Structural effects of neutral lipids on the divalent cation-induced interactions of phosphatidylserine-containing bilayers

by

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ABSTRACT

As Ca^{2+} and phosphatidylserine (PS) are known to induce the adhesion of bilayer vesicles and form collapsed multibilayer structures *in vitro*, it was the aim of this study to examine how that interaction and the resultant structures might be modified by neutral lipid species. X-ray diffraction data from multilamellar systems suggest that phosphatidylcholine (PC) and diacylglycerol (DG) might be in the collapsed phase up to a concentration of ~30 mole % and that above this concentration these neutral lipids may modify Ca^{2+}-induced bilayer interactions. Using large unilamellar vesicles and long incubations in excess Ca^{2+} to ensure equilibration, similar preliminary results were again obtained with PC, and also with phosphatidylethanolamine (PE). A combination of X-ray diffraction, thin-layer chromatography, density gradient centrifugation and freeze-fracture electron microscopy, used in conjunction with an osmotic stress technique, showed that (i) ~30 mole % PC can be accomodated in the $Ca(DOPS)_2$ phase; and (ii) higher PC levels modify Ca^{2+}-induced bilayer interactions resulting in single lamellar phases of larger dimension and reduced tendency for REV collapse. Importantly, the data suggest that PC is dehydrated during the rapid collapse process leading to $Ca(DOPS)_2$ formation and exists with this dehydrated phase. Similar results were obtained using PS isolated from bovine brain. Preliminary studies using two different phosphatidylethanolamine (PE) species indicated accomodation by $Ca(DOPS)_2$ of ~25-30 mole % PE and bulk phase separation, of species favouring a non-bilayer phase, at higher levels. Significantly, all PS/PE vesicles appear to undergo a complete Ca^{2+}-induced collapse, even with contents of up to 90 mole % PE. These data suggest that PE may have an important role in fusion mechanisms *in vivo.* In sum the data lend both structural and stoichiometric evidence for the existence of laterally segregated neutral lipid molecules within the same bilayers as PS domains exposed to Ca^{2+}.

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exactly what they have given to me

—— and especially to Susie for being there and making all the goals so much more worthwhile ——

INTRODUCTION

The use of phospholipid model systems provides a rational, *in vitro* , reductionist approach to the study of the more complex membranes found *in vivo*. Such systems can be used to model specific domains within natural membranes allowing for the more systematic study of the structural organization of individual components as well as the structural effects of introducing other molecules into the domain or its surrounding medium. The information obtained from such studies can be used to gain an understanding of the structural organization, function, and behaviour of complex natural membranes (Das and Rand, 1986; Duzgunes et al., 1987b; Gruner et al., 1985).

Domains of negatively charged phospholipids are known to serve as binding sites for cations and for several proteins, including protein kinase C. This enzyme becomes membrane-bound and primed as a result of cooperative interactions with Ca^{2+} and phosphatidylserine (Bell, 1986; Snoek et al., 1986). However, to be fully activated this enzyme complex must bind with a molecule of diacylglycerol, a second-messenger product of phosphatidylinositol turnover during intracellular signal transduction (Berridge, 1987). As the local, transient presence of diacylglycerol is difficult to study directly the present research involved the use of artificial membrane systems. Interestingly, other model system studies have revealed that (i) Ca^{2+} and phosphatidylserine form a dehydrated, collapsed $Ca(PS)_2$ lamellar phase (Feigenson, 1986) that has been suggested to exclude other molecules (Feigenson, 1988; Florine and Feigenson, 1987a,b); and (ii) diacylglycerol is a potent perturber of bilayer structure (Coorssen and Rand, 1987; Das and Rand, 1984, 1986; Epand, 1985). Thus, by employing large unilamellar vesicles and prolonged exposures to solutions containing excess Ca^{2+}, it was the primary aim of this research to determine whether neutral lipids, even strongly hydrated phosphatidylcholine, could be in laterally segregated domains within $Ca(PS)_2$ bilayers without disrupting their structure, or whether the neutral lipid was actually bulk segregated to form a phase of its own. Silvius and Gagne (1984a,b) identified three distinct phase regions of Ca^{2+} - PS - neutral lipid

interactions. Using a combination of X-ray diffraction, thin-layer chromatography, density gradient centrifugation, and freeze-fracture electron microscopy, in conjunction with an osmotic stress technique, three phase regions were also identified in this study. Preliminary studies using phosphatidylethanolamine yielded similar results and indicated that these vesicles were susceptible to Ca^{2+}-induced collapse even with contents of up to 90 mole % phosphatidylethanolamine. The implications of a trial experiment using phosphatidylserine/ diacylglycerol multilamellar systems are also discussed.

LITERATURE REVIEW

(A) Lipid Bilayers and Biomembranes: Structural and Functional Considerations

Based upon the results of chemical compositional studies, structural evidence from electron-microscopy, and the similarity in properties of phospholipid bilayers (described below) and natural membranes, Singer and Nicolson (1972) proposed the now widely accepted fluid-mosaic model of biological membranes. Quite generally, this model suggests that the constituent lipids (primarily phospholipids) assume a closed bilayer conformation yielding a structural matrix with which functional proteins may be associated (Fig. 1). Since the bilayer is fluid the hydrocarbon region is thought to consist of an appropriate mixture of saturated and unsaturated fatty acid chains that is disordered at physiological temperatures. Consequently, all constituent bilayer molecules including the proteins are assumed to be capable of free lateral diffusion.

Phospholipids, cholesterol, and glycolipids constitute the three principal constituent lipid classes of most animal cell plasma membranes. The first two are by far the most abundant classes while the glycolipids comprise about five per cent of the lipid molecules in the outer monolayer. The common characteristic of all three classes is that they are amphipathic; each molecule has a hydrophilic (polar) end and a hydrophobic (nonpolar) end. In the case of the phospholipids this general structure comprises a polar head group consisting of glycerol and a phosphate linked to a residue that can be either choline, ethanolamine, serine , glycerol, or inositol. The nonpolar hydrocarbon end consists of two fatty acid chains linked to the head group via the glycerol moiety. Each chain may vary both in length (generally from twelve to twenty-four carbon atoms) and degree of unsaturation (from fully saturated to varying numbers of double bonds). Thus, the primary membrane lipids are the phospholipids phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylinositol (PI), as well as sphingomyelin which has a ceramide (sphingosine plus a fatty acid side chain) hydrocarbon end and either a phosphocholine or phosphoethanolamine head group. Both PS and PI carry net negative charges while the other three phospholipids are electrically neutral at physiological pH (Alberts et al., 1983; Bretscher, 1985; Lehninger, 1975).

Figure 1: The Fluid-Mosaic Model of Cell Membranes

phospholipid molecule

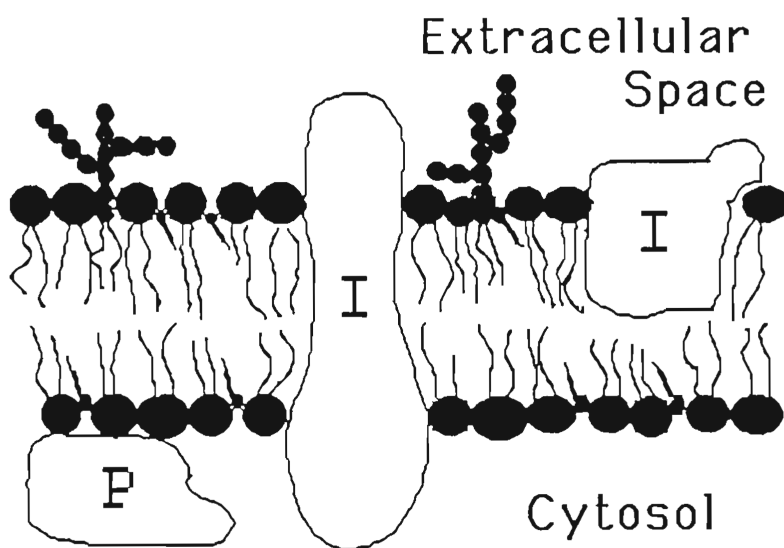
sterol (cholesterol) molecule

glycolipid molecule

I integral membrane protein

P peripheral protein

-Adapted from Singer and Nicolson (1972).


Extracellular
Space
I
I
P
Cytosol

In excess aqueous medium amphipathic molecules aggregate in such a manner as to minimize the contact between their nonpolar ends and the surrounding water while presenting the maximum polar surface possible to this environment. Such aggregation results from the tendency of water molecules to force hydrophobic groups together thus minimizing their disruptive effects on the hydrogen bonded water and polar group networks (Alberts et al., 1983; Lehninger, 1975; Tanford, 1972). As with other physical properties of amphipathic molecules such hydrophobic bonding into larger structures (self-assembly) is dependent upon the nature of the head group, the length and degree of saturation of the hydrocarbon chains (Tardieu et al., 1973), and the concentration of the molecules (Cullis et al., 1983; Gruner et al., 1985). At extremely low concentrations phospholipids exist as dispersed monomers and at some level designated the "critical micellar concentration" (CMC) they aggregate into larger structures such as spherical micelles. At concentrations above the CMC a variety of molecular assemblies are possible and the major membrane phospholipids tend to spontaneously adopt the bilayer or lamellar conformation upon hydration (Fig. 2c) (Cullis et al., 1983; Gruner et al., 1985; Luzzati, 1962, 1974, Tanford, 1972).

Bangham et al. (1965) demonstrated the formation of large multilamellar vesicles (liposomes) upon hydration of dry phospholipids and the sonication of such suspensions was soon found to yield unilamellar vesicles (Papahadjopoulos and Miller, 1967). These latter preparations with diameters in the range of 200-300 Å are described as small unilamellar vesicles (SUV). Vesicles with larger diameters (≥ 1000 Å) can be multilamellar (MLV) or unilamellar (LUV) (Nir et al., 1983; Szoka, 1987) and a variety of preparatory techniques have been established (Szoka and Papahadjopoulos, 1980). These vesicles are usually irregular in shape and generally more stable than SUV since there is less strain in their membrane (Duzgunes et al., 1983; Nir et al., 1983). As with other phospholipid bilayers, vesicles are quite impermeable to most biological molecules such as amino acids, sugars, proteins, and nucleic acids, as well as ions (Bretscher, 1985; Lehninger, 1975; Nir et al., 1983).

Aqueous dispersions of lamellar lipid bilayers consist of three distinct regions, (i) the water

region which may be perturbed by the polar groups; (ii) the polar group region which interacts chemically, electrostatically, and sterically both within itself and with the water; and (iii) the hydrocarbon region which can exert pressure on the lipid-water interface as a result of fluctuations in the flexible hydrocarbon chains (Kirk et al., 1984). Such fluctuations are possible when the hydrocarbon chains are in a disordered, fluid state above a specific temperature, usually in the range of 0-40°C depending on the lipid species. This characteristic order-disorder transition in the packing of the hydrocarbon chains is known as the main hydrocarbon chain phase transition (T_m) (Nagle, 1980). At temperatures below T_m (Fig. 2a) the chains are packed rigid and parallel (gel phase) and may be either perpendicular to the lamellar surface (L_β phase) or tilted at some angle to the surface normal ($L_{\beta'}$ phase). Above T_m (Fig. 2c) the hydrocarbon chains are said to melt or undergo cooperative rotameric disordering; many of the C-C bonds will undergo a *trans* to *gauche* configurational change resulting in the appearance of "kinks" in the chains. However, the average orientation of the chains remains perpendicular to the plane of the bilayers. This is the liquid crystalline or L_α phase which most naturally occurring and synthetic phospholipids spontaneously adopt upon hydration at physiological temperature and pH (Bach, 1983; Chapman, 1975; Cullis and de Kruijff, 1979; Gruner et al., 1985; Makowski and Li, 1983; Nagle, 1980; Verma and Wallach, 1983). Within such bilayers the individual phospholipid molecules are capable of rapid lateral diffusion, rapid rotation about their long axes, and as mentioned above, flexion of their hydrocarbon chains (which increases with distance from the polar head group) (Alberts et al., 1983; Bretscher, 1985).

While subsequent research has supported the fluid-mosaic model it has also raised interesting questions regarding the intramembrane distribution and possible functions of particular bilayer components. If the membrane lipids *in vivo* serve only in a structural capacity, providing the basic bilayer framework, then almost any single phospholipid species

Figure 2: Structural Organizations of Phospholipid Molecules and Their Characteristic X-Ray Diffraction Patterns; The Gel Lamellar (L_β), Liquid-Crystalline Lamellar (L_α), and Inverse Hexagonal (H_{II}) Phases

d total repeat spacing; sum of d_l and d_w

d_l thickness of the hydrocarbon layer

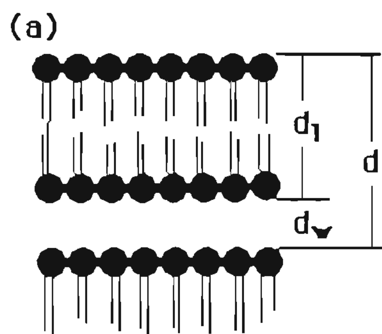
d_w thickness of the water layer

n lattice repeat

Structural Organization

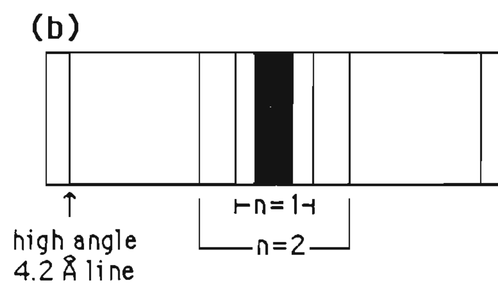
Characteristic X-ray Diffraction Pattern

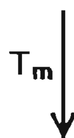
(a)



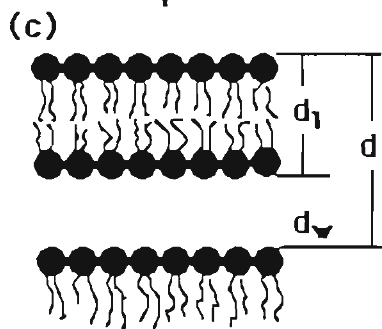
Gel Lamellar (L_β)

(b)



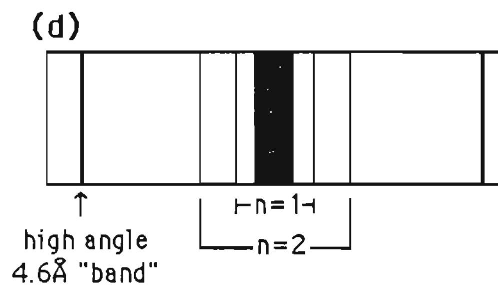


(c)

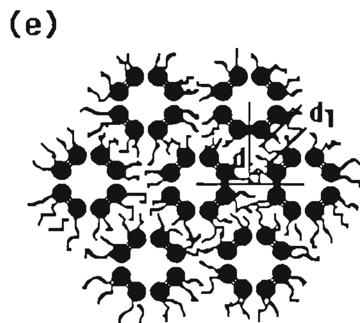


Liquid-Crystalline Lamellar (L_α)

(d)

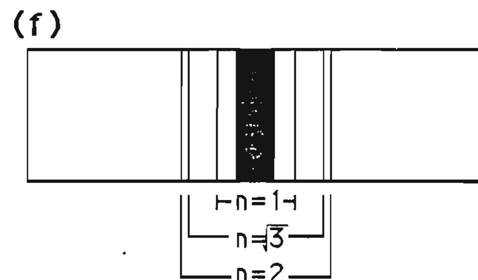


(e)



Inverse Hexagonal (H_{II})

(f)



could satisfy the conformational requirements; there would be no need for the vast diversity of lipids that constitute most natural membranes. This diversity coupled with inherent differences in the distribution of phospholipids in membranes (see below) implies the potential for other functional roles. For example, the pioneering work of Luzzati and Husson (1962) demonstrated that the bilayer lipids are capable of specific structural rearrangements to non-bilayer configurations. More recent research indicates that both the gel and fluid phases, as well as the potential to adopt non-bilayer phases, may be intrinsic to such dynamic cellular processes as membrane fusion, exo- and endocytosis, and enzyme, receptor, or carrier protein activities (Carruthers and Melchior, 1988; Cullis and de Kruijff, 1979; Cullis et al., 1983, 1985; Das and Rand, 1986; de Kruijff, 1987; Deuticke and Haest, 1987; Fong and McNamee, 1986; Killian and de Kruijff, 1986, 1988; Mayer and Nelsestuen, 1981; Rand, 1981; Rand and Parsegian, 1984; Snoek et al., 1986).

When considering the structural dynamics inherent in lyotropic liquid crystalline lipid systems one immediately notes an extreme sensitivity to thermodynamic variables. As already noted, when temperature is increased beyond T_m the structure passes through a series of phase changes, named according to the nomenclature introduced by Luzzati (Luzzati and Tardieu, 1974; Tardieu et al., 1973). The most common sequence involves the change from gel (L_β) to liquid crystalline (L_α) state through which the lamellar bilayer organization is maintained. Further change, beyond a transition temperature designated T_h (Siegel, 1984, 1986a,b, 1987), usually involves the L_α - H_{II} transition yielding a distinctly different phase or polymorph (Cullis and de Kruijff, 1978; Gruner et al., 1985; Kirk et al., 1984). Originally characterized by Luzzati and Husson (1962), the inverse hexagonal phase (H_{II}) may well be the most common non-bilayer structural organization adopted by hydrated lipid systems. This phase has been described as a liquid crystalline hydrocarbon matrix penetrated by long, hexagonally packed, parallel aqueous channels that are stacked on a two dimensional lattice (Fig. 2e) (Cullis

et al., 1983, 1985; Gruner et al., 1985; Luzzati and Husson, 1962; Rand, 1981). The H_{II} phase per se has nothing to do with the two dimensional structure of natural membranes; its formation is an *in vitro* phenomenon that is of considerable interest since it indicates a destabilization of bilayer organization. It is this perturbation of bilayer structure that may be of significance at specific sites *in vivo* . More complex, three-dimensionally periodic structures, designated cubic and rhombic phases respectively, have also been identified in lipid-water systems but unambiguous structural determinations have been difficult (Cullis et al., 1983; Eriksson et al., 1985, 1987; Gruner et al., 1985; Lindblom et al., 1986; Luzzati and Husson, 1962; Luzzati and Tardieu, 1974).

The transition from the L_α to the H_{II} phase (the L-H transition) is a temperature-dependent property of several phospholipid species, and this dependence is related to the length and degree of unsaturation of the hydrocarbon chains. Thus, the introduction of unsaturated *cis* bonds results in "kinking" of the hydrocarbon chains and such disorder or splaying is even more pronounced in longer chains (Gruner et al., 1985). Furthermore, these disordering effects can be compounded by the progressive introduction of *gauche* rotamers at saturated bonds as the temperature of the system is increased (Gruner et al., 1985; Nagel, 1980).
However, specific agents may control the occurrence of both the gel-liquid and the L-H transitions in bilayer systems. Such control would allow for the localized destabilizations that might be of some functional use in biomembrane mechanisms. In general, research extending over the last two decades indicates that under appropriate conditions almost all of the major lipid species found in biomembranes adopt either L_β, L_α, or H_{II} phase structure depending upon the specific influences of such physiologically relevant factors as hydration, local pH, local ion composition, the presence of membrane proteins, and the presence of other lipid species (Cevc, 1987; Coorssen and Rand, 1987; Cullis and de Kruijff, 1979; Cullis et al., 1983, 1985; Das and Rand, 1984, 1986; Duzgunes et al., 1987b; Gruner et al., 1985).

Qualitatively, the structural transitions can be explained on the basis of the shape of the

average volume occupied by each lipid molecule. When a molecule possesses a cylindrical shape it presents the same cross-sectional area at the head group-water interface (hydrophilic volume) as in the hydrocarbon zone (hydrophobic volume) and can pack within a plane and form a bilayer. Agents that tend to increase the hydrophobic volume relative to the hydrophilic volume —— increases in temperature, chain length, or the degree of chain unsaturation —— or decrease the hydrophilic volume relative to the hydrophobic volume —— dehydration, local pH, and divalent cation binding in some species —— yield a molecular shape that has been described as a cone. Such molecules cannot pack on a plane and form surfaces of high curvature that can accommodate the relatively large volume of the hydrocarbon region; thus, these molecules tend to pack as inverted cylindrical micelles yielding the H_{II} phase (Cullis et al., 1983, 1985; Luzzati and Husson, 1962; Rand, 1981). Interestingly, in anionic lipid species the binding of divalent cations generally results in an isothermal or ionotropic transition from the liquid-crystalline phase to a gel state (Boughriet et al., 1988; Duzgunes et al., 1987b; Loosley-Millman, 1980; Papahadjopoulos et al., 1976, 1977; Rand, 1981; Sundler, 1984; Trauble and Eibl, 1974). The effects of membrane proteins and other lipid species on conformational preferences is less well understood and underscores the reasons for studying mixed liquid-crystalline model systems. In general, lipid-protein interactions are relatively dynamic processes that range from the bilayer stabilizing qualities of certain integral membrane proteins (Cullis and Grathwohl, 1977; Cullis et al., 1985) to the abilities of some channel proteins to induce H_{II} phase formation in associated lipids (Cullis et al., 1985; Killian and de Kruijff, 1986, 1988).

A more quantitative approach to the concept of molecular shape and lipid packing has been attempted by Kirk et al. (1984). Their theoretical model of the L-H phase transition considers four contributions to the total free energy/molecule: elastic curvature, hydrocarbon packing, hydration repulsion, and electrostatic effects. Each of these was expressed as a free

energy/molecule at a given lipid to water ratio for each of three specific phases: L_α, H_{II}, and C_{II} (a close packed array of inverted spherical lipid micelles). The phase with the lowest total free energy was considered to be the energetically favored phase at that particular lipid concentration. At low hydration the H_{II} phase is of lowest free energy and above the critical concentration of 20% water, L_α is the favored phase. C_{II} is never predicted to be the stable phase, which suggests that it should not occur in binary systems. These general trends conform well to experimental observations (Gruner, 1985; Gruner et al., 1985). However, elaboration of the model will require an introduction of temperature dependence and a better understanding of head group conformation and interactions (Kirk et al., 1984).

In addition to these findings of phase preferences among the endogenous lipid species, research has also revealed some additional features of biological membranes. The various lipid species have been found to be asymmetrically distributed across the bilayer structure. In the erythrocyte membrane PC and sphingomyelin appear to be preferentially localized in the outer monolayer while PE, PS, and PI are the principal constituent species of the inner monolayer (Bretscher, 1972; Bretscher and Raff, 1975; Farooqui et al., 1987; Op den Kamp, 1979; Rothman and Lenard, 1977). Similar distributions have been characterized in secretory cell types (Fontaine et al., 1980; Sandra and Pagano, 1978; Zwaal and Bevers, 1983), with a reverse asymmetry inherent in secretory granules (Deutsch and Kelly, 1981; Ekerdt et al., 1981; Szoka, 1987 and references therein). An ATP-dependent translocation mechanism that maintains this asymmetric distribution of PS has recently been identified in the human erythrocyte membrane (Middelkoop et al., 1988). In addition, cholesterol is apparently most abundant in the outer monolayer (Op den Kamp, 1979) but some model membrane studies have indicated that this sterol prefers the inner monolayer at concentrations above 30 mole per cent (de Kruijff et al., 1976). Therefore, while there is no unique distribution of lipids the findings, particularly those indicating an active maintenance of asymmetry, indicate a general characterisitc that strongly suggests a functional role (Farooqui, 1987).

