lipid varied. It was shown in Fig. 13 that enzyme activity was independent of micelle concentration up to 0.5 mM Triton X-100. In Fig. 14, this observation was extended to include concentrations of Triton X-100 close to its CMC of 0.24 mM (110) and at various fixed mol% of PMME (similar results were obtained for PE and PDME). Here it is evident that no minimum activity is displayed even at the CMC of Triton X-100, and thus no saturation curves related to micelle concentration can be constructed in the range of surfactant concentrations where micelles exist. This is contrary to the kinetic model for *N. naja naja* venom phospholipase A_2

(283,284) or *E. coli* phosphatidyserine decarboxylase (285) for which true saturable binding to micelles occurs. Fig. 15 shows a substrate velocity curve and double reciprocal plot (insert) for fixed PMME and increasing Triton X-100 concentration. In this case the micelle concentration is constant but the surface concentration of PDME is varied. A sigmoidal substrate velocity curve and resultant parabolic inverse plot again indicate *apparent* cooperativity for lipid substrate methylation. Identical results were obtained for PE and PMME. For situation (iii), double reciprocal plots of initial velocity versus lipid substrate concentration (at fixed Triton X-100) were found to be non-linear and highly cooperative. Thus, for situations (ii) and (iii) similar inital velocity responses were obtained. Plots of PE-dependent activity at 0.5 mM Triton X-100 (Fig. 16) gave a Hill number of 3.7. Similar behaviour was noted for PMME and PDME. In the case of PMME, there was a marked increase in the Hill number with higher concentrations of detergent (Fig. 17). Hill numbers for 0.5, 1.0 and 2.0 mM Triton X-100 were 3.1, 4.7 and 6.4, respectively. Similarly, Hill numbers of 2.5, 2.8, 6.1 and 12.8 were determined for PDME methylation at 0.5, 1.0, 2.0 and 3.0 mM Triton X-100, respectively (Fig. 18). This type of cooperative kinetic behaviour has been described (283,284) using phospholipase A_2 and been attributed to changes in the size and shape of the mixed micelle as the concentration of phospholipid in the mixed micelle increases. Another plausible theory is that integral membrane proteins (like PE *N*-methyltransferase) have boundary layers of lipid that must be filled to achieve full catalytic activity. The nonlinear inital velocity curves may be the result of partial filling of this domain.

3.2.4 Effect of Phosphatidylcholine on Initial Velocities

Curve fitting of data from Figs. 15-18 is complex (286). The Hill equation and coefficients were formulated under the assumption of infinite

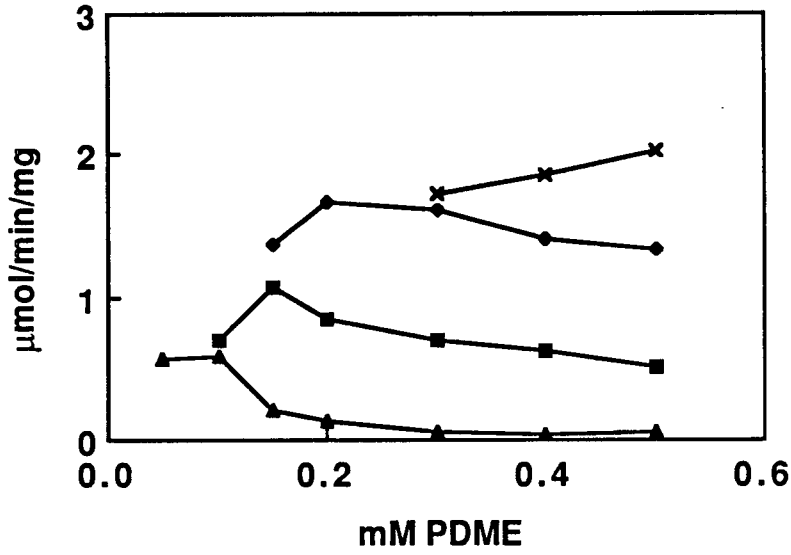

Figure 14. **Lack of influence of micelle concentration on PDME methylation.** Methyltransferase activity was assayed at increasing micelle concentrations and at fixed PDME/Triton X-100 ratios of 1:1 (✖), 1:2 (◆), 1:3 (■) and 1:5 (▲).

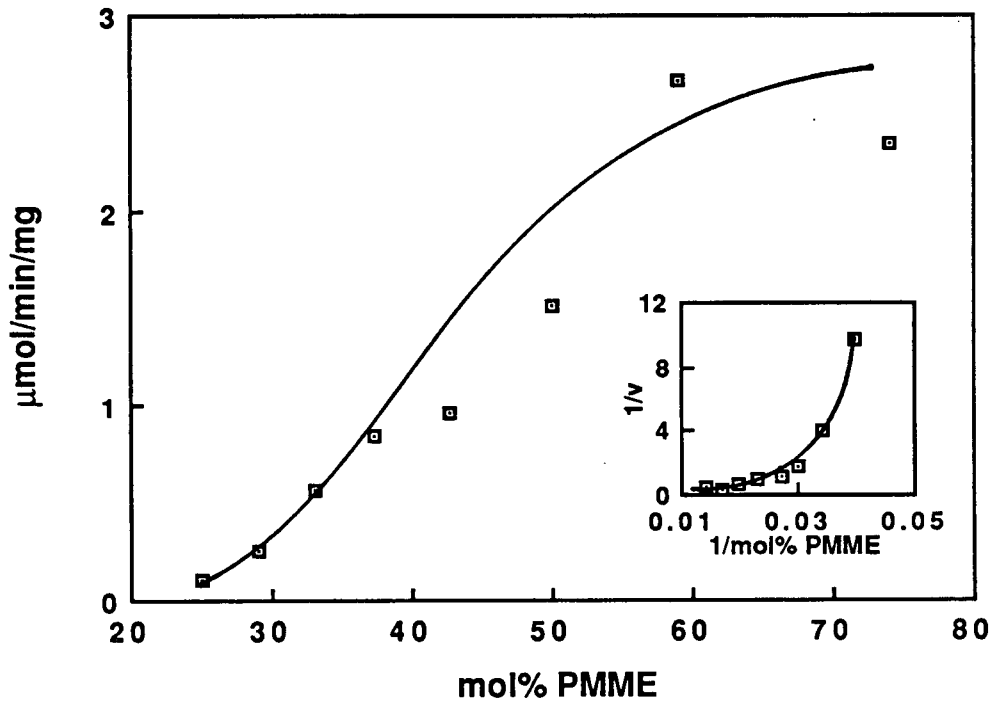

Figure 15. **Cooperative methylation at fixed PMME and increasing Triton X-100 concentration.** Methyltransferase activity was determined at a fixed PMME concentration of 0.75 mM and titrated with 0.5 to 2.2 mM Triton X-100. The corresponding inverse plot is shown in the insert.

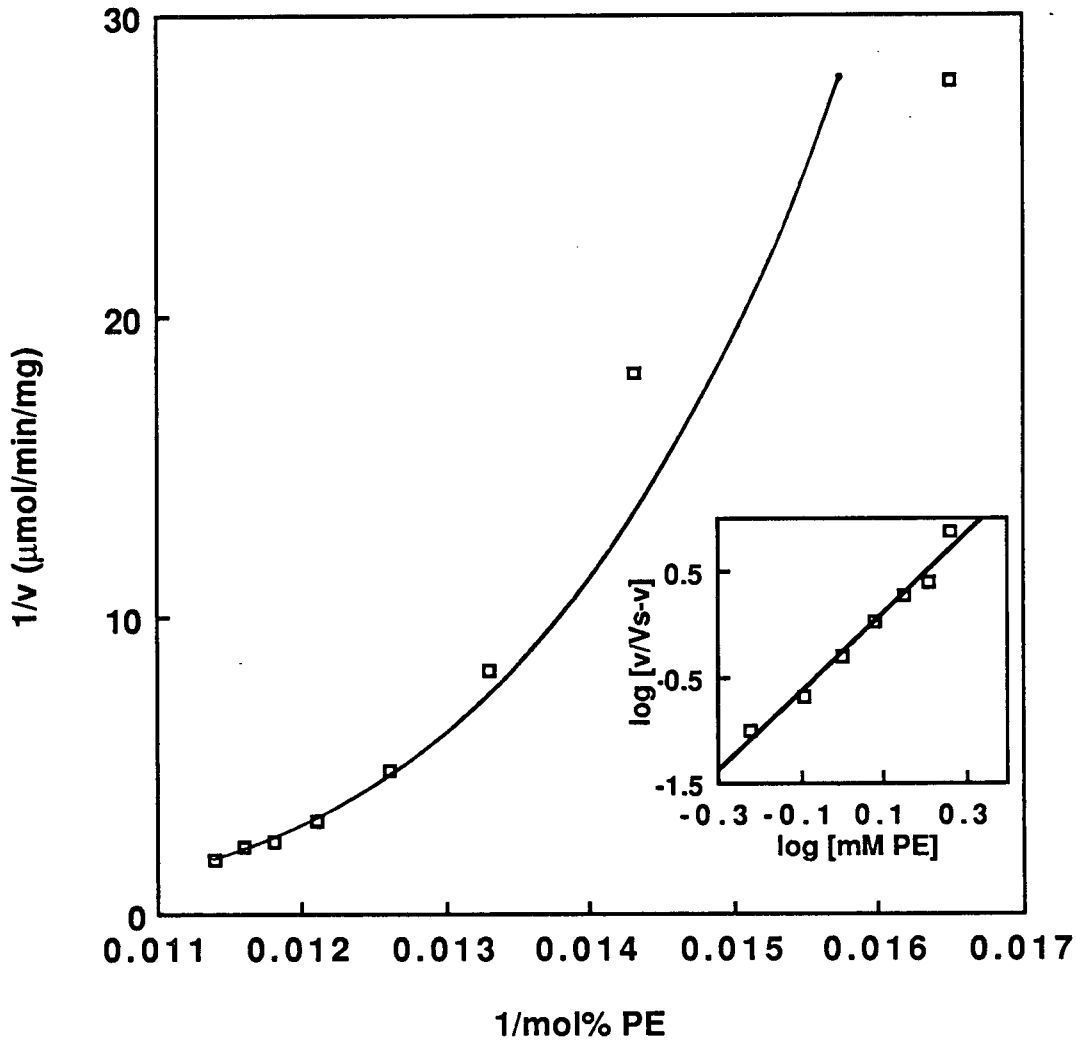

Figure 16. **Cooperativity of PE-dependent methylation.** PE *N*-methyltransferase activity was assayed in the presence of various PE concentrations at a fixed Triton X-100 concentration of 0.5 mM. The data is expressed in the form of a double reciprocal plot. The insert shows a Hill plot of the PE initial velocity data.

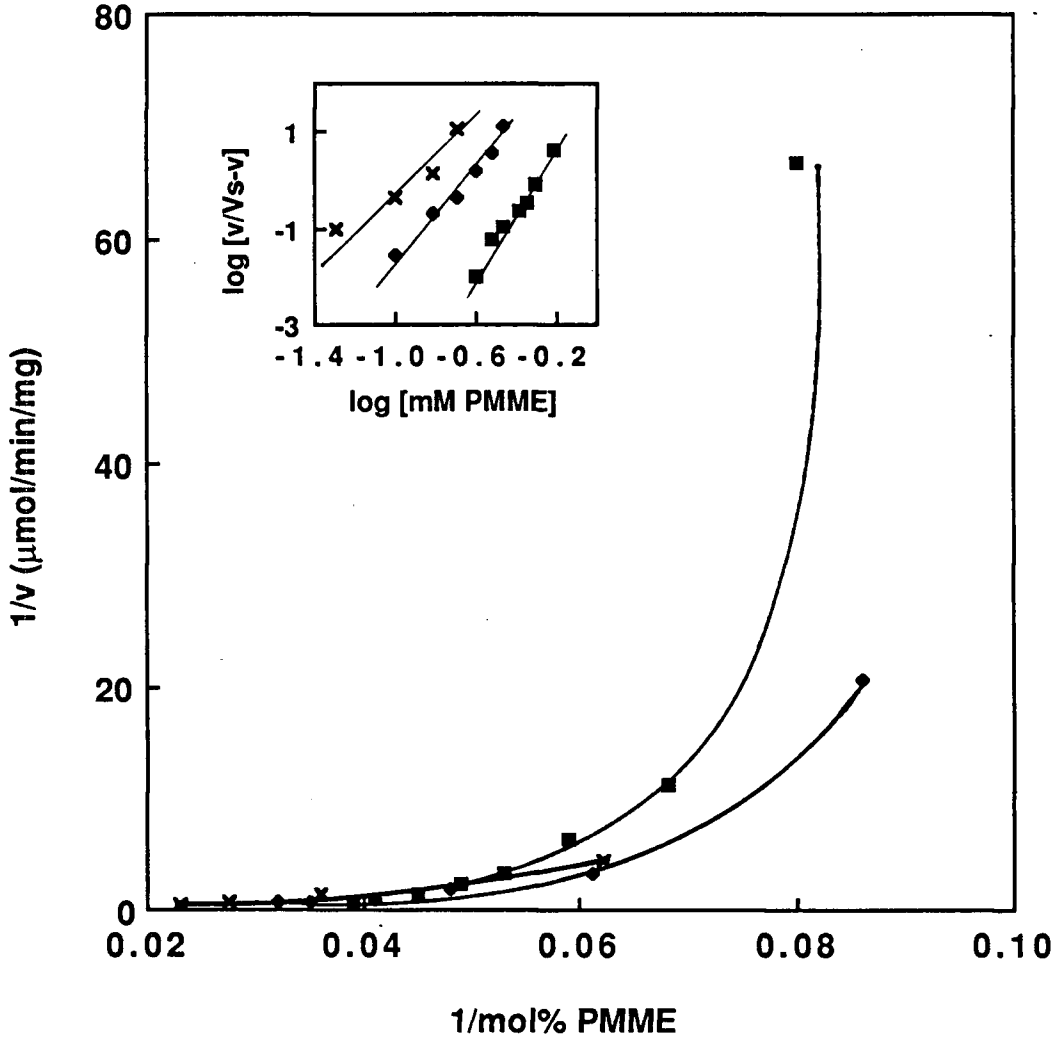

Figure 17 **Cooperativity of PMME-dependent methylation.** PE *N*-methyltransferase activity was determined in the presence of various concentrations of PMME at 0.5 mM (✖), 1.0 mM (◆) and 2.0 mM (■) Triton X-100 and expressed in the form of a double reciprocal plot. The insert shows the corresponding Hill plot for the three Triton X-100 concentrations.

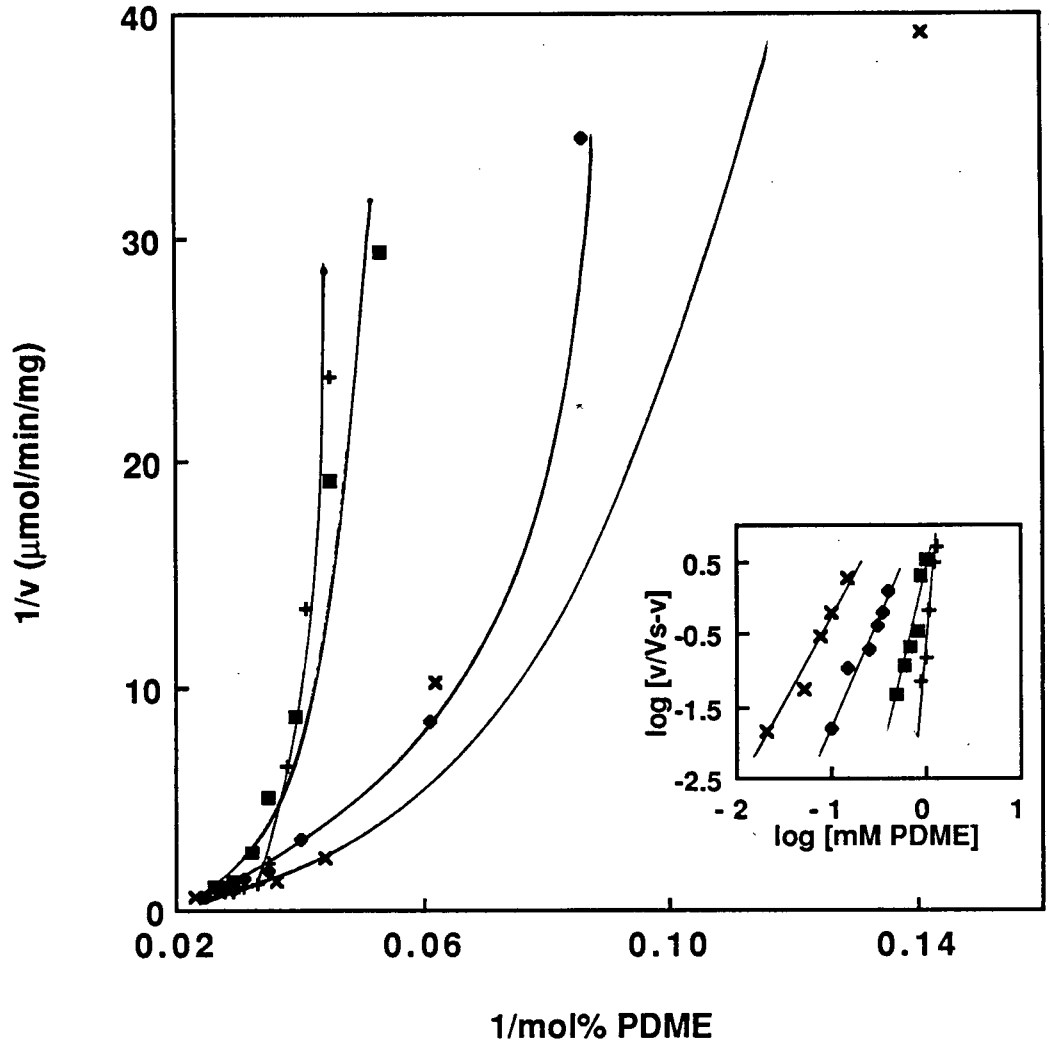

Figure 18. **Cooperativity of PDME-dependent methylation.** PE *N*-methyltransferase activity was determined in the presence of various concentrations of PDME at 0.5 mM (✖), 1.0 mM (◆), 2.0 mM (■) and 3.0 mM (✚) Triton X-100. The insert shows the corresponding Hill plot for the four Triton X-100 concentrations.

cooperativity, a situation that would not apply to steady-state enzyme kinetics. In addition, the *apparent* cooperativity observed for activation of integral membrane proteins is due to non-cooperative binding of phospholipid (287). Still, Hill coefficients are useful empirical measures of cooperativity.

The *apparent* cooperativity of PE, PMME, or PDME methylation is reminiscent of previous work on *E.coli* diglyceride kinase (288) and (Na^+-K^+)ATPase (289), both of which require nonsubstrate lipids for activation. Fig. 19 shows sigmoidal initial velocity curves of PE-, PMME- and PDME-dependent PE *N*-methyltransferase activities at fixed Triton X-100 concentration and increasing bulk substrate. However, this sigmoidicity is largely obviated when a fixed mol% of egg PC is included in the micelle. As noted in Section 3.2.1, PE methylation reaches a maximum out of the range where a uniform population of mixed micelles exist. However, it should be noted that in Fig. 19 a small peak of PE-dependent activity occurred in the mixed micelle range of the substrate velocity curve.

The return to Michaelis-Menten kinetics afforded by egg PC is further illustrated in Fig. 20. This figure shows inverse- and Hill-plots (insert) for the data in Fig. 19. Hill coefficients for PE, PMME and PDME in the absence of egg PC were 3.6, 2.5 and 4.7 (average of 2 experiments), respectively. However, with the addition of an increasing fixed mol% of egg PC (refer to Fig. 19 for details) the coefficients were reduced to 1.2, 1.1 and 0.9 (average of 2 experiments) for PE, PMME and PDME, respectively.

