Kinetic Model of the Protein-Mediated Phosphatidylcholine Exchange between Single Bilayer Liposomes†

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ABSTRACT: The phosphatidylcholine exchange protein from beef liver catalyzes the exchange of phosphatidylcholine between single bilayer liposomes (Hellings et al. (1974), *Eur. J. Biochem. 47,* 601). A model has been proposed which describes the kinetics of this exchange. Steady-state equations have been derived from the model and have been used for the derivation of the theoretical rate equation. Computer analysis shows a good fit with the experimental results. It follows from the analysis that the apparent dissociation constant of the exchange protein–liposome complex decreases with an increasing phosphatidic acid content of the liposomes. This suggests that in this model system it is the phospholipid composition of the membranes involved that regulates the amount of exchange protein available to function as a carrier of phosphatidylcholine.

Mammalian tissues contain soluble proteins which catalyze the transfer of phospholipids between membrane structures in vitro (Wirtz and Zilversmit, 1969; McMurray and Dawson, 1969; Wirtz, 1971; Miller and Dawson, 1972). It is thought that in situ these proteins, denoted as phospholipid exchange proteins, may be involved in the biogenesis of subcellular membranes (Wirtz, 1974). To date, phospholipid exchange proteins have been isolated from bovine liver (Kamp et al., 1973), bovine heart (Ehnholm and Zilversmit, 1973), and bovine brain (Helmkamp et al., 1974). In vitro, the proteins from bovine liver and heart catalyzed particularly the transfer of phosphatidylcholine between membranes while the proteins isolated from bovine brain showed a great preference for phosphatidylinositol.

After purification, 1 mol of the bovine liver protein contained 1 mol of noncovalently bound phosphatidylcholine (Kamp et al., 1973; Demel et al., 1973). Experiments with phospholipid monolayers have demonstrated that the protein can exchange its bound phosphatidylcholine molecule for one present in the monolayer (Demel et al., 1973). By this mode of action the protein functions as a carrier by shuttling phosphatidylcholine back and forth between membrane interfaces.

In the present paper we propose a model for the carrier action of the phosphatidylcholine exchange protein from bovine liver. This model is based on the following assumptions: (1) the formation of a collision complex between protein and membrane precedes the actual transfer of phosphatidylcholine; (2) the protein does not leave the membrane without being associated with a phosphatidylcholine molecule. This implies that net transfer of phosphatidylcholine to the membrane involved does not occur under the experimental conditions used in this study.

One of the crucial events in the transfer of phosphatidylcholine between membranes is the interaction of the exchange protein with the membrane. In order to be able to study in a systematic way the factors that govern this interaction, one should have at one's disposal a membrane system in which the physicochemical properties of the membrane can be varied. Single bilayer liposomes of known phospholipid composition provide such a system (Huang, 1969; Johnson, 1973; Litman, 1973).

Recently a technique has been described by which one can measure the exchange of phosphatidylcholine between single bilayer liposomes (Hellings et al., 1974). In this system exchange of phosphatidylcholine was only observed in the presence of the phosphatidylcholine exchange protein. The protein-mediated exchange followed the kinetics of an isotope exchange reaction (McKay, 1938). Furthermore, it was shown that the rate of exchange diminished with increasing negative surface charge of the liposomes. Incorporation of up to 20 mol % phosphatidic acid or phosphatidylinositol in the phosphatidylcholine liposomes resulted in a virtually complete inhibition of phosphatidylcholine exchange.

The kinetic model proposed in this paper has been tested with the single bilayer liposome system. It will be shown that the inhibition of phosphatidylcholine exchange observed with negatively charged liposomes can be attributed to changes in the association constants between protein and membrane.

Experimental Section

Materials. Phosphatidylcholine was isolated from egg yolks following the procedure of Papahadjopoulos and Miller (1967). Phosphatidic acid was prepared from egg yolk phosphatidylcholine by degradation with phospholipase D extracted from Savoy cabbage (Davidson and Long, 1958). A [^{14}C]methyl group was introduced into the polar headgroup of phosphatidylcholine by the method of Stoffel et al. (1972). [7α-^{3}H]Cholesteryl oleate was prepared from [^{3}H]cholesterol (The Radioachemical Centre, Amersham, England) and oleoyl chloride by the method of Swell and Treadwell (1955). Phosphatidylcholine exchange protein was purified to homogeneity from beef liver according to the procedure of Kamp et al. (1973). The protein (65 μg/ml) was stored at −20° in a 50% glycerol buffer (10 m*M* sodium citrate–20 m*M* disodium hydrogen phosphate (pH

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5.0)). Before use, the protein solution was diluted fivefold with 12.5 m*M* sodium phosphate buffer (pH 7.0).

