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Transbilayer diffusion of phospholipids: dependence on headgroup structure and acyl chain length

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The kinetics and thermodynamics of the transmembrane movement (flip-flop) of fluorescent analogs of phosphatidic acid (PA), phosphatidylglycerol (PG), phosphatidylcholine (PC), and phosphatidylethanolamine (PE) were investigated to determine the contributions of headgroup composition and acyl chain length to phospholipid flip-flop. The phospholipid derivatives containing *n*-octanoic, *n*-decanoic or *n*-dodecanoic acid in the *sn*-1 position and 9-(1-pyrenyl)nonanoic acid in the *sn*-2 position were incorporated at 3 mol% into sonicated single-bilayer vesicles of 1-palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphocholine (POPC). The kinetics of diffusion of the pyrene-labeled phospholipids from the outer and inner monolayers of the host vesicles to a large pool of POPC acceptor vesicles were monitored by the time-dependent decrease of pyrene excimer fluorescence. The observed kinetics of transfer were biexponential, with a fast component due to the spontaneous transfer of pyrenyl phospholipids in the outer monolayer of labeled vesicles and a slower component due to diffusion of pyrenyl phospholipid from the inner monolayer of the same vesicles. Intervesicular transfer rates decreased approx. 8-fold for every two carbons added to the first acyl chain. Correspondingly, the free energy of activation for transfer increased approx. 1.3 kcal/mol. With the exception of PE, the intervesicular transfer rates for the different headgroups within a homologous series were nearly the same, with the PC derivative being the fastest. Transfer rates for the PE derivatives were 5- to 7-fold slower than the rates observed for PC. Phospholipid flip-flop, in contrast, was strongly dependent on headgroup composition with a smaller dependence on acyl chain length. At pH 7.4, flip-flop rates increased in the order PC < PG < PA < PE, where the rates for PE were at least 10-times greater than those of the homologous PC derivative. Activation energies for flip-flop were large, and ranged from 38 kcal/mol for the longest acyl chain derivative of PC to 25 kcal/mol for the PE derivatives. Titration of the PA headgroup at pH 4.0 produced an approx. 500-fold increase in the flip-flop rate of PA, while the activation energy decreased 10 kcal/mol. Increasing acyl chain length reduced phospholipid flip-flop rates, with the greatest change observed for the PC analogs, which exhibited an approx. 2-fold decrease in flip-flop rate for every two methylene carbons added to the acyl chain at the *sn*-1 position. Our results suggest that the insolubility of the polar headgroup in the membrane interior is the major barrier to flip-flop. The contribution of the apolar portion of the phospholipid to flip-flop is less significant.

Abbreviations: C_8, octanoic acid; C_{10}, decanoic acid; HDL, high-density lipoproteins; lysoPC, 1-acyl-*sn*-glycero-3-phosphocholine; Mops, 4-morpholinepropanesulfonic acid; PN, 9-(1-pyrenyl)nonanoic acid; POPC, 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphocholine; PA, phosphatidic acid, PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; C_{12}PNPC, C_{12}PNPE, C_{12}PNPG, C_{12}PNPA, 1-lauroyl-2-[9-(1-pyrenyl)nonanoyl]-phosphatidylcholine, -phosphatidylethanolamine, -phosphatidylglycerol and -phosphatidic acid, respectively.

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Introduction

The transport of lipophilic molecules to and among the various compartments of living cells is an important part of molecular sorting and processing. Some of these processes involve the breaking and forming of covalent bonds. Others occur through the action of specific transport proteins. These activities occur against a constant background of spontaneous transport events that are governed by (1) the structure of the transported species and (2) its microenvironment. These processes include diffusion in the membrane plane, spontaneous transfer among lipid surfaces, and transbilayer migration (flip-flop).

The lateral diffusion and intermembrane transfer of phospholipids have been the subject of numerous studies (for review, see Ref. 1). Many of the physical and chemical determinants that govern these processes have been elucidated. In general, these studies have shown that the lateral diffusion of membrane lipids is strongly dependent on the fluidity and composition of the host membrane, but relatively independent of the chemical nature of the diffusing species. In contrast, intermembrane diffusion rates are strongly dependent on the composition and length (i.e., hydrophobicity) of the phospholipid acyl chains. Studies of phospholipid exchange between bilayer membranes [2] or the surface monolayer of phospholipids surrounding model lipoproteins [3] have shown that the free energy of activation for transfer increases approx. 650 cal/mol for every methylene unit added to the acyl chains. This is reflected in an almost 10-fold decrease in the rate of transfer for every two methylenes added. Furthermore, transfer rates increase 3- to 5-fold with the addition of double bonds. The electrolyte composition of the aqueous phase also modifies intermembrane transfer rates through headgroup charge neutralization and salting in/salting out effects [3]. These observations are consistent with a mechanism in which the rate-limiting step for transfer is dissociation of phospholipid monomers from the membrane into the aqueous phase.

Information of similar scope is lacking for phospholipid flip-flop. A variety of techniques, including enzymatic modification, chemical reaction and spectroscopy, have been used to quantify the transmembrane mobility of various phospholipids in both model and biological membranes (for review, see Refs. 4 and 5). The results vary widely but, in general, it can be concluded that flip-flop rates are exceedingly slow in model membranes, with half-lives ranging from hours to days, whereas in several of the biological membrane systems studied, half-lives of minutes or less have been reported [6–8]. The more rapid diffusion kinetics may be due to specific carrier proteins or to nonspecific lipid-protein interactions which provide lower energy pathways for the migration of polar lipids across the membrane.

