Phosphatidylethanolamine N-Methyltransferase in Human Red Blood Cell Membrane Preparations

KINETIC MECHANISM*

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The biosynthesis of phosphatidylcholine (PC) via the methylation of phosphatidylethanolamine (PE) was first described by Bremer and his colleagues (1, 2). The activity of this pathway in the liver is 10-1000-fold greater than that found in most, if not all, other cells (3). Thus, the physiological importance of this pathway may be located mainly in the liver. On the other hand, this activity has been reported in many other tissues and cells suggesting that simply because it is in existence it may have importance. In a previous publication, we have presented data which suggest that an altered phospholipid methylation in synaptic plasma membranes may be related to the onset of physical dependence to ethyl alcohol (4). Mato and Alemany (3) have reviewed other possible functions of the methylation pathway for the synthesis of PC. There is evidence to support a function in biological signal transduction (5-7), but suggestions that phospholipid methylation plays a role in membrane fluidity (8) and that the PC formed via the methylation of PE provides a source for arachidonic acid (6) have been questioned (3, 9). In any case, the methylation of PE has been reported in many cells, albeit low compared to liver, and further study is necessary to clarify its role in cellular metabolism.

As pointed out by Audubert and Vance (10), the number of enzymes required for the sequential methylation of PE has been controversial, but kinetic considerations strongly suggest one enzyme in animal tissues (10). Purification of this enzyme by Mato’s group (11, 12) and by Ridgway and Vance (13) have clearly established that there is only one enzyme in liver. Kodaki and Yamashita (14) have demonstrated that two genes are involved in the methylation of PE in yeast; one for the enzyme that methylates PE and one for the enzyme that methylates the next two substrates. These data strongly suggest two enzymes in certain microorganisms.

The kinetic characteristics of PE N-methyltransferase (EC 2.1.1.17) have been reported for several tissues (10-13, 15-18), but the low activity in tissues other than liver has made these studies difficult. Also, the TLC separation of PE, PME, PDE, and PC used by most investigators is very time-consuming and tedious. We have used a HPLC separation, which incorporated a flow-through scintillation detector. In a preliminary communication (19), we reported that the stepwise incorporation of [H]methyl groups from S-adenosylmethionine (AdoMet) into exogenous PME and PDE can be measured in the RBC membrane from human erythrocytes using these radio-HPLC techniques. Ridgway and Vance (13) have presented kinetic data on their purified preparation of this enzyme. They noted complicated kinetics which were mainly due to the micellar character of the enzyme and its lipid substrates in the purified state, but they did not report a kinetic mechanism for the methylation of phospholipid. We
have observed relatively straightforward kinetics with this enzyme from human RBC membrane preparations; however, due to the high concentration of PE present in the membranes, kinetic studies involving this phospholipid were not feasible using the techniques reported herein. In this publication, we present evidence to suggest a kinetic mechanism for the methylation of both PME and PDE.

**EXPERIMENTAL PROCEDURES**

Materials—[H]AdoMet was purchased from Du Pont-New England Nuclear and used without further purification. Unlabeled AdoMet and AdoHcy were purchased from Sigma. The unlabeled AdoMet was purified on Dowex 1-X8 (HCO₃⁻ form) anion exchange resin at 4 °C according to Shapiro and Ehninger (22). After purification, the unlabeled AdoMet was lyophilized, dissolved in water, and adjusted to pH 3.0 with HCl. This was dispensed into small fractions and frozen. The thawed aliquot was not used again. None of the AdoMet was used after 6 months without repurifying. The concentration of the AdoMet was measured spectrophotometrically (molar absorptivity in H₂O = 15,400 at 280 nm) (22). Dowex 1-X8, CHAPS, dimethyl sulfoxide, and buffer were obtained from Sigma. Dioleoyl-PME and -PDE were obtained from Avanti Polar Lipids, Inc., Pelham, AL.

**RBC Membrane Preparation**—Venous blood (50–100 ml) was obtained multiple times from four healthy male subjects who were 25–45 years of age. Prior to obtaining the blood, consent forms were signed by the University of Nevada Biomedical Human Subjects Review Board and by the individual donors were obtained. The blood was drawn in 10-ml Vacutainer tubes containing K₂EDTA (Becton-Dickinson and Co., Rutherford, NJ). These tubes were centrifuged at 2500 rpm in a Sorvall GLC-1 table-top centrifuge for 15 min. The plasma and buffy coat were removed by aspiration. RBC membrane preparations were made using a modification of the procedure of Dodge et al. (20). The packed RBCs were resuspended and washed 3 times in isotonic saline. The ratio of packed cells to washing solution was approximately 1:15 for all lysing and washing steps. The packed RBCs were resuspended and washed in 5 mM Tris/renalin buffer, pH 10.0. This suspension was centrifuged at 50,000 × g for 1 mg in a refrigerated centrifuge (Beckman J21) with the brake on. The resulting pellet was resuspended in 0.5 mM Tris phosphate buffer, pH 10.0, and centrifuged at 5 μl for 10 min at 50,000 × g with the brake off. This washing step was repeated 3 times. The membrane vesicles were washed once more after resuspension in the same buffer, but a higher centrifugal force was required to obtain a firm pellet (106,000 × g for 40 min). The resulting hemoglobin-free pellet was resuspended in 50 mM Tris-HCl buffer, pH 6.8, and centrifuged at 106,000 × g for 12 min. The supernatant was removed in both the ultracentrifugation and these samples were washed once more in the same buffer, pH 6.8, to about 10 mg/ml protein concentration and frozen at −70 °C until use. This preparation of RBC membranes should yield mainly sealed inside-out vesicles as noted by Wheeler (21).

