Partitioning of a Fluorescent Phospholipid Between Fluid Bilayers: Dependence on Host Lipid Acyl Chains

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ABSTRACT The partition coefficient K_p was measured for a headgroup-labeled phospholipid (12:0,12:0)-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-PE (12-NBD-PE), equilibrated between LUV of a series of phosphatidylcholines (PC). Fluorescence resonance energy transfer between the 12-NBD-PE and a headgroup-rhodamine-labeled PE was used to find the equilibrium concentration of the 12-NBD-PE in the different LUV. Reliable equilibrium concentrations were obtained by monitoring the approach to equilibrium starting from a concentration below and from a concentration above the ultimate values. Using (16:0,18:1Δ9)-PC as the reference lipid, K_p ranged from a high value of 1.65 favoring (16:0,18:1Δ9)-PC over (16:1Δ9,16:1Δ9)-PC, to a low value of 0.90, favoring (22:1Δ13,22:1Δ13)-PC over (16:0,18:1Δ9)-PC. The K_p values enabled calculation of the acyl chain contribution to the excess free energy of mixing for (12:0,12:0) acyl chains at infinite dilution in the L_α phase of PC having acyl chains of (16:0,18:1Δ9), (16:1Δ9,16:1Δ9), (18:1Δ9,18:1Δ9), (18:1Δ6,18:1Δ6), (20:1Δ11,20:1Δ11), and (22:1Δ13,22:1Δ13). (14:1Δ9,14:1Δ9)-PC was found to transfer so rapidly between LUV as to preclude reliable K_p measurement.

INTRODUCTION

Because phospholipids are key components of cells, it is important to find relationships between structure and behavior. Thermodynamic approaches are promising, since equilibrium thermodynamic properties, such as the chemical potential, have predictive power. A measurable parameter of thermodynamic significance is a phase transition temperature, T_t. A wealth of phase information now exists for (usually) binary mixtures of lipids as a function of temperature and lipid composition (Caffrey et al., 1996; Tenchov, 1985). A great majority of these studies make use of more-or-less readily detectable phase transitions such as $L_\beta \rightleftharpoons P_\beta' \rightleftharpoons L_\alpha$ or H_{II}. By use of models such as the Bragg-Williams approximation, a fit of calculated liquidus (and solidus) boundaries to the experimental boundaries could enable correlation of lipid structure to microscopic interaction energies. This approach has yielded both promising molecular models (Lin et al., 1996) and also evidence of limitation to small differences between the lipids (Nibu et al., 1995). This use of phase transition modeling also restricts observations to particular categories of lipids. It could be the case that some key correlations of lipid structure with behavior require direct examination of lipid behavior *within* the L_α phase commonly found in real biomembranes.

One general way to examine lipid behavior in the fluid L_α phase is to measure the rate of transfer of a labeled lipid between different types of lipid vesicles (Roseman and Thompson, 1980; Nichols and Pagano, 1981). Studies of this type have been especially useful for examining the correlation of lipid desorption rate with lipid structure and vesicle properties (Pownall et al., 1991; Wimley and Thompson, 1991; Shin et al., 1991; Silvius and Leventis, 1993; Shahinian and Silvius, 1995). Equilibrium partitioning of a labeled lipid between bilayer vesicles has also been measured (Nichols and Pagano, 1982; Shin et al., 1991; Shahinian and Silvius, 1995). An especially convenient model system was developed by Nichols and Pagano (1981; 1982) based on fluorescence energy transfer between an NBD (*N*-[7-nitrobenz-2-oxa-1, 3-diazol-4-yl])-labeled phospholipid and a rhodamine-labeled lipid.

In this study a fluorescence energy transfer method is used to show that in a favorable system the acyl chain contribution to a phospholipid's activity coefficient at infinite dilution can be determined for (*sn*1,*sn*2) = (12:0,12:0) acyl chains in a series of L_α phase phosphatidylcholines (PC) of varying acyl chain properties. The partition coefficient, K_p, of a headgroup NBD-labeled phospholipid is measured after its equilibration between two different types of L_α phase vesicles, each composed of an essentially pure PC. The observed K_p values vary with acyl chain length and unsaturation. In addition to the particular pattern of K_p behavior observed, the relatively direct method described here might be useful for developing and testing other less direct but more widely applicable ways to understand phospholipid mixing.

MATERIALS AND METHODS

Phospholipids were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL) with the exception of the fluorescent headgroup-labeled probe (12:0,12:0)-NBD-N-PE (12-NBD-PE), which we synthesized. Phospholipids were tested for purity from time to time by loading 100 μg (or 10 μg of fluorescent lipid) on an Adsorbosil Plus 1 TLC plate (Alltech Associates, Inc., Deerfield, IL), developing with chloroform/methanol/water = 65:25:4, v/v for PCs or chloroform/methanol = 2:1 v/v for 12-NBD-PE or (16:0,16:0)-(lissamine rhodamine B)-N-PE (16-R-PE). By comparison with a 1 μg spot of the same lipid, each lipid was >99% pure.

12-NBD-PE was synthesized following the procedure of Gruber and Schindler (1994), except that reaction was allowed to proceed 3–5 h, and

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purification was effected by fractionation on a column of Bio Sil A silicic acid (Bio-Rad Laboratories, Richmond, CA) followed by TLC on Adsorbosil-5 soft-layer plates, eluting with chloroform/methanol = 2:1. A chloroform solution of the purified lipid was filtered through a 0.22 μm Teflon filter (Micron Separations, Inc., Westboro, MA), then stored at −80°C. This 12-NBD-PE had an extinction coefficient in methanol of $\epsilon_{465} = 2.2 \times 10^4$ cm^{-1} M^{-1} and an rf value identical to that of commercial (14:0,14:0)-NBD-PE.

The buffer for most samples was 5 mM Pipes/200 mM KCl at pH 7.00. The buffer was filtered through a 0.1 μm filter (Millipore Corp., Marlborough, MA) to remove dust. For some centrifugation experiments, KCl was replaced by CsCl.

LUV were prepared by extrusion (Lipex Biomembranes Inc., Vancouver, B.C.) 10× through a stack of polycarbonate filters consisting of two 0.1 μm filters preceded by one each of 0.4 μm, 1.0 μm, 3.0 μm, and 10 μm filters. This filter stack effectively removed dust, and enabled many extrusions without replacing the filters.

The multilamellar vesicle samples applied to the extruder were prepared by one of the following protocols, which differ in how solvent is removed.

Dry films

Chloroform solutions of lipids were measured out by use of a Hamilton syringe with Teflon-tipped plunger. After vortex mixing, solvent was removed in a stream of N_2 gas followed by vacuum pumping for a half day at 5–20 mTorr. Buffer was added to the dry films, and samples vortexed vigorously.

Lyophilization A

Samples were prepared before hydration as above, except that 100 μl cyclohexane was added to the dry films. The lipid solution in cyclohexane was then sprayed into the bottom of a 12 mm × 100 mm glass tube cooled in a bath of liquid N_2. Frozen samples were placed in a vacuum desiccator that rested on dry ice, with vacuum pumping at this temperature for a half day, followed by water ice temperature for a half day, and finally room temperature for a half day. Samples were then hydrated in buffer.

