Structural Effects of Neutral Lipids on Divalent Cation-Induced Interactions of Phosphatidylserine-Containing Bilayers

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ABSTRACT Ca^{2+} is known to induce the adhesion and collapse of phosphatidylserine (PS) bilayers into dehydrated multilamellar structures. The aim of this study was to examine how that interaction and the resultant structures might be modified by neutral lipid species. A combination of rapid mixing, x-ray diffraction, thin-layer chromatography, density gradient centrifugation, and freeze-fracture electron microscopy was used in conjunction with osmotic stress techniques to characterize the structures formed by the Ca^{2+}-induced interaction of multilamellar liposomes and of large unilamellar vesicles. The results showed that dioleoylphosphatidylcholine and dioleoylphosphatidylethanolamine at concentrations of up to ~30 mol % are accommodated in a single dehydrated multilamellar structure. Similar results were obtained using mixed PS species isolated from bovine brain. Principally, the data indicate that neutral lipid is both dehydrated during the rapid collapse process of $Ca(PS)_2$ formation and accommodated within this dehydrated structure. The large energies available on formation of the $Ca(PS)_2$ bilayers contribute to the dehydration of neighboring neutral lipids that likely form continuous bilayers with them. Higher concentrations of these neutral lipids modify Ca^{2+}-induced bilayer interactions, leading to progressively weaker interactions, larger bilayer separations, and in some cases separation into two structures; phosphatidylethanolamine species favoring nonbilayer structures tended to promote such separation at lower concentrations than bilayer lipids.

INTRODUCTION

Traditionally, the nonspecific physical forces determining interactions between all large molecules, molecular aggregates, and surfaces in aqueous media were accounted for by the combination of electrostatic and van der Waals forces. The requirement for an additional force became evident when neutral surfaces were observed to take up water against van der Waals attraction (LeNeveu et al., 1976). This strong, exponential repulsive hydration force now appears to be universal and dominates interactions between all large hydrophilic surfaces in water within 10 to 20 Å separation (Leikin et al., 1993).

There are means of overcoming hydration repulsion. The addition of divalent cations to acidic phospholipids, in the form of either fully hydrated multilayers or unilamellar vesicles, triggers their collapse into dehydrated multilamellar arrays. The acidic phospholipid phosphatidylserine (PS) has been the object of a variety of studies investigating its physical properties in the presence and absence of various divalent cations. Micromolar concentrations of Ca^{2+} are sufficient to produce interactions between PS bilayers leading to collapse and formation of a dehydrated $Ca(PS)_2$ multilamellar complex, stable to temperatures well over 100°C (Hauser and Shipley, 1984, 1985; Newton et al., 1978; Portis et al., 1979). Particularly noteworthy is the elegant work of Feigenson (1989), which proves that PS:Ca^{2+} is the 2:1 binding ratio in the dehydrated complex.

Do PS bilayers mixed with neutral lipids, which themselves bind very little Ca^{2+} and are subject to hydration repulsion, also collapse together upon addition of Ca^{2+}? There have been numerous studies to characterize the behavior of mixed PS/neutral phospholipid systems, both with and without Ca^{2+}, using a variety of physical techniques (see Prestegard and O'Brien (1987) for a review). These studies suggest that Ca^{2+} induces a segregation of acidic phospholipids from neutral ones. This is indicated by (1) the appearance of individual endothermic peaks in differential scanning calorimetry thermograms (Hui et al., 1983; Papahadjopoulos et al., 1974; Silvius and Gagne, 1984a, b); (2) changes in the motional behavior of probe molecules (Florin and Feigenson, 1987) or self-quenching of fluorescence (Hoekstra, 1982a, b); and (3) shifts in ^{31}P NMR signal shapes accompanied by decreases in signal intensity (Hui et al., 1983; Tilcock et al., 1984, 1988). The studies by Silvius and Gagne (1984a, b) indicate (1) that Ca^{2+}-induced lipid segregation occurs predominantly at PS contents of ~40 mol % and ~15–20 mol % in PS/phosphatidylcholine (PC) and PS/phosphatidylethanolamine (PE) systems respectively; and (2) that PC and PE will be accommodated in $Ca(PS)_2$ at ~10–40 mol % and ~20 mol %, respectively. The work of Florine and Feigenson (1987) on DOPS/DOPC multilayers using fluorescent and spin-label probes indicates that Ca^{2+} induces thermodynamic phase separation into a rigid PS-rich phase and a PC-rich fluid phase. In similar systems, PE seems to favor the fluid as well as the interfacial regions between $Ca(PS)_2$ gel and PS/PE fluid phase domains (Florin-Casteel and Feigenson, 1988).

Lipid segregation or separation in most of these studies is interpreted in terms of lipid segregation within continuous bilayers, often without good evidence for the latter. The

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possibility of lipid segregation into discrete unconnected phases, frequently and unambiguously observed by using x-ray diffraction, is difficult to rule out. The term "phase" is used both in the thermodynamic sense, as in the observations of Feigenson (1989), and in the structural sense, as in the x-ray diffraction observation of a single multilamellar structure. In many cases, the structural phases are considered to be in thermodynamic equilibrium. However, Fig. 7 illustrates the essence of one question this study attempts to address, the possibility of lipid segregation within a single multilamellar structure. In this paper, then, we have used the terms "phase" and "phase separation" to describe thermodynamically defined states, and "structures" in referring to structural states defined by x-ray diffraction and other measurements employed in this study.

We have addressed the question, then, of what happens in mixed PS/PC and PS/PE bilayers when the Ca^{2+}-induced attraction involving formation of $Ca(PS)_2$ competes with the hydration repulsion of the neutral lipid. DOPC and DOPS mixtures were used initially to (1) maintain homogeneity in the hydrocarbon region; (2) minimize the likelihood of lipid demixing; and (3) add a phospholipid with a high degree of polar group hydration.

The affinity for Ca^{2+} of interacting bilayer surfaces that contain acidic lipids is much higher than for similar, isolated surfaces. This affinity difference has been translated into the adhesion energy that is available as the surfaces collapse together (Parsegian and Rand, 1983). For PS bilayers, that adhesion energy is on the order of 100 ergs/cm^2. On the other hand, we have also measured the energy required to dehydrate neutral phospholipid bilayers. It is on the order of 10–100 ergs/cm^2 for PE and PC bilayers, respectively; PS itself appears to have a lower value, more like PE than PC in that respect. It would thus appear that energy released on the formation of $Ca(PS)_2$ could be of a magnitude sufficient to dehydrate surrounding neutral lipids. Conversely, the presence of enough neutral lipid might modify or preclude the collapse or dehydration of such mixtures.