Preparation of Single Bilayer Liposomes. Phosphatidylcholine was mixed with phosphatidic acid at the desired molar ratio in chloroform. After evaporation of the chloroform in vacuo the lipid was suspended in 12.5 m*M* sodium phosphate buffer (pH 7.0) by mixing on a Vortex. The suspension was sonicated for 30 min at 0° under nitrogen with a Branson sonifier (energy output, 50 W). The phospholipid concentration of the suspensions varied between 2 and 6 μmol/ml. The liposomes were prepared 1 day before use. Chromatography of phosphatidylcholine liposomes containing 2 mol % phosphatidic acid on a Sepharose 4B column indicated that the liposomes eluted from the column as a single peak behind the void volume. This is a criterium for a homogeneous single bilayer liposome population (Huang, 1969). Since all the lipid suspensions used in this study contained phosphatidic acid (2–12 mol %), a 30-min sonication sufficed to obtain single bilayer liposomes (Huang, 1969; Johnson, 1973).

Assay of Phosphatidylcholine Exchange between Liposomes. The assay was a slight modification of the one published previously (Hellings et al., 1974). The exchange of phosphatidylcholine between liposomes was determined by measuring the transfer of [^{14}C]phosphatidylcholine from "donor" liposomes labeled with [^{14}C]phosphatidylcholine to "acceptor" liposomes containing a trace of [^{3}H]cholesteryl oleate (0.01% by weight). The ^{3}H label served as internal standard as it does not redistribute between acceptor and donor liposomes under the conditions of incubation.

The incubations were performed at 25° in 1.5 ml of 12.5 m*M* sodium phosphate buffer (pH 7.0). Incubations were started by addition of the donor liposomes to the medium containing acceptor liposomes and exchange protein. Incubations were carried out for 10 min and stopped by addition of 25 μl of a saturated $HgCl_2$ solution (approximately 250 m*M*) at which concentration the exchange protein was completely inhibited. Acceptor liposomes were separated from donor liposomes by applying the mixture to a small DEAE-cellulose column (bed volume 0.2–1 ml) which was equilibrated with the phosphate buffer. The column was washed with 4.5 ml of phosphate buffer. By this procedure the donor liposomes containing 6.5–12 mol % phosphatidic acid were completely adsorbed to the DEAE-cellulose while the acceptor liposomes containing 2 mol % phosphatidic acid eluted from the column. The separation was completed within 5 min. The recovery of the acceptor liposomes as measured by the ^{3}H radioactivity varied between 40 and 70% and was found to depend on the proportion of liposomal phospholipid to ion exchanger used. Losses were due to nonspecific adsorption to the DEAE-cellulose. To the eluent (6 ml total volume) was added 12 ml of chloroform–methanol (1:2, v/v). After thorough mixing on a Vortex the upper and lower phases were separated by centrifugation. The upper phase was removed by suction and the lower phase containing the acceptor liposome lipids concentrated to dryness in vacuo. The lipid residue was dissolved in 16 ml of toluene (0.5% 2,5-diphenyloxazole and 0.03% 1,4-bis[2-(5-phenylaxazolyl)]benzene) and the radioactivity measured with a Packard TriCarb liquid scintillation spectrometer. The ^{14}C/^{3}H ratio of the acceptor liposomes was calculated and corrected for control experiments without exchange protein. The ^{14}C radioactivity transferred in the control experiments never exceeded 3% of the total donor ^{14}C radioactivity. The corrected ^{14}C/^{3}H ratio was converted into nanomoles of phosphatidylcholine exchanged between donor and acceptor liposomes as described (Hellings et al., 1974).

Kinetic Treatment of the Phosphatidylcholine Exchange. The exchange of phosphatidylcholine is considered to occur between two liposomal populations L_1 and L_2 which consist of phosphatidylcholine. It is assumed that every exchange protein molecule which is not associated with a liposome is associated with a phosphatidylcholine molecule. A free protein molecule which carries phosphatidylcholine originating from L_1 (L_2) is designated P_1 (P_2). It is postulated that the formation of a protein–liposome complex is necessary for exchange. Such a complex formed by the interaction of P_1 with a liposome of population L_2 is denoted P_1L_2. Similarly the complexes P_1L_1, P_2L_1, and P_2L_2 are defined. The following reaction steps (eq 1–6) characterized by specific rate constants are considered. L_1* and L_2* are the liposomal populations L_1 and L_2 in which a phosphatidylcholine molecule has been replaced by a molecule originating from populations L_2 and L_1, respectively. Reaction steps 1 and 2 (4 and 5) have the same rate constant k_1 (k_2) of complex formation as the subscripts 1 and 2 in P_1 and P_2 indicate the origin of the phosphatidylcholine molecule bound to the protein and not a chemical difference. Furthermore, it is assumed that P_2L_1 (P_1L_2) dissociates completely in P_1 and L_1* (P_2 and L_2*). See Appendix I for the instance where a certain proportion of P_2L_1 (P_1L_2) dissociates in P_2 and L_1 (P_1 and L_2).