Furthermore, the existence of lateral segregation in both model (He and Hui, 1985; Hui, 1981; Hui and Parsons, 1975; Luna and McConnell, 1977; Ohnishi and Ito, 1974; Papahadjopoulos et al., 1974; Tokutomi et al., 1981; see also Duzgunes, 1985 and Duzgunes et al., 1987b for reviews) and biomembranes (Bearer and Friend, 1980, 1982; Illsley et al., 1987; Karnovsky et al., 1982; Severs and Robenek, 1983; Wolf et al., 1981; Yeagle et al., 1987) has been observed. Specific proteins have been found to associate preferentially with either the fluid (Florin and Feigenson, 1987b; London and Feigenson, 1981b) or the gel phase lipids (Mayer and Nelsestuen, 1981). Such lateral accumulations of lipids have been termed "clusters" when the lipids are of the same species, with the term "domain" referring to a lateral membrane area where the different lipids have the same phase state (Gaestel et al., 1983). Evidence indicates that such domains may in fact be specific biophysical and/or biochemical functional zones analogous to (i) sites of protein attachment, anchoring, insertion, and/or activation (Ganong et al., 1986; Mayer and Nelsestuen, 1981; Resnick and Nelsestuen, 1980; Rosing et al., 1988; Shiffer et al., 1988; Snoek et al., 1986), or (ii) "active zones" (with associated protein components) specialized for membrane fusion (as for the exocytotic release of neurotransmitters from presynaptic nerve terminals) (Boughriet et al., 1988; Morris et al., 1979; Ornberg and Reese, 1981; Pumplin et al., 1981). The precise roles of such specific groupings of lipids are only now becoming more apparent. In the case of enzyme functioning research on adenylate cyclase indicates that neutral lipids, particularly PC, are essential for receptor-mediated stimulation while PI, if present in the external monolayer, can act as a negative modulator of the coupling between the active receptor-ligand complex and the stimulatory GTP binding regulatory protein (Depauw et al., 1988; McOsker et al., 1983). Rosing et al. (1988) confirm the necessity of PS and Ca^{2+} for the complex protease-mediated activation of the vitamin K-dependent coagulation factors. Phospholipids with phosphocholine headgroups have been shown to inhibit phospholipases activated by DG, while PE potentiates the activated hydrolysis (Dawson et al., 1985). Vemuri and Philipson (1987) have characterized

the requirements for anionic phospholipids (particularly PS, cardiolipin, or phosphatidic acid) and cholesterol in the activation and optimal activity of the Na^{+}-Ca^{2+} exchange complex. Furthermore, a recent review by Deuticke and Haest (1987) stresses an important role for bilayer thickness in the functioning of membrane-intercalated proteins. However, in terms of both enzyme binding and activation, and membrane fusion the intracellular signalling system involving the receptor-mediated turnover of inositol phospholipids (Berridge and Irvine, 1984; Hokin, 1985; Nishizuka, 1984b, 1986) has in the past decade made the importance of small specific groups of lipids quite clear.

Within the plasma membrane of neurons and other secretory cell types there exists a small labile pool of polyphosphoinositides produced via the sequential phosphorylation of phosphatidylinositol. In response to a wide variety of extracellular signals the rapid metabolic turnover of inositol phospholipids has been found to serve as a major signal transduction mechanism generally associated with an increase in the free intracellular Ca^{2+} concentration and the activation of a Ca^{2+}-PS-dependent protein kinase (protein kinase C, PKC). Depolarization or the formation of an active receptor-ligand complex stimulates the hydrolysis of triphosphoinositide (phosphatidylinositol 4,5-bisphosphate, PIP_2) by a Ca^{2+}-dependent phosphodiesterase (phospholipase C). The phospholipid is thus cleaved to yield demyo-inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DG), both of which serve as intracellular second messengers (Berridge, 1984, 1985, 1987; Berridge and Irvine, 1984; Hokin, 1985; Michell, 1983; Michell and Putney, 1987; Nishizuka, 1984a,b, 1986). Model system studies suggest that such PI turnover might also result in significant structural changes to bilayer membranes (Ohki et al., 1981, 1982a).

IP_3 is the putative ligand which is thought to interact with a specific receptor on the membrane of the endoplasmic reticulum and thus facilitate the mobilization of stored intracellular calcium (Berridge, 1984, 1985, 1987; Berridge and Irvine, 1984; Hokin,

1985; Michell and Putney, 1987; Nishizuka, 1984b). Recent evidence (Guillemette et al., 1987; Somlyo et al., 1985; Streb et al., 1983; Volpe et al., 1985; Worley et al., 1987) strongly supports this concept of IP_3 as the intracellular Ca^{2+} mobilizing factor.

In vitro studies implicate DG in the activation of a variety of phospholipases (Dawson et al., 1983, 1984) and its production is tightly correlated with the fusion of membranes (Allan and Michell, 1975; Allan et al., 1976; Knight et al., 1982; Rink et al., 1983; Wakelam, 1983, 1985; Whitaker and Aitchison, 1985). The primary function of DG *in vivo* appears to be the activation of protein kinase C (PKC) (Bell, 1986; Ganong et al., 1986; Kishimoto et al., 1980; Michell and Putney, 1987). This enzyme is a 81Kd polypeptide with two functional domains; one regulatory (hydrophobic) with phospholipid and one or more Ca^{2+} binding sites, and the other hydrophilic that carries the catalytically active centre (Parker et al., 1986). The enzyme is the product of a gene family that is widely distributed across vertebrate and invertebrate species and in various tissues and organs, but the highest concentrations are found in the brain where a large quantity of the enzyme (or subtypes) is associated with synaptic membranes (Coussens et al., 1986; Kikkawa et al., 1982). *In vitro* and *in vivo* studies indicate a broad substrate specificity and implicate PKC in several functions including roles in secretion, synaptic plasticity, and axon terminal growth (Haslam and Davidson, 1984; Kishimoto et al., 1980; Michell and Putney, 1987; Nishizuka, 1984a, 1986). Alkon and Rasmussen (1988) have reviewed this literature and present a theory for sustained or long-term cellular responsiveness involving Ca^{2+} and PKC (see also Besterman et al., 1986).

Bell (1986) has summarized the requirements for activation of PKC. Ca^{2+} and a minimum of four PS molecules (but less than ten) are required for membrane binding and a primed enzyme complex. PE will also act as a suitable adjunct . DG increases the affinity of PKC for both PS and Ca^{2+} thereby rendering the the enzyme fully active without a net increase in the intracellular Ca^{2+} concentration (Kaibuchi et al., 1981; Kishimoto et al., 1980). This specificity of PKC,

lipid, and Ca^{2+} interaction appears to be more than a simple surface phenomenon (Walker and Sando, 1987). DG is active within the plane of the membrane only transiently and within a minute of formation it disappears, returning to inositol phospholipids via conversion to phosphatidic acid (PA), or becoming hydrolyzed to yield arachidonic acid (Berridge, 1984, 1985, 1987; Hokin, 1985; Michell and Putney, 1987; Nairn et al., 1985; Nishizuka, 1984a, 1984b). This latter metabolite may in turn activate guanylate cyclase to produce the intracellular messenger cGMP, or may be further metabolized to yield eicosanoids which are now being investigated as both intracellular second messengers and local hormones (Berridge, 1987; Bevan and Wood, 1987; Piomelli et al., 1987). As well, Besterman et al. (1986) have characterized an *in vivo* pathway for the rapid production of DG by hydrolysis of PC.

DG has also been shown to possess potent membrane destabilizing properties that permit it to induce major structural transitions (to the H_{II} phase) in phospholipid-water systems (Das and Rand, 1984 and 1986; Epand, 1985); cholesterol has been found to potentiate this effect (Coorssen and Rand, 1987). Since it is produced at specific membrane locations (Besterman et al., 1986) the destabilizing effects of DG may be focused in a manner intrinsic to the process of membrane fusion, suggesting that this molecule may serve as a local agent to induce the required structural changes at appropriate times.

Thus, the breakdown of a small portion (10-20%) of inositol phospholipids (or PC) generates a bifurcating signal transduction mechanism, the two pathways of which appear to function synergistically, mediating several cellular processes including exocytosis (Berridge, 1987; Besterman et al., 1986; Michell and Putney, 1987; Nishizuka, 1986).

Considering the dynamic nature of many membrane-mediated processes, and the inherent functional requirements of many proteins, a fixed fluid bilayer structure for biomembranes as inferred by the fluid mosaic model seems less tenable. Inherent differences in the lateral and transbilayer distributions of endogenous lipids, and the ability of physiologically relevant "local agents" to induce transitory or long-lived gel, fluid, or non-bilayer arrangements

provides for a better understanding of the molecular mechanisms inherent in more complex processes such as fusion. But how are the properties, mechanisms, and organization of such inherently diverse biomembranes to be characterized? Considering this complexity of natural membranes, arising not only from the diversity of lipid species but also from their distributions and possible interactions, the attractiveness of the relatively simple lipid-water systems originally used to characterize natural membrane structure is obvious. From a reductionist perspective such aqueous lipid dispersions represent the principal means available to circumvent the complexity of biological membranes in order to more systematically analyze the biochemical and biophysical properties and interactions of the constituent molecules (Duzgunes, 1985; Duzgunes et al., 1987b; Gruner et al., 1985; Hong et al., 1987; Rand and Parsegian, 1984; Szoka, 1987). In effect these lipid-water systems represent a specific class of lyotropic liquid crystals; they are composed of more than one component (the resulting structure being dependent on the ratios of the components) and possess the crystalline aspect of periodicity in one, two, or three dimensions without having the molecules rigidly locked in a lattice (Kirk et al., 1984; Gruner et al., 1985).

It is this quality of periodic ordering of the lipid molecules on a lattice that can most effectively be exploited in the investigation of the structural properties of these hydrated lipid model systems. X-ray diffraction is the classical technique in this field of research allowing for the unambiguous determination of molecular organization (Cullis and de Kruijff, 1979; Cullis et al., 1983, 1985; Gruner et al., 1985; Luzzati and Husson, 1962). Simply stated, this technique involves the utilization of a narrow, well-defined, monochromatic beam of X-rays directed through the specimen, and a means of recording (either film or a position-sensitive detector) the distribution and intensity of the resulting X-ray scatter. In the case of one-dimensionally periodic phases such as parallel lamellar bilayers separated by water layers of uniform thickness (Fig. 2a,c), the diffraction pattern typically consists of a series of reflection lines in a characteristic ratio 1:1/2:1/3:1/4:1 ... In addition, the L_β phase is characterized by

high angle Bragg reflections at 4.2 Å (Fig. 2b) while the L_α phase may yield a broad, diffuse band at about 4.6 Å (Fig. 2d) (Gruner et al., 1985; Luzzati and Husson, 1962; Makowski and Li, 1983). Many researchers complement the use of X-ray diffraction with very rapid freezing and freeze-fracture electron microscopy as this technique can detect structures such as intact and aggregated unilamellar vesicles that are amorphous to X-ray diffraction. Rapid freezing is advantageous since it does not require cryoprotectants which may alter the processes being studied (Heuser et al., 1979). In the freeze-fracture technique the specimen is ultra-rapidly frozen, cracked open under high vacuum, and a thin metal film is evaporated at an oblique angle onto the freshly cleaved surface. The resulting replica of the specimen surface topography is the record of lipid structure and may be examined in detail using the electron microscope. As the fracture tends to propagate preferentially along the length of the hydrocarbon matrix (at the interface between opposing terminal methyl groups of the fatty acid chains) this surface is a reliable indicator of the lipid organization. Characteristically, L_α bilayers yield smooth fracture faces (Cullis et al., 1983 and 1985; Gruner et al., 1985; Makowski and Li, 1983). In the case of the H_{II} phase (Fig. 2e), such a structural arrangement (packing on a two dimensional lattice) yields an X-ray diffraction pattern with reflection lines in a characteristic ratio of $1{:}1/\sqrt{3}{:}1/2{:}1/\sqrt{7}{:}1$ ... (Fig. 2f) (Luzzati and Husson, 1962) and a freeze-fracture face that appears as a series of parallel, concentric ridges arranged in distinct layers (Gruner et al., 1985). The third most widely utilized investigative technique is nuclear magnetic resonance (NMR), particularly ^{31}P-NMR for phospholipids. It should suffice here to say that the shape of the ^{31}P-NMR spectra reflects the rate, extent, and axes of motion experienced by a phospholipid molecule in a given structural organization (for a more thorough description of the technique and its theory see Cullis and de Kruijff, 1979). Thus, the shape of the ^{31}P resonance is not uniquely related to molecular organization. The NMR technique does not provide unambiguous structural information, only definitive, direct structural methods such as X-ray

diffraction can supply such data. However, recent systematic X-ray and NMR comparisons have shown excellent agreement and thus indicate that the technique can be utilized with some degree of confidence (Cullis and de Kruijff, 1979; Cullis et al., 1983, 1985; Gruner et al., 1985). Recently, ^{13}C solid-state NMR has been used to characterize the overall molecular conformation, as well as the effects of temperature and dehydration on the head group and acyl chain regions of phospholipids in oriented bilayers (Braachs-Maksvytis & Cornell, 1988; Braach-Maksvytis et al., 1988). Other investigative techniques including electron paramagnetic resonance (EPR) and fluorescence spectroscopy using spin-labelled and fluorescent probe molecules, differential scanning calorimetry, infrared spectroscopy, and Raman spectroscopy are also widely used (Chapman, 1983; London and Feigenson, 1981a, 1981b; Nir et al., 1983). Fusion assays have recently also been in vogue (Wilschut and Hoekstra, 1984, 1986). The most widely used system (Wilschut et al., 1980, 1983) follows vesicle aggregation by light scattering, and measures the mixing of internal contents by the fluorescence of a terbium-dipicolinate complex, and leakage by release of carboxyfluorescene fluorescence quenching. Mixing of vesicle lipids is measured by the mixing of fluorescent-labeled lipids (Hoekstra, 1982b) and self-quenching of fluorescence is used to monitor phase separations (Hoekstra, 1982a). These assays have been used to probe the kinetics of the fusion and phase separation processes in model systems but provide no detailed structural information per se unless coupled with another technique(Ababei and Hildenbrand, 1984; Morris et al., 1985; Nir et al., 1983; Wilschut et al., 1985b). Furthermore, the results obtained by such assays have recently been questioned based on the results of more direct observations (Kachar et al., 1986; Miller and Dahl, 1982; Rand et al., 1985b), and comparative studies (Duzgunes et al., 1987a).

The term fusion, as it relates to membranes *in vivo*, implies the occurrance of topological changes requiring two critical steps, (i) the very close approach of the two membranes (adherence); and, (ii) the destablization, rupture, and coalescence of the two membranes only

in the contact area (ie. leakless) (Alberts et al., 1983; De Lisle and Williams, 1986; Kachar et al., 1986; Rand, 1981; Rand and Parsegian, 1986; Rand et al., 1985a,b). The major barrier to the close approach of phospholipid bilayers, and likely any hydrophilic surface, is a strong (kilocalories/mole) hydration repulsion arising from the energy required to remove intervening water. Extensively characterized by Rand and colleagues (LeNeveu et al., 1976, 1977; Rand, 1981), this force has been observed within both neutral (Chernomordik et al., 1987; Horn, 1984; Lis et al., 1982) and anionic phospholipid systems (Cowley et al., 1978). This ubiquitous force dominates intersurface interactions at all separations of less than about 20-30 Å, growing exponentially with a characterisitc distance of 2-3 Å, and thus preventing closer approach unless bilayers undergo a structural reorganization (Parsegian and Rand, 1983; Parsegian et al., 1985; Rand, 1981; Rand and Parsegian, 1984; Rand et al., 1985a). Such interfacial forces thus effect a coupling between inter- and intrabilayer forces by modulating the interactions between neighboring lipid molecules within the same monolayer. Molecular shape and hence structural preferences at close dimensions is thereby affected by this coupling (Cevc, 1987; Gruner et al., 1985; Rand 1981).

Charges on bilayers will perturb the distribution of intervening mobile ionic species (Parsegian and Gingell, 1972; Verwey and Overbeek, 1948) yielding the electrostatic forces that dominate the repulsion between bilayers at separations beyond about 30 Å. Van der Waals attractive forces, arising from differences in polarization of the bilayers and the aqueous medium, counter those of repulsion and are responsible for stabilizing the approach of neutral bilayers (Parsegian and Rand, 1983; Rand, 1981). However, thermal-mechanical fluctuations resulting in a fluctuation-enhanced repulsive force have been suggested to overwhelm van der Waals attraction and prevent stable adhesion in some systems (Evans and Parsegian, 1986). Evidence for the existence of attractive hydration forces has also been presented (Parsegian and Rau, 1984; Parsegian et al., 1985; Rand and Parsegian, 1988; Rand et al., 1988). It is suggested that the spatial correlation of complementary groups between bilayers may result in water forming interbilayer hydrogen-bonded bridges (Parsegian and Rau, 1984; Rand et al.,

1988). Stable contact between membrane surfaces is therefore a matter of reducing or neutralizing the strong hydration barriers (dehydrating hydrophilic groups) (Parsegian and Rand, 1983; Rand and Parsegian, 1984, 1986).

For bilayers of neutral or zwitterionic lipids van der Waals attraction results in stable separations of 20-30 Å (Lis et al., 1982). Adsorption of charged ionic species (Eisenberg et al., 1979; Lis et al., 1981; McLaughlin et al., 1981) or the addition of two to three mole percent charged (acidic) lipid results in an electrostatic repulsion sufficient for such bilayers to separate indefinitely, sensitive to the usual effects of charge density and ionic conditions (Cowley et al., 1978; Loosley-Millman et al., 1982; Rand, 1981). In solutions of monovalent ions (Cowley et al., 1978; Loosley-Millman et al., 1982) charged multibilayers experience mutual repulsion dominated by electrostatics at long distances (>30 Å) and hydration repulsion limiting approach to about 20-30 Å. Similarly, liposomes of acidic phospholipids experience mutual electrostatic repulsion and therefore cannot form stable aggregates. This barrier to close approach is reduced in solutions of cations by both the screening of the negative surface charges and the binding of cations (decreasing the magnitude of the surface charge density) (Duzgunes, 1985; Nir et al., 1983; Ohki et al., 1982b). Interestingly, exposure to millimolar levels of divalent cations results in the collapse of most pure acidic phospholipid systems into multilamellar complexes containing little or no intervening water (Boughriet et al., 1988; Hauser and Shipley, 1984; Loosley-Millman, 1980; Newton et al., 1978; Portis et al., 1979). It appears that (i) the binding of divalent cations to apposed bilayers can displace the water of hydration at the polar surfaces; and (ii) Ca^{2+} appears to be the most effective divalent metal in achieving this result (Bentz and Duzgunes, 1985; Duzgunes, 1985; Duzgunes et al., 1984, 1987b; Nir et al., 1983).

In a wide variety of cell types there exist specialized organelles for the storage and release of specific compounds. Such secretory vesicles, containing proteins or neurotransmitters, release their contents into the extracellular space via fusion with the plasma membrane (Heuser et al.,

1979; Ornberg and Reese, 1981a; Palade and Bruns, 1968). In the case of neurons, neurotransmitters are stored in synaptic vesicles and are released by the fusion of these vesicles to the presynaptic plasma membrane at specialized regions known as active zones (De Lisle and Williams, 1986; Pumplin et al., 1981; Reichardt and Kelly, 1983). Other secretory cells seem to be (or become) fusion competent at random sites on their plasma membranes. In the case of compound exocytosis multiple fusion events, between secretory organelles, can also occur in addition to fusion with the plasma membrane (De Lisle and Williams, 1986; Duzgunes, 1985; Ornberg and Reese, 1981b). Exocytotic release is triggered by a transient increase in the intracellular free Ca^{2+} concentration of the cell as a result of Ca^{2+} influx and/or release from intracellular stores. This Ca^{2+} flux is a well documented necessity for virtually all exocytotic fusion events (Andrews et al., 1988; Charlton et al., 1982; Creutz, 1984; Creutz et al., 1987; Lipscombe et al., 1988; Plattner, 1987; Reichardt and Kelly, 1983). Nayar et al. (1982) utilizing the Ca^{2+} - stimulated exocytotic release of chromaffin granule contents as a model, found that pure anionic phospholipids, and mixtures such as PE-PS, and those approximating the erythrocyte inner monolayer, can act as adjuncts to this release process. As well, significant reductions in the threshold Ca^{2+} concentrations required to induce phase separation and fusion/collapse in several model systems have been observed in the presence of Mg^{2+} (Bally et al., 1983; Duzgunes et al., 1981b; Nir, 1984; Nir et al., 1983; Portis et al., 1979; Wilschut et al., 1981), cholesterol (Bally et al., 1983; Braun et al., 1985; Stamatatos and Silvius, 1987; Tilcock et al., 1988) and physiological levels of phosphate (Duzgunes et al., 1981b; Farooqui et al., 1987; Fraley et al., 1980; Zakai et al., 1977). This suggests that such lipid and ionic compositions could result in the bilayer destabilization necessary to explain the exocytotic fusion process. Such observations prompt the suggestion that active zones or other fusion competent sites of plasma membranes (and the outer monolayer of secretory vesicles) possess similar lipid complements with strong tendencies for isothermal or ionotropic phase transitions.

However, these model systems all require Ca^{2+} concentrations in the range of 2mM or more in order to trigger such transitions (Cullis et al., 1983, 1985; Nayar et al., 1982). During neurotransmitter release the transient, local, free intracellular Ca^{2+} concentration is approximately 10μM (Reichardt and Kelly, 1983) although higher functional concentrations may exist in submembrane compartments immediately adjacent to the inner monolayer (Charlton et al., 1982; Creutz, 1984; Gorman et al., 1984; Pumplin et al., 1981; Rasmussen and Barrett, 1984). The timely studies by Morris et al. (1979) and Ekerdt et al. (1981) confirmed that other membrane constituents are of principal importance in conferring the Ca^{2+} - sensitivity to the fusion event. With the likelihood that such factors are proteinaceous in nature, numerous liposome studies have been done to characterize the fusion-promoting properties of several water soluble, Ca^{2+} - binding or Ca^{2+} - independent proteins (reviewed recently by Hong et al., 1987; Meers et al., 1988). With the exception of known fusion-inducing viral proteins, no other integral membrane proteins have yet been shown to function in any manner other than promoting liposome aggregation.

Therefore, while high divalent cation concentrations are required in model systems, specific cellular constituents may promote interbilayer contacts and hence negate repulsive forces *in vivo.* Considering then (i) the enrichment of PE and acidic phospholipids in the cytoplasmic monolayer of both the plasma membrane and secretory vesicles; (ii) the presence of membrane sterols; (iii) the presence of cytoplasmic Mg^{2+} and phosphate; and (iv) the presence of contact-promoting membrane components, it would seem quite possible that Ca^{2+} - induced lipid phase rearrangements (destabilization) are partly responsible for fusion *in vivo* .

(B) Lipid Molecules of Interest: PS, DG, PC, and PE

The aim of this section is to provide a brief but comprehensive review of the physical structure and molecular characteristics of the lipids phosphatidylserine, diacylglycerol, phosphatidylcholine, and phosphatidylethanolamine. Inherently, such a review will also allow

for an examination of the experimental considerations that shaped the design and objectives of this study.

Of the major phospholipids of mammalian membranes the acidic molecule PS (Fig. 3a) is unique in its susceptibility to ionotropic phase transitions. It has thus been the object of a variety of studies investigating its physical properties in the presence and absence of various di- and trivalent metals (Bentz et al. 1988; Browning and Seelig, 1980; Cevc et al., 1981; Deleers et al., 1985; MacDonald et al., 1976; McLaughlin et al., 1981; Ohki and Duax, 1986; Papahadjopoulos et al., 1976, 1977; Portis et al., 1979; Puskin, 1977; Puskin and Martin, 1979; Rehfeld et al., 1981). In the extensively studied example of Ca^{2+}, the observed effects include lateral phase separations in mixed lipid systems (Duzgunes et al., 1984; Hoekstra, 1982a,b; Hui et al., 1983; Ohnishi and Ito, 1974; Silvius and Gagne, 1984a,b; Tilcock and Cullis, 1981), fusion in liposome and liposome-planar bilayer systems (Duzgunes and Ohki, 1977; Duzgunes et al., 1981a,b, 1984; Papahadjopoulos et al., 1976; Silvius and Gagne, 1984a,b; Struck et al., 1981; Wilschut et al., 1980, 1981, 1983, 1985a,b) and the formation of collapsed multilamellar complexes containing little or no intervening water (Hauser and Shipley, 1984, 1985; Hauser et al., 1977a; Jacobson and Papahadjopoulos, 1975; Loosley-Millman, 1980; Newton et al., 1978; Portis et al., 1979).