Lyophilization B

Samples were prepared as in protocol 2, except that chloroform was used for lyophilization. During vacuum pumping, the vacuum desiccator containing the samples rested on dry ice for 1–2 days, followed by water ice for a half day, and finally room temperature for a half day. Samples were then hydrated in buffer.

Rapid solvent exchange

Samples were prepared as in protocol 2, except that dichloromethane was added to the dry films. The dichloromethane solution of lipids was then sprayed from a small orifice through a vacuum against the rapidly vortexing hydration buffer in a Teflon centrifuge tube. This procedure, which we term RSE, forms an MLV suspension in buffer directly from a lipid solution in organic solvent without an intervening dry lipid state. Details of this method will be described in a manuscript in preparation.

After extrusion, aliquots of the aqueous LUV suspension, taken in quadruplicate, were assayed for phosphate concentration (Kingsley and Feigenson, 1979). Then, unless vesicle concentration was under study, experimental samples were prepared at 0.500 mM for each type of lipid. Still at room temperature, fluorescence of each freshly prepared sample was then measured, including background samples. The spectrofluorimeter was Hitachi Model 3010 (Hitachi Instruments, Inc., Danbury, CT) interfaced to a MacIntosh computer. Slits were 10 nm for excitation and emission. Exciting light at 470 nm passed through a 470 nm interference filter (Corion Corp., Holliston, MA). Emitted light passed through a 480 nm long-pass filter (Fuji Photo Film Co., Tokyo, Japan). A water Raman peak was collected at the start of each series of time point measurements in order to monitor any instrumental changes over the 4-day period of most experiments. After an aliquot was taken to determine fluorescence at zero time, the sample tubes containing 0.500 mM of each of two types of LUV were sealed under argon and placed in a heating block in the dark at 40°C.

Experiments requiring separation of LUVs used a Beckman TL-100 centrifuge with the 100.3 rotor (Beckman Instruments, Inc., Palo Alto, CA). Polycarbonate tubes containing 2 ml spun at 90K rpm provided an average force of ~350,000 × g, sufficient for LUV separation in <0.5 h. In order to effect separation of the LUV, samples were prepared in either 5 mM Pipes/200 mM KCl, or else in 5 mM Pipes/200 mM CsCl, yielding aqueous densities of ~1.008 and 1.025, respectively (Freier, 1978). A mid-density separation buffer was prepared as an equal volume mixture of the two buffers, so that ultracentrifugation should cause one type of LUV to rise toward the air/water interface, while the other type of LUV should pellet at the bottom of the tube. A 300–400 μl sample of the LUV mixture was mixed with 2 ml of the mid-density separation buffer. After centrifugation, samples were aspirated from the top, middle, and bottom of each tube, and quantitated by weighing. An aliquot from these samples was taken for phosphate assay, and another aliquot for F_{535} assay. Total lipid recovery was 80–90%, with unrecovered lipid in a faint streak visible on the tube wall.

RESULTS

Experimental design

The experimental goal was to measure the concentration of 12-NBD-PE in each of two types of PC vesicles between which the 12-NBD-PE had equilibrated. The experimental strategy was to monitor the approach to these equilibrium concentrations starting from both above and below the equilibrium concentration. LUV of (16:0,18:1Δ9)-PC as the reference PC were mixed with LUV of one of eight different PC types, having acyl chains (16:0,18:1Δ9), (12:0,12:0), (14:1Δ9,14:1Δ9), (16:1Δ9,16:1Δ9), (18:1Δ9,18:1Δ9), (18:1Δ6,18:1Δ6), (20:1Δ11,20:1Δ11) or (22:1Δ13,22:1Δ13).

In one type of sample, all 12-NBD-PE was initially in LUV of (16:0,18:1Δ9)-PC, together with LUV of the test PC containing the fluorescence quencher. An initially unquenched fluorescence signal decreases with time as 12-NBD-PE transfers to the LUV of the PC containing the fluorescence quencher. In a second type of sample, all 12-NBD-PE was initially in LUV of the test PC containing the fluorescence quencher, together with LUV of (16:0,18:1Δ9)-PC. The initially strongly quenched fluorescence signal increases with time as 12-NBD-PE transfers to the LUV of (16:0,18:1Δ9)-PC.

The LUV of these eight PCs under study contained 16-R-PE as a quencher of NBD fluorescence. Fig. 1 shows the quenching of 12-NBD-PE fluorescence as a function of 16-R-PE mol fraction in LUV of (16:0,18:1Δ9)-PC. In other PCs, the initial quenching varies, but the curve shapes at higher 16-R-PE mol fractions are similar. The precise amount of residual 12-NBD-PE fluorescence in the quenching environment was determined from a zero-time fluorescence measurement with all added 12-NBD-PE located in the quenching LUVs. For most of the experiments shown, 16-R-PE was fixed at mol fraction 0.015, giving ~90% quenching.

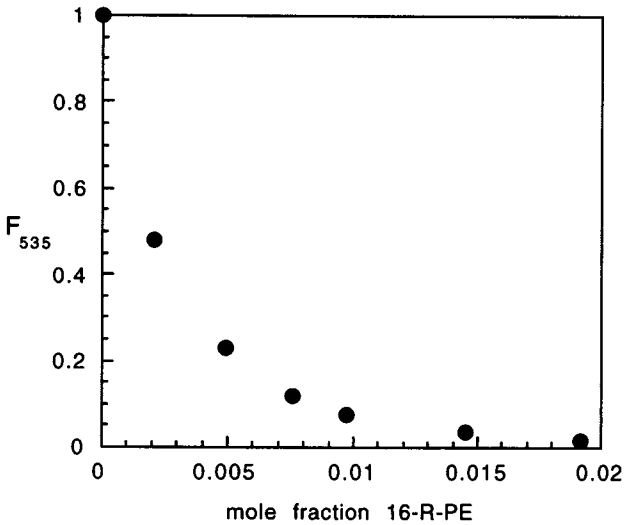

FIGURE 1 Fluorescence quenching of 12-N-PE by 16-R-PE. Normalized fluorescence per 12-N-PE at mol fraction 0.002 in LUV of (16:0,18:1Δ9)-PC containing 16-R-PE. Excitation 470 nm, emission 535 nm.

In a successful experiment, when populations of LUV were mixed only the 12-NBD-PE equilibrated between the different LUV. Experiments did not yield useful data either when the PC transferred between LUV (as happened with (14:1Δ9,14:1Δ9)-PC) or when the temperature was greater than 45°C, which allowed transfer of 16-R-PE on a time scale of hours. In preliminary experiments (data not shown), equilibration of 12-NBD-PE was found to be too slow below 40°C, so this temperature was selected for all experiments shown.

12-NBD-PE equilibration and K_p calculation

Fig. 2 shows the approach to equilibrium of 12-NBD-PE transfer between LUV populations. In these paired experiments, the 12-NBD-PE started at time 0 either in LUV of (16:0,18:1Δ9)-PC, or else in LUV of a PC containing the fluorescence quencher 16-R-PE. As the 12-NBD-PE concentrations equilibrate in the two types of LUV, the fluorescence at 535 nm approaches a nearly constant value. Fig. 2 *C* shows a slight upward drift of the fluorescence at longer times. The nature of this drift, which thwarted determination of K_p for (14:1Δ9,14:1Δ9)-PC, was examined in more detail (see Appendix).