3.3 Kinetic Mechanism of Purified Phosphatidlyethanolamine *N*-Methyltransferase

3.3.1 Evaluation of the Kinetic Pathway

The kinetic mechanism of PE *N*-methyltransferase was studied by evaluation of initial velocity- and product inhibition-patterns as described by Cleland (290). It would be virtually impossible to determine the kinetic

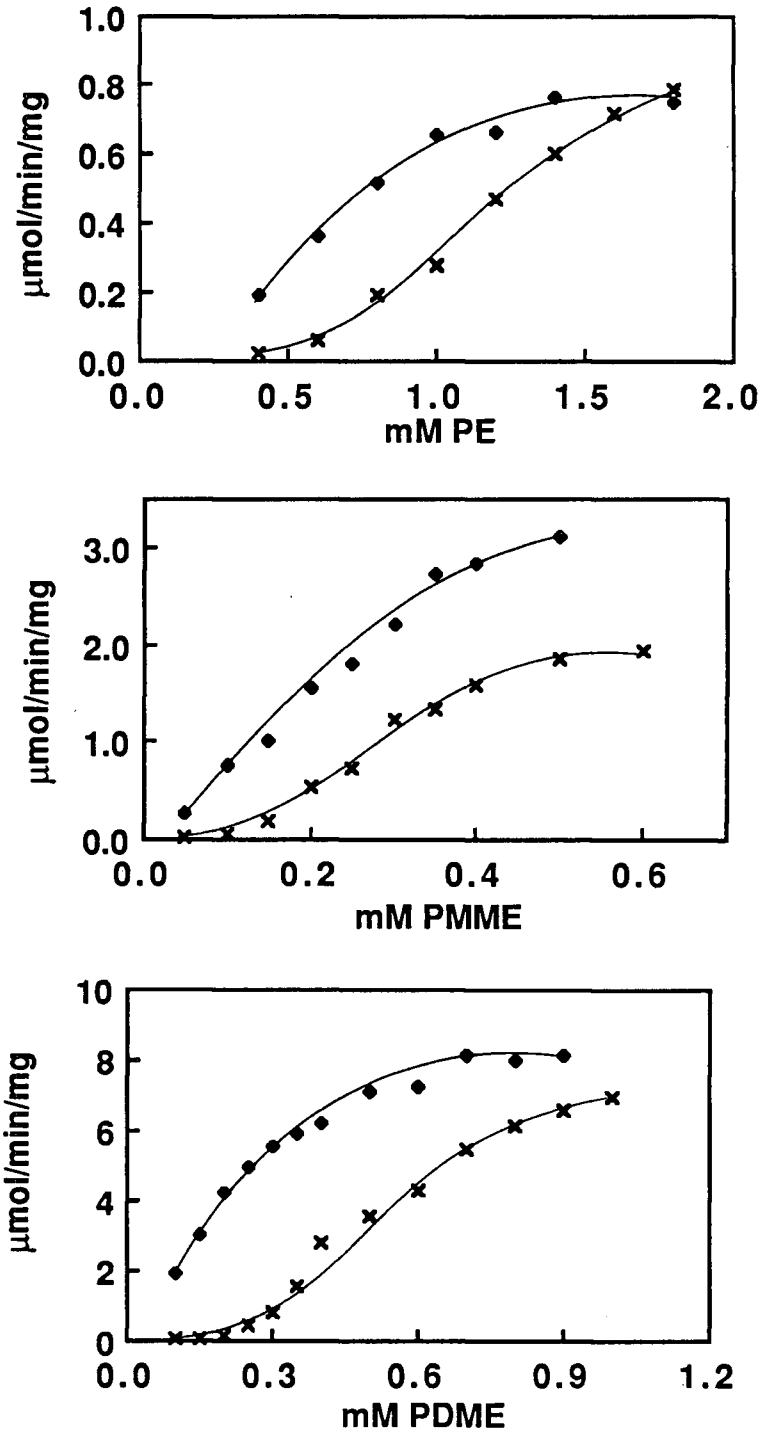

Figure 19. **Influence of egg PC on PE, PMME, and PDME initial velocity curves.** Increasing PE, PMME and PDME were methylated at fixed Triton X-100 concentrations of 0.5, 1.0, and 1.0 mM, respectively (✖). PMME and PDME were methylated in the presence of 30 mol% egg PC and PE methylation was with 40 mol% egg PC (◆).

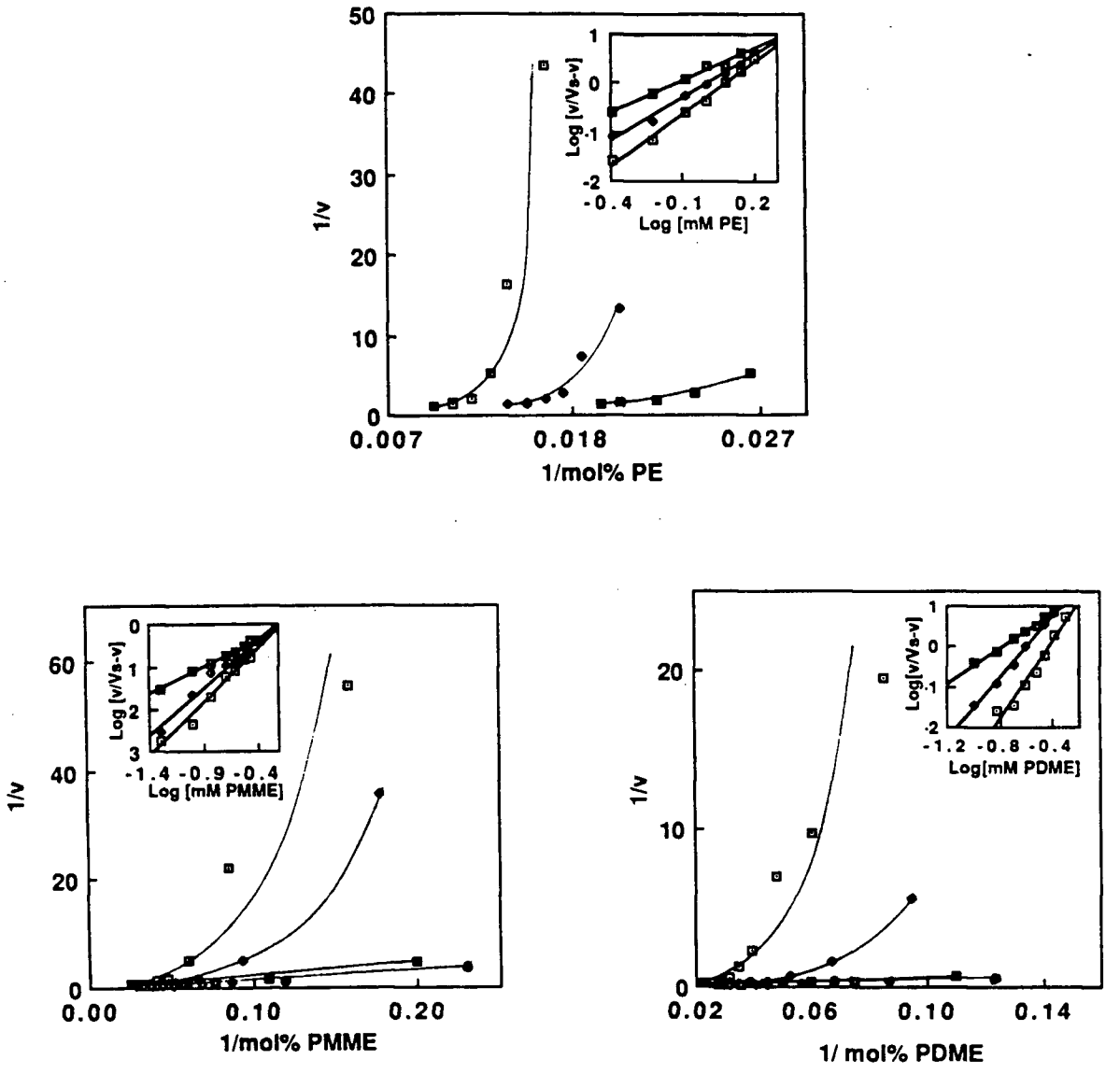

Figure 20. **Influence of egg PC on inverse and Hill plots for PE, PMME and PDME.** A, varied mol% of PE was methylated at a fixed Triton X-100 concentration of 0.5 mM and fixed egg PC of 0 (⊡), 20 (◆) and 40 (■) mol%. B, varied mol% of PMME, 1.0 mM Triton X-100 and 0 (⊡), 10 (▲), 20 (■), and 30 (●) fixed mol% egg PC was methylated. C, a varied mol% of PDME was methylated in the presence of 1.0 mM Triton X-100 and 0 (⊡), 10 (▲), 20 (■), and 30 (●) fixed mol% of egg PC. In all instances, the corresponding Hill plot is shown as an insert. Refer to the text for Hill coefficients.

mechanism on the basis of PE methylation alone since steady state is reached in 1 min, after which no accumulation of PMME or PDME occurs (88). As shown in product analysis (Section 3.1.2), the two intermediate methylation steps could be assayed individually with PMME and PDME; in both instances 95-100 % of the product was PDME and PC, respectively. The conversion of PE to PMME has yet to be assayed as a discrete step. However, the rate-limiting nature of the first step allows it to be assayed on the basis of PE to PC conversion provided no intermediates are present or accumulate.

Inverse plots for variable AdoMet at several fixed concentrations of PMME (Fig. 21A) or PDME (Fig. 21B) are shown. In both instances two sets of intersecting lines were obtained, which is partial evidence for a sequential addition of substrates. Varying PMME or PDME (Fig. 22) at several fixed concentrations of AdoMet, but in the presence of 30 mol% egg PC, also yielded intersecting lines. Evidence for a sequential addition of substrates for PMME and PDME methylation by partially pure PE *N*-methyltransferase has previously been reported (89). Inverse plots of variable PE (plus 40 mol% egg PC) at several fixed concentrations of AdoMet were also intersecting (Fig. 23). Slope or intercept replots of initial velocity patterns, without egg PC and with PE, PMME and PDME fixed and varied, were highly irregular or parabolic in nature, consistent with the previous discussion detailing the nonlinearity of inverse plots and lipid binding requirements of PE *N*-methyltransferase. Replots of variable PMME (with 30 mol% PC) versus fixed AdoMet (Fig. 22) were used to determine kinetic constants and will be discussed in a latter section.

Product inhibition patterns for PE, PMME and PDME methylation, at variable AdoMet and several fixed concentrations of AdoHcy, revealed a set of lines intersecting on the horizontal axis (Fig. 24). Noncompetitive inhibition

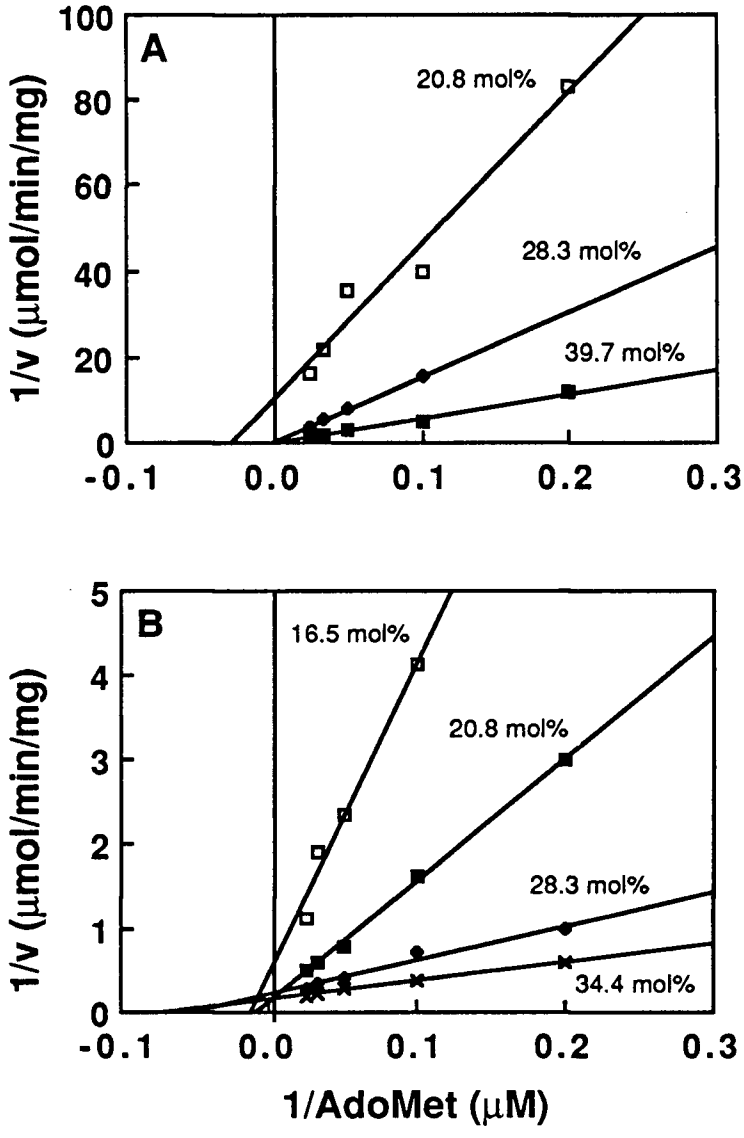

Figure 21. **Double reciprocal plots of initial methylation rates versus AdoMet at various fixed concentations of PMME and PDME.** The indicated fixed mol% of PMME (A) and PDME (B) was methylated at varied AdoMet concentrations.

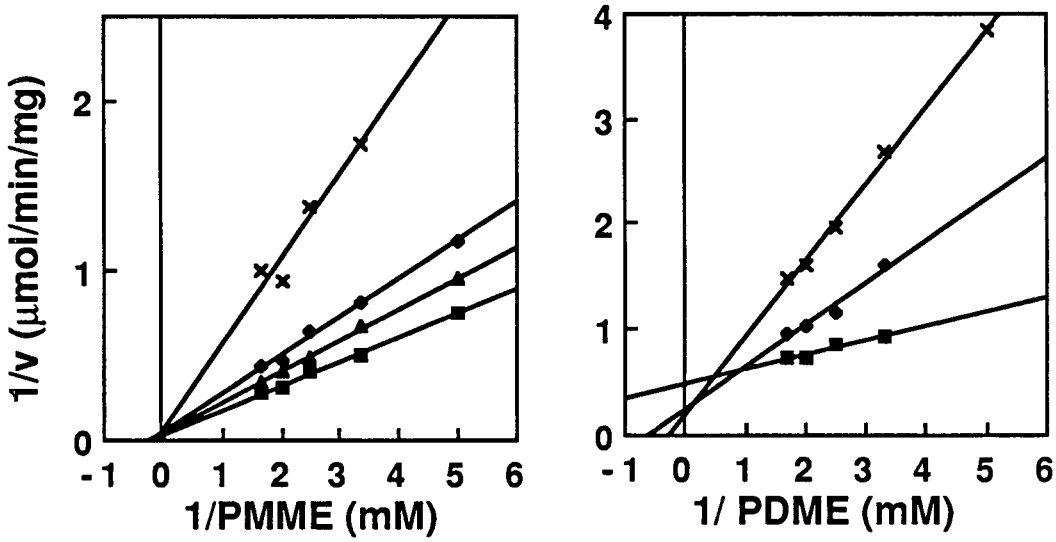

Figure 22. **Double reciprocal plots of initial methylation rates versus PMME and PDME at various fixed concentrations of AdoMet.** Methylation rates at 10 (✖), 20 (◆), 30 (▲), and 40 (■) µm AdoMet were determined in the presence of 30 mol% egg PC as described in Section 2.3.1. PDME methylation was not analyzed at 30 µM AdoMet.

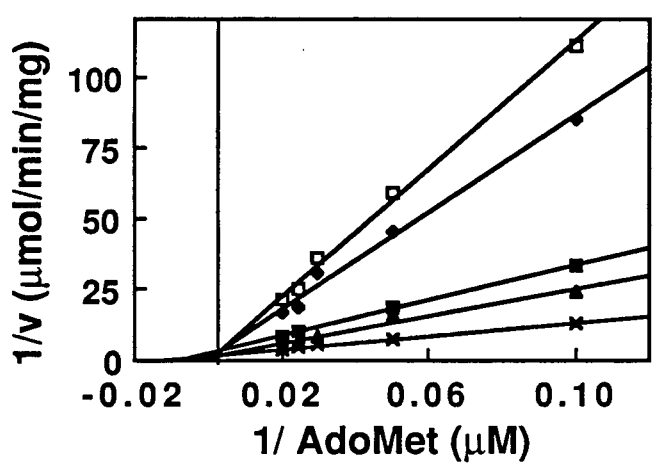

Figure 23. **Double reciprocal plots of initial PE methylation rates versus various AdoMet concentrations.** Methylation rates at 0.3 (□), 0.4 (◆), 0.6 (■), 0.8 (▲), and 1.0 (✖) mM PE were determined in the presence of 40 mol% egg PC and variable AdoMet concentrations as described in Section 2.3.1

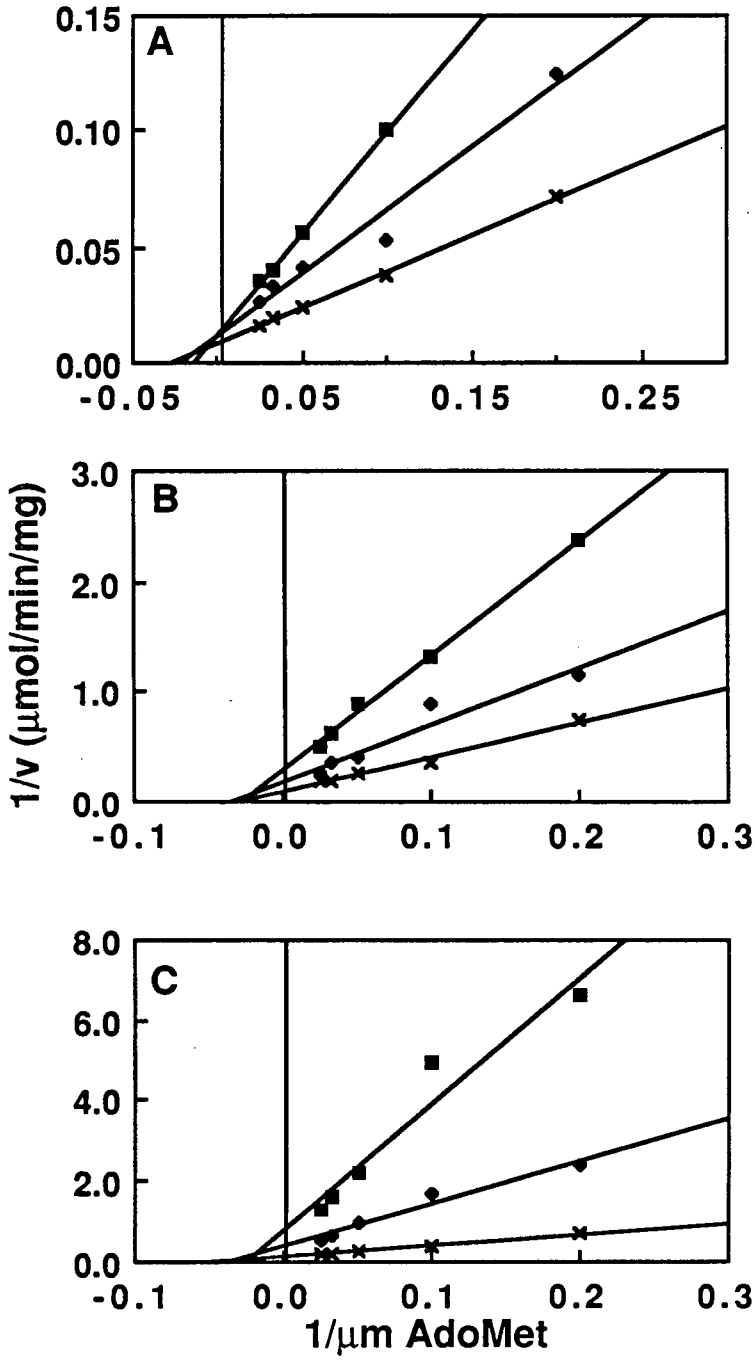

Figure 24. **Inhibition of PE, PMME and PDME methylation by AdoHcy at variable AdoMet.** Methylation of 88 mol% PE (A), 49 mol% PMME (B), and 61 mol% PDME (C) was determined at various fixed AdoHcy concentrations of 0 (✕), 100 (◆) and 400 (■) µm and variable AdoMet.

indicates that the two ligands are separated by a reversible step, but do not bind the same enzyme form (290). Product inhibition patterns for several fixed mol% of PE, PMME and PDME at variable AdoHcy were uncompetitive (Fig. 25), and therefore separated in the kinetic scheme by an irreversible step made so by the presence of 200 μM AdoMet. Consistant with uncompetitive inhibition was the reversion to noncompetitive kinetics when AdoMet was lowered to nonsaturating levels (25 μM).

One remaining piece of evidence to indicate an ordered Bi-Bi mechanism is direct competition between PMME and PDME for the free enzyme form. Data in support of this is shown in Fig. 26A for the inhibition of PMME methylation by PDME and is presented in the form of a Dixon plot (291). A slope replot (insert) was found to be parabolic, but points at high mol% phospholipid intersect the origin, indicative of competitive inhibition of PMME methylation by PDME. Similarly, PMME was observed to inhibit competitively PDME methylation (Fig. 26B). Experiments to show what type of inhibition PMME and PDME exert over PE methylation were fraught with technical problems due to the low activity of PE *N*-methyltransferase toward PE in mixed micelles and the high concentration of PE necessary to achieve full activity. However, as shown in Fig. 27, PMME and PDME inhibit the conversion of [^{3}H]PE to [^{3}H]PC. This inhibition was not the result of nonspecific surface dilution since PC lacked effect at concentrations of PMME and PDME that produced 50% inhibition of PC formation. No accumulation of labeled PMME or PDME was observed at high concentration of cold PMME or PDME, indicative of inhibition of PE methylation at the first methyl group addition. The results of these initial velocity and product inhibition patterns are summarized in Table 5.