**Incubation Conditions and Lipid Extraction**—The incubation mixture contained in final concentrations: 1 mM MgCl₂, 25 mM Tris/glycylglycine buffer (pH 10.5), 6 mM CHAPS, and 7.5 μCi of [H]AdoMet. The phospholipids were dissolved first in 60 mM CHAPS by warming the solution and then added to the incubations. The concentration of each of the phospholipids was varied according to the experiment. The two phospholipid substrates, unlabeled AdoMet and AdoHcy, were added in various concentrations depending on the experiment. These data are shown in the legends to the figures. The incubation volume was 1.2 ml, and the incubation temperature was 37 °C. The reaction was linear between 2 and 8 mg of protein for 50 min. Generally, 6 or 7 mg of protein were used per assay, and the reaction was allowed to proceed for 45 min before being terminated by adding 6 ml of ice-cold 40% trichloroacetic acid. This mixture was centrifuged at 50,000 × g for 10 min at 5 °C. The supernatant was decanted, and the precipitate was washed twice with 6 ml of ice-cold 20% trichloroacetic acid. The precipitate was extracted twice with 3 ml of chloroform/methanol (1:2) containing 0.02% butyldihydroyxylolute according to the method of Bligh and Dyer (23). The chloroform phase was stored at −10 °C overnight. After drying under N₂ at 80 °C, 90 μl of chloroform were added, and 70 μl were injected into the HPLC for analysis. The chloroform and methanol were distilled prior to the HPLC analysis because the PDE substrate is methylethyllysine, the time to conversion to PDE and on to PC must be taken into consideration; therefore, the formulas described by Audubert and Vance (10) were used in all calculations. Because Bansal and Kanfer (24) have reported chemical methylation of PE by AdoMet, we used a boiled enzyme control in addition to either a zero time or a zero control protein. None of these controls gave products with labeled methyl groups.

**Kinetics of Methylation Products**—A Spectra-Physics SP7800 delivery system was used with an Altitech LiChrosphere column (250 × 4 mm) containing S1 100/II (10 μm). The phospholipids were separated using the solvent suggested by Geurts Van Kessel et al. (25), and Fig. 1A shows a typical separation of the three radioactive products from an incubation in which only endogenous phospholipid was used as substrate. A, B, and C show the conversion pattern when exogenous PME or PDE was added. Initially, the technique was checked using a Kratos variable wavelength UV monitor at 205 nm and unlabeled standards of PE, PME, PDE, and PE. The solvent was hexane/isopropl alcohol/water, and the instrument was programmed at 1 ml/min to allow a 15-min gradient to be run which started at an initial mixture of 44:50:6 and continued to a mixture of 54:40:6. This final mixture was run isocratically until the PC was eluted. The entire chromatogram took about 30 min. The effluent from the HPLC column was directed into a Fle-Ome Beta model PC flow-through scintillation counter from RadioAnalytic, Inc., Tampa, FL, and the radioactivity in each phospholipid product was quantitated. The two organic solvents were HPLC grade obtained from Fisher. The water was deionized and filtered through a 0.45-μm nylon 66 filter before use.

Statistical analysis of the data was made by the use of the MINITAB® software package and the Stata statistical package (University of Nevada, Reno). For analysis of kinetic constants, the values of [P] and [Q] in Equation 1 (below) were set equal to zero as appropriate.

**Kinetic Analysis**—The analysis of enzyme kinetics used the approach and nomenclature developed by Cleland (26, 27). The kinetic behavior was simulated by a computer program written by one of the authors. The number of iterations/min of simulated time was increased until reactant concentrations were independent of the number of iterations. The assumptions of the model were that the conversion of PDE to PE and the conversion of PE to PC were catalyzed by two completely separate enzyme activities and that all reactants freely equilibrated with the solvent. Both enzyme activities were assumed to follow Equation 1 below.

\[
v = \frac{1}{(V_{\text{max}}[A][B]/(K_A K_B)) + (1/A) + (1/B) + (1/[Q]/K_Q) + (1/[P]/K_P) + ([A]/K_A [P]) + ([B]/K_B [Q]) + ([Q]/K_Q [P]) + ([P]/K_P [Q])}
\]

where \(V_{\text{max}}\) = maximum velocity in the forward direction, \(V_{\text{max}}\) = maximum velocity in the reverse direction, \(A\) = AdoMet, \(B\) = either PME or PDE, \(P\) = PDE or PC, and \(Q\) = AdoHcy. The Greek letters represent the ratio of the dissociation constant of the reactant from a ternary complex to the dissociation constant of the same reactant from a binary complex (for example, \(K_A\) = the Michaelis constant for A).