This experiment provides the data needed to calculate a partition coefficient, K_p, between LUV of (16:0,18:1Δ9)-PC and LUV of a test PC, (x,y)-PC. From the initial value of the fluorescence, F_O, in the nonquenching reference (16:0,18:1Δ9)-PC together with the equilibrium value of the fluorescence, F_{EQ}, and the molar amounts of the two phases, $N_{(x,y)PC}$ and $N_{(16:0,18:1)PC}$, the ratio of 12-NBD-PE concentrations can be expressed as the partition coefficient,

$$K_p = \frac{[\text{12-N-PE}]_{(16:0,18:1)PC}}{[\text{12-N-PE}]_{(x,y)PC}} = \frac{F_{EQ}}{(F_O - F_{EQ})} \frac{N_{(x,y)PC}}{N_{(16:0,18:1)PC}} \quad (1)$$

F_{EQ} is obtained from the equilibrium value of the fluorescence F'_{EQ} of the upper curves in Fig. 2 by subtracting the background scattered light, B, and the fluorescence from the 12-NBD-PE in the quenching LUV at equilibrium, F^Q_{EQ}. We chose a uniform way of evaluating F'_{EQ} as simply the mean value of the upper and lower approaches to equilibrium at 35 h,

$$F'_{EQ} = \frac{(F^{upper}_{35h} + F^{lower}_{35h})}{2} \quad (2)$$

F^Q_{EQ} is not measured directly, but instead is obtained from the measured fluorescence F^Q_O at time $(t) = 0$ for a sample with all 12-NBD-PE in the quenching LUV (that is, fluorescence at $t = 0$ for the lower curves in Fig. 2), multiplied by the fraction of 12-NBD-PE in the quenching LUV at equilibrium,

$$F^Q_{EQ} = (F^Q_0 - B) \Big/ \left(K_p \frac{N_{(x,y)PC}}{N_{(16:0,18:1)PC}} + 1 \right) \quad (3)$$

We can then write Eq. 4

$$F_{EQ} = F'_{EQ} - F^Q_{EQ} - B \quad (4)$$

There are no free parameters other than K_p. Equation 1 is solved for each pair of PCs by iteration, starting with an approximation of $K_p = 1$. These K_p values are depicted in Fig. 3.

A measure of the influence of the quencher 16-R-PE on the properties of that LUV can be found from the dependence of K_p on mol fraction of 16-R-PE. The range of 16-R-PE concentrations explored was limited by signal-to-noise and by consideration of what is an acceptable level of 16-R-PE as an impurity in the PC. Values of K_p at 16-R-PE mol fractions 0.015 and 0.030 are shown in Fig. 3, labeled as A^1 and A^3.

Validity of K_p model

Simple tests verified that these calculated values of K_p have the properties expected for an equilibrium partition coefficient at infinite dilution. Partition between (16:0,18:1Δ9)-PC and (16:1Δ9,16:1Δ9)-PC was examined in detail, since the magnitude of 12-NBD-PE partition is greatest between these two types of LUV. In these experiments, instead of obtaining the full time course of equilibration as shown in Fig. 2, only the initial time point and a time point at 40 h were used for the K_p calculation. Duplicate samples were examined, and the K_p values given in Table 2 are the mean of these duplicates, with the range indicated.

The K_p values can be interpreted in a simple way if they are the infinite dilution values. In order to examine the possible dependence of K_p on concentration of 12-NBD-PE in the low concentration regime of interest here, samples were prepared as described above, using protocol 4 (RSE) for removal of organic solvent. The concentration of 12-NBD-PE ranged from an initial mol fraction of 0.0002 for

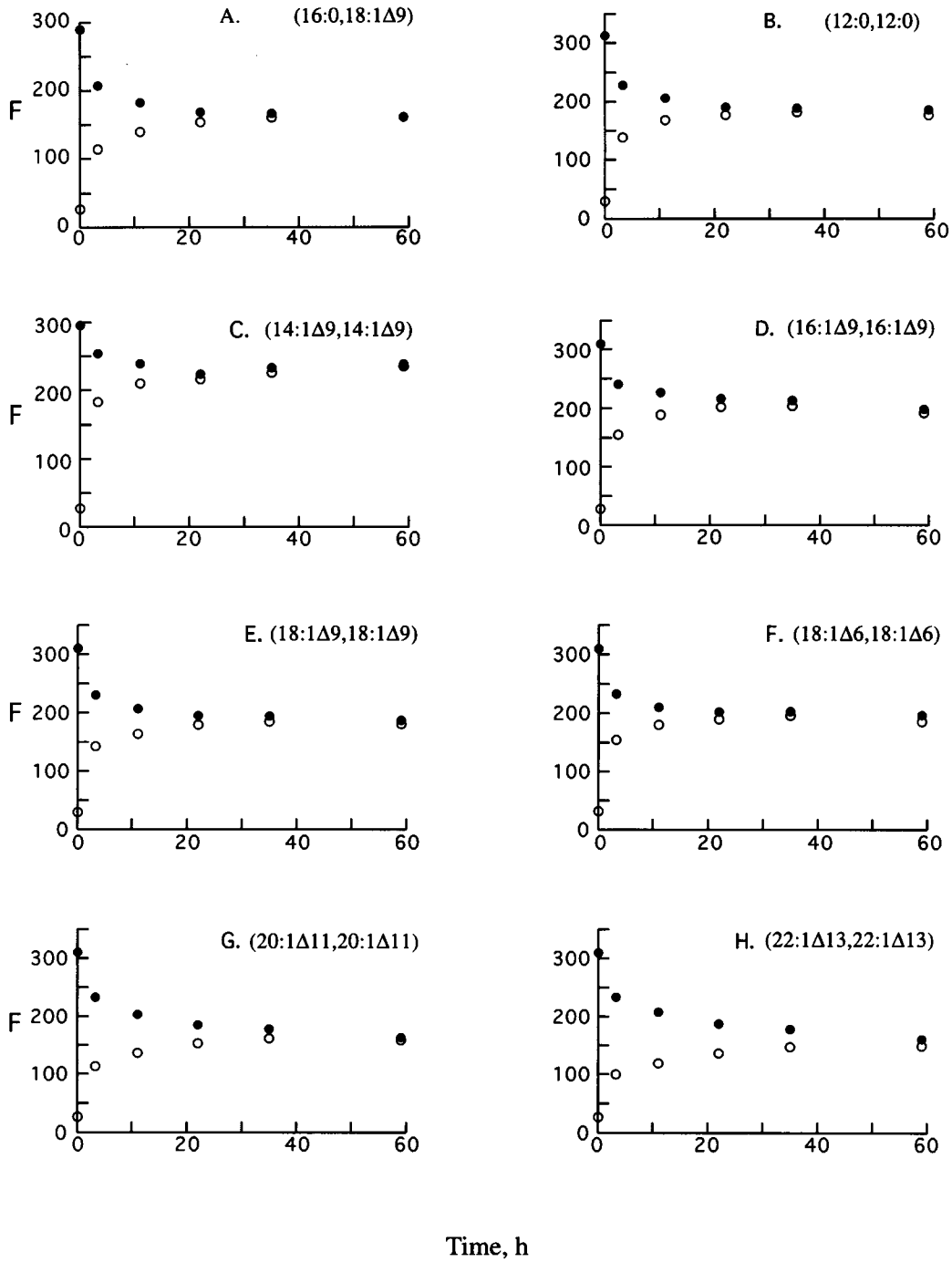

FIGURE 2 Fluorescence shows approach to equilibrium concentrations of 12-N-PE in coexisting LUV of (16:0,18:1Δ9)-PC and (x,y)-PC. At the initial time, 12-N-PE at mol fraction 0.002 is located entirely in LUV of (16:0,18:1Δ9)-PC containing no quencher (*upper curves*), or else in LUV of (x,y)-PC containing mol fraction 0.015 of 16-R-PE as a fluorescence quencher (*lower curves*). Each type of LUV at 0.50 mM, T = 40°C. The error in these fluorescence measurements is estimated to be smaller than the symbol size.

all of the probe in the LUV of (16:0,18:1Δ9)-PC, to a mol fraction of 0.02, a range of 100-fold.

To find out whether it is valid for K_p determinations to neglect any trace of 12-NBD-PE outside of the LUV (e.g., on the tubes walls or in solution), and if K_p is independent of LUV concentration, the concentration of LUV was varied over a 10-fold range. K_p was measured for samples with each LUV PC at either 0.10 mM, 0.50 mM, or 1.00 mM. These results are in Table 1.

To verify that these K_p values are at equilibrium and therefore independent of equilibration mechanism, the possible influence of the kinetics of 12-NBD-PE desorption or adsorption was tested. The ratio of LUV of the two different PC types was varied from 2:1 to 1:4. The ratio was restricted

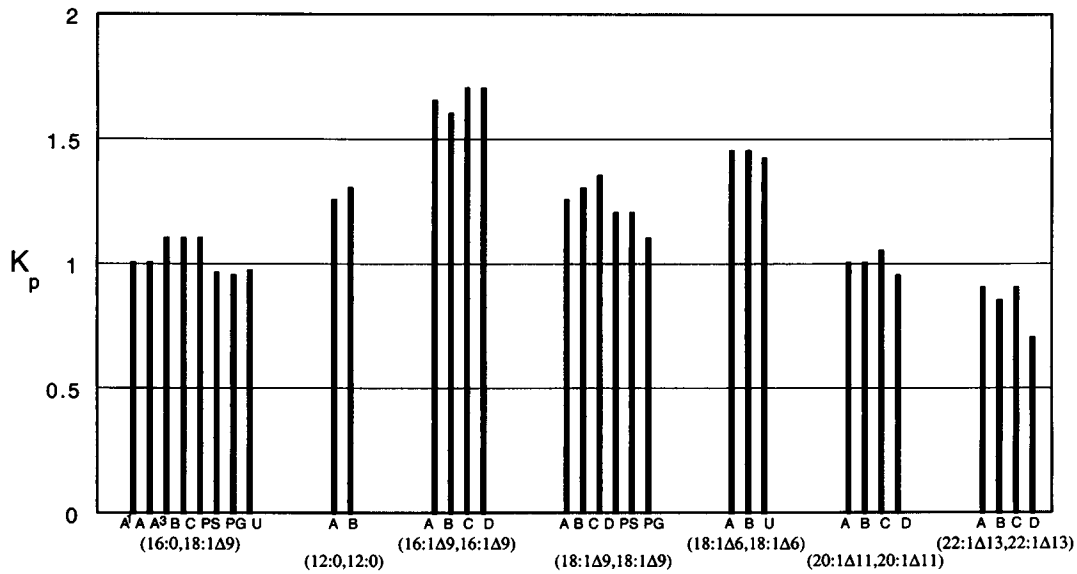

FIGURE 3 Partition coefficients of 12-N-PE between (16:0,18:1Δ9)-PC and (x,y)-PC. K_p values >1 reflect preferential partitioning into (16:0,18:1Δ9)-PC. LUV extruded from samples prepared by: rapid solvent exchange, A, PS, and U; lyophilization from chloroform, B and PG; lyophilization from cyclohexane, C; dried film, D. K_p determined by resonance energy transfer, A, B, C, D, PS, and PG. K_p determined by ultracentrifugal separation of LUV, U. 16-R-PE mol fraction 0.015 except for A^1, where it is 0.010, and A^3, where it is 0.030.

TABLE 1 Invariance of K_p of 12-N-PE between LUV of (16:0,18:1Δ9)-PC and LUV of (16:1Δ9,16:1Δ9)-PC

	Ratio of LUV* (16:0,18:1Δ9)-PC/(16:1Δ9,16:1Δ9)-PC				Total lipid#, mM			Mol fract. 12-N-PE§			
	1/0.5	1/1	1/2	1/4	0.2	1.0	2.0	0.0002	.002	.01	.02
K_p	1.4 ± .05	1.65 ± .05	1.6 ± .1	1.7 ± .05	1.52 ± .03	1.7 ± .05	1.6 ± .01	1.55 ± .05	1.7 ± .05	1.55 ± .05	1.65 ± .05

*12-N-PE at initial mol fraction 0.002 in LUV of (16:0,18:1Δ9)-PC at 0.50 mM.
#12-N-PE at initial mol fraction 0.002 in LUV of (16:0,18:1Δ9)-PC mixed 1:1 with LUV of (16:1Δ9,16:1Δ9)-PC.
§12-N-PE initially in LUV of (16:0,18:1Δ9)-PC, mixed 1:1 with LUV of (16:1Δ9,16:1Δ9)-PC, total lipid 1.0 mM.

to an 8-fold range because the difference between F_O and F_{EQ} (see Eq. 1) had to be measured with sufficient accuracy, and because F_{EQ} had to be accurately determined after subtracting the background. These K_p values are given in Table 1.

Alternative K_p measurement

Although the determination of K_p described above is relatively straightforward, with few intervening assumptions, a check by another method was sought. LUV were separated by ultracentrifugation, and the amount of 12-NBD-PE in each type of LUV was quantitated by measuring F_{535}. As described above (Materials and Methods) replacing KCl with CsCl provided sufficient density difference to enable vesicle separation. The limiting factor for several of the PCs examined in this type of experiment proved to be the leakiness of the LUV at 40°C. For (14:1Δ9,14:1Δ9)-PC, even at 20°C for less than a half hour, LUV were so leaky to ions in the buffer that these LUV would not float. For (16:1Δ9,16:1Δ9)-PC after 24 h at 40°C, leakiness led to spreading of the LUV throughout the centrifugation volume, precluding reliable K_p determination. Not all PCs were studied by this method, since a large K_p value was needed, given the observed lower precision compared to the fluorescence quenching procedure above. Reproducible values using this ultracentrifugation procedure, labeled as *U* in Fig. 3, were obtained for 12-NBD-PE equilibration between LUV pairs of (16:0,18:1Δ9)-PC/(16:0,18:1Δ9)-PC and (16:0,18:1Δ9)-PC/(18:1Δ6,18:1Δ6)-PC.

Headgroup effect

The influence of phospholipid headgroup on partition of 12-NBD-PE was examined in phophatidylserine (PS) and phosphatidylglycerol (PG). Again using (16:0,18:1Δ9) acyl chains as the reference, LUV of (16:0,18:1Δ9)-PS were mixed with LUV of either (16:0,18:1Δ9)-PS or (18:1Δ9,18:1Δ9)-PS. Similarly, LUV of (16:0,18:1Δ9)-PG were mixed with LUV of either (16:0,18:1Δ9)-PG or (18:1Δ9,18:1Δ9)-PG. PS samples were prepared by protocol 4, the RSE method. PG samples were prepared by protocol 1, using a thin film dried from $CHCl_3$ solution (the PG was not sufficiently soluble in dichloromethane to use the RSE meth-

od). One LUV population contained 0.015 mol fraction 16-R-PE as the quencher of 12-NBD-PE fluorescence, and the 12-NBD-PE was initially present in either the quenching or else the nonquenching LUV. The time-dependent fluorescence curves are shown in Fig. 4, and the calculated K_p values in Fig. 3, labeled as PS and PG.

Different reference LUV

A test was made of the reproducibility of the pattern of K_p dependence for 12-N-PE partition on PC acyl chain properties. A series of experiments was performed, essentially identical to that shown in Fig. 2, but with (22:1Δ13, 22:1Δ13)-PC as the reference LUV for every measurement. These K_p values are shown in Table 2. Because of the slower probe equilibration with (22:1Δ13,22:1Δ13)-PC, the equilibrium values could not be determined as precisely as those obtained with (16:0,18:1Δ9)-PC as the reference.

TABLE 2 K_p of 12-N-PE between (22:1Δ13,22:1Δ13)PC and (x,y)PC

PC	K_p
(12:0,12:0)	1.4
(16:0,18:1Δ9)	1.2
(16:1Δ9,16:1Δ9)	1.8
(18:1Δ9,18:1Δ9)	1.4
(18:1Δ6,18:1Δ6)	1.6
(20:1Δ11,20:1Δ11)	1.4
(22:1Δ13,22:1Δ13)	0.9

DISCUSSION

Probe equilibration

This method requires that the probe equilibrate between LUV populations. 12-NBD-PE transfers much faster than does 14-NBD-PE (which we did not study, but which is described by Arvinte et al., 1986) or (16:0,18:1Δ9)-NBD-PE, which we found to move only ~25% of the way to its equilibrium value in 4 days at 45°C (data not shown). Silvius and Leventis (1993) prepared a fluorophor derivative (bimaine) of PE that transfers between vesicles much faster than does the NBD derivative used here, but which equilibrates slowly between the bilayer leaflets. The 12-N-PE equilibration time scale of days is slow enough that the fluorescence at the initial time can be accurately determined immediately after mixing LUV populations. Most important for the probe equilibration question, the experiments graphed in Fig. 2 are unequivocal that equilibrium is closely approached. No assumptions are needed, e.g., about transbilayer flip-flop rates or adsorption rates. The time scales observed here should not be assumed to apply to biomembranes or lipoproteins, because of the influence of proteins on the kinetics (sorption and flip-flop).

Since rates of desorption can be measured, and even used to infer equilibrium values, the experimental system was tested for any kinetic influence on K_p values. Desorption

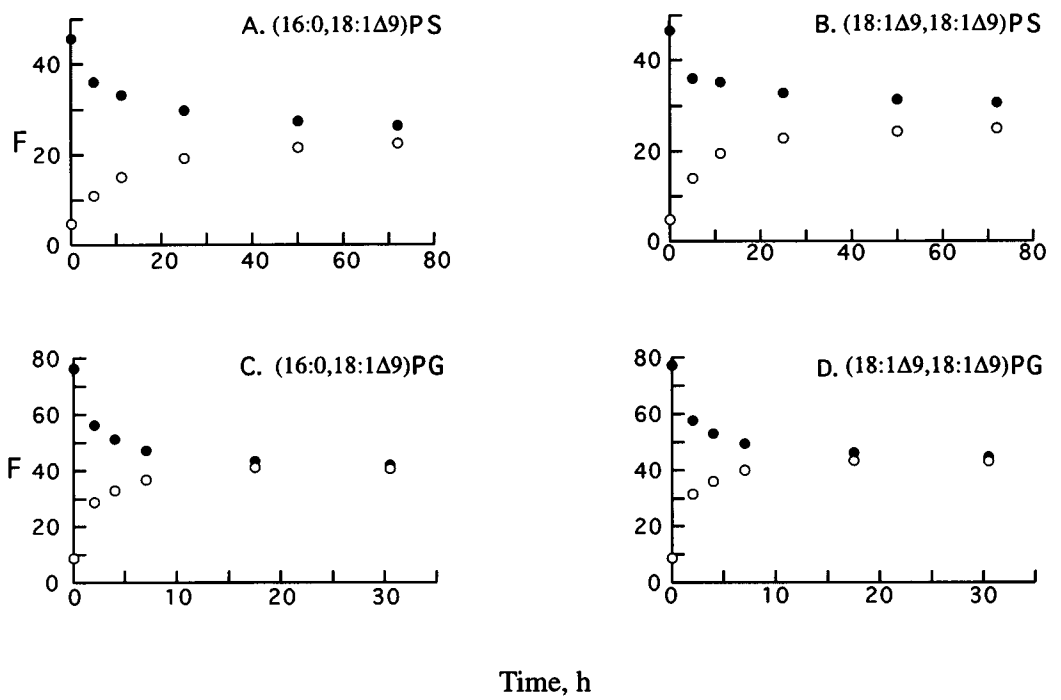

FIGURE 4 Fluorescence shows approach to equilibrium concentrations of 12-N-PE in coexisting LUV of (16:0,18:1Δ9)-PS and (16:0,18:1Δ9)-PS (*A*) or (18:1Δ9,18:1Δ9)-PS (*B*) and coexisting LUV of (16:0,18:1Δ9)-PG and (16:0,18:1Δ9)-PG (*C*) or (18:1Δ918:1Δ9)-PG (*D*). At the initial time, 12-N-PE at mol fraction 0.002 is located entirely in LUV of (16:0,18:1Δ9)-PS or (16:0,18:1Δ9)-PG containing no quencher (*upper curves*), or else in LUV of PS or PG containing mol fraction 0.015 of 16-R-PE. Each type of LUV at 0.50 mM, T = 40°C.

(and adsorption) rates depend on rate constants multiplied by LUV area (Nichols and Pagano, 1982). If rates of desorption (or adsorption) were to creep into the "equilibrium" values, then these values should change as the relative amounts of the LUV populations are varied. Table 1 shows that the K_p values do not depend on relative amounts of LUV, hence have no contribution from rates of desorption (or adsorption). This conclusion is confirmed by keeping the relative LUV amounts equal, but changing the concentration of PC from 0.20 mM to 2.0 mM. The constancy of these K_p values, shown in Table 1, also confirms that there is no significant lipid pool other than the LUV populations, i.e., that lipid adsorbed on the walls or at the aqueous interface or soluble in the buffer can be neglected when the lipid concentration is 0.20 mM or greater.

Sample preparation and reproducibility

In order to detect if preparation artifacts influence the results of the studies reported here, samples were prepared by 1) removal of the chloroform solvent in a stream of N_2 gas followed by vacuum; 2) lyophilization from cyclohexane; 3) lyophilization from chloroform; and 4) rapid solvent exchange from a lipid solution in dichloromethane to an aqueous MLV suspension. Fig. 3 shows that the K_p values are not dependent upon the details of sample preparation. Thus the independent sets of experiments shown in Fig. 3 can be taken to indicate the reproducibility of the pattern of K_p dependence on acyl chain properties. Even the relatively small variation in K_p observed, from ~0.9 to 1.6, can be measured reliably.

An alternative procedure for finding K_p is to allow equilibration of 12-NBD-PE, then separate the populations of LUV and analyze each for fluorophor content. We did not explore a variety of ways to separate LUV, but rather found that adjusting the specific gravity of both the LUV internal volume and the external aqueous medium by use of CsCl or KCl enabled some of the types of PC examined above to be separated by ultracentrifugation. For the two favorable cases studied, (16:0,18:1Δ9)-PC and (18:1Δ6,18:1Δ6)-PC, values determined after LUV separation by ultracentrifugation agree with those found in the LUV mixture having fluorescence quencher in one LUV population, as shown in Fig. 3.

Henry's law regime

The mol fraction of 0.002 for all 12-NBD-PE in one LUV population might seem to be rather dilute, but any inferred independence of K_p with concentration (Henry's law regime) must be tested. We chose the lipid showing the largest K_p value of 1.65, (16:1Δ9,16:1Δ9)-PC, and varied the mol fraction of 12-NBD-PE from 0.0002 to 0.02. As shown in Table 1, these K_p values range from 1.55 ± 0.05 to 1.7 ± 0.05 without showing a pattern. We interpret this result as showing that K_p is indeed reporting on the Henry's law regime for 12-NBD-PE at mol fraction 0.002. Thus, there are no confounding interactions between acyl chains of 12-NBD-PE molecules.

Relation to activity coefficients

The starting point for analyzing the dependence of K_p on acyl chain properties is to describe the lipid transfer experiment explicitly in terms of the environment of the 12-NBD-PE in the two different LUV,

$$(\text{12-NBD-PE})_{\text{in(x,y)PC}} \rightleftharpoons (\text{12-NBD-PE})_{\text{in(16:0,18:1}\Delta\text{9)PC}} \quad (5)$$

Equating the chemical potential of 12-NBD-PE in the two LUV lipid phases at equilibrium,

$$\mu^{12}_{\text{(x,y)PC}} = \mu^{12}_{\text{(16:0,18:1}\Delta\text{9)PC}} \quad (6)$$

Since $\mu^{12}_{\text{(x,y)PC}} = \mu^{*12} + RT\ln(\gamma^{12}_{\text{(x,y)PC}})(\chi^{12}_{\text{(x,y)PC}})$, where χ is the mol fraction, the ratio of activity coefficients is

$$(\gamma^{12}_{\text{(x,y)PC}})/(\gamma^{12}_{\text{(16:0,18:1}\Delta\text{9)PC}}) = (\chi^{12}_{\text{(16:0,18:1}\Delta\text{9)PC}})/(\chi^{12}_{\text{(x,y)PC}}) = K_p \quad (7)$$

Headgroup interactions

A key simplifying assumption is that the free energy of transfer of 12-NBD-PE between LUV of different PC types includes separable contributions from the headgroup and from the acyl chains. This assumption has experimental support for desorption free energy (Silvius and Leventis, 1993; Pownall et al., 1991). As far as headgroup/headgroup interactions are concerned, transfer between LUV populations takes the NBD-phosphoethanolamine between two structurally identical environments. If the headgroup orientations in the PCs studied are the same, then perhaps the only other important consideration is the headgroup area (or volume). If the headgroup area (or volume) is sufficiently different for these PCs and is a strong factor in these headgroup interactions, then we would mistakenly assign all effects to the acyl chains.

The limited variety of commercially available phospholipids other than PCs makes difficult the systematic study of any such coupling between headgroup and acyl chains. However, both PS and PG are available with (16:0,18:1Δ9) and with (18:1Δ9,18:1Δ9) acyl chains. Between LUV of PS (one determination), K_p = 0.95 between (16:0,18:1Δ9)-PS and (16:0,18:1Δ9)-PS, and K_p = 1.2 between (16:0, 18:1Δ9)-PS and (18:1Δ9,18:1Δ9)-PS. Between LUV of PG (two determinations), K_p = 0.97 ± 0.02 between (16:0, 18:1Δ9)-PG and (16:0,18:1Δ9)-PG, and K_p = 1.10 ± 0.02 between (16:0,18:1Δ9)-PG and (18:1Δ9,18:1Δ9)-PG. These values fit the pattern depicted in Fig. 3 for LUV of PCs but do not rule out a small coupling of acyl and headgroup energies. If headgroup-headgroup interactions were both significant and also dependent on the nature of the acyl chains, then we would have expected differences among the LUV of PC, PS, and PG.

The time scale for approach to equilibrium can be compared for PC, PS, and PG, all having either (16:0,18:1Δ9) or (18:1Δ9,18:1Δ9) acyl chains. Comparing Fig. 2 *A* with Fig. 4, *A* and *C*, and Fig. 2 *E* with Fig. 3, *B* and *D*, for both types of acyl chains the rate of approach to equilibrium is PG > PC > PS. These rates are affected by both desorption and by transbilayer flip-flop, and could be a strong function of the NBD moiety. We have not attempted additional analysis.

Interpretation

These arguments that our partition experiment reflects only acyl chain effects imply that we obtain information *only* about that contribution to thermodynamic activity from the acyl chains. That is, Eq. 7 can be written to show explicitly that K_p has no contribution from the PC headgroup,

$$\gamma^{12}_{(x,y)}/\gamma^{12}_{(16:0,18:1\Delta9)} = K_p \qquad (8)$$

If K_p indeed measures only acyl chain effects, then $\gamma^{12}_{(12:0,12:0)} = 1.00$. Since $K_p = 1.25$ in (12:0,12:0)-PC, and using Eq. 8, we can calculate the activity coefficient for 12-NBD-PE in (16:0,18:1Δ9)-PC, $\gamma^{12}_{(16:0,18:1\Delta9)} = 1/1.25 = 0.80$. The acyl chain components of the activity coefficients for the other PCs can be calculated in the same way.

Rather than to keep track of the behavior of (12:0,12:0) acyl chains in terms of a "component of the activity coefficient," we prefer to use the excess free energy of mixing, since the components of the free energy can be simply additive. In a binary mixture of a phospholipid and 12-N-PE, the component of the excess free energy of mixing of the (12:0, 12:0) acyl chains with the phospholipid's (x, y) acyl chains is given by

$$(\Delta G^E_{mix})^{12}_{(x,y)} = RT \ln \gamma^{12}_{(x,y)}$$

The values of these excess free energies at infinite dilution are tabulated in Table 3. For comparison with the bulk of the experiments that used (16:0,18:1Δ9)-PC as the reference LUV, Table 3 includes the set of results calculated from Table 2, using (22:1Δ13,22:1Δ13)-PC as the reference LUV. The pattern of $(\Delta G^E_{mix})^{12}_{(x,y)}$ values is the same using either reference PC, but the values using (16:0,18:1Δ9)-PC were essentially replicated in several independent experiments. All of these values are smaller in magnitude than *RT*. The significance of these acyl chain interaction energies between (12:0,12:0) and its surroundings perhaps can be gauged by noting that an excess free energy of ~+0.5 *RT* would be required to induce phase separation at mol fraction 0.50 (Guggenheim, 1952).

TABLE 3 Excess free energy of mixing of 12-N-PE at infinite dilution in PC, acyl chain contribution

PC	ΔG^E_{mix}, kJ/mol(RT)	
	(16:0,18:1Δ9)PC as reference LUV	(22:1Δ13,22:1Δ13)PC as reference LUV
(12:0,12:0)	0	0
(16:0,18:1Δ9)	−0.58 (−0.22)	−0.4 (−0.15)
(16:1Δ9,16:1Δ9)	+0.72 (+0.28)	+0.7 (+0.3)
(18:1Δ9,18:1Δ9)	≈0	≈0
(18:1Δ6,18:1Δ6)	+0.39 (+0.15)	+0.4 (+0.15)
(20:1Δ11,20:1Δ11)	−0.58 (−0.22)	−0.4 (−0.15)
(22:1Δ13,22:1Δ13)	−0.85 (−0.33)	−0.9 (−0.3)

The estimated error of these free energies is ±0.05 kJ/mol (±0.02 RT).

In considering the molecular-level origins of the differences in excess free energy of mixing, it is important to notice that the process of transfer of 12-NBD-PE from (x,y)-PC involves formation of new (x, y)-(x, y) acyl contacts, together with loss of (x, y)-(12:0,12:0) acyl contacts. That is, the observed K_p values reflect *both* the free energy of (x, y)-(x, y) contacts and the free energy of (x, y)-(12:0, 12:0) contacts. The experiments reported here do not enable resolving these two different contributions. However, making use of the gel-fluid transition temperature of the PC as a simple indicator of the energy of the acyl chain interactions in the pure lipid, together with the excess free energies of mixing reported in Table 3, some comments might be useful:

1. $(\Delta G^E_{mix})^{12}_{(x,y)} = +0.72$ kJ/mol in (16:1Δ9,16:1Δ9) and ≈ 0 kJ/mol in (18:1Δ9,18:1Δ9). In other words, for (16:1Δ9,16:1Δ9)-PC, replacing host PC with (12:0,12:0) acyl chains is unfavorable by +0.72 kJ/mol, whereas for (18:1Δ9,18:1Δ9)-PC this transfer involves no change in free energy. These PCs have the same location of the double bond and differ only in the length of the acyl chain below the level of the extended probe acyl chain. Seeking a molecular-level description consistent with these free energies, a possibility that can be discounted is that (12:0,12:0) interactions replace *more* favorable interactions of (16:1Δ9,16:1Δ9)-(16:1Δ9,16:1Δ9) compared with (18:1Δ9,18:1Δ9)-(18:1Δ9,18:1Δ9). This explanation is improbable because the lower temperature for the gel-fluid transition of (16:1Δ9,16:1Δ9)-PC, −36°C, compared to that for (18:1Δ9,18:1Δ9)-PC, −21°C (Marsh, 1990), implies less favorable acyl interactions for (16:1Δ9,16:1Δ9)-PC compared with (18:1Δ9,18:1Δ9)-PC at the temperature of the experiment, 40°C. We are left with the likelihood that interactions between (12:0,12:0)-(18:1Δ9,18:1Δ9) are more favorable than are interactions between (12:0,12:0)–(16:1Δ9,16:1Δ9). Without measuring the temperature dependence of K_p we cannot know whether enthalpy or entropy is dominating.
2. Comparing $(\Delta G^E_{mix})^{12}_{(x,y)} \approx 0$ kJ/mol in (18:1Δ9,18:1Δ9) with +0.39 kJ/mol in (18:1Δ6,18:1Δ6), we first note the lower temperature for the gel-fluid transition of (18:1Δ9,18:1Δ9)-PC, −21°C, compared to that for (18:1Δ6, 18:1Δ6)-PC, +1°C (Marsh, 1990). Thus, disruption of favorable interactions among (18:1Δ6,18:1Δ6) acyl chains could be a significant factor to explain the decreased partition of 12-N-PE, but we have no independent observations to confirm this explanation. A different but also plausible explanation is that the packing energy of (12:0,12:0) acyl chains against Δ6 is unfavor-

able compared with Δ9, without a compensating gain in entropy.

3. Since $(\Delta G^{E}_{mix})^{12}_{(x,y)} = -0.85$ kJ/mol in (22:1Δ13, 22:1Δ13), it is apparent that the packing of the 12-carbon saturated acyl region is not greatly disrupted by the presence of (12:0,12:0) chains, which have lowest free energy in this host than in the others studied. With a gel-fluid transition temperature of +13°C (Marsh, 1990), (22:1Δ13, 22:1Δ13)-PC must have relatively favorable interactions among its acyl chains, hence interaction of (12:0,12:0) with these chains must be particularly favorable.
4. Since $(\Delta G^{E}_{mix})^{12}_{(x,y)} = -0.58$ kJ/mol in (20:1Δ11, 20:1Δ11), the transfer free energy is indistinguishable from that in (16:0,18:1Δ9). These PCs have virtually identical gel-fluid transition temperatures of about −4°C (Marsh, 1990), implying similar interaction energy among their acyl chains. If this is true then apparently the "average" of (12:0,12:0) contacts with 16:0 and with 18:1Δ9 are quite similar to the contacts of (12:0,12:0) with (20:1Δ11,20:1Δ11). (16:0,18:1Δ9) has one saturated 16:0 acyl chain having no unfavorable contacts nor packing disruptions that would be caused by (12:0,12:0) chains, whereas the 18:1Δ9 chain is likely to have slightly more unfavorable contact with (12:0,12:0) than would 20:1Δ11.

Despite the above speculations, without a reliable model these thermodynamic studies do not establish the microscopic interaction energies between acyl chains. Instead, the excess mixing free energies tabulated in Table 2 simply represent the acyl chain contributions, at infinite dilution in the L_α phase, to the chemical potential of a phospholipid with (12:0,12:0) acyl chains: the tendency of this phospholipid, to chemically react, cluster, change phase, or transfer to another bilayer.

The experimental system described here was optimized to give a reliable set of K_p determinations as a function of phospholipid acyl chain properties in L_α phase phospholipids. Experiments were not designed to yield particular biological relevance. For that reason, we have decided not to pursue more detailed information for (12:0,12:0) acyl chains, such as K_p values in other phospholipid types, or the temperature dependence of K_p in order to evaluate ΔH and ΔS. We cannot say whether the excess free energies that we have calculated here are representative of the range for more biologically interesting cases, such as (16:0,20:4)-PC in a matrix of (16:0,18:1)-PC, or cholesterol at high concentration in various phospholipids.

This method relies upon the equilibration of a probe phospholipid between populations of LUV. Disadvantages are that the acyl chains of interest must be part of a sufficiently rapidly transferring probe, and that the time scale of days might not be suitable for unstable lipids. Now that we have a set of reliable values of K_p we can proceed to develop methods that are applicable to the cases of greatest biological interest.

APPENDIX

(14:1Δ9,14:1Δ9)-PC behavior

Fig. 2 *C* shows that 12-NBD-PE fluorescence in the presence of (14:1Δ9,14:1Δ9)-PC LUV, starting either with all fluorophor in (16:0, 18:1Δ9)-PC or in (14:1Δ9,14:1Δ9)-PC, does not approach a constant value, but instead rises slowly but continuously with time. If we were to estimate a value for F'_{EQ} as described above, the resulting $K_p \approx 2.6$. However, the deviation from the approach to constant fluorescence observed with the other PCs merits scrutiny.

Since the experimental model is of LUV populations whose lipids do not equilibrate, with one LUV type containing 16-R-PE as a quencher of NBD fluorescence, it is necessary that these lipids do not transfer sufficiently to change the calculations. A simple experimental system to detect lipid transfer consists of 16-R-PE at 0.015 mol fraction where self-quenching is ~90%, mixed with a population of acceptor LUV. Transfer of 16-R-PE would relieve self-quenching and result in a fluorescence increase. In contrast, transfer of a PC from the 16-R-PE-containing LUV would increase the concentration of 16-R-PE and result in a fluorescence decrease. Fig. A1 shows a transfer experiment for 16-R-PE with all the PCs studied. For seven of the PCs, a small rise is indeed observed. This same very gradual rise of 16-R-PE fluorescence (~1% increase in F_{593} in 100 h) also occurs in the absence of acceptor LUV, and in either glass or Teflon tubes (data not shown).

The exceptional lipid is (14:1Δ9,14:1Δ9)-PC. Fig. A1 shows that in (14:1Δ9,14:1Δ9)-PC the 16-R-PE fluorescence decreases markedly starting from its partially self-quenched state. A plausible explanation is that (14:1Δ9,14:1Δ9)-PC is sufficiently water-soluble, or desorbs from LUV sufficiently rapidly, that it equilibrates, which in this sample means that it mixes with the (16:0,18:1Δ9)-PC and thus leaves the 16-R-PE more concentrated.

In order to test this model of (14:1Δ9,14:1Δ9)-PC transfer, LUV of (16:0,18:1Δ9)-PC containing 0.015 mol fraction 16-R-PE were prepared and incubated with LUV composed solely of (14:1Δ9,14:1Δ9)-PC. The results of this experiment are shown in Table A1. The F_{593} increased over time, about 10× more than the increases seen in Fig. A1. Hence, trace 16-R-PE transfer to a less-quenching environment is not a satisfactory explanation. Instead, what occurs is massive transfer of (14:1Δ9,14:1Δ9)-PC to the LUV of (16:0,18:1Δ9)-PC, thereby diluting the 16-R-PE. An esti-

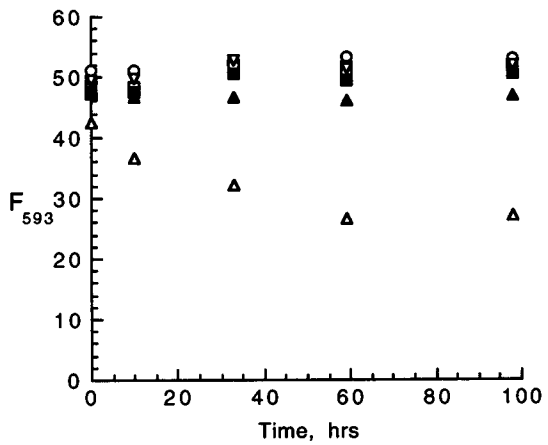

FIGURE A1 Fluorescence of 16-R-PE changes slowly with time in LUV of most PCs incubated with LUV of (16:0,18:1Δ/9)-PC, but drops markedly in (14:1Δ9,14:1Δ)-PC (△). 16-R-PE initially at mol fraction 0.015 in LUV of 0.50 mM (16:0,18:1Δ9)-PC (○); (12:0,12:0)-PC (●); (16:1Δ9, 16:1Δ9)-PC (▶); (18:1Δ9,18:1Δ9)-PC (□); (18:1Δ6,18:1δ6)-PC (■); (20:1Δ11,20:1Δ11)-PC (▼); (22:1Δ13,22:1Δ13)-PC (▽), all incubated with LUV of 0.50 mM (16:0,18:1Δ9)-PC at 40°C. Excitation 470 nm, emission 593 nm.

TABLE A1 Time-dependent relief of 16-R-PE self-quenching reveals movement of (14:1Δ9,14:1Δ9)-PC

LUV composition, Ratio (16:0,18:1Δ9)-PC/(14:1Δ9,14:1Δ9)-PC	F_{593}
1/0	9.2
1/0.5	10.5
1/1	11.6
1/0 + 0/1, t = 0	9.2
1/0 + 0/1, t = 6.5h	10.0
1/0 + 0/1, t = 23h	10.1
1/0 + 0/1, t = 95h	10.3

16-R-PE is present at mol fraction 0.015 of the (16:0,18:1Δ9)-PC in each sample. A single type of LUV of varying ratio (16:0,18:1Δ9)PC/(14:1Δ9,14:1Δ9)-PC, first three entries. LUV of (16:0,18:1Δ9)-PC containing 16-R-PE are mixed at t = 0 with equimolar LUV of (14:1Δ9,14:1Δ)-PC, last four entries.

mate can be made of the extent of (14:1Δ9,14:1Δ9)-PC movement by calibrating the F_{593} per mM 16-R-PE at 0.015 mol fraction in (16:0,18:1Δ9)-PC, and in (16:0,18:1Δ9)-PC diluted with (14:1Δ9,14:1Δ9)-PC to 2:1 and 1:1. These calibrations are shown in Table A1. This experiment shows that almost half of the (14:1Δ9,14:1Δ9)-PC, present initially as single-lipid LUV that were equimolar with single-lipid LUV of (16:0,18:1Δ9)-PC, transfers into the latter PC in a few days. This massive transfer changes both the acyl chain character and the volume of the different LUV populations, precluding a reliable estimate of K_p. Thus, although it might be the case that the transfer of (12:0,12:0) acyl chains into (14:1Δ9,14:1Δ9) acyl chains is unfavorable, we cannot conclude this from the data.

We were surprised that (14:1Δ9,14:1Δ9)-PC transfers so rapidly between vesicles. This observation implies a high rate of desorption in the pure lipid. We have not determined whether the desorption rate is also high for (14:1Δ9,14:1Δ9)-PC that is a dilute component in a bilayer matrix of another lipid. We note that (12:1Δ11,12:1Δ11)-PC, which does form bilayer vesicles (Caffrey and Feigenson, 1981) seems to transfer extremely rapidly, quickly lowering aqueous surface tension (Buboltz, unpublished experiments).

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