The quantitative relationship between the composition and structure of phospholipids and flip-flop rates is unknown. In this report we present kinetic and thermodynamic data for the rates of flip-flop of fluorescent phospholipid analogs across bilayers of POPC as a function of headgroup composition and acyl chain length in the diffusing species. Fluorescent analogs of PA, PC, PE, and PG containing 9-(1-pyrenyl)nonanoic acid esterified to the *sn*-2 position of the glycerol backbone and either an 8, 10- or 12-carbon saturated fatty acid in the *sn*-1 position were studied. Inclusion of the pyrene fluorophore enabled us to continuously monitor time-dependent changes in the concentration of labeled lipid in both monolayers of vesicle bilayers by fluorimetric analysis of pyrene excimer emission. The utility of this spectroscopic technique for measuring lipid transfer kinetics has been demonstrated in previous studies [3,9–11].

Experimental procedures

Materials

POPC, 1-octanoyl-, 1-decanoyl-, and 1-dodecanoyllysoPC were purchased from Avanti Polar Lipids, (Birmingham, AL). 9-(1-Pyrenyl)nonanoic acid (PN) was obtained from Molecular Probes, (Eugene, OR). 4-Pyrollidinopyridine and trimethylacetylchloride were obtained from Aldrich Chemical Co. (Milwaukee, WI). Phospholipase D (cabbage) was supplied by Sigma (St. Louis, MO). The solvents used were reagent or HPLC grade. All other materials were reagent grade.

Methods

Lipid synthesis. The structures of the lipids used

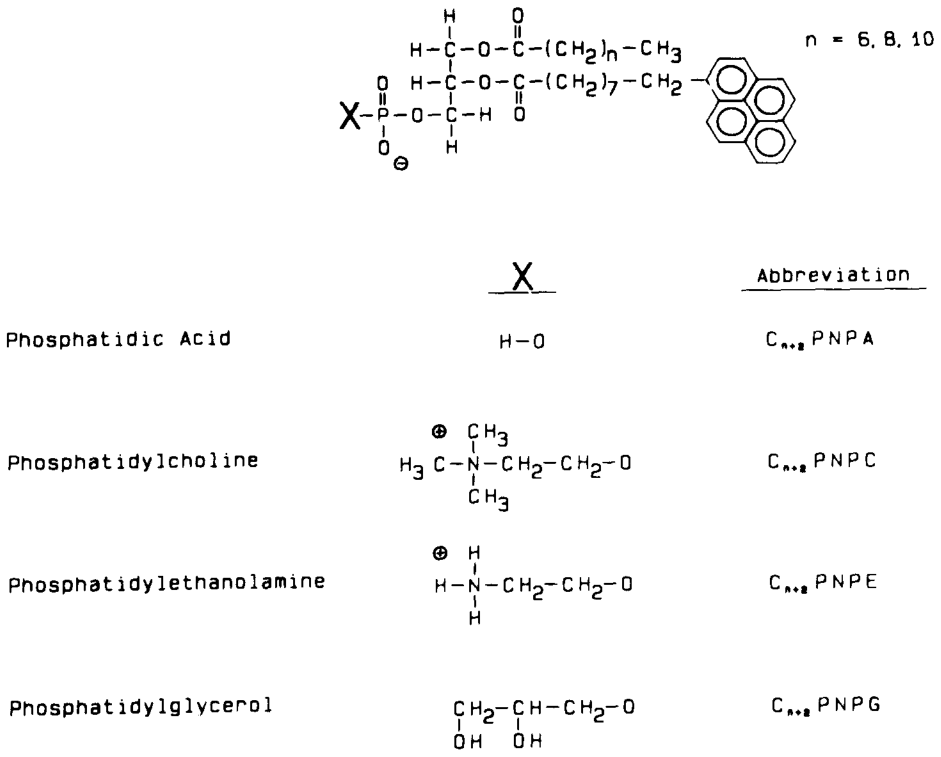

Fig. 1. Structures of pyrene-labeled phospholipids used in this study.

in this study are given in Fig. 1. The synthesis of 1-lauroyl-2-[9-(1-pyrenyl)nonanoyl]PC (C_{12}PNPC) outlined below is representative of the acylation procedure used for all the lipids in this study.

1.1 mol equiv. of trimethylacetyl chloride were added to 100 mg of PN in 5 ml of methylene chloride (dry), which also contained an equivalent of triethylamine (distilled over KOH). After 2 h of stirring in a sealed screwcap test-tube at ambient temperature, 2 ml of methylene chloride containing 0.2 equiv. 1-lauroyllysoPC and 0.01 equiv. 4-pyrollidinopyridine (catalyst) were added. Stirring of the reaction mixture was continued at 40 ° C, overnight. The absence of a lysoPC band in separations of reaction mixture on silica coated TLC plates, developed in chloroform/methanol/ammonium hydroxide (65 : 35 : 5), indicated complete acylation. C_{12}PNPC was isolated by elution of methylene chloride solution through a column of Silicar CC4 (Mallinkrodt, Paris, KT) in chloroform with an increasing gradient of methanol. The product eluted at 40% methanol. Purity was determined by TLC and HPLC.

The PG and PE analogs were obtained by the enzymatic transphosphatidylation of the corresponding PC derivative with phospholipase D in the presence of glycerol and ethanolamine, respectively [12]. Triton X-100 was included in the aqueous phase (2%, v/v) to facilitate dispersion of the phospholipid. The PA derivatives were obtained as side-products of the transphosphatidylation.