However, Ca^{2+} concentrations in the millimolar range are required to produce significant binding to isolated fluid state PS bilayers, one of the critical determinants of fusion in these model systems (Bentz and Duzgunes, 1985; Bentz et al., 1983, 1985a, 1988; Duzgunes et al., 1981a; Nir, 1984; Nir et al., 1983; Wilschut et al., 1981, 1985a). On the other hand, for phospholipid bilayers already in close apposition micromolar concentrations of Ca^{2+} are sufficient to produce the necessary binding (Feigenson, 1986, 1988). The resulting complex of Ca^{2+} and PS is dehydrated and stable at temperatures well over 100°C (independent of saturated acyl chain length) (Hauser and Shipley, 1984, 1985; Hauser et al., 1977a; Newton et al., 1978; Portis et al., 1979) compared to DOPS and PS from bovine brain (BBPS) with T_m of

-11°C (Browning and Seelig, 1980) and -1 to 15°C, respectively (Boggs et al., 1977). X-ray diffraction studies reveal a short lamellar repeat spacing and sharp high-angle lines indicative of highly ordered ("frozen") acyl chains. This gel phase appears to be unique in that Ca^{2+} binds only to the phosphate of the head-group, specifically displacing its waters of hydration; the resulting conformational change leaves the head-group lying approximately perpendicular to the plane of the bilayer (Casal et al., 1987a,b). Particularly noteworthy is the recent work of Feigenson (1986, 1988) which confirms the 2:1 PS/Ca^{2+} binding ratio in the dehydrated complex, firmly establishes that Ca^{2+} binds between PS lamellae (trans) to form the $Ca(PS)_2$ phase, and that the aqueous Ca^{2+} concentration must be supersaturated for this to occur. These critical concentrations of free Ca^{2+} range from 3.0 μM for DOPS to 0.25-0.7 μM for BBPS with its heterogeneous hydrocarbon region. This verifies earlier suggestions regarding the nature and stochiometry of the binding and its difference from Ca^{2+} binding at an aqueous PS surface (Ekerdt and Papahadjopoulos, 1982). This phase transition is also known to occur within the time scale of the fusion event (Miller and Dahl, 1982; Rand et al., 1985b).

In biomembranes PS coexists with numerous lipids, the zwitterionic phospholipids being the most prominent. Accordingly, there have been numerous studies to characterize the behaviour of mixed PS/neutral phospholipid systems, with and without Ca^{2+}, using a variety of physical techniques (Florine and Feigenson, 1987a; Hoekstra, 1982a; Hui et al., 1983; Luna and McConnell, 1977; Ohki et al., 1981, 1982a,b; Papahadjopoulos et al., 1974, 1976, 1977; Silvius and Gagne, 1984a,b; Stewart et al., 1979; Tilcock et al., 1981, 1984, 1988; Wilschut et al., 1980, 1981, 1983, 1985a,b; see also Duzgunes et al., 1984, 1987b, and Prestegard and O'Brien, 1987, for thorough reviews). These studies indicate a Ca^{2+} - induced lateral phase separation of acidic phospholipids from neutral ones resulting in the formation of domain boundaries (Papahadjopoulos, 1978; Papahadjopoulos et al, 1974, 1976, 1977). Lateral phase separation is said to be indicated by the appearance of individual endothermic peaks in

differential scanning calorimetry thermograms (Hui et al., 1983; Papahadjopoulos et al., 1974; Silvius and Gagne, 1984a, 1984b; van Dijck et al., 1978), changes in motional behaviour of probe molecules (Florin and Feigenson, 1987a; Ito and Ohnishi, 1974; Ohnishi and Ito, 1974) or self-quenching of fluorescence (Hoekstra, 1982a, 1982b), coexisting morphologies observed by freeze-fracture electron microscopy (Hui et al., 1983; Stewart et al., 1979; van Dijck et al., 1978), shifts in ^{31}P NMR signal shapes and decreases in signal intensity (Hui et al., 1983; Tilcock et al., 1984, 1988); however, these do not appear to rule-out the possibility of partial or even complete bulk phase separation. Furthermore, while such macroscopic ionotropic phase transitions and separations do not appear to be essential for the fusion event (Duzgunes et al., 1984a; Hoekstra, 1982b; Silvius and Gagne, 1984b; Sundler, 1984; Wilschut et al., 1985; see also Duzgunes et al., 1987b and Prestegard and O'Brien, 1987 for reviews of the evidence) it is possible that phase transitions and phase separations occur locally in the area of intermembrane attachment. Low Ca^{2+} concentrations (micromolar range) will phase separate lipids in the zone of contact between phospholipid bilayers already in close apposition (such as would be achieved by specific proteins) (Duzgunes, 1985; Feigenson, 1988; Ohki et al., 1982a; Tokutomi et al., 1981). Formation of microdomains of PS and dipalmitoylphosphatidylcholine (on the order of 10nm diameter) in the presence of Ca^{2+} have been observed by ^{31}P-NMR and microprobe X-ray analysis (Hui et al., 1983). Such microdomains exhibit no macroscopic or morphological evidence of phase separation by DSC or freeze-fracture electron microscopy respectively. Wilschut et al. (1985a) have suggested that laterally fluctuating phase boundaries between microdomains of condensed and fluid phase lipids might act as transient, local destabilization sites (point defects) required for membrane fusion. The Ca^{2+} and anionic phospholipid requirements of several other membrane processes such as enzyme activation may also be a reflection of this ability to form dehydrated domains (Snoek et al., 1986).

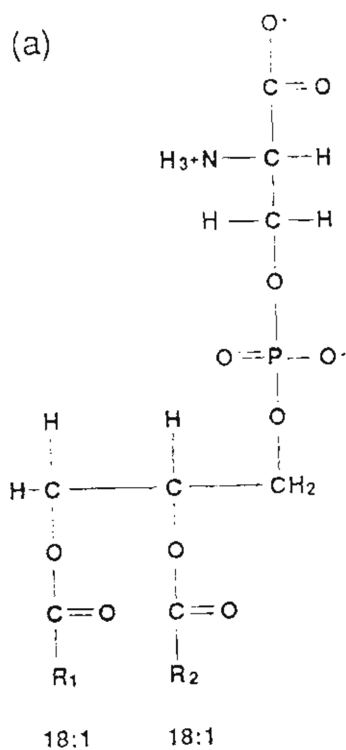
The introduction of PC into PS LUV results in the requirement of increasing Ca^{2+}

concentrations to induce fusion in accord with the finding that the amount of bound Ca^{2+} per PS decreases with increasing mole fractions of PC. By 50-60 mole % PC (species dependent), PS/PC LUV do not undergo fusion even when exposed to very high (50 mM) Ca^{2+} concentrations; 7 mM Ca^{2+} or higher will induce aggregation (Duzgunes et al., 1981a,b; Ekerdt and Paphadjopoulos, 1982; Kachar et al., 1986; Papahadjopoulos et al., 1974; Silvius and Gagne, 1984a). On the other hand, LUV fusion in the presence of PE (50 mole %) requires Ca^{2+} concentrations only slightly higher than the threshold for pure PS, and contents as high as 75 mole % do not effect fusion susceptibility (Duzgunes et al., 1981b). Silvius and Gagne (1984b) using several synthetic PE and PS species in LUV systems, found fusion efficiency to increase up to ~75 mole % PE; however, the decrease in fusion efficiency at PE concentrations > 75 % was accompanied by a corresponding decrease in the threshold Ca^{2+} concentration. They note that at 92.5 % PE the Ca^{2+} threshold for fusion of DEPE/DEPS LUV is only about 30% of that for pure DEPS LUV. Furthermore, (i) Mg^{2+} can also initiate fusion in LUV composed of equimolar mixtures of PS and PE (Duzgunes et al., 1981b) while it is ineffective in fusing similar pure PS systems (Wilschut et al., 1981); and (ii) the addition of PE will confer Ca^{2+} - induced fusion competency to PI LUV (Sundler et al., 1981), the pure PI systems being insensitive to even high Ca^{2+} concentrations (Sundler and Papahadjopoulos, 1981). These differences in the abilities of PC and PE to promote Ca^{2+} - induced responses in liposome model systems have been confirmed by a variety of techniques (Kachar et al., 1986; Leonards and Dhers, 1987; Stamatatos and Silvius, 1987; Uster and Deȧmer, 1981; Vanderwerf and Ullman, 1979). Kachar et al. (1986) attribute these differences to inherent differences in adhesion energies; those for PE being about an order of magnitude larger than for PC (Mara and Israelachvili, 1985; Parsegian and Rand, 1983).

However, the question of lateral or bulk phase separation still exists. The recent studies by Silvius and Gagne (!984a and 1984b) indicate (i) that Ca^{2+} - induced lateral phase separation

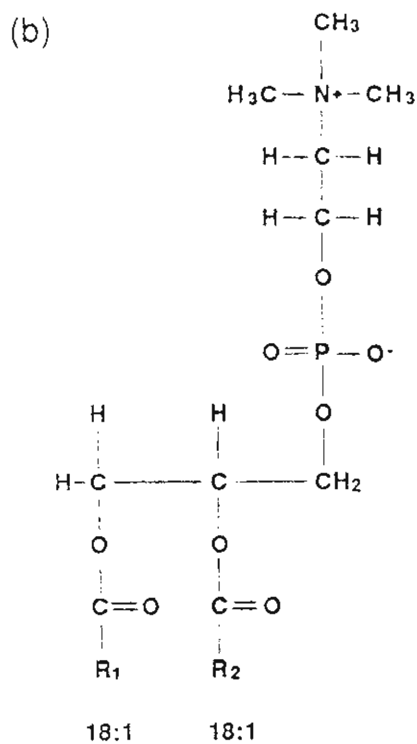
occurs predominantly at PS contents of ~40-70 mole % and ~20-80 mole % in PS/PC and PS/PE systems respectively; (ii) that PC and PE will be accomodated in $Ca(PS)_2$ at ~30 mole % and ~20 mole %, respectively; and (iii) that ~40 mole % (with PC) or ~20 mole % (with PE) PS will be accomodated in liquid-crystalline neutral lipid bilayers. The results of van Dijck et al. (1978) and Hui et al. (1983) support these findings. Sun et al. (1979) have also noted that in PS/PC vesicles containing up to 33 mole percent PC fusion is essentially identical to that of pure PS in excess Ca^{2+}. Conversely, the work of Florine and Feigenson (1987) on DOPS/DOPC MLV systems, using fluorescent and spin-label probes indicates that Ca^{2+} induces a bulk phase separation into a rigid PS phase that excludes PC, and a PC-rich fluid phase. In similar systems, PE seems to favour the fluid as well as the interfacial regions between $Ca(PS)_2$ gel and PS/PC fluid phase domains (Florin-Casteel and Feigenson, 1988). Kachar et al. (1986) confirm by X-ray diffraction the segregation of DOPE H_{II} , and $Ca(PS)_2$ lamellar bulk phases following the Ca^{2+} - induced fusion/collapse in similar LUV systems. Such Ca^{2+}-induced bulk separations into H_{II} and lamellar phases have also been observed in unsaturated PS/PE systems (Tilcock and Cullis, 1981). However, in systems of more unsaturated species of PS, or other anionic phospholipids, or with added cholesterol, Ca^{2+} does not induce lateral phase separation but rather effects an L_α-H_{II} transition in which all component lipids adopt the non-bilayer phase (Tilcock et al., 1984, 1988). These authors suggest that in heterogeneous biomembrane systems with both PE and cholesterol constituents, Ca^{2+}-induced phase segregation of PS may not be the norm. Indeed, the large content of polyunsaturated PS in bovine retinal rod outer segment membranes has been suggested to minimize the effects of its interactions with calcium (Sklar et al., 1979). Analogously, Cevc (1987) notes that any chain unsaturation renders membranes more sensitive to the modulation of their properties by the head-group region and ionic solution effects, suggesting that this may be part of the reason for hydrocarbon heterogeneity *in vivo* . A correlation between high levels of polyunsaturated lipids in cell

Figure 3: The Chemical Structures of Phosphatidylserine, 1,2-Diacylglycerol, Phosphatidylcholine, and Phosphatidylethanolamine



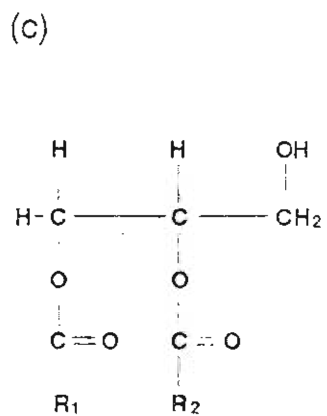
DOPS

Dioleoyl Phosphatidylserine



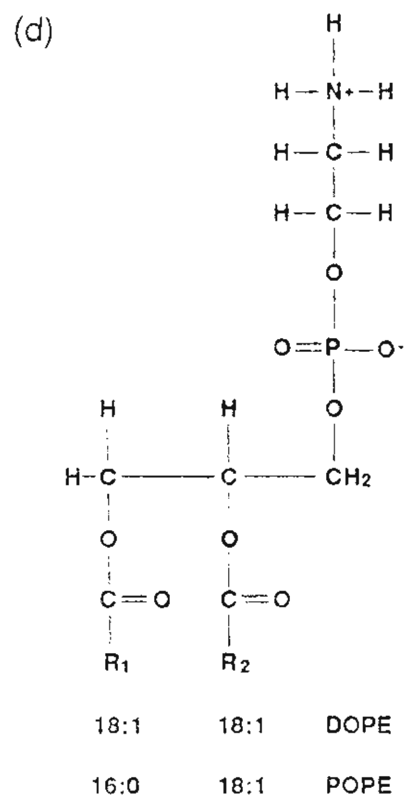
DOPC

Dioleoyl Phosphatidylcholine



DG

1,2-Diacylglycerol



Dioleoyl Phosphatidylethanolamine

1-Palmitoyl-2-Oleoyl Phosphatidylethanolamine

membranes and increased susceptibility to PEG-induced fusion has also recently been demonstrated (Roos, 1988; Roos et al., 1987, and references therein).

The DG molecule consists of a variable two chain hydrocarbon region as possessed by the membrane phospholipids, but does not have the hydrophilic head group that is associated with the normal membrane constituents (Lehninger, 1975) (Fig. 3c). DG thus promotes the L-H transition by adding hydrocarbon volume to the phospholipid bilayer in such a manner (spreading of polar groups) that the molecules must reorganize to the high curvature H_{II} phase in order to maintain an effective polar group interface with the aqueous environment (Das and Rand, 1986). This effect is potentiated by the presence of cholesterol (Coorssen and Rand, 1987). Ohki et al. (1981, 1982a) have also noted a lower threshold for Ca^{2+}-induced phase separation in PC/PS/DG (3:5:2) membranes compared to PS/PC (1:1) and PC/PS/PI (3:5:2) systems. These results suggest that at physiological calcium concentrations phase separation can be induced by the conversion of PI to DG.

Of the major membrane phospholipids the most prominent and most widely studied is PC (Fig. 3b). The large, strongly hydrated headgroup of this neutral phospholipid makes it ideal as a stabilizer of bilayer structure (Cullis and de Kruijff, 1979; Cullis et al., 1983, 1985; Hauser et al., 1981; Macdonald and Seelig, 1987; Sundler, 1984). Binding of divalent cations to the headgroup results in charging of the bilayers and their mutual repulsion (Inoko et al., 1975; Lis et al., 1981; McLaughlin et al., 1978). This binding is also known to induce structural changes (alter the phase state) at higher Ca^{2+} concentrations; packing is distorted for several nanometers about the binding centre resulting in a more rigid membrane and a slight upward shift in T_m (Inoko et al., 1975; Conti et al., 1987). The results of Altenbach and Seelig (1984) suggest that these effects may arise from specific Ca^{2+} binding to two PC molecules on the same bilayer (cis binding). Furthermore, this Ca^{2+} binding is enhanced in the presence of Cl^- ions (Hauser et al., 1977b). The T_m of DOPC, the species used in this study, is

known to be -22°C (Ladbrooke and Chapman, 1969).

PE, another of the major phospholipid constituents of natural membranes has been well characterized in model systems (for a review see Cullis and de Kruijff, 1979). Compared to PC, the headgroups of PE (Fig. 3d) form a more tightly packed lattice via electrostatic interactions and hydrogen bonding (Boggs, 1987; Hauser et al., 1981), and appear to have a lower affinity for water (Jendrasiak and Hasty, 1974). In accord with their relatively small polar volumes, various PE species (natural and synthetic) are capable of well defined, reversible L-H transitions as temperature is increased; more unsaturated species tending to preferentially adopt the H_{II} organization over the L_α (Cullis and de Kruijff, 1978; Hauser et al., 1981; Tilcock and Cullis, 1982). Recent evidence (Duzgunes et al., 1984; Silvius and Gagne, 1984b; see also Duzgunes, 1985 and Duzgunes et al. 1987b for reviews) appears to rule-out the earlier hypothesis that this non-bilayer phase is involved in the fusion mechanism (Cullis and de Kruijff, 1979; Cullis and Hope, 1978; Cullis et al., 1983, 1985; Hope and Cullis, 1981; Hope et al., 1983; Rand, 1981; Siegel, 1984, 1986a,b; Verkleij et al., 1979a,b, 1980, 1985). However, under the dehydrating conditions of the contact zone between adhering membranes, the transient destabilization inherent to the initial formation of such high curvature structures might serve as a "point defect" (Hui et al., 1981; Portis et al., 1979) to promote fusion (Bentz et al., 1985, 1988; Chernomordik et al., 1987; Das and Rand, 1986; Duzgunes, 1985; Duzgunes et al., 1987b; Ellens et al., 1986; Nir et al., 1983; Prestegard and O'Brien, 1987; Siegel, 1987). Furthermore, according to the theoretical work of Siegel (1987), such structures would exist only transiently (half-lives on the submillisecond to millisecond time scale), and would thus be extremely difficult to image using available techniques; assuming more than one possible mechanism of fusion, the existence of inverted micellar intermediates (single H_{II} micelles between apposed bilayers) as fusion sites is probable for some systems (Hui

et al., 1981; Prestegard and O'Brien, 1987; Siegel, 1987; Siegel et al., 1987; Sundler, 1984; Tilcock and Cullis, 1981).

The specific PE species used in this study (Fig. 3d) were chosen for their inherently different qualities. POPE forms stable lamellar bilayers at 23°C (T_m=31°C; T_h=69°C) while DOPE prefers the H_{II} phase under similar conditions (T_m=-8°C; T_h=10°C), reflecting the effects of having two unsaturated acyl chains. It is now generally accepted that in mammalian plasma membranes PE is a member of a class of lipids which act to modulate membrane-membrane contact and promote the destabilization or perturbation of bilayer structure (Cullis and de Kruijff, 1978b; Ellens et al., 1986; Kolber and Haynes, 1979). Evidence indicates that PE, like PS and PI, is located primarily on the inner monolayer of plasma membranes and the outer monolayer of secretory vesicles, suggesting a possible localized disruption of bilayer structure during Ca^{2+} fluxes and the transient presence of DG generated from PI. Interestingly, as noted earlier, PS is necessary for PKC activation, but when PE is also present, the enzyme is still fully activated by DG at the 10^{-7}M range of Ca^{2+} concentration. PC and sphingomyelin are inhibitory (Nishizuka, 1984a).

(C) Model Systems and Their Relevance to Biomembranes

Several authors have recently presented critical commentaries on the applicability of model membrane studies to the understanding of biomembrane functions and processes (Chernomordik et al., 1987; Cullis et al., 1985; Gruner etal., 1985; Rand and Parsegian, 1984, 1986; Parsegian et al., 1984). They have noted a failure of these systems to mimic the finely controlled specificity and leakless quality of fusion *in vivo*. Particularly noteworthy are the findings of Miller and Dahl (1982) and Rand and colleagues (Kachar et al., 1986; Rand et al., 1985b). By direct assessment of vesicle morphology using rapid-mixing freeze-fracture electron microscopy and video-enhanced light microscopy they have found significant short-comings or inconsistencies in the liposome models of fusion compared to the process *in vivo*.

Primarily these studies have found the model process to be unfocused and leaky; that fusion (rupture in the contact zone between apposed membranes) and deflation (rupture outside of the contact zone) are almost equally likely events (Kachar et al., 1986; Rand et al., 1985b; Rand and Parsegian, 1988). Furthermore, such model fusion events are uncontrolled, resulting in the rapid (<10 ms) and complete collapse of vesicular structures. Duzgunes et al. (1987a) have also recently noted problems with specific fusion assays, cautioning that aggregation can yield data indicative of fusion and thus result in overestimates of the reaction kinetics. In addition to their intrinsic interest the most reasonable correlations of the lipid-water systems is therefore not in modeling the global behaviour of membranes but rather the behaviour of specific local domains (Das and Rand, 1986; Duzgunes et al., 1987b; Wilschut and Hoekstra, 1984, 1986). The present study concerns PS-containing domains exposed to Ca^{2+} and the suggested segregation of neutral phospholipids away from such domains; is this a lateral or a bulk segregation? Are there single phases of neutral lipid-PS-Ca^{2+} coexistence?

(D) Objectives

An intimate complex of Ca^{2+}, PS, and DG is required to fully activate PKC (Bell, 1986 and references therein). However, (i) Ca^{2+} and PS are known to form a completely dehydrated collapsed lamellar phase (Feigenson, 1986; Hauser and Shipley, 1984; Loosley-Millman, 1980; Newton et al., 1978; Portis et al., 1979) that has been suggested to exclude neutral phospholipids (Florine and Feigenson, 1987a; Kachar et al., 1986; Rand and Parsegian, 1988; Tilcock et al., 1981); and (ii) DG is known to be a potent perturber of bilayer structure (Coorssen and Rand, 1987; Das and Rand, 1984, 1986; Epand, 1985). Our original interest therefore was in exploring the possible effects of DG on $Ca(PS)_2$ and how these two components might interact. Would the presence of DG modify Ca^{2+} - PS binding and thus affect the formation of the $Ca(PS)_2$ phase? Could DG be in the $Ca(PS)_2$ phase and is this collapsed phase stable in its presence? How might the interactions be related to PKC activation or membrane fusion?

Initial studies were done with synthetic phospholipid species to control the nature of the hydrocarbon region, and high Ca^{2+} concentrations (10-11mM) to ensure Ca^{2+}/lipid ratios of 10:1. These saturating Ca^{2+} levels were used to ensure the complete formation of the $Ca(PS)_2$ phase according to the mechanism described by Feigenson (1986). The preliminary experiments with DOPS/DG mixtures suggested that up to 30 mole % DG might be in the $Ca(PS)_2$ phase and that higher DG levels modified Ca^{2+} - PS binding. However, since it does not form ordered structures in aqueous solutions, any bulk segregated DG could not be identified in these systems. Thus, questioning the initial results led us to the use of PC as this neutral lipid could be identified by ionic screening and osmotic stress if it became bulk segregated; it was considered quite unlikely that this strongly hydrated headgroup would readily exist in the dehydrated phase. However, comparable to the samples with DG, this study suggested that ~30 mole % PC was in the $Ca(PS)_2$ phase and that higher DG concentrations modified Ca^{2+}-induced bilayer interactions. It was assumed that the absence of bulk phase segregation after exposure to Ca^{2+} was a spurious or artificial effect reflecting a faulty experimental technique and most of our effort went into attempts to eliminate this phenomenon rather than study it. As a result progressively more stringent preparative and verification criteria were established, including the use of (i) osmotic stress; (ii) LUV preparations; (iii) overnight incubations in Ca^{2+} solution; (iv) freeze-fracture electron microscopy; (v) thin-layer chromatography of each sample both before and after experimental procedures; (vi) heterogeneous PS from a natural source; (vii) sonication after incubation in Ca^{2+}; (viii) sucrose density gradient analysis; and (ix) a freeze-thaw protocol. Having thus verified and characterized the phenomenon we reapproached the issue with the aim of discovering how much neutral lipid, associated with PS, can be dehydrated by the formation of $Ca(PS)_2$; how much neutral lipid can be accomodated by this collapsed phase? Preliminary studies involving two PE species will also be discussed.