The kinetic constants were derived as much as possible from the data presented herein. However, the values of some of the kinetic constants (e.g. \(K_P\)) were not determined experimentally and were estimated from the work of others (15, 17). Previous work has shown that neither PDE nor PC is a strong inhibitor of the methyltransferase reaction (17). A concentration of 0.14 mM PDE had no effect on PME methylation, while 0.3 mM PC reduced the velocity of PC formation roughly one-half (15). The values of the kinetic constants were set no larger than required to achieve this same level of inhibition.

**RESULTS**

**Effect of Exogenous PE**—The conversion of PE to PC requires three methyl transfer steps. We added various concentrations of dipalmitoyl PE (5–50 μM) to the reaction, but the reaction rate remained constant (Fig. 2). We also added dioleoyl PE (50–200 μM) and made a similar observation regarding the reaction rate (data not shown). These data suggest that, because we were using RBC membrane preparations, the endogenous PE concentration was sufficient to saturate the enzyme. Because the calculated endogenous concentration of PE is 0.8–3.0 mM (30), there is about 4–15 times more endogenous PE than exogenous. The maximal velocity
with exogenous dipalmitoyl-PE was 0.080 ± 0.009 pmol/min/mg, and the maximal velocity with exogenous dioleoyl-PE was 0.068 ± 0.006 pmol/min/mg; thus, there was a slight (15%) decrease in rate with the more unsaturated form of PE. Because exogenous PE had little effect on enzyme velocity, kinetic analysis of the first methylation step was not possible. To analyze the following two methylation reactions, the concentrations of PME and PDE were varied at different concentrations of AdoMet. The dependence of the velocity of methylation of the phospholipids upon AdoMet concentration is shown in Fig. 3. Double-reciprocal plots were linear with both lipids.

*Endogenous PME and PDE*—Low levels of PME and PDE have been shown to be present in liver (28); therefore, it is probable that low levels of the intermediates, PME and PDE, also may be present in RBC membrane preparations. To estimate these concentrations we used a simple iterative procedure using the Michaelis-Menten equation and data generated experimentally. At saturating concentrations of AdoMet, the phospholipid concentration was varied, the velocities were
determined, and the \( V_{\text{max}} \) and \( K_m \) values were estimated from the slopes and y intercepts of double-reciprocal plots. Using the velocity obtained in the absence of added phospholipid and the calculated \( K_m \) and \( V_{\text{max}} \) values, the Michaelis-Menten equation was solved to determine the substrate concentration. This endogenous substrate concentration was added to the exogenous substrate concentrations to recalculate a new \( K_m \), \( V_{\text{max}} \) and a new endogenous substrate concentration. This iterative process was repeated until these three values remained constant. Usually within three iterations the values of kinetic constants had ceased to change. The validity of the procedure was checked by computer simulation using the initial forward velocity form of Equation 1. As estimated by this procedure, the endogenous levels of PME and PDE were 0.244 and 0.118 nmol/mg protein, respectively. For a typical assay which contained 7 mg of protein, the endogenous concentration levels of PME and PDE were 1.42 and 0.69 \( \mu \)M, respectively. These concentrations were used in calculating the actual concentrations of the phospholipids used in subsequent experiments. Normally we recovered about 2 mg of RBC membrane protein/ml of packed RBCs. Therefore, the concentrations of PME and PDE in red cells would be about 0.5 and 0.25 \( \mu \)M, respectively. These values compare favorably with the concentrations determined by chemical analysis in rat liver (0.55 and 0.25 \( \mu \)M, respectively (28)).

**Kinetic Constants**—The first step in the evaluation of the kinetic constants was to determine separately the kinetic mechanisms most consistent with the observed methylation of PME and PDE. The velocities of the reactions at variable concentrations of the two phospholipid substrates at fixed concentrations of AdoMet are shown in Fig. 4. The linear intersecting lines with both PME and PDE are consistent with a sequential mechanism in both cases thus eliminating possible ping-pong mechanisms. Replots of the slopes and y intercepts of the double-reciprocal plots were linear. The linearity of both the primary and secondary plots is emphasized by comparing the values of slopes and intercepts obtained using velocities at all phospholipid concentrations (endogenous + exogenous) to those which omitted the rate for

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**Fig. 2.** Effect of phosphatidylethanolamine concentration on methylation. The incubation mixture contained in final concentration: 1 mM MgCl\(_2\), 25 mM Tris/glycylglycine buffer (pH 10.3), 6 mM CHAPS; RBC membranes (7.5 mg of protein) and 200 \( \mu \)M (7.5 \( \mu \)Ci) \[^{3}H\]AdoMet. The PE concentration was varied from 0 to 50 \( \mu \)M, and the reaction volume was 1.2 ml. The mixture was incubated for 45 min at 37 °C and stopped by adding 6 ml of ice-cold 40% trichloroacetic acid. The total rate represents the combined rate of incorporation into all methylated products, and the first methylation refers to only the incorporation into PME from PE as calculated by the formula described by Audubert and Vance (10).

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**Fig. 3.** The effect of AdoMet concentration on velocity with constant concentrations of PME (A) or PDE (B). The reaction mixture was similar to that noted for Fig. 2 except that the PME and PDE concentrations were 125 \( \mu \)M and that the AdoMet concentration was varied at 5, 10, 20, and 100 \( \mu \)M. Five mg of protein were used with the PME incubation, and 5.9 mg of protein were used with the PDE. The remainder of the conditions was identical to Fig. 2. Endogenous concentrations of 0.244 nmol/mg for PME and 0.118 nmol/mg for PDE were added to the concentrations of all exogenous phospholipid substrates in all subsequent calculations.

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The reaction in the absence of exogenous phospholipid. The slopes and intercepts calculated in these two ways agreed within 1 S.D. The linearity over such a wide range of concentrations means that steady-state mechanisms involving the random addition of substrates are highly unlikely.

To distinguish between the possibilities of a rapid-equilibrium random mechanism and an ordered steady-state mechanism, product inhibition by AdoHcy was measured. Fig. 5 shows the primary plots of AdoHcy inhibition at five concentrations of phospholipid. The double-reciprocal plots gave a pattern essentially that of a classical noncompetitive inhibitor. Replots of slopes and intercepts were made, and AdoHcy was shown to be a linear noncompetitive inhibitor when phospholipid was the variable substrate. The double-reciprocal plots when AdoHcy was the variable substrate (Fig. 6) intersected near but not on the 1/\( V_{\text{max}} \) axis. The pattern of the apparent \( V_{\text{max}} \) and \( K_{\text{MAdoHcy}} \) is much more consistent with a competitive inhibitor than with either a noncompetitive or partially uncompetitive inhibitor. The slopes were a linear function of inhibitor concentration, whereas intercepts varied slightly, in a random manner, about the mean value of the intercept. The F test indicated a nonsignificant deviation from a zero-order dependence of y intercept on inhibitor concentration. Hanes-Woolf plots of the data (not shown) lead to the same conclusion, i.e. the AdoHcy concentration does not have a significant effect on apparent \( V_{\text{max}} \). Therefore,
we conclude that AdoHcy is a linear competitive inhibitor of AdoMet for both of the methylation steps studied.

AdoHcy is competitive against AdoMet and noncompetitive against both phospholipids. Because the product inhibition patterns for the two substrates (phospholipid and AdoMet) are clearly different, a simple (i.e. no dead-end complexes) rapid-equilibrium random mechanism is ruled out. However, a rapid-equilibrium random mechanism with E-PME-AdoHcy and E-PDE-AdoHcy being dead-end complexes is consistent with the data as is an ordered steady-state mechanism. Because the phospholipids PME and PDE are both substrates and products of the reaction it is reasonable to propose that PME and PDE can bind to the same enzyme form both as a product and as substrate. It is possible (29) to distinguish between ordered and random mechanisms if the dissociation constant for release of AdoHcy from an E. AdoHcy binary complex (product inhibition) is different from the dissociation constant for release of AdoHcy from an E-phospholipid-AdoHcy ternary complex (dead-end inhibition). In such a case, the apparent $K_{\text{app}}(\text{AdoHcy})$ would be a function of phospholipid concentration. In the ordered mechanism alternative, there would be no E-phospholipid on the reaction path (because phospholipid binds only to E-AdoMet); therefore, the apparent $K_{\text{app}}(\text{AdoMet})$ would be independent of the concentration of phospholipid. The apparent $K_{\text{app}}(\text{AdoHcy})$ was found to decrease with increasing substrate (PDE) concentration (Table I). Therefore, a rapid-equilibrium sequential mechanism with a dead-end complex is the most consistent with the data.

The kinetic constants were evaluated assuming this random mechanism (Equation 1) and are reported in Table II. Both a saturated (16:0) and an unsaturated acyl chain (18:1) phospholipid were used in the experiments. Depending on the acyl chain, the $K_n$ for PME was 20-40 pM, while that for PDE was less than half this value, 9-14 pM. The unsaturated chain gave the higher values in both cases. The $K_n$ for AdoMet was 5-9 uM with PME and 4 pM with PDE. The $V_{\text{max}}$ values for the PME methylation were identical when determined by treating either PME or AdoMet as the variable substrate. Similar results were obtained for PDE. The $V_{\text{max}}$ for methylation of PME was about 50% greater than that for PDE regardless of the acyl chain used. Further, the $K_{\text{app}}(\text{AdoMet})$ (the apparent dissociation constant of AdoMet) was essentially the same for the methylation of either of the 16:0 phospholipids, but the $K_{\text{app}}(\text{AdoMet})$ was about 2-fold higher for 18:1 PDE than for 18:1 PME. The $K_i(\text{lipid})$ (the apparent dissociation constant for phospholipid) was about 2-fold higher for 18:1 phospholipids than 16:0 phospholipids paralleling the difference in the Michaelis constants. The observation that the dissociation constants of the reactants were consistently higher than the Michaelis constants indicated some cooperativity of reactant binding to the enzyme; the enzyme binds
the second reactant somewhat more strongly than the first. The acyl chain species had a small but definite effect on the Michaelis and inhibition constants, whereas the effect on \( V_{\text{max}} \) was minimal. As judged by the \( V_{\text{max}}/K_m \) parameter, there is little preference for either acyl chain.

The inhibition constants for AdoHcy are shown in Table III. When AdoMet was the variable substrate, the \( K_i \) was 1.0 \( \mu M \) with PME and 1.6 \( \mu M \) with PDE. When the phospholipids were the variable substrates, the \( K_i \) was 1.4 \( \mu M \) with PME and 1.1 \( \mu M \) with PDE.

**Product Ratios**—Whereas the data for both PME and PDE methylation are consistent with a rapid-equilibrium sequence:

### Table II

**Kinetic constants of the methylation reaction**

The kinetic constants were obtained using di16:0 and di18:1 phospholipids as substrates (see "Experimental Procedures"). The phospholipid used is indicated by the designation di16:0 or di18:1 under the variable substrate concentration. The data were obtained by varying one substrate concentration at fixed concentrations of the other as indicated. Values of \( V_{\text{max}} \), \( K_m \), and \( K_i \) were estimated from secondary plots of slopes and intercepts of the primary (double-reciprocal) plots. Where two values for the Michaelis constants of phospholipid and AdoMet are given, the values listed were obtained when phospholipid (upper value) and AdoMet (lower value) were the variable substrates. The \( K_{i\text{AdoHcy}} \) and the \( K_{i\text{AdoMet}} \) are the inhibitor constants for the respective substrate. The values of the Michaelis constants represent the dissociation of the indicated reactant from the ternary enzyme complex (that is the Michaelis constant for AdoMet is equal to \( K_A \) in Equation 1) whereas the \( K_{i\text{AdoHcy}} \) repre-
sents the dissociation of the reactant from the binary enzyme complex (that is the inhibition constant for AdoMet is equal to \( K_A \) in Equation 1).

![Graph](image)

**Fig. 6. AdoHcy inhibition as a function of AdoMet concentration.** The concentrations of both PME and PDE were 125 \( \mu M \), but the concentrations of AdoMet were 7.5, 15, and 30 \( \mu M \) with PME and 25, 50, and 100 \( \mu M \) with PDE. The protein concentration was 6.5 mg with PME and 5 mg with PDE. The remainder of the conditions was the same as in Fig. 2. Endogenous concentrations of PME and PDE were used as noted in Fig. 3.

**Table I**

**Effect of increasing PDE concentrations on the apparent inhibitor constants for AdoHcy**

The PDE concentration was varied as indicated; the concentration of AdoMet was 7.5, 10, 15, and 30 \( \mu M \), and the concentration of AdoHcy was 0, 1.5, or 15 \( \mu M \). The incubation was for 45 min at 37 °C, and the other components of the incubation are indicated in Fig. 2.

| PDE \( \mu M \) | PME \( K_i \) \( \mu M \) | PME \( V_{\text{max}} \) pmol/min/mg protein | PDE \( K_i \) \( \mu M \) | PDE \( V_{\text{max}} \) pmol/min/mg protein |
|---|---|---|---|---|
| 10 | 4.7 | 20.0 ± 1 | 1.0 ± 1 | 0.60 ± 0.1 |
| 15 | 2.9 | 22.5 ± 0.5 | 2.9 ± 1 | 0.63 ± 0.05 |
| 30 | 1.2 | 25.0 ± 0.9 | 10.0 ± 1 | 0.75 ± 0.04 |
| 100 | 1.7 | 27.5 ± 1.0 | 15.0 ± 1 | 0.85 ± 0.04 |

| Phospholipid substrate | PME | PDE |
|---|---|---|
| K<sub>m</sub> \( \mu M \) | V<sub>max</sub> pmol/min/mg protein | K<sub>m</sub> \( \mu M \) | V<sub>max</sub> pmol/min/mg protein |
| Phospholipid | 20 ± 2 | 20 ± 1 | 19 ± 2 | 18 ± 1 |
| di16:0 | 48 ± 4 | 81 ± 0.7 | 41 ± 4 | 82 ± 0.4 |

**Table III**

**Inhibitor constants for AdoHcy**

Incubation conditions are indicated in Fig. 2, and the \( K_i \) data were calculated from Figs. 5 and 6.

| Fixed substrate | Variable substrate | PME (16:0) | PDE (16:0) | AdoMet | PME | PDE |
|---|---|---|---|---|---|---|
| PME (16:0) | AdoMet | 1.0 | Competitive |
| PDE (16:0) | AdoMet | 1.6 | Competitive |
| AdoMet | PME | 1.4 | Noncompetitive |
| AdoMet | PDE | 1.1 | Noncompetitive |

In both cases, the ratios of PDE:PC produced by methylation of PME are consistent with a rapid-equilibrium sequence. Using Equation 1, the values of the kinetic constants listed in Table II, and a digital computer, the predicted product ratio of PDE to PC produced by two separate enzymes (one reacting only with PME, the other reacting only with PDE) can be calculated under a variety of conditions. The computer-simulated enzymic activities faithfully reproduced the experimental data when PDE concentration was varied. However, when PME was the variable phospholipid, there was a striking difference in the product ratios of PDE to PC compared with those obtained experimentally. The ratios of PDE:PC obtained from computer simulation are compared with those from actual experiments in Table IV. In all cases, the experimentally derived ratio was considerably less than that from computer simulation using a two-enzyme model. The nature of the acyl chain has a large effect on the ratio of observed PDE:PC (Table IV) but little effect on the observed
The computer model estimates the amount of PDE and PC that would be formed from PME (either di16:O or di18:O) under the reaction conditions assuming the methylations are performed by separate enzymes. The data are from two separate experiments. The 1.4 μM concentration of PME represents the endogenous concentration of this substrate, and the other values for PME concentration represent that which was added exogenously. Each of these reactions will have an additional 1.4 μM of endogenous PME. The reaction time was set at 45 min. The initial PDE concentration was set at either 0 or 9.69 μM (data in parentheses). For more details about the assumptions in the computer model see "Experimental Procedures."

| [AdoMet] | [PME] | Simulation | Experimental |
|----------|-------|------------|--------------|
| μM       | μM    |            |              |
| Exp. 1   |       |            |              |
| 5        | 1.4   | 800(1.3)   | 0.3          |
| 5        | 10    | 800(0.2)   | 2.6          |
| 5        | 100   | 810(15)    | 6.5          |
| 100      | 10    | 220(7.7)   | 1.0          |
| 100      | 100   | 220(15)    | 2.4          |
| Exp. 2   |       |            |              |
| 7.5      | 125   | 300(15)    | 5.2          |
| 15       | 125   | 400(15)    | 3.6          |
| 30       | 125   | 300(15)    | 2.9          |

kinetic constants shown in Table II (and therefore on the predicted product ratio). We conclude that, while exogenous PDE can react with the methyltransferase, in the normal reaction sequence the PDE intermediate does not equilibrate with the solvent and that the methylations required for the conversion of PME to PC do not require release of PDE from the enzyme.

**Discussion**

In this study the methylation of phospholipid was examined in purified but intact membranes derived from human red cells. The intact membrane can catalyze the methylation of PE to PC, and the methylations of PME and PDE are partial reactions in the overall metabolic conversion of PE to PC. When the methylations of PME and PDE are examined individually, the kinetic data is consistent with a random Bi-Bi sequential mechanism. Several possibilities exist for the catalysis of these three methylations. 1) Each methylation could be catalyzed by a separate enzyme specific for PE, PME, or PDE. However, studies from liver (11–13) indicate that a single protein can account for all three methylations. 2) Each methylation occurs at a separate, independent, specific site on a triple-sited protein. This possibility is kinetically equivalent to three separate enzymes. 3) All three methylations occur at the same enzymic site. This possibility can be distinguished kinetically since PE, PME, and PDE would compete for methylation in a competition assay. 4) Substrate channeling may occur in the sense that the intermediates PME and PDE are ordinarily not released from the enzyme surface. Substrate channeling may occur with either a triple-sited enzyme (no. 2 above) or a single-sited enzyme (no. 3 above).

We have not tested for the presence of a trifunctional or identical-site enzyme, but from an examination of product ratios, substrate channeling appeared to have occurred. It is possible that the product ratios arise as an artifact of accumulation of products (PME and PDE) near the enzyme surface so that the recently formed intermediate products are preferentially bound to the enzyme for the next round of methylation. In other words, the newly released products do not have time to equilibrate with the bulk phase before being rebound to the enzyme. As discussed in one of the following sections, the rate of production of product/unit surface area was so low in the red cell membrane it is unlikely that there was any net accumulation of products in the vicinity of the enzyme, i.e. the rate of product formation is much slower than the rate of diffusion of the products. Therefore, the product ratios appear to be due to channeling.

**Relative Rates of Methylation**—The high concentration of endogenous PE prevented study on the first methylation, i.e. the conversion of PE to PME. However, the maximal velocities obtained (Fig. 2) strongly suggest that this first methylation is the rate-limiting step in the complete methylation of PE to PC; it was one-fifth to one-eighth that of either of the remaining methylations. This finding is consistent with other reports (15–17). The relative V_<sub>max</sub> values reported here for PE, PME, and PDE are similar to the relative velocities obtained by Ridgway and Vance (13) for the pure rat liver enzyme in the presence of near-saturating concentrations of all substrates.

**Comparison of Kinetic Constants**—Panagia et al. (17) have studied the phospholipid methyltransferase in heart sarclemma, and they report a K<sub>m</sub> for AdoMet of 3.6 μM for the methylation of PME and 119 μM for the methylation of PDE. This value for PME methylation is similar to that which we observed for RBC membranes (Table II), but the K<sub>m</sub> for PDE methylation was 10-fold higher than we observed. In the absence of added phospholipid, the K<sub>m</sub> for rat intestine phospholipid methylation (30) was 40 μM compared to an apparent Michaelis constant of 31 μM, which we obtained under similar conditions (data not shown). Using a partially purified preparation from rat liver, Schneider and Vance (15) have reported K<sub>m</sub> values for AdoMet of 22 and 16 μM for the methylation of PME and PDE, respectively. They also report K<sub>i</sub> values of 4.9 and 6.7 μM for AdoHcy for these same two reactions. It is interesting that the K<sub>i</sub> values reported for liver are similar to those for the RBC, and it also appears that the inhibition by AdoHcy is competitive toward AdoMet in both of these tissues.

The data from two previous studies (13, 15) clearly showed that the third methylation was always slower than the second and that the first methylation was always the slowest by at least an order of magnitude. The results which we report are consistent with these findings. In the intestine, Panagia et al. (17) observed that the third methylation was faster than the second and that the first methylation was considerably slower than either the second or third. Thus, there appears to be subtle differences between tissues with respect to the kinetics of this enzyme.

**Endogenous Levels of PME and PDE**—We have estimated the endogenous concentration of both PME (1.42 μM) and PDE (0.69 μM) in RBC membrane vesicles. For comparison the calculated concentration of endogenous PE is 0.8–3.0 mM (31).

**Reaction Rate at Physiological Reactant Concentrations**—Using the value for endogenous PME concentration from this paper and the values from Oden and Clark (32) regarding the concentration of AdoMet and AdoHcy in the RBC, we calculate an in vivo rate of methylation to be 5 pmol/min/liter packed cells starting with PME compared to 1240 pmol/min/liter at V<sub>max</sub>. Assuming a 50% hematocrit, 5 pmol/min/liter translates into a rate of 2.5 pmol/min/liter of whole blood and about 11.25 pmol/min for an individual. Obviously this rate is very slow, and the contribution of the methylation pathway in the RBC toward the total synthesis of PC should be minimal.
Product Ratios and Substrate Channeling—The product ratio (PDE:PC) is a measure of the degree to which an intermediate (in this case PDE) is “channeled.” If channeling were complete, the PDE:PC ratio would be much less than 1. A computer model was used to estimate the product ratio under the reaction conditions employed in this work assuming that the conversion from PME to PC was catalyzed by two completely separate and independent enzymes (or catalytic sites). Any values lower than the computer estimates would indicate partial substrate channeling. From the large difference between experiment and model (Table IV), one infers that substrate channeling occurred. The channeling increased as AdoMet concentration increased, while increasing PME concentration decreased channeling. The observation can be intuitively rationalized as follows. Increasing the AdoMet concentration accelerates both the methylation of PME and PDE, thus favoring the precessive methylation of substrate to PC (i.e. channeling). Increasing the PME concentration increases the rate of the first methylation only, leading to an accumulation of PDE and thus increasing the observed product ratio (decreased channeling).

Nonspecific Origins of Product Ratios—We have argued from the results presented in Table IV that the distribution of products is consistent with substrate channeling. However, there are two experimental factors which should be considered in interpreting the data. The first arises from the low amount of product formed experimentally. If this low activity is due to a low turnover number, the amount of enzyme in the membrane may be comparable to the amount of product formed. For example, from the data of Ridgway and Vance (13), it can be estimated that there are 35 pmol of enzyme/g of liver microsomal protein. The PME and PDE formation measured in the assay may actually be enzyme-bound intermediates which are released during the work-up and do not exist as free entities. Therefore, substrate channeling may be far more complete than indicated by the experimentally observed product ratios. However, the finding of 33 pmol of PDE/g of brain (33) and the report of significant amounts of PME (0.55 μM) and PDE (0.93 μM) in the liver (28) indicate that PME and PDE are released from the enzyme under normal physiological conditions.

The second factor is more complicated. Goldman and Katchalski (34) have shown that geometry can be important in the product distribution of enzymes catalyzing consecutive reactions. They treated the case where two membrane enzymes catalyzed consecutive reactions with soluble substrates. A analogous situation is possible here if one assumes preferential interactions between enzyme and PDE (e.g. boundary lipid). However, the channeling due to nonspecific effects such as those discussed by Goldman and Katchalski (34) is not important in this case for three reasons. 1) The rate of PDE production is extremely slow. We estimate that the rate of PDE production is 1 × 10^{-18} mol s^{-1} cm^{-2} of purified RBC membrane which is slower than the rate at which thermal motion will equilibrate the newly formed phospholipid with the bulk solvent. By using the equations of Ngo and Laidler (35), the concentration of PDE in the immediate vicinity of the enzyme is estimated to be only 6 × 10^{-23} M higher than that in the bulk phase. Therefore, there will be no accumulation of intermediate in the vicinity of the enzyme to favor the second methylation. 2) Using our experimental conditions and kinetic values, the equation of Goldman and Katchalski (34) predicted a constantly increasing product ratio (which was always higher than any of those listed in Table IV over the 45-min assay period), whereas the product ratio observed experimentally was constant (data not shown). 3) We observed that the acyl chain had a large effect on product ratio but little effect on the kinetic constants (Tables II and IV). Such a finding is not expected for a nonspecific physical process like that proposed by Goldman and Katchalski (34). Experimental observations also argue against accumulation of intermediates spatially close to the enzyme. 1) The greatest channeling is seen at low reaction rates (for example when PE is the substrate), a result opposite from what is predicted if the channeling were an artifact of accumulation of intermediates. 2) The PDE and PC accumulate in constant ratio, whereas the diffusion model would predict an initial increase in PDE followed by an increase in PC.

A Possible Kinetic Mechanism—A mechanism which will explain both the pattern of reaction velocities and product ratios is needed. We have shown that with either substrate (PME or PDE) the data are consistent with a random Bi-Bi (sequential) mechanism. (The PME reaction is followed by measuring the weighted sum of the PDE and PC formed.) However, the experimentally determined product ratios when PME is the substrate indicate that a simple Bi-Bi sequential mechanism cannot account for the observed results. Combining this data into a mechanism which would be consistent with the product ratios has led us to propose the mechanism in Scheme 1.

The scheme is written for the conversion of PE to PC and is based on data obtained for enzyme in native RBC membranes (high endogenous PE and PC levels). Three enzyme forms (E, F, and G) are shown in the mechanism for clarity; however, we do not specify the identity of these forms. They may represent different catalytic sites on a single enzyme or they may represent different isomeric forms of the same active site. The vertical lines in the diagram indicate the channeling of the phospholipid intermediate from one enzyme form to another. In the proposed mechanism, AdoMet and AdoHcy always combine with the same enzyme forms; therefore, inhibition between these two compounds is competitive. AdoHcy is noncompetitive with the phospholipid substrate because of the formation of an E-phospholipid-AdoHcy dead-end complex (not shown in Scheme 1). Therefore, saturation with PME or PDE cannot relieve the inhibition due to AdoHcy. Once on the enzyme, the phospholipid can undergo multiple methylations before release.

Effect of AdoMet on Product Ratio—Increasing the concentration of AdoMet decreased the value of the PDE:PC product.
Kinetics of Phosphatidylethanolamine N-Methyltransferase

The effect of PME concentration on the product ratio also suggests the presence of multiple binding sites for phospholipid which become increasingly occupied by the normal intermediate of methylation (PME) when present at an abnormally high concentration as in our experiments. The accumulation of enzyme-PME complexes blocks the normal precessive methylation of phospholipid and causes premature release of partially methylated phospholipid. Schemes 1 and 2 are consistent with the experimental observations. For example in Scheme 2 the multiple methylations required for the conversion of PE to PC involve the migration of PDE from FPQ to GA. A high concentration of an intermediate such as PME may lead to PME binding to all phospholipid sites, i.e. PME may bind to G to form a G-PME complex, thus lowering the levels of G-AdoMet and the rate of PC formation relative to the rate of PDE formation. As the concentration of PME increases the value of $k_{s'}/k_{l}$ decreases (Equation 2) thus increasing the value of the PDE:PC product ratio.

The mechanism proposed here is sequential and precessive as is that recently proposed by Ridgway and Vance (37) for the rat liver enzyme; however, it differs in many other aspects. We have no evidence supporting either PME or PDE as the leasing substrate in an ordered mechanism but do have evidence for a random order of addition of reactants. It is not clear if these differences are due to species or organ differences or due to the radically different nature of the preparations (intact membranes versus reconstituted purified enzyme). The mechanism proposed here is more complete and better explains the data. For example according to the mechanism of Ridgway and Vance (37), all substrates (PE, PME, PDE) must be converted to PC with no phospholipid intermediates (because no intermediates are permitted to leave the enzyme). In contrast, our mechanism explains the large accumulation of PDE always seen when PME is the substrate.

In summary, the results of studies on the initial velocity, product inhibition, and product ratios are consistent with a random Bi-Bi sequential mechanism for the methylation of both PME and PDE and with the catalysis of both methylations by a single multi-site enzyme using partial substrate channeling. We have proposed a testable model based on these observations to guide future experiments.

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