Thermodynamics of Mixing of Phosphatidylserine/Phosphatidylcholine from Measurements of High-Affinity Calcium Binding†

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ABSTRACT: A simple model system is described that allows measurement of equilibrium Ca^{2+} binding to multilamellar vesicle mixtures of palmitoyloleoylphosphatidylserine (P,O-PS) and dimyristoleoylphosphatidylcholine (MO,MO-PC). The constraint of the chemical equilibrium among aqueous Ca^{2+}, hydrated P,O-PS/MO,MO-PC, and $Ca(PS)_2$, together with measurements of the Ca^{2+} concentration in equilibrium with defined PS/PC ratios, enables the determination of the thermodynamic activity of the lipids. The activity coefficient of dilute P,O-PS in PC is analyzed in terms of the partial molal free energy to transfer P,O-PS from an environment of PS to an environment of PC. This study of P,O-PS/MO,MO-PC, by comparison with the earlier study of P,O-PS/P,O-PC [Feigenson, G. W. (1989) *Biochemistry 20*, 1270–1278], reveals that the excess partial molal free energy to transfer P,O-PS from P,O-PS to P,O-PC is −0.7 kcal mol^{-1}. This free energy change arises in part from the favorable transfer of the negatively charged phosphoserine headgroup from an environment of negative charges to an environment of zwitterions. The contribution of acyl chain mismatch to the partial molal free energy to transfer P,O-PS from P,O-PS to MO,MO-PC is found to be approximately +0.7 kcal mol^{-1}. This value is much larger than that of the excess partial molal free energy of mixing in isotropic solutions of linear hydrocarbons that differ in chain length or unsaturation.

Biomembranes have an impressive variety of lipid types. One source of this variety is the acyl chains (Baldassare et al., 1977; Ansell & Spanner, 1982). The type of acyl chain influences the phase behavior of the lipid (Chapman et al. 1969; Tenchov, 1985), the rate and range of local lipid motion (Seelig & Seelig, 1980; McElhaney, 1989), the thickness of the bilayer (Caffrey & Feigenson, 1981; Seddon et al., 1984), the membrane permeability (DeGier et al., 1968; McElhaney, 1989), and the membrane enzymatic activities [see review by McElhaney (1989)].

One way to understand lipid behavior is to use thermodynamic principles. Determination of thermodynamic parameters in a lipid system carries the promise of predicting the tendency of a lipid to change its physical or chemical state, without requiring a correct description of the details of conformation and motion of the molecule. The thermodynamic approach requires first a description of the phase behavior of the system. For a mixture of lipids, a second important characteristic of the system is the thermodynamic activity of the components. It is this activity that measures the tendency of a lipid to change its state.

Until recently, lipid thermodynamic activity in a lipid mixture could not be determined by a direct experiment and, instead, required untestable assumptions in order to apportion enthalpic and entropic contributions to the free energy of mixing (Silver, 1985; Tenchov, 1985). Furthermore, experiments aimed at thermodynamic characterization were largely confined to lipids having experimentally accessible phase transition temperatures, and thus could not establish the characteristics for most of the biologically interesting lipids. These problems of undetermined parameters and of high phase transition temperatures have now been overcome by the use of a mixed lipid system that is constrained by a heterogeneous chemical (rather than thermal) equilibrium (Feigenson, 1989):[1]

$$Ca(PS)_2 \rightleftharpoons Ca^{2+}(aq) + 2PS^{-}(aq) \quad (1)$$

The equilibrium reaction involves three distinct phases: $Ca(PS)_2$, hydrated PS/PC multilayers, and aqueous Ca^{2+}. The equilibrium aqueous Ca^{2+} concentration, termed $[Ca^{2+}]^*$, is characteristic of the PS/PC mole ratio. The thermodynamic activity (a_{PS}) and activity coefficient (γ_{PS}) are obtained directly from measurements of $[Ca^{2+}]^*$ in equilibrium with defined ratios of PS/PC. For P,O-PS/P,O-PC[2] multilayers (i.e., matched acyl chains on PS and PC), γ_{PS} approaches 0.3 as the PS concentration approaches zero.

These studies of P,O-PS/P,O-PC used Millipore filters to support the lipid multilayers in order to facilitate Ca^{2+} binding at micromolar $[Ca^{2+}]$. Presumably, the multilayers did not

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[1] Abbreviations: PS, 1,2-diacyl-*sn*-glycero-3-phosphoserine; PC, 1,2-diacyl-*sn*-glycero-3-phosphocholine; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); BAPTA, 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; BrBAPTA, 1,2-bis(*o*-amino-5-bromophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; K_D, dissociation constant; M^{n+}, multivalent metal cation; A_0, absorbance in the absence of M^{n+}; A_1, absorbance in excess M^{n+}; A_0',A_0 divided by the absorbance at the isosbestic point; A_1', A_1 divided by the absorbance at the isosbestic point; a_n, thermodynamic activity of n; γ_n, activity coefficient of n; μ_n, chemical potential of n; μ°_n, chemical potential of n at its standard state; μ^E_n, excess chemical potential of n.

[2] With this study we embark on a systematic investigation into the influence of fatty acyl chain types of lipid properties. We find no simple shorthand in the literature that is convenient for signifying different acyl chains in *sn*-1 and *sn*-2. Therefore, we use an abbreviation system that identifies each acyl chain by one or two letters, separated by a comma, with the *sn*-2 chain abbreviation followed by a hyphen and the lipid headgroup type, also abbreviated. Thus, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine = P,O-PS (C16:0, C18:1 acyl chains), 1-palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphocholine = P,O-PC, and 1,2-dimyristoleoyl-*sn*-glycero-3-phosphocholine = MO,MO-PC (C14:1, C14:1 acyl chains).

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form completely closed vesicles when supported on Millipore filters, thereby giving Ca^{2+} access to PS binding sites. However, the filters required extensive washing to remove detergent contamination, and the washed filters still released a very slightly absorbant substance. Furthermore, the filters complicate the description of the system.

In this work, we investigated the effect of mismatched acyl chain composition of PS and PC on the high-affinity binding reaction between Ca^{2+} and PS in PS/PC multilayers. We developed a simple method to prepare PS/PC multilayers which does not require the use of Millipore filters and which enables the binding reaction to be monitored and near-equilibrium conditions to be reached.

Materials and Methods

Materials

Lipids were from Avanti Polar Lipids, Inc. (Birmingham, AL); the calcium chelator/indicators 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA) and 1,2-bis(*o*-amino-5-bromophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BrBAPTA) were from Molecular Probes (Junction City, OR); piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (Pipes), puriss grade, was from A. G. Fluka Corp. (Hauppauge, NY). Calcium carbonate, ultrex grade, and the phosphate standard, analytical grade, from J. T. Baker (Bricktown, NJ). Chelex-100 ion-exchange resin was from Bio-Rad (Rockville Center, NY). Water was purified through a Milli-Q water purifying system. All other chemicals were of reagent grade.

Methods

Preparation of Solutions. Chelex was washed with 1 N HCl and then with 1 N KOH, with three Milli-Q water rinses after each acid and base wash. The Chelex was then repeatedly washed with Pipes–KCl buffer (20 mM Pipes, 100 mM KCl, pH 7.00) until the pH of the buffer with Chelex had stabilized at 7.00. The Pipes–KCl buffer (pH 7.00) was stored over a bed of Chelex in a polypropylene bottle at 4 °C, which reduced multivalent cation contamination to below 1 μM (Feigenson, 1986). Prior to use, the Pipes–KCl buffer was warmed to room temperature and then filtered through an acid-rinsed sintered glass funnel to remove Chelex bead fragments. All borosilicate culture tubes, 10 × 75 mm, were also rinsed with 10% HCl prior to use.

The calcium standard solution and the calcium chelator/indicator standard solutions of BAPTA and BrBAPTA were prepared following the procedures outlined by Feigenson (1986). The calcium standard concentration was 12.44 mM, and the nominal concentrations for BAPTA and BrBAPTA were 10 and 20 mM, respectively. The actual concentrations of the BAPTA and BrBAPTA standards were determined by titration with the Ca^{2+} primary standard according to either of the following methods: (i) a Ca^{2+}-sensitive electrode (Radiometer) together with the pH meter (Fisher Accumet Model 810) operating in the millivolt mode or (ii) a Cary 219 spectrophotometer and measurement of the absorbance of the chelator/indicator dye at a peak in the difference spectrum (Yeager & Feigenson, 1988; unpublished experiments).

The molar concentrations of P,O-PS and MO,MO-PC in chloroform were determined spectrophotometrically by phosphate assay as described by Kingsley and Feigenson (1979).

Preparation of Multilayer Dispersions. A Hamilton gastight syringe was used to measure chloroform solutions of the lipids into tubes to yield a specified mole ratio of PS/PC. This stock chloroform solution of the desired PS/PC mole ratio was dried under a gentle stream of N_2 followed by 3 h of mechanical vacuum pumping in the dark.

Preparation I. The dried PS/PC mixture was then redissolved in a predetermined volume of chloroform, and a 50-μL aliquot containing the appropriate quantity of lipid was transferred into the tubes. These 50-μL aliquots were dried to thin films by use of N_2 and slow rotation. Chloroform was completely removed by 20–24 h of mechanical vacuum pumping, in the dark.

Preparation II. The dried PS/PC mixture was redissolved in a predetermined volume of cyclohexane, and 50–100-μL aliquots were transferred into culture tubes. The aliquots were frozen in thin films and then lyophilized by mechanical vacuum pumping at room temperature, in the dark, for 24 h. Gas chromatographic (GC) analysis of hydrated lyophilized lipid powder (PS/PC = 0.5/0.5) showed no contamination with cyclohexane, with a detection limit of 1 cyclohexane per 10000 phospholipids. The GC conditions were as follows: temperature of injector, 150 °C; detector, 250 °C; column, 50 °C; stainless steel column (6 ft × 125 in.) packed with SP-2330 silica (Supelco, Inc., Bellefonte, PA).

For both preparations I and II, the vacuum was released with argon, and aqueous Ca^{2+} in Pipes–KCl buffer was added directly to the dried film or after a 0.5- or 2-h hydration period in Pioes–KCl buffer without added Ca^{2+}. During the 0.5- or 2-h hydration period without Ca^{2+}, samples were stored under argon, in the dark at 20 °C. An initial incubation volume of 1 mL was necessary to obtain reproducible Ca^{2+} binding.

Measurement of Ca^{2+}. BAPTA and BrBAPTA were used as Ca^{2+} chelator/indicators to measure free aqueous Ca^{2+} concentrations following Tsien (1980) as described by Feigenson (1986). Briefly, absorbance measurements were obtained at the peak of the difference spectrum and also at the isosbestic point, for the specific chelator/indicators with and without Ca^{2+}. Ca^{2+} concentration was then calculated from

$$[Ca^{2+}] = K_D \frac{[Ca^{2+}\ \text{chelator}]}{[\text{free chelator}]} = K_D \frac{A' - A_0'}{A_1' - A'} \qquad (2)$$

The K_D values used for Ca^{2+}–BAPTA and Ca^{2+}–BrBAPTA were 0.24 and 1.8 μM, respectively, as previously determined for this buffer system at 20 °C (Feigenson, 1986). A_0 is the absorbance of the free chelator, A_1 is the absorbance of the Ca^{2+}–chelator complex, and A is the absorbance of the chelator at a given $[Ca^{2+}]$ in a sample. The prime indicates division of measured peak absorbance by the measured absorbance at the isosbestic point. Peak absorbances and isosbestic point absorbances were at 253 and 236.6 nm for BAPTA and at 262 and 247.7 nm for BrBAPTA, respectively. A_0' values used for BAPTA and BrBAPTA were 1.62 and 1.49, respectively, and A_1' values were 0.163 and 0.24, respectively (Feigenson, 1989).

At various times in the course of Ca^{2+} binding/dissolving, 90–140-μL aliquots were removed and centrifuged at 22000*g* for 15–30 m in a SW-27 swinging-bucket rotor at 20 °C in 400-μL polypropylene centrifuge tubes which were supported in 2 M sorbitol. These centrifuge conditions are sufficient to pellet 99–100% of the lipid. Supernatant was then removed and used for spectrophotometric determination of free $[Ca^{2+}]$ and total aqueous $[Ca^{2+}]$, as described above.

Time Course for Ca^{2+} Binding and Dissolving. Ca^{2+} binding was started when Ca^{2+}–chelator/indicator was added to the lipid multilayers. After this addition, samples were flushed with argon, sealed with Parafilm, and rotated slowly in the dark in a constant-temperature water bath at 20 °C. Aliquots (100–140 μL) were removed at specified times, and the free $[Ca^{2+}]$ was determined as detailed above. Samples

Table I: Comparison of the Characteristic Equilibrium Free [Ca^{2+}] (μM) for P,O-PS/MO,MO-PC = 1/0, 0.6/0.4, and 0.4/0.6 Multilamellae Prepared by Four Different Methods

		cyclohexane		
PS/PC mole ratio	chloroform nonhydrated	nonhydrated	0.5-h hydration	2-h hydration
1.0/0.0	0.042 ± 0.003	0.043 ± 0.003	0.043 ± 0.003	0.041 ± 0.002
0.6/0.4	0.098 ± 0.004	0.110 ± 0.009	0.107 ± 0.009	0.105 ± 0.008
0.4/0.6	0.185 ± 0.006	0.200 ± 0.018	0.203 ± 0.028	0.206 ± 0.027

were handled gently in order that lipid loss be minimal when aliquots were taken for spectrophotometric measurement. The binding period continued for 15–18 days.

An aliquot of BAPTA or BrBAPTA, sufficient to dissolve 10–100% of bound Ca^{2+}, was added to each sample, thereby starting the dissolving period. Incubation of samples continued as in the binding period. Aliquots (90–120 μL) were removed at specified times, and the free [Ca^{2+}] was then determined. Again, it was important to handle samples gently so as not to disturb the loosely settled lipid at the bottom of each tube. However, for the first time point, the lipid had not settled to the bottom of the tube prior to the removal of this aliquot; hence, some lipid was lost. This loss was estimated and then taken into account when the amount of Ca^{2+} bound per PS was calculated. The dissolving period continued for 14–16 days.

Results

We examined the time course of Ca^{2+} binding/dissolving for four different preparations: (1) PS/PC dried from chloroform, with direct addition of aqueous Ca^{2+} in Pipes–KCl buffer; (2) PS/PC lyophilized from cyclohexane, with direct addition of aqueous Ca^{2+} in Pipes–KCl buffer; (3) PS/PC lyophilized from cyclohexane, with a 0.5-h hydration period in Pipes–KCl buffer prior to aqueous Ca^{2+} addition; (4) PS/PC lyophilized from cyclohexane, with a 2-h hydration period in Pipes–KCl buffer prior to aqueous Ca^{2+} addition. The binding period lasted 8 days and the dissolving period 14 days in this comparison of preparation methods.

The total amount of Ca^{2+} bound and the free [Ca^{2+}] values were virtually identical for a given PS/PC ratio in all four preparations at the end of the dissolving time course, with PS/PC ratios of 1/0, 0.6/0.4, and 0.4/0.6. However, differences in the values for free [Ca^{2+}] among the four preparation were observed in the binding period. For example, after 8 days of Ca^{2+} binding, for multilayers dried from chloroform of PS/PC ratios of 1/0, 0.6/0.4, and 0.4/0.6, the free [Ca^{2+}] values were 0.045, 0.15, and 0.34 μM, respectively. In comparison, the lyophilized preparations with PS/PC ratio of 1/0 showed essentially the same free [Ca^{2+}], whereas the lyophilized preparations with PS/PC = 0.6/0.4 and 0.4/0.6 had higher free [Ca^{2+}] than their chloroform-dried counterparts (0.23 and 0.65 μM, respectively). However, following the dissolution period of 14 days the equilibrium free [Ca^{2+}] values at each PS/PC mole ratio were the same for the four preparations (Table I). Since it is the dissolving period that consistently brings the samples to equilibrium, the differences observed during the binding period were not a concern (Feigenson, 1986). Because drying lipid mixtures from chloroform would seem to have more likelihood to induce demixing, we chose to use the protocol of lyophilizing PS/PC and hydrating for 2 h.

We then investigated the effects of acyl chain mismatching of P,O-PS and MO,MO-PC on high-affinity calcium binding in multilamellar dispersions of P,O-PS/MO,MO-PC of ratios from 1/0 to 0.2/0.8. The total lipid concentration, initial free [Ca^{2+}], and chelator/indicator concentrations form each P,O-PS/MO,MO-PC mole ratio are listed in Table II. The time course is divided into two parts, which we term "binding" and "dissolving".

Change in Free [Ca^{2+}] during Binding. The time course of the change in the free [Ca^{2+}] up to 18 days after the addition of Ca^{2+}–BAPTA or Ca^{2+}–BrBAPTA to hydrated multilayers of PS/PC ratios of 1/0, 0.5/0.5, and 0.2/0.8 is shown in panels A–C of Figure 1. The time courses for Ca^{2+} binding at all other PS/PC mole ratios studied followed curves similar to those shown in Figure 1. The three PS/PC mole ratios presented show the range of the changes in [Ca^{2+}] as the mole fraction of PS decreases. For PS/PC = 1/0, the [Ca^{2+}] does not change after about 10 days of binding (Figure 1A) whereas for PS/PC = 0.2/0.8 the free [Ca^{2+}] is still decreasing even after 18 days of binding (Figure 1C).

Changes in Free [Ca^{2+}] during Dissolving. In order to dissolve a portion of the Ca^{2+} bound to PS, and thereby to determine the true equilibrium free [Ca^{2+}], BAPTA or BrBAPTA was added to the samples that had been exposed to aqueous Ca^{2+} for 15–19 days. The free [Ca^{2+}] was thus reduced to a value below the equilibrium [Ca^{2+}]. The time courses of the change in the free [Ca^{2+}] over the dissolving period for PS/PC = 1.0, 0.5/0.5, and 0.2/0.8 are shown in panels A–C of Figure 1. The first aliquot was removed 15–20 min after the addition of chelator in order to observe the initial drop in free [Ca^{2+}]. However, the dissolution of Ca^{2+} was so rapid for PS/PC = 1/0 that enough Ca^{2+} had dissolved, even in this brief period, to raise the free Ca^{2+} close to the equilibrium concentration (inset of Figure 1A). For PS/PC = 0.5/0.5 or 0.2/0.8 a significant drop in free [Ca^{2+}] was observed as the chelator rapidly binds a small amount of accessible Ca^{2+}, followed by a slow rise in [Ca^{2+}] as bound Ca^{2+} dissolves and approaches the equilibrium value (insets of panels B and C of Figure 1). We also found that the free [Ca^{2+}] had reached an unchanging value after about 1 day of dissolving for all other PS/PC sets. Therefore, the equilibrium [Ca^{2+}]* for each PS/PC ratio was achieved quite rapidly by dissolving bound Ca^{2+}.

For PS/PC = 0.4/0.6, separate sample sets were prepared with BAPTA and with BrBAPTA in order to test for any chelator dependence of the measurements.

Equilibrium Isotherms. The dependence of the fraction of PS bound by Ca^{2+} on the free [Ca^{2+}] is shown in Figure 2. Experiments were designed to measure low fractions of binding, in order to identify accurately the concentration where Ca^{2+} binding begins (that is, where the PS/PC ratio is not perturbed from the starting value). For each PS/PC ratio, no binding occurs until [Ca^{2+}]* is reached. At [Ca^{2+}]*, added Ca^{2+} binds to PS with little increase in aqueous [Ca^{2+}]. The value of [Ca^{2+}]* is seen to be characteristic for each PS/PC mole ratio, with [Ca^{2+}]* increasing as the PS concentration decreases.

Discussion

Comparison of Sample Preparation Methods. This study began with only a slight modification of our earlier method, in which detergent-free Millipore filters were used in order to eliminate the laborious washings needed to remove the detergent. However, the detergent-free Millipore filters proved

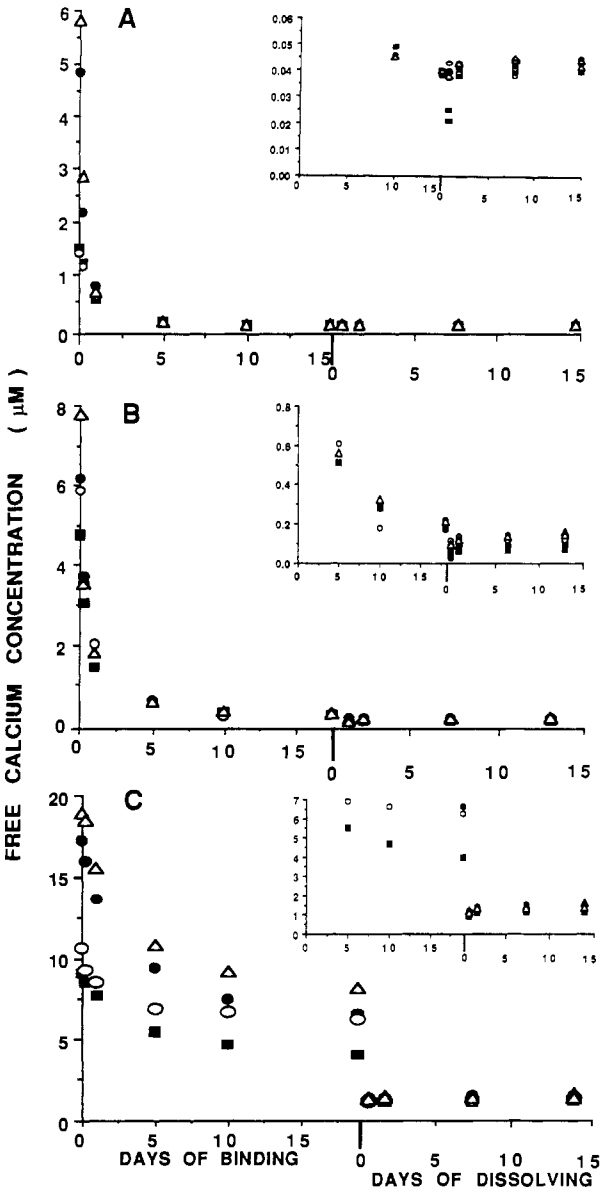

FIGURE 1: Time course of Ca^{2+} equilibration with P,O-PS/MO,-MO-PC multilamellar dispersions. Total lipid concentration is 4 mM. The time course is divided into two parts, termed binding and dissolving, by the wide solid line. The initial [Ca^{2+}] ranged from 1.4 to 18.5 μM. Dissolving was initiated by adding sufficient Ca^{2+}-free chelator solution to reduce the free [Ca^{2+}] to below the equilibrium value. (A) P,O-PS/MO,MO-PC = 1.0/0.0. Initial [Ca^{2+}] of 1.4 μM (○), 1.5 μM (■), 4.8 μM (●), and 5.8 μm (△). Inset shows an enlargement of the binding and dissolving transition. (B) P,O-PS/MO,MO-PC = 0.5/0.5. Initial [Ca^{2+}] of 4.7 μM (■), 5.9 μM (○), 6.1 μM (●), and 7.8 μM (△). Inset shows an enlargement of the binding and dissolving transition. (C) P,O-PS/MO,MO-PC = 0.2/0.8. Initial [Ca^{2+}] of 8.5 μM (■), 10.5 μM (○), 16.0 μM (●), and 18.5 μM (△). Inset shows an enlargement of the binding and dissolving transition.

to be contaminated with Ca^{2+} ions, which slowly leached into the Pipes–KCl buffer during the incubation period (data not shown). Extensive washings of the filters in Ca^{2+} chelators were ineffective in removing the contaminating Ca^{2+} ions.

We then explored more simple systems, having no devices to aid Ca^{2+} binding, consisting of lipid films or else lyophilized lipid powders, together with buffer or buffer with Ca^{2+}. We sought a protocol wherein the following would be met: (1) appreciable Ca^{2+} would bind to P,O-PS; (2) this binding period would not be longer than a few weeks; (3) during the dissolving period, we should be able to detect an initial drop in the free [Ca^{2+}] to below the equilibrium value upon chelator addition,

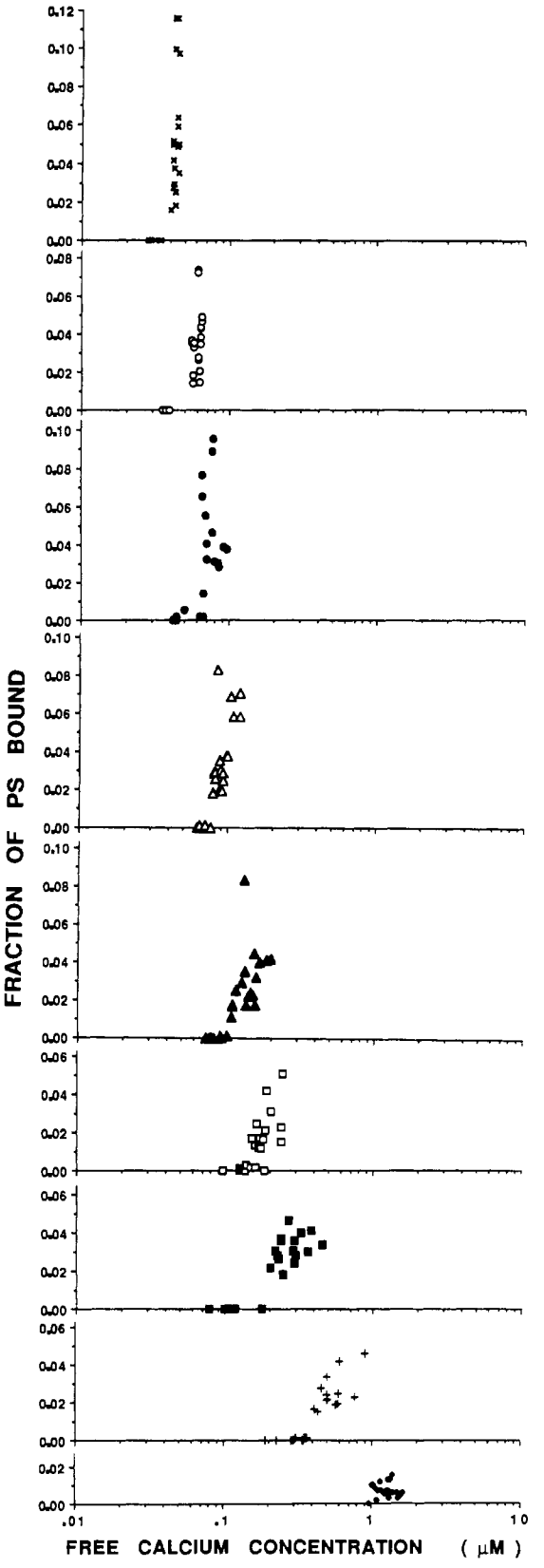

FIGURE 2: Isotherms of Ca^{2+} binding to different mole ratios of P,O-PS/MO,MO-PC. Isotherms were obtained for total lipid concentrations ranging from 2 to 8 mM (see Table II). Samples were incubated in Ca^{2+}–chelator solutions for 15–18 days, during which time Ca^{2+} binding took place. Ca^{2+}-free chelator solutions were then added to lipid dispersions and incubated for 2 weeks, and the final [Ca^{2+}] values shown in the figure were measured. P,O-PS/MO,-MO-PC ratios are 1.0/0.0 (×), 0.8/0.2 (○), 0.7/0.3 (●), 0.6/0.4 (△), 0.5/0.5 (▲), 0.4/0.6 (BAPTA; □), 0.4/0.6 (BrBAPTA; ■), 0.3/0.7 (+), and 0.2/0.8 (◆).

Table II: Starting Mole Ratios, Total Lipid Concentrations, and Ca^{2+}/Chelator Combinations

set	mole ratios of P,O-PS/MO,MO-PC	total lipid (mM)	per each total lipid concn: initial free Ca^{2+} (μM)	total Ca^{2+} (mM)	chelator concn	no. of samples in set
1	1.0/0.0	2, 4, 6	1.5	0.12	0.15 mM BAPTA	12
			4.8	0.14	0.15 mM BAPTA	
			1.4	0.24	0.30 mM BAPTA	
			5.8	0.28	0.30 mM BAPTA	
2	0.8/0.2	2, 4, 6	1.5	0.12	0.15 mM BAPTA	12
			4.8	0.14	0.15 mM BAPTA	
			1.4	0.24	0.30 mM BAPTA	
			5.8	0.28	0.30 mM BAPTA	
3	0.7/0.3	2, 4, 6	1.5	0.12	0.15 mM BAPTA	12
			4.8	0.14	0.15 mM BAPTA	
			1.4	0.24	0.30 mM BAPTA	
			5.8	0.28	0.30 mM BAPTA	
4	0.6/0.4	2, 4, 6	4.7	0.14	0.15 mM BAPTA	12
			6.1	0.15	0.15 mM BAPTA	
			5.9	0.29	0.30 mM BAPTA	
			7.8	0.29	0.30 mM BAPTA	
5	0.5/0.5	2, 4, 6	4.7	0.14	0.15 mM BAPTA	12
			6.1	0.15	0.15 mM BAPTA	
			5.9	0.29	0.30 mM BAPTA	
			7.8	0.29	0.30 mM BAPTA	
6	0.4/0.6	2, 4, 6	4.7	0.14	0.15 mM BAPTA	12
			6.1	0.15	0.15 mM BAPTA	
			5.9	0.29	0.30 mM BAPTA	
			7.8	0.29	0.30 mM BAPTA	
7	0.4/0.6	4, 6, 8	8.5	0.17	0.20 mM BrBAPTA	12
			16.0	0.19	0.20 mM BrBAPTA	
			10.5	0.30	0.35 mM BrBAPTA	
			18.5	0.34	0.35 mM BrBAPTA	
8	0.3/0.7	4, 6, 8	8.5	0.17	0.20 mM BrBAPTA	12
			16.0	0.19	0.20 mM BrBAPTA	
			10.5	0.30	0.35 mM BrBAPTA	
			18.5	0.34	0.35 mM BrBAPTA	
9	0.2/0.8	4, 6, 8	8.5	0.17	0.20 mM BrBAPTA	12
			16.0	0.19	0.20 mM BrBAPTA	
			10.5	0.30	0.35 mM BrBAPTA	
			18.5	0.34	0.35 mM BrBAPTA	

whereupon sufficient $Ca(PS)_2$ would dissolve so that the free $[Ca^{2+}]$ could rise and stabilize at an equilibrium value. Briefly, the method must yield a $[Ca^{2+}]$ that is the same for binding as for dissolving, and that value does not change with time. Figure 1 shows that the new protocol meets these criteria.

We can compare our earlier procedure with the new protocol: Feigenson (1989) reported the characteristic free equilibrium $[Ca^{2+}]$ for P,O-PS supported on Millipore filters as 0.040 ± 0.003 μM. In this study, we find this value to be 0.043 ± 0.003 μM for unsupported, lyophilized P,O-PS multilayers in the same buffer. We take these values to be the same and conclude that this value is a true, method-independent characteristic of the Ca^{2+}–P,O-PS binding reaction in 20 mM Pipes–100 mM KCl at 20 °C and pH 7.00.

Thermodynamic Analysis. We can analyze the Ca^{2+} concentration, $[Ca^{2+}]^*$, in equilibrium with each P,O-PS/MO,-MO-PC mole ratio in order to uncover the lipid mixing behavior. Our starting point is the binding/dissociation reaction for Ca^{2+} and PS^-, which includes the important finding that PC does not enter the $Ca(PS)_2$ rigid phase (Feigenson, 1989). The dissociation reaction for eq 1 is written as

$$K_D = \frac{\mathbf{a}_{Ca^{2+}}\mathbf{a}^2_{PS^-}}{\mathbf{a}_{Ca(PS)_2}} \qquad (3)$$

A standard state is defined at the measurement temperature of 20 °C, for each substance. For aqueous Ca^{2+}, the standard state is 1 M. The thermodynamic activity of a pure phase of $Ca(PS)_2$ is a constant, set at unity by choice of the standard state. The relationship between the thermodynamic activity and the measured concentration for Ca^{2+} in 20 mM Pipes and 100 mM KCl can be written as $\mathbf{a}_{Ca^{2+}} = \gamma'[Ca^{2+}]$, where γ' is the activity coefficient for aqueous Ca^{2+}. The activity of PS can be expressed in terms of the PS mole fraction in the PS/PC bilayer as $\mathbf{a}_{PS} = \gamma_{PS}[PS]$. When we use the convention that $\mathbf{a}_{PS} = 1$ when $[PS] = 1$, then from Figure 2 and eq 3 $K_D = \gamma'[Ca^{2+}]^* = \gamma'(0.043\ \mu M)$. For other values of PS/PC and $[Ca^{2+}]^*$

$$\frac{K_D}{\mathbf{a}_{Ca^{2+}}} = \frac{0.043\ \mu M}{[Ca^{2+}]^*} = \mathbf{a}^2_{PS} = (\gamma_{PS}[PS])^2 \qquad (4)$$

A plot of $\mathbf{a}_{PS}$ vs [PS], that is, $(0.043\ \mu M/[Ca^{2+}]^*)^{1/2}$ vs [PS], is shown as the filled circles in Figure 3. The activity at each mole fraction appears to follow ideal behavior. The meaning of this apparent ideal mixing of P,O-PS/MO,MO-PC is revealed, as discussed below, by a comparison with measurements for P,O-PS/P,O-PC, shown as the open circles in Figure 3.

The chemical potential of P,O-PS at any mole fraction [PS] in a PS/PC mixture can be written as

$$\mu_{PS} = \mu^\circ_{PS} + RT \ln (\gamma_{PS}[PS])$$

For the transfer of PS from pure fluid-phase PS to a PS/PC mixture

$$\mu^{transfer}_{PS} = \mu_{PS} - \mu^\circ_{PS} = RT \ln[PS] + RT \ln \gamma_{PS}$$

For *ideal* mixing of P,O-PS with a PC, the partial molal free energy of transfer is given by

$$\mu^{transfer,ideal}_{PS} = RT \ln [PS]$$

and is always favorable. We are interested in the free energy

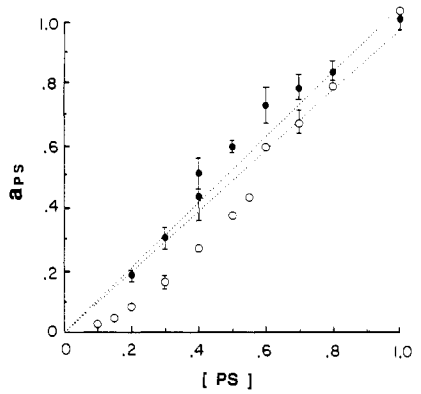

FIGURE 3: Thermodynamic activity of PS as given by (0.043 μM/$[Ca^{2+}]^*)^{1/2}$ (see text), plotted against the mole fraction of PS. The dotted lines show the range of ideal mixing, given the experimental uncertainty of $[Ca^{2+}]^*$ for pure P,O-PS of 0.043 ± 0.003 μM. P,-O-PS/MO,MO-PC (●). Data for P,O-PS/P,O-PC (○) taken from Feigenson (1989) are also shown. Error bars are shown when the estimated uncertainty in $[Ca^{2+}]^*$, determined from the binding isotherms, exceeds the symbol size.

of transfer of P,O-PS from pure fluid-phase P,O-PS to infinitely dilute P,O-PS in PC, *exclusive* of the (entropically) favorable ideal mixing contribution. We now write

$$\mu_{PS}^{transfer} - RT \ln [PS] = \mu^{E}_{PS} = RT \ln \gamma_{PS}$$

where μ^E is the *excess* chemical potential. Therefore, the partial molal free energy that describes the (entropic and enthalpic) effects of transferring a PS molecule from PS to PC exclusive of the dilution is just the excess chemical potential for mixing, in the dilute (Henry's law) PS region. For the mixture P,O-PS/P,O-PC, as [PS] approaches zero, $\mu^{E}_{PS\rightarrow 0} = RT \ln 0.3 = -0.7$ kcal mol^{-1}. For P,O-PS/MO,MO-PC, $\mu^{E}_{PS\rightarrow 0} = RT \ln (\cong 1) \cong 0$ kcal mol^{-1}.

To this simple treatment of the data we now add an assumption that will enable an interpretation of the free energy of transfer of the PS in terms of molecular properties: the free energy of phospholipid mixing can be represented as a sum of contributions from acyl chain mixing and from headgroup mixing. We consider this to be a reasonable first-order approximation, even though changes in headgroup area must be coupled to changes in acyl chain interactions. Accordingly, for the mixture P,O-PS/P,O-PC the entire −0.7 kcal mol^{-1} is ascribed to nonideal headgroup mixing, because the different lipids have the same acyl chains. We attribute much of the −0.7 kcal mol^{-1} to the relief of electrostatic repulsion. This transfer of P,O-PS from an environment of negative charges to an environment of zwitterions must correspond to one of the larger possible changes in electrostatic environment that a phospholipid can undergo. However, the actual apportioning of the headgroup contribution among electrostatic, other headgroup–headgroup, and solvation differences between pure fluid-phase P,O-PS and dilute fluid-phase P,O-PS in P,O-PC will require additional experiments.

In contrast, the transfer of P,O-PS from P,O-PS to MO,-MO-PC shows no excess partial molal free energy. Since the change in headgroup environment of the P,O-PS is the same as in the transfer to P,O-PC, for which $\mu^{E}_{PS\rightarrow 0} = -0.7$ kcal mol^{-1}, then a compensating free energy change must be occurring. We conclude that the "mismatching" of P,O acyl chains in an environment of MO,MO acyl chains leads to an unfavorable contribution of approximately +0.7 kcal mol^{-1} for the combined enthalpic and entropic effects at 20 °C.

We can compare our results on the mixing of P,O chains with MO,MO chains to the available data on hydrocarbon mixtures [see Tiegs et al. (1986) for an extensive compilation]. Representative examples of data at infinite dilution of the solute are as follows: decane in hexadecane at 70 °C, γ = 0.96 and $\mu^E \cong -0.03$ kcal mol^{-1} (Snyder & Thomas, 1968); heptane in 1-octadecene at 41 °C, γ = 0.95 and $\mu^E \cong -0.03$ kcal mol^{-1} (Alessi et al., 1982); heptane in 1-eicosane ($C_{20}H_{40}$) at 56 °C, γ = 1.11 and $\mu^E \cong +0.07$ kcal mol^{-1} (Weidlich & Gmehling, 1985). Apparently, data from mixing of linear hydrocarbons in an isotropic liquid have little predictive value for the much larger deviations from ideality for phospholipid chain mixing.

The acyl chains found in biological membranes vary in chain length, unsaturation, double bond number, location and type, and branching. For example, in phospholipids of mammalian membranes the length of the hydrocarbon chain ranges from 14 to 24 carbons with 0 to 6 double bonds found primarily in the cis conformation (Stubbs & Smith, 1984). These structural differences could have a large influence on lipid behavior. What we can say at this stage of our study is that a lipid having P,O acyl chains has approximately 3-fold greater activity when surrounded by M,O acyl chains than when surrounded by P,O acyl chains. Since much larger acyl chain differences are found naturally, we expect that lipid behavior in real biological membranes in the fluid state could be even more strongly influenced by the acyl chain environment than we have yet measured. In contrast, purely electrostatic effects on lipid activity seem unlikely to be much larger than the 3-fold effect that we have measured for transfer of a negatively charged lipid from an environment of negative charges to one of zwitterions.

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Registry No. P,O-PS, 40290-44-6; MO,MO-PC, 18194-24-6; Ca, 7440-70-2.

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Gramicidin A Induced Fusion of Large Unilamellar Dioleoylphosphatidylcholine Vesicles and Its Relation to the Induction of Type II Nonbilayer Structures†

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ABSTRACT: The fusogenic properties of gramicidin were investigated by using large unilamellar dioleoylphosphatidylcholine vesicles. It is shown that gramicidin induces aggregation and fusion of these vesicles at peptide to lipid molar ratios exceeding 1/100. Both intervesicle lipid mixing and mixing of aqueous contents were demonstrated. Furthermore, increased static and dynamic light scattering and a broadening of ^{31}P NMR signals occurred concomitant with lipid mixing. Freeze–fracture electron microscopy revealed a moderate vesicle size increase. Lipid mixing is paralleled by changes in membrane permeability: small solutes like carboxyfluorescein and smaller dextrans, FD-4(M_r ~4000), rapidly (1–2 min) leak out of the vesicles. However, larger molecules like FD-10 and FD-17 (M_r ~9400 and 17200) are retained in the vesicles for >10 min after addition of gramicidin, thereby making detection of contents mixing during lipid mixing possible. At low lipid concentrations (5 μM), lipid mixing and leakage are time resolved: leakage of CF shows a lag phase of 1–3 min, whereas lipid mixing is immediate and almost reaches completion during this lag phase. It is therefore concluded that leakage, just as contents mixing, occurs subsequent to aggregation and lipid mixing. Although addition of gramicidin at a peptide/lipid molar ratio exceeding 1/50 eventually leads to hexagonal H_{II} phase formation and a loss of vesicle contents, it is concluded that leakage during fusion (1–2 min) is not the result of H_{II} phase formation but is due to local changes in lipid structure caused by precursors of this phase. By making use of gramicidin derivatives and different solvent conformations, it is shown that there is a close parallel between the ability of the peptide to induce the H_{II} phase and its ability to induce intervesicle lipid mixing and leakage. It is suggested that gramicidin-induced fusion and H_{II} phase formation share common intermediates.

Membrane fusion is an essential event in many processes in cellular physiology and in viral infectivity. Studies on enveloped viruses in particular have provided strong evidence for direct involvement of proteins and (poly)peptides in the initiation, specificity, and control of biological fusion processes. Despite enormous progress in this field, the molecular mechanism of the dynamic structural reorganization during this process remains elusive.

Basic concepts concerning lipid structure during fusion have been derived from extensive explorations of model membrane systems. For pure lipid membranes, which in close resemblance to biomembranes are rich in type II lipids (preferring inverted nonbilayer structures, e.g., hexagonal H_{II} phase), consensus is reached that inverted micellar intermediates which have been proposed a decade ago (Verkleij et al., 1979a,b) play an important role in the fusion process (Ellens et al., 1989; Gruner et al., 1988).

Model membrane studies have also documented the lipid vesicle fusion[1] modulating activity of a variety of proteins and (poly)peptides [see for reviews Hong et al. (1987), Blumenthal (1987), and Bentz and Ellens (1988)]. A common denominator for (poly)peptide fusogens seems to be the ability of the molecule to destabilize the bilayer. This can be accomplished by either predominantly electrostatic [e.g., polylysines (Walter et al., 1986; Gad et al., 1985)] or hydrophobic [e.g., alamethicin (Lau & Chan, 1975)] interactions, but most often by a combination of both [e.g., melittin (Morgan et al., 1983; Batenburg et al., 1987a)]. Interestingly, for a number of cases, positive correlations have been found between the ability of a polypeptide to modulate vesicle fusion and the formation of type II nonbilayer lipid structures. Examples are glycophorin (Taraschi et al., 1982a,b), myelin basic protein (Smith & Cornell, 1985; Lampe & Nelstuen, 1982), a cardiotoxin (Batenburg et al., 1985), and melittin (Batenburg et al., 1987a,b). However, a satisfactory molecular picture connecting these observations has not been obtained because of

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[1] In the references quoted in the introduction, the term fusion is not always well-defined and is often used if, e.g., only lipid mixing is observed. Throughout the present paper, fusion refers to the process leading to both mixing of lipids and mixing of contents of vesicles.

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