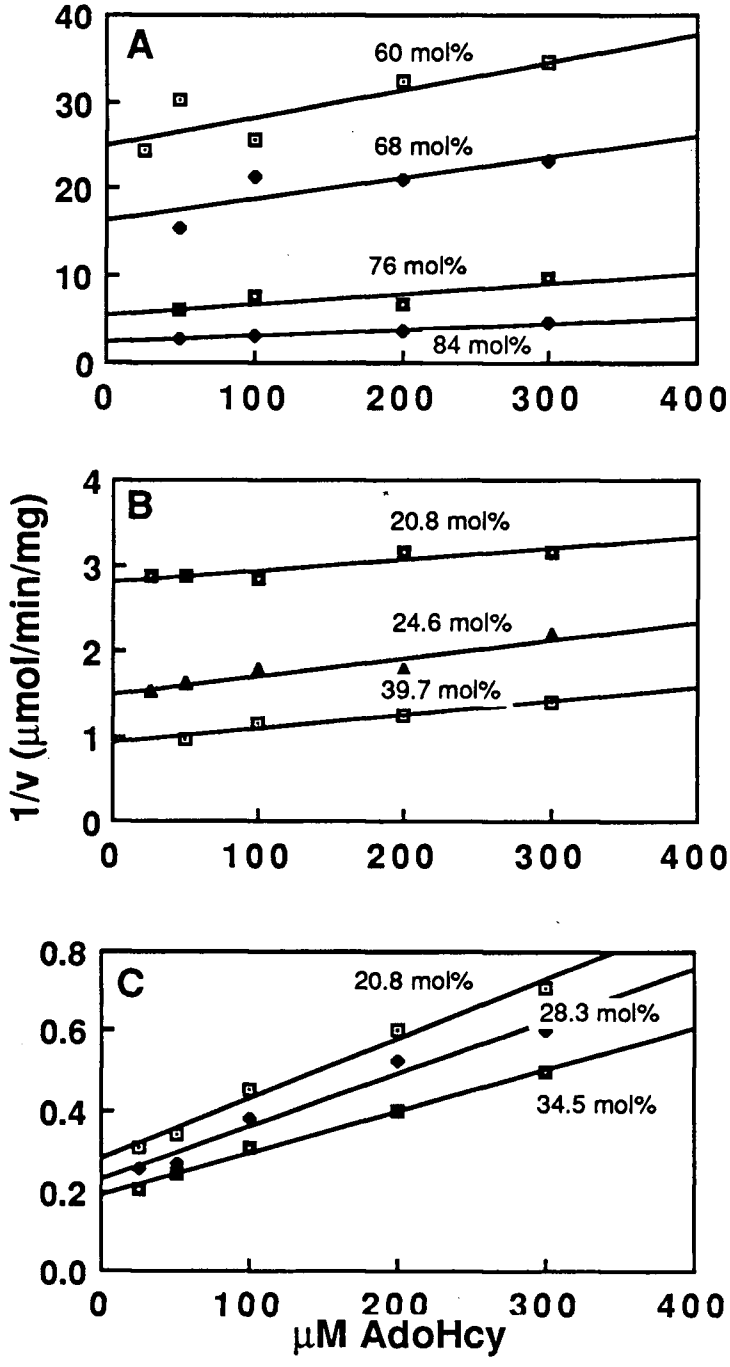

Figure 25. **Inhibition of PE, PMME, and PDME methylation by AdoHcy at fixed and saturating AdoMet.** PE (A), PMME (B) and PDME (C) methylation rates, at the indicated fixed mol%, were determined at variable AdoHcy concentrations and constant AdoMet of 200 µM. Data is presented in the form of a Dixon plot.

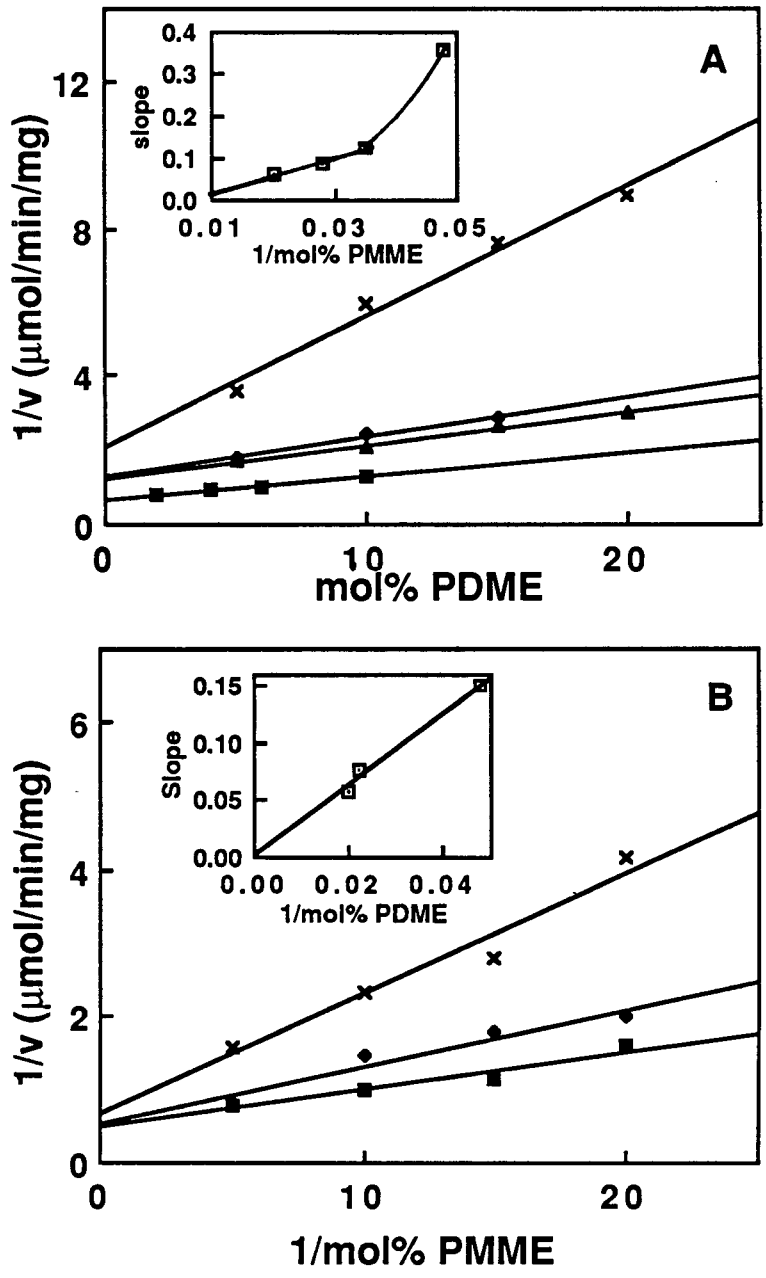

Figure 26. **Dixon Plots showing co-inhibition of PDME and PMME Methylation.** 20.8 (✖), 28.3 (◆), 34.5 (▲) and 49.9 (■) mol% PMME (panel A) was methylated at various mol% of PDME. Radioactive PDME was isolated by TLC and radioactivity determined as described in Section 2.3.3. A replot of slope versus 1/mol% PMME is shown in the insert. 20.8 (✖), 44.0 (◆) and 50.0 (■) mol% PDME (panel B) was methylated in the presence 5 to 20 mol% PMME and the radioactivity in PC determined. A replot is shown in the insert.

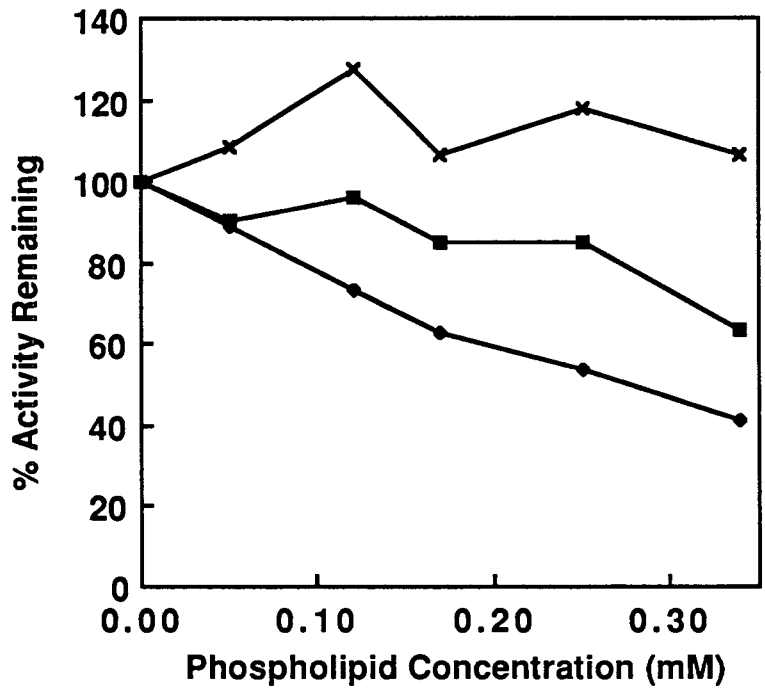

Figure 27. **Inhibition of PE methylation by PMME and PDME.** [^{3}H]PE (2 mM) was methylated in the presence of 0.5 mM Triton X-100, 200 μM AdoMet, and various concentrations of PMME (◆), PDME (■) and PC (✖) (Section 2.3.2). Products were isolated by TLC and counted as described in Section 2.3.3. The radioactivity recovered in PC is shown.

Table 5. TYPES OF COSUBSTRATE AND PRODUCT INHIBITION PATTERNS OBSERVED FOR PE, PMME AND PDME METHYLATION BY PURIFIED PE *N*-METHYLTRANSFERASE

PHOSPHOLIPID SUBSTRATE	COSUBSTRATE EFFECT OF ADOMET	PRODUCT EFFECT OF ADOHCY ON v_f(ADOMET)	PRODUCT EFFECT OF ADOHCY ON v_f(PMME,PDME)	PRODUCT EFFECT OF PMME,PDME on v_f(PDME,PMME)
PE	INTERSECTING	NONCOMPETITIVE	UNCOMPETITIVE	----
PMME	INTERSECTING	NONCOMPETITIVE	UNCOMPETITIVE	COMPETITIVE
PDME	INTERSECTING	NONCOMPETITIVE	UNCOMPETITIVE	COMPETITIVE

v_f, forward reaction velocity

The various combinations and permutations of substrates and inhibitors used to generate the inverse- and Dixon-plots herein are indicative of an ordered Bi-Bi mechanism for the methylation of PMME to PDME and PDME to PC (290). Evidence for a single lipid substrate active site and product inhibition patterns for PE methylation were used to formulate a complete model (Fig. 28) for the methylation of PE to PC based on the nomenclature of Cleland (292).

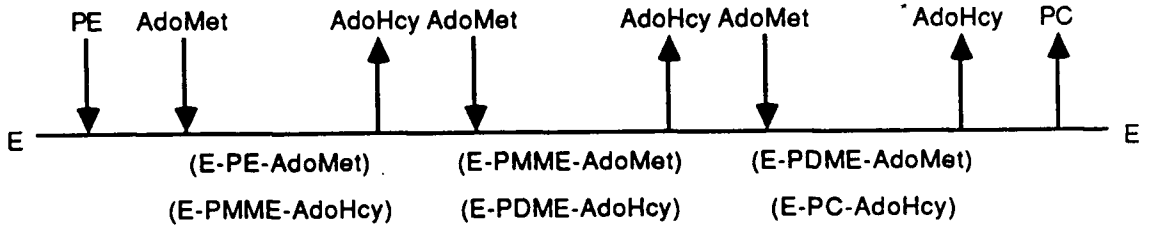

Figure 28. A concerted kinetic mechanism for PE methylation.

Briefly, the model predicts PE initally binds to a common lipid substrate binding site followed by AdoMet. PMME is formed and the first AdoHcy dissociates. The next two steps are essentially two ordered Bi-Bi mechanisms linked together. The only deficiency in this model is the inability to show the effect of PC on substrate binding. As shown previously, instead of PC acting as a classic product inhibitor it actually activates PE, PMME and PDME methylation. By a process of elimination, PC has tentatively been placed as the last product to depart.

3.3.2 Kinetic Constants

Table 6 is a compilation of kinetic constants determined for PE, PMME and PDME methylation. The K_m values for AdoMet are similar to those reported for microsomes (88). AdoHcy K_i values (determined from slope and intercept replots of Fig. 24) appear to decrease as the PE molecule is progressively

methylated. The K_i value of 277 μM for the inhibition of PE methylation is about 100-fold greater than that found for microsomes (3 μM). In addition, the K_i values for inhibition of PMME and PDME methylation by AdoHcy are 10- to 20-fold greater than those previously reported for the partially pure enzyme (89). The 5- and 10-fold greater *Vmax* values for PMME and PDME methylation, respectively, compared to that for PE methylation, support the notion that PE to PMME conversion is rate-limiting. Furthermore, the apparent K_m for PE was greater than values for PMME or PDME. *Vmax* values determined in the presence of egg PC followed the pattern PDME > PMME > PE, but were increased 2- to 10-fold over values determined in the absence of egg PC. Slope replots of data from Figs. 21 and 25 were nonlinear, consistent with the sigmoidal curves found when lipid substrate was variable. Only slope and intercept replots of PMME data (Fig. 22) in the presence of 30 mol% egg PC were linear. A $K_{m\text{-}AdoMet}$ of 11.9 μM , *Vmax* of 32.3 μmol/min/mg and $K_{m\text{-}PMME}$ of 0.4 mM were calculated. These data are similar to values presented in Table 6.

Table 6. KINETIC CONSTANTS FOR PE *N*-METHYLTRANSFERASE[1]

Lipid Substrate	$K_{m\text{-}AdoMet}$[2] (μM)	$K_{m\text{-}PL}$[3] (mM)	$K_{i\text{-}AdoHcy}$[2] (μM)	V_{max}2 (μmol/min/mg)	V_{max}3 (μmol/min/mg)
PE	36.6±14.7	5.00(1.02)	277.5±175.9	0.95±0.20	3.38(0.78)
PMME	39.7(14.9)	0.74±0.30	138.3(23.5)	9.70(0.95)	20.36±6.35
PDME	13.6(3.5)	2.12(0.07)	73.9(9.7)	4.25(1.10)	40.95(5.11)

[1] Values are the average 3 determinations (±S.D) or 2 determinations (range).
[2] Values determined from replots of Lineweaver-Burk data with AdoHcy fixed and variable and AdoMet variable. PE, PMME and PDME were held fixed at 88, 49 and 61 mol%, respectively.
[3] Constants determined from Lineweaver-Burk plots at 200 μM AdoMet, variable concentrations of PE, PMME or PDME and a fixed mol% of egg PC.

3.3.3 Free Sulfhydryls are Required for Methylation

The role of sulfhydryls in PE *N*-methyltransferase catalysis was re-evaluated using the purified enzyme. As reported previously (84,85), a free

sulfhydryl(s) is required for methylation of microsomal PE and it is essential to have a reducing agent, such as DTT, present during purification (Section 2.2). DTT was removed from purified PE *N*-methyltransferase during concentration on a Mono S column exactly as described in Section 2.2.7 (using buffer A minus DTT). The enzyme was completely inactive when assayed for PE-, PMME- and PDME-dependent activities in the absence of DTT (Fig. 29). However, when DTT was titrated into the assay there was a complete recovery of all activities. Maximum reactivation was achieved at about 10 mM DTT (Fig. 29). It is possible that removal of DTT results in rapid oxidation of two or more cysteine residues and formation of intramolecular disulfide bridges. Formation of intermolecular disulfide linkages cannot be ruled out, however.

As expected, methylation was inhibited 70-90% by a 2.5 mM excess of IAA and greater then 95% by 2.5 mM DTNB (Table 7). DTT (a 2-fold excess) alleviated DTNB inhibition, but not inhibition by IAA. Based on these preliminary results it seems that cysteine(s) are required for catalytic activity.

Table 7. Inhibition of PE *N*-Methyltransferase by Sulfhydryl Modifying Reagents

	Assay Substrate (%Activity)		
Reagent[1]	PE	PMME	PDME
IAA	33.4 (3.3)	7.7 (4.8)	29.3 (8.3)
IAA+DTT	46.9 (7.5)	13.9 (7.8)	40.9 (1.8)
% Reactivation	13.5% (10.8)	6.2% (3.0)	11.7% (6.5)
DTNB	2.2 (2.0)	1.5 (1.7)	1.2
DTNB+DTT	81.9 (2.9)	64.0 (12.0)	90.4 (3.4)
% Reactivation	80.1% (0.9)	62.5% (10.3)	89.2% (2.8)

[1]Purified enzyme was incubated with 5 mM IAA or DTNB. A 10 min reactivation with 10 mM DTT was initiated after a 10 min incubation on ice with inhibitor. Results are the average of two experiments with the range in brackets.

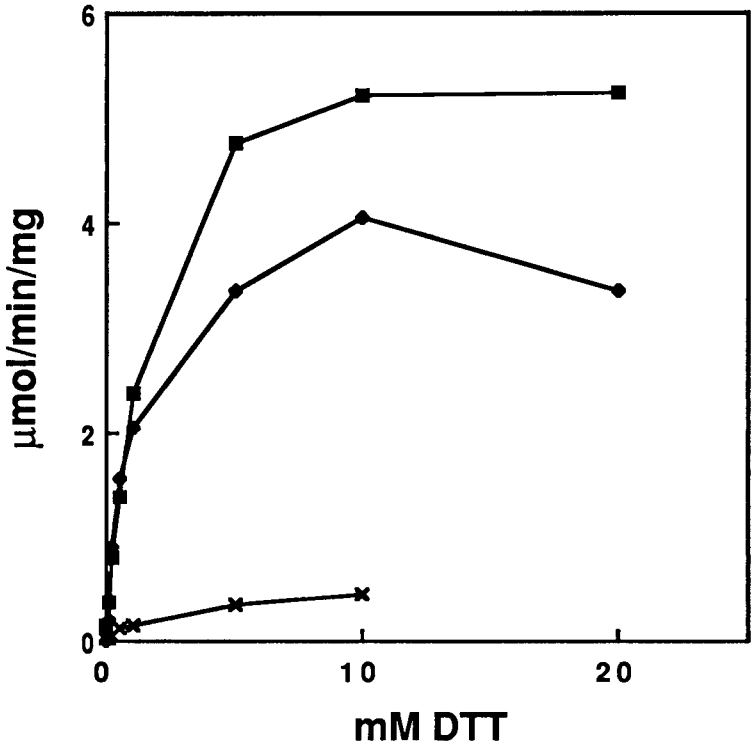

Figure 29. **Activation of PE, PMME and PDME methylation by DTT.** DTT-free methyltransferase was incubated with the indicated concentrations of DTT for 10 min on ice and assayed as described in Section 2.3.1. The distribution of products using PE (✖), PMME (◆) and PDME (■) as substrates was similar to that described in Table 4.

3.4 Photoaffinity Labeling of Microsomal Phosphatidylethanolamine *N*-Methyltransferase

3.4.1 Photolabeling of Microsomes

Photolabeling of liver microsomal proteins using AdoMet was investigated as a means of identifying PE *N*-methyltransferase in tissues and cellular fractions. Irradiation of microsomes in the presence of [*methyl*-^{3}H]AdoMet, and identification of the labeled proteins by SDS-PAGE and fluorography, revealed the presence of four major bands (Fig. 30). The most heavily labeled was a protein of 19 kDal. The proteins of 19 and 30 kDal were labeled as the result of photoactivation since both were absent in incubations which were not irradiated. The band at 25 kDal was labeled in the absence of irradiation, suggesting it appears as a result of direct methyl group incorporation (carboxymethylation). The 19 kDal protein was almost identical in molecular mass to the PE *N*-methyltransferase purified from microsomes (Section 3.1.4). A band was observed slightly above the 19 kDal protein, but this was not a consistent observation.

3.4.2 Photolabeling of the Microsomal 19 kDal Methyltransferase

The time course of photolabeling was essentially linear for at least 30 min (Fig. 31). The efficiency of labeling was estimated to be 0.01-0.005% (for a 20 min irradiation) and labeling was independent of pH between 6 and 10. As illustrated in Fig. 32, photolabeling was inhibited completely by the addition of a 300-fold excess of cold AdoMet or AdoHcy, but not by adenosine or adenine. Distribution of the 19 kDal protein was examined in various organs and correlated with the activity of PE *N*-methyltransferase (Fig. 33). Methylation activities were the greatest in liver microsomes, and addition of PMME resulted in a 3.6-fold stimulation of activity. No such stimulation was observed for methylation in other organs. The 19 and 30 kDal proteins were observed in liver, however, the former was completely absent from kidney, lung and heart.

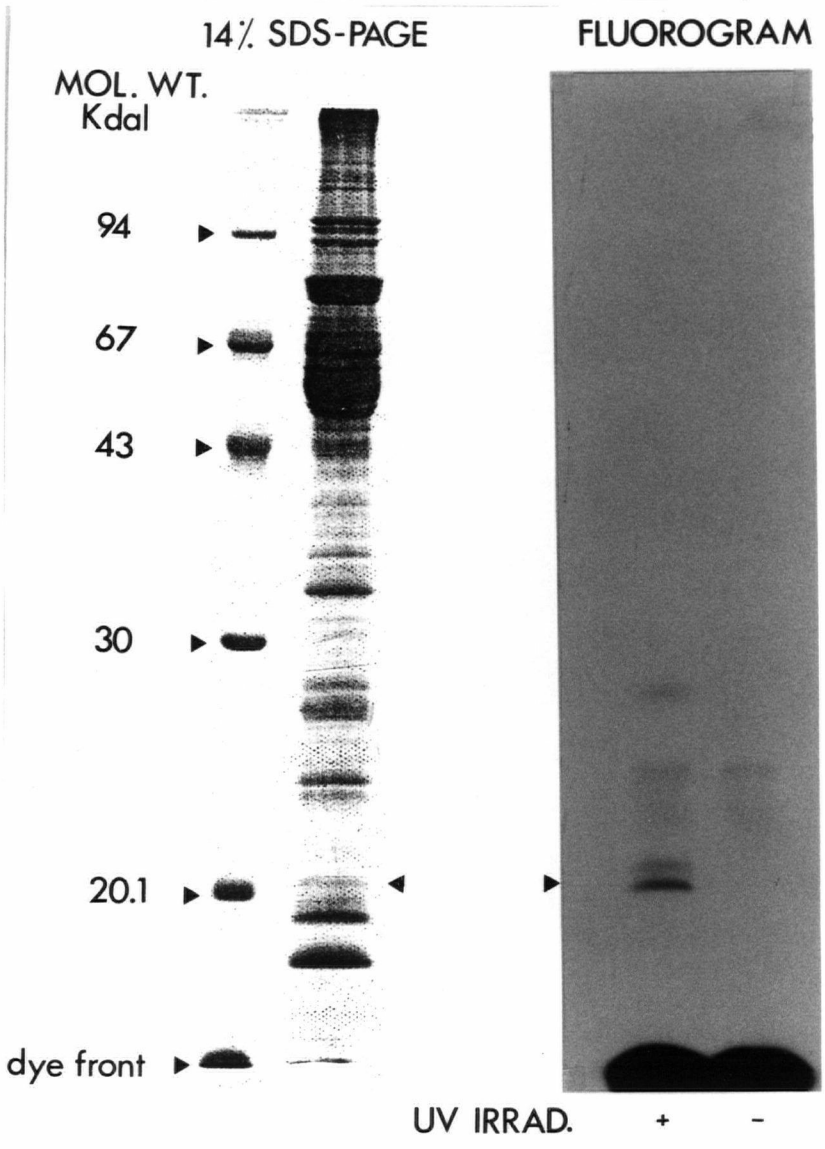

Figure 30. **Photolabeling of microsomal proteins with [*methyl*-^{3}H]AdoMet and identification by SDS-PAGE.** Microsomes were photolabeled for 20 min and subjected to SDS-PAGE. The gel was dried and exposed to film for 6 days. Non-irradiated microsomes were kept in the dark at 4°C for 20 min.

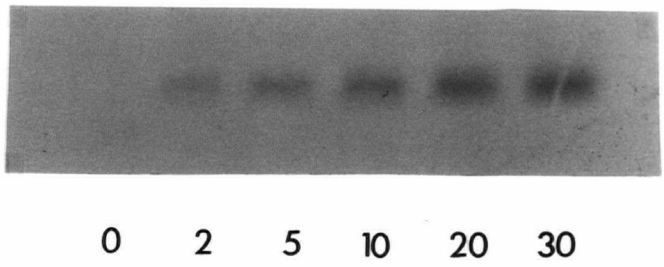

Figure 31. **Time course of 19 kDal protein photolabeling.** Microsomes (100 μg) were labeled with [*methyl*-^{3}H]AdoMet for the indicated times (in min) and identified as described in Section 2.11. The fluorogram shown in the Figure was the result of a 7 day exposure.

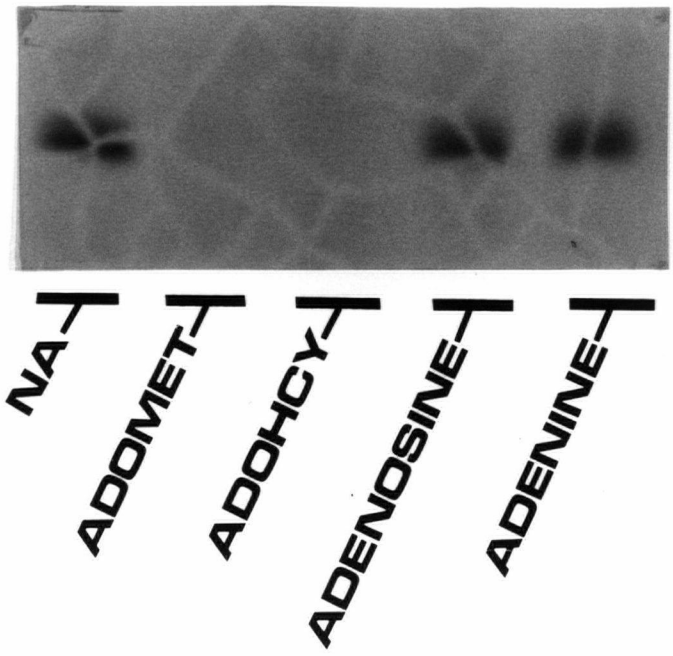

Figure 32. **Inhibition of 19 kDal protein photolabeling by AdoMet and AdoHcy.** Microsomes (100 μg) were photolabeled with [*methyl*-^{3}H]AdoMet for 20 min in the presence of 100 μm unlabeled AdoMet, AdoHcy, adenosine or adenine (these concentrations are in 300-fold excess compared to labeled AdoMet). Following electrophoresis and treatment with fluor, the gel was exposed to film for 8 days. NA, no addition.

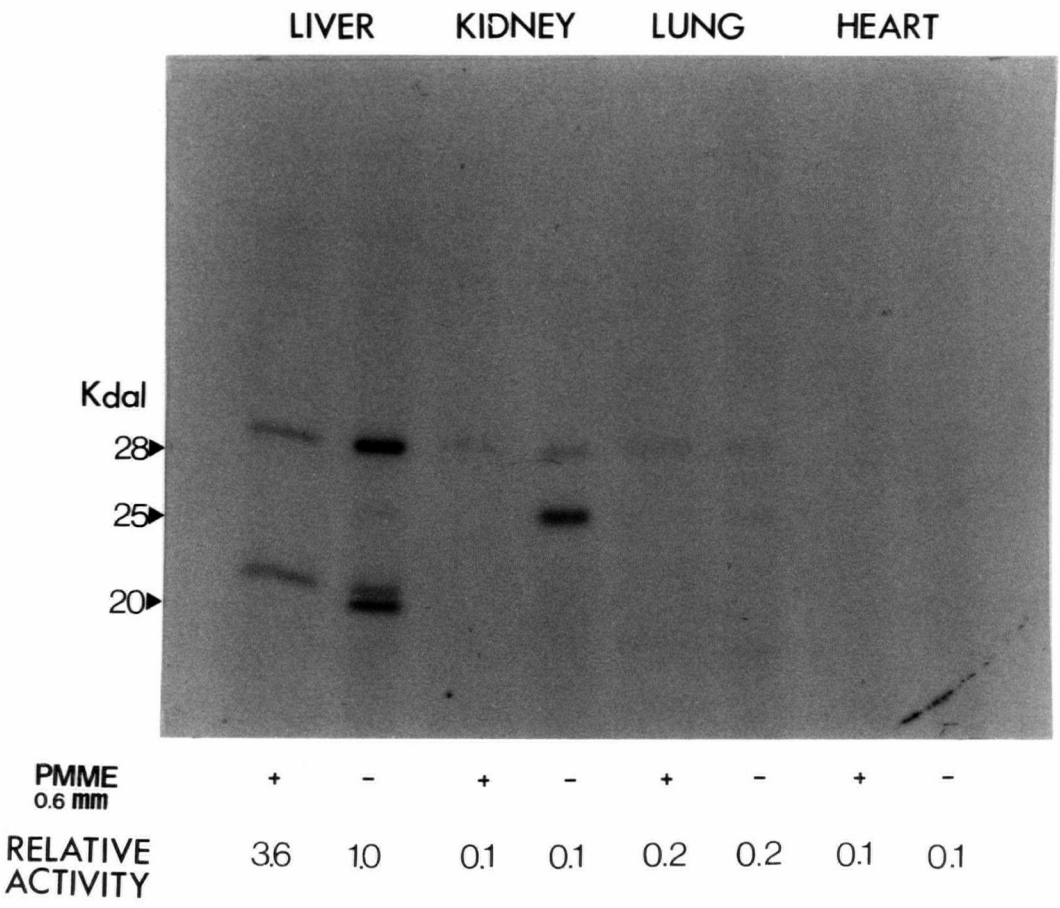

Figure 33. **Distribution of [*methyl*-^{3}H]AdoMet labeled proteins in extrahepatic tissues.** 100,000 x *g* fractions of tissue post-mitochondrial supernatants (100 µg) were photolabeled for 20 min, separated by SDS-PAGE, soaked in fluor and exposed to film for 11 days. The corresponding fluorogram is shown. PE *N*-methyltransferase activity was assayed with and without PMME as described in Section 2.3.1. Triton X-100 was not included in the photolabeling cocktails.

Traces of the 30 kDal band were observed in kidney and lung. Inclusion of PMME in the irrradiation cocktail inhibited labeling of the 19 kDal protein in liver and the 30 kDal band to a lesser extent (Fig. 33). The identity of the 25 and 30 kDal proteins is unknown, but based on molecular mass similarities it seems likely that the 19 kDal AdoMet-labeled protein is indeed PE *N*-methyltransferase. Attempts to label the purified PE *N*-methyltransferase with [*methyl*-^{3}H]AdoMet have been unsuccessful owing to the 'quenching' effect of Triton X-100 on the photolabeling process. Once reconstitution of the enzyme into phospholipid vesicles is achieved this inhibition will be alleviated and photolabeling should establish the identity of the microsomal 19 kDal protein.

3.5 Specificity of Phosphatidylethanolamine *N*-Methyltransferase.

3.5.1 High Performance Liquid Chromatographic Separation of Phospholipids

Typical elution profiles of microsomal PC, microsomal PE and egg PC molecular species from a 4.5 x 150 mm C18 reverse-phase column are shown in Fig. 34. The resolution of molecular species is similar to previous reports on fractionation of whole liver PE and PC (123) and egg PC (124). The shorter C18 column allows separation times to be reduced from 2 h (123) to 1 h, but lessened resolution of peaks 9-10 and 15-16 for microsomal PE and PC. This did not interfere in the interpretation of results.

Peak identification and mol% distribution of molecular species are shown in Table 8. Microsomal PE and PC were both found to be enriched in 1-palmitoyl- and 1-stearoyl-2-docosahexaenoyl species relative to that reported for whole liver (123). Microsomal PE and PC contain 85% and 91%, respectively, of their mass in two groups of molecular species; those with palmitate or stearate in the one position and linoleate, arachidonate or docosahexaenoate in the two position. Interestingly, microsomal PE contains only traces of saturated

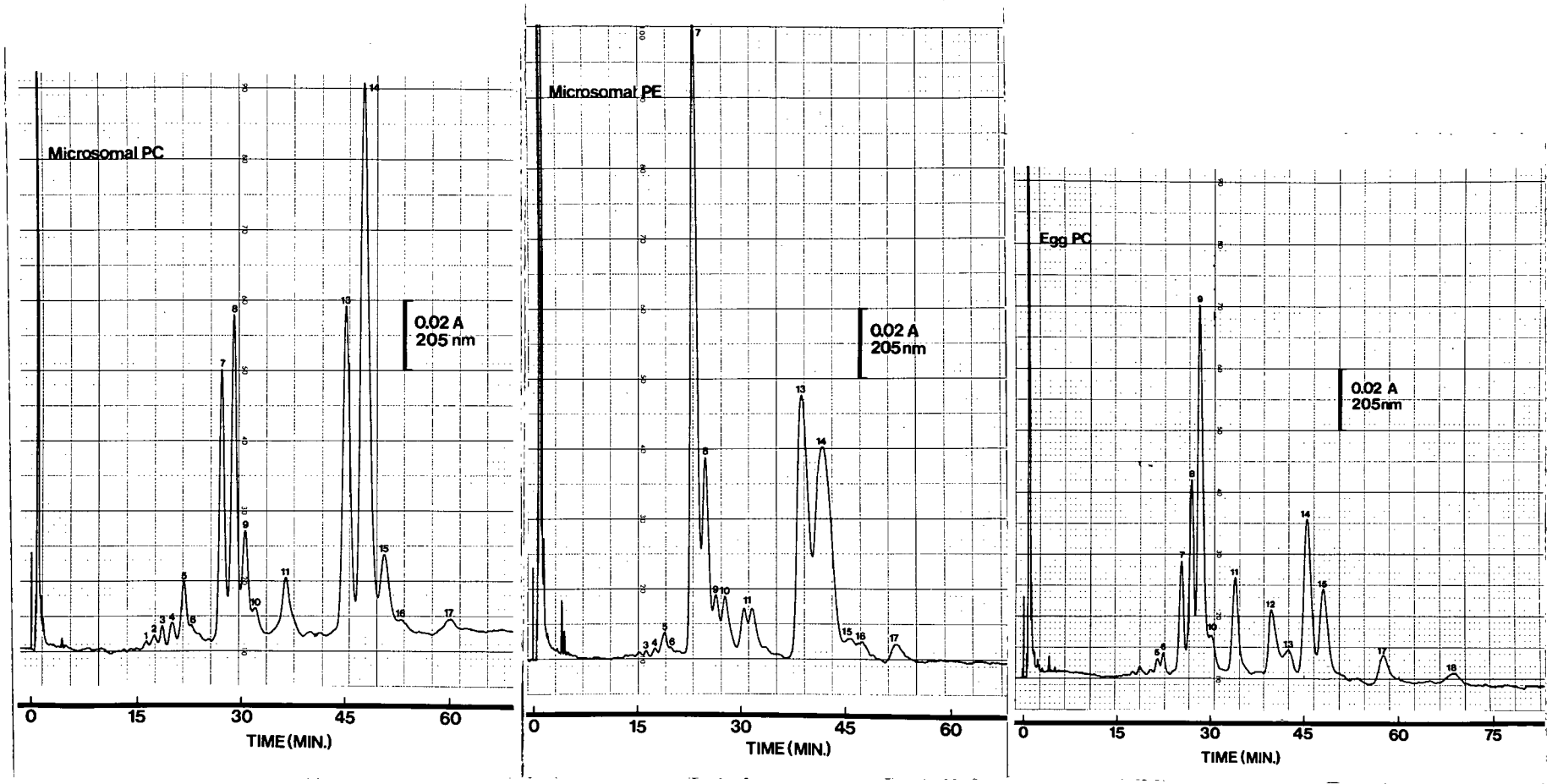

Figure 34. **Separation of microsomal PE, microsomal PC and egg PC by reverse-phase HPLC.** Eighty µg microsomal PE, 120 µg microsomal PC, and 220 µg of egg PC were dissolved in absolute ethanol and separated by reverse-phase HPLC as described in Section 2.4.2. Peak identification is given in Table 8.

Table 8. Mol% Distribution of Microsomal PC, Microsomal PE and Egg PC Molecular Species

Peak Number	Molecular Species	Microsomal PC (n=3)	Microsomal PE (n=7)	Egg PC or TP-Egg PE (n=3)
1,2	18:2-18:3,14:0-22:6	1.5±1.4	ND[1]	ND
3	16:1-18:2	0.7±0.4	0.5±0.3	ND
4	18:2-22:6	1.5±1.0	↓[2]	ND
5	18:2-20:4	3.4±0.2	1.5±0.4	2.6±1.1
6	18:2-18:2	↓	↓	↓
7	16:0-22:6	4.9±0.5	19.1±2.2	3.4±1.0
8	16:0-20:4	9.7±1.5	10.5±1.6	4.4±1.4
9,10	16:0-18:2,18:1-18:2	12.0±1.5	8.5±1.5	20.5±2.7
11	18:0-20:5,16:0-22:5	4.6±0.7	5.1±0.9	4.1±1.4
12	16:0-18:1	ND	ND	41.6±4.6
13	18:0-22:6	11.7±2.8	14.2±4.5	↓
14	18:0-20:4	30.5±3.3	32.0±4.8	6.6±1.4
15,16	18:0-18:2,18:0-22:5	15.9±0.9	6.2±1.3	5.4±4.6
17	18:0-20:3,18:0-22:5	2.5±1.1	1.7±0.3	3.3±1.9
18	18:0-18:1	ND	ND	8.9±0.7

Values are expressed as the average of the indicated number of determinations ± S.D. Peak distribution was determined by pooling individual phospholipid species and quantitating lipid phosphorus as described under "Experimental Procedures".

[1]ND, not detected

[2]↓, indicates species mol% distribution was pooled with preceding value.

or monoenoic molecular species (293). TP-egg PE contains 60 mol% of 1-palmitoyl with oleate or linoleate in the 2 position.

3.5.2 In Vitro Molecular Species Specificity

Because of their molecular species composition, TP-egg PE and microsomal PE are excellent PE *N*-methyltransferase substrates for testing enzyme specificity. Table 9 shows the results of such experiments. When microsomes were incubated with [*methyl*-^{3}H]AdoMet, and the PC fractionated by HPLC, it was found that the % distribution of radioactivity did not parallel PC mol% distribution but was almost identical to that of microsomal PE (Table 8). A similar pattern was obtained when pure PE *N*-methyltransferase was incubated with microsomal PE and the PC molecular species analyzed. Methylation of TP-egg PE, PMME and PDME resulted in a pattern of labeling that was similar to the mol% composition of molecular species in egg PC. The one exception noted was an apparent preferential labeling of 1-palmitoyl-2-linoleoyl and 1-oleoyl-2-linoleoyl species of PC and PDME. These results indicated that PE *N*-methyltranferase shows little substrate specificity, and methylates molecular species of PE, PMME and PDME on the basis of their mol% within a mixture.

3.5.3 Methylation Rates with Synthetic Phosphatidylethanolamines

Specificity studies were extended to examine the rates of methylation of various synthetic PEs at saturating concentrations of 2 mM. First, it should be noted that while there was no selectivity for any given species of PE between microsomal- and TP-egg-PE, the former was methylated at about 3 times the rate (Fig. 35B). In addition, 1-palmitoyl-2-oleoyl PE (which makes up 41% of TP-egg PE) and TP-egg PE were methylated at approximately the same rate. Enhanced methylation of dilinolenoyl-, dilinoleoyl- and dioleoyl-PE compared to

Table 9. % Distribution of Labeled PC and PDME Molecular Species Synthesized by PE *N*-Methyltransferase *In Vitro*

	Substrate Methylated/Product Analyzed				
Peak Number[1]	Microsomes/PC[2] (n=3)	Microsomal PE/PC[3] (n=3)	TP-Egg PE/PC[3] (n=3)	PDME/PC[3] (n=3)	PMME/PDME[3] (n=4)
1,2	0.4±0.1	0.3±0.1	ND[4]	ND	ND
3	0.3±0.1	0.3±0.2	ND	ND	ND
4	0.4±0.1	0.5±0.1	ND	ND	ND
5,6	2.6±0.6	1.9±0.6	1.0±0.4	0.8±0.1	0.8±0.5
7	23.9±1.8	23.0±4.1	2.8±0.7	1.8±0.1	1.4±0.3
8	12.5±1.5	11.9±1.6	4.3±1.3	1.9±0.4	1.8±0.3
9,10	8.9±0.8	8.9±0.6	32.8±0.7	26.5±1.0	28.1±2.6
11	3.6±0.2	2.9±1.6	3.2±0.2	1.6±0.2	2.2±0.2
12	ND	ND	36.4±3.3	49.6±1.0	45.7±3.2
13	18.0±1.2	17.6±1.8	↓[5]	↓	↓
14	22.3±2.1	25.2±2.9	5.2±1.2	2.9±0.3	5.3±1.8
15,16	5.9±0.1	5.5±0.7	8.7±0.5	7.9±0.5	9.6±1.0
17	1.0±0.2	1.3±0.7	1.2±0.5	0.8±0.1	0.7±0.2
18	ND	ND	4.4±0.6	6.2±0.7	4.2±1.2
% Recovery	76.6±10.4	81.9±18.2	90.2±0.2	72.9±7.2	88.9±13.5

Values are expressed as the average of the indicated number of determinations ±S.D.

[1]Refer to Table I for molecular species identification

[2]Rat liver microsomes were incubated with [*methyl*-^{3}H]AdoMet and the labeled PC analyzed by HPLC as described in Section 2.3.1.

[3]Purified PE *N*-methyltransferase was incubated with purified microsomal PE, TP-egg PE, PMME, or PDME and the indicated product analyzed by HPLC as described in Section 2.4.

[4]ND, not detected.

[5]↓, indicates % distribution of label in species was included in preceding value.

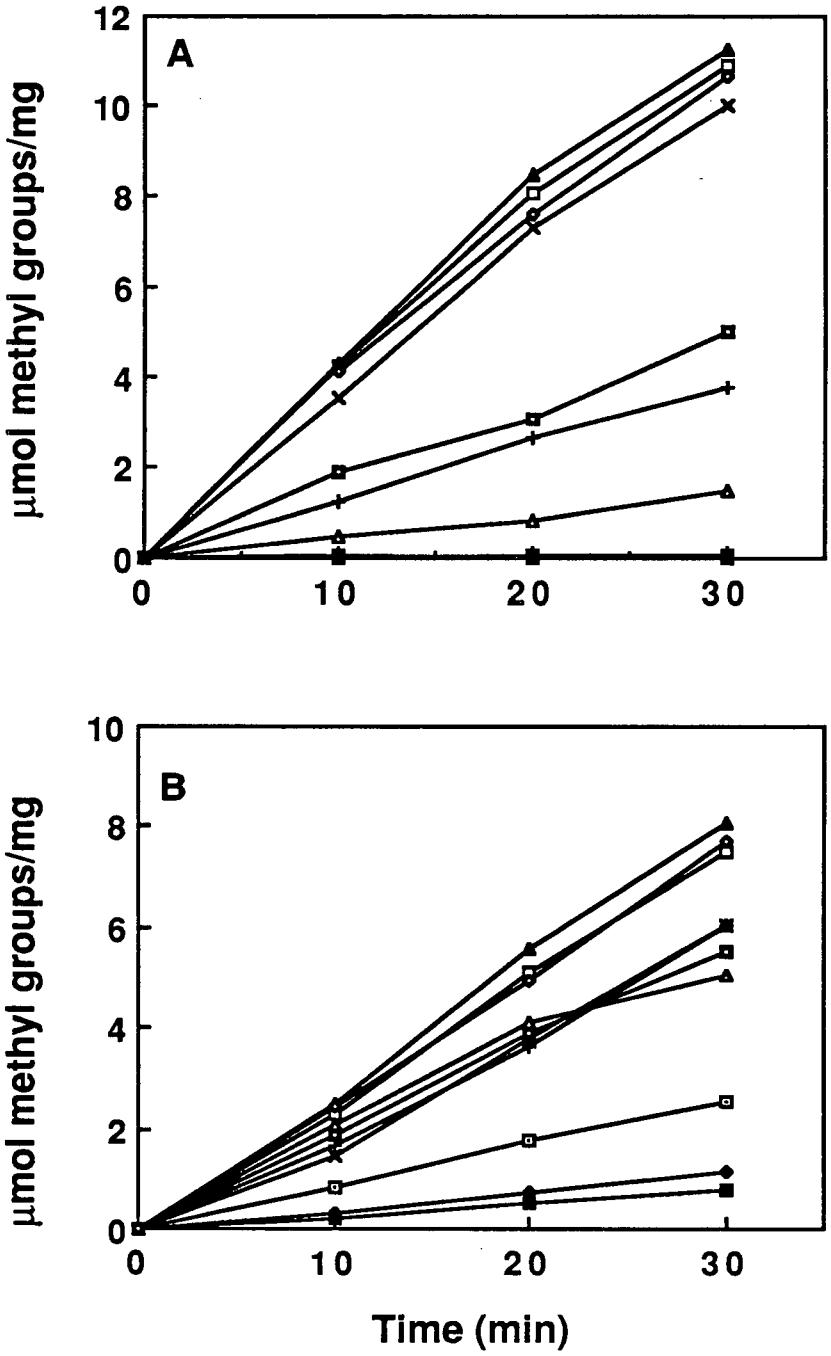

Figure 35. **Rates of methylation for various PEs using purified PE *N*-methyltransferase** A, PE *N*-methyltransferase (0.12 mg) was assayed with 2 mM microsomal-(◇), dilauroyl-(△), dimyristoyl-(⊡) dipalmitoyl-(◆), distearoyl-(■), 1-palmitoyl-2-oleoyl-(◘), dioleoyl-(✖), dilinoleoyl-(□), dilinolenoyl-(▲) and TP-egg-PE (✚) for up to 30 min. Product analysis revealed 90-95% of the label in PC in all cases. B, methylation rates were determined as described above except 40 mol% microsomal PC was included in the assay.

distearoyl-PE was observed, indicating that the introduction of double bonds in the PE molecule markedly enhanced methylation rates. There was only a 16% difference (average of 2 exp.) in methylation rates between the species containing 6 double bonds (dilinolenoyl) and the species containing two (dioleoyl). All the disaturated PEs tested had minimal activity, with the exception of dilauroyl PE. Dipalmitoyl, distearoyl and dimyristoyl were the only PEs tested that had phase transition temperatures (60-66^{o}C, 71^{o}C and 49.5-47.5^{o}C, respectively) above the assay temperature (293). This may indicate a preference by PE *N*-methyltransferase for substrate and bulk phase phospholipids in the liquid-crystalline phase.