MATERIALS AND METHODS

MATERIALS

Dioleoylphosphatidylserine (DOPS), bovine brain phosphatidylserine (BBPS), Dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylethanolamine (DOPE), and 1-palmitoyl-2-oleoylphosphatidylethanolamine (POPE) were purchased from AVANTI POLAR LIPIDS INC. (Birmingham, Alabama). 1,2 Diacylglycerol (DG) derived from egg phosphatidylcholine was obtained from SERDARY RESEARCH LABORATORIES (London, Ontario). To eliminate divalent contaminants DOPS and BBPS stocks were converted to their sodium salts using a chloroform-methanol aqueous two-phase system. All other lipids were used without further purification. Thin-layer chromatography showed all lipids to be >99% pure, and they were stored under nitrogen at -18°C. Polyethylene glycol (PEG) and N-tris[Hydroxymethyl]methyl-2-aminoethane sulfonic acid (TES) were obtained from SIGMA CHEMICAL COMPANY (St. Louis, MO), and Dextran T2000 was purchased from PHARMACIA (Uppsala, Sweden). Sucrose was purchased from CALBIOCHEM (La Jolla, California). Uni-pore polycarbonate membranes (1.0 μm) were obtained from BIO-RAD LABORATORIES (Richmond, California). All water used was double-distilled in glass. Salts were all of analytical grade.

METHODS

(A) Thin Layer Chromatography

The purity of lipid stocks and experimental mixtures was checked by chromatography on silica gel G plates in chloroform : methanol : 7N NH_4OH (65:35:4 v/v). The chromatograms were developed with iodine vapour.

(B) Sample Preparation

i) Multilamellar Systems

Defined lipid mixtures were produced by combining the appropriate amounts of the component lipids in organic solvent, removing the solvent by rotary evaporation, and then vacuum drying. Each gravimetric sample was hydrated by weighing the dry lipid and 2mM TES

buffer (pH 7.3) into small weighing bottles and then allowing the sealed mixture to equilibrate in the dark, at room temperature, for three days. For the larger samples designated "fully hydrated multilamellar systems" the dry lipid was transferred to washed 30 ml polycarbonate centrifuge tubes and hydrated overnight (conditions as above) with 2mM TES. Appropriate volumes of 10-11mM divalent cation stock solutions (prepared with the TES buffer) were added to give final divalent to lipid ratios of 10:1 and samples were again allowed to equilibrate, for specified periods (1-2 or 12 hours), under identical conditions. All samples were centrifuged at 35K for one hour. Lipid pellets were recovered and supernatants discarded. Each sample was combined with some powdered teflon (an X-ray calibration standard) and sealed between mica windows 1 mm apart in X-ray sample holders.

ii) Unilamellar Vesicle (REV) Systems

Samples of defined lipid mixture were established as described above and large unilamellar vesicles were formed according to the reverse-phase evaporation method of Szoka and Papahadjopoulos (1978). Aside from the ease of preparation the advantages of using such reverse-phase evaporation vesicles (REV) include the characteristic yield of primarily unilamellar vesicles, little evidence of bilayer asymmetry and associated packing constraints as found in SUV, and the ability to prepare small volumes of vesicles (Bergelson and Barsukov, 1977; Duzgunes et al., 1983; Huang and Mason, 1978; Parente and Lentz, 1984; Szoka and Papahadjopoulos, 1978). At room temperature lipid samples were dissolved in diethyl ether that had been freshly redistilled over sodium bisulphite to remove peroxides. 2mM TES buffer (pH 7.3) was added to give a 3:1 organic/aqueous phase ratio and the sample was briefly sonicated to produce an emulsion. Under low vacuum a rotary evaporator was used to remove most of the ether resulting in a gel-like consistency. Additional TES buffer was added and the suspension was dispersed by vortexing. Traces of remaining solvent were removed by rotary evaporation under high vacuum for ~15-20 minutes. Vesicles were sized by extrusion through 1.0 μm polycarbonate membranes, and these suspensions were collected in 30 ml polycarbonate

centrifuge tubes. These samples were then exposed to excess calcium (10-11mM stock solutions), recovered by centrifugation, and mounted for X-ray diffraction as described above. In some experiments an additional sonication step was added after the incubation in Ca^{2+} medium in an attempt to liberate any bulk separated neutral lipid that might have been trapped within collapsed $Ca(PS)_2$ structures.

(C) Additional Protocols

i) Osmotic Stress

In effect this is a method of controlled dehydration used to measure interbilayer forces (LeNeveu et al., 1976, 1977; see Rand, 1981 for a review). In this case it is used not as a method of measurement, but rather to characterize the structures formed by the lipid mixtures and to verify the presence of any amorphous, bulk segregated lipid. The technique involves equilibrating the lipid with water of known chemical potential (controlled by osmotic or vapour pressure); the lipid must compete for water against an external pressure. Therefore, at equilibrium the total internal pressure between bilayers is equal to the external pressure applied to the bilayers,

$$P_{ext} = P_{rep} - P_{att}$$

where P_{ext} is the external pressure and P_{rep} and P_{att} are the repulsive and attractive pressures beween bilayers, respectively. The external pressure was provided by either the osmotic pressure of a PEG or dextran solution (π);

$$\pi = P_{ext} = -\mu_w/V_w \quad \text{(dynes/cm}^2\text{)}$$

or the vapour pressure generated by a saturated salt solution (T);

$$T = P_{ext} = -\mu_w/V_w \quad \text{(dynes/cm}^2\text{)}$$

where μ_w is the chemical potential of water between the bilayers relative to bulk water, P_{ext} is the external pressure compressing the system, and V_w is the molar volume of water (Parsegian et al., 1986; Rand, 1981).

For lower pressures (0 to 10^7 dynes/cm^2) PEG and dextran solutions of known concentrations were prepared using Ca^{2+}-TES stock solutions identical to those in which the respective samples had originally been incubated. Within the sealed X-ray sample holder the lipid was separated from such polymer solutions by a dialysis membrane and left to equilibrate in the dark, at room temperature, for three days. Polymer solutions were changed twice daily to ensure that no hydrostatic pressure differences built-up across the membrane. The final equilibrated polymer concentration was measured within ±0.2% using an Abbe refractometer. The osmotic pressure was then obtained from established standard curves (see Parsegian et al., 1986 for example). The polymer solutions were discarded, the samples resealed in their holders, and X-ray diffraction used to analyze the resulting structure(s). Such lower pressures (log P=~6-7 dynes/cm^2) are considered a mild dehydration protocol, and were used to collapse and order amorphous structures. Alternatively, for the higher pressures ($10^{8.2}$ to $10^{9.2}$ dynes/cm^2) used to further characterize ordered phases, the lipid sample was removed from the holder and equilibrated with a known vapour above a specific saturated salt solution. After three days the sample was resealed in the holder and analyzed by X-ray diffraction.

ii) Rapid-Mixing, Spray-Freezing, Freeze-Fracture Electron Microscopy

This technique was used in parallel with the first experimental REV series to more fully characterize the inherent morphologies of these lipid mixtures. Using a Berger ball mixer (Commonwealth Technology Inc., Alexandria, VA) a suspension of vesicles was rapidly mixed with an equal volume of Ca^{2+} stock solution to yield a final Ca^{2+}/lipid ratio of 10. This mixture was allowed to equilibrate at room temperature for ~1 hour prior to rapid spray-freezing, without cryoprotectant, on standard Balzers equipment (Balzers, Hudson, NH). The freeze fracturing was done on Balzers 400T equipment with both cleaving and quartz-crystal monitored shadowing (45°) and replicating at $5x10^{-8}$ mbar and -150°C. Examination and micrographs were done on a Philips EM 30 electron microscope (Philips, Holland).

iii) Continuous Sucrose Density Gradient Centrifugation

In order to confirm the presence of neutral lipid in the $Ca(PS)_2$ phase, and in an attempt to separate bulk-segregated lipid phases (as identified by X-ray diffraction), we adopted the use of density gradients. Sucrose solutions were prepared using Ca^{2+}-TES stock solutions identical to those in which the respective samples had originally been incubated. Continuous gradients were quickly and easily established using equipment graciously supplied by Dr. D. Bruce. Following incubation with Ca^{2+} and recovery by centrifugation as described above, sample pellets (or portions thereof) were quickly resuspended in <1 ml of Ca^{2+}-TES, and this fine suspension was then layered on the top of a continuous sucrose density gradient. All samples were centrifuged at 17,000 x g for one hour in a swinging-bucket head. Resulting separations were recorded photographically and, assuming ideal continuity throughout the gradient, densities were estimated by measuring the location of any given band on the gradient. Separate bands or fractions recovered from the gradient were washed twice in Ca^{2+}-TES at 35K for one hour. These samples were then mounted for X-ray diffraction as described above.

iv) Freeze-Thaw Protocol

In order to confirm that our 12 hour incubation procedure allowed for full access of Ca^{2+} to all the lipid in a sample we performed a freeze-thaw protocol (Feigenson, 1986) to compare results. Following the 12 hour incubation in Ca^{2+} medium samples were (i) incubated at -10°C for 10-15 minutes in a constant temperature bath of KCl/ice (1/4 by weight); (ii) nucleated to freeze by touching the centrifuge tube just above the meniscus with a pipe cleaner dipped in liquid nitrogen; (iii) returned to the -10°C bath for ~10 minutes; and (iv) removed to a water bath to thaw at room temperature. Following ten such cycles each sample was briefly sonicated and then centrifuged for one hour at 35K. Recovered pellets were then analyzed by density gradient centrifugation and X-ray diffraction, as described above.

(D) Analyses of Structural Parameters

X-ray diffraction was used to characterize the structures formed by the various lipid mixtures under specific temperature and osmotic conditions. The $CuK\alpha 1$ line (λ = 1.540 Å), produced at a fixed anode tube, was isolated using a bent quartz crystal monochromator. The diffraction patterns of each sample were recorded photographically on Kodak DEF-392 film using Guinier X-ray cameras operating *in vacuo* . Temperature was maintained to ±0.5 °C using thermoelectric control. Samples were allowed to equilibrate for 20-30 minutes after a temperature change before their diffraction patterns were recorded. Characterizing the phase structures was done by matching the diffraction patterns to known lattice geometry. This was accomplished by measuring the diffraction spacings using a Scherr Tumico (St. James, Minnesota) optical comparator. According to the Bragg equation,

$$1/d = 2 \sin \theta / n\lambda$$

where λ is the wavelength of the X-rays, n is the order of the reflection, and θ is the angle of incidence of the beam on the bilayer planes, d is the distance between the planes; equal to the sum of d_l, the thickness of the hydrocarbon layer, and d_w, the thickness of the water layer (in the nomenclature of Luzzati). Repetitive measurements of the sample films indicated a d spacing measuring error of ±0.5 Å for normal, sharp reflections, and up to ±1.0 Å for diffuse reflections.

RESULTS

All samples were hydrated in excess 2mM TES buffer (pH=7.3) unless otherwise stated. All exposures to divalent cations (all chloride salts) were in this TES medium at an excess cation to lipid ratio of 10:1 (10-11mM solutions, unless otherwise stated). Incubation under all circumstances was in the dark under conditions of room temperature and pressure. Data tables for all figures in this section appear in the Appendix. All samples yielded sharp X-ray diffraction patterns to 3 or 4 lattice orders unless otherwise described. All samples are referred to by their mole percent neutral lipid content. As stated in the Methods, all reflections have measurement errors of ±0.5 Å, and this error increases to ±1.0 Å for those reflections described as " diffuse."

(A) Characterizing the Phases Formed by DOPS with Divalent Cations

These initial experiments were conducted to characterize the collapsed phase formed by DOPS and Ca^{2+} as well as the complexes formed by DOPS and other divalent cations. Pure DOPS hydrated gravimetrically in limited 2mM TES buffer yielded a single lamellar (L) phase (d spacing of 137 Å); dehydration by vacuum desiccation resulted in the formation of two separate L phases of 44.5 Å and 53.1 Å respectively (Table 1). A sample of DOPS exposed to Ca^{2+} (Sample 2, Table 1) yielded a single L phase of 51.4 Å that was insensitive to dehydration and showed a weak high angle (4.2 Å) line indicative of frozen (crystalline) acyl chains; the work of Feigenson (1986) suggests that the composition of this phase is $Ca(DOPS)_2$. Sample 3 (Table 1) confirmed that there were no problems with sample hydration prior to exposure to Ca^{2+} since DOPS hydrated for one week before Ca^{2+} addition still yielded the characteristic L phase (51.8 Å) with frozen chains. Sample 4 (Table 1), which was fully hydrated DOPS exposed to 1.0M Ca^{2+}, also yielded the characteristic collapsed phase (51.3 Å with frozen chains). Exposure of hydrated DOPS to Mg^{2+} yielded a single L phase of 50.9 Å which shrank to 49.7 Å after desiccation, with no indications of chain ordering in either case. Ba^{2+} produced a fluid L

TABLE 1:
FULLY HYDRATED DOPS MULTILAMELLAR SYSTEMS EXPOSED TO VARIOUS DIVALENT CATIONS

Sample #	Divalent cation	Temp. (°C)	log P (dynes/cm^2)	Lipid phase(s)	d Spacing(s) (Å)	Comments
1	-----	23	-----	L	137	29.6 wt.% in 2mM TES.
		23	desiccated	LL	44.5 53.1	vacuum desiccated 2 hrs.
2	Ca	23	-----	L	51.5	frozen chains
		23	8.49	L	51.3	frozen chains
3	Ca	23	-----	L	51.8	frozen chains; hydrated for 1 wk. without Ca^{2+}
4	Ca	23	-----	L	51.3	exposed to 1M Ca^{2+}; frozen chains
5	Mg	23	-----	L	50.9	
		23	desiccated	L	49.7	vacuum desiccated 2 hrs.
6	Ba	23	-----	L	49.2	
		23	8.87	L	48.1	
7	Mn	23	-----	L	52.5	frozen chains
		23	8.49	L	52.5	frozen chains
8	Mn	23	-----	L	52.5	frozen chains
		23	8.49	L	52.6	frozen chains

- Sample 1 allowed to equilibrate for 3 days.
- Samples 2-8 hydrated overnight in 1 ml of 2 mM TES (pH 7.3) and exposed to excess divalent cation (10:1 Me^{2+}/phospholipid) for 1-2 hours (Sample 4 in 1000:1 Ca^{2+}/phospholipid).
- Sample 1 prepared gravimetrically; all others centrifuged at 35K for 1 hour.
- All divalent cations used were chloride salts.
- L: lamellar phase.
- Lipid phase and d spacing values were determined from measurements of each diffraction pattern.
- Each phase symbol represents an individual X-ray experiment.

phase of 49.2 Å which shrank to 48.1 Å under osmotic stress (8.87 dynes/cm^2). In two separate samples Mn^{2+} yielded a collapsed phase of 52.5 Å (frozen chains) that was insensitive to osmotic stress (Samples 7 and 8, Table 1). Thus, the $Ca(DOPS)_2$ phase did have weakly ordered hydrocarbon chains, compared to the well ordered hydrocarbon region 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). The phases formed by DOPS with Mg^{2+} or Ba^{2+} were not as dehydrated as that formed with Ca^{2+}, while the phase formed with Mn^{2+} appeared to be dehydrated but had a larger repeat spacing than $Ca(DOPS)_2$.

(B) Characterizing the Collapsed $Ca(DOPS)_2$ Phase Using Osmotic Stress

In an effort to better characterize the $Ca(DOPS)_2$ complex we were curious as to its degree of hydration; could water be removed from the structure and the dimension modified as a consequence? Six samples of $Ca(DOPS)_2$ were subjected to different osmotic stresses (Table 2). PEG solutions were used to produce the lower osmotic stresses (7.76-7.93 dynes/cm^2) and vapour pressures the higher (8.35-9.19 dynes/cm^2). Vacuum desiccation for ~2 hours was used as the strongest dehydration protocol. No degree of osmotic stress had any effect; all samples yielded identical, sharp lamellar diffraction patterns with d spacings of 51.4-51.6 Å, even after dessication. This suggested that the $Ca(DOPS)_2$ system contained no free water. Could such a dehydrated phase form with neutral lipids present; could it accomodate any neutral lipid?

(C) Fully Hydrated DOPS/DG Multilamellar Systems Exposed to Ca^{2+}

This experimental series was designed as a preliminary study to characterize the behaviour of fully hydrated DOPS/DG multilamellar systems exposed to excess Ca^{2+} (Table 3). Pure DOPS yielded the characteristic single L phase of 51.4 Å corresponding to the collapsed $Ca(DOPS)_2$ complex. An identical phase was obtained with samples containing 17 and 33 mole % DG respectively, although the first and second order reflections of the latter sample were not quite

TABLE 2:
FULLY HYDRATED DOPS MULTILAMELLAR SYSTEMS EXPOSED TO Ca^{2+}: CHARACTERIZING THE COLLAPSED PHASE USING OSMOTIC STRESS

Sample #	Temp. (°C)	log P (dynes/cm^2)	Lipid phase(s)	d Spacing(s) (Å)	Comments
1	23	7.76	L	51.4	
2	23	7.89	L	51.6	
3	23	7.93	L	51.5	
4	23	8.35	L	51.6	
5	23	8.49	L	51.5	
	23	desiccated	L	51.5	
6	23	8.89	L	51.6	
	23	9.19	L	51.6	

- All samples in a solution of 2 mM TES (pH 7.3) and excess $CaCl_2$ (10:1 Ca^{2+}/phospholipid) for 12 hours.
- All samples centrifuged at 35K for 1 hour.
- L: lamellar phase; LL: coexistence of two lamellar phases.
- Lipid phase and d spacing values were determined from measurements of each diffraction pattern.
- Each phase symbol represents an individual X-ray experiment.

TABLE 3:
FULLY HYDRATED DOPS/DG MULTILAMELLAR SYSTEMS EXPOSED TO Ca^{2+}

Mole % DG	Temp. (°C)	log P (dynes/cm^2)	Lipid phase(s)	d Spacing(s) (Å)	Comments
0	23	-----	L	51.4	
17	23	-----	L	51.5	
33	23	-----	L	51.5	slightly diffuse reflections
44	23	-----	L	55.3	diffuse reflections & central scattering
50	23	-----	L	64.2	diffuse reflections & central scattering

- All samples hydrated overnight in 1 ml of 2mM TES (pH 7.3).
- All samples exposed to excess $CaCl_2$ (10:1 Ca^{2+}/lipid) for 1-2 hours.
- All samples centrifuged at 35K for 1 hour.
- L: lamellar phase.
- Lipid phase and d spacing values were determined from measurements of each diffraction pattern.
- Each phase symbol represents an individual X-ray experiment.

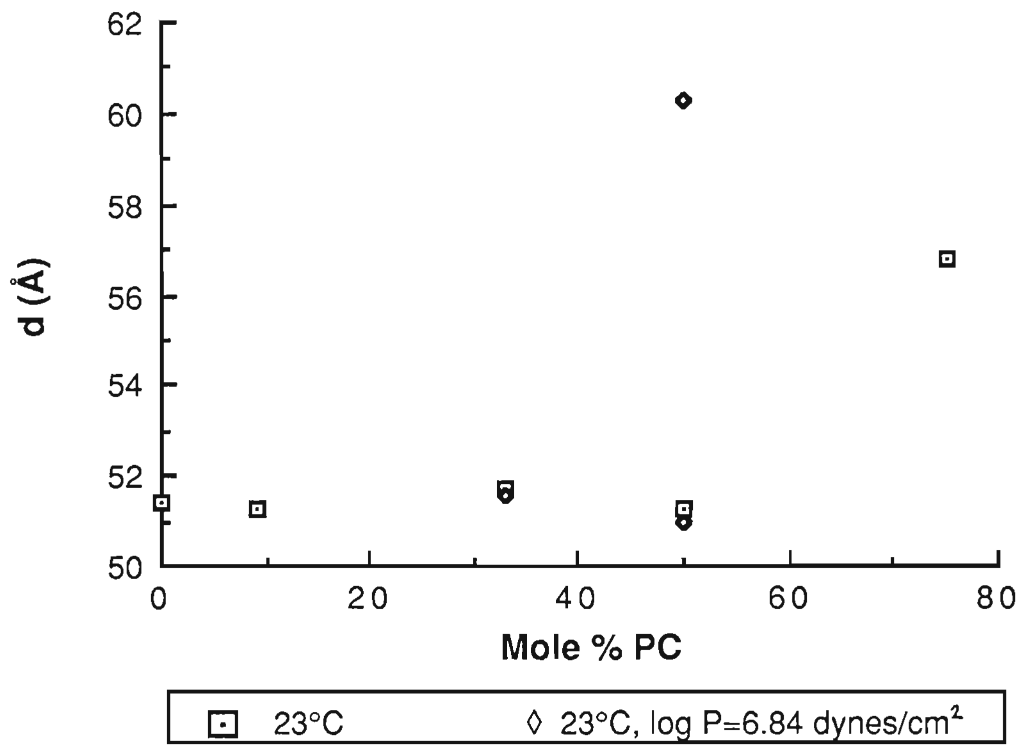
as sharp as those of the 0 and 17% DG samples indicating a slightly disordered phase. A sample with a DG content of 44 mole % yielded 2 orders of diffuse reflections indicating a disordered L phase of 55.3 Å; central scattering suggested that much of the lipid might either be contained in amorphous structures, or be fully hydrated (swollen) without exposure to Ca^{2+}. Reflections of an equimolar DOPS/DG sample were almost identical to those of the previous sample but with a d spacing of 64.2 Å. Although it would be difficult to detect any DG that was excluded from this phase, these results suggested that up to ~30 mole % DG could be accomodated in the $Ca(DOPS)_2$ phase and that higher DG concentrations modified the Ca^{2+}-induced bilayer interactions resulting in lamellar phases of larger dimensions (proportional to the DG content). Central scatter indicated the presence of amorphous structures; was this evidence of bulk lipid segregation? Was Ca^{2+} access to all the lipid being limited by these multilamellar systems?

(D) Fully Hydrated DOPS/DOPC Multilamellar Systems Exposed to Ca^{2+}

Since it does not form ordered structures in aqueous solution, the presence of bulk segregated DG could not have been detected in the previous series. Accordingly, we decided to use PC as a neutral lipid since its behaviour at different levels of hydration and in the presence of Ca^{2+} has been well documented by this lab and others. In order to match hydrocarbon chains and prevent nonideal mixing we chose DOPC; thus, the species of the binary mixtures differed only in headgroup structure. This series was designed as a preliminary X-ray study to characterize the behaviour of fully hydrated DOPS/DOPC multilamellar systems exposed to excess Ca^{2+} for 1-2 hours (Fig. 4; see also Appendix Table 1). Pure DOPS in Ca^{2+} at 23°C again yielded the 51.4 Å L phase. A phase with identical dimensions was identified in samples with molar DOPC contents of 9, 33, and 50%, respectively. The first and second order reflections of the sample containing 33 mole % DOPC were slightly diffuse, and all reflections from the equimolar sample were quite diffuse. A sample containing 75 mole % DOPC yielded two orders of diffuse reflections indicating a single 56.8 Å L phase, with central scatter suggesting that much of the lipid might

Figure 4: The Effects of Excess Ca^{2+} on Fully Hydrated DOPS/DOPC Multilamellar Systems

Graph showing the effect of mole percent PC content on lamellar phase d spacings following 1 to 2 hour incubations of fully hydrated DOPS/DOPC multilamellar systems in Ca^{2+} solution (Ca^{2+}/phospholipid=10). Samples were recovered by centrifuging at 35K for 1 hour and analyzed by X-ray diffraction. The samples of 33 mole % and 50 mole % PC were also subjected to an osmotic stress of log P=6.84 dynes/cm^2 and reanalyzed. In samples where bulk phase separation was indicated an identical symbol appears twice, indicating the d spacings of the two separate L phases determined by X-ray diffraction.