HPLC analysis of pyrenyl phospholipids. HPLC was performed with a Spectra-Physics 8000B liquid chromatograph (Spectra-Physics, Santa Clara, CA) coupled to Spectra-Physics 8400 UV/VIS absorbance detector and a Spectroflow 980 fluorescence detector (Kratos Analytical, Ramsey, NJ). Retention times on a C_8 reverse-phase column (Spectra-Physics RP-8) were determined for each of the C_8 and C_{12} phospholipid derivatives. Lipids were eluted at a 1 ml/min flow rate with a gradient of 2-propanol : 5 mM $(NH_4)_3PO_4$ (pH 7.4) increasing from a ratio of 45 : 55 to 50 : 50 over 60 min.

Vesicle preparation. Donor vesicles were prepared by combining aliquots of POPC and pyrene phospholipid in chloroform at a molar ratio of 97 : 3. Acceptor vesicle lipid solutions contained only POPC. The solvent was evaporated under a stream of N_2 followed by a minimum of 4 h of high vacuum to remove traces of solvent. The dried lipid mixture was dispersed in 100 mM NaCl/10 mM MOPS/5 mM EDTA/1 mM NaN_3 (pH 7.4, unless noted otherwise) and sonicated with a microtop probe on a Branson sonifier (Branson, Danbury, CT). During sonication, the lipid dispersion was kept under an N_2 atmosphere and cooled by an ice-bath. The sonication and isolation of vesicles was done by the procedure of Barenholz et al. [13].

Kinetic fluorimetry. Donor vesicle solutions (40 μM POPC) in 1 cm^2 fluorescence cuvettes, sealed with a Teflon stopper, were equilibrated to the appropriate temperature in the thermostated cuvette holder (external circulating water bath) of an SLM 8000 spectrofluorimeter (SLM, Urbana IL). A Teflon-coated magnetic stir bar, included in the cuvette, was driven by a magnetic stirrer under the cuvette holder. A concentrated aliquot of acceptor vesicles was added (final [POPC] = 0.8 mM) and simultaneously a time base scan of the fluorescence at excitation and emission wavelengths of 342 nm and 470 nm, respectively, was begun on an X-Y chart recorder. The width of the slits between the excitation source and the sample was kept narrow (2 nm) to minimize photolysis of the fluorophore. Photolysis rates were less than 10% of the slowest rate observed for excimer fluorescence decay. A thermocouple and a Bailey BAT-8 digital thermometer (Bailey Instruments, Saddle Brook, NJ) were used to measure the temperature of the cuvette contents. The thermocouple was precalibrated to correct for the nonlinearity of response. The lipids were solvent-extracted from several samples following the decay experiments and analyzed by TLC on silica for lipid degradation. Only bands corresponding to the starting lipids could be detected.

Analysis of data. The fluorescence scans were converted to digital format with a digitizing tablet (Summagraphics, Fairfield, CT). The kinetic parameters were determined by fitting the fluorescence decay data to Eqn. 1 using a nonlinear regression program based on the Marquardt algorithm [14].

$$F(t) = A_1 \exp(-k_1 t) + A_2 \exp(-k_2 t) + C \qquad (1)$$

A_1 and A_2 represent the initial fluorescence intensities observed in two kinetically distinct compartments. k_1 and k_2 are the rates at which A_1 and A_2 decay. The static background fluorescence and the total excimer fluorescence remaining after complete equilibration of the probe lipid between all the lipid compartments (i.e., $t = \infty$) are represented by C. Many scans were obtained under conditions where only one component in the decay kinetics could be observed. These scans were fitted to a form of Eqn. 1 in which one exponential term was set equal to zero (Eqn. 2).

$$F(t) = A \exp(-kt) + C \qquad (2)$$

The two rate constants for the kinetic components in C_{12}PNPC fluorescence decays were different by greater than two orders of magnitude. In this case, the kinetic parameters for the long phase were determined by fitting the tail of the scan to Eqn. 2. The kinetic parameters of the short phase were obtained by fitting the difference between the observed decay and the long component to Eqn. 2. The accuracy of the fits was determined by examination of residual plots and by the size of χ^2. Fits were accepted when $\chi^2 < 1$.

Thermodynamic parameters. Activation energies, E_{act}, were calculated from the slopes of the Arrhenius plots. The remaining thermodynamic parameters were calculated from absolute rate theory [15]. The enthalpy of formation of the activated state, $\Delta H^\ddagger$; the entropy of formation of the activated state, $\Delta S^\ddagger$; and the free energy of activation, $\Delta G^\ddagger$, are determined as shown:

$$\Delta H^\ddagger = E_{act} - RT$$

$$\Delta S^\ddagger = R \ln(NhX/RT)$$

$$X = k \exp(\Delta H^\ddagger/RT)$$

$$\Delta G^\ddagger = \Delta H^\ddagger - T\Delta S^\ddagger$$

R is the gas constant, T is the temperature (K), N is Avogadro's number, h is Planck's constant and k is the rate constant at 37°C extrapolated from the Arrhenius data.

Results

Between 0 and 5%, the intensity of excimer fluorescence of the pyrene-labeled phospholipids is linear with their respective concentrations in the POPC bilayer [3]. Addition of a large molar excess of vesicles composed of only POPC (acceptor vesicles) to vesicles containing 3 mol% pyrene lipid (donor vesicles) resulted in depletion of the pyrene lipid in donor vesicles by spontaneous transfer to the acceptor vesicles. The time-course of decrease in pyrene lipid concentration in the donor vesicles was detected through the decay in excimer fluorescence. Representative traces of excimer fluorescence decay for C_{12}PNPA and C_{12}PNPC (Fig. 2) are biphasic and best described as the sum of two exponentials plus a constant (Eqn. 1). Similar results were obtained for the PG and PE analogs. Fluorescence scans were obtained at a series of temperatures between 40 and 70 ° C. Fits of the data to Eqn. 1 yield values for A_1 and A_2 that are proportional to the initial fluorescence intensities associated with the constants k_1 and k_2, respectively. The ratio of A_1/A_2 obtained for donor vesicles containing C_{12}PNPC, C_{12}PNPE, C_{12}PNPG or C_{12}PNPA was 2.9, 1.3, 1.7 and 1.2, respectively. These ratios were independent of the amount of acceptor vesicles added. However, the residual fluorescence remaining after the steady-state had been reached (C in Eqn. 1 and Eqn. 2) was directly dependent on the final lipid concentration. These results are expected if A_1 and A_2 are the initial fluorescence from separate compartments in the donor vesicles and C represents the fluorescence that remains when the probe lipid has equilibrated at a finite concentration between all the lipid compartments.

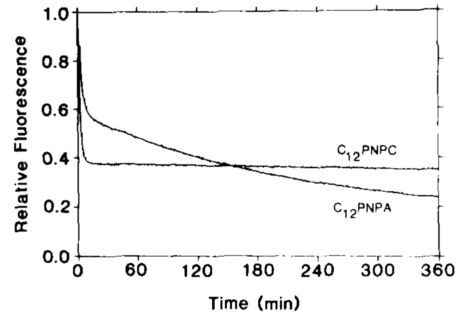

Fig. 2. Decay of excimer fluorescence as a function of time at 50 ° C following addition of POPC acceptor vesicles to donor vesicles containing 3 mol% C_{12}PNPA or C_{12}PNPC. The solid lines represent the least-squares sit of the data to Eqn. 1 with k_1, k_2, A_1, A_2 and C as the free parameters. Plots of the residuals (not shown) showed a variation of less than 0.2% between the data and the fits.

Rate constants calculated for the fast component in fluorescence decays obtained for the C_{10} and C_8 derivatives increased approx. 8-fold with a

TABLE I

SUMMARY OF KINETIC DATA AND THERMODYNAMIC PARAMETERS OF THE ACTIVATED STATE FOR THE FAST COMPONENT IN TRANSFER OF PYRENYL PHOSPHOLIPIDS BETWEEN POPC VESICLES

Headgroup	n [a]	k_1 [b] (min^{-1})	$t_{1/2}$ (min)	E_{act} [c] (kcal/mol)	$\Delta H^{\ddagger}$ (kcal/mol)	$\Delta G^{\ddagger}$ (kcal/mol)	$\Delta S^{\ddagger}$ (cal/mol per K)
Choline	8	6.80	0.10	16.1	15.5	19.5	−13
	10	1.28	0.54	18.8	18.2	20.7	−8
	12	0.15	4.62	22.3	21.7	21.9	−6
Glycerol	8	5.27	0.13	18.6	18.0	19.7	−5
	12	0.06	11.6	22.3	21.7	22.4	−2
Acid	8	6.50	0.11	14.2	13.6	19.5	−19
	12	0.08	8.7	23.3	22.7	22.2	2
Ethanolamine	8	0.99	0.70	17.7	17.1	20.7	−12
	12	0.03	23.1	26.0	25.4	22.8	8

[a] Number of carbons in acyl chain at position one.

[b] The rate constants (k_1) and half-times ($t_{1/2}$) are extrapolated to 37 ° C from the Arrhenius data.

[c] The activation energies have an experimental error of ±5%.

decrease in acyl chain length at position one of two methylene units (Table I). As a consequence, the rapid phase kinetics of the C_8 and C_{10} derivatives were examined in the temperature range of 0 to 30 °C where the rates were sufficiently slow to allow accurate analysis by our fluorescence assay. In this temperature range, contributions from the second decay component were undetectable. The effect of temperature on the rates of the slow component were derived from fluorescence traces obtained between 40 and 70 °C. At these higher temperatures, the decay of the fast component was nearly complete in the time required to add acceptor vesicles (approx. 30 s). In both cases, the kinetic traces were monoexponential and were fit well by Eqn. 2.

Contributions to the observed fluorescence decays due to vesicle fusion were not significant. The decay kinetics for donor vesicles containing C_8PNPA and incubated at donor/acceptor ratios of 1 : 10 to 1 : 80 gave less than a 10% change in the kinetics of transfer. Furthermore, when the non-exchangeable cholesteryl ester analog, cholesteryl [12-(1-pyrenyl)]dodecanoate, was substituted for pyrene-labeled phospholipid in the donor vesicles, no time-dependent decrease in excimer fluorescence could be detected following the addition of acceptor vesicles (data not shown).

The uniqueness of the two decay components is indicated in the temperature dependence of the rates. Arrhenius plots of the rates for the rapid transfer components of the PC analogs are shown in Fig. 3. The activation energies of transfer obtained from the slopes are 23, 19 and 16 kcal/mol for the C_{12}, C_{10} and C_8 derivatives, respectively. The same effect of acyl chain length on activation energy was observed for the PA, PG, and PE analogs. Massey et al. [3] reported an activation energy of 23.7 kcal/mol for the transfer of C_{12}PNPC between the membranous surfaces of model lipoproteins, which is similar to our result for this lipid. The thermodynamic parameters for the transition state of the fast component are given in Table I. The most notable trend is in $\Delta G^{\ddagger}$, which exhibits an average change of 550 to 650 cal/methylene unit, irrespective of the identity of the headgroup.

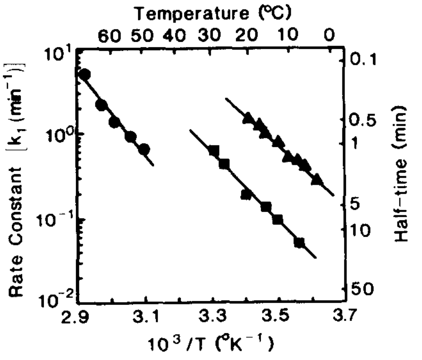

Fig. 3. Arrhenius plots of the rate constants for the fast decay component (k_1) observed in fluorescence scans of C_8PNPC (▲), C_{10}PNPC (■) and C_{12}PNPC (●) transfer. Activation energies, calculated from the slopes of the linear regression fits (solid lines), are given in Table I.

Arrhenius plots of decay rates for the slow phase are displayed in Fig. 4. With the exception of the PE analogs, the activation energies reveal a

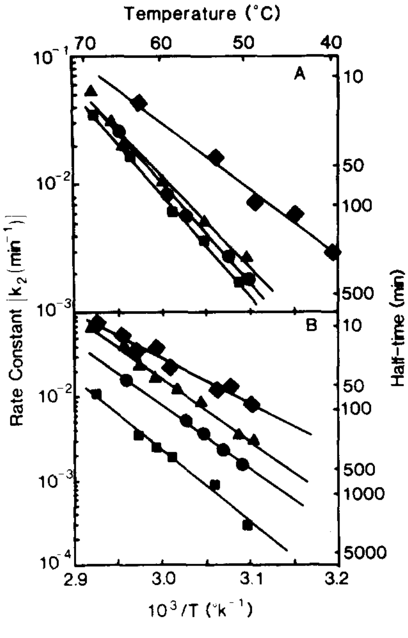

Fig. 4. Arrhenius plots of the rate constants calculated for the slow decay component (k_2) observed in fluorescence scans of the transfer of the (A) C_8 and (B) C_{12} pyrene-labeled phospholipid analogs. The rates for the C_8 analogs were obtained from fits of the fluorescence data to Eqn. 2. The fluorescence data for the C_{12} analogs of PA, PG and PE were fit to Eqn. 1 to obtain values for k_2. The values of k_2 for C_{12}PNPC were determined as described in Experimental Procedures. Data given are for the PC (■), PA (▲), PG (●) and PE (◆) analogs. Activation energies, calculated from the slopes of linear regression fits (solid lines), are given in Table II.

TABLE II

SUMMARY OF KINETIC DATA AND THERMODYNAMIC PARAMETERS OF THE ACTIVATED STATE FOR THE SLOW COMPONENT IN TRANSFER OF PYRENYL PHOSPHOLIPIDS BETWEEN POPC VESICLES

Headgroup	n [a]	k_2 [b] (h^{-1})	$t_{1/2}$ (h)	E_{act} [c] (kcal/mol)	$\Delta H^\ddagger$ (kcal/mol)	$\Delta G^\ddagger$ (kcal/mol)	$\Delta S^\ddagger$ (cal/mol per K)
Choline	8	0.008	87	37	36	26	32
	10	0.004	173	36	35	27	28
	12	0.002	347	38	37	27	33
Glycerol	8	0.01	69	34	34	26	25
	12	0.01	69	35	34	26	26
Acid	8	0.02	35	29	28	26	8
	12	0.02	35	30	30	26	13
Ethanolamine	8	0.11	6	25	25	25	0
	12	0.07	10	25	25	25	0

[a] Number of carbons in acyl chain at position one.

[b] The rate constants (k_2) and half-times ($t_{1/2}$) are extrapolated to 37 °C from the Arrhenius data.

[c] The activation energies have an experimental error of ± 8%.

thermodynamic barrier which is 10–20 kcal/mol larger than that for the fast component (Table II). However, the differences between the activation energies for the flip-flop of the C_8 and C_{12} phospholipids suggest that there is an increase in the energy of the transition state for the slow phase of only about 1 kcal/mol for every four methylene units added to the first acyl chain. In contrast, there was more than a 10 kcal/mol variation in activation energy with the identity of the polar headgroup. The range of activation energies extended from 25 kcal/mol for the PE analogs to 38 kcal/mol for the pyrenyl PC's *.

The relative contributions of lipid structure to decay rates is presented in Fig. 5 as a histogram. These data show that rates for the rapid phase (A) are a sensitive function of the acyl chain length but nearly independent of the headgroup structure. The opposite correlation was found for the second component, where rates were more strongly dependent on the nature of polar headgroup than the acyl chain composition.

The hydrophobicity of the headgroup may influence the kinetics of phospholipid transfer in the

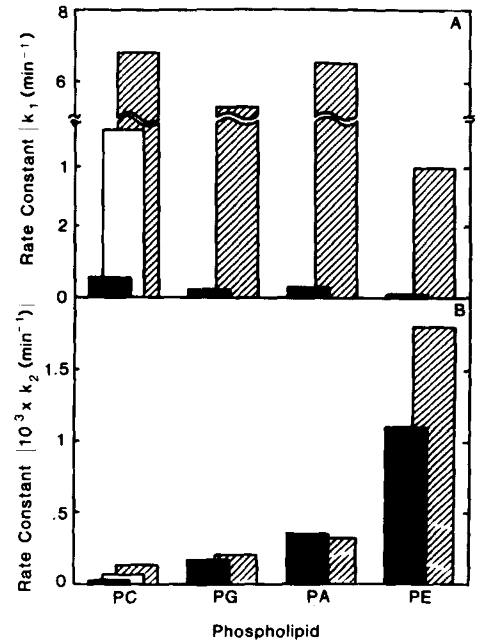

Fig. 5. Histogram of the rates (A) k_1 and (B) k_2 at 37 °C as a function of headgroup composition and acyl chain length for the C_8 (hatched bar), C_{10} (open bar), and C_{12} (solid bar) phospholipid analogs. The values for the rates were extrapolated from the Arrhenius data. Note that the rates in (A) are in reciprocal minutes and the rates in (B) are in reciprocal hours.

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* These values are significantly higher than the activation energy of 23.7 kcal/mol reported by De Kruijff and Van Zoelen [34] for dimyristoylPC flip-flop in sonicated vesicles of the same lipid.

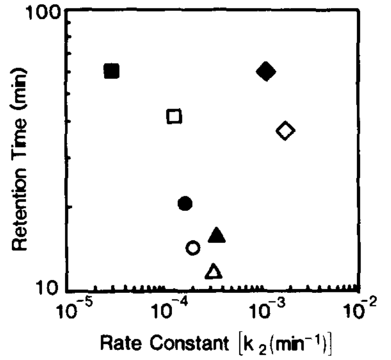

Fig. 6. Log-log plot of the retention times on a reverse-phase HPLC column of the C_8 (open symbols) and the C_{12} (closed symbols) pyrene-labeled phospholipid analogs versus the phospholipid flip-flop rates at 37 °C. □, PC, ◇, PE, △, PA and ○, PG.

same way that the length of acyl chains does [2,3]. Therefore, we determined the relative hydrophobicity of the pyrene-labeled phospholipids by reverse-phase HPLC. The logarithm of the retention time of lipophilic compounds on reverse-phase HPLC columns can be directly correlated with their relative hydrophobicity [16,17]. For both the C_8 series of phospholipids and the more hydrophobic C_{12} derivatives, their ranking according to hydrophobicity, obtained from the retention times on a C_8 column, was PA < PG < PE < PC. In Fig. 6, the logarithm of the retention times is plotted versus k_2 at 37 °C.

The contribution of charged moieties in the phospholipid headgroup to the kinetics of transfer was examined for C_8PNPA in donor vesicles at pH 4.0. Rates were calculated from fits of the fluorescence decays to Eqn. 1, since the two kinetic components could not be isolated at any temperature. As shown in Table III, the activity energy for the slow component (k_2) is reduced approx. 10 kcal/mol at pH 4.0 relative to that observed at pH 7.4. The activation energy for the rapid transfer component (k_1) is increased by about 4 kcal/mol at pH 4.0, which is reflected in the almost 4-fold decrease in the rate extrapolated to 37 °C.

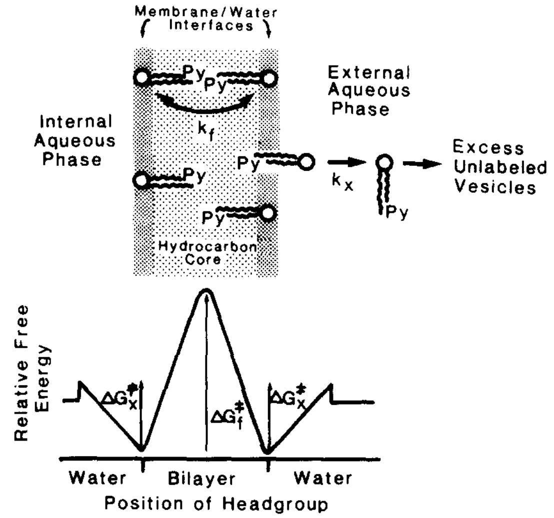

Fig. 7. Free-energy diagram of pyrenyl phospholipid translocation through the interior of the bilayer and into the aqueous phase. The free energy of activation is represented as a function of the phospholipid headgroup position along the horizontal axis. The position of the pyrenyl phospholipids in the membrane is shown in the upper diagram. The magnitude (arrow) of $\Delta G_x^\ddagger$, the free energy of activation for transfer into the aqueous phase, increases, primarily, with the acyl chain length of the diffusing species. The magnitude of $\Delta G_f^\ddagger$, the free energy of activation for flip-flop, is a function of headgroup composition and, to a lesser extent, the acyl chain length in the translocated lipid.

TABLE III

KINETICS AND THERMODYNAMICS OF C_8PNPA TRANSFER AT pH 4.0 [a]

Kinetic component	Rate constant [b] (min^{-1})	$t_{1/2}$ (min)	E_{act} (kcal/mol)	$\Delta H^\ddagger$ (kcal/mol)	$\Delta G^\ddagger$ (kcal/mol)	$\Delta S^\ddagger$ (cal/mol per K)
First	1.76	0.39	18	17	20	−11
Second	0.14	5	20	19	22	−9

[a] Donor and acceptor vesicles were prepared as described in Experimental Procedures in 100 mM NaCl/10 mM sodium acetate/5 mM EDTA/1 mM NaN_3 (pH 4.0).

[b] The rate constants and half-times are extrapolated to 37 °C from the Arrhenius data.

Discussion

As shown above, the kinetics of transfer of pyrene-labeled lipids are composed of two components which can be assigned on the basis of (1) our understanding of the structure of single bilayer vesicles and (2) the current knowledge about the structural determinants that regulate lipid transfer between lipid surfaces. We assign the fast component to desorption of pyrene-labeled lipid from the outer monolayer of donor vesicles into the surrounding aqueous phase. This is followed by rapid diffusion-controlled association of the phospholipid with the acceptor vesicles which are always present in excess. There is a good correlation between our results for the fast component and the data of Massey et al. [3] who measured the desorption of pyrene-labeled lipids from the phospholipid surface of reconstituted HDL to various acceptors. In both cases, each additional methylene unit that was added to the first acyl chain of pyrene-labeled PC decreased the rate constant and increased the free energy of activation by about 650 cal/mol. Furthermore, the activation energies reported for transfer of C_{12}PNPA (common to both studies) between model lipoproteins, and those determined for the fast decay component in this study are nearly the same (approx. 23–24 kcal/mol). Since the model HDL have a discoidal architecture in which there is only one monolayer of phospholipid, all of which is exposed to the surrounding aqueous phase, the transfer kinetics can only be from this surface into the water. On this basis, we infer that the same is true for the fast component observed in our transfer data. Moreover, the qualitative changes produced by changing the polar headgroup parallel those reported by Massey et al. [9] for phospholipid transfer between model HDL.

We assign the slower decay component to the transbilayer migration of the pyrene-labeled lipid from the inner to the outer monolayer of the vesicles. This assignment is based upon two arguments. First, the inner monolayer is the only remaining distinct compartment in the bilayer of donor vesicles. Second, the intensity ratios, $A_1/A_2 = 2.9$, for the initial fluorescence intensities of both components are similar to the outside/inside distribution of PC in single bilayer vesicles. Although A_1 and A_2 are subject to some unknown differences in quantum yields, there is no reason to believe that there would be a large difference. In addition, the lower A_1/A_2 values observed for the PA, PG and PE analogs are consistent with the observation that phospholipids with headgroups that are smaller than the PC headgroup will preferentially partition into the inner monolayer [18].

The kinetic model

A number of complex kinetic models have been proposed to describe the kinetics of transfer for lipophiles that reversibly migrate between monolayers of the membrane and traverse the aqueous phase separating vesicles [19,20]. In the general solution to the full kinetic model given by Storch and Kleinfeld [20], the observed rates of depletion of probe lipid in the donor vesicles are not identical to the true lipid transfer rates, but are functions of both the flip-flop rates, the on and off rates for aqueous phase transfer, and the total lipid concentration. Furthermore, the pre-exponential terms (A_i values in Eqn. 1) are not directly equal to the concentrations of probe lipid in any phase but are complicated functions of these concentrations and the true rate constants for transfer. However, in systems such as ours, where back-exchange of labeled lipid from acceptor to donor lipid pools is negligible due to the large excess of acceptor lipid, and where transmembrane diffusion rates are more than an order of magnitude smaller than the transaqueous exchange rates, the observed rates of probe lipid exchange are nearly identical to the true rates of lipid transfer. Therefore, the rates of flip-flop and intervesicle transfer are obtained directly from fits of the observed excimer fluorescence decays to Eqn. 1 and Eqn. 2. Similarly, the A_i values obtained are an accurate indication of the relative probe concentration in each kinetically distinct compartment *.

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* Reversible flip-flop may be significant in the transfer kinetics observed for C_8PNPA at pH 4.0 where the apparent flip-flop rates were less than 10-fold smaller than the intervesicle transfer rates.

Transmembrane diffusion and phospholipid structure

Previous studies have shown that removal of the headgroup from a phospholipid results in a dramatic enhancement of the transmembrane movement of the diacylglycerol that remains [21,22]. In our study, the largest changes in phospholipid flip-flop were produced by changes in the headgroup composition. Together, these observations indicate that the major determinants of flip-flop lie in the headgroup region of the phospholipid. In contrast, our results for intervesicular phosphpolipid exchange and those reported previously [9] show that the rate at which phospholipids enter the aqueous phase is governed primarily by the acyl chain composition (i.e., hydrophobicity) in the apolar segment of the lipid.

Movement of phospholipids across the membrane is a permeation process. It is well established that permeation of the phospholipid bilayer by solutes in the aqueous phase is a function of the lipid solubility, size, charge and hydrogen-bonding potential of the solute [23]. With the exception of hydrogen-bonding *, our data give direct evidence that these same factors are important in flip-flop.

Permeation rates increase with the lipid solubility of the permeating species. We obtained a qualitative measure of the headgroup solubility in a hydrocarbon-like environment by measuring the partitioning of the pyrenyl lipids between a stationary hydrophobic phase and a mobile aqueous phase by reverse-phase HPLC (fig. 6). For a given acyl chain composition, the affinity of the phospholipid for the hydrophobic phase is the order PA < PG ≪ PE < PC. The ranking of headgroup types according to flip-flop rates does not correlate directly with the ranking derived from the hydrophobic phase affinity given by the retention times. However, certain trends are apparent. The PA and PG analogs are similar in terms of flip-flop rates and retention times. In comparison to the equivalent PE, the retention times of PA and PG are significantly shorter, indicating reduced partitioning into the hydrophobic phase, which correlates directly with the lower flip-flop rates observed for these two lipids. The direct opposite results were obtained for the PC analogs suggesting that other factors must be considered.

It is well established that membrane permeability decreases as permeant size increases [23]. Therefore, lipid size may be a factor which accounts for the slow permeation of the bilayer by PC despite the relatively high affinity of the PC headgroup for the hydrophobic phase. The approximate headgroup volumes of PA, PE, PG and PC are 41, 67, 83 and 101 Å^3, respectively, as determined from Corey-Pauling-Koltun models. The slower flip-flop rate of PC may, in part, be due to the lower probability that a space large enough to accommodate the passage of the PC headgroup will open in the bilayer. Similar minimums in permeation rates, as a result of the opposing effects of lipid solubility and size, have been reported previously for the bilayer permeabilities of a homologous series of amides which varied in the number of carbons linked to the carboxyl carbon [24].

The water of hydration surrounding the headgroup will contribute to the energetics of flip-flop and may also ccount for the large difference we observe between PC and PE flip-flop rates. We do not know whether, in the migration of phospholipid across the bilayer, the water of hydration is shed or remains associated with the headgroup to some extent. The energetics of both processes are unfavorable in that dehydration energy is required for the removal of solvating water and introduction of water into the hydrophobic membrane interior by association with a headgroup raises the energy of the transition state for flip-flop. These factors may favor PE flip-flop since several studies have shown that the shell of water molecules hydrating the PE headgroup is smaller than the hydration sphere surrounding the PC headgroup [25,26]. Therefore, the diffusion of PC across the bilayer will require more energy, either for dehydration or for the opening of a larger space in the bilayer to accommodate the PC headgroup and the associated hydration shell. The large positive entropy change calculated for formation of the activated state in PC flip-flop, in comparison to

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* Hydrogen bonding between adjacent phospholipids and with water at the membrane surface will also contribute to the thermodynamics of flip-flop [23]. Headgroup constituents which can participate in hydrogen bonds include the phosphoryl group, the amino group of PE, and the hydroxyl groups of PG.

the negligible entropy of activation for PE flip-flop (Table II), may partially result from the release of water of hydration from the PC headgroup.

Neutralization of charged moieties in the headgroup by binding of counterions in the aqueous phase enhances flip-flop rates. We found that the flip-flop rate of C_8PNPA in the fully protonated state * was nearly 500-fold greater than that observed for the dissociated form at a bulk pH of 7.4. Further, protonation reduced the apparent activation energy for flip-flop by approx. 10 kcal/mol. These effects can be attributed to the enhanced lipid solubility of uncharged PA. Our results are similar to previous studies which showed that binding of Ca^{2+} in the aqueous phase by PA in the membrane results in the rapid translocation of the neutral complex across the bilayer [27,28]. Whereas uncharged PA migrated rapidly through the membrane, we observed that the rate of intervesicle transfer was reduced. This may result from a decrease in the aqueous solubility of fully protonated PA.

The decrease in phospholipid flip-flop rates observed for the PC and PE analogs when the length of the acyl chain at the *sn*-1 position was increased (Table II) may also result from the dependence of membrane permeability on permeant size described above. The net result of flip-flop, in addition to transfer of phospholipid between opposing monolayers, is the inversion of the long axis orientation of the phospholipid in the bilayer. Rotation of a rod-like phospholipid molecule within the membrane around axes perpendicular to the long axis of the phospholipid is sterically hindered by the close-packed array of neighboring rod-like phospholipids. Therefore, intramembrane inversion of phospholipids with shorter acyl chains (i.e., shorter rod) would be favored since less displacement of neighboring phospholipids would be required. A similar structural selectivity may function in biological membranes as indicated in a recent report by Middelkoop et al. [29] who found that the acyl chain composition of PC determines its flip-flop rate in the erythrocyte membrane.

A diagram of the free energy for transfer of a pyrene-labeled phospholipid through the membrane interior and into the surrounding aqueous phase is given in Fig. 7. The lowest energy conformation of phospholipid in the membrane is with the polar constituents at the membrane/water interface and the acyl chains buried in the membrane interior. Transfer of phospholipid out of the membrane into the aqueous phase exposes hydrophobic acyl chains to water raising the energy barrier to transfer by 550 to 750 cal/mol for every methylene in contact with water. Transfer of phospholipid through the membrane interior also has a large, positive energy of transition which is primarily due to the unfavorable passage of the polar headgroup through the nonpolar core of the membrane.

Biological significance

On the time-scale of metabolic events in the cell, passive phospholipid flip-flop is very slow. It could not account for the rapid translocation of membrane lipids during membrane synthesis. The enzymes involved in membrane lipid synthesis are located on the cytoplasmic surfaces of the endoplasmic reticulum [30], suggesting that there must be a transport mechansim to carry newly synthesized membrane lipids from that surface to the lumenal surface. Bishop and Bell [31] have detected a 'flippase' activity in microsomal membranes from rat liver which facilitates the transport of PC. In contrast to the rapid flip-flop rates ($t_{1/2}$ = min) observed in microsomal membranes, lipid flip-flop in the plasma membrane of such cells as the erythrocyte is slow ($t_{1/2}$ = hours–days). The kinetic barrier to phospholipid translocation may be important for maintaining the asymmetric transmembrane distribution of phospholipids found in these membranes [32]. Specialized metabolic pathways may exist to transport phospholipids across the plasma membrane. Zachowski et al. [33] have found evidence for an ATP-dependent transport pathway for spin-labeled analogs of aminophospholipids in the erythrocyte membrane. Pagano and Longmuir [22] determined that a fluorescent derivative of PA was transferred across the

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* The pK_a of PA in the bilayer is approximately 3.5 [35]. PA carries a net charge of -1 at pH 7.4. The concentration of protons at the phosphate headgroup of PA in the bilayer at a bulk pH of 4.0 is not directly known. We expect, however, that the pH in the vicinity of PA is close to the pK_a such that flip-flop of the fully protonated PA predominates over the flip-flop of the dissociated form.

plasma membrane of fibroblasts by enzymatic conversion to the nonpolar diacylglycerol, which permeates the membrane much more rapidly.

In the absence of specialized proteins or other factors to provide low energy pathways for transmembrane movement, phospholipids are confined to the host monolayer by a high energy barrier within the membrane. The major determinants in the barrier size are the polarity, size and charge of the phospholipid headgroup with lesser contributions from the acyl chain length.

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