$$P_1 + L_1 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} P_1L_1 \quad (1)$$

$$P_2 + L_1 \xrightarrow{k_1} P_2L_1 \quad (2)$$

$$P_2L_1 \xrightarrow{k_{-1}} P_1 + L_1^* \quad (3)$$

$$P_2 + L_2 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} P_2L_2 \quad (4)$$

$$P_1 + L_2 \xrightarrow{k_2} P_1L_2 \quad (5)$$

$$P_1L_2 \xrightarrow{k_{-2}} P_2 + L_2^* \quad (6)$$

The initial rate, V_0, at which phosphatidylcholine is transferred from L_1 to L_2, is given by $V_0 = k_{-2}[P_1L_2]$. By applying steady-state approximations to the concentrations of the reaction components, it is possible to express $[P_1L_2]$ in $[L_1]$, $[L_2]$, P_{tot}, and the rate constants. This yields the following expression for the initial rate

$$V_0 = \frac{k_1[L_1]k_2[L_2]P_{tot}}{(k_1[L_1] + k_2[L_2])(1 + k_1/k_{-1}[L_1] + k_2/k_{-2}[L_2])} \quad (I)$$

in which $[L_1]$ and $[L_2]$ are the total concentrations of phosphatidylcholine present in liposomes L_1 and L_2, and P_{tot} the total amount of exchange protein. Ideally, V_0 should be related to the amount of phosphatidylcholine present only in the outer liposomal monolayer (see Discussion). For the derivation of V_0, see Appendix I. In the present study L_1 and L_2 correspond with the donor and acceptor liposomes, respectively. The experimentally determined initial rates (V_{exp}) were fitted with the theoretical rate equation (eq I) by means of the least-squares method. For a mathematical treatment of the kinetic data see Appendix II. The values estimated for the kinetic parameters (k_1, k_2, k_1/k_{-1}, and k_2/k_{-2}) of eq I are expressed in the total liposomal phosphatidylcholine concentration. The relative significance of these values will be discussed (see Discussion).

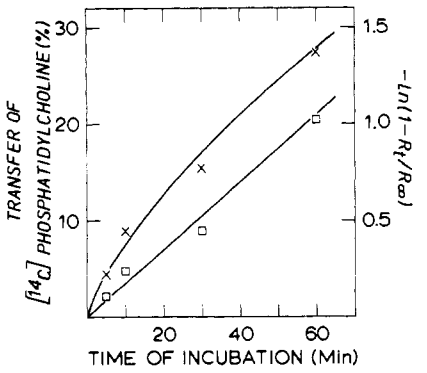

FIGURE 1: Effect of time of incubation on the transfer of [^{14}C]phosphatidylcholine from ^{14}C-labeled donor liposomes containing 6.5 mol % phosphatidic acid, to acceptor liposomes containing 2 mol % phosphatidic acid. Incubations were performed as outlined in the Experimental Section. The medium contained 0.25 μmol of donor phosphatidylcholine, 0.63 μmol of acceptor phosphatidylcholine, and 0.65 μg of phosphatidylcholine exchange protein. (X) Transfer of [^{14}C]phosphatidylcholine in percent; (□) $-\ln (1 - R_t/R_\infty)$, in which R_t and R_∞ are the percent of ^{14}C label transferred to the acceptor liposomes at time t and infinity.

Results

Exchange of phosphatidylcholine between liposomes was determined by measuring the transfer of [^{14}C]phosphatidylcholine from "donor" to "acceptor" liposomes as outlined in the Experimental Section. The transfer as catalyzed by the phosphatidylcholine exchange protein is dependent on time (Figure 1). It is seen that a linear relationship exists between time and $-\ln (1 - R_t/R_\infty)$ in which R_t and R_∞ are the percent of [^{14}C]phosphatidylcholine transferred to the acceptor at time t and infinite time. It was pointed out by McKay (1938) that such a relationship underlies an isotope exchange reaction. In most kinetic experiments performed, transfer of [^{14}C]phosphatidylcholine was approximately linear with time up to 10% (Figure 1). Routinely initial rates (nmol of phosphatidylcholine min^{-1}) were calculated from a 10-min incubation in the presence of such an amount of exchange protein that a transfer of 10% was not surpassed. The initial rate is also linear with the protein concentration.

Initial rates of phosphatidylcholine exchange were determined under three sets of experimental conditions. In all cases the acceptor liposomes contained phosphatidylcholine and 2 mol % phosphatidic acid; however, the donor liposomes contained phosphatidylcholine and varying amounts of phosphatidic acid, namely 6.5, 9, or 12 mol %. In Figure 2A-C, for example, the experimentally determined initial rates (V_{exp}) using the system containing 2 and 9 mol % phosphatidic acid liposomes are plotted against the donor concentration at three different acceptor concentrations. The curves drawn have been calculated fitting all the values of V_{exp} (n in Table I) to the theoretical rate equation (eq I) derived from the proposed model. The average standard deviation (T) in the values of V_{calcd} which make up these curves is 0.4 nmol min^{-1}. It is seen that all curves pass through a maximum, the position and magnitude of which varies with the proportion of acceptor to donor concentration. Similar curves have been calculated from the values of V_{exp} obtained with 2 and 6.5 mol % phosphatidic acid and 2 and 12 mol % phosphatidic acid liposomes.

The V_{exp} of the three sets of experiments have been fitted with the theoretical rate equation (eq I). Table I summarizes the estimated parameters with their standard deviations. The parameters k_1/k_{-1}, which may be interpreted as the association constant of the exchange protein–donor liposome complex, increase with increasing phosphatidic acid content of the liposomes. The association constant k_2/k_{-2} for the acceptor liposome is lower under all circumstances. The parameters k_1 and k_2 have the dimension of a second-order rate constant. The value of these parameters increases with more negatively charged liposomes. The two sets of parameters estimated for the 2 mol % phosphatidic acid liposomes are all within the standard deviation and independent of the kind of donor liposome used.

The reciprocal of the association constant, i.e., the dissociation constant of the various liposome–exchange protein complexes, is given in Table II. Increasing the phosphatidic acid content of the liposomes from 2 to 12 mol % results in a 150-fold decrease of the dissociation constant. The parameters k_1 and k_2 in Table I are expressed in nmol of phosphatidylcholine transferred per μg of protein per mmol of liposomal phosphatidylcholine per min. In the model proposed k_1 and k_2 are comparable to the rate constants of association. By taking into consideration a molecular weight of 20,000 for the exchange protein, these rate constants have been recalculated the values of which are given in Table II. From these rate constants and the dissociation constant the rate constant of dissociation follows. It may be seen from Table II that the rate constant of association increases and that of dissociation decreases with increased liposomal phosphatidic acid content.

The values of V_{exp} are plotted against the donor concen-

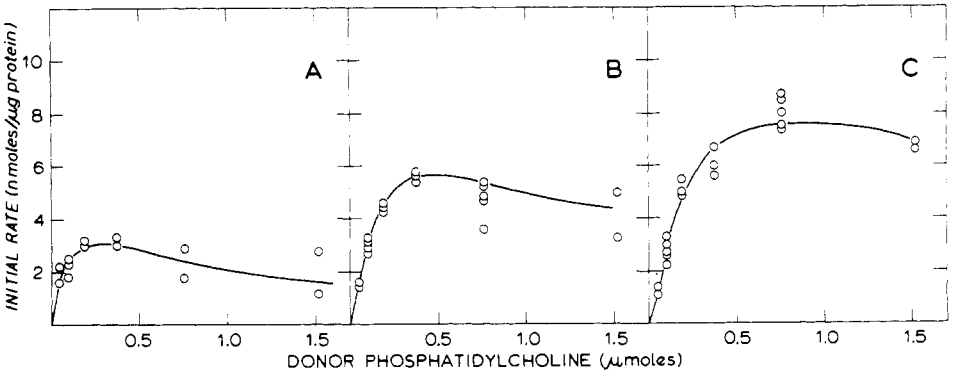

FIGURE 2: The initial rate of phosphatidylcholine exchange as a function of donor and acceptor phosphatidylcholine concentration. The donor and acceptor liposomes contained 9 and 2 mol % phosphatidic acid, respectively. The incubation medium contained 0.65 μmol (A), 1.95 μmol (B), and 3.90 μmol (C) acceptor phosphatidylcholine. (○) The experimentally determined initial rate, V_{exp}. The parameters of the computer-calculated curves are based on 49 values of V_{exp} (see Table I).

Table I: Kinetic Parameters Derived from the Theoretical Rate Equation I.

(mol % Phosphatidic Acid)					(nmol μg^{-1} mM^{-1} min^{-1})		T^d
Donor	Acceptor	n^a	$k_1/k_{-1}{}^b$ (mM^{-1})	$k_2/k_{-2}{}^b$ (mM^{-1})	$k_1{}^c$	$k_2{}^c$	(nmol min^{-1})
6.5	2	40	1.28 ± 0.45	0.09 ± 0.18	43 ± 9	24 ± 7	0.6
9	2	49	2.37 ± 0.64	0.18 ± 0.12	85 ± 17	16 ± 3	0.4
12	2	31	11.2 ± 6.3	0.27 ± 0.08	111 ± 69	20 ± 6	0.6

[a] Number of values of V_{exp} fitted in rate equation. [b] Values ± standard deviation, expressed per mM liposomal phosphatidylcholine. [c] Values ± standard deviation, expressed in nmol of phosphatidylcholine transferred per μg of exchange protein per mM of liposomal phosphatidylcholine per min. [d] Standard deviation in V_{calcd}.

Table II: Dissociation and Rate Constants of the Phosphatidylcholine Exchange Protein from Beef Liver with Respect to Various Liposomes.

Liposome (mol % Phosphatidic Acid)	Dissociation Constant[b] (M)	Association Rate Constant[b] (mM^{-1} min^{-1})	Dissociation Rate Constant (min^{-1})
2[a]	6×10^{-3}	4×10^2	22×10^2
6.5	8×10^{-4}	9×10^2	7×10^2
9	4×10^{-4}	17×10^2	7×10^2
12	9×10^{-5}	22×10^2	2×10^2

[a] The values of the constants with respect to this liposomal population are the averages of the values given in Table I. [b] As for the relative nature of the estimated values, see Discussion.

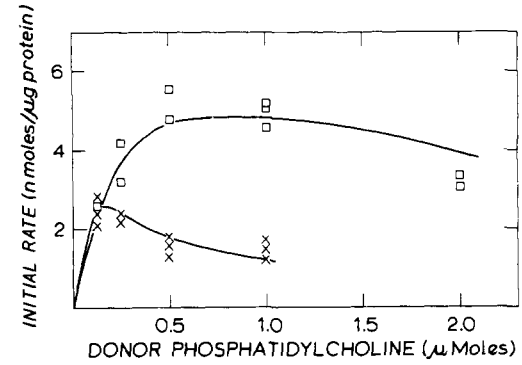

FIGURE 3: Effect of phosphatidic acid content of the donor liposomes on the initial rate of phosphatidylcholine exchange. The medium contained 0.84 μmol of acceptor phosphatidylcholine. (□) V_{exp} with 6.5 mol % phosphatidic acid donor liposomes; (X) V_{exp} with 12 mol % liposomes. The parameters of the computer-calculated curves are based on 40 values of V_{exp} for the 2–6.5 mol % and on 31 values of V_{exp} for the 2–12 mol % phosphatidic acid liposome system (see Table I).

tration of 6.5 and 12 mol % phosphatidic acid liposomes at a constant acceptor concentration, 0.84 μmol of phosphatidylcholine (Figure 3). The curves drawn have been calculated in the usual manner. The maximal value of V_{calcd} is attained with about 0.15 μmol of the 12 mol % liposomes in comparison with about 1.0 μmol of the 6.5 mol % liposomes; the maximal values themselves differ by a factor of 2. Furthermore, the decline in V_{calcd} with increasing donor concentration is much more pronounced with the 12 mol % liposomes. These differences reflect to a large extent the much higher association constant for the 12 mol % liposomes. It can be seen from the theoretical rate equation (eq I) that the decrease of V_{calcd} with increasing $[L_1]$ is particularly related to the parameter k_1/k_{-1}.

Discussion

The phosphatidylcholine exchange protein from beef liver catalyzes the exchange of phosphatidylcholine between membranes without affecting the physical nature of the membranes involved. This has been demonstrated in experiments where the exchange was measured between a [^{14}C]phosphatidylcholine labeled and an unlabeled monolayer and between such a ^{14}C-labeled monolayer and unlabeled phosphatidylcholine liposomes (Demel et al., 1973). In both instances the exchange protein by functioning as a carrier of phosphatidylcholine caused the dilution of the surface radioactivity without affecting the surface pressure of the ^{14}C-labeled monolayer, i.e., [^{14}C]phosphatidylcholine had been replaced by phosphatidylcholine from the unlabeled monolayer or liposomes. Likewise, the protein-mediated exchange of phosphatidylcholine between liposomes may be understood. To make sure that indeed we were dealing with exchange, a set of experiments were performed where the transfer of [^{14}C]phosphatidylcholine from ^{14}C-labeled donor liposomes to [^{3}H]cholesteryl oleate acceptor liposomes was compared with the transfer in opposite direction from ^{14}C,^{3}H-labeled acceptor liposomes to unlabeled donor liposomes. From the increase of the ^{14}C/^{3}H ratio of the acceptor liposomes in the former instance and the decrease of this ratio in the latter instance, it was concluded that the nmol of phosphatidylcholine transferred from donor to acceptor liposome equaled the quantity transferred in opposite direction. This is supported by the observation that the kinetics of an ideal isotope exchange reaction underlie the liposome–liposome assay (Hellings et al., 1974).

The standard deviation in V_{calcd} as determined by computer is 0.4–0.6 nmol min^{-1}. Since this value compares favorably with the error in V_{exp} found by repeating a specific incubation several times, it can be concluded that the fit of the experimental data with the model is satisfactory. The values of V_{exp} plotted in Figure 2 demonstrate the reliability of this computer-assisted fit. It is shown in Table I that the kinetic parameters of the 2 mol % phosphatidic acid liposomes determined from the 2–6.5 mol %, 2–9 mol %, and 2–12 mol % phosphatidic acid liposome systems have similar values within the calculated standard deviation. Clearly, then, the kinetic parameters of the acceptor liposomes are independent of the kind of donor liposome used. This is in further support of the model proposed.

Johnson (1973) reported that the diameter of single bilayer liposomes increased approximately twofold as the proportion of phosphatidic acid to phosphatidylcholine increased from 4 to 33 mol %. However, the area occupied per phospholipid molecule was virtually unchanged, namely 67 Å^2 vs. 63 Å^2. This shows that the phosphatidic acid content of the liposomes does not affect the number of phospholipid molecules per unit surface area, i.e., the surface density. Michaelson et al. (1973) provided evidence that single bilayer liposomes containing equal amounts of phosphatidyl-

choline and phosphatidylglycerol were characterized by an asymmetric distribution of these phospholipids over the bilayer: the outer monolayer contained twice as many phosphatidylglycerol as phosphatidylcholine molecules. The authors propose that a counterbalancing of the relatively high electrostatic repulsion between the negatively charged phosphatidylglycerol molecules on the inner liposomal monolayer underlies this asymmetric distribution. In the present study the phosphatidic acid content of the liposomes varied from 2 to 12 mol %. At the pH of incubation (pH 7) phosphatidic acid has a negative charge comparable to phosphatidylglycerol (Abramson et al., 1964). Given the relatively low concentrations of phosphatidic acid it is assumed that a substantial asymmetric distribution of phosphatidic acid and phosphatidylcholine over the bilayer of these liposomes can be excluded. Then, in summary, it may be concluded that the quantity of phosphatidylcholine present in the outer monolayer, and the total surface area occupied by it, are proportional to the total phosphatidylcholine concentration and independent of the liposomal phosphatidic acid content. Taking this conclusion into consideration it is valid to compare directly the estimated parameters for the different liposomes which are expressed in terms of total liposomal phosphatidylcholine concentration (Tables I and II).