62
60
58
56
54
52
50
d (Å)
0
20
40
60
80
Mole % PC
23°C
23°C, log P=6.84 dynes/cm²

be contained in amorphous structures. When osmotically stressed (log P=6.84 dynes/cm^2) the sample with 33 mole % DOPC yielded the same 51.6 Å L phase. With an identical osmotic stress the equimolar sample showed bulk phase separation into two L phases, one corresponding to the collapsed 51.4 Å complex and the other, with a spacing of 60.3 Å, corresponding closely to PC, as determined by Lis et al. (1981) (see also Appendix Table 8).

Similar to the previous experiments with DG this study suggested that ~30 mole % PC was accomodated in the dehydration-insensitive Ca(DOPS)$_2$ phase. Higher levels of PC modified Ca^{2+}-induced bilayer interactions resulting in more disordered lamellar phases, increases in lattice dimensions at PC contents >50 mole %, and amorphous lipid structures. A possible interpretation however is that the multilamellar model systems might be limiting the access of Ca^{2+} to only a portion of the lipid present which collapses and forms a lamellar phase while the central scattering may be indicative of swollen bilayers exposed to little or no Ca^{2+}.

In the equimolar sample, it appeared that when stressed osmotically to collapse and order any amorphous structures, either the amorphous lipid was becoming ordered and yielding a second phase, or the osmotic stress technique itself was causing bulk phase segregation (this latter possibility was subsequently verified using sucrose density gradients; see Fig. 9).

In order to circumvent these difficulties we decided on the use of (i) 1.0 µm unilamellar vesicles, produced by reverse phase evaporation (REV), to ensure immediate access of Ca^{2+} to all the lipid in a sample; and (ii) twelve hour incubation times to ensure complete equilibration of Ca^{2+} with all the lipid present in a sample.

(E) DOPS/DOPC REV Exposed to Ca^{2+}

This series of X-ray experiments was designed as a trial using the new unilamellar vesicle system and incubation protocol (Fig. 5; see also Appendix Table 2). Aliquots from these DOPS/DOPC REV samples were examined using rapid-mixing, spray-freezing, freeze-fracture electron microscopy to characterize the lipid morphology responsible for the central scatter

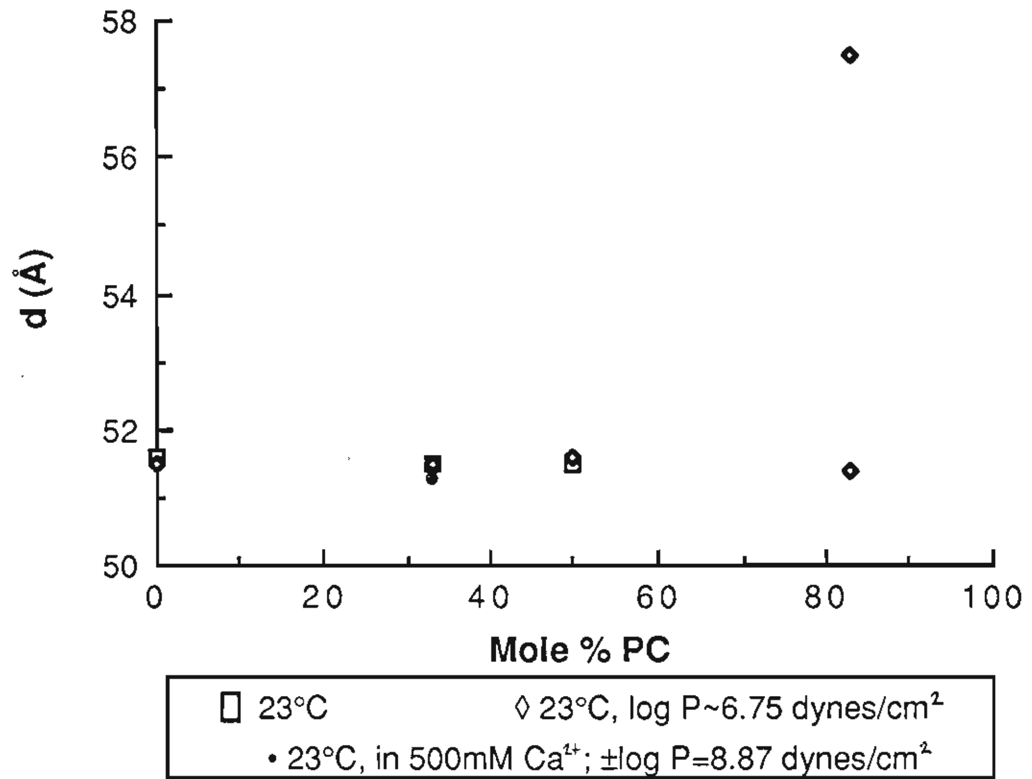
and diffuse X-ray reflections previously observed at higher PC contents (Appendix Table 1). X-ray diffraction of these same samples indicated a L phase of ~51.4 Å up to 50 mole % DOPC, however the reflections obtained from this equimolar sample were diffuse. The freeze-fracture studies revealed dispersions of separated unilamellar vesicles of approximately uniform size in all samples before the addition of Ca^{2+} (Fig. 6a). After rapid-mixing and one hour incubations in 10mM Ca^{2+} the samples revealed multilamellar structures at 0, 33, and 50 mole % PC with coexisting aggregations of uncollapsed vesicles in the 33 and 50 mole % samples (Fig. 6b, c, d). At higher PC contents (83 mole %) X-ray diffraction yielded only central scatter and the micrographs revealed only aggregated vesicles (Fig. 6e).

After mild osmotic stress (log P=~6.75 dynes/cm^2) there was no change in the diffraction patterns obtained from the samples containing 0, 33, and 50 mole % PC. There were indications from this X-ray analysis of bulk phase separation in the 83 mole % PC sample, although there was only a first order reflection for each phase; assuming these both to be lamellar, then one corresponded to 51.4 Å and the other to 57.5 Å. Central scatter suggested that much of the lipid was still contained in vesicles.

A neutral lipid such as PC will bind cations such as Ca^{2+} and form charged bilayers that repel each other to indefinite separations (Rand, 1981). At high ionic concentrations however, cations remaining in solution will "screen" such charged bilayers thus reducing electrostatic repulsion and resulting in their separation to some equilibrium spacing (Lis et al., 1981; Rand, 1981). If at lower concentrations of Ca^{2+}, a DOPC-rich phase is segregated out of the collapsed phase and forms amorphous structures then the use of high cation concentrations to ensure ionic screening should allow for the detection of such phases by X-ray diffraction. Following this rationale, a sample of REV containing 33 mole % DOPC was exposed to 500mM Ca^{2+}. This sample yielded X-ray diffraction data that was quantitatively and qualitatively identical to that of the sample exposed to the lower Ca^{2+} concentration (Fig. 5; Appendix Table

Figure 5: The Effects of Excess Ca^{2+} on DOPS/DOPC REV Systems

Graph showing the effect of mole percent PC content on lamellar phase d spacings following 12 hour incubations of DOPS/DOPC REV suspensions in Ca^{2+} solution (Ca^{2+}/phospholipid=10). Samples were recovered by centrifuging at 35K for 1 hour and analyzed by X-ray diffraction. The samples of 0, 33, 50, and 83 mole % PC content were also subjected to an average osmotic stress of log P=6.75 dynes/cm^2 and reanalyzed. A second 33 mole % PC sample was exposed to 500mM Ca^{2+} and analyzed before and after an osmotic stress of log P=8.87 dynes/cm^2. In samples where bulk phase separation was indicated an identical symbol appears twice, indicating the d spacings of the two separate L phases determined by X-ray diffraction.



58
56
54
52
50
d (Å)
0
20
40
60
80
100
Mole % PC
23°C
◊ 23°C, log P~6.75 dynes/cm²
• 23°C, in 500mM Ca²⁺; ±log P=8.87 dynes/cm²

Figure 6: The Effects of Excess Ca^{2+} on DOPS/DOPC REV Systems; A Rapid-Mixing, Spray-Freezing, Freeze-Fracture Electron Microscopy Study

Electron micrographs of DOPS/DOPC REV suspensions before (a) and after ~1 hour in Ca^{2+} solution (Ca^{2+}/phospholipid=10). All samples were rapid-mixed and freeze-fractured using standard techniques and Balzers equipment.

(a) Uniform REV dispersion seen in all samples before Ca^{2+} incubation; magnified 9,000 times.

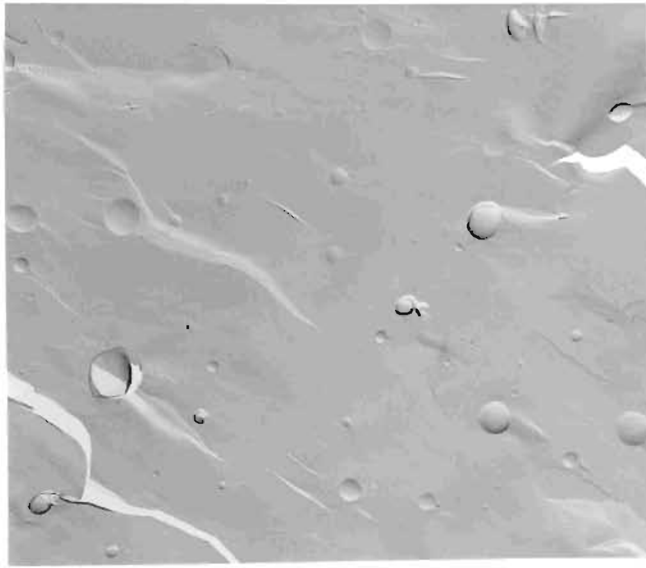
(b) DOPS REV; magnified 53,000 times.

(c) DOPS/DOPC REV (33 mole % PC); magnified 35,000 times.

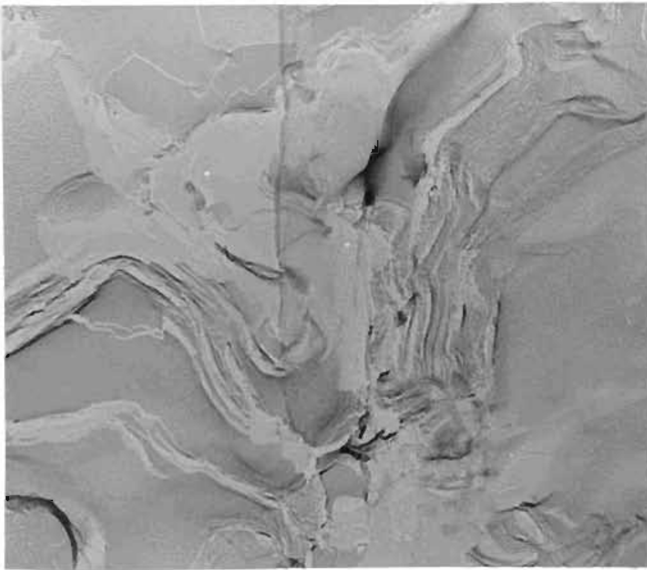
(d) DOPS/DOPC REV (50 mole % PC); magnified 22,000 times.

(e) DOPS/DOPC REV (83 mole % PC); magnified 22,000 times.

(a)

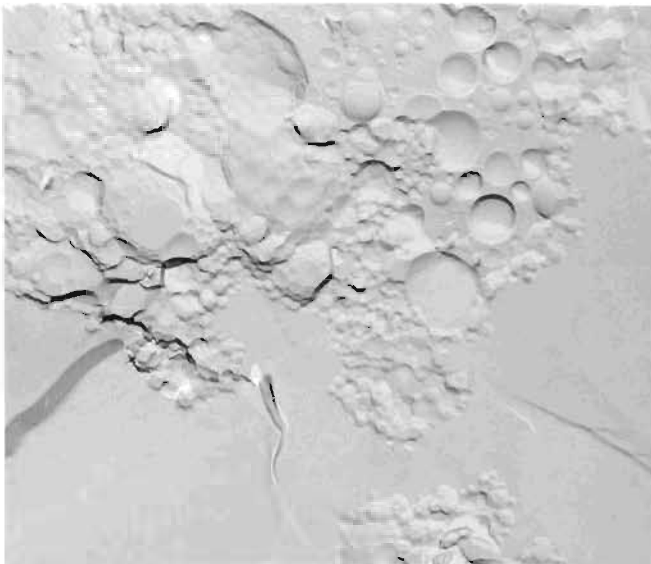


(b)

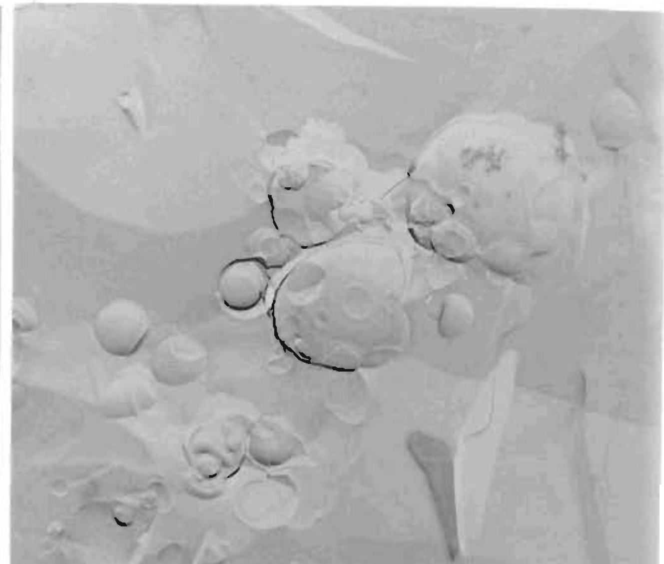


(c)

(d)



(e)



2). This sample also proved to be insensitive to even strong osmotic stress (log P=8.87 dynes/cm^2). These results therefore appeared to confirm that up to ~30 mole % PC is accomodated in the $Ca(DOPS)_2$ phase. Higher PC levels seemed to modify the vesicle interactions yielding less collapse and fusion of REV, as confirmed by freeze-fracture electron microscopy. In order to verify the lipid compositions of the binary systems it was decided that subsequent samples would be examined by TLC both before REV preparation and after final X-ray analysis to qualitatively confirm the presence and proportions of PS and neutral lipid in each.

(F) Further Protocols and a Trial Using Sucrose Density Gradients

In order to further verify that DOPC was, as the data seemed to indicate, in the collapsed $Ca(DOPS)_2$ phase and that no bulk lipid segregation occurred at the higher levels of PC, two more protocols were established. First, if PC was segregated (bulk) from the $Ca(DOPS)_2$ we attempted to separate these phases by density gradient centrifugation. It was hoped that this method, in conjunction with X-ray diffraction and TLC, would (i) confirm the presence of PC in the collapsed phase by a change in density; or (ii) allow us to isolate bulk separated phases, a long-standing problem in this field. Second, each sample of Ca^{2+}-precipitated lipid was briefly sonicated and recovered by centrifugation in order to free any trapped bulk segregated PC before density gradient centrifugation. A trial experiment was performed to establish the best procedure for optimizing the gradient centrifugation technique (Table 4). A suspension of DOPS/DOPC REV containing 33 mole % PC was incubated in Ca^{2+}. An aliquot of this sample yielded X-ray reflections of a single 51.5 Å L phase that was stable against osmotic stress (log P=6.88 dynes/cm^2). A second aliquot was centrifuged on a density gradient of 0 to 25 weight percent sucrose and yielded a single band. This band was recovered from the gradient and one aliquot was immediately analyzed by X-ray diffraction while the other was washed twice in fresh Ca^{2+}-TES solution prior to X-ray analysis (see Table 4). The sample in sucrose yielded a 52.5 Å L phase which became sharper and shrank to 51.4 Å (with frozen chains) when

TABLE 4:
DOPS/DOPC REV EXPOSED TO Ca^{2+}: TRIAL EXPERIMENT USING A CONTINUOUS SUCROSE DENSITY GRADIENT

Mole % PC	Temp. (°C)	log P (dynes/cm^2)	Ca^{2+} Precipitate Lipid phase	d Spacing (Å)	In Sucrose Sol'n Lipid phase	d Spacing (Å)	Washed in Ca^{2+}-TES Lipid phase	d Spacing (Å)
33	23	-----	L	51.5 *	L	52.5 *	L	51.5 *
	23	6.88	L	51.5 *	L	51.4 +	L	51.3 +

- Each sample was an aliquot from a stock of DOPS/DOPC REV's exposed to a solution of 2 mM TES (ph 7.3) and excess $CaCl_2$ (10:1 Ca^{2+}/phospholipid) for 12 hours (sucrose gradients were also made with this Ca^{2+}-TES stock).
- All samples centrifuged at 35K for 1 hour; sucrose gradients centrifuged at 10K for 1 hour; washed samples centrifuged at 35K for 1 hour in the Ca^{2+}-TES solution (repeated twice).
- Examination of the sample by TLC before REV preparation, and after final X-ray analysis qualitatively confirms the presence and proportions of DOPS and DOPC.
- L: lamellar phase; *: slightly diffuse reflections; +: faint indications of frozen chains.
- Lipid phase and d spacing values were determined from measurements of each diffraction pattern.
- Each phase symbol represents an individual X-ray experiment.

osmotically stressed (log P=6.88 dynes/cm^2). The washed sample yielded a 51.5 Å L phase which became sharper following an identical dehydration protocol (log P=6.88 dynes/cm^2). Therefore, we established a protocol involving two washes in fresh Ca^{2+} solution for samples recovered from sucrose gradients. The data in Table 4 also indicate that the sonication before centrifugation yielded no changes in structure (as determined by X-ray diffraction) compared to previous findings. These results indicate that sonication was either ineffective in separating bulk segregated L phases or that DOPS and DOPC existed in the same phase.

(G) Density Gradient Analyses of DOPS/DOPC REV Exposed to Ca^{2+}

All density gradient centrifugations yielded single bands with specific sample densities proportional to PC content (see Figs. 7, 8, and 9). This was further confirmed by pooling Ca^{2+}-preprecipitated aliquots of 0, 17, and 33 mole % PC samples which separated on the gradients according to their PC content and which had similar d spacings, equal to the 51.4 Å collapsed phase (Appendix Table 5, fractions designated f3, f2, and f1, respectively). Thus, collating all density data from three studies that utilized sucrose gradients (see Appendix Table 6) and examining the relationship between sample density and mole % PC content, Figure 9 shows that the results of all gradient analyses were similar. The line of best fit for the density values in Figure 9 was determined by simple linear regression and has a correlation coefficient (R) of 0.98. In all experiments TLC confirmed the approximate proportions of DOPS and DOPC in each sample.

A single lamellar phase with ordered acyl chains and a d spacing of about 51.4 Å was observed at PC contents of up to 17 mole %, following incubation in excess Ca^{2+} (Fig. 10a, see also Appendix Tables 3 and 4). An identical L phase was observed at PC contents of 33 and 50 mole % although the reflections were diffuse and there were no detectable high angle indications of chain ordering; central scatter in the equimolar DOPS/DOPC samples indicated the presence of amorphous lipid structures. At contents of 75, 83, 89, and 100 mole % DOPC each sample produced central scattering, indicating that much of the lipid was contained in amorphous

Figure 7: The Effects of Excess Ca^{2+} on DOPS/DOPC REV Systems; Results of Exposure to a Continuous Density Gradient of 0 to 11 Wt. % Sucrose

Preparation and X-ray data appear in Appendix Table 4 .

Gradient #1 - DOPS/DOPC (50 mole % PC)

Gradient #2 - DOPS/DOPC (75 mole % PC)

Gradient #3 - DOPS/DOPC (83 mole % PC)

Gradient #4 - DOPS/DOPC (89 mole % PC)

Gradient #5 - DOPC

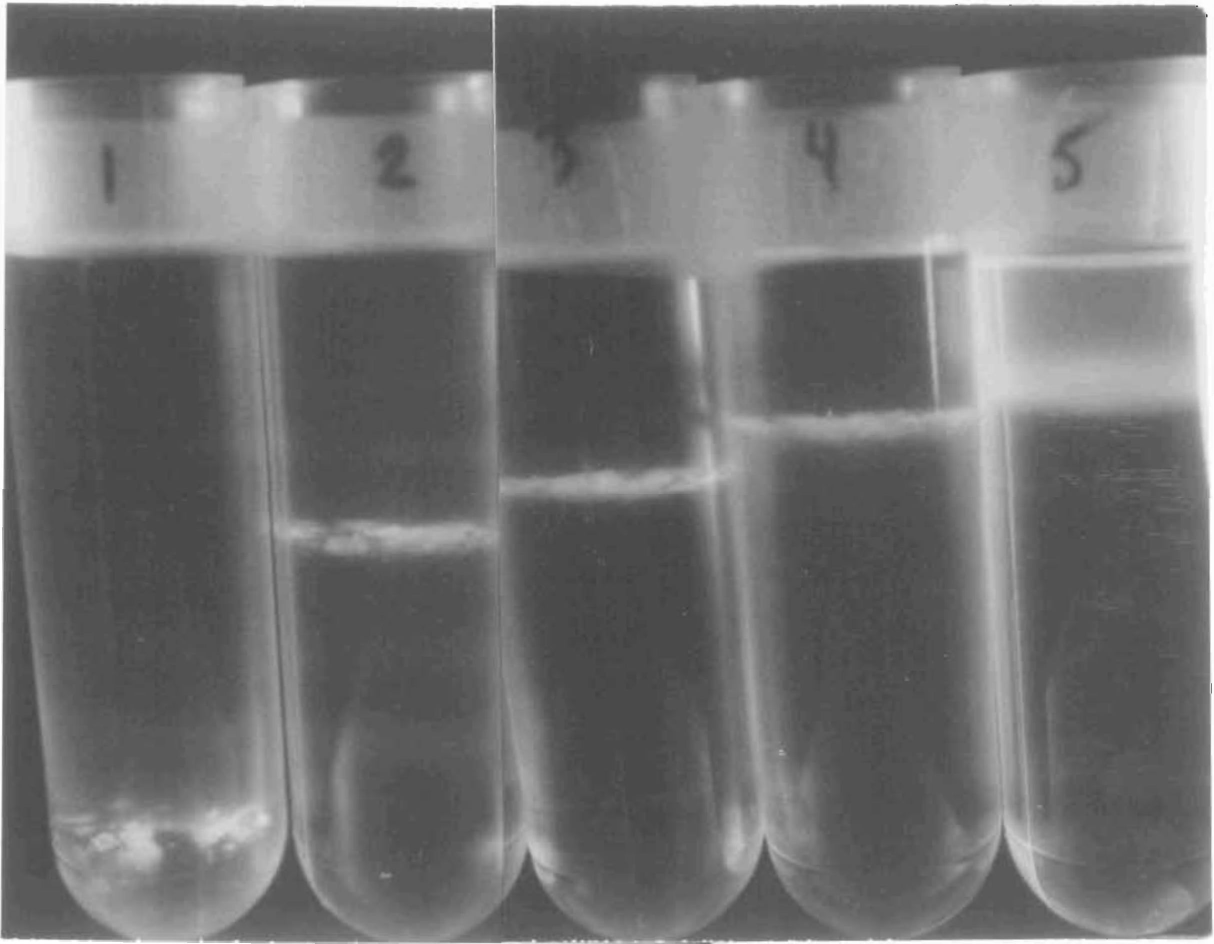

1
2
3
4
5

Figure 8: The Effects of Excess Ca^{2+} on DOPS/DOPC REV Systems After Exposure to a Freeze-Thaw Protocol; Results of Exposure to a Continuous Density Gradient of 1 to 22 Wt. % Sucrose

Preparation and X-ray data appear in Appendix Table 5 .

Gradient #1 - DOPS

Gradient #2 - DOPS/DOPC (17 mole % PC)

Gradient #3 - DOPS/DOPC (33 mole % PC)

Gradient #4 - DOPS/DOPC (50 mole % PC); note bulk phase separation

Gradient #5 - DOPS/DOPC (83 mole % PC)

Gradient #6 - DOPC

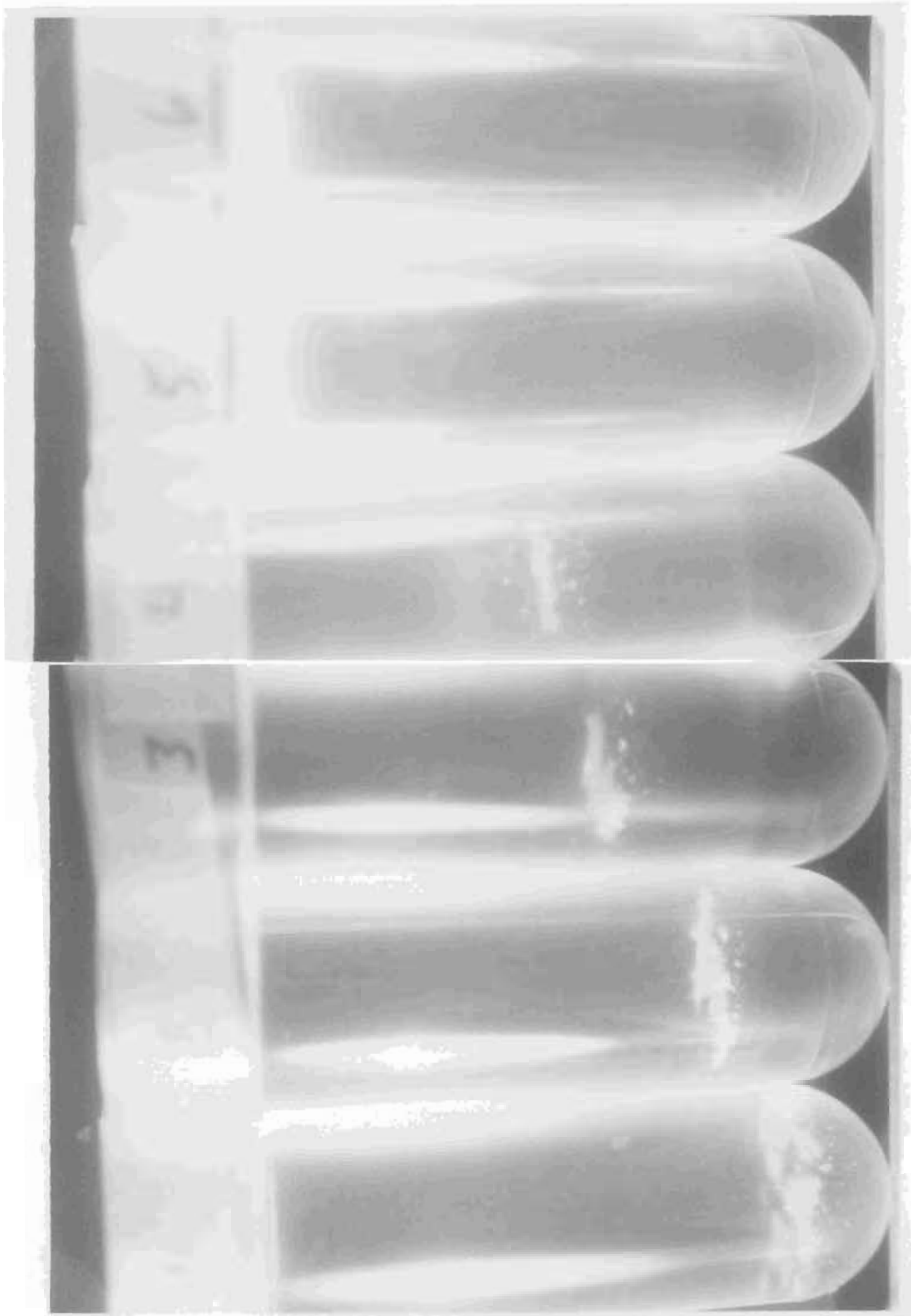
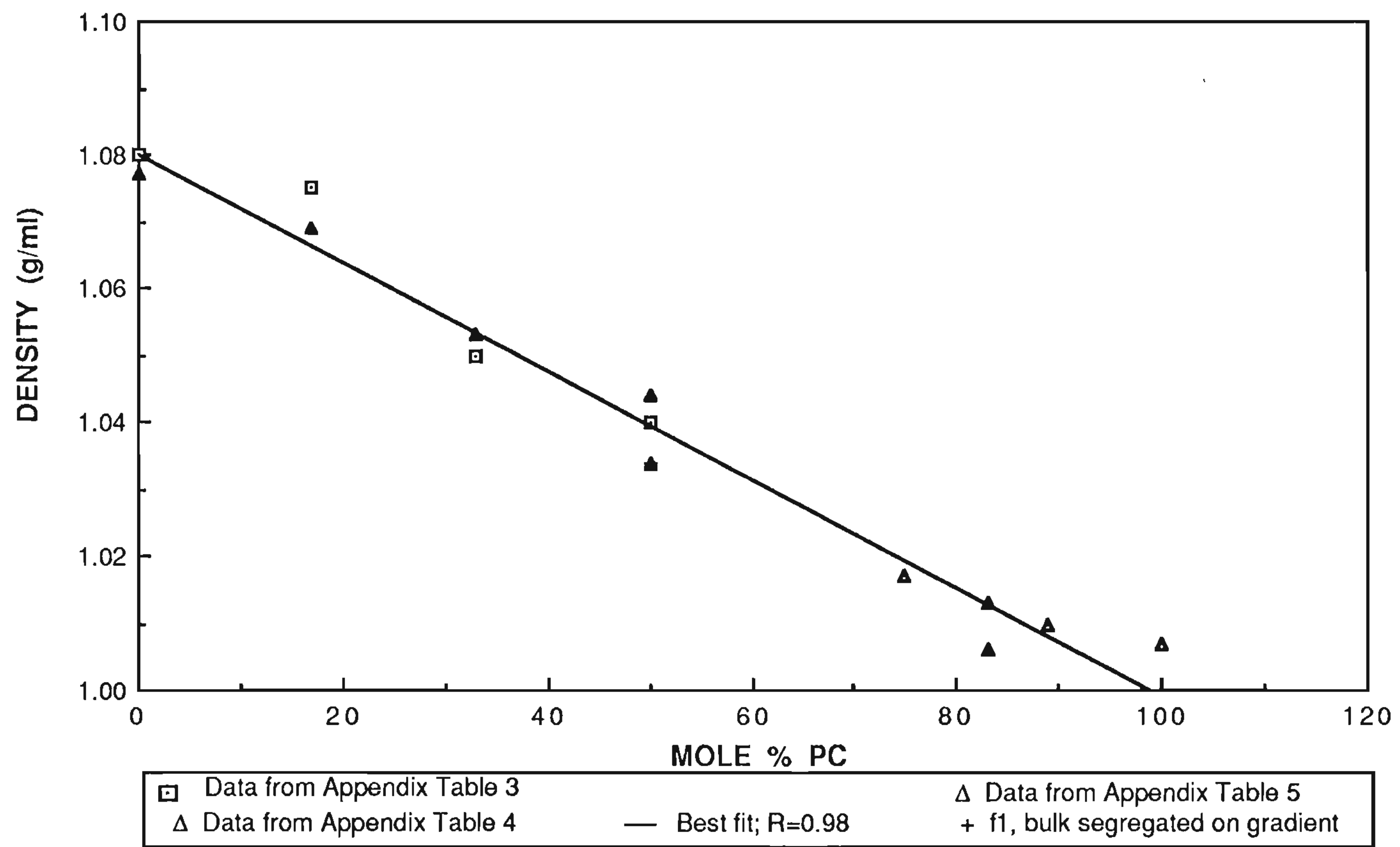


Figure 9: Effect of Mole Percent PC Content on Densities of DOPS/DOPC REV Exposed to Excess Ca^{2+}

Graph showing the relationship between DOPS/DOPC sample density and mole percent PC content following incubations in excess Ca^{2+} (see Appendix Table 6). Line of best fit has a correlation coefficient (R) of 0.98.


1.10
1.08
1.06
1.04
1.02
1.00
DENSITY (g/ml)
0
20
40
60
80
100
120
MOLE % PC
Data from Appendix Table 3
Δ Data from Appendix Table 4
— Best fit; R=0.98
Δ Data from Appendix Table 5
+ f1, bulk segregated on gradient

structures, and diffuse reflections indicating a single L phase, the dimension of which increased with the increasing PC levels up to ~59 Å for pure DOPC.

Using gentle osmotic stress (log P=~6.86 dynes/cm^2) to collapse and order any amorphous structures in these samples revealed the 51.4 Å phase to be dehydration-insensitive up to PC contents of 33 mole % (Fig. 10b). The central scatter was eliminated from the equimolar sample which yielded bulk segregated phases of 51.5 Å and 57.6 Å. The pure DOPC samples each yielded a single, diffuse 56.6 Å L phase. The samples containing 75-89 mole % PC were damaged during application of the osmotic stress technique and thus no dehydration data is available for these samples.

A control sample of pure DOPC REV in Ca^{2+}, not exposed to a sucrose gradient, yielded similar data. This sample yielded sharp reflections of a 60.3 Å L phase, with the presence of central scatter (data in Appendix Table 4) suggesting some vesicle aggregation rather than complete collapse. Gentle osmotic stress revealed a 55.0 Å L phase with some central scatter remaining.The similarity in the behaviour of this DOPC sample and those exposed to the sucrose gradient indicate that the collapse of pure PC REV was not an artifact resulting from the use of a sucrose gradient, although sucrose almost certainly provides some osmotic stress (log P=~6-7 dynes/cm^2 for the sucrose concentrations used in these gradients). This additional stress, though slight, may account for the complete collapse of these systems observed following the osmotic stress protocol, while the control sample exposed to the same protocol still shows some central scattering.

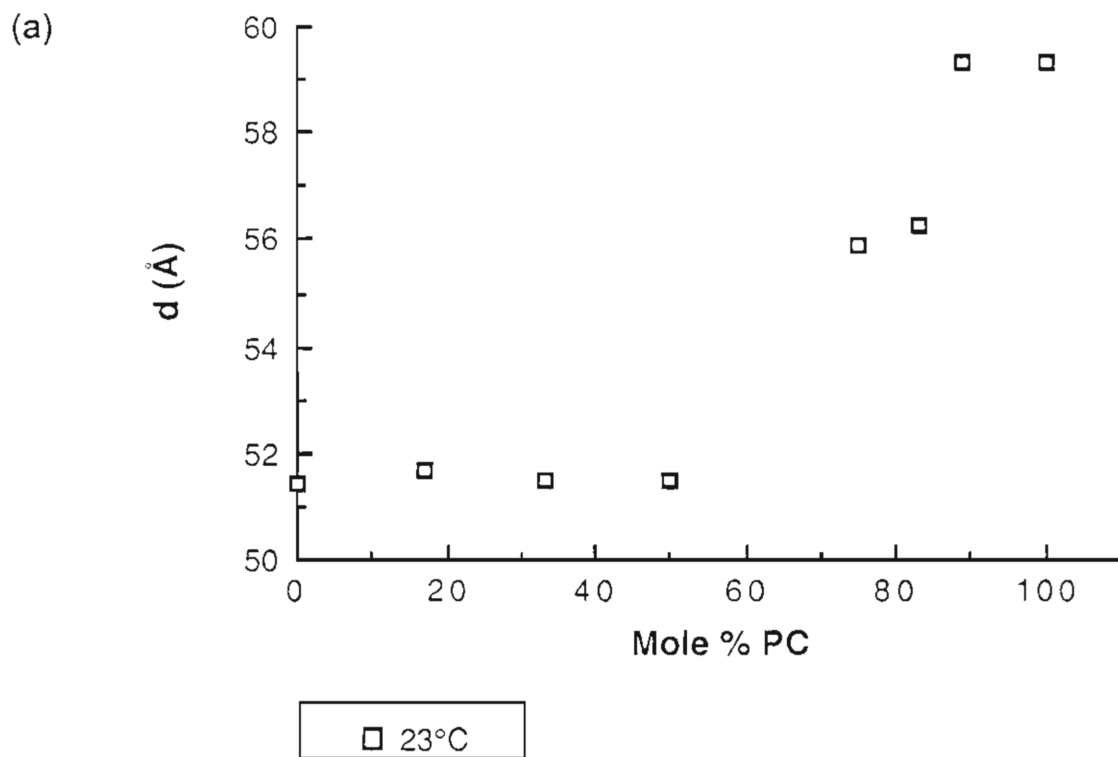
Together the results of this study suggested that up to ~30 mole % PC could be accomodated in the dehydration-insensitive $Ca(PS)_2$ phase. Higher PC levels modify vesicle interactions resulting in a reduced capacity for REV fusion/collapse and the presence of disordered liquid-crystalline L phases that increase in dimension at PC contents above 50 mole %.

The only sample analyzed by density centrifugation to show bulk phase separation on a

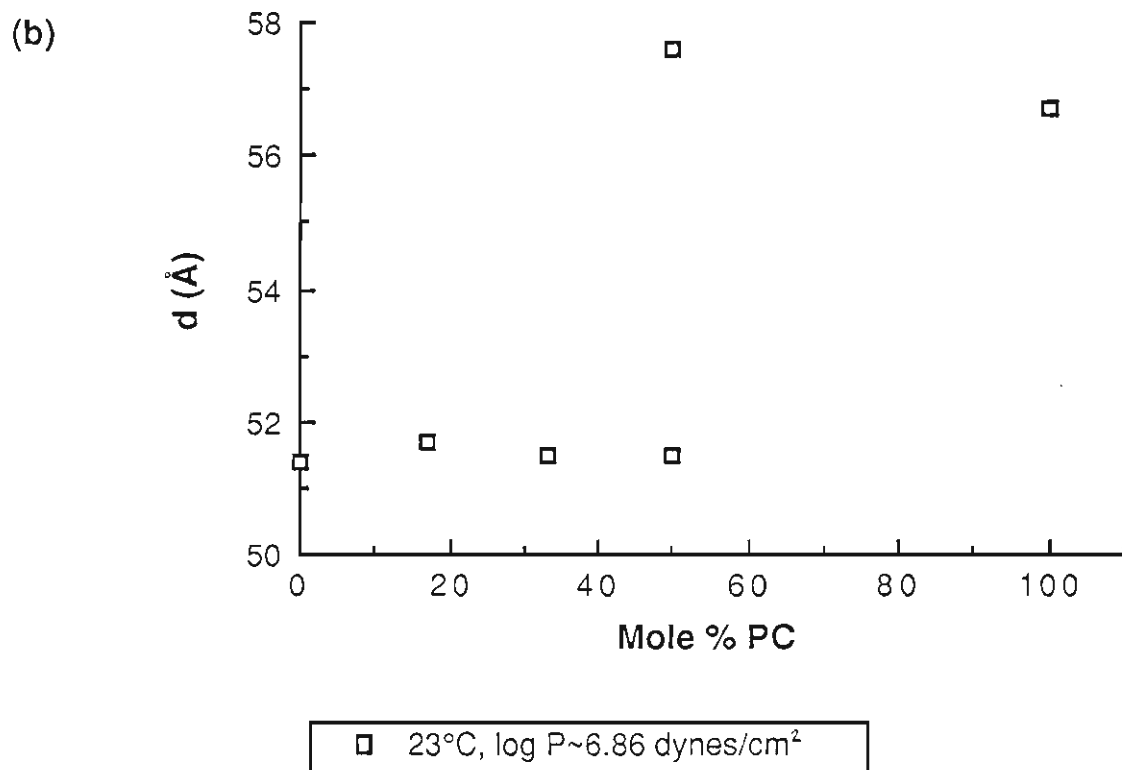
Figure 10: The Effects of Excess Ca^{2+} on DOPS/DOPC REV Systems; X-Ray Diffraction Study Following Sucrose Density Gradient Analyses

Graph (a) showing the effect of mole percent PC content on lamellar phase d spacings following standard 12 hour Ca^{2+} incubation, recovery by centrifugation, and subsequent analysis by sucrose density gradient centrifugation. The samples of 0, 17, 33, and 50 mole % PC and 75, 83, 89, and 100 mole % PC were analyzed on continuous density gradients of 6-20 wt. % and 0-11 wt. % sucrose, respectively. Samples were recovered by washing twice in Ca^{2+} solution at 35K for 1 hour and reanalyzed by long exposure (~10-12 hours/sample) X-ray diffraction. Samples of 0, 17, 33, 50, and 100 mole % PC were also subjected to an osmotic stress of log P=6.86 dynes/cm^2 and reanalyzed (b). In samples where bulk phase separation was indicated an identical symbol appears twice, indicating the d spacings of the two separate L phases determined by X-ray diffraction.

(a)



(b)



gradient (Figs. 8, 9, and 11) was an equimolar DOPS/DOPC sample that had been subjected to a freeze-thaw protocol (to ensure Ca^{2+} equilibration) prior to centrifuging on a sucrose gradient. The dimension of one diffuse phase (f2) corresponded to the collapsed $Ca(PS)_2$ complex, with central scatter indicating that much of the lipid was in amorphous structures, while the other (f1; less dense) was a slightly diffuse L phase of 53.7 Å (Fig. 11 and Appendix Table 5). Feigenson (1988a) has quite recently shown that freeze-thaw cycling alone (in the absence of Ca^{2+}) can induce lipid phase segregation in similar PS/PC systems, indicating that it is no longer the equilibration method of choice in binary lipid systems. This suggests that the bulk phase segregation observed in this equimolar sample may have been temperature-induced, rather than being indicative of any Ca^{2+} effect.

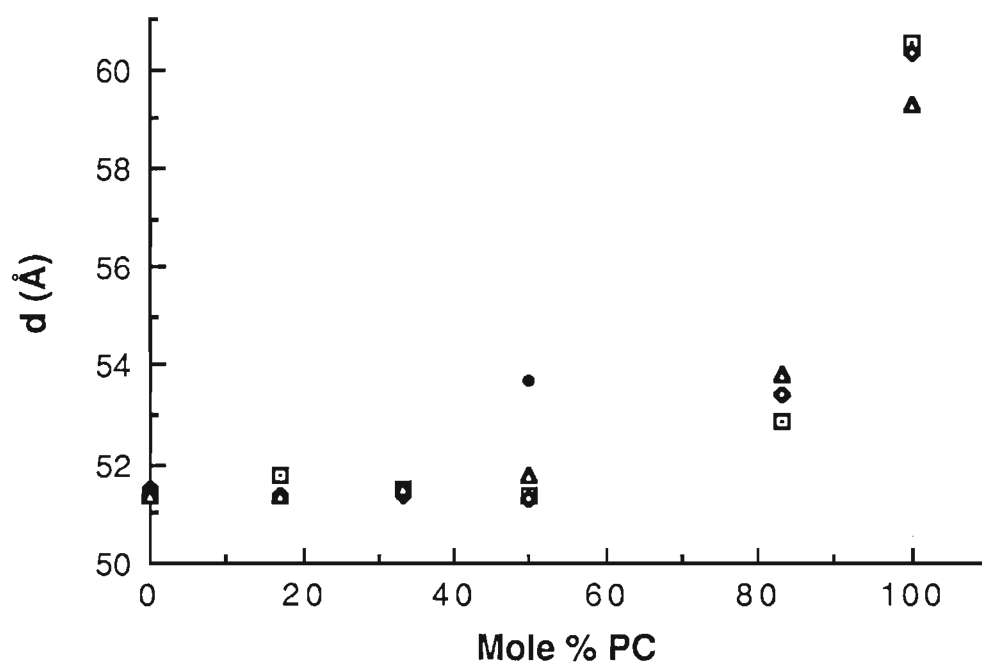
(H) Ensuring Ca^{2+} Equilibration

The protocol using the combination of density gradient, X-ray diffraction, and TLC, in conjunction with the osmotic stress technique, provided further evidence that up to 30 mole % DOPC can be accomodated in the anhydrous $Ca(DOPS)_2$ phase and that no bulk lipid segregation occurs at any PS/PC ratio from REV. However, there were still some suggestions that Ca^{2+} may not be equilibrated with all the lipid in a given sample (Feigenson, personal communication). As freeze-thaw cycles were, until quite recently, the accepted method for such equilibration a comparative study was conducted (Fig. 11; see also Appendix Table 5). Figure 11 indicates that d spacing data are all similar regardless of whether a sample had been incubated in Ca^{2+} for 12 hours, or had additionally been through 10 freeze-thaw cycles, or had been spun on a sucrose gradient. The data in Appendix Table 5 confirm the qualitative similarities (chain ordering, central scatter, and quality of reflection) between samples having the same PC content. TLC was again used before and after the experimental protocols to confirm the lipid contents and approximate proportions in each sample.

All data were qualitatively and quantitatively similar to previous results; lower PC

Figure 11: The Effects of Excess Ca^{2+} on DOPS/DOPC REV Systems; Comparing the Twelve Hour Incubation, Freeze-Thaw, and Density Gradient Protocols

Graph showing the effect of mole percent PC content (DOPS/DOPC REV suspensions) on lamellar phase d spacings following either (i) the standard 12 hour incubations in Ca^{2+} solution (Ca^{2+}/phospholipid=10); (ii) an added 10 freeze-thaw cycles in the Ca^{2+} solution; or (iii) a subsequent exposure to a density gradient of 1 to 22 wt. % sucrose. Note bulk phase separation in equimolar PS/PC sample (see also Fig. 10, gradient #4). Samples were recovered from sucrose by washing twice in Ca^{2+} solution at 35K for 1 hour and all samples were analyzed by long exposure (~10-12 hours/sample) X-ray diffraction. In samples where bulk phase separation was indicated an identical symbol appears twice, indicating the d spacings of the two separate L phases determined by X-ray diffraction.



⊡ 23°C, incubated in Ca^{2+}	Δ 23°C, after sucrose gradient
◊ 23°C, after freeze-thaw protocol	• f1, bulk segregated on gradient

concentrations (≤~50 mole %) appear to be accomodated in the collapsed 51.4 Å L phase, although the phases become disordered and coexist with amorphous structures above ~30 mole % PC, and the lamellar repeat increases at PC levels above 50 mole %. Again, the X-ray data indicated that pure DOPC REV systems yield a characteristic L phase of ~60.4 Å (59.3 Å from the sucrose gradient). Central scattering, at least from the freeze-thawed sample, indicated that some of the DOPC was contained in amorphous structures.

(I) PS/PC Systems: Cumulative Results of X-Ray and Density Analyses

Fig. 12 collates all the data on lamellar phase dimensions in fully hydrated DOPS/DOPC systems in relation to mole % PC content at 23°C. The density gradient and X-ray diffraction data in both Fig. 12 and Appendix Table 6 suggest that there is no bulk lipid segregation between 0-100 % PC from REV. The lamellar repeat spacings correspond to the collapsed $Ca(DOPS)_2$ phase up to equimolar PS/PC; reflections becoming diffuse at contents above ~30 mole % PC, with central scattering at PC levels ≥ 50 mole %. Freeze-fracture studies (Fig. 6) suggest that this central scattering corresponds to intact REV, which are amorphous to X-ray analysis. Furthermore, all samples showed a consistent decrease in density in proportion to their PC content (Fig. 9, and Appendix Table 6), confirming that PS and PC were in the same phase. No bulk lipid segregation into two lamellar phases, at any PS/PC ratio, was ever found by X-ray diffraction prior to the application of osmotic stress. Similarly, no bulk segregated lipid phases were identified on the sucrose gradients.

To summarize, the results indicate that DOPC levels above ~30 mole % modify Ca^{2+}-induced vesicle interactions resulting in reduced REV fusion/collapse but that up to 50 mole % DOPC can be accomodated in a collapsed, 51.4 Å lamellar phase. At higher DOPC contents (>50 mole %) those REV that do collapse yield L phases of larger dimension. This does not imply bulk lipid segregation since the density gradient analyses reveal only one band at all DOPS/DOPC ratios; the lipid compositions of all REV (intact and collapsed) must be identical from the start. The

Figure 12: The Effects of Excess Ca^{2+} on PS/PC Systems; Cumulative Results of X-Ray Studies at 23°C

Graph showing the effect of mole percent PC content on lamellar phase d spacing following exposures of fully hydrated DOPS/DOPC systems to excess Ca^{2+}. In samples where bulk phase separation was indicated an identical symbol appears twice, indicating the d spacings of the two separate L phases determined by X-ray diffraction.

□ DOPS/DOPC multilamellar systems (Fig. 4)

◊ DOPS/DOPC REV (Fig. 5)

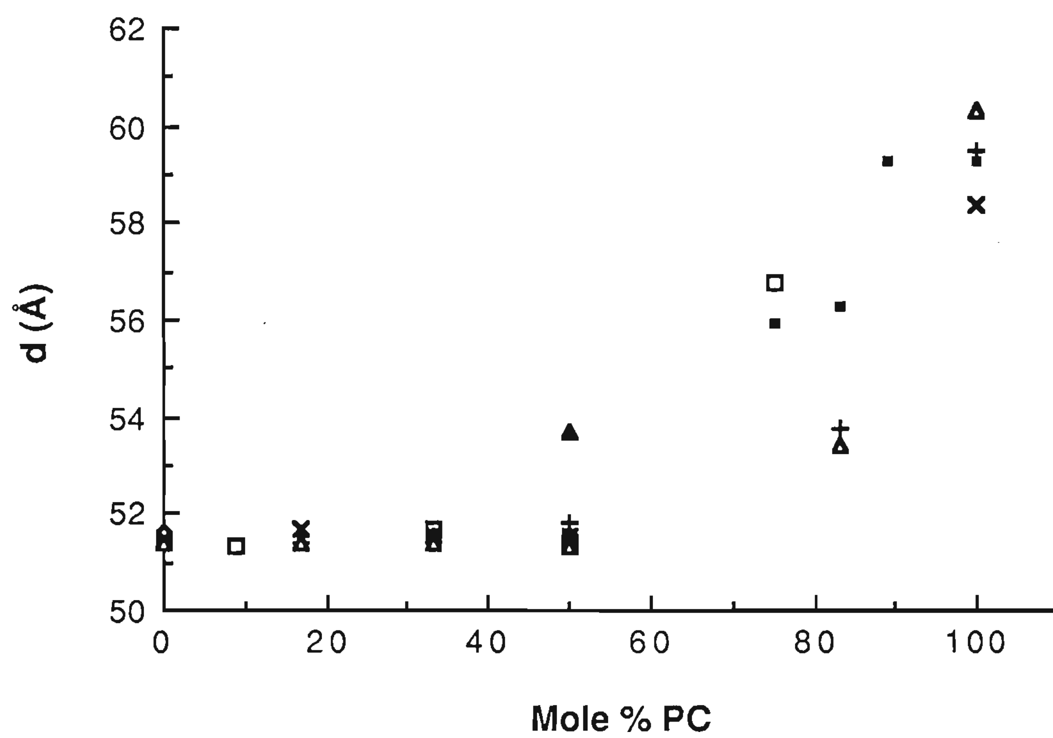
Δ DOPS/DOPC REV with freeze-thaw protocol (Fig. 11)

X DOPS/DOPC REV recovered from gradient (Fig. 10 & Appendix Table 3)

■ DOPS/DOPC REV recovered from gradient (Fig. 10 & Appendix Table 4)

+ DOPS/DOPC REV with freeze-thaw protocol, recovered from sucrose gradient (Fig. 11)

▲ Bulk segregated fraction of DOPS/DOPC (1:1) recovered from sucrose gradient after freeze-thaw protocol (Fig. 11)


62
60
58
56
54
52
50
d (Å)
0
20
40
60
80
100
Mole % PC

results imply a reduced tendency for REV to fuse and/or collapse at higher (>~50 mole %) PC contents. With increasing PC/PS ratios, the L phases that do arise are progressively PC-enriched as indicated by the corresponding decrease in densities (Fig. 9); consistent with this is the increase in d spacings at PC levels above ~50 mole %. At a content of 89 mole % DOPC the d spacing matches that of pure DOPC (Fig. 12, and Appendix Table 4). The original data from hydrated multilamellar systems follows a similar general trend (Fig. 12), indicating (i) accomodation of DOPC in the dehydration-insensitive, 51.4 Å collapsed phase up to a content of ~30 mole %; (ii) a disordered 51.3 Å L phase at equimolar DOPS/DOPC, subject to osmotically-induced bulk segregation (yielding the 51 Å and a 60 Å phase); and (iii) a disordered L phase of about 57 Å at a content of 75 mole % DOPC, with central scatter indicating that much of the lipid is contained in amorphous structures (Appendix Table 1).

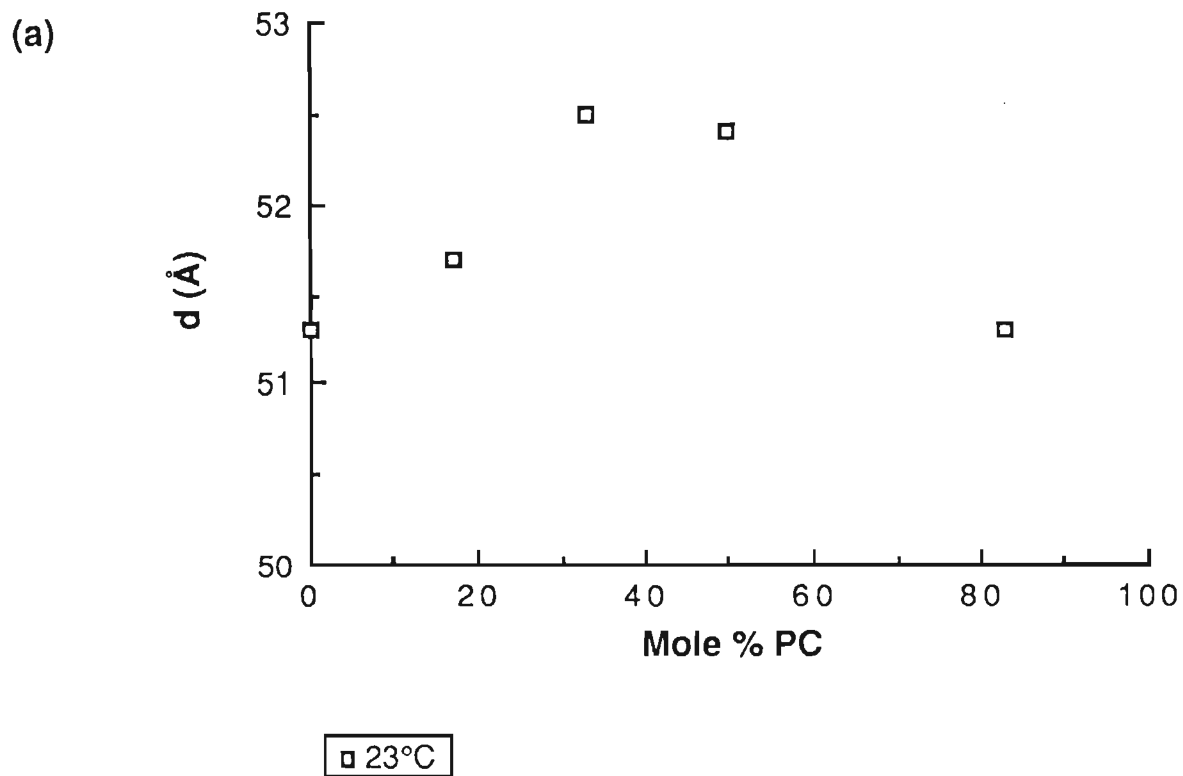
(J) BBPS/DOPC REV Exposed to Ca^{2+}: A Preliminary Study

Considering all the evidence indicating that low levels of DOPC were not bulk segregated during the ordering of the PS hydrocarbon region inherent in the formation of the $Ca(PS)_2$ phase, we sought to verify that this was not simply a species-specific phenomenon associated with DOPS. Since the hydrocarbon region of PS extracted from bovine brain (BBPS) becomes much more well-ordered in the presence of Ca^{2+} than does that of DOPS (Jacobson and Papahadjopoulos, 1975; Newton et al., 1978; Papahadjopoulos et al., 1977; Portis et al., 1979) it would be expected to more likely exclude PC from the dehydrated $Ca(PS)_2$ phase. At both 23 and 37°C a collapsed L phase (51.3 Å) with frozen chains existed up to PC contents of at least 17 mole %, reflections being slightly diffuse at this content (Fig. 13a and Appendix Table 7). At a molar PC content of 33% reflections were slightly diffuse and a single L phase of 52.5 Å, with frozen chains, existed at both temperatures. An equimolar BBPS/DOPC sample yielded diffuse reflections of a 52.4 Å L phase, with central scatter, at 23 and 37°C. With 83 mole % DOPC diffuse reflections of a 51.3 Å phase could be identified at 23°C, and at 37°C this phase

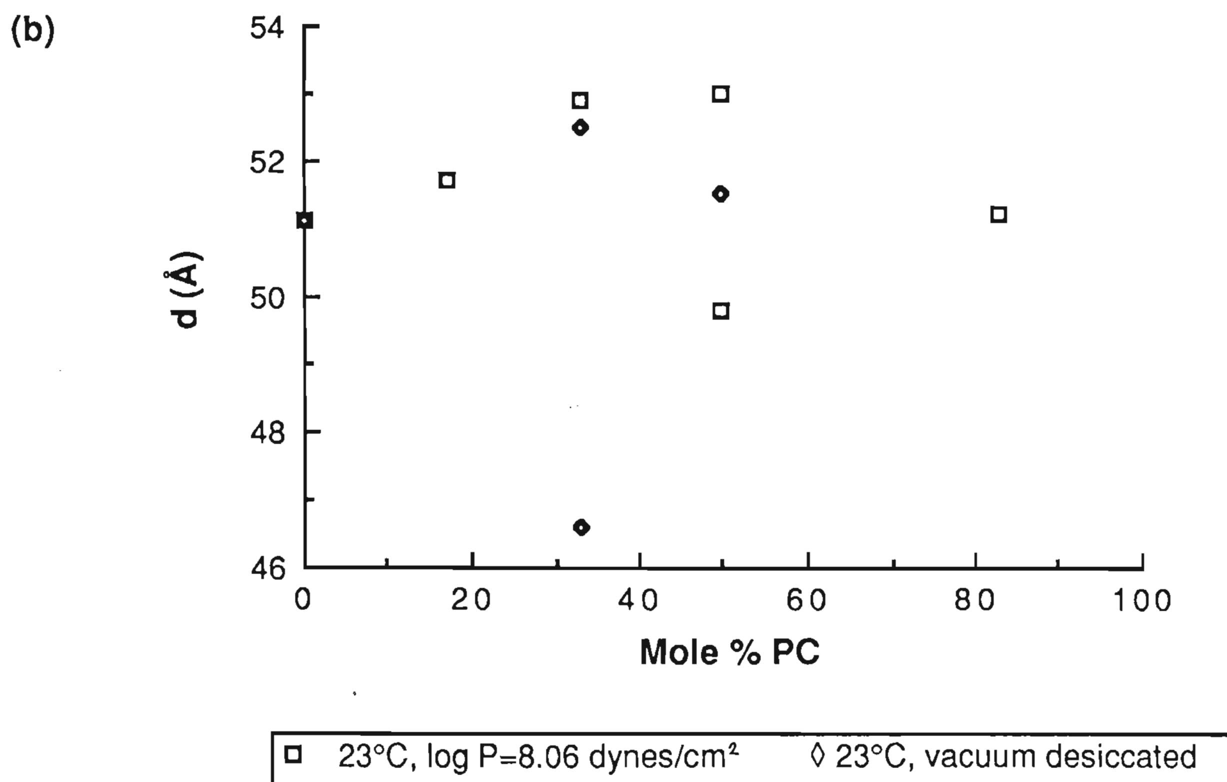
Figure 13: The Effects of Excess Ca^{2+} on BBPS/DOPC REV Systems

Graph (a) showing the effect of mole percent PC content on lamellar phase d spacings following 12 hour incubations of BBPS/DOPC REV suspensions in Ca^{2+} solution (Ca^{2+}/phospholipid=10). Samples were recovered by centrifuging at 35K for 1 hour and analyzed by long exposure (~10-12 hours/sample) X-ray diffraction. All samples were also subjected to an average osmotic stress of log P=8.06 dynes/cm^2 and reanalyzed (b). In samples where bulk phase separation was indicated an identical symbol appears twice, indicating the d spacings of the two separate L phases determined by X-ray diffraction.

(a)



(b)



shrank to 50.6 Å; central scatter indicated that much of the lipid was in amorphous structures.

The use of osmotic stress (log P=8.06 dynes/cm^2) to collapse and order the amorphous structures, and vacuum desiccation to further characterize the resulting L phases at 23°C, (Fig. 13b) revealed that the collapsed 51.3 Å L_β phase was stable up to PC contents of 17 mole %. The 52.5 Å phase formed at 33 mole % PC was stable under osmotic stress, but desiccation resulted in bulk phase separation into one phase of this same dimension and one of dimension 46.6 Å (only a first order reflection), with high angle lines indicative of strongly ordered chains. The equimolar sample yielded bulk separated L phases of 49.8 Å and 53.0 Å under osmotic stress, accompanied by diffuse reflections and strong indications of frozen chains. Only diffuse reflections of a 51.5 Å L phase with frozen chains remained following desiccation. A single 51.2 Å L phase with sharp reflections was identified in the osmotically stressed sample containing 83 mole % PC. The approximate proportions of BBPS and DOPC in each sample were confirmed by TLC. Although more detailed studies will be required to confirm the similarity to the DOPS system these results suggested that PC levels of up to ~30 mole % could be accomodated in the $Ca(BBPS)_2$ phase and that higher concentrations of this neutral lipid modified the Ca^{2+}-induced interactions of bilayer vesicles; the shorter d spacings of these BBPS/DOPC systems at high PC contents suggest that more DOPC can be accomodated by dehydrated BBPS compared to DOPS.

(K) PE and the $Ca(DOPS)_2$ Phase

We undertook some preliminary studies using PE to study the effect of its smaller headgroup and lower hydration in this Ca^{2+}-induced collapse process; might PE be accomodated in a collapsed phase at even higher levels than PC? Considering the strong tendency for this phospholipid to adopt non-bilayer configurations we thought to try two different species, POPE (lamellar at room temperature) and DOPE (H_{II} at room temperature).

Due to their strong preference for the H_{II} phase, single component PE vesicles cannot be

prepared at neutral pH (Szoka, 1987). POPE (T_m=~31°C) forms coexisting L_β and L_α phases in aqueous solution at 23°C and a single L_α phase at 31°C (Appendix Table 9); the presence of Ca^{2+} has no effect (Appendix Table 8), although this may be indicative of an equilibration problem. Ca^{2+}-free DOPS/POPE gravimetric control samples (see Appendix Table 9) showed that POPE had fluid chains in these mixtures (25, 33, and 50 mole % PE) at 23°C, and that d_l was constant. DOPE undergoes the L-H transition at ~10°C in the presence or absence of Ca^{2+} (Appendix Table 8 ; Shyamsunder et al., 1988).

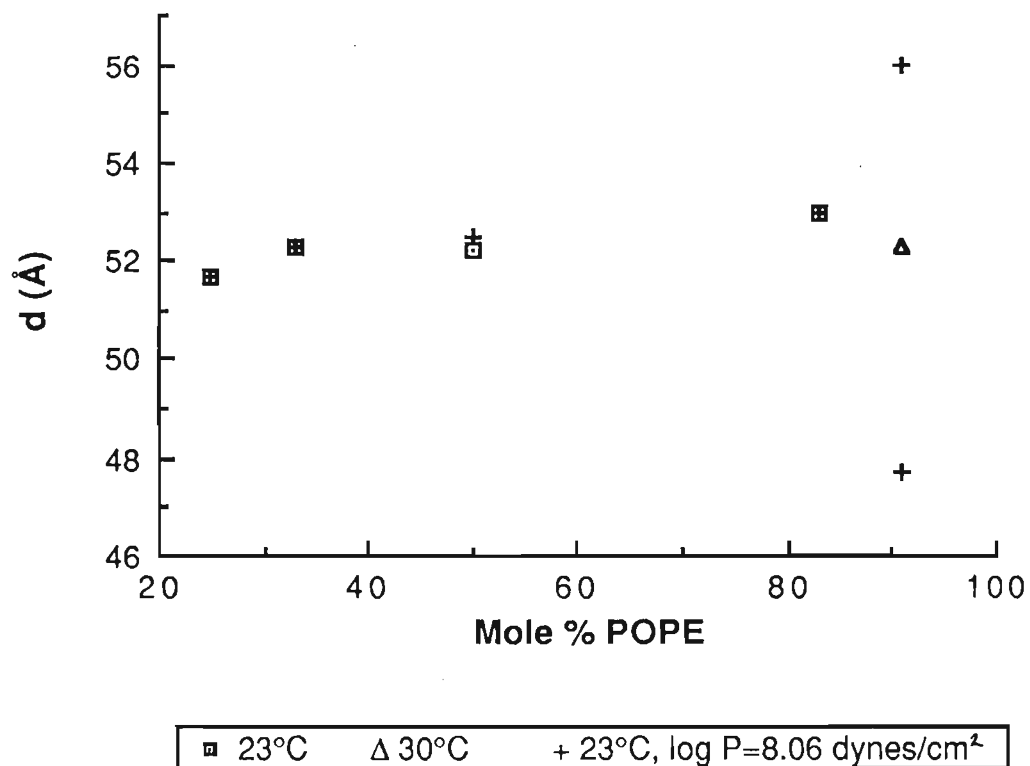
Fig. 14 (a) (see also Appendix Table 10) presents the data from a trial experiment of DOPS/POPE REV exposed to Ca^{2+}. At 25, 33, 50, and 83 mole % PE there were single L_β phases of 51.7, 52.3 Å, 52.2 Å, and 53.0 Å, respectively. Increasing to a PE content of 91 mole % resulted in complicated lipid segregation and frozen chains at 23°C, while at 30°C a single L_α phase of 52.3 Å was observed. A strong osmotic stress (log P=8.06 dynes/cm^2) did not affect the d spacings of the L phases observed in the samples containing 25, 33, 50, and 83 mole % PE. With stronger osmotic stress (log P=8.87 dynes/cm^2) the reflections from the equimolar sample became sharper and a bulk segregated L phase of 52.5 Å was observed to coexist with a smaller amount of a second phase having only a single reflection (~37 Å). With 83 mole % PE, the d spacing increased to 55.1 Å and the chains were frozen, at log P=8.6 dynes/cm^2. At log P=8.06 dynes/cm^2 and 23°C the 91 mole % PE sample showed indications of frozen chains and bulk phase segregation to a prominent 56.0 Å L phase and a L phase of ~48 Å. Under the same pressure a single 51.3 Å L phase with frozen chains was observed at 30°C.

Fig. 14 (b) (see also Appendix Table 11) presents the data from a similar trial experiment exposing DOPS/DOPE REV to Ca^{2+}. Samples containing either 25 or 33 mole % DOPE each yielded a L phase of ~51.6 Å with faint indications of frozen chains. At 50% DOPE there was

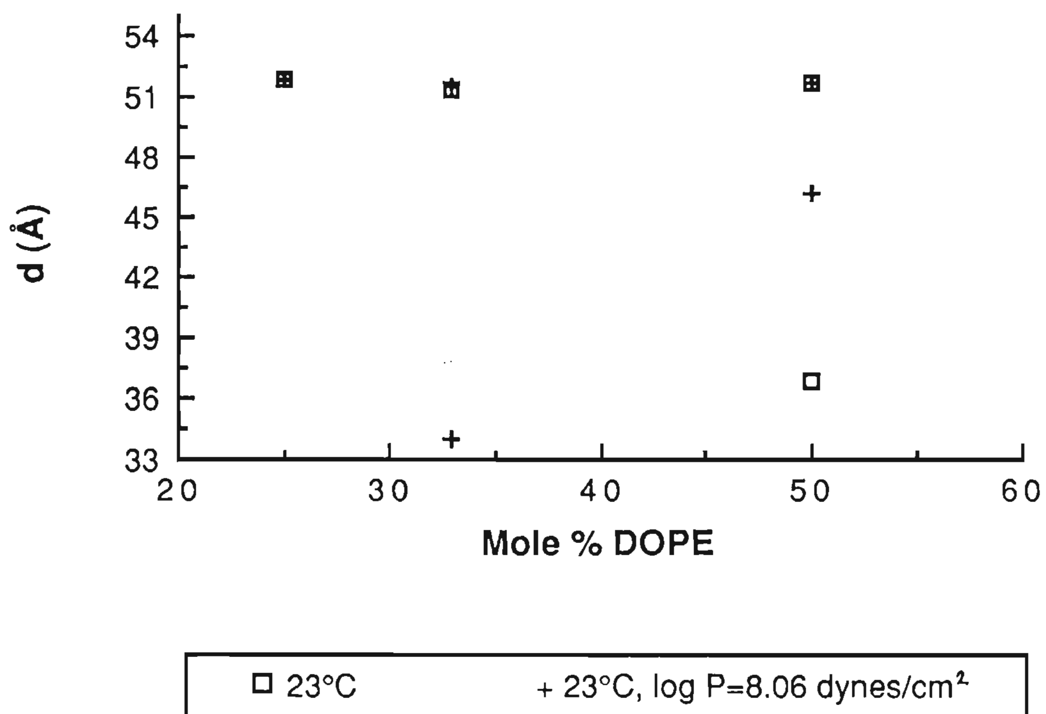
Figure 14: The Effects of Excess Ca^{2+} on DOPS/POPE and DOPS/DOPE REV Systems

Graphs showing the effect of mole percent PE content on phase dimension following 12 hour incubations of (a) DOPS/POPE and (b) DOPS/DOPE REV suspensions in Ca^{2+} solution (Ca^{2+}/phospholipid=10). Samples were recovered by centrifuging at 35K for 1 hour and analyzed by X-ray diffraction. All samples were also subjected to an osmotic stress of log P=8.06 dynes/cm^2 and reanalyzed. In samples where bulk phase separation was indicated an identical symbol appears twice, indicating the d spacings of the two separate phases determined by X-ray diffraction.

(a)



(b)



bulk phase separation into the collapsed phase (51.7 Å) and a second L phase of 36.8 Å. At log P=8.06 dynes/cm^2 there were no changes in the dimensions of the lamellar phases produced in the 25 and 33 mole % DOPE samples. There was also a small amount of a second phase in the 33 mole % PE sample, a phase producing only a single reflection of ~34 Å. The collapsed 51.7 Å phase in the 50% DOPE sample proved to be dehydration-insensitive while the dimension of the second bulk segregated L phase increased to 46.2 Å; the reflections were slightly diffuse, and the chains were frozen following the osmotic stress.

Since the d spacings of the POPE L_α phase and the $Ca(DOPS)_2$ phase differ by only 1-2 Å it is difficult to accurately determine differences between the dimensions of the L phases observed in these preliminary DOPS/POPE systems. Such problems in describing the behaviour of these systems containing POPE emphasizes the importance of using neutral lipid species with equilibrium spacings quite different from the 51.4 Å collapsed phase. Such well-defined changes in lamellar phase dimension were observed in the DOPC-containing systems described above. However, the initial results presented in Figures 14 (a) and (b) (Appendix Tables 10 and 11) suggest that ~25-30 mole % PE may be accomodated in the $Ca(DOPS)_2$ phase, and that even at low (<10 mole %) PS levels, PE-containing REV all collapse when exposed to excess Ca^{2+}. TLC confirmed the approximate proportions of PS and PE in each sample. The data also indicate that high concentrations (≥50 mole %) of lipids preferring non-bilayer configurations (ie. T_h= ~10°C for DOPE) will not form single L phases of larger dimension, as was observed in the systems containing PC, but rather will spontaneously bulk phase separate in the presence of Ca^{2+}. Further studies are required to better characterize these PS/PE systems before definite comparisons to PC-containing systems can be made.

(L) <u>DOPS/DOPC REV Exposed to Mg^{2+}</u>

Considering the tendency for DOPS/DOPC lamellar phases with high PC contents to be disordered and to phase segregate into the 51.4 Å phase and a second L phase of larger dimension

when osmotically stressed, we thought that a more hydrated divalent cation-PS complex might maintain a single phase. Since DOPC behaves much the same in Mg^{2+} as it does in Ca^{2+} (Lis et al., 1980; see also Appendix Table 8) and the complex of PS and Mg^{2+} is not as dehydrated as that with Ca^{2+} (Hauser and Shipley, 1984; Newton et al., 1978; Portis et al., 1979; see also Table 3), it was thought that the Mg^{2+} system might more readily accomodate PC. As indicated by the data in Appendix Table 12 , Mg^{2+} did not appear to cause significant collapse of systems containing 33 or 50 mole % DOPC; central scatter predominates in the X-ray scatterings obtained at both 23 and 37°C. A diffuse bulk segregated phase of ~41 Å also appeared in the equimolar sample at 37°C. This is consistent with the more hydrated nature of Mg^{2+}/PS bilayers (Newton et al., 1978; Portis et al., 1979). Application of osmotic stress (log P=8.06 dynes/cm^2) to collapse these REV to ordered phases yielded clean, sharp reflections of a single L phase in both the 33 and 50 mole % PC samples, with no indications of phase separation at either 23 or 37°C. These results therefore confirmed the prediction that bulk phase segregation of neutral lipid would not be observed in this more hydrated cation/PS complex. TLC before REV preparation and after final X-ray analysis qualitatively confirmed the approximate proportions of DOPS and DOPC in each sample.

DISCUSSION

Initial experiments using DOPS/DOPC multilamellar systems suggested that there were problems with Ca^{2+} equilibration in such systems, particularly at higher PC levels (75 mole %); central scattering indicated that some of the lipid was fully hydrated but not exposed to Ca^{2+}. In order to ensure the exposure of all the lipid to Ca^{2+}, we turned to large unilamellar vesicles (REV) as a model system. These vesicles tend to rupture when they interact in excess Ca^{2+}, allowing for the equilibration of Ca^{2+} with all the lipid of the outer and inner monolayers. Any intact vesicles that remain can subsequently be ruptured osmotically to ensure Ca^{2+} equilibration. Based upon the results of systematic studies using a combination of X-ray diffraction, thin-layer chromatography, density gradient centrifugation and freeze-fracture electron microscopy, in conjunction with an osmotic stress technique, we have constructed two idealized schemes (Figs. 15 and 16) suggested by the results. The first shows the equilibrium structures and their dimensions formed by PS/PC REV exposed to excess Ca^{2+}. The second shows the structural changes that these undergo when osmotically stressed. The results show that up to ~30 mole %, DOPC is in the Ca^{2+}-precipitated lamellar (L_1) phase (Fig. 15a, section 1) and that higher PC contents yield L phases with d spacings that increase as PC contents increase beyond ~50 mole %, approaching the 60 Å limit characteristic of DOPC from these systems (Fig. 15a, section 2). These L phases of larger dimension coexist with intact vesicles suggesting that the REV (Fig. 15a, section 2) do not achieve complete equilibration with Ca^{2+} by the precipitation/incubation protocol used; X-ray diffraction yields central scattering and freeze-fracture electron microscopy (Fig. 6) reveals intact vesicles. In the following we summarize the rational and evidence that yields these idealized schemes.

(A) Identifying the Collapsed $Ca(PS)_2$ Phase

In 70 wt. % buffer solution pure DOPS multilayers swell to an equilibrium spacing of 137 Å (Table 3; Loosley-Millman, 1980; Loosley-Millman et al., 1982). Vacuum desiccation (this

study) results in separation into two L phases of 44.5 Å and 53.1 Å. The X-ray studies (Tables 1 and 2, and Fig. 13) showed that exposure of DOPS or BBPS to excess Ca^{2+} yields a lamellar phase of short spacing (51.4 Å), comparable with previous studies (Feigenson, 1988; Hauser and Shipley, 1984, 1985; Hauser et al., 1977a; Hui et al., 1983; Loosley-Millman, 1980; Newton et al., 1978; Portis et al., 1979). With BBPS this $Ca(PS)_2$ phase has well ordered acyl chains whereas the 51.4 Å phase formed by Ca^{2+} and DOPS is less ordered in the hydrocarbon region. As with previous studies (Loosley-Millman, 1980; Newton et al., 1978; Portis et al., 1979) the $Ca(PS)_2$ phase was found to be free of water; its dimension is insensitive even to prolonged vacuum desiccation (Tables 2 and 3; Appendix Table 7). Conversely, the phases formed by DOPS with Mg^{2+} or Ba^{2+} contained some water that could be removed by vacuum desiccation (Table 3) similar to the results of Hauser and Shipley (1984), Loosley-Millman (1980), Newton et al. (1978), and Portis et al. (1979) with different PS species. The phase formed with Mn^{2+} was collapsed and dehydrated but ~1 Å larger than $Ca(DOPS)_2$. As well as being anhydrous and thus stable against even rigorous dehydration measures, the $Ca(PS)_2$ complex is also stable to temperatures greater than 100°C (Hauser and Shipley, 1984, 1985; Hauser et al., 1977a; Newton et al., 1978; Portis et al., 1979; van Dijck et al., 1978). Thus, the characteristics of a pure $Ca(DOPS)_2$ phase is a d spacing of ~51.4 Å, insensitive to dehydration or increases in temperature, and with only weak high angle lines indicative of chain ordering. In Ca^{2+} solution the L phase formed by BBPS is different only in that it has more well-ordered (frozen) acyl chains.

(B) <u>Identifying Bulk Segregated Neutral Lipid</u>

In 50 wt. % buffer solution pure DOPC multilayers swell to an equilibrium spacing of about 60 Å (Lis et al., 1982). In excess divalent cation solutions these DOPC lamellae become charged and swell indefinitely due to electrostatic repulsion (Lis et al., 1981). When osmotically stressed (log P=~5.5-6.0 dynes/cm^2), these charged systems also yield an equilibrium spacing

of about 60 Å (Lis et al., 1981). Both the quantitative and qualitative X-ray diffraction data (central scattering and phase separation) from the pure DOPC systems (multilamellar and REV) exposed to excess Ca^{2+} or Mg^{2+} in the present study suggest that these samples were not achieving cation equilibration. The DOPC REV systems in this study showed evidence of some collapse to disordered L phases of about 60 Å. The presence of central scattering suggests that a fraction of the lipid did not bind Ca^{2+} but remained in the form of intact REV. The exposure of DOPC REV to excess $CaCl_2$ solution may result in some collapse, probably due to osmotic shrinkage and packing distortions induced by Ca^{2+} binding to the external monolayer (asymmetric Ca^{2+} distribution). Upon centrifuging through a continuous sucrose density gradient any remaining vesicles would be expected to deflate osmotically. The ~1 Å shorter d spacings characteristic of the collapsed DOPC REV recovered from the sucrose gradients may be indicative of the mild dehydration induced by the sucrose solutions.

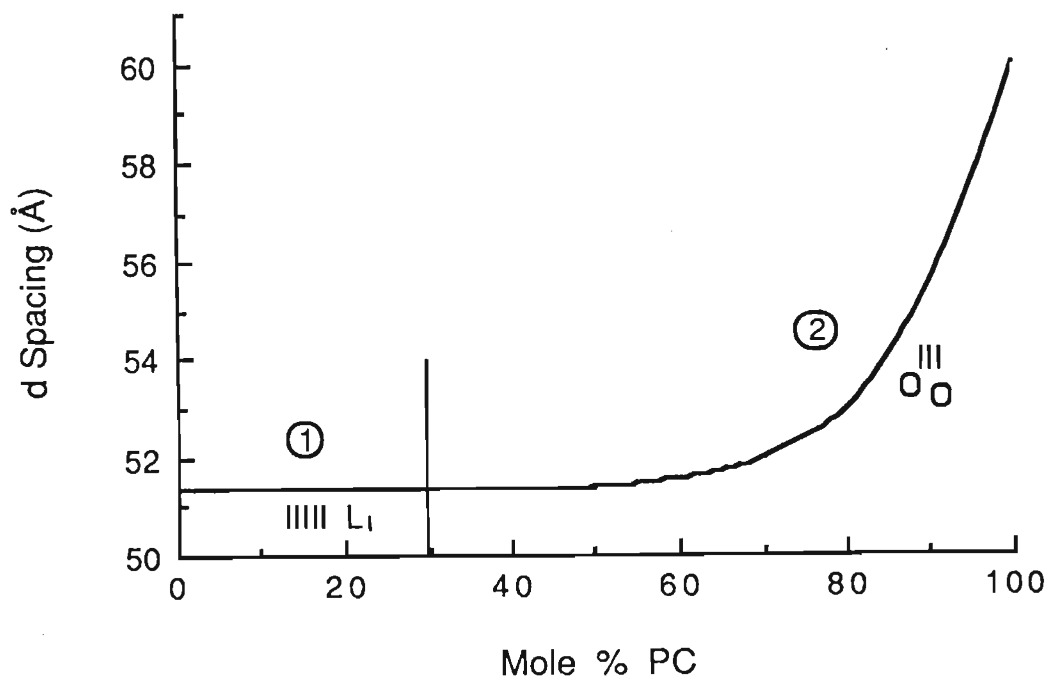
(C) Structural Effects of Exposing PS-containing Lipid Bilayer Vesicles to Ca^{2+} Solutions

Exposing DOPS/DOPC REV to excess Ca^{2+} (Fig. 16) results in the formation of (i) collapsed, 51.4 Å lamellae (L_1) when the PC levels are below ~30 mole % (Fig. 15a, section 1); and (ii) liquid-crystalline lamellae of increasing dimensions (d=51.4-60 Å, depending on PC content) coexisting with amorphous structures at PC levels of ~30-100 mole %. The amorphous structures consist of intact, aggregated uncollapsed vesicles (Fig. 15a, section 2), as confirmed by freeze-fracture electron microscopy (Fig. 6). These coexisting L and vesicular structures could indicate that Ca^{2+}-induced lipid segregation has occurred (ie. that the two structures have different compositions). However, since every DOPS/DOPC sample yielded only one band on the density gradients (Figs. 7 and 8) and densities decreased in proportion to increasing PC-content (Fig. 9) we conclude that no lipid segregation occurred in section 2 (Fig. 15a) and that the coexisting structures have the same composition. For this reason we turned to the osmotic stress technique since all the lipid in a sample will be structured into a lamellar phase by this

Figure 15: Idealized Schemes Showing the Lamellar Phases Formed by DOPS/DOPC REV Systems: The Effects of Excess Ca^{2+} and Osmotic Stress

Figures (a) and (b) compare the global compositions of the lipid samples prior to Ca^{2+}-precipitation with the dimensions of the resulting lamellar phases. Figure (a) is the schematic diagram for Ca^{2+}-precipitated systems. Region 1 represents the collapsed, 51.4 Å lamellar phase (L_1) formed at PC contents of less than ~30 mole %. Region 2 represents REV of higher PC content and limited capacity for collapse, a region of increasing lamellar phase dimensions and coexisting intact vesicles. Figure (b) is the schematic diagram for Ca^{2+}-precipitated systems following osmotic stress. Stable, single lamellar phase regions exist at high and low PC contents, respectively. The dehydration-insensitive 51.4 Å L_1 phase is found at PC contents below ~30 mole %, while a single liquid-crystalline lamellar phase, with a repeat spacing close to that of pure PC, exists at PC levels above ~80 mole %. The intervening region 3 represents systems susceptible to osmotically-induced bulk phase segregation yielding the 51.4 Å L_1 phase and L_2 phases with higher d spacings.

(a)



(b)

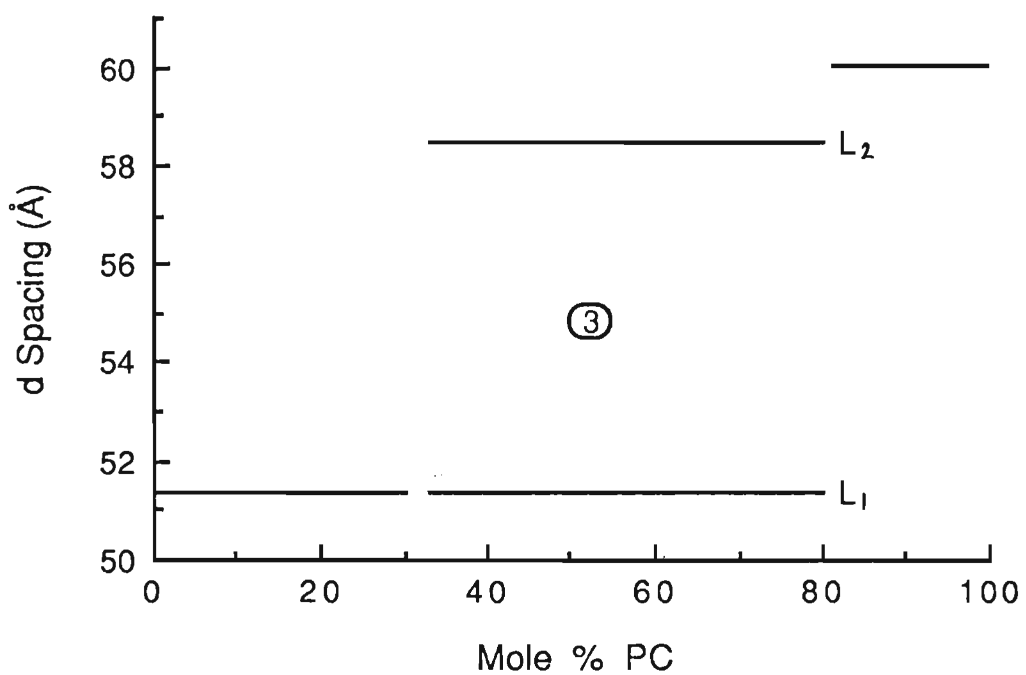
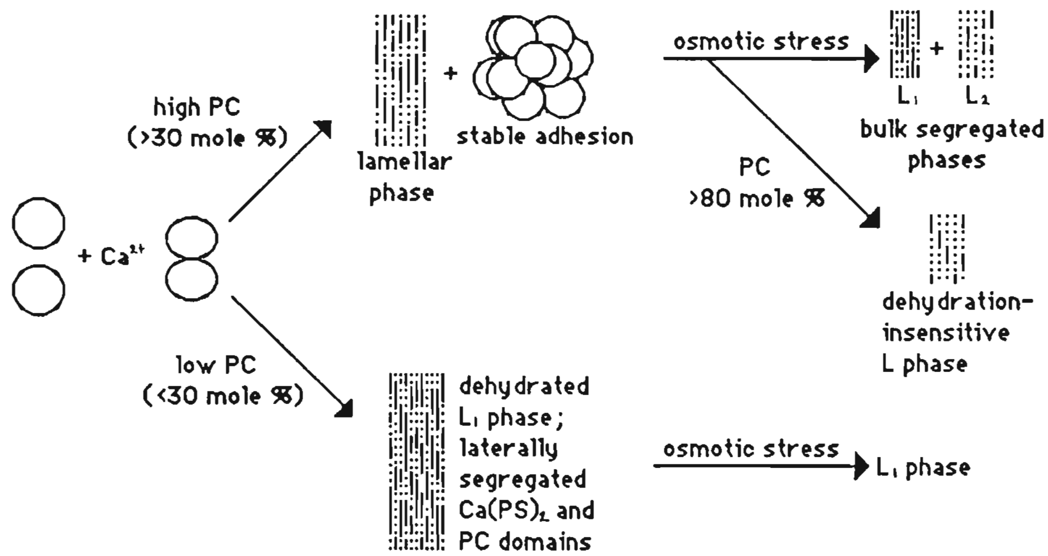


Figure 16: Structural Changes and Equilibrium States of PS/PC REV Systems Exposed to Excess Ca^{2+} and Osmotic Stress

Schematic diagram summarizing the changes that specific bilayer vesicles undergo when induced to interact by the addition of excess $CaCl_2$.

- adapted from Rand and Parsegian (1988).


+ Ca2+
high PC
(>30 mole %)
lamellar
phase
+
stable adhesion
osmotic stress
+
L1
L2
bulk segregated
phases
PC
>80 mole %
dehydration-
insensitive
L phase
low PC
(<30 mole %)
dehydrated
L1 phase;
laterally
segregated
Ca(PS)2 and
PC domains
osmotic stress
L1 phase

method and thus be identifiable by X-ray diffraction; ie. vesicles and any other amorphous structures will collapse to L phases. Applying the osmotic stress technique to the DOPS/DOPC REV systems after equilibration in excess Ca^{2+} (Fig. 16) yielded (i) the collapsed, dehydration -insensitive 51.4 Å L_1 phase at PC contents of less than ~30 mole % (Figs. 15b and 16); (ii) coexisting L_1 and L_2 phases (bulk phase segregation) at PC contents of ~30-80 mole % (Fig. 15b, section 3); and (iii) a single L phase at PC contents above 80 mole %.

Since the compositional similarity of the coexisting L and vesicular structures (Fig. 15a, section 2) had already been confirmed, we concluded that the observed bulk phase segregation (Fig. 15b, section 3) was an artifact of dehydration. In all examples of osmotically-induced bulk phase segregation, X-ray diffraction revealed the 51.4 Å L_1 phase and an L_2 phase of higher d spacing (Fig. 15b, section 3, and Fig. 16). Feigenson (1988a) has recently suggested that the freeze-thaw protocol is inappropriate for simply equilibrating Ca^{2+} in such mixed PS/PC systems since temperature changes alone can induce phase separation, even in the absence of Ca^{2+}. The one instance of bulk phase segregation observed on a density gradient, without osmotic stress, is thus likely the result of employing this freeze-thaw technique. Interestingly, a DOPS/DOPC sample containing ~80 mole % PC showed no evidence of bulk phase segregation, even after the freeze-thaw protocol. Similarly, a BBPS/DOPC system containing ~80 mole % PC did not separate into two bulk phases upon dehydration. This supports the simple interpretation (Fig. 15b) that there is a second <u>single</u> phase region, at PC levels >~80 mole %, in these osmotically stressed DOPS/DOPC mixtures.

One way to confirm these results at intermediate to high PC contents would be to perform a systematic study on Ca^{2+}-precipitated DOPS/DOPC REV (containing ~30-100 mole % PC) over a range of osmotic stresses. Beginning at very low levels (log P = 3-4 dynes/cm^2) and progressively increasing the amount of applied osmotic stress would allow us to gradually induce vesicle aggregation and collapse; we could thus identify both those DOPS/DOPC ratios

prone to bulk phase segregation and those that form a stable single phase. Thus, while the behaviour of these REV systems has been well characterized at low neutral lipid levels, the behaviour at higher levels (ie. >~50 mole % PC), as idealized in Figures 15 and 16, remains tentative. Furthermore, while the results rule-out bulk phase segregation in these systems, the possibility of lateral segregation can not be excluded. It is possible that in all PS/PC mixtures exposed to Ca^{2+}, even those in section 1 (Fig. 15a) that form the dehydration-insensitive L_1 phase, laterally segregated $Ca(DOPS)_2$ and PC-rich domains coexist within the same bilayers.

Florine and Feigenson (1987a) observe segregation in similar PS/PC samples and interpret this as indicating two separate phases (personal communication). This interpretation represents a fundamental difference in nomenclature compared to that used to describe the results obtained using X-ray diffraction. The probe molecules employed by Florine and Feigenson (1987a) differentiate between phases on the basis of the lipid environment in which the probe is located. As such, these probe molecules provide evidence of separated $Ca(PS)_2$ and PC-rich phases but do not differentiate between bulk and lateral segregation. Conversely, according to the X-ray diffraction results there are only single multilamellar structures with specific dimensions present subsequent to Ca^{2+}-precipitation (Figs. 15 and 16); therefore, PS and PC are said to be in the same structural phase. The implications of these interpretations will be discussed in (D).

Preliminary studies using either POPE or DOPE as the neutral lipid in these REV systems exposed to excess Ca^{2+} revealed behaviour similar to that seen in the DOPS/DOPC systems. It appeared that up to ~25-30 mole % PE could be accomodated in a L_1 phase (Figs. 14 a and b). Contrary to the DOPS/DOPC systems, higher PE levels still allowed for the complete collapse of DOPS/PE REV (ie. no central scattering indicative of intact REV in these systems); these higher PE levels yielded L phases with d spacings approaching that of the pure PE species. However, as the d spacings of $Ca(DOPS)_2$ and the POPE L_α phase are quite similar, more carefully controlled

studies are required to ensure the accuracy of this distinction. The disordered L phases identified at POPE levels above ~30 mole % were also susceptible to osmotically-induced bulk phase segregation (Fig. 14a), although in some systems (ie. equimolar DOPS/DOPE) the segregation was Ca^{2+}-induced (Fig. 14b). The shorter (~34-37 Å) dimension of the bulk segregated phase in these mixtures, identified by only one reflection, is too short to be an L phase but could represent the $\sqrt{3}$ reflection of a PE-rich H_{II} phase. As with the PC-containing systems, a more systematic study of DOPS/PE REV containing high PE levels would be useful to better characterize the behaviour of these systems.

Noting the similarity in the results obtained in DOPS/DOPC multilamellar and REV systems, and in PS/PC and PS/PE systems we can accept the results of the original binary system study on DOPS/DG multilayers with some degree of confidence. As a neutral lipid, DG follows the general scheme of Figure 15a (see Table 1); similar to the other neutral lipids, DG appears to be accomodated in an L_1 phase up to levels of ~30 mole %, and higher DG contents yield single L phases of higher d spacing.

(D) Comparisons, Contrasts, and Implications

Extensive systematic studies on a variety of PS/PC multilamellar systems exposed to excess Ca^{2+} (≥10mM) have also identified three distinct phase 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 mole % PC can be accomodated in the $Ca(PS)_2$ phase and that ~30-40 mole % PS is accomodated in a single, PC -rich, liquid-crystalline phase. Between these boundaries bulk phase segregation of the $Ca(PS)_2$ and PC-rich liquid-crystalline phases was observed. In contrast our REV systems exhibited bulk phase segregation only when osmotically stressed. As these studies (Hui et al., 1983; Silvius and Gagne, 1984a; van Dijck. et al., 1978) utilized 2-3 fold higher Ca^{2+} concentrations than the present study, they indicate either that multilamellar and REV systems

behave differently, and/or that Ca^{2+} concentration makes a difference. Considering the short (~2 hour) incubation times used by Hui et al. (1983) and van Dijck et al. (1978), and the absence of any protocol to ensure Ca^{2+} equilibration in these studies, it is possible that the examples of bulk phase segregation observed in those studies may be indicative of samples not fully equilibrated with Ca^{2+}. Hui et al. (1983) did in fact note some problems regarding Ca^{2+} equilibration across the lamellae. The calorimetry studies by Silvius and Gagne (1984a) could not distinguish between bulk and laterally segregated lipid, relying on the results of Hui et al. (1983) and van Dijck et al. (1978) to make the distinction. Furthermore, the fusion assays used by these authors (Silvius and Gagne, 1984a) could also not provide such structural information. The results of the present study confirm that bulk phase segregation is not the norm in these systems.

X-ray microprobe analysis of BBPS/DPPC MLV systems revealