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Phase behavior and arrangement of molecular species in mixtures of a mixed chain and a symmetric phosphatidylethanolamine in the gel and fluid phases

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1-Octadecanoyl, 2-decanoylphosphatidylethanolamine (C(18:0)C(10:0)PE) has been reported to exhibit mixed interdigitated gel-phase packing of the phospholipid acyl chains (Mason, J.T. and Stephenson, F.A. (1990) Biochemistry 29, 590–598). In contrast, ditetradecanoylphosphatidylethanolamine (C(14:0)C(14:0)PE) packs without significant interdigitation of the phospholipid acyl chains across the bilayer center. In this report, the gel–fluid transition temperatures of C(18:0)C(10:0)PE and C(14:0)C(14:0)PE in multilamellar dispersions were determined by fluorescence anisotropy of *cis*-parinaric acid and *trans*-parinaric acid with a descending temperature scan rate of 0.67°C/min. The transition mid-points detected for C(18:0)C(10:0)PE with *cis*-parinaric acid were 19°C in water, 18°C at pH 8.1, and 14°C at pH 10. The phase diagram for C(14:0)C(14:0)PE and C(18:0)C(10:0)PE at pH 10 suggests complete mixing in the fluid phase and considerable immiscibility in the gel phase. Cross-linking of equimolar mixtures of C(14:0)C(14:0)PE and C(18:0)C(10:0)PE with dimethylsuberimidate at pH 10 revealed a random arrangement of the two species in the fluid phase, confirming the notion that C(18:0)C(10:0)PE and C(14:0)C(14:0)PE are miscible in the fluid phase, as determined from the phase diagram. In contrast, cross-linking of the equimolar mixture of C(18:0)C(10:0)PE and C(14:0)C(14:0)PE in the gel phase at 0°C revealed a non-random arrangement, demonstrating and confirming immiscibility in the gel phase.

Introduction

The existence of lipid domains in mixtures of fluid-phase phospholipids is a matter of some controversy. Evidence in favor of immiscibility in simple fluid lipid mixtures includes the work of Melchior [1] who used DSC to examine C(18:0)C(18:0)PC/C(14:0)C(14:0)PC mixtures which were quickly frozen from the fluid phase. These quickly frozen lipids exhibited two distinct endotherms, which were interpreted to mean that the lipids were found in distinct domains which were trapped by the extremely fast cooling technique employed. Recently Krisovitch and Regen detected immiscibility in some fluid-phase mixtures of artificial lipids by examining cross-linked nearest neighbor pairs [2]. Lentz and co-workers [3] interpreted the presence of a horizontal fluidus line in their phase diagram of C(15:0)C(15:0)PG/C(14:0)C(14:0)PC derived from DSC and fluorescence polarization measurements as evidence of inhomogeneities in the composition of this fluid-phase mixture.

While horizontal fluidus lines in phase diagrams are fairly rare, other deviations from the predicted diagrams for ideal mixtures have been interpreted as evidence of non-homogeneities in fluid-phase mixtures. From a statistical mechanical analysis of deviations from ideality in phase diagrams, Von Dreele [4] predicted deviations from random in the arrangement of fluid-phase mixtures of C(18:0)C(18:0)PC and C(14:0)C(14:0)PC, C(18:0)C(18:0)PC and C(16:0)C(16:0)PC, and C(16:0)C(16:0)PC and C(14:0)C(14:0)PC. Another analysis of phase diagrams for six mixtures in terms of lipid arrangement by Lee [5] also

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Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; C(18:0)C(10:0), 1-octadecanoyl, 2-decanoyl; C(12:0)C(12:0), dilauroyl or didodecanoyl; C(14:0)C(14:0), dimyristoyl or ditetradecanoyl; C(15:0)C(15:0), dipentadecanoyl; C(16:0)C((16:0), dipalmitoyl or dihexadecanoyl; C(18:0)C(18:0), distearoyl or dioctadecanoyl; C(18:1c)C(18:1c), dioleoyl or 9,10-*cis*-octadecaenoyl; C(18:1t)C(18:1t), dielaidoyl or 9,10-*trans*-octadecaenoyl; cPnA, *cis*-parinaric acid or 9,11,13,15-*cis,trans,trans,cis*-octadecatetraenoic acid; tPna, *trans*-parinaric acid or 9,11,13,15-all *trans*-octadecatetraenoic acid.

predicted significant deviations from random mixing in the fluid phase for each of the mixtures, including C(18:1c)C(18:1c)PC/C(16:0)C(16:0)PE.

In contrast, in previous work from our laboratory, using dimethylsuberimidate cross-linking to analyze nearest neighbor pairing, we have not detected inhomogeneities in pH 10 fluid-phase mixtures of C(14:0)C(14:0)PE/C(18:1t)C(18:1t)PE and C(18:1t)C(18:1t)PE/C(12:0)C(12:0)PE in the fluid phase [6]. We found only slight immiscibility in C(16:0)C(16:0)PE/C(18:1c)C(18:1c)PE fluid-phase mixtures.

In the past several years, a significant body of work has examined the structure adopted by phosphatidylcholines in which one acyl chain is roughly twice as long as the other. Perhaps the most studied lipid has been 1-octadecanoyl,2-decanoylphosphatidylcholine (C(18:0)C(10:0)PC). X-ray diffraction, Raman spectroscopy, and DSC data are all consistent with the notion that C(18:0)C(10:0)PC adopts an interdigitated structure in the gel phase in which the area per head group is large, corresponding to the area of three acyl chains [7–12]. The structure of C(18:0)C(10:0)PC in the fluid phase is less well-understood than the gel-phase structure, but several lines of evidence suggest a partially interdigitated arrangement with the area per head group corresponding to only two acyl chains [9–12].

The thermotropic behavior of mixtures of C(18:0)C(10:0)PC and the symmetric species of the same molecular weight, C(14:0)C(14:0)PC, was investigated by Lin and Huang [13] using high-resolution differential scanning calorimetry. The phase diagram indicates that these lipids form a eutectic mixture with a eutectic point at 40% C(14:0)C(14:0)PC. The two lipids were completely miscible in the liquid crystalline phase, but partially immiscible in the gel phase. Ali et al. published different data on an equimolar mixture of C(18:0)C(10:0)PC and C(14:0)C(14:0)PC, also suggesting considerable immiscibility in the gel phase [12]. Recently, Raman spectroscopy studies on this mixture have demonstrated that one of the two gel phases present in the mixed system exhibits mixed interdigitation (three chains per head group) and the other gel phase exhibits partial interdigitation (two chains per head group) [14].

The corresponding phosphatidylethanolamine, C(18:0)C(10:0)PE, has been characterized by DSC and ^{31}P-NMR spectroscopy, demonstrating that this lipid also adopts a mixed interdigitated structure in the gel phase, like the PC with the same acyl chains [15].

In this report, we describe the phase diagram for C(14:0)C(14:0)PE and C(18:0)C(10:0)PE mixtures at pH 10 determined by using fluorescence anisotropy of *cis*-parinaric and *trans*-parinaric acids to detect the gel–liquid crystalline-phase transition. We also used the bifunctional reagent dimethylsuberimidate to analyze the nearest neighbor pairs in C(18:0)C(10:0)PE/C(14:0)C(14:0)PE mixtures at temperatures both above and below the liquid crystalline–gel-phase transition of both lipids at pH 10. We performed these studies at pH 10 in order to be certain to maintain a bilayer liquid crystalline, rather than a hexagonal (H_{II}) phase [16]. While, as expected, inhomogeneity was detected in the gel phase, no immiscibility was observed in the liquid crystalline phase. The immiscibility seen in the gel phase is analyzed with respect to the phase diagram which we have obtained.

Materials and Methods

Materials. C(14:0)C(14:0)PE was purchased from Avanti Polar Lipids (Alabaster, AL). *cis*-Parinaric and *trans*-parinaric acids were obtained from Molecular Probes (Eugene, OR). C(18:0)C(10:0)PE was synthesized starting with 1–18:0 lyso PC (Avanti) and capric (decanoic) acid (NuChek Prep, Elysian, MN), as previously described [17]. The C(18:0)C(10:0)PE was pure by TLC on silica-gel G in chloroform/methanol/water (65:25:4, v/v) and HPLC on a reverse-phase column [18].

Phospholipid dispersions for fluorescence measurements. Phospholipid concentration was determined by phosphate assay [19]. The phospholipids (400 nmol) in chloroform/methanol (2:1, v/v) were mixed, the solvent was evaporated under nitrogen, and the samples were placed under vacuum for 30 min. Then, at 0°C, 3.2 ml of water or 0.05 M KCl, 5 mM EDTA, 10 mM Tris (pH 8.1) or 0.1 M NaCl, 0.05 M sodium bicarbonate (pH 10) were added to each sample. The samples were vortexed 1 min at 0°C. Samples in water or at pH 8.1 were heated to 60°C and samples at pH 10 were heated to 52°C, then sonicated for 15 s in a bath. All samples were then cooled to 10°C, reheated to the same temperature as previously and again sonicated for 15 s. The cooling, heating, sonication cycle was repeated one more time. Finally the samples were cooled to 10°C and then kept at 0°C for 2 to 3 days before taking fluorescence measurements. In the case of the pH 10 samples used for the phase-diagram measurements, a modification was added in which the samples were dissolved in chloroform after the initial drying, redried under nitrogen, redissolved in benzene and lyophilized. Buffer was then added and the samples were treated as described above. According to Lin and Huang [13], the second procedure yielded vesicles in which the mixing of the two corresponding PCs was greater than other methods that they employed. In our hands, there was no significant difference in the fluorescence data obtained from the two procedures we employed, as determined by trials on an equimolar mixture of the two PEs.

Fluorescence measurements. The data were obtained using a Spex Fluorolog spectrofluorometer, essentially as previously described [20]. 2 nmol of *cis-* or *trans-*parinaric acid in 10 μl ethanol were injected into the vesicle suspension and mixed thoroughly. The cooling rate was 0.67°C/min. Transition onsets were chosen as the highest temperature at which the anisotropy increased above the values at higher temperatures and continued to increase at lower temperatures. Transition completion temperatures were taken as the last value (cooling) before the anisotropy plateaued. The temperature halfway between the transition onset and completion temperatures was taken as the transition midpoint.

Phospholipid dispersions for cross-linking. C(14:0)C(14:0)PE (1 μmol) and/or C(18:0)C(10:0)PE (1 μmol) in chloroform/methanol (2:1, v/v) were mixed and vesicles were prepared in 2 ml of 100 mM NaCl, 50 mM sodium bicarbonate (pH 10) by the same vortexing, heating, sonicating, and cooling cycle procedure described for the fluorescence studies. In the experiment in which the lipids were present in separate vesicles, vesicles were formed of single lipids at the same concentration and under the same conditions. The vesicles were then mixed prior to the cross-linking reaction.

Cross-linking reaction. PE vesicles were equilibrated at the indicated temperature. The ratio of dimethylsuberimidate to PE was 1 to 5 for the samples at 45°C, 1 to 2 for the samples at 40°C, 50°C, and 60°C, and 1 to 1 for the samples at 0°C. Immediately before adding it to the vesicles, the dimethylsuberimidate was dissolved in pH 10 buffer to which 2 mol of sodium hydroxide per mol dimethylsuberimidate dihydrochloride had been added. The reaction times were 30 min at 60°C and 45°C, 1 h at 50°C, 2 h at 40°C, and 24 h at 0°C. The reactions were stopped by addition of 50 μl glacial acetic acid and extracted with chloroform and methanol. The water phase was reextracted twice with chloroform and the combined organic phase was evaporated under nitrogen.

Analysis of cross-linked products. The samples containing cross-linked products were redissolved in 4 ml chloroform and filtered through a solvent-resistant 0.4 μm filter. The filtrate was again dried under nitrogen and the lipids redissolved in 100 μl chloroform/methanol (2:1). 50 μl were chromatographed on three 4.6 × 250 mm Altex Ultrasphere ODS columns (C_{18} reverse phase) in series. Elution was isocratic in 20 mM choline chloride in ethanol/water/hexane (78:14:10) at 0.2 or 0.4 ml/min. The homodimer of C(14:0)C(14:0)PE eluted first, the heterodimer second, and the homodimer of C(18:0)C(10:0)PE third. At 0.4 ml/min, the peaks eluted in 6 to 8 h. Detection was by absorbance at 205 nm. At this wavelength, absorption coefficients of the various dimers are similar [21]. Dimers were quantitated by weighing the sample peaks. In all cases triplicate samples were analyzed. The values after the "±" represent the standard deviation of the triplicate samples.

Determination of pK_a of PEs. The solvent was evaporated from 4 μmol PE. 5 ml of 8 mM Triton X-100 (electrophoresis grade from Fisher Scientific) was added and the sample was mixed until optically clear. The sample was titrated with 0.1 M NaOH and the pH was measured after each addition. A control sample of 5 ml 8 mM Triton X-100 was titrated similarly. The volume of titrant required to reach each pH value was calculated [22]. The pK_a of each PE was determined from a plot of the corrected volume of titrant added (vol_t) vs. $vol_t \times [H^+]$ [22].

Results

Transition temperatures from fluorescence anisotropy of C(14:0)C(14:0)PE and C(18:0)C(10:0)PE in water, at pH 8.1, and at pH 10.0

Table I shows the transition mid-points for C(14:0)C(14:0)PE and C(18:0)C(10:0)PE. For comparison, when Mason and Stephenson cooled a sample and took DSC measurements in the same pH 8.1 buffer, they detected two transitions for C(18:0)C10:0)PE at 17.9°C and 20.1°C. On heating a hydrated sample, they detected a single transition at 21.1°C [15].

Fluorescence intensity of cPnA

As expected, the intensities of *cis-* and *trans-*parinaric acids, increased with decreasing temperatures in C(14:0)C(14:0)PE. Similarly, the intensity of tPnA in C(18:0)C(10:0)PE increased with decreasing temperature. However, in C(18:0)C(10:0)PE, the fluorescence intensity of cPnA in both the pH 8.1 and 10 buffers decreased as the lipid was cooled through the transition temperature. The average decrease in fluo-

TABLE I

Gel–fluid-phase transition temperatures as determined by steady-state fluorescence anisotropy

Lipid	Probe	T_m(°C) [a]		
		at pH 10 [b]	at pH 8.1 [c]	in H_2O
C(14:0)C(14:0)PE	cPnA	37	47	49
	tPnA	39	n.d. [d]	49
C(18:0)C(10:0)PE	cPnA	14	18	18
	tPnA	14	19	19

[a] T_m represents the midpoint of the transition as determined by fluorescence anisotropy of cPnA or tPnA, as described in Materials and Methods.
[b] The buffer was 0.1 M NaCl, 0.05 M sodium bicarbonate (pH 10).
[c] The buffer was 0.05 M KCl, 5 mM EDTA, 10 mM Tris (pH 8.1).
[d] Not determined.

rescence intensity was about 30% in either buffer. In contrast, the intensity of cPnA in C(18:0)C(10:0)PE increased with cooling through the phase transition in water.

pK_a of C(18:0)C(10:0) PE, C(18:1c)C(18:1c)PE, and C(14:0)C(14:0)PE

The pK_as of C(18:0)C(10:0)PE and C(18:1c)C(18:1c)PE in Triton X-100 dispersions at 25°C and the pK_a of C(14:0)C(14:0)PE in a Triton X-100 dispersion at 50°C were each determined to be 9.6.