We have used x-ray diffraction, freeze-fracture electron microscopy (EM), density gradient centrifugation, and osmotic stress to analyze the final equilibrium structures formed when PS/PC unilamellar vesicles are triggered to interact by the addition of $CaCl_2$. Under these conditions and depending on PC content, large vesicles undergo very rapid and complex structural rearrangements including adhesion, rupture, fusion, and the formation of collapsed multilamellar phases (Kachar et al., 1986). It is usually only the very early events and the final equilibrium structures that can be analyzed with confidence. It is these final structures that are investigated here to determine the relative disposition of the neutral and $Ca(PS)_2$ lipid molecules.

MATERIALS AND METHODS

Dioleoylphosphatidylserine (DOPS), bovine brain phosphatidylserine (BBPS), dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylethanolamine (DOPE) and 1-palmitoyl-2-oleoylphosphatidylethanolamine (POPE) were purchased from Avanti (Birmingham, AL). 1,2-Diacylglycerol (DG) derived from egg PC was obtained from Serdary (London, Ont., Canada). Polyethylene glycol (PEG) and *N*-tris[hydroxymethyl] methyl-2-aminoethane sulfonic acid (TES) were from Sigma Chemical Co. (St. Louis, MO), and Dextran T2000 was from Pharmacia (Uppsala, Sweden). Sucrose was purchased from Calbiochem (La Jolla, CA). Unipore polycarbonate membranes (1.0 μm) were from Bio-Rad (Richmond, CA). All water used was double-distilled in glass. Salts were all of highest analytical grade.

All lipids were confirmed to be >99% pure by thin layer chromatography and were stored under nitrogen at −20°C. To eliminate divalent contaminants, DOPS and BBPS stocks were converted to their sodium salts. In most cases, the lipid composition of samples was checked by thin layer chromatography, both before further processing (see below) and after the final x-ray analysis to confirm the integrity of the lipid. All procedures were conducted at 23°C, unless otherwise indicated.

Sample preparation

Multilamellar systems

Defined lipid mixtures and gravimetric samples were produced as previously described (Coorssen and Rand, 1990), hydrated with 2 mM TES buffer (pH 7.3), and equilibrated for 3 days in the dark. Larger samples were hydrated overnight in 30-ml polycarbonate centrifuge tubes. Appropriate volumes of divalent cation stock solutions in TES buffer were then added to give a final divalent cation to lipid ratio of 10. After a specified equilibration period (1–2 or 12 h), samples were centrifuged at 100,000 × *g* for 1 h. Lipid pellets were recovered, combined with some powdered Teflon (an x-ray calibration standard), sealed in sample holders between mica windows 1 mm apart, and analyzed by x-ray diffraction as previously described (Coorssen and Rand, 1990). All samples yielded sharp x-ray diffraction patterns to 3 or 4 lattice orders unless otherwise described; repeated measurements of the films indicated a measuring error of ±0.5 Å for normal, sharp reflections and up to ±1.0 Å for broader reflections.

Unilamellar vesicle systems

Samples of defined lipid mixture were established as described above and from these large unilamellar vesicles were produced by the reverse-phase evaporation (REV) method of Szoka and Papahadjopoulos (1978). Briefly, lipid samples were dissolved in freshly redistilled diethyl ether, TES buffer was added to give a 3:1 organic/aqueous phase ratio, the sample was briefly sonicated to produce an emulsion, and most of the ether was then removed by rotary evaporation. Additional TES buffer was added, the suspension dispersed by vortexing, and traces of remaining solvent removed by rotary evaporation under high vacuum for ~15–20 min. Vesicles were sized by extrusion through 1.0-μm polycarbonate membranes, and these suspensions were collected in 30-ml polycarbonate centrifuge tubes. Samples were then exposed to excess calcium, recovered by centrifugation, and mounted for analysis by x-ray diffraction as described above. In some experiments an additional sonication step was added after the incubation with calcium in an attempt to liberate any bulk segregated neutral lipid that might have been trapped within the phase formed by Ca^{2+} and PS.

Additional analytical techniques

Osmotic stress was used to verify the presence of any disordered, bulk segregated lipid that could not be characterized by x-ray diffraction alone, and to effect any dehydration that might be possible in a given sample. The degree of lipid hydration was modified or regulated by equilibrating the samples with water of known chemical potential as determined by the osmotic pressure of a PEG or dextran solution, or by the vapor pressure generated by a saturated salt solution (Rand, 1981; Parsegian et al., 1986). For simplicity, both are referred to here as osmotic stress.

For lower pressures (0–10^7 dyn/cm^2) PEG and dextran solutions of known concentration were prepared using Ca^{2+}-TES stock solutions identical to those in which the respective samples had been incubated. Within

the x-ray sample holders the lipid was separated from such polymer solutions by a dialysis membrane and left to equilibrate in the dark for 3 days. Polymer solutions were changed twice daily to ensure that no hydrostatic pressure differences built-up across the membrane. The final equilibrated polymer concentrations were measured to within 0.2% using an Abbe refractometer and the corresponding osmotic pressures obtained from established standard curves (see, e.g., Parsegian et al. (1986)). Samples were again sealed in their holders, and x-ray diffraction used to analyze the resulting structure(s). Low pressures ($\log P = \sim 6$–7 dyn/cm^2) produced a mild dehydration and were used to collapse and order amorphous lipid structures. (We refer to lipid samples that produce no coherent x-ray diffraction as "amorphous"; EM showed that such samples usually contain aggregated unilamellar vesicles). Higher pressures ($10^{8.2}$ to $10^{9.2}$ dyn/cm^2) were used to further characterize ordered phases. The lipid samples were removed from the holder and equilibrated for 3 days with a known vapor above a specific saturated salt solution, and resealed for x-ray diffraction. Vacuum desiccation for 2 h was used as the strongest dehydration protocol.

A rapid-mixing, spray-freezing, freeze-fracture procedure (Kachar et al., 1986) for sample preparation was used to more fully characterize the structures found in the lipid-Ca^{2+} mixtures by EM. Using a Berger ball mixer (Commonwealth Technology, Alexandria, VA), vesicle suspensions were rapidly mixed with equal volumes of Ca^{2+} stock solution to a final Ca^{2+}/lipid ratio of 10. The mixture was equilibrated in the dark at room temperature for 1 h before rapid spray-freezing using standard Balzers equipment (Balzers, Hudson, NH). The freeze-fracturing was done on Balzers 400T equipment; cleaving, quartz-crystal monitored shadowing (45°C) and replicating were carried out at 5×10^{-8} mbar and −150°C. Samples were viewed using a Philips EM 300 electron microscope (Philips, Eindhoven, Holland).

To better characterize the structures, continuous sucrose density gradients were used (Coorssen and Rand, 1988). Sucrose solutions were prepared using Ca^{2+}-TES stock solutions identical to those in which the respective samples had been incubated. After incubation with Ca^{2+} and recovery by centrifugation, sample pellets (or portions thereof) were quickly resuspended in <1 ml of Ca^{2+}-TES, and this fine suspension was applied to the top of the gradient. All samples were centrifuged at 17,000 × g for 1 h. Separate bands recovered from the gradient were washed twice in Ca^{2+}-TES at 100,000 × g for 1 h before analysis by x-ray diffraction.

RESULTS

Ca^{2+}-induced structures formed by DOPS in the absence and presence of neutral lipid

DOPS multilayer systems

Initial experiments were done to characterize the structures formed by DOPS when exposed to solutions containing a large molar excess of Ca^{2+} or other divalent cation. Na-DOPS, hydrated gravimetrically to ~30 wt % lipid in TES buffer, yielded a single multilamellar (L) structure with a repeat spacing d of 137 Å. Subsequent vacuum desiccation resulted in the formation of two separate L structures of 44.5 and 53.1 Å. Na-DOPS exposed to Ca^{2+} yielded a single L structure of 51.4 Å that produced a broad band centered on 4.5 Å, and a weak but distinct high-angle line at 4.2 Å indicative of gel acyl chain ordering. Feigenson (1986) has shown that the composition of this collapsed L structure is a $Ca(DOPS)_2$ phase. The weak high-angle line indicated that the $Ca(DOPS)_2$ lamellar phase had a significantly smaller amount of hydrocarbon chain order in comparison with that of the $Ca(PS)_2$ phase formed by other PS species (Loosley- Millman, 1980; Newton et al., 1978; Portis et al., 1979; Tilcock et al., 1988).

No degree of dehydration had any effect on the 51.4 Å dimension of this collapsed phase. All samples yielded identical sharp lamellar diffraction patterns even after vacuum desiccation, suggesting that the $Ca(DOPS)_2$ contained no removable water. DOPS suspended in buffer for 1 week before Ca^{2+} addition also yielded this characteristic collapsed L phase, as did fully hydrated DOPS exposed to a 1.0 M Ca^{2+} solution (Ca^{2+}-to-lipid ratio of 1000). This collapsed phase was insensitive to temperatures up to at least 90°C and could not be rehydrated by prolonged incubation in TES buffer.

As summarized in Table 1, the combination of spacing, chain order, and removable water was also found to be unique to Ca^{2+}, consistent with findings involving other PS species (Hauser and Shipley, 1984, 1985). The structures formed by DOPS with Mg^{2+} or Ba^{2+} decreased in dimension with dehydration, i.e., had removable water. The structure formed with Mn^{2+}, although of larger repeat spacing than $Ca(DOPS)_2$, did not.

DOPS/DOPC multilayer systems

Mixtures over a wide range of mole ratios were used to characterize the behavior of fully hydrated DOPS/DOPC multilamellar systems exposed to excess Ca^{2+} in solution for 1–2 h. Their final structure and x-ray dimension are shown in Fig. 1. A single L structure of dimension identical to that of the collapsed $Ca(DOPS)_2$ phase was formed at DOPC contents of 9–50 mol %. At 33 mol % DOPC, the first- and second-order reflections were slightly broadened and all reflections from the equimolar sample were quite broad. A single 56.8 Å L structure with two orders of broad reflections was identified at 75 mol % DOPC; central scatter suggested that much of the lipid in these samples was disordered.

Mild osmotic stress had no effect on the lamellar spacing of the samples containing up to 33 mol % DOPC (Fig. 1). However, it produced two L structures in the equimolar PS/PC sample, one corresponding to the collapsed complex and the other, with repeat spacing of 60.3 Å, corresponding closely to pure PC (Lis et al., 1981; this paper). This raised the possibility that osmotic stress either ordered previously amorphous lipid into a second lamellar structure, or itself caused lipid segregation. We interpret these initial results to indicate that up to ~30 mol % DOPC was accommodated in a dehydration-insensitive multilamellar structure. Higher levels of DOPC-modified Ca^{2+}-induced bilayer interactions resulting in more disordered L structures, increased lattice

TABLE 1 X-ray repeat spacings of the multilamellar structures formed by divalent-cation-induced interaction of DOPS bilayers, and their change under osmotic stress

Divalent cation	Osmotic stress (log P-dyn/cm^2)	Spacing (Å)	Comment
Ca	0	51.5	Frozen chains
	8.49	51.3	Frozen chains
	Vacuum	51.5	Frozen chains
Mg	0	50.9	Disordered chains
	Vacuum	49.7	
Ba	0	49.2	
	8.87	48.1	
Mn	0	52.5	Frozen chains
	8.49	52.6	Frozen chains

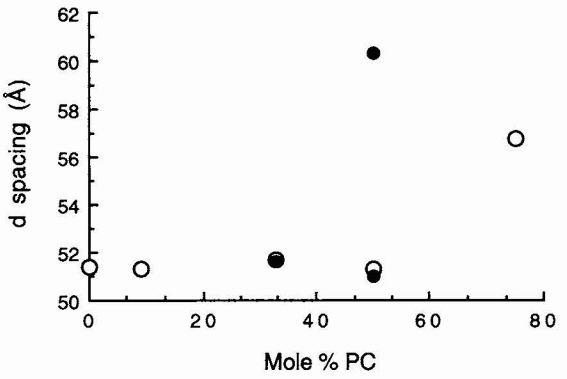

FIGURE 1 Multilamellar *d* spacings of fully hydrated DOPS/DOPC multilamellar systems after 2 h incubation in excess Ca^{2+} buffer (Ca^{2+}/phospholipid = 10). The samples of 33 and 50 mol % PC were subsequently subjected to an osmotic stress of log P = 6.84 dyn/cm^2 and reanalyzed. The 50 mol % sample separated into two discrete multilamellar structures, indicated by the identical symbols at the same PC content.

dimensions at PC contents above ~50 mol %, and led to the presence of amorphous lipid structures.

DOPS/DOPC unilamellar vesicles

To eliminate the possibility that the multilamellar structures might limit access of Ca^{2+} to a portion of the lipid, large unilamellar vesicles (REV) were incubated for 12 h in the $CaCl_2$ solutions. These samples yielded x-ray data identical to that of the multilamellar systems, with the exception that higher DOPC contents (83 mol %) produced only central scattering (described below). Identical results were also found when DOPS/DOPC REV samples were processed using the rapid-mixing protocol to initiate the lipid-Ca^{2+} interaction and thus further ensure full access of cation to the lipid.

Aliquots of the rapid-mixed samples were examined by freeze-fracture EM to characterize the lipid structures responsible for the central scatter and broadened x-ray reflections observed at higher (>50 mol %) PC contents (Fig. 2). Dispersions of separate, unilamellar vesicles of approximately uniform size were seen by EM in all samples before the addition of Ca^{2+} (Fig. 2 *a*). After rapid mixing and 1-h incubations with Ca^{2+}, multilamellar structures as determined with x-ray diffraction were seen at 0, 33, and 50 mol % DOPC, with some coexisting aggregates of uncollapsed vesicles most clearly evident in the 50-mol % samples (Fig. 2, *b–d*). At higher DOPC contents (83 mol %), where x-ray diffraction yielded only central scatter, the micrographs showed only aggregated vesicles.

After mild osmotic stress (log P = 6.84 dyn/cm^2), there was no change in the diffraction patterns obtained from samples containing 0–50 mol % DOPC. There was some indication of two structures in the 83 mol % DOPC sample, although only a first-order reflection for each could be seen; central scatter indicated that much of the lipid was still contained in the nonordered structures.

Exposure of REV containing 33 mol % DOPC to 500 mM Ca^{2+} yielded x-ray diffraction data that were quantitatively and qualitatively identical to that of the sample exposed to the ~50-fold lower Ca^{2+} concentration normally used. In addition, strong osmotic stress revealed no other structures. These results confirm that up to ~30 mol % DOPC is present in the Ca-induced multilamellar structure.

Density gradient centrifugation

To further characterize the Ca^{2+}-induced structures, two other protocols were adopted. First, in conjunction with x-ray diffraction and thin-layer chromatography, density gradient centrifugation was used to separate structures (Coorssen and Rand, 1988) and measure their relative density. Second, each Ca^{2+}-precipitated sample was briefly sonicated and recovered by centrifugation to separate any segregated lipid structures before density gradient centrifugation. Control samples confirmed that sonication itself did not disrupt the $Ca(DOPS)_2$ complex, as characterized by x-ray diffraction.

Ca^{2+}-induced interactions of REV composed of all lipid mixtures yielded single bands in the density gradients, with sample densities proportional to PC content (Fig. 3, *a* and *b*) and with no indications of lipid segregation. The combined density gradient data from three separate experiments showed identical, direct (correlation coefficient of 0.99) relationships between sample density and mol % PC (Fig. 4). Pooling Ca^{2+}-precipitated aliquots from samples containing 0, 17, and 33 mol % PC, showed that these separated on a sucrose gradient according to their PC content, even though x-ray analysis showed each to be a collapsed 51.4-Å L structure.

To confirm that separation could be effected, an equimolar sample of DOPS/DOPC was subjected to freeze-thaw cycling, which has previously been shown to induce pronounced lipid segregation in similar PS/PC systems (Feigenson, 1989). Subsequent sonication and density gradient centrifugation yielded two physically separated structures, one of density corresponding to the collapsed phase and another of lower density. X-ray analysis confirmed that the denser band was the collapsed multilamellar structure and that the other was of a larger lattice dimension (Fig. 3 *a*).

X-ray diffraction of samples recovered from the density gradients revealed the following: (1) a single L structure of 51.4 Å, with ordered acyl chains, at DOPC contents up to 17 mol %; (2) an identical L phase with broader reflections at DOPC contents of 33 and 50 mol % (no indication of chain ordering) but with central scatter indicative of some disordered lipid in the equimolar samples; and broad reflections indicating a single L structure between 75 and 100 mol % DOPC, the dimension of which increased with the increasing PC levels, up to ~59 Å for pure DOPC. Central scatter indicated that a considerable portion of the lipid was disordered.

Osmotic stress (log P = ~6.86 dyn/cm^2) of these samples confirmed that the 51.4 Å structure was dehydration-insensitive up to PC contents of 33 mol % and eliminated central scatter from the equimolar sample, yielding segregated L structures of 51.5 and 57.6 Å. The pure DOPC

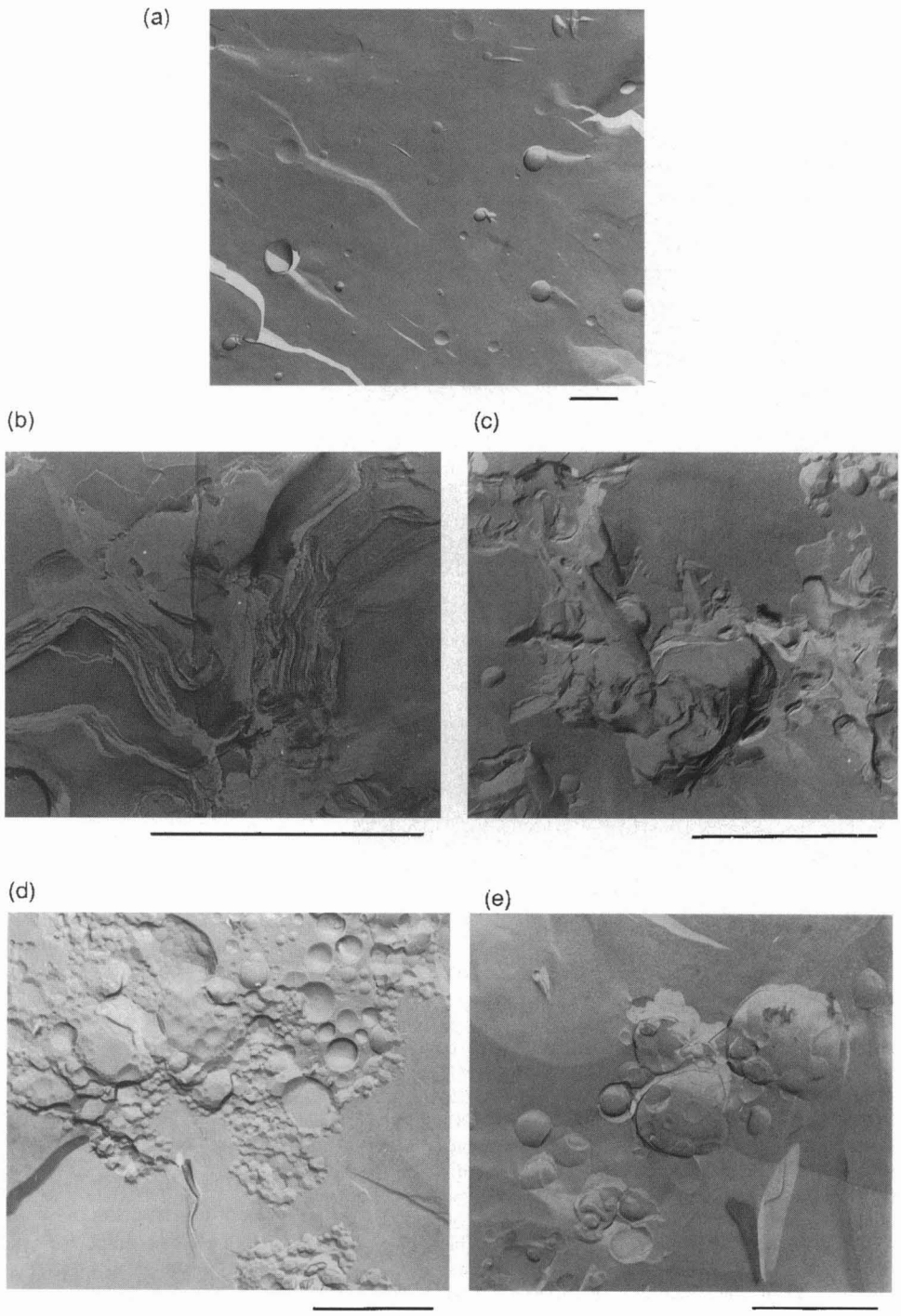

FIGURE 2 Rapid mixing, spray-freezing, freeze-fracture EM. Electron micrographs of DOPS/DOPC REV suspensions before (*a*) and after (*b–e*) ~1 h in Ca^{2+} solution (Ca^{2+}/phospholipid = 10). The bar represents ~1000 nm. (*a*) Uniform REV dispersion seen in all samples before Ca^{2+} incubation. (*b*) DOPS: x-ray diffraction showed a single 51.6 Å lamellar structure with sharp reflections. (*c*) DOPS/DOPC (33 mol % PC): x-ray diffraction, 51.5 Å lamellar structure. (*d*) DOPS/DOPC (50 mol % PC): x-ray diffraction, 51.5 Å lamellar phase broadened reflections and some central scatter (*e*) DOPS/DOPC (83 mol % PC): x-ray diffraction yielded only central scatter.

samples yielded single, 56.6 Å L structures after osmotic stress. Samples of pure DOPC REV in Ca^{2+}, which had not been exposed to the sucrose gradient, yielded similar data, reflections of a 60.3 Å L structure, with central scatter, and a 55.0 Å L structure after gentle osmotic stress, with some central scatter remaining. The similarity in the behavior of this DOPC sample and those exposed to the sucrose gradient indicated that the collapse of pure DOPC REV was not an artifact resulting from the use of a sucrose gradient, although sucrose certainly provides some osmotic stress (log P = ~6–7 dyn/cm^2 through the gradients used). Although slight, this additional stress probably accounts for the more complete collapse of these systems by osmotic stress, whereas the control sample still showed some central scattering. The added osmotic stress of the sucrose is likely to have promoted the lipid segregation into two L structures seen in the equimolar DOPS/DOPC sample as well, given that such segregation was not normally seen in similar preparations subjected only to mild osmotic stress (see above).

The results obtained with REV are similar to those from the multilamellar systems. All the data concerning the multilamellar dimensions have been plotted in Fig. 5. From this

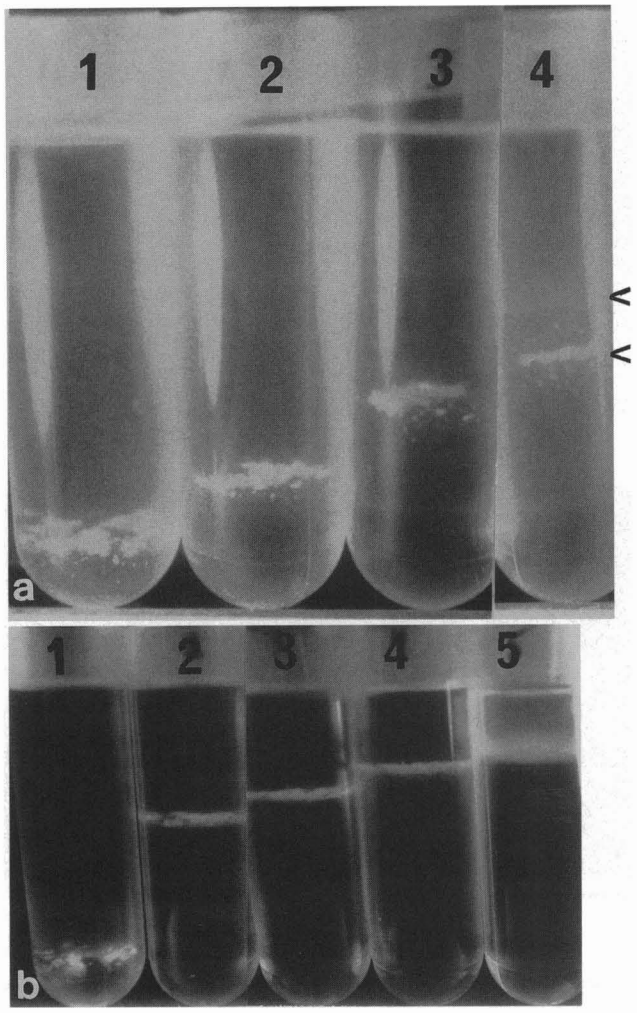

FIGURE 3 (*a*) The effects of excess Ca^{2+} on DOPS/DOPC REV systems of low to equimolar PC content; separation on a continuous density gradient of 1 to 22 wt. % sucrose. Multilamellar repeat spacings from sharp x-ray diffraction reflections of these samples are given. Except for 50 mol %, frozen hydrocarbon chains were detected. Gradient #1: DOPS, d = 51.5 Å; gradient #2: DOPS/DOPC (17 mol % PC), d = 51.4 Å; gradient #3: DOPS/DOPC (33 mol % PC), 51.5 Å; gradient #4: DOPS/DOPC (50 mol % PC), freeze-thaw-induced separation into two lamellar structures (arrows), each shown by diffraction to be of dimensions d = 51.8 and 53.7 Å. (*b*) The effects of excess Ca^{2+} on DOPS/DOPC REV systems of equimolar to full PC content; separation on a continuous density gradient of 0 to 11 wt.% sucrose. Multilamellar repeat spacing from the broadened x-ray diffraction reflections of these samples are given, and each sample also showed central x-ray scattering. Gradient 1: DOPS/DOPC (50 mol % PC), d = 51.5 Å; gradient 2: DOPS/DOPC (75 mol % PC), d = 55.9 Å; gradient 3: DOPS/DOPC (83 mol % PC), d = 56.3 Å; gradient 4: DOPS/DOPC (89 mol % PC), d = 59.3 Å; gradient 5: DOPC, d = 59.3 Å.

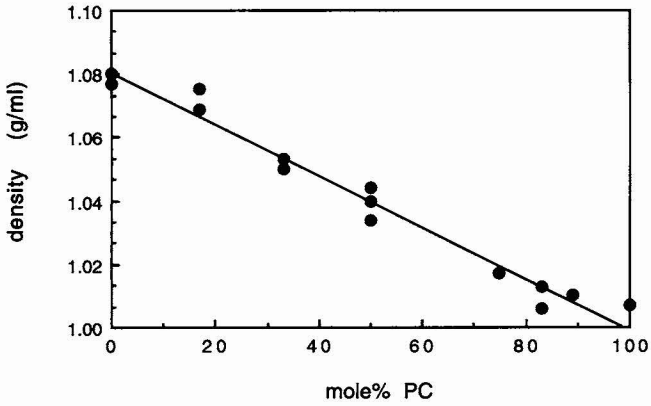

FIGURE 4 The relationship between DOPS/DOPC sample density and mol % PC content after incubations in excess Ca^{2+} (three separate experiments). Line of best fit has a correlation coefficient (R) of 0.99.

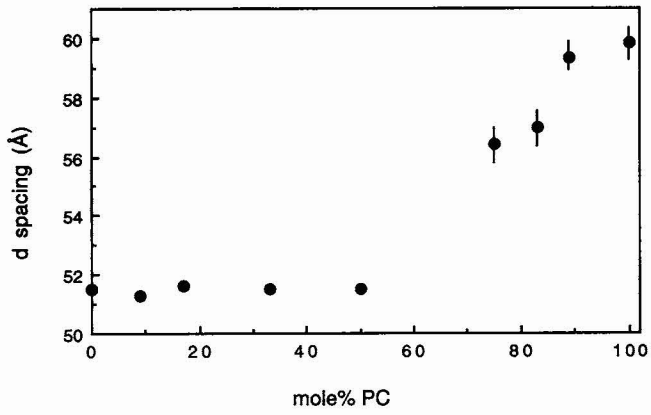

FIGURE 5 The effect of mol % PC content on multilamellar repeat spacing after exposures of fully hydrated DOPS/DOPC systems to excess Ca^{2+}. d spacings are averages from all multilayer and REV experiments with their full range shown where it exceeds the size of the symbol.

compilation we conclude that (1) DOPC at levels up to ~50 mol % is accommodated in a collapsed 51.4 Å lamellar structure that becomes progressively disordered with increasing neutral lipid, and coexists with uncollapsed vesicles at levels of DOPC between 30 and 50 mol %; (2) the lamellar repeat spacing of the Ca^{2+}-induced L structure progressively increases at DOPC levels above 50 mol %; (3) at ~50 mol % DOPC, the lipid has a tendency, under osmotic stress, to segregate into a collapsed L structure and a largely DOPS-free L structure.

Other divalent cations and neutral lipids

DOPS/DOPC unilamellar vesicles exposed to Mg^{2+}

To test the specificity of Ca^{2+} on the interaction structures, similar experiments were done using Mg^{2+}. This cation did not cause collapse of systems containing either 33 or 50 mol % DOPC. Application of osmotic stress (log P = 8.06 dyn/cm^2) yielded sharp reflections of a single 50.5 Å L structure similar to that of $Mg(DOPS)_2$ itself (Table 1), in both the 33 and 50 mol % PC samples, and showed no indications of phase segregation or central scatter.

BBPS/DOPC REV exposed to Ca^{2+}

To determine whether the limits of accommodation of PC in a collapsed $Ca(PS)_2$ phase were specific to DOPS, we tested PS extracted from bovine brain (BBPS). As shown in Table 2, a dehydration-insensitive L structure of 51.5 Å, with well-ordered acyl chains, was formed at DOPC contents of up to at least 17 mol %. At 33 mol % DOPC, a single 52.5 Å L structure with well-ordered acyl chains and slightly broad-

TABLE 2 X-ray repeat spacings of the multilamellar structure formed by calcium-induced interaction of bilayers, and their change under osmotic stress

Mole % neutral lipid	osmotic stress (log P-dyn/cm^2)	BBPS/ DOPC spacing (Å)	DOPS/ POPE spacing (Å)	DOPS/ DOPE spacing (Å)
0	0	51.3*		
	8.06	51.1*		
17	0	51.7*		
	8.06	51.7*		
25	0		51.7*	51.8*
	8.06		51.7*	51.8*
33	0	52.5*	52.3*	51.4
	8.06	52.9*	52.3*	51.5*
50	0	52.4	52.2*	36.8 (H), 51.7
	8.06	49.8, 53.0*	52.5*	46.2 (H), 51.7
83	0	51.3	53.0*	
	8.06	51.2	53.0*	
91	0		lipid segregation	
	8.06		56.0, 47.7	
100	0	60.5	61.2*	

*Hydrocarbon chains in the gel state.

ened reflections was seen; this structure was stable under osmotic stress but strong desiccation resulted in structural segregation, although only a weak first-order reflection of the second structure could be seen. Equimolar BBPS/DOPC yielded broad reflections of a 52.5 Å L structure with central scatter; osmotic stress caused a segregation into two lamellar structures of 49.8 and 53.0 Å, with broad reflections and strong chain ordering. Broad reflections of a single 51.5 Å L structure with frozen chains remained after desiccation. BBPS with 83 mol % DOPC yielded broad reflections of a 51.3 Å L structure with no chain ordering; central scatter indicated that much of the lipid was still in uncollapsed structures. Osmotic stress resulted in a sharp, single L structure of 51.2 Å with no indication of chain ordering, suggesting DOPC-rich fluid bilayers.

Although more detailed studies will be required to fully characterize these systems, the similarity to the results obtained with DOPS confirm that (1) DOPC levels of up to ~30 mol % can accommodated in the collapsed structure; (2) intermediate concentrations of neutral lipid (~30–80 mol %) modify the Ca^{2+}-induced fusion/collapse of bilayer vesicles resulting in sensitivity to osmotically induced structural segregation; and (3) high levels of PC (>~80 mol %) result in a fluid lamellar structure that is largely insensitive to the Ca^{2+}-induced interactions that result in dehydration and collapse.

PE and the Ca(DOPS)$_2$ phase

PE was used to study the effect of its smaller headgroup, lower hydration and non-bilayer-forming tendencies (DOPE specifically) on the Ca^{2+}-induced collapse process. POPE alone forms a 54 Å lamellar structure in excess buffer. Ca^{2+}-free DOPS/POPE gravimetric control samples (25, 33, and 50 mol % POPE) form lamellar structures with indications of acyl chain ordering and diffraction spacings that increase indefinitely with increasing water content (data not shown). DOPE alone forms inverted hexagonal structures at temperatures above ~10°C, in both the presence and absence of Ca^{2+} (Shyamsunder et al., 1988; J. R. Coorssen and R. P. Rand, unpublished observations).

At 25–83 mol % POPE, DOPS/POPE REV that had been incubated with Ca^{2+} all formed single collapsed L structures with well-ordered hydrocarbon chains, and d spacings that did not change with strong osmotic stress (Table 2). Increasing POPE to 91 mol % still yielded acyl chain ordering but in the presence of complicated lipid segregation; strong osmotic stress resulted in segregation into a prominent 56.0 Å L structure and a faint second L structure of much smaller dimension. These results showed that a much larger amount of POPE than DOPC is accommodated in the Ca^{2+}-induced collapsed structure. This is consistent with the smaller size, degree of hydration, and repulsive force of this neutral lipid.

In DOPS/DOPE REV incubated with Ca^{2+}, samples containing either 25 or 33 mol % DOPE formed collapsed structures with faint indications of acyl chain ordering and d spacings that were insensitive to strong osmotic stress (Table 2). However, weak indications of a second, segregated structure were apparent in the 33 mol % DOPE sample after osmotic stress. At 50 mol % DOPE, there was segregation into the collapsed L structure and a second structure of dimension consistent with the hexagonal phase of pure DOPE. These results show that the non-bilayer-forming tendency of this lipid led to its segregation from the Ca^{2+}-induced collapsed L structure at much lower levels than either POPE or DOPC.

DISCUSSION

We have constructed two idealized schemes, Fig. 6, *a* and *b*, suggested by the results. The first shows the equilibrium structures and their dimensions, formed by large unilamellar vesicles of various PS/PC mixtures, after exposure to excess Ca^{2+}. The second shows changes that these structures undergo when dehydrated through osmotic stress. The results show that up to ~30 mol % DOPC is accommodated in the Ca^{2+}-precipitated L structure. Higher PC contents yield L structures with d spacings that increase as PC content increases beyond ~50 mol %, approaching the 60 Å limit characteristic of DOPC itself. These L structures of larger dimension coexist with aggregated vesicles, which have the same density and, we conclude, the same global composition. Osmotic stress of the structures only in the intermediate range of PC content resulted in separate multilamellar structures.

The collapsed Ca(DOPS)$_2$ phase

The x-ray studies showed that exposure of DOPS or BBPS vesicles to excess Ca^{2+} yields a lamellar phase of short spacing (51.4 Å), consistent with previous studies (Hauser and Shipley, 1984, 1985; Hauser et al., 1977; Loosley- Millman, 1980; Newton et al., 1978; Portis et al., 1979). As with BBPS, the Ca(DOPS)$_2$ phase was found to be free of removable

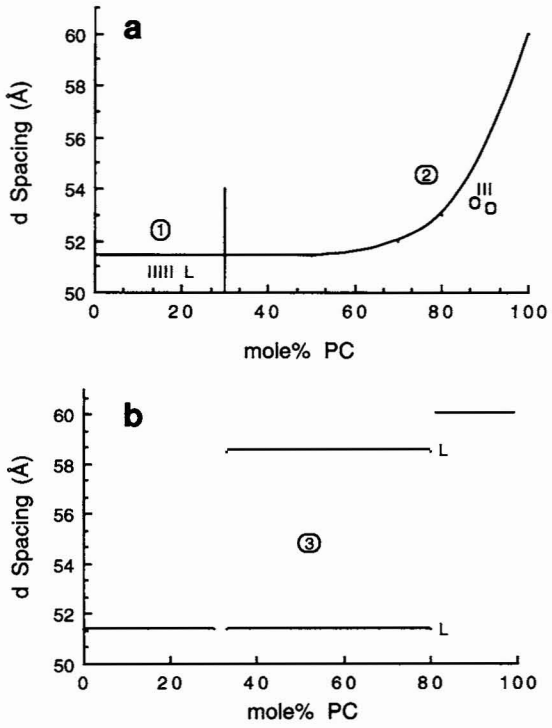

FIGURE 6 Idealized schemes showing the lamellar structures formed by DOPS/DOPC REV systems in excess Ca^{2+} (*a*) and the effects of osmotic stress on them (*b*). The global compositions of the lipid samples before Ca^{2+} precipitation are plotted against the dimensions of the resulting lamellar structures. (*a*) Region 1 represents the collapsed, 51.4 Å lamellar structure formed at PC contents of less than ~30 mol %. Region 2 represents an area of increasing multilamellar dimensions and coexisting partially collapsed and adhering vesicles. (*b*) Stable, single multilamellar regions exist at high and low PC contents, respectively. The dehydration-insensitive 51.4 Å structure is found at PC contents below ~30 mol %; a single liquid-crystalline lamellar structure, with a repeat spacing close to that of pure PC, exists at PC levels above ~80 mol %. The intervening region, 3, is susceptible to osmotically-induced separation into lamellar structures of 51.4 Å and one with higher repeat spacings.

water. The acyl chains of $Ca(BBPS)_2$ are practically all in the well-ordered gel state. However, in the 51.4 Å phase formed by Ca^{2+} and DOPS, far less of the hydrocarbon region is gel state.

Structural effects of exposing PS/PC-containing lipid bilayer vesicles to Ca^{2+} solutions

A variety of studies on PS/PC multilamellar systems exposed to excess Ca^{2+} (10 mM) have identified three distinct regions (Hui et al., 1983; Silvius and Gagne, 1984a; van Dijck et al., 1978). Similar to the findings of the present study, these authors suggest that, depending on the PS and PC species used, ~30–40 mol % PC can be accommodated in the Ca^{2+}-induced structure, and that ~30–40 mol % PS is accommodated in a single, PC-rich liquid-crystalline structure. Between 40 and 60% PC in PS, segregation into $Ca(PS)_2$ and PC-rich liquid-crystalline regions was observed. In contrast, our REV systems exhibited structural segregation only when osmotically stressed. Considering the short incubation times used by both Hui et al. (1983) and van Dijck et al. (1978), it is possible that their lipid segregation resulted from problems with Ca^{2+} equilibration.

Exposing DOPS/DOPC REV to excess Ca^{2+} results in the formation of a collapsed, 51.4 Å multilamellar structure when the PC levels are below ~30 mol %. Multilamellar structures of increasing dimensions (d = 51.4–60 Å, depending on PC content) coexist with amorphous structures form at PC levels of ~30–100 mol %. The amorphous structures consist of intact, aggregated uncollapsed vesicles, as observed by freeze-fracture EM. Since every DOPS/DOPC sample yielded only one band on the density gradients, with density proportional to the PC content, we conclude that the coexisting aggregated vesicles and multilamellar structures have the same global composition.

Lipid segregation

Studies using fluorescent and spin-label probes on PS/PC multilamellar vesicle systems, combined with measurements of PS and PC thermodynamic activity, show that in excess Ca^{2+} there is segregation of PC-rich liquid-crystalline and $Ca(PS)_2$ domains at any PS/PC ratio (Ohnishi and Ito, 1974; Feigenson, 1989; Florine and Feigenson, 1987; Swanson and Feigenson, 1990; Huang et al., 1993). The later studies have included fastidious protocols to ensure Ca^{2+} equilibration, and have measured the equilibrium aqueous calcium concentration for these phases, and provided a measure of the activity coefficients of the lipids in these mixtures. The essential results of these studies have led to a detailed description of phase separation into $Ca(PS)_2$ and fluid PC domains in these mixed lipid systems.

The essential result of our studies is that the lipid mixture is contained within one single multibilayer structure. Inasmuch as these structures were stable over a period of months, and unchanging in dimension, we believe they represent the equilibrium structures of similar phases observed by Feigenson (1989). We conclude therefore that $Ca(PS)_2$ and fluid PC domains coexist within one single multilamellar structure over a substantial range of PS/PC ratios. This combination of findings leads to the schematic picture shown in Fig. 7.

The sharp x-ray diffraction lines for DOPC contents up to 30 mol %, and the insensitivity of such samples to dehydration, show that both the water content and any variation in the repeat spacing is extremely small. This means that DOPC must become largely dehydrated during the process that results in the formation of the $Ca(PS)_2$ domains. That the DOPS and DOPC domains are of identical interbilayer dimension shows that DOPC in its domain is held dehydrated and cannot express its much larger interbilayer dimension. This strongly suggests that DOPS and DOPC bilayers are continuous, as drawn in Fig. 7. It further suggests that any mutual alignment of DOPS and DOPC domains between bilayers is not well developed, because large three-dimensional DOPC domains would be expected to take on the much

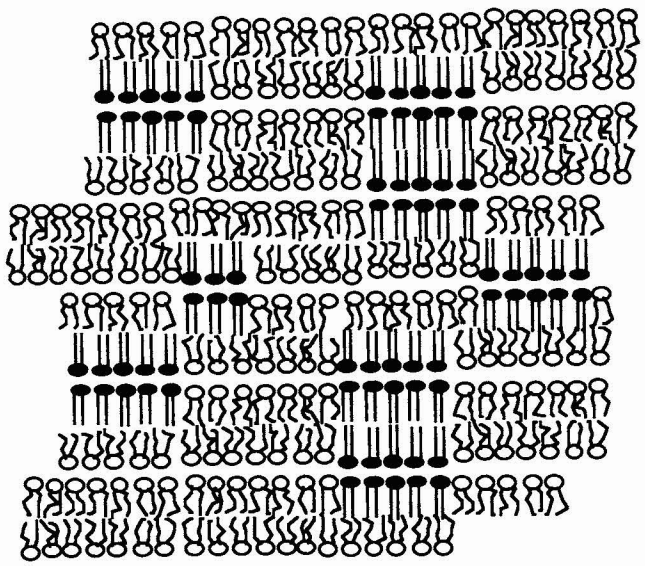

FIGURE 7 Schematic representation of the equilibrium structures formed when bilayer vesicles are induced to interact by the addition of excess $CaCl_2$. Lipids with filled polar heads represent PS that segregated from PC by calcium binding (Florine-Casteel and Feigenson, 1988) and their chains frozen. PC regions coexist with PS in continuous bilayers within the single multilayer, or in adhering uncollapsed vesicles. PC must be considerably dehydrated in the Ca-induced adhesion.

larger interbilayer dimension characteristic of DOPC multilayers. No such evidence of coalescence of domains is seen even some months after sample preparation.

Even at the higher DOPC contents of 30–50% (Fig. 5) the average lamellar spacing does not change. Only at DOPC contents above 50 mol % does the multilamellar spacing increase, indicative of separation of the bilayers. Apparently in this region the adhesion energy of the $Ca(PS)_2$ bilayers begins to be balanced by the repulsive hydration energy of the DOPC bilayers.

Energy of bilayer interactions

We have attempted to estimate the two opposing energies contributing to the formation and stability of these multilamellar structures, the adhesion energy available from the Ca^{2+}-DOPS reaction, and the energy required to dehydrate these surfaces in bringing them into close apposition.

The affinity for Ca^{2+} of interacting bilayer surfaces, K_m, is much higher than that for isolated surfaces, K_o, as measured by the dissociation constants for Ca^{2+}. Adhesion energy, G_a, is estimated from the relative affinities of the isolated bilayer surface and the interacting surfaces (Parsegian and Rand, 1983).

$$G_a = kT \ln K_m/K_0$$

K_0 has been measured to be on the order of 100 mM from electrophoresis of PS liposomes (McLaughlin et al., 1981). K_m has been measured by Feigenson (1989) for POPS; it is on the order of 1 μM and rather sensitive to the ionic strength. This gives an adhesion energy in the pure PS phase on the order of 65 ergs/cm^2. In the final adhered multilamellar phase, or even for adhered but uncollapsed vesicles, we assume the adhesion energy is dominated by contact between the segregated $Ca(PS)_2$ domains (pure PC adhesion energy is negligible in comparison, on the order of 0.01 ergs/cm^2 (Rand and Parsegian, 1989)). Then adhesion energy in the mixtures might be given by the proportional area of the $Ca(PS)_2$ domains.

We have measured the energy required to dehydrate phospholipid bilayers, G_h, and bring them into close apposition (Rand and Parsegian, 1989).

$$G_h = P_o \cdot \lambda \cdot (\exp(-d_w/\lambda) - \exp(-d_{wo}/\lambda))$$

For DOPC $P_o = 10^{10.6}$, $\lambda = 2.1$ Å, $d_{wo} = 28.1$ Å, and d_w is ~11 Å for the 51.4 Å collapsed phase. Repulsive energy, G_h, then is on the order of 4 ergs/cm^2.

G_a far exceeds G_h at higher contents of PS and becomes comparable to it only as the PC content increases to ~90%. Fig. 5 shows that above 50–75 mol % PC, the bilayers begin to separate as hydration repulsion moves the interaction energy minima out.

These crude estimates are consistent with the observations. However, a fuller analysis of the stability of such a structure as shown in Fig. 7 would have to take into account lateral interactions between lipids and the entropic effects of lipid segregation.

Other neutral lipids

Silvius and Gagne (1984b) characterized the behavior of PS/PE dispersions in excess Ca^{2+}. This work demonstrated that ~20 mol % PE would be accommodated in the Ca^{2+} structure, whereas 15–20 mol % PS would saturate the liquid-crystalline PE structure. Whether Ca^{2+}/PS microdomains exist at these low concentrations of PS in PE has not been established, although the work of Tokutomi et al. (1981) suggested that PE also undergoes lateral phase separation from $Ca(PS)_2$ domains. Our preliminary studies using either POPE or DOPE as the neutral lipid in these REV systems exposed to excess Ca^{2+} revealed behavior similar to that seen in the DOPS/DOPC system. At least ~25–30 mol % PE could be accommodated in a condensed lamellar structure.

Contrary to the DOPS/DOPC systems, at higher PE levels there was complete collapse of the DOPS/PE REV (i.e., no central scattering indicative of intact REV in these systems). Interestingly, Tokutomi et al. (1981) noted a higher degree of lipid separation for PE compared with PC and an order of magnitude lower Ca^{2+} threshold for phase separation in PS/PE compared to PS/PC membranes. This all suggests that the lower hydration repulsion expressed by PE enhances these activities of Ca^{2+}, although all those factors that contribute to the stability of these structures will eventually have to be assessed.

In equimolar DOPS/DOPE multilamellar systems 31P-NMR, freeze-fracture EM, and x-ray diffraction data indicate a Ca^{2+}-induced lipid segregation of DOPE into a hexagonal

and $Ca(DOPS)_2$ lamellar phase (Tilcock et al., 1984). Kachar et al. (1986) obtained similar x-ray and freeze-fracture data at a DOPE concentration of 50 mol %. However, systems using more unsaturated PS species did not undergo Ca^{2+}-induced lipid separation (Tilcock et al., 1984, 1988).

Noting the similarity in the results obtained in DOPS/DOPC multilamellar and REV systems, and in PS/PC and PS/PE systems we believe similar effects exist for DOPS/diacylglycerol multilayers (Coorssen, 1988). As a neutral lipid, diacylglycerol follows the general scheme of Fig. 5. Although isolated diacylglycerol does not form a recognizable lipid-water phase, it appears to be accommodated in a condensed multilamellar structure up to levels of ~30 mol %, and higher diacylglycerol contents yield single L structures of higher *d* spacing.

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