In studying the interaction of phospholipase A with phosphatidylcholine present in monolayers, Verger et al. (1973) used in the kinetic treatment of this interaction the surface density of phosphatidylcholine, i.e., the number of molecules per unit surface area as the "substrate concentration". In the present study also it would probably be more realistic to express the dimension of the kinetic parameters in the surface density of phosphatidylcholine present in the outer liposomal monolayer. Then, however, one still faces the problem inherent to any heterogeneous phase system; namely, that the estimated parameters have only relative significance (Roholt and Schlamowitz, 1961; Wells, 1972). For this reason, in calculating the kinetic parameters for the single bilayer liposome system it was felt unnecessary to correct for the approximately 30% phosphatidylcholine present in the inner liposomal monolayer which very likely does not participate in the exchange reaction (Hellings et al., 1974; Kornberg and McConnell, 1971; Finer et al., 1972). From the above argument it is clear that the estimated parameters given in Tables I and II serve only a comparative purpose and that no absolute significance should be attached to the values. One, however, should make an exception for the dissociation rate constant (Table II, k_{-1} and k_{-2} in Table I) which characterizes all molecular events after the formation of the collision complex and lacks the liposomal phosphatidylcholine concentration term.

As for this moment it is thought that phosphatidic acid present in the outer monolayer functions as a "neutral" spacer; in this context "neutral" means that the exchange protein has no affinity for phosphatidic acid as an exchangeable phospholipid upon interaction with the liposomal surface. Therefore, the amount of phosphatidic acid present in the liposomes was not considered in the calculation of the kinetic parameters. In the present study, however, an increase of the phosphatidic acid content from 2 to 12 mol % will decrease the surface density of the outer monolayer phosphatidylcholine by approximately 10%. Computer analysis has indicated that variation of up to 10% in the phosphatidylcholine concentration has little significant effect on the estimated parameters.

In the acceptor-donor liposome system the exchange protein carries phosphatidylcholine between the liposomal vesicles without altering the identity of these vesicles, except that a redistribution of ^{14}C radioactivity is observed. The phosphatidylcholine molecule released by the protein in the membrane is chemically identical with the one extracted by the protein from the membrane. This exchange process, which is described by the kinetic model proposed in this study, differs from an enzymic reaction since no covalent bonds are formed or broken. However, it is thought that upon interaction of the protein with the liposomal surface the protein comes from a hydrophilic into a more hydrophobic environment. This may induce a conformational change in the protein to the extent that the phosphatidylcholine molecule noncovalently bound to the protein mixes with the liposomal phosphatidylcholine pool. The specificity of the exchange protein for phosphatidylcholine suggests that in addition to hydrophobic forces, electrostatic interactions between the polar moiety of the phosphatidylcholine molecule and the protein also play a role in the above process (Kamp et al., 1973).

The theoretical rate equation (eq I) derived from the model is characterized by four unknown variables: (a) k_1/k_{-1} and k_2/k_{-2} which may be considered as the association constants of the collision complex between exchange protein and liposome L_1 and L_2; and (b) k_1 and k_2 which are the rate constants of association. As for the association constants, additional techniques should be used to confirm the values computed from the model. Whether the values computed for k_1 and k_2 are the actual rate constants of association or relate to a composite term of three rate constants (k_1k_3/k_{-1}, and k_2k_4/k_{-2}) depends on the probability (k_3/k_{-1} and k_4/k_{-2}) that the complexes P_2L_1 and P_1L_2 dissociate in P_1 and L_1*, and P_2 and L_2* (see Appendix I). Phosphatidylcholine molecules undergo a lateral diffusion in the plane of a liposomal membrane with a diffusion constant in the order of 10^{-8} cm^2 sec^{-1} (Devaux and McConnell, 1972). From this it may be calculated that the probability per unit time that a lipid molecule jumps from one site to any one of its neighboring sites is in the order of magnitude of 10^7 sec^{-1}. If after formation of the exchange protein–liposome complex the exchange frequency of the protein-bound phosphatidylcholine molecule with the liposomal phosphatidylcholine pool is of the same order, and high with regard to the lifetime of the collision complex, then k_3/k_{-1} (k_4/k_{-2}) may equal one. This would render k_1 and k_2 in Table I directly interpretable as the rate constants of association. On the other hand, if k_3/k_{-1} (k_4/k_{-2}) is not equal to one, the values computed relate to k_1k_3/k_{-1} (k_2k_4/k_{-2}).

It is shown in Figure 2 that the initial rate, V_0, passes through a maximum, the position and value of which is determined by the proportion of donor to acceptor concentration. Furthermore, it can be seen from the theoretical rate equation (eq I) that the donor concentration at which V_0 has its maximal value and the decrease of V_0 with increasing donor concentration are greatly dependent on the value of k_1/k_{-1}, i.e., the association constant. This latter point has been illustrated in Figure 3 with donor liposomes of 6.5 and 12 mol % phosphatidic acid liposomes. At a donor concentration above 0.15 μmol of phosphatidylcholine, V_0 has a lower value with the more negatively charged liposomes. The dissociation constant of the exchange protein–liposome complex decreases from 6×10^{-3} to 9×10^{-5} M as the liposomal phosphatidic acid content increases from 2 to 12 mol % (Table II). Therefore, the decrease of V_0 observed with more negatively charged liposomes may be understood

as a consequence of a smaller dissociation constant; less exchange protein will be free in the medium to function as a carrier of phosphatidylcholine. This suggests that the lipid composition of the membrane may exert a regulatory role on the activity of the exchange protein. Similar observations have been made with the proteins isolated from beef brain which catalyze particularly the transfer of phosphatidylinositol between membranes (Helmkamp et al., 1974). The protein-mediated transfer of phosphatidylinositol between rat liver microsomes and single bilayer liposomes was inhibited by increasing the phosphatidylinositol to phosphatidylcholine molar ratio of these liposomes (Harvey et al., 1974). It is of interest to note that in this instance the level of phosphatidylinositol in the liposomes controls the rate of phosphatidylinositol transfer to these membranes.

Although phosphatidic acid is considered a "neutral" spacer with regard to its affinity for the exchange protein, incorporation of this negatively charged phospholipid into the liposomal bilayer will have a pronouced effect on the interfacial properties. Recently it was demonstrated that the presence of phosphatidic acid in single bilayer phosphatidylcholine liposomes affected the packing of the polar region of these liposomes (Flanagan and Hesketh, 1973). On the other hand, surface charge had much less of an effect on the forces which dictate the dynamic properties of the hydrocarbon region in lipid bilayers (Shinitzky and Barenholz, 1974). A possible explanation of the inhibitory effect of phosphatidic acid on the exchange of phosphatidylcholine observed in the present study is that the negatively charged polar headgroups repel each other so that the exchange protein penetrates more easily into the liposomal surface. Consequently, this may be reflected in a decrease of the dissociation constant (Table II). This is supported by the observation that divalent ions such as Ca^{2+} and Mg^{2+} which will neutralize the negative surface charge counteract the inhibitory effect of phosphatidic acid (K. W. A. Wirtz and J. A. Hellings, unpublished observation). The inhibitory effects of other negatively charged phospholipids, such as phosphatidylinositol, phosphatidylglycerol, and phosphatidylserine, on the exchange of phosphatidylcholine may be understood in a similar way (Wirtz, 1972). The specificity of the exchange protein with regard to the transfer of phosphatidylcholine suggests that an electrostatic interaction between the protein and the polar headgroup of phosphatidylcholine is involved (Kamp et al., 1973). As yet it is not known how the surface charge of the interface and the ionic composition of the medium relate to this interaction.

Appendix I

For a definition of the abbreviations P_1, P_2, P_1L_1, P_2L_2, P_1L_2, and P_2L_1, see the Experimental Section. The kinetic model of the protein-mediated phosphatidylcholine exchange between liposomes is based on the reaction steps 1–6b. L_1* and L_2* are the liposomal populations L_1 and L_2 in which a phosphatidylcholine molecule has been replaced by a molecule originating from population L_2 and L_1, respectively. k_3/k_{-1} may be interpreted as the fraction of P_2L_1 that dissociates into P_1 and L_1*, i.e., the probability that the phosphatidylcholine molecule associated with the protein molecule is exchanged for a phosphatidylcholine molecule of liposome 1. Similarly, $(k_{-1} - k_3)/k_{-1}$ is that fraction of P_2L_1 which will not exchange its phosphatidylcholine molecule upon dissociation.

$$P_1 + L_1 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} P_1L_1 \quad (1)$$

$$P_2 + L_1 \xrightarrow{k_1} P_2L_1 \quad (2)$$

$$P_2L_1 \xrightarrow{k_3} P_1 + L_1^* \quad (3a)$$

$$P_2L_1 \xrightarrow{k_{-1}-k_3} P_2 + L_1 \quad (3b)$$

$$P_2 + L_2 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} P_2L_2 \quad (4)$$

$$P_1 + L_2 \xrightarrow{k_2} P_1L_2 \quad (5)$$

$$P_1L_2 \xrightarrow{k_4} P_2 + L_2^* \quad (6a)$$

$$P_1L_2 \xrightarrow{k_{-2}-k_4} P_1 + L_2 \quad (6b)$$

The transfer of [^{14}C]phosphatidylcholine as actually measured in this study relates to reaction step 6a. The initial rate, V_0, at which phosphatidycholine molecules originating from L_1 (donor) appear in L_2 (acceptor) is given by $V_0 = k_4[P_1L_2]$. $[P_1L_2]$ is expressed in $[L_1]$, $[L_2]$, P_{tot}, and the rate constants by applying steady-state approximations to the exchange proteins P_1 and P_2 and the complexes P_1L_1, P_2L_1, P_1L_2, and P_2L_2. Under the steady-state conditions eq 7–13 can be set up. By substituting in eq 7 or 8, $[P_1L_2]$,

$$d[P_1]/dt = k_{-1}[P_1L_1] + k_3[P_2L_1] + (k_{-2} - k_4)[P_1L_2] - k_2[P_1][L_2] - k_1[P_1][L_1] = 0 \quad (7)$$

$$d[P_2]/dt = k_{-2}[P_2L_2] + k_4[P_1L_2] + (k_{-1} - k_3)[P_2L_1] - k_1[P_2][L_1] - k_2[P_2][L_2] = 0 \quad (8)$$

$$d[P_1L_2]/dt = k_2[P_1][L_2] - k_{-2}[P_1L_2] = 0 \quad (9)$$

$$d[P_2L_2]/dt = k_2[P_2][L_2] - k_{-2}[P_2L_2] = 0 \quad (10)$$

$$d[P_1L_1]/dt = k_1[P_1][L_1] - k_{-1}[P_1L_1] = 0 \quad (11)$$

$$d[P_2L_1]/dt = k_1[P_2][L_1] - k_{-1}[P_2L_1] = 0 \quad (12)$$

$$P_{tot} = [P_1] + [P_2] + [P_1L_1] + [P_2L_1] + [P_2L_2] + [P_1L_2] \quad (13)$$

$[P_2L_2]$, $[P_1L_1]$, and $[P_2L_1]$, which follow from eq 9 to 12 we obtain

$$[P_2] = \frac{k_{-1}}{k_1}\frac{k_2}{k_{-2}}\frac{k_4}{k_3}[P_1]\frac{[L_2]}{[L_1]}$$

By substituting $[P_2]$, $[P_1L_2]$, $[P_2L_2]$, $[P_1L_1]$, and $[P_2L_1]$ in eq 13 we obtain:

$$P_{tot} = [P_1]\left(1 + \frac{k_{-1}}{k_1}\frac{k_2}{k_{-2}}\frac{k_4}{k_3}\frac{[L_2]}{[L_1]}\right)\left(1 + \frac{k_1}{k_{-1}}[L_1] + \frac{k_2}{k_{-2}}[L_2]\right)$$

By substituting P_{tot} in eq 9 we obtain:

$$[P_1L_2] = \frac{(k_3k_1/k_{-1})[L_1](k_2/k_{-2})[L_2]P_{tot}}{[(k_3k_1/k_{-1})[L_1] + (k_4k_2/k_{-2})[L_2]][1 + (k_1/k_{-1})[L_1] + (k_2/k_{-2})[L_2]]}$$

Since the initial rate $V_0 = k_4[P_1L_2]$, the theoretical rate equation follows. In the Experimental Section it is assumed that $k_3/k_{-1}(k_4/k_{-2})$ equals one, i.e., $P_2L_1(P_1L_2)$ dissociates completely in P_1 and L_1* (P_2 and L_2*). By eliminating k_3/k_{-1} and k_4/k_{-2} from the theoretical rate equation, we obtain:

$$V_0 = \frac{k_1[L_1]k_2[L_2]P_{tot}}{(k_1[L_1] + k_2[L_2])(1 + k_1/k_{-1}[L_1] + k_2/k_{-2}[L_2])}$$

Appendix II

The experimentally determined initial rates (V_{exp}) were fitted with the theoretical rate equation (eq I) by means of the least-squares method. This rate equation is characterized by the unknown parameters k_1, k_2, k_1/k_{-1}, and k_2/k_{-2} which are abbreviated as P_1, P_2, P_3, and P_4, respectively. A computer program was used which minimized the sum F of the squared differences between measured (V_{exp}) and calculated initial rates (V_{calcd}) by adjusting the above parameters:

$$F(P_1, P_2, P_3, P_4) = \sum_n (V_{exp} - V_{calcd})^2 = \text{minimal}$$

in which n is the number of data. V_{calcd} is calculated with eq I for a definite set of parameters. The minimization technique of Fletcher and Powell (1963) was used. The values P_1, P_2, P_3, and P_4 corresponding with the minimum of F are the best estimates of the parameters. The standard deviation of V_{calcd} is given by the square root of the variance T^2: $T = [F_{min}/(n-4)]^{1/2}$ in which F_{min} is the minimal sum and $n-4$ the number of degrees of freedom. The standard deviation of the parameters can be found from the covariance matrix (Wentworth, 1965). This matrix is formed as follows. The elements b_{ij} of the 4×4 matrix $\bar{\mathbf{B}}$ are given by:

$$b_{ij} = \sum_n \left(\frac{\partial F}{\partial P_i}\right)\left(\frac{\partial F}{\partial P_j}\right)$$

The covariance matrix $\bar{\mathbf{C}}$ is obtained by multiplication of T^2 and the inverse of $\bar{\mathbf{B}}$: $\bar{\mathbf{C}} = T^2 \times \bar{\mathbf{B}}^{-1}$. The variance of the parameters are given by the elements c_{ii} forming the diagonal of the covariance matrix $\bar{\mathbf{C}}$. The standard deviation of parameter P_i is the square root of the corresponding variance: $T_{P_i} = (c_{ii})^{1/2}$.

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