Phase diagram

The phase diagram for C(14:0)C(14:0)PE/C(18:0)C(10:0)PE in the pH 10 buffer is shown in Fig. 1. Most of the points were obtained using *cis*-parinaric acid, since this partitions nearly equally between gel- and fluid-phase lipids [23]. Thus changes in the anisotropy of cPnA occur as soon as gel phase is present and can be detected as long as more gel phase is being formed. In contrast, *trans*-parinaric partitions preferentially into gel phase, making it a sensitive indicator of small amounts of gel-phase formation (transition onsets during cooling), but tPnA may reach essentially the high limiting anisotropy value, while there is still fluid-phase present [23], making it less suitable for detection of transition completion temperatures. Therefore, tPnA was used for accurate detection of some transition onsets, particularly those of mixtures with small amounts of C(14:0)C(14:0)PE. The data are shown both uncorrected and corrected for the width of transition of the pure components as described by Mabrey and Sturtevant [24]. This rather arbitrary correction was fairly large for the fluorescence data, as changes in anisotropy were detected over a fairly broad range.

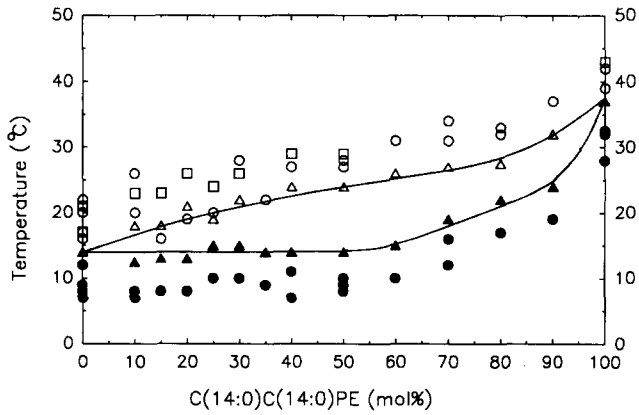

Fig. 1. C(18:0)C(10:0)PE/C(14:0)C(14:0)PE phase diagram in 0.1 M NaCl, 0.05 M sodium bicarbonate (pH 10) by fluorescence anisotropy of *cis*- and *trans*-parinaric acids. Open squares, transition onset temperatures observed with tPnA; open circles, transition onset temperatures observed with cPnA; closed circles, transition completion temperatures observed with cPnA; open triangles, transition onset temperature of average or tPnA data after correction [24]; closed triangles, transition completion temperature of average data after correction [24].

TABLE II

Dimeric species formed from dimethylsuberimidate cross-linking of equimolar fluid-phase mixtures of C(14:0)C(14:0)PE and C(18:0)C(10:0)PE at pH 10

Dimer	Percent of dimeric species formed [a]			
	at 40°C	at 45°C	at 50°C	at 60°C
C(14:0)C(14:0)PE-C(14:0)C(14:0)PE	25±1	23±0	25±1	25±0
C(14:0)C(14:0)PE-C(18:0)C(10:0)PE	48±1	50±2	48±1	48±1
C(18:0)C(10:0)PE-C(18:0)C(10:0)PE	27±1	27±2	26±1	26±1

[a] Values may not total 100% because of rounding to nearest whole percent.

The solidus line appears essentially flat from 0% C(14:0)C(14:0)PE to about 60% C(14:0)C(14:0)PE, indicating complete immiscibility in the gel phase at these compositions. In contrast, the fluidus line appears to vary continuously with com-position, implying no immiscibility in the fully fluid mixtures.

Cross-linking of equimolar mixtures of C(14:0)C(14:0)PE and C(18:0)C(10:0)PE at pH 10

Analysis of the dimeric species resulting from dimethylsuberimidate cross-linking of equimolar fluid-phase mixtures of C(14:0)C(14:0)PE and C(18:0)C(10:0)PE is shown in Table II. Controls in which the lipids were cross-linked in separate vesicles demonstrated that dimethylsuberimidate is approximately equally reactive with each of the two lipids in the fluid phase at pH 10. If the lipids were randomly arranged, the cross-linking pattern of the dimeric species would have been 25% of each homodimer and 50% of the heterodimer [6]. Thus, at all fluid-phase temperatures investigated, the cross-linking of the two lipids was essentially random, indicating complete miscibility of

TABLE III

Dimeric species formed from dimethylsuberimidate cross-linking of equimolar gel-phase mixtures of C(14:0)C(14:0)PE and C(18:0)C(10:0)PE at pH 10 and 0°C

Dimer	Percent of dimeric species formed	
	Two lipids in separate vesicles	Two lipids in the same vesicles
C(14:0)C(14:0)PE-C(14:0)C(14:0)PE	25±1	22±2
C(14:0)C(14:0)PE-C(18:0)C(10:0)PE	0	20±1
C(18:0)C(10:0)PE-C(18:0)C(10:0)PE	75±1	58±1

TABLE IV

Nearest neighbors predicted for dimethylsuberimidate cross-linking of equimolar gel-phase mixtures of C(14 : 0)C(14 : 0)PE and C(18:0)C(10:0)PE at pH 10 and 0°C (calculated from data in Table III)

Nearest neighbor pair	Percent of nearest neighbor contacts predicted from amount of dimer formation in separate vesicles simultaneously reacted with dimethylsuberimidate	
	Assuming heterodimer reactivity equals that of C(18:0)C(10:0)PE homodimer	Assuming heterodimer reactivity equals that of C(14:0)C(14:0)PE homodimer
C(14:0)C(14:0)PE-C(14:0)C(14:0)PE	46	36
C(14:0)C(14:0)PE-C(18:0)C(10:0)PE	14	33
C(18:0)C(10:0)PE-C(18:0)C(10:0)PE	40	31

the two lipids in the fluid phase, as predicted by the phase diagram.

Cross-linking of an equimolar mixture of the two lipids in the gel phase at pH 10 is shown in Table III. When the lipids were present simultaneously in a mixture of separate vesicles, C(18 : 0)C(10 : 0)PE was about three times more reactive than C(14 : 0)C(14 : 0)PE. Table IV shows the cross-linking patterns which one would expect if the two PEs were equally reactive with dimethylsuberimidate in the gel phase. In other words, these are the nearest neighbor pairs, determined after taking the reactivity of dimethylsuberimidate with the two lipids into account. These values were calculated from the data in Table III. These data can again be compared with the 25% of each homodimer and 50% of the heterodimer which were predicted for a mixture in which the nearest neighbor lipids were randomly paired. In one case, it was assumed that the heterodimer was as likely to form as C(18 : 0)C(10 : 0)PE homodimers and in the second case, it was assumed that the heterodimer was as likely to form as C(14 : 0)C(14 : 0)PE homodimers. In either case, it can be seen clearly that the two lipids were not paired randomly as neighbors. There was significant clustering of like lipid species, as predicted by the phase diagram which suggests immiscibility in the gel phase for the equimolar mixture.

Discussion

The transition temperature determined for C(18 : 0) C(10 : 0)PE at pH 8.1 agrees reasonably well with that previously reported by Mason and Stephenson [15], although, with steady-state fluorescence anisotropy measurements, we were unable to resolve the transition on cooling into two endotherms as was possible by DSC. As was discussed by Mason and Stephenson, highly asymmetric PEs appear to be much closer in transition temperature to the corresponding PCs than symmetric PEs are. They determined that the transition temperature of C(18 : 0)C(10 : 0)PE was about 2°C higher than C(18 : 0)C(10 : 0)PC [15], as compared to 15 to 20°C higher for symmetric PCs than PEs. Similarly, the depression in transition temperature which occurs when PE is shifted from below to above its pK_a is smaller for the highly asymmetric PE, C(18 : 0) C(10 : 0)PE, than for symmetric PEs. In this report, we showed that the transition temperature of C(18 : 0)C(10 : 0)PE was decreased 4 to 5°C at pH 10 as compared to in water, while in this and a previous report [20], we showed that the transition temperatures of the symmetric saturated PEs, C(12 : 0)C(12 : 0)PE, C(14 : 0)C(14 : 0)PE, and C(16 : 0)C(16 : 0)PE, were decreased 9 to 10°C at pH 10 as compared to in water. The difference in amount of transition temperature change is not due to a difference in the pK_as of the asymmetric and symmetric PEs, since we determined that the pK_as of both C(18 : 0)C(10 : 0)PE and C(14 : 0) C(14 : 0)PE, as well as that of C(18 : 1c)C(18 : 1c)PE, were 9.6. This pK_a value corresponds to the intrinsic pK_a value determined for egg PE [25]. Instead, the difference in the amount of transition temperature change must reflect the fact that C(18 : 0)C(10 : 0)PE is undergoing a change from a liquid crystalline to an interdigitated gel phase rather than from a liquid crystalline to a non-interdigitated gel phase. The zwitterionic form of PE lends stability to a non-interdigitated gel phase by allowing both strong electrostatic and hydrogen-bonding interactions between PE head groups [26,27]. Neither the gel phase of PCs, whose fully methylated ammonium groups do not allow such close PO...N contacts, nor pH 10 PE, whose negatively-charged head groups probably do not form intermolecular hydrogen bonds due to charge repulsion, show similar stability. Likewise, strong interactions between PE head groups are probably not possible in the interdigitated gel phase, even at neutral pH where PE is zwitterionic, because of the expanded head group area relative to the non-interdigitated bilayer. Mason and Stephenson [15] suggested this lack of strong PE head group interactions in the gel phase as the reason that asymmetric PEs and PCs with the same acyl chains have similar transition temperatures. It seems a likely explanation for the similarity in transition temperature of asymmetric PEs above and below their transitions also.

We observed about a 30% decrease in fluorescence intensity of cPnA at pH 8.1 and 10 in C(18 : 0)C(10 : 0) PE at the transition temperature. Since parinaric acids are not fluorescent when free in aqueous solutions [23], a possible explanation for these data is that the partition coefficient of cPnA between lipid and the aqueous

phase is shifted in favor of the aqueous phase below the transition temperature of C(18:0)C(10:0)PE in the pH 8.1 and pH 10 buffers. Fluorescence quenching of over 50% was observed with diphenylhexatriene in an interdigitated gel phase as compared to a noninterdigitated gel phase [28,29]. One explanation previously offered is that the diphenylhexatriene was unable to orient itself parallel to the plane of the bilayer in the interdigitated bilayer. Thus only an orientation perpendicular to the plane of the bilayer was allowed. If so, the diphenylhexatriene in the parallel orientation was either released from the bilayer as the interdigitated phase was formed or moved to the perpendicular orientation. However, since quenching also occurs with *cis*-parinaric acid (which is oriented perpendicular to the bilayer [30]), there must be other factors involved besides the inability of the interdigitated phase to accommodate a probe parallel to the plane of the bilayer. In fact, some quenching of diphenylhexatriene attached to a PC acyl chain also occurred in the previous studies, and it was suggested that this may be due to increased exposure of the fluors to the aqueous phase when the lipids are in an interdigitated arrangement [28,29]. Either this explanation or the movement of the fluors into the aqueous phase seem plausible based on our data, but it is interesting to note that this phenomenon was not observed with tPnA or as C(18:0)C(10:0)PE underwent a gel–fluid-phase transition in water.

Both from the phase diagram and by our cross-linking results, we show that C(18:0)C(10:0)PE and C(14:0)C(14:0)PE are completely miscible and arranged randomly in the fluid phase at pH 10. We also found complete or nearly complete miscibility in other fluid-phase PE mixtures by examining cross-linking patterns [6]. These results contrast with the predictions of Von Dreele [4] and Lee [5] who interpret non-ideality in the phase diagrams of binary phospholipid mixtures as suggestive of fluid as well as gel-phase inhomogeneities. It also has been concluded that fluid-phase mixtures of the corresponding PCs are completely miscible [13], although it is believed that pure asymmetric lipids adopt a partially interdigitated structure [9–12]. We failed to detect a eutectic point in the C(18:0)C(10:0)PE/C(14:0)C(14:0)PE system at pH 10 by steady-state fluorescence anisotropy measurements with *cis*- and *trans*-parinaric acids. A eutectic point was detected by both DSC and Raman spectroscopy in a mixture of the corresponding PCs [13,14].

In contrast to the results for the fluid-phase mixtures, both the phase diagram and our cross-linking results suggest gel-phase immiscibility of the two lipids. The horizontal solidus line starting at 0% C(14:0)C(14:0)PE suggests that, in an equimolar mixture of the two lipids below the line at 0°C, each phase is composed of a single lipid. Interestingly, the C(18:0)C(10:0)PE in separate vesicles was about three times more reactive with dimethylsuberimidate than C(14:0)C(14:0)PE, consistent with the explanation that these lipids assume different structures in the gel phase. We have previously reported that symmetric gel-phase PEs are about four times less reactive than fluid-phase PEs, while all fluid-phase PEs react similarly with dimethylsuberimidate [20]. We hypothesized that the lower reactivity of the gel phase was due to the excessive length of dimethylsuberimidate (about 1.1 nm) as compared to the average distance between PEs in the gel phase (about 0.7 nm). This explanation would concur with the present data as mixed interdigitated gel phases are associated with a 1.5-fold increased area per head group as compared to non-interdigitated gel phases [7,9]. This corresponds to about a 1.2-fold increase in inter-head group distance.

Assuming that the two lipids coexist in separate phases in the same membrane, the cross-linking data can be used to estimate the sizes of the clusters of like lipids [31]. Theoretically, such information also can be obtained from an analysis of the deviations from ideality of the phase diagram [4,5,31], and recently information on cluster shape and size has been obtained using fluorescence recovery after photobleaching [32]. Uncertainty arises in our analysis since the relative reactivity of dimethylsuberimidate with heterogeneous nearest neighbor pairs as compared to each of the like pairs is not known. It may be most likely that the reactivity is similar to the reactivity of the C(14:0)C(14:0)PE homodimers, since the heterogeneous nearest neighbor pairs are probably not interdigitated. In that case, the amount of gel-phase immiscibility would be similar to that predicted for an equimolar C(16:0)C(16:0)PC/C(18:0)C(18:0)PC mixture (34% heterogeneous nearest neighbors) and the average cluster size would be about 31 molecules [4,31]. The present phase diagram does appear to be considerably less ideal than the C(16:0)C(16:0)PC/C(18:0)C(18:0)PC mixture, however [4]. The C(14:0)C(14:0)PC/C(18:0)C(18:0)PC gel-phase mixture is also less ideal; in this case, about 23% heterogeneous nearest neighbors are predicted, corresponding to an average cluster size of 39 molecules [4,31]. This would correspond to a situation in which, for the C(18:0)C(10:0)PE/C(14:0)C(14:0)PE mixture, the reactivity of the heterogeneous nearest neighbor pairs was intermediate between the homogeneous nearest neighbor pairs.

In summary, we find no evidence for immiscibility in fluid-phase mixtures of a highly asymmetric lipid, C(18:0)C(10:0)PE, and the symmetric lipid of the same molecular weight. These lipids are, however, non-randomly arranged in the gel phase, as demonstrated both by determination of the phase diagram by steady-state fluorescence anisotropy of parinaric acids

and by dimethylsuberimidate cross-linking. Importantly, these results also demonstrate the ability of the cross-linking technique to detect non-random PE arrangements when such arrangements exist.

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