Results presented in Section 3.2.4 indicated a role for boundary lipids in the modulation of PE *N*-methyltransferase activity. If this were true, methylation rates for the saturated and moneonic PEs tested in Fig. 35B should be affected in a positive manner by the inclusion of unsaturated PC. The results of this experiment are shown in Fig 35B. Dimyristoyl-, dipalmitoyl- and distearoyl-PE methylation was stimulated by the inclusion of 40 mol% microsomal PC. This stimulation was no doubt due to a fluidizing effect of the microsomal PC such that the PE-PC mixture assumes a T_m below the assay temperature of 37^{o}C. Stimulation of dilauryl-, 1-palmitoyl-2-oleoyl- and TP-egg-PE methylation is not due to a shift in T_m values but is a reflection of some structural requirement for lipids in the boundary layer of PE *N*-methyltransferase. The requirement would be for PE or PC molecules with more than one double bond in their acyl chains. In agreement with these observations, methylation rates for the more unsaturated PEs were not altered by inclusion of PC.

3.5.4 Molecular Species Specificity In Vivo

It could be argued that the lack of PE *N*-methyltransferase specificity *in*

Table 10. Distribution of [*methyl*-3H]Methionine-Labeled PC Molecular Species During a 24 Hour Chase.

Peak number	Time (hours) 0	2	6	12	18	24	mol% cellular PC
1,2,3	1.2±0.8	0.9±0.5	0.8±0.4	1.5±0.7	1.4±0.6	1.1±0.2	0.9±1.2
4	0.6±0.2	0.5±0.1	0.6±0.2	0.6±0.1	0.7±0.4	0.6±0.2	1.4±0.9
5,6	1.6±1.2	1.2±0.3	1.0±0.2	1.1±0.5	0.9±0.3	0.9±0.4	1.9±0.5
7	21.0±6.2	16.0±5.7	12.3±4.9	10.4±4.4	9.4±3.1	8.6±4.0	10.2±4.0
8	13.9±0.9	17.0±3.4	15.1±1.3	14.7±1.9	15.7±1.8	17.2±1.9	16.3±3.5
9,10	7.5±1.3	8.3±1.4	7.8±2.7	9.5±1.1	9.2±0.8	9.6±1.3	10.1±0.9
11	2.8±0.4	2.5±0.3	2.7±0.5	2.1±0.1	2.1±0.4	2.2±0.5	2.8±0.2
13	18.2±2.4	17.6±1.7	17.5±2.8	16.6±2.2	15.2±2.1	14.0±1.4	14.1±1.8
14	27.7±6.2	27.9±4.6	30.9±5.6	33.2±6.6	32.4±7.2	34.3±8.1	29.4±3.8
15	6.±1.4	6.8±2.6	9.8±2.1	8.5±2.6	11.3±1.3	10.0±1.5	10.9±1.4
16	0.8±0.4	0.8±0.4	1.1±0.4	1.2±0.4	1.2±0.1	1.2±0.3	1.6±1.1
%Recovery	78.9±5.0	86.7±9.1	87.2±4.6	76.3±11.0	81.6±3.6	83.6±6.4	79.2±6.4

Results are expressed as the average of 4 separate pulse-chase experiments ± S.D.

vitro using complex mixtures of PE was due to pH, detergent or buffer effects and does not represent true *in vivo* conditions. Hence, PE *N*-methyltransferase specificity *in vivo* was tested by pulsing monolayers of rat hepatocytes for 1 h with [*methyl*-^{3}H]methionine. Reverse-phase HPLC was used to monitor the fate of PC molecular species produced via methylation during a 24 h chase period. Table 10 shows the % distribution of radioactivity in PC molecular species over the 24 h chase period. At the end of the 1 h pulse, the distribution of PC species was very similar to the mol% distribution in microsomal PE (Table 8) and to the label distribution observed for microsomal PE methylation *in vitro* (Table 9). During the course of the chase, however, there was a progressive reduction in the % of label in 1-palmitoyl-2-docosahexaenoyl PC. Concomitant with this loss were less significant increases in 1-palmitoyl and 1-stearoyl species with oleate and arachidonate in the two position. By 24 h the % distribution of label in PC molecular species was almost identical to the mol% distribution in total cell PC (Table 10). HPLC elution profiles for PC molecular species at 0, 12 and 24 h reveal in a graphic manner the striking decay of label from 1-palmitoyl-2-docosahexaenoyl PC (Fig. 36). The loss of this species was not the result of preferential secretion, since % distribution of label in medium PC was identical to that in the hepatocyte from 6 to 24 h.

The remodeling of PE derived PC to conform to total cell molecular species composition is further demonstrated in Table 11. For simplicity only the 6 major species are shown. It is evident that immediately following the pulse period 1-palmitoyl-2-docosahexaenoyl PC had the highest specific activity, while species containing 2 and 3 double bonds had the lowest. Similar to % label distribution data, there was a decay in 1-palmitoyl-2-docosahexaenoyl PC and increases in the other species specific activity. The one exception being 1-stearoyl-2-docosahexaenoyl PC. It should be noted that

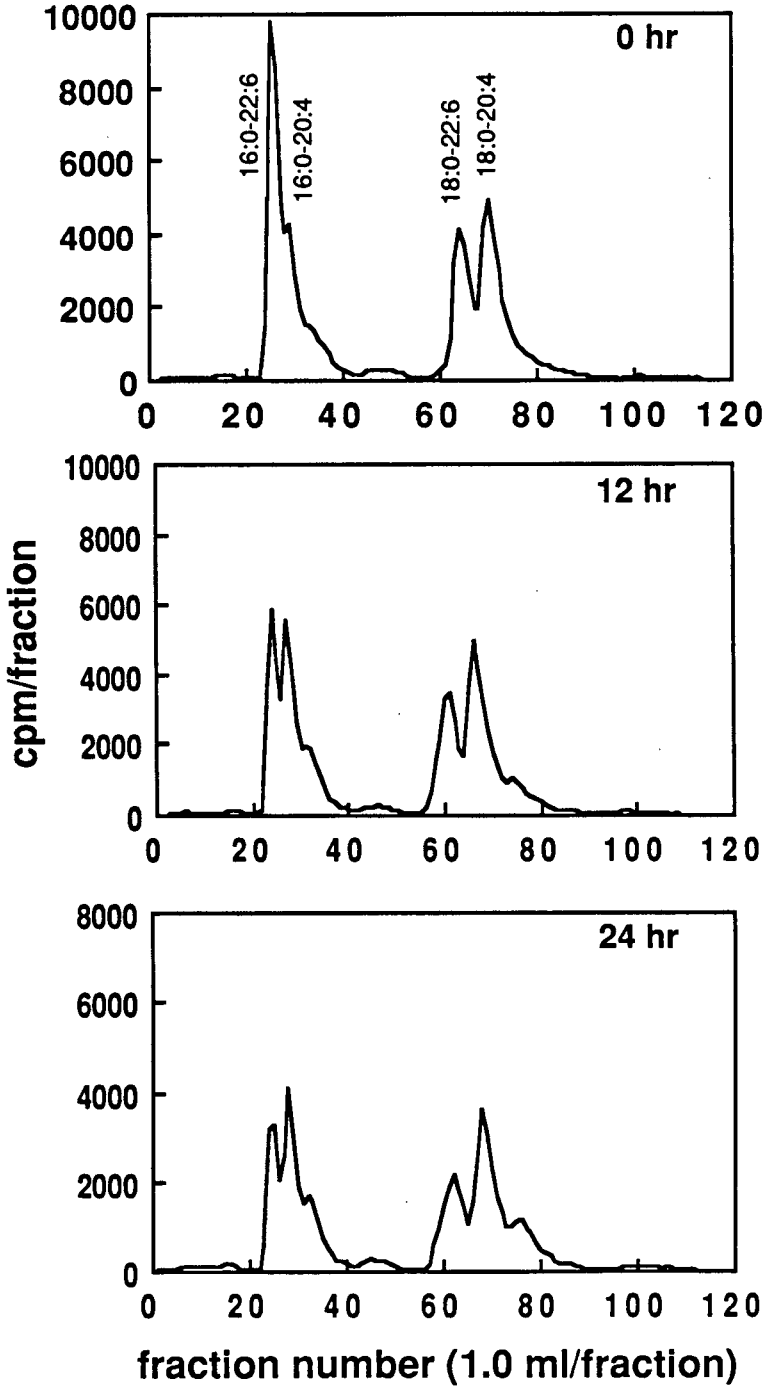

Figure 36. **HPLC elution profiles of [*methyl*-^{3}H]methionine-labeled cellular PC molecular species during a 24 hour chase period.** Cellular PC (90,000 cpm) was fractionated by HPLC, collected fractions allowed to evaporate to dryness and radioactivity was measured. Recoveries of radioactivity were 85-95%. Identification of the major molecular species is shown in the 0 h frame.

decay in 1-palmitoyl-2-docosahexaenoyl PC occurred even though total cell PC specific activity increased. The increases in 1-palmitoyl-2-arachidonyl- and 1-stearoyl-2-arachidonyl-PC specific activity followed closely the net increase in cell PC specific activity. At 24 h the specific activities of the 6 fractions were not significantly different, indicating that PE-derived PC is being remodeled to conform to the molecular species composition of total cell PC.

Table 11. Specific Activity of Hepatocyte [*methyl*-^{3}H]PC Molecular Species

Molecular Species	Time (hours) 0	12	24
	Specific Activity[1]		
16:0-22:6	1575±294	1194±36	899±83
16:0-20:4	627±58	753±180	912±54
16:0-18:2,18:1-18:2	501±144	609±296	806±59
18:0-22:6	823±330	894±303	813±114
18:0-20:4	774±41	932±294	931±63
18:0-18:2,18:0-22:5	331±73	669±205	735±128
Specific Activity of Total PC	619±30	750±47	857±7

[1] cpm/nmole PC (counting efficiency was constantly between 35 and 40%).

3.6 *In Vitro* Phosphorylation of Phosphatidylethanolamine *N*-Methyltransferase by cAMP-Dependent Protein Kinase.

3.6.1 Phosphorylation and Phosphoamino Acid Analysis

As an initial step toward determining if phosphorylation plays a role in PE *N*-methyltransferase regulation *in vivo*, its viability as a substrate for the catalytic subunit of cAMP-dependent protein kinase was tested *in vitro*. Fig. 37 shows an autoradiogram of phosphorylated fractions from the PE *N*-methyltransferase purification scheme separated by SDS-PAGE. Both microsomes (A and B) and microsomal membranes (C and D) contain a plethora of proteins phosphorylated by cAMP-dependent kinase or endogenous kinases.

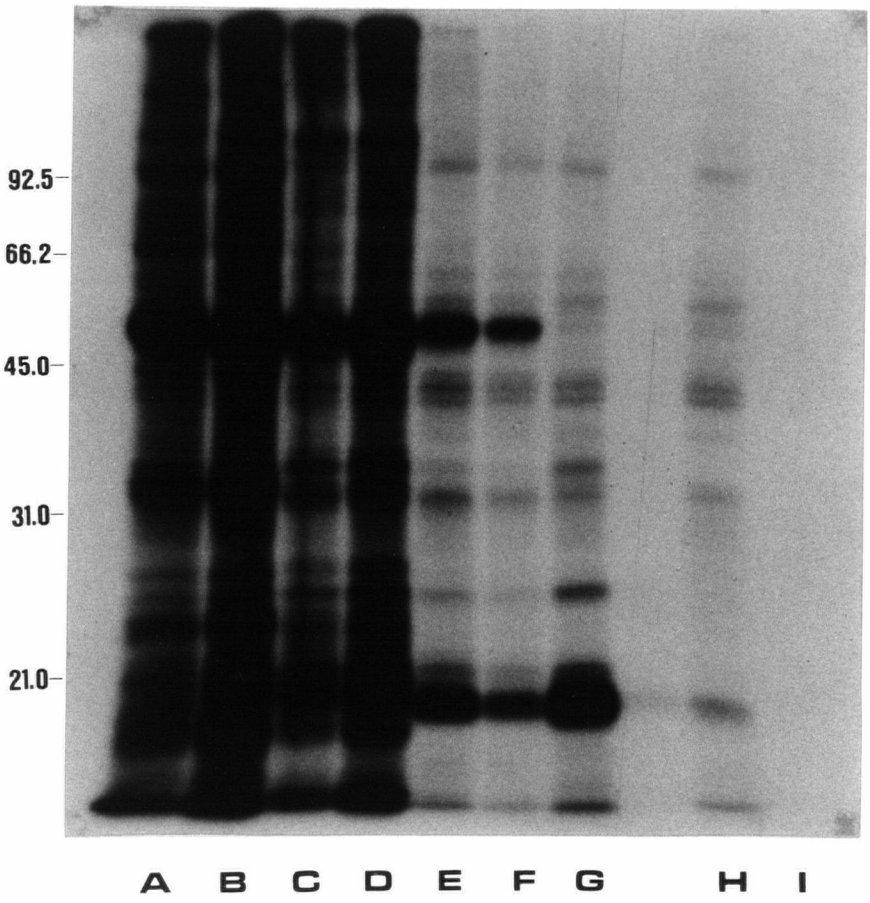

Figure 37. **Phosphorylation of partially purified and purified PE *N*-methyltransferase by cAMP-dependent protein kinase.** Samples were incubated with cAMP-dependent protein kinase for 60 min, separated by 10% SDS-PAGE, stained with Coomassie Blue and exposed to Kodak XAR-5 film for 18 h. Lane A, microsomes (25 μg); lane B, microsomes (50 μg); lane C, soluble microsomal membranes (25 μg); lane D, soluble microsomal membranes (50 μg); Lane E, octyl Sepharose step (1 μg); lane F, octyl Sepharose step (0.5 μg); lane G, Pure PE *N*-methyltransferase (0.45 μg); lane H, minus methyltransferase; lane I, minus kianse.

One of the most abundant is a 50 kDal protein. The octyl Sepharose fraction (E and F) contains both a 50 and 18.3 kDal phosphoprotein, while the purified enzyme (lane G) is composed of only the 18.3 kDal methyltransferase. The minor bands in lane G, with the exception of minor contaminantes at 25 and 30 kDal, are due to proteins in the kinase preparation since a control incubation (lane H) with no PE *N*-methyltransferase also had these contaminants. This figure not only showed that PE *N*-methyltransferase is a kinase substrate, but also revealed that the 50 kDal protein previously thought to be the methyltransferase (114,115,159,162) is a phosphorylated contaminant.

Phosphoamino acid analysis of the ^{32}P-labeled PE *N*-methyltransferase revealed that cAMP-dependent kinase phosphorylates on a serine residue (Fig. 38, lane A). Identity was based on both the mobility of authentic phosphoserine and of labeled phosphoserine from hydrolyzed histone (lane D). Hydrolyzed preparations containing only kinase or methyltransferase showed no phosphoamino acids (lanes B and C).

Phosphorylated PE *N*-methyltransferase was subjected to two-dimensional gel electrophoresis and the position of the phosphoenzyme isoproteins compared to the stained gel. The result of such an experiment is shown in Fig. 39 for 20 and 60 min incubations with cAMP-dependent protein kinase. Evidently, phosphorylation shifts both the major isoproteins and a third minor isoprotein to the anode on the NEPHGE gel, indicative of an increase in negative charge. Futile attempts were made to shift the mobility or alter the distribution of isoproteins by treatment with potato acid- or alkaline-phosphatase. This gives an early indication that the isoproteins are not the result of phosphorylation prior to or during purification.

3.6.2 Stoichiometry of Phosphorylation and Effect on Activity

Phosphate incorporation into PE *N*-methyltransferase reached a

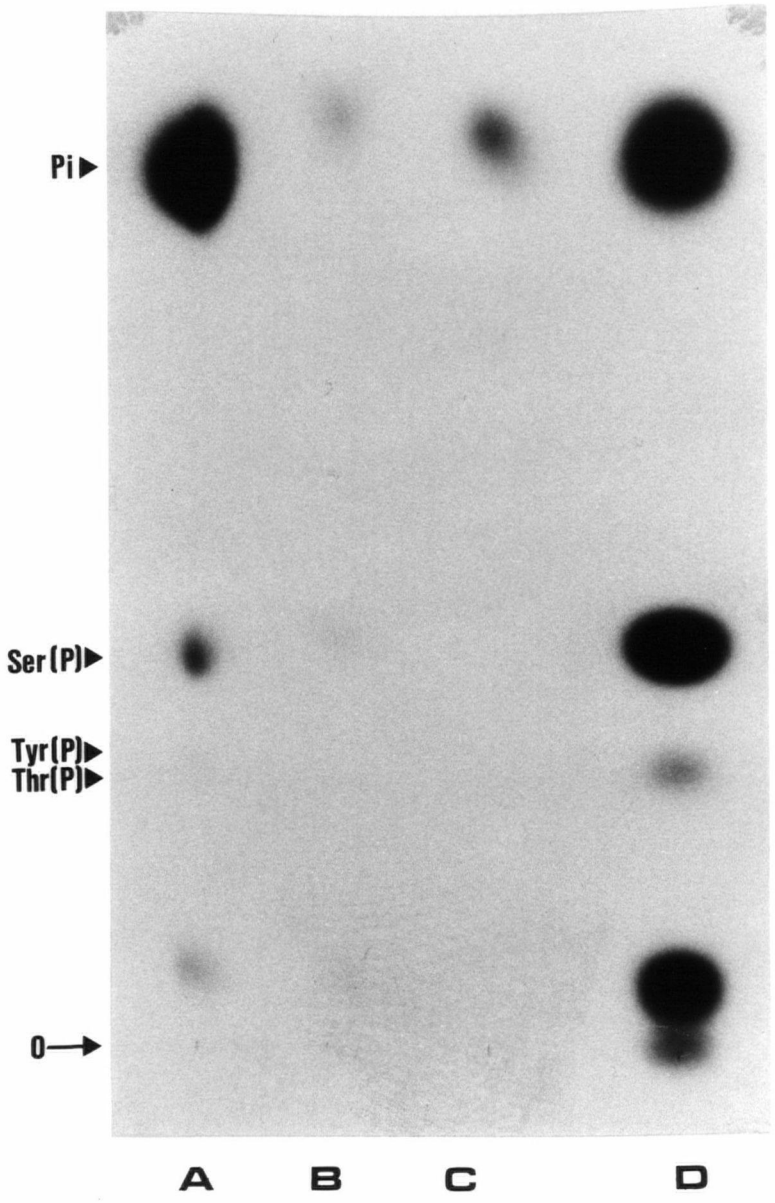

Figure 38. **Phosphoamino acid analysis of ^{32}P-labeled PE *N*-methyltransferase.** Purified PE *N*-methyltransferase (0.18 μg) was phosphorylated, hydrolyzed and subjected to TLE as described in Section 2.10.2. The TLE plate was exposed to Kodak XAR-5 film for 14 h. Lane A, PE *N*-methyltransferase plus kinase; lane B, PE *N*-methyltransferase only; lane C, kinase only; lane D, 20,000 cpm of phosphorylated, hydrolyzed histone. O, point of sample application.

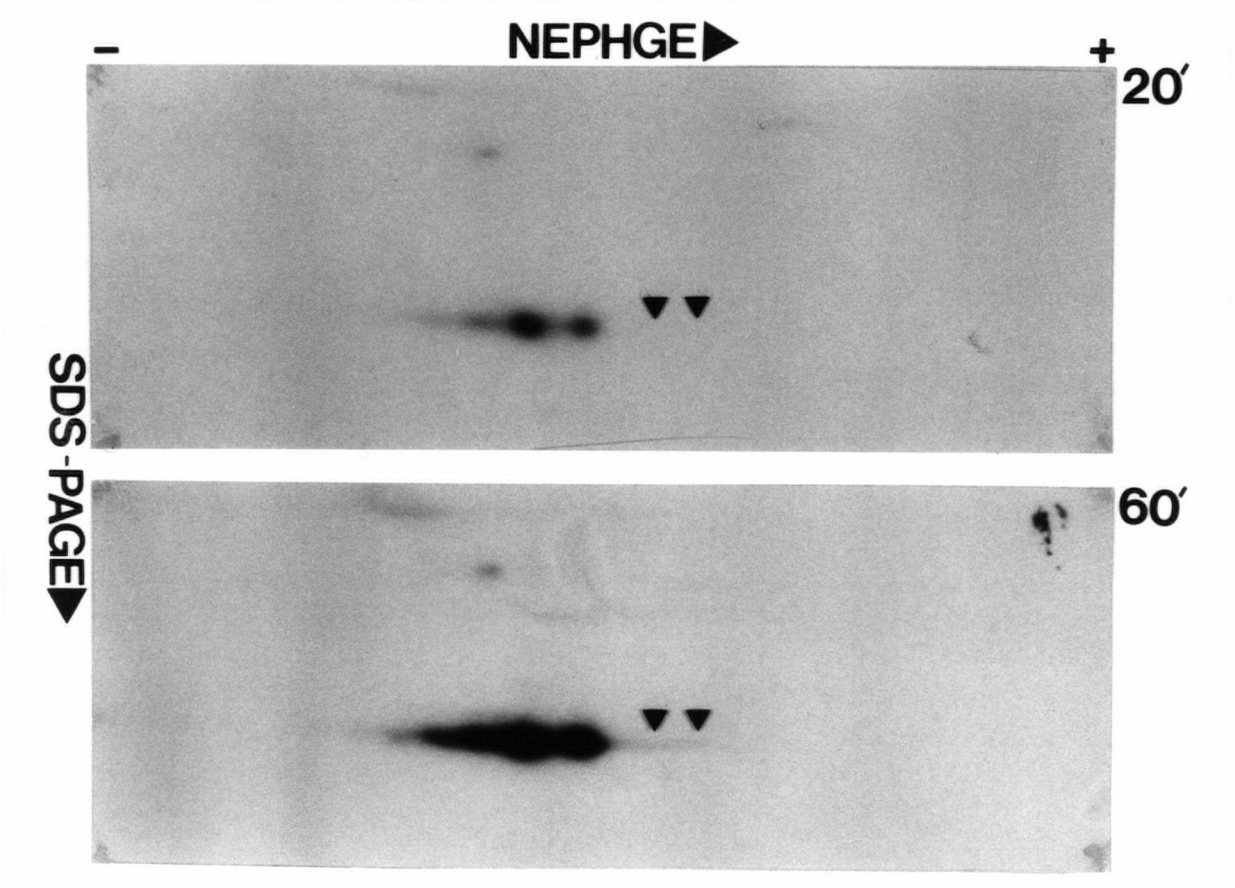

Figure 39. **Two-dimensional gel analysis of phosphorylated PE *N*-methyltransferase.** 0.39 μg of phosphorylated PE *N*-methyltransferase was subjected to two-dimensional gel electrophoresis as described in Section 2.5. The dried gel was exposed to Kodak XAR-5 film for 18 h. Triangles indicate the positions of the stained isoproteins.

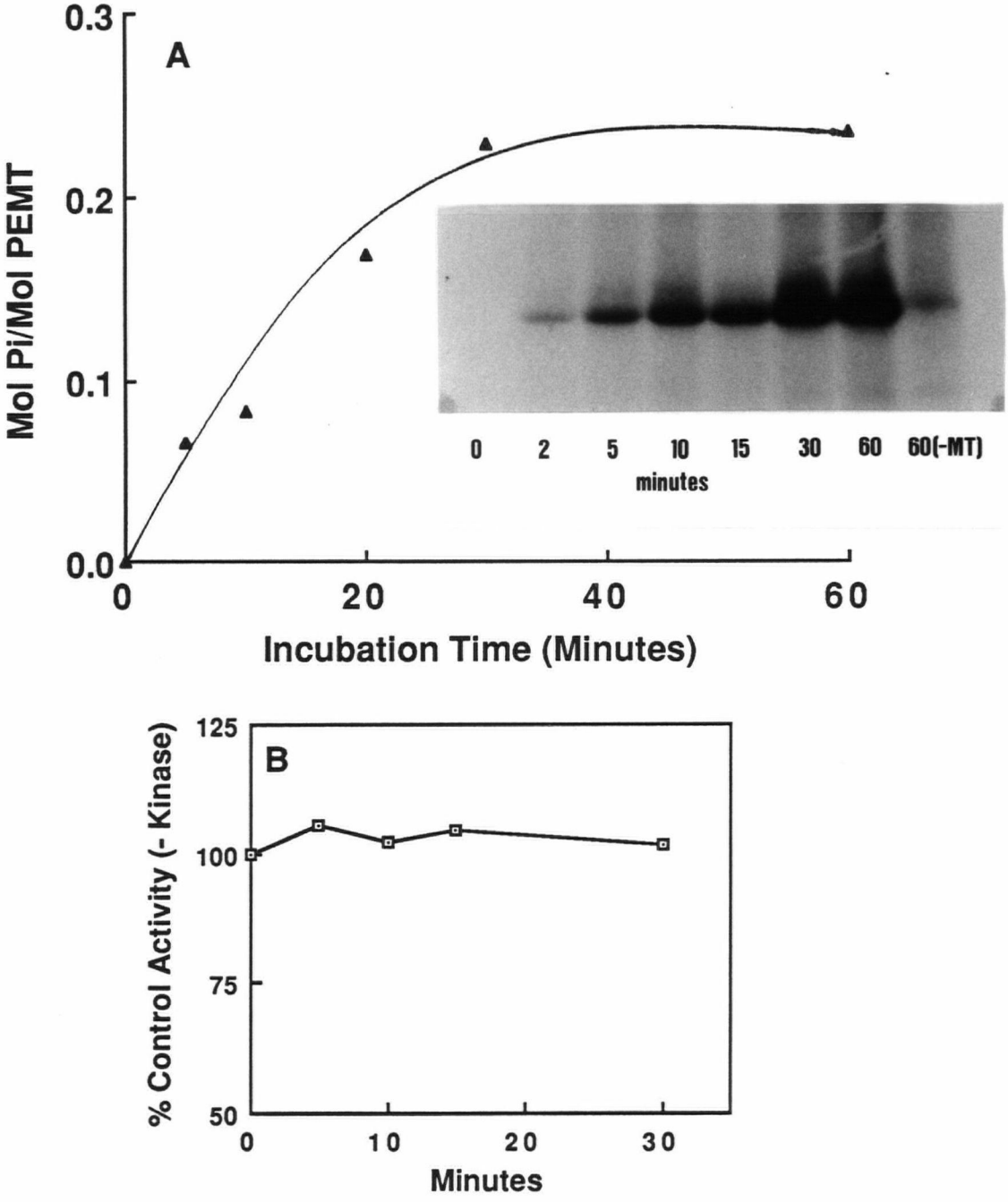

Figure 40. **Time course of PE *N*-methyltransferase phosphorylation *in vitro*.** *Panel A*, enzyme was phosphorylated with the catalytic subunit or cAMP-dependent protein kinase as described in Section 2.10.1. Radioactivity in the PE *N*-methyltransferase band was determined and expressed as mole phosphate/mole enzyme (average of four experiments). The insert shows a representive autoradiogram from an experiment in Panel A. *Panel B*, PE *N*-methyltransferase was phosphorylated for 30 min and then assayed for PMME-dependent activity. Identical results were obtained for PE-dependent activity.

maximum at 30 min with a stoichiometry of only 0.25 mol Pi/mol enzyme (Fig. 40A). The addition of PE, PMME or PDME had no effect on incorporation nor did varying the Triton X-100 concentration. Interestingly, no effect of phosphorylation was observed on enzyme activity (Fig. 40B insert) in the presence of saturating AdoMet and PMME. The same lack of effect was observed for PE-dependent activity.

3.7 Phosphatidylethanolamine *N*-Methyltransferase in Choline-Deficient Rat Liver.

3.7.1 Effect of Choline Deficiency on Activity and Mass

It has previously been reported that microsomes from choline-deficient rat livers were increased in PE *N*-methyltransferase activity about 2-fold (186,187). However, the reason for this elevation in activity had not been elucidated. Using new information on the enzyme assay and an anti-PE *N*-methyltransferase antibody, PE methylation in the choline-deficient rat was again addressed.

A 2-fold elevation of PE *N*-methyltransferase activity was observed in choline-deficient microsomal fractions when endogenous PE was a substrate (Table 12). However, when a saturating quantity of exogenous methyl acceptor (PMME) and 1.0 mM Triton X-100 was added the observed differences in activity between the choline-deficient and -supplemented liver microsomes were no longer significant. It is apparent that methylation activity is being affected by the level of substrate PE in the microsome. This observation was further corroborated by the reaffirmation (185) that choline-deficiency results in a slight elevation in hepatic PE (Table 13). PC levels were reduced in the deficient liver, and more importantly the PC/PE ratio was reduced from 1.88 to 1.22. A characteristic accumulation of triglyceride (185, 295) was also noted (23.9 ± 6.0 compared to 3.7 ± 0.9 µmol/g liver in choline-deficient and -

Table 12. Activity of PE *N*-Methyltransferase in Endoplasmic Reticulum (Microsomes) from Choline-Deficient (CD) and Choline-Supplemented (CS) Rat Livers

	Endogenous PE		Exogenous PMME	
	CD	CS	CD	CS
ER I	1.45±0.34	0.74±0.19	8.06±2.85	6.70±1.68
	(0.01<p>0.02)		(n.s)	
ER II	1.09±0.17	0.61±0.15	6.15±1.05	5.36±0.91
	(0.001<p<0.01)		(n.s)	

ER I and II were isolated by the method of Croze and Morre (297). Both CD and CS fractions were enriched 10-fold and had identical NADPH-cytochrome *c* reductase activity. Methyltransferase activity is expressed as nmol/min/mg±S.D. Results are the average of 4 determinations.
n.s., not significant.

Table 13. Phospholipid Levels in Choline-Deficient (CD) and Choline-Supplemented (CS) Rat liver Homogentates

	PE[1]	PC	PC/PE
CD	9.36±0.79	11.40±2.13	1.22
CS	8.13±0.82	15.27±1.02	1.88
	p<0.1	p<0.02	

[1]PE and PC levels are expressed as μmol/g liver±S.D. 55-60 g rats were maintained on deficient or 0.3% (w/w) choline supplemented diet for 3 days prior to sacrifice.

supplemented livers, respectively).

As further confirmation that enzyme activity was affected by substrate availability, the amount of methyltransferase protein in choline-deficient and -supplemented rat liver microsomes was determined by immunoblot analysis using a rabbit polyclonal anti-PE *N*-methyltransferase antibody. Fig. 41 shows an autoradiogram of a nitrocellulose blot of two different amounts of deficient and supplemented rat liver microsomes plus an enzyme standard. No difference in immuno-detectable mass was observed between choline-deficient or -supplemented microsomes. The molecular mass of PE *N*-methyltransferase (18.3 kDal) in microsomes was identical to that of the purified enzyme standard.

3.7.2 Activity and Mass in Choline- and Methionine-Deficient Rat Hepatocytes.

Choline- and methionine-deficient rat hepatocytes were cultured in the presence of choline or methionine, and changes in PE *N*-methyltranferase mass and activity were examined. Supplementation of deficient hepatocytes with 100 μM choline for a 4 h period resulted in a 12% decrease in activity when assayed with endogenous PE (Fig. 42A). Activity measured with saturating PMME was unchanged. During this time the cellular content of PC increased from 100 to 130 nmol/mg protein, while PE levels remained constant (Fig. 42B). With addition of 200 μM methionine, however, there was a 50% decrease in activity (Fig 43A) accompanied by a similar drop in cellular PE mass (Fig. 43B). Again, immunoblotting showed enzyme protein to be constitutive even though activity was halved upon methionine supplementation (Fig. 44). These results provide strong support for the role of PE in auto-regulation of PE *N*-methyltransferase in choline and methionine deficiency.

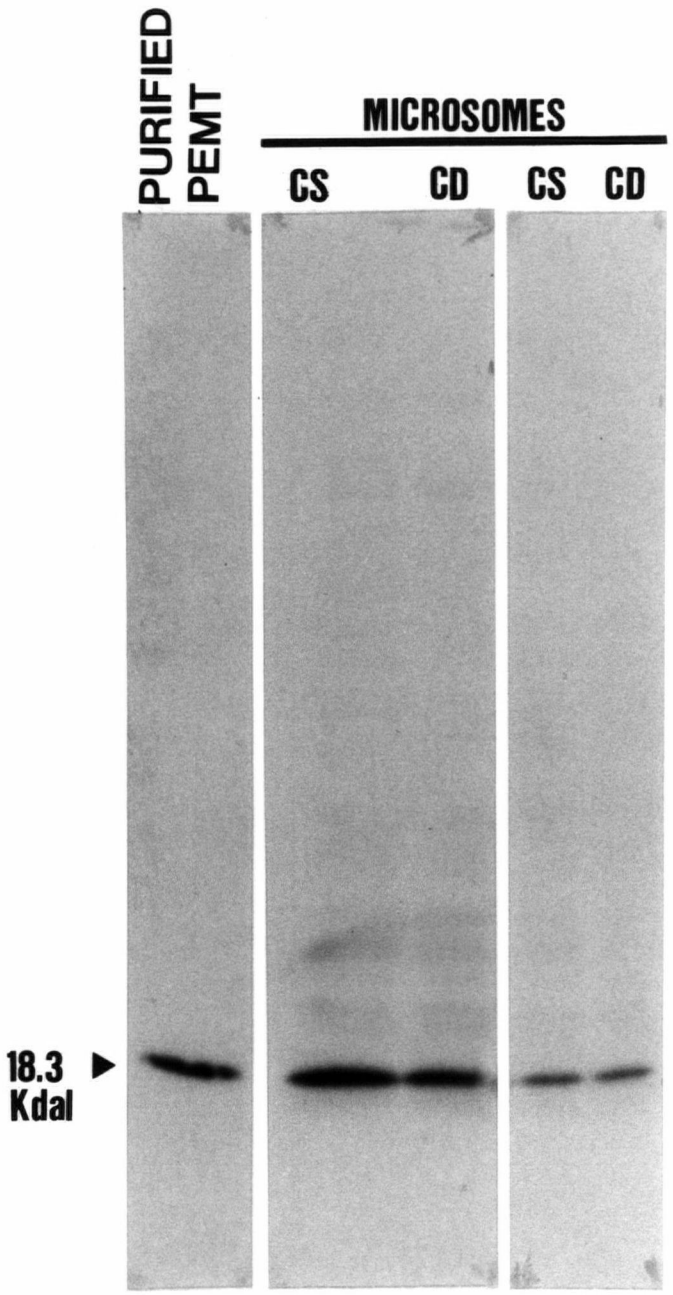

Figure 41. **Immunoblot of PE *N*-methyltransferase in choline-deficient and choline-supplemented rat liver microsomes.** 100 and 300 μg of microsomes were separated on 5-15% gels, transferred to a nitrocellulose membrane and probed with an anti-PE *N*-methyltransferase antibody. The autoradiogram was exposed for 6 h. A purified enzyme standard is shown. The recovery of microsomes from choline-deficient and choline-supplemented liver homogenates was 76.5±9.8% and 80.9±4.7% (n=3), respectively, based on NADPH-cytochrome *c* reductase activities (298).

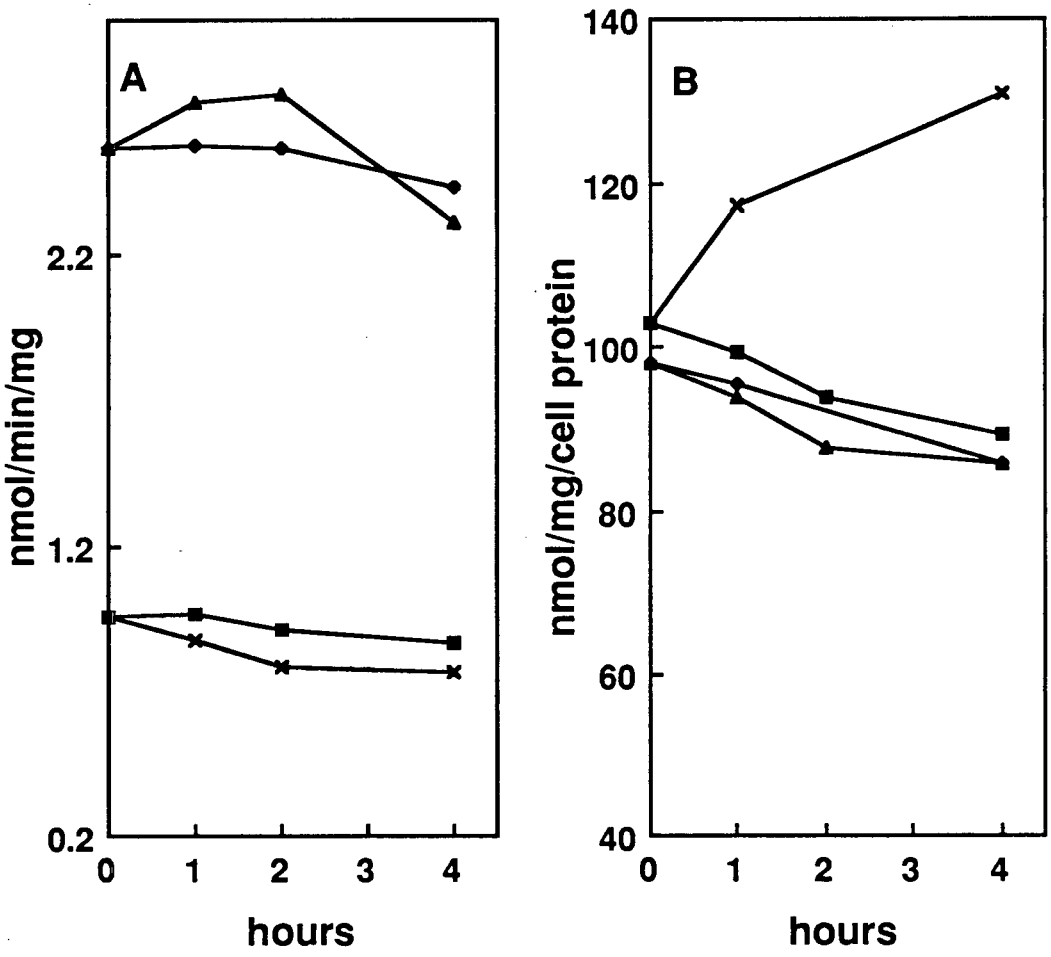

Figure 42. **Effect of choline supplementation on PE *N*-methyltransferase activity in choline- and methionine-deficient hepatocytes.** Panel A, at the indicated times hepatocytes were collected, homogenized and a membrane fraction isolated by centrifugation. PE *N*-methyltransferase activity was assayed for 20 min under the following conditions; ▲, choline-deficient plus PMME; ◆, choline-supplemented plus PMME; ■, choline-deficient with endogenous PE; ✖, choline-supplemented with endogenous PE. Panel B, hepatocyte PE and PC were extracted, separated by TLC and lipid phosphorous determined. ▲, choline-deficient PE; ◆, choline-supplemented PE; ■, choline-deficient PC; ✖, choline-supplemented PC.

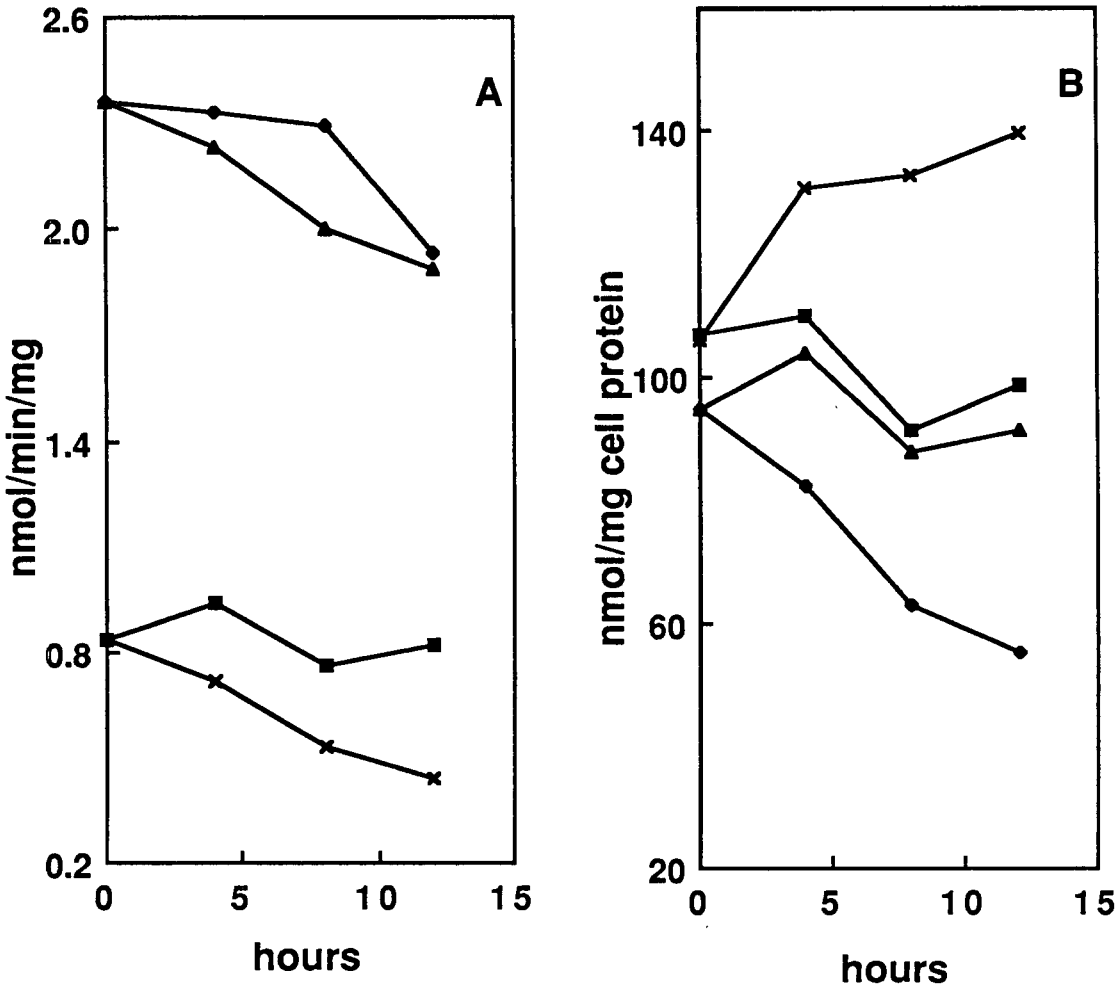

Figure 43. **Effect of methionine supplementation on PE *N*-methyltransferase activity in choline- and methionine deficient hepatocytes.** Panel A, at the indicated times hepatocytes were collected, homogenized and a membrane fraction isolated by centifugation. PE *N*-methyltranferase activity was assayed for 20 min under the following conditions; ▲, methionine-deficient plus PMME; ◆, methionine-supplemented plus PMME; ■, methionine-deficient with endogenous PE; ✖, methionine-supplemented with endogenous PE. Panel B, hepatocyte PE and PC were extracted, separated by TLC and lipid phosphorous determined. ▲, methionine-deficient PE; ◆, methionine-supplemented PE; ■, methionine-deficient PC; ✖, methionine-supplemented PC.

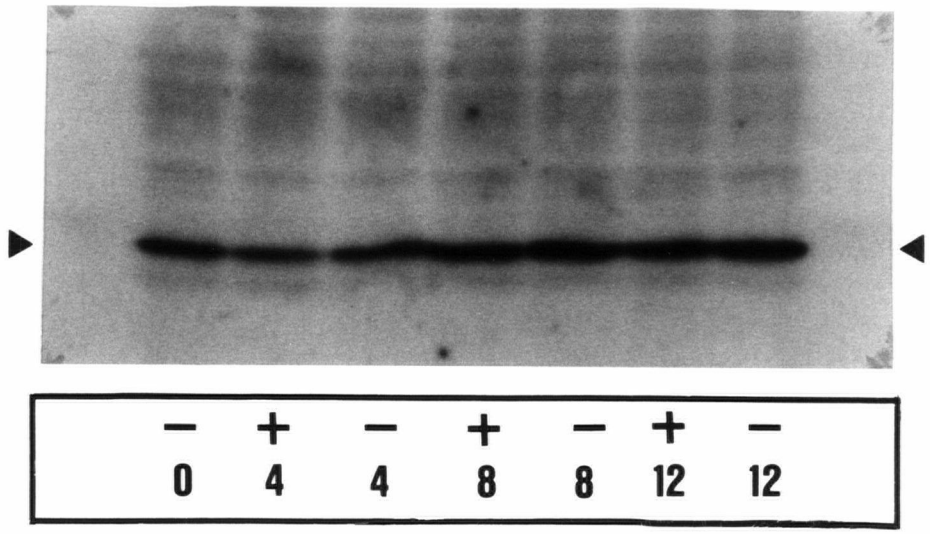

Figure 44. **Immunoblot of methionine-supplemented and methionine-deficient hepatocyte homogenates with an anti-PE *N*-methyltransferase antibody.** 200 μg of methionine-supplemented and -deficient hepatocyte membranes was probed with an anti-methyltransferase antibody. The nitrocellulose blot was exposed to Kodak XAR-5 film for 12 h.

3.8 *N*-Terminal Sequence Analysis of Phosphatidylethanolamine *N*-Methyltransferase

The first 30 *N*-terminal amino acids of purified rat liver PE *N*-methyltransferase are shown in Fig. 45. The sequence was identical for two separate determinations to residue 15. Residues 15-30 are from one determination and the chance of a misread is greater. Only 2 charged amino acids are observed in this sequence; the remainder being neutral or hydrophobic. Significant identity (35%) was noted between this sequence and the predicted *N*-terminal amino acid sequence of the 21 kDal *S. cerevisiae* PEM 2 (195). The sequence of rat liver enzyme started at residue 10 of the yeast sequence indicating possible post- or co-translational cleavage of some of the *N*-terminal amino acids. Homology (25%) between the rat liver *N*-terminal sequence and that of bovine phenylethanolamine *N*-methyltransferase (296) was also noted.

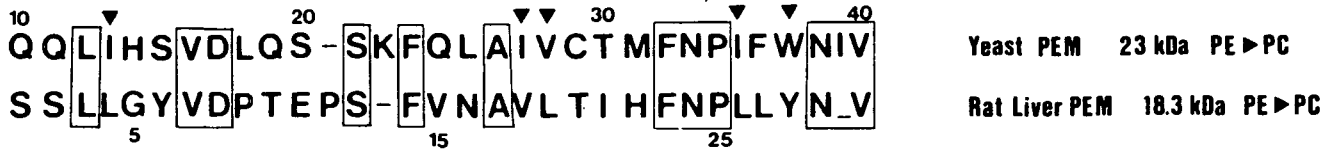

Figure 45. ***N*-terminal amino acids of rat liver PE *N*-methyltransferase.** Homology with the *S. cerevisiae* PEM 2 (195) *N*-terminal is indicated by the enclosed areas. Conservative substitutions are indicated by inverted triangles.

DISCUSSION

4.1 Purification and Molecular Properties

PE methylation is considered to be a minor route for the synthesis of PC in liver, but may be more important in situations where PC synthesis via the CDP-choline pathway is compromised (186,187). Alternatively, it has been demonstrated that factors that stimulate the CDP-choline pathway inhibit PE methylation (148,178,183,232,233). As a prelude to understand better the factors that regulate hepatic PC synthesis, PE *N*-methyltransferase was purified to apparent homogeneity from rat liver microsomes.

The 7 step purification scheme illustrates some properties of this enzyme. Contrary to previous reports from this laboratory (89), we were successful in using the nonionic detergent Triton X-100 as a solubilizing agent after substituting 20 mM potassium phosphate and 10% glycerol for the Tris HCl buffers used in other purification attempts. Purified PE *N*-methyltransferase showed no perceivable loss in activity for at least 2 months when stored in buffer A plus 0.1% Triton X-100 at 4°C. This is in sharp contrast to the 80% loss of activity reported for the partially pure enzyme after 16 h at 2°C (89).

A second property that became apparent during our purification efforts was that PE *N*-methyltransferase is a very basic protein. PE *N*-methyltransferase passed unretained through an anion exchange resin (PBE 94, step 7) at pH 9.4 and can only be resolved into two isoproteins when electrophoresised toward the cathode in a NEPHGE system. What relation this alkaline pI has to enzyme function is yet unknown. Interestingly, the *S. cerevisiae* PEM 2 is also a basic protein (195). This enzyme contains 23 basic residues (13 lysine, 7 arginine and 3 histidine residues) and only 14 acidic amino acids (9 glutamate and 5 aspartate residues). The pI of the native

purified enzyme has not been determined. Other *N*-methytransferases which possess basic pH optima (8.5-9.5) do not have basic pIs (299-303). Thus, the basic residues in PE *N*-methyltransferase may have a structural function, perhaps for charge-pairing with the phosphate groups in the surrounding phospholipids.

One apparent cytosolic domain (residue 46-61) of the yeast methyltransferase contains six basic residues and two cysteines. This may represent the catalytic domain since two cysteines have been implicated in methylation in the rat liver enzyme. The hypothetical role of these two cysteines in methyl transfer is shown in Fig. 46. This mechanism involves enhancement of the nucleophilicity of the PE amino group by general base catalysis. The two cysteines would abstract both amino group protons during PDME formation, and then, due to the electron-donating capacity of the two methyl groups on PDME, final methylation would occur without general base catalysis. General base catalysis has been suggested to be the probable mechanism in enzymatic *O*-methyltransferase reactions (304,305). This conclusion was based on studies using various compounds that undergo intramolecular transalkylation (305a-305c). An example of such a reaction is shown in Fig. 47. The intramolecular reaction rate shows a strong pH dependence above 9.0, indicative of ionization of the nucleophilic *cis*-alcohol. Also, the reaction is catalyzed by oxyanion buffers (phosphate), but not by amines, which acted as nucleophiles and abstracted the sulfonium methyl group (305a). An ionized cysteine residue (in the methyltransferase active site) could act as a general base catalyst in much the same way as phosphate. Most of the *N*-methyltransferases examined possess essential cysteine residues, but their exact role in catalysis has not been elucidated (299-303).

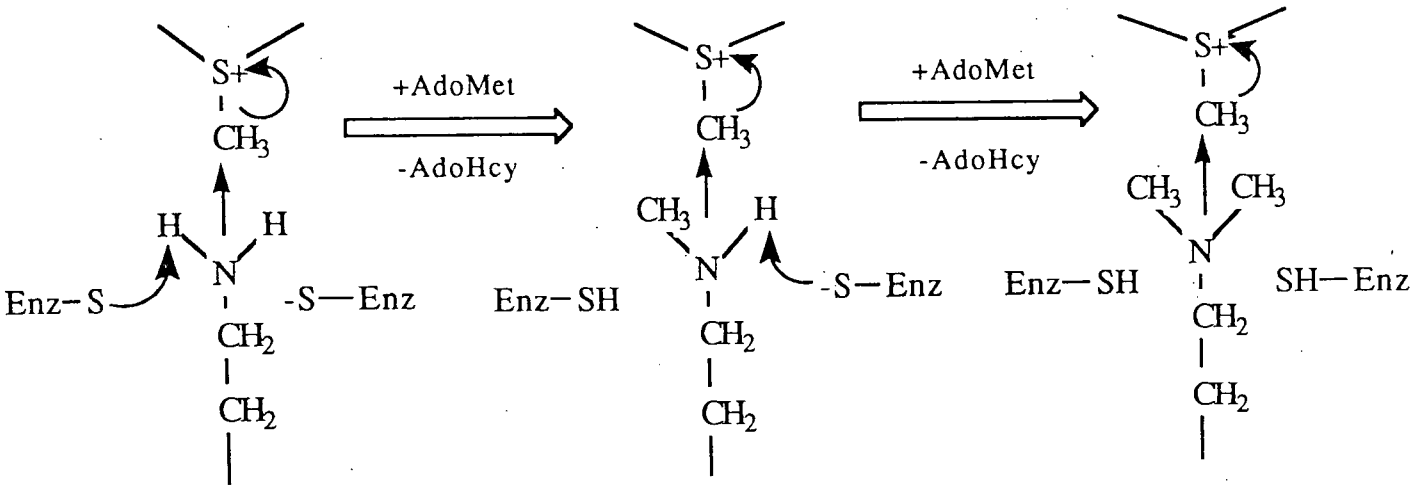

Figure 46. **Hypothetical catalytic mechanism for PE *N*-methyltransferase.**

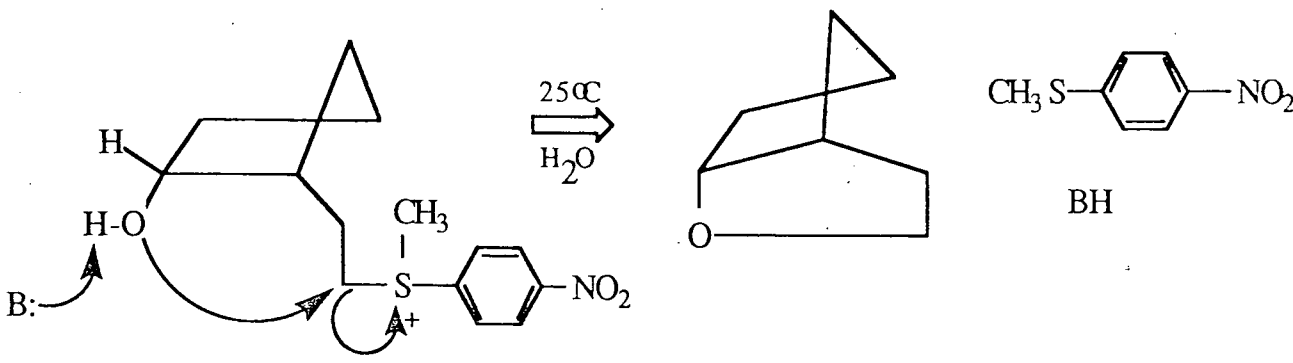

Figure 47. **Intramolecular transalkylation of 1-(2-methoxycyclopentyl)-2-*p*-nitro-methylsulfoniumphenylethane.** The Figure was adopted from Ref. 305.

It is feasible that the cysteines are not involved directly in the catalytic mechanism but have some regulatory role. Short term regulation of PE methylation could be achieved by thiol-disulfide exchange mechanisms in the cell by a variety of biologically active disulfides. Such a mechanism has been proposed for regulation of rat liver guanidoacetate *N*-methyltransferase (302).

The extremely alkaline pH optimum of PE *N*-methyltransferase has long been a point of contention. Assay of activity *in vitro* consistently revealed a pH optimum between 9 and 10.5 (84,87-89) for the methylation of PE, but workers still insist on assaying at 'physiological' pH (7.4) or at values between 8.0 and 8.5 (refer to Table 1 for specifics). The alkaline lability of AdoMet has often been used as an excuse to assay at suboptimal pH, but this premise seems unfounded (88). Activity measurements at 7.4 would be credible if measurements were at the same time done at pH 9.5-10.0.

Why both the microsomal and purified methyltransferase have such alkaline pH optima is unknown. Does the enzyme within the intact hepatocyte also function at an alkaline pH and could pH act to regulate enzyme activity? These are questions that at this moment remain unanswered. Interestingly, the ethanolamine headgroup of PE would have a pKa in the range of the PE *N*-methyltransferase pH optimum (pKa of the ethanolamine amino group is 9.5). This indicates that the deprotonated species of PE maybe the active nucleophile involved in methyl group abstraction from AdoMet. Activity does increase beyond pH 9.5, as would be expected if activity were related to the concentration of unprotonated PE, and then declines sharply (Fig. 7), maybe as the result of protein denaturation. Alternatively, the pH optimum could reflect the ionization state of amino acids in the enzyme (cysteines or basic groups) involved in catalysis. Since the amino group pKa for PE in

endoplasmic reticulum membranes and of catalytic residues in the enzyme has not been determined, the dependence of activity on these factors is unknown.

In a highly speculative vein, perhaps PE *N*-methyltransferase is localized in a portion of the endoplasmic reticulum where hydroxide ions are concentrated as the result of ion translocation processes. The bulk cellular pH is perhaps the sum of that in various microenvironments.

The subunit molecular mass (18.3 kDal) of PE *N*-methyltransferase is unusually small considering the fairly complex series of methylation reactions it catalyzes. Analysis of the pure enzyme in Triton X-100 micelles by gel filtration indicated that a single subunit is present per micelle. No information is yet available concerning the subunit structure in phospholipid membranes. Low molecular masses have been reported for several other phospholipid biosynthetic enzymes: the 34 kDal phosphatidylinositol synthase (306) and 23 kDal phosphatidylserine synthase (282) purified from *S. cerevisiae* and the 13.2 kDal diglyceride kinase (307) and 27 kDal CDP-diglyceride synthetase (308) from *E.coli*.

Two reports by Pajares *et al.* (114,115) have claimed purification of PE *N*-methyltransferase from rat liver. These researchers have co-purified PE *N*-methyltransferase activity with a 50 kDal protein. The specific activity of this preparation (assayed in the presence of a mixture of PE, PMME and PDME) was 0.27μmol/min/mg protein (115). Based on the data presented herein, several lines of argument would indicate that the 50 kDal protein bears no relation to PE *N*-methyltransferase. First, we have achieved final specific activities with PE, PMME and PDME as substrates that are 2.3-, 32- and 14-fold higher, respectively, than that reported by Pajares *et al.* (115). Although these authors assay PE *N*-methyltransferase activity at 20°C, pH 8.35 and 100 μM AdoMet, all of which are suboptimal assay conditions in our hands, direct

comparison would indicate a substantially greater purification in our case. Second, examination of Fig. 8 and Table 3 would indicate that the 50 kDal protein is the major protein in partially purified fractions (steps 2-5), but is less abundant in step 6 relative to the 18.3 kDal protein and completely absent in the pure fraction. Third, a polyclonal anti-PE *N*-methyltransferase antibody raised against the purified enzyme recognized only the 18.3 kDal methyltranferase in liver microsomes (Figs. 11 and 41) and in hepatocyte membranes (Fig. 44). No cross-reactivity with proteins of 50 kDal was observed. Fourth, *N*-terminal sequence of the rat liver methyltransferase was 35% homologous with PEM 2 of *S. cerevisiae,* an enzyme of similar molecular mass which catalyzes an identical reaction. Fifth, experiments indicated that a microsomal methyltransferase of 19 kDal was the major protein photolabeled by [*methyl*-^{3}H]AdoMet. The molecular mass of this photolabeled protein was identical to the purified microsomal PE *N*-methyltransferase and no proteins of 50 or 25 kDal were photolabeled. These five points taken together indicate that the 50 kDal protein is only a persistent contaminant and that a microsomal protein of 18.3 kDal is PE *N*-methyltransferase.

The distribution of products of PE, PMME and PDME methylation was observed to remain constant throughout the purification of the microsomal enzyme. As with the microsomal enzyme, the major product of PE and PMME methylation by the purified enzyme was PC and PDME, respectively. Another interesting observation from Table 4, that is not immediately apparent, was that PMME appeared to inhibit the formation of PC from endogenous PE in microsomes. Implications for a single active site for phospholipid substrates and the competition among them for this site is discussed further in Section 4.3.

The time course of product formation from PE and PDME (Fig. 6) using the purified enzyme is similar to that reported for microsomes (88). The time course for the formation of PMME methylation products revealed a gradual increase in the formation of PC and reduction of PDME formation. As the concentration of newly formed PDME approaches 0.04 mM in the assay, it competes efficiently with PMME for methylation, resulting in enhanced PC production. These results would seem to indicate that PMME and PDME compete for methylation by PE *N*-methyltransferase. This result was corroborated by kinetic analysis (Section 4.2). It is interesting to note that, like methylation of microsomal PE, methylation of pure microsomal PE by purified PE *N*-methyltransferase results in the formation of PC (92%). Clearly, PE methylation is the rate-limiting step in the reaction sequence and PE does not compete with and release the two partially methylated intermediates.

4.2 Kinetic Properties of Phosphatidylethanolamine *N*-Methyltransferase

A common problem encountered in the assay of PE *N*-methyltransferase has been the efficient delivery of lipid substrates to the enzyme. Previously, exogenous lipid substrates have been added to assay mixtures as vesicle preparations with no detergent. Under these conditions, enzyme activity would be limited by the rate of exchange of vesicle phospholipids into microsomes. PE *N*-methyltransferase assayed in the presence of Triton X-100 has been reported to be activated by PMME and PDME (86). Tanaka *et al.* (86) also reported that exogenous PE was methylated by Triton X-100 solubilized microsomes. Here, a simple Triton X-100 mixed micelle assay has been developed that can be used to assay all three methylation activities. The addition of increasing concentrations of surfactant inhibited methylation of PE, PMME and PDME by dilution of phospholipid on the micelle surface. This inhibition was found to be independent (displaying apparent first order

kinetics) of the concentration of mixed micelles when a fixed mol% of phospholipid substrate was used. The apparent first-order kinetics with relation to micelle concentration indicated that methylation is an intramicellar reaction. The linearity of PE, PMME and PDME methylation (Fig. 5), and more importantly the absence of an initial lag in rates, indicates that exchange of phospholipid between micelles is not rate-limiting.

Optimal PE methylation, unlike PMME or PDME, occurs at a mol% where non-micellar structures no doubt exist. It is conceivable that pure PE, rather than PE in a micellar form, is more readily methylated by PE *N*-methyltransferase. Pure PE in solution is proposed to exist in a hexagonal II array (309). This is probably not the case in our assay where the pH is >9.0 and 12 mol% of Triton X-100 is present. Both pH >9.0 (59) and a Triton X-100/PE ratio of 0.1 or 9 mol% Triton X-100 (280) favour the formation of bilayer structure. Robinson and Waite (310) reported a lysosomal phospholipase A_2 that preferentially hydrolyzed PE in what appears to be hexagonal II phase. PC, PI and phosphatidylglycerol underwent maximal hydrolysis when in mixed micelles. The propensity of PE *N*-methyltransferase for PE bilayers versus micellar structures is quite intriguing and requires more study in a reconstituted system free of detergent.

Catalysis by purified PE *N*-methyltransferase in PE-, PMME- or PDME-Triton X-100 mixed micelles has been systematically studied to determine the kinetic mechanism of PE methylation. During the construction of initial velocity curves it became apparent that PE, PMME and PDME methylation, either at fixed Triton X-100 or phospholipid, was sigmoidal and cooperative in nature (Fig. 15-19). There could be a variety of reasons for this type of response; a noncatalytic binding site that interacts with the catalytic site, multiple interacting catalytic sites on the same subunit or inhibition by Triton

X-100 that is relieved when its mol% in micelles is reduced. Theoretical and practical experimentation has lead to the formulation of a model which explains kinetic cooperativity in terms of non-cooperative binding of boundary- or annular-lipid to integral membrane enzymes (287,289,311). Electron spin resonance studies have confirmed that PC molecules bind to the (Na^+-K)ATPase at multiple non-interacting sites (312), and binding is sensitive to the lipid headgroup charge (313,314). Sarcoplasmic reticulum Ca^{2+}-ATPase activities were reported to be sensitive to the fatty acid chain length of PC in membranes in which it has been reconstituted (315). These studies indicated that integral membrane enzymes required phospholipid fatty acyl chains and/or polar head groups localized in a boundary lipid layer. If even 1-5 of the 20-100 (287,289,316) lipid binding sites of integral membrane proteins are unoccupied, catalytic efficiency could be greatly diminished. PE *N*-methyltransferase would seem to fall into this catagory of enzymes, judging from the sigmoidal velocity curves for PE, PMME, and PDME methylation and Hill kinetics shown in Figs. 16-18 and 20. The effect of egg PC on PE *N*-methyltransferase activity at nonsaturating concentrations of PE, PMME and PDME indicates occupation of the boundary layer by non-substrate phospholipid is sufficient for activation. Triton X-100 is not an activator of PE *N*-methyltransferase, and it exerts inhibition both in terms of classic surface dilution and by occupying phospholipid binding sites in the boundary layer. In this regard, Triton X-100 does not have the structural features necessary for PE *N*-methyltransferase activation. This two-fold inhibition could explain why a high mol% of PE, PMME or PDME is required for full activity.

The structural features of phospholipids required for activation of PE *N*-methyltransferase have not been systematically evaluated. It was observed that monoenoic PEs were methylated at a lower rate than species containing

two or more double bonds (Fig. 35). At the same time the enzyme exhibited no molecular species specificity. This was interpreted to indicate that monoenoic- and saturated-PEs occupied the enzymes boundary layer and there affected methylation rates in a negative fashion by influencing enzyme conformation. This interpretation is supported by the increased PE methylation rates, particularly of the saturated species, upon inclusion of 40 mol% microsomal PC. The effect of other phospholipids and cholesterol on methylation rates in mixed micelles has not yet been examined. It is feasible that the anionic phospholipids, PI and PS, could charge-pair with basic residues on PE *N*-methyltransferase and provide a more suitable lipid boundary layer. Interestingly, it has been reported that reconstituted *S. cerevisiae* PS synthase is modulated by the PI/PS ratio in PC and PE containing vesicles (317).

Whether positive cooperativity prevailed or not (as when egg PC was present), analysis of PE *N*-methyltransferase initial velocity and product inhibition patterns were reproducible and fit the mechanism proposed in Fig. 28. The more salient features of this mechanism are: (i) PE and the two intermediates compete for a common active site, (ii) separate methylation of PMME or PDME follows an ordered Bi-Bi mechanism and (iii) PE methylation to PMME is rate-limiting; a conclusion supported by the product distribution data discussed in Section 4.1 and comparison of K_m, $Vmax$ and inital rate values for PE, PMME and PDME. Now, a more systematic analysis has shown conclusively that PMME and PDME compete with one another for a common active site. Inhibition of PE methylation by PMME and PDME was demonstrated, but we cannot say with complete certainty that it is competitive. Key to understanding the kinetic mechanism is individual analysis of PMME and PDME methylation, and fit of initial velocity- and product inhibition-patterns to an ordered Bi-Bi mechanism. During concerted methylation of PE, both

mechanisms are linked and neither PMME or PDME diffuse from the active site. As with any concerted pathway it is advantageous for the rate-limiting step to be the first. This allows for control over the accumulation of intermediates (which could be potential feed-back inhibitors) and prevents unnecessary expenditure of substrates on a partial process. There is compelling evidence that PE methylation is regulated by AdoHcy levels *in vivo* (144,146,148,149), but it now appears that PC plays no such role, at least for catalysis in mixed micelles. It is feasible that at high concentrations of PC (>40 mol%) surface dilution of substrate would occur.

In conclusion, evidence has been presented that hepatic PE methylation is catalyzed by a single enzyme and occurs via a concerted mechanism. *Apparent* cooperativity related to the binding of substrate phospholipids and nonsubstrate PC in Triton X-100 mixed micelles, and structural requirements by the enzyme with regard to activation, could have some relevance to regulation of PE *N*-methyltranferase *in vivo*.

4.3 Molecular Species Specificity *In Vitro* and *In Vivo*

This work is the first report on the specificity of pure PE *N*-methyltransferase for molecular species of PE and to extend *in vitro* observations to cultured rat hepatocytes. From these studies we can conclude that the enzyme does not display specificity for molecular species of PE, PMME or PDME. The molecular species of these lipids are methylated according to the mol% in the membrane or micelle. This result is obtained with microsomal enzyme, pure enzyme or in intact hepatocytes. Experiments on PE methylation rates showed that the PE *N*-methyltransferase is affected by the fluidity of the lipid environment. The enzyme is inactive in a gel phase lipid environment, such as dipalmitoyl PE, and its activity in the liquid-crystalline phase is

proportional to the degree of unsaturation of the activating and substrate lipids.

The estimated 20% of total cellular PC produced by methylation in hepatocytes (68) is small and its function is unknown. An attractive hypothesis regarding methyltransferase function would be to supply polyunsaturated-rich PC to the cellular pool. This would depend both on the molecular species composition of microsomal PE and on the specificity of the methyltransferase. Previous reports, which utilized isotopic labeling of hepatic PC and PE in whole rats and molecular species fractionation by argentation TLC, generally concluded that tetraenoic or hexaenoic PC were the major products formed by methylation (117-119). The conclusions presented herein, though more specific in molecular species identification, are in agreement with an enrichment in docosahexaenoate containing species at the expense of more saturated species. The content in microsomal PE and PC of arachidonate containing species with palmitate and stearate in the *sn*-1 position are very similar so methylation would not enrich the PC pool significantly in these species. In this regard, results herein showed no enrichment in PC tetraenoic species as was alluded to previously (117,118).

Studies on PE *N*-methyltransferase in rat brain reached conclusions similar to those for liver. PDME, an intermediate in the methylation process, was reported to a have a fatty acid composition similar to its precurser PE (318). *In vitro* labeling of synaptosomal PC (121) and *in vivo* labeling of whole rat brain PC (122) with [*methyl*-^{3}H]AdoMet and [*methyl*-^{3}H]methionine, respectively, indicated preferential synthesis of PC rich in tetraenoic, pentaenoic and hexaenoic species. PE methylation activity in brain is 100- to 500-fold lower than liver, and the contribution to net PC synthesis is very small.

Reverse-phase HPLC offers a distinct technical advantage over argentation TLC in that individual species, and not pooled fractions, can be identified. Using this technique to identify the PC molecular species products formed from two complex mixtures of PE, no difference in % distribution of label relative to the abundance of a particular substrate PE could be identified. Similar results using PMME, PDME and microsomes as substrates lend credence to the proposal that PE *N*-methyltransferase shows little specificity with regards to acyl chain length and degree of unsaturation. As shown previously, choline- (120) and fatty acid-deficiency (117) caused marked alterations in the molecular species composition of hepatic PE and PC. In both instances, PC formed by methylation reflected the molecular species in precursor PE.

The lack of molecular species specificity using pure enzyme and crude microsomes was also observed *in vivo* using monolayers of rat hepatocytes. The % distribution of label in PC molecular species immediately following the [*methyl*-^{3}H]methionine pulse period was almost identical to the mol% distribution of PE in microsomes. An interesting finding in the hepatocyte studies was the rapid remodeling of PE derived PC such that by 12 h it no longer resembled PE in its molecular species composition, but was very similar to that of cellular PC. The molecular species that turned over most rapidly was 1-palmitoyl-2-docosahexaenoyl PC. Work in whole animals (117,120) has also shown that a methyl-labeled hexaenoic fraction of PC decayed to a new steady-state by 5-6 h. In the present study 1-stearoyl-2-docosahexaenoyl PC showed only a 23% decrease in distribution, and no change in specific activity, compared to a 59% decrease in distribution of 1-palmitoyl-2-docosahexaenoyl PC, and a 1.8-fold decrease in specific activity. The fate of 1-palmitoyl-2-docosahexaenoyl PC and the mechanisms by which it is metabolized are unknown. A possible fate could be hydrolysis by the action of various

phospholipases to constitutive components. If such were the case, 1-palmitoyl-2-docosahexaenoyl PC could be an immediate source of choline for the cell in case of dietary insufficiency. Another scenario would involve the concerted action of a phospholipase A_2 and acyl CoA:lysophosphatidylcholine acyltransferase (28,29) to generate a new PC molecular species with the choline label intact. The decay from 21.0% to 8.6% (Table III) in 1-palmitoyl-2-docosahexaenoyl PC more than accounts for the net 5.4% increase in other 1-palmitoyl containing species (peaks 8, 9, 10). The decay in 1-stearoyl-2-docosahexaenoyl PC distribution from 18.2% to 14.0% cannot account for the net 10.5% net increase in the major 1-stearoyl containing species (peaks 14, 15, 16 & 17). These two points indicate that, besides deacylation-reacylation at the *sn*-2 position, more complete degradation of 1-palmitoyl-2-docosahexaenoyl PC and recycling of the choline head group is occuring. Alternatively, remodeling at the *sn*-1 fatty acid may occur. Remodeling in this fashion would lead to redistribution of label and specific activity for PE-derived PC so that it eventually conforms to cell PC composition. It is of interest to speculate that 1-palmitoyl-2-docosahexaenoyl PC could be prone to oxidation of its *sn*-2 acylchain, and thus be hydrolyzed by a phospholipase A_2 specific for oxidized or fragmented, short-chain phospholipids (319,320).

Based on pulse-chase studies, PE *N*-methytransferase supplies the hepatocyte with PC containing more than twice its complement of 1-palmitoyl-2-docosahexaenoyl PC, the majority of which seems to be degraded or remodeled. Preliminary pulse-chase experiments in hepatocytes have shown that 14% of label, at the end of a 1 h pulse with 15 μCi of [*methyl*-^{3}H]choline, is in 1-palmitoyl-2-docosahexaenoyl PC. During the chase period this value decays to the 10% normally found in cell PC. Indeed, *in vitro* experiments on diglyceride utilization by CDP-choline:1,2-diacyl-*sn*-glycerol

cholinephosphotransferase have shown 1-palmitoyl-2-docosahexaenoyl PC synthesis from the corresponding diglyceride (31). This would seem to indicate that the CDP-choline pathway can also supply the hepatocyte with this PC species. Since the CDP-choline pathway appears to be quantitatively more important for PC synthesis, the role of PE methylation in polyunsaturated PC synthesis is still open to debate.

In conclusion, PE *N*-methyltransferase shows little substrate specificity *in vitro* or *in vivo,* and methylates PE at maximal rates provided the molecular species contains two or more double bonds. The variation in methylation rates for synthetic PEs is further evidence for a role of the lipid environment in modulating activity *in vitro.*

4.4 *In Vitro* Phosphorylation of Phosphatidylethanolamine *N*-Methyltransferase

Controversy surrounding the molecular nature of the methylation system has also extended to possible modes of regulation. PE *N*-methyltransferase is regulated by cellular levels of AdoMet and AdoHcy (148,149), fatty acids (178) and PE/PC ratios (139). There is a growing body of evidence that suggests PE methylation is regulated by phosphorylation (refer to Section 1.8.2). Most of the studies in question have correlated elevated cellular cAMP with an increase in phosphorylation state; implicating a cAMP-dependent protein kinase. It should be reiterated that the cAMP mediated effects are small and often inconsistent. As well, the direct phosphorylation studies indicated a 50 kDal protein (presumed to be the methyltransferase) was a cAMP-dependent protein kinase substrate. The 50 kDal protein is indeed a kinase substrate (Fig. 37), but this phosphorylated protein is completely absent from purified PE *N*-methyltransferase. We can now say with certainty that this 50 kDal protein is not PE *N*-methyltransferase and most conclusions claiming identity require re-evaluation.

As an initial step toward delineating the role of reversible phosphorylation in PE *N*-methyltransferase regulation, the enzyme was tested as a substrate for cAMP-dependent protein kinase *in vitro*. The enzyme was found to be a substrate for the kinase, albeit a poor one (only 0.25 mol Pi/mol methyltransferase was incorporated in 30 min). Enzymes such as the branched-chain 2-oxoacid dehydrogenase, adipocyte hormone-sensitive lipase, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase, tyrosine-3-mono-oxygenase and ornithine decarboxylase (reviewed in Ref. 321) all undergo rapid (<10 min) phosphorylation by their respective kinases *in vitro*. In most instances the incorporation of phosphate was stoichiometric. Sluggish phosphorylation of PE *N*-methyltransferase could occur for two obvious reasons. Although various assay conditions, such as the inclusion of PE, PMME and PDME and manipulation of the Triton X-100 concentration, did not affect phosphorylation, conditions may not yet be ideal for maximum phosphorylation. Also, the methyltransferase may be in a highly phosphorylated state following purification. This seems unlikely since phosphatase treatment did not enhance phosphorylation or alter isoprotein distribution. Clearly more work is needed to solve this problem.

Phosphorylation was observed to have no effect on methylation of PE or PMME when assayed at saturating concentration of substrates. However, it is possible that phosphorylation may alter the K_m for one of the methyltransferase substrates. A good example of this type of effect can be seen with liver pyruvate kinase. cAMP-dependent protein kinase was observed to cause a 3-fold increase in the K_m for phosphoenolpyruvate (in the absence of the allosteric effector fructose-1,6-diphosphate) without changing the V*max* (322,323). Perhaps phosphorylation alters the pH optimum of PE *N*-

methyltransferase. Effects of phosphorylation on kinetic constants and pH optima are areas of future consideration.

Consistent with the specificity of cAMP-dependent kinase (324), PE *N*-methyltransferase was phosphorylated on a serine residue. The nature of the site and number of sites has yet to be delineated. The consensus sequence of the cAMP-dependent kinase phosphorylation site was shown to consist of two basic residues (one of which is arginine) adjacent or several residues *N*-terminal to a serine or (rarely) a threonine residue (324). Considering the extremely basic pI of PE *N*-methyltransferase, the presence of multiple arginine residues would not be unexpected. No potential sites for phosphorylation were evident in the enzymes *N*-terminal region (Fig. 45). Amino acid sequencing of the phosphopeptide from the methyltransferase should identify the cAMP-dependent kinase phosphorylation site and indicate if more than one site is involved.

Two-dimensional gel electrophoresis of the phosphorylated methyltransferase indicated that *in vitro* phosphorylation could not shift the distribution of the two isoproteins. Instead, both isoproteins were shifted to the anode. This indicated that if the isoproteins were the result of phosphorylation then different sites were being labeled *in vitro*.

Much work is required to show conclusively that phosphorylation regulates PE *N*-methyltransferase. Of paramount importance is the generation of a precipitating antibody that can be used to analyze the enzyme's phosphorylation state in hepatocytes. Exposure of hepatocytes to hormones which modulate cAMP levels should also alter PE *N*-methyltransferase phosphorylation state.

4.5 Phosphatidylethanolamine *N*-Methyltransferase in Choline- and Methionine-Deficiency

Choline deficiency produces a variety of gross physiological changes in liver lipid metabolism and in the levels of circulating lipoproteins. The levels of all plasma lipids (cholesterol ester, cholesterol, triglyceride and phospholipids) were depressed when rats were maintained on a choline-deficient diet (184,318). The reduction in circulating triglyceride levels is concurrent with an 5- to 10-fold accumulation of this lipid in liver (137,185,295,325,326). The accumulation is probably related to a cessation of VLDL secretion (137,184). Yao and Vance (137) noted that supplementation of choline or methionine to deficient hepatocytes resulted in a resumption of VLDL secretion, but did not change cellular triglyceride mass or synthetic rates (monitored by [9,10-^{3}H]oleate labeling). Maintenance of choline-deficient rats on a choline-supplemented diet for 3 days reduced triglyceride levels from 220 to 142 μmol/g body weight (control value was 35 μmol/g body weight, Ref. 327). Thus, triglyceride levels and synthetic rates are slow to normalize upon administration of choline.

The mass of hepatic PC was shown to be reduced in choline-deficiency (185,319,327,328,329). The primary reason for this effect is lack of substrate for the CDP-choline pathway. The activities of the CDP-choline pathway enzymes appear to be largely unchanged (186). However, Hoffman *et al.* (187,328) reported that microsomal cholinephosphotransferase activity was reduced 5-fold by choline, methionine, folate and vitamin B_{12} deficiency. Preliminary results have suggested that phosphocholine cytidylyltransferase activity undergoes redistribution from cytosol to microsomes in the choline-deficient state (188), but total activity is unchanged.

An elevation in the activities of the PE biosynthetic enzymes cannot explain the elevated hepatic levels of PE (185,319,326,328,329) since Schneider

and Vance (186) observed no changes in the CDP-ethanolamine pathway enzymes. The elevated PE levels are, no doubt, the result of lack of conversion to PC via methylation. While PE *N*-methyltransferase activity is elevated in choline-deficient microsomes (186,187,329), the *in vivo* methylation of [1,2-^{12}C]ethanolamine-labeled PE to PC is impaired (329). The impaired methylation is related to the reduced levels of AdoMet and the low AdoMet/AdoHcy ratio in choline-deficient livers (329). Supplementation of choline- and methionine-deficient hepatocytes with methionine was found to result in a 2-fold reduction in PE concentration (137), indicating that methyl group availability was limiting the reaction.

Armed with new information on the molecular properties of PE *N*-methyltransferase, the regulation of this enzyme in choline-deficient rat liver was re-examined. The auto-regulation of PE *N*-methyltransferase by PE levels and the mass of enzyme was monitored under conditions where the concentrations of PC and PE changed significantly.

Similar to previous reports, choline-deficiency resulted in a marked reduction in liver PC concentrations and a less significant elevation in PE. More importantly, the ratio of PC to PE in hepatic membranes was reduced in the deficient state. Concomitant with the increase in PE was a 2-fold elevation in microsomal endogenous PE-dependent methyltransferase activity in choline-deficient liver microsomes. Differences between deficient and supplemented microsomes were obviated when an excess of PMME was added to the assay. The methyltransferase, which possesses a single active site for all lipid substrates, is clearly regulated by PE levels. Furthermore, the concentration of PE (or its mol% in membranes) in normal liver microsomes is not saturating and increases, due to choline deficiency, results in elevated *in vitro* activities. This dependency of methylation on PE levels was previously

observed in ethanolamine-supplemented hepatocytes and hypothesized to occur in the choline-deficient liver (139). Previously, Hoffman *et al.* (187) reported that PDME-dependent methyltransferase activity was reduced 1.2-fold and activity with endogenous PE was stimulated 1.4-fold by choline and methionine deficiency. However, these researchers were under the impression that two separate enzymes, under different regulatory controls, were involved in PE methylation. We now know that this is not the case.

The regulation of PE *N*-methyltransferase by PE levels in choline deficiency is not related to changes in enzyme mass. This is illustrated most effectively by the immunoblot in Fig. 41. Clearly, immuno-detectable enzyme mass was not altered by maintenance on a choline-deficient diet for three days. The relationship between the endogenous PE assay versus exogenous PMME assay can now be understood in light of the constitutive nature of enzyme mass. The PMME-dependent assay will give enzyme activities that are proportional to enzyme mass, while the endogenous PE assay is a reflection of PE substrate levels. The discovery of conditions under which PE *N*-methyltransferase mass and substrate is altered (ie. developmental induction) should give further insights into regulation.

The relationship between PE levels and methyltransferase activity was further established in choline- and methionine-deficient hepatocytes. In hepatocytes, supplementation of these phospholipid precursors, and their effects on lipid levels, could be correlated to changes in enzyme mass and activity. It was observed that supplementation with methionine alone caused a 55% decrease in cellular PE concentrations (Fig. 43B). This corresponded to a 47% decrease in endogenous PE-dependent methyltransferase activity and no change in PMME-dependent activity (Fig. 43A). Enzyme protein was not altered by methionine supplementation. Under identical conditions, only a

12% decrease in PE-dependent activity was observed upon choline supplementation (Fig. 42A). Thus, in a well defined culture system, changes in enzyme activity are related to altered PE levels and not to changes in enzyme mass.

Are there any conditions that may be expected to change expression of enzyme mass? By analogy to *S.cerevisiae* PE methylation, choline and methionine do not repress PE methylation (190,192), but choline does in the presence of inositol. This does not seem to be the case in choline-deficient hepatocytes, when addition of choline to inositol containing medium (0.04 mM in MEM) did not affect enzyme activity (Fig. 42). Multivalent regulation at the level of substrate availability, phosphorylation, transcription and translation may be involved in regulation of rat liver PE *N*-methyltransferase. While there is evidence for the first two regulatory mechanisms, the latter two are only hypothetical.

Conclusions and Future Considerations

The impetus of this work was to promote research on the molecular properties and regulation of PE *N*-methyltransferase. Neither of these goals can be addressed properly without purification of the enzyme. To this end, PE *N*-methyltransferase was purified from rat liver microsomes following solubilization with Triton X-100. A single 18.3 kDal protein catalyzed the complete conversion of PE to PC. The kinetic mechanism occurs in a concerted manner with partially methylated intermediates remaining in a common active site until PC is produced.

Studies on modes of regulation showed that cAMP-dependent protein kinase phosphorylated the methyltransferase *in vitro*. Phosphorylation had no effect on enzyme activity when assayed at saturating substrate levels. No information is yet available on phosphorylation *in vivo*. Work with the choline-deficient rat model reiterated previous observations on PE *N*-methyltransferase regulation by substrate levels. Changes in enzyme activities were found to be related to PE levels, and not to changes in enzyme mass. In this regard, enzyme mass appeared to be constitutive, even when dietary choline and methionine were withheld.

With the availability of purified enzyme, knowledge of molecular properties and preliminary results on regulation, work can begin in earnest on enzyme structure and regulation. The following approaches could be quite fruitful.

1. The enzyme could be cloned from a rat liver cDNA library using an oligonucleotide probe complimentary to the *N*-terminal of the enzyme or by the use of polyclonal antibodies. If this were unsucessful, the protein could be sequenced. This is not such a daunting task considering the size of the enzyme (aprox. 160-170 amino acids).

2. While it is apparent that different molecular species of PE influence the rate of methylation, the effect of other phospholipid classes has not been determined. These experiments could be done in mixed micelles or in a reconstituted system.

3. More indepth analysis of enzyme phosphorylation sites, effects on catalytic properties and possible endogenous kinases and phosphatases could be performed.

4. *In vivo* phosphorylation and cellular half-live of the methyltransferase could be determined as soon as a precipitating antibody is raised. Oddly, all antibodies that have been raised against the enzyme do not immunoprecipitate activity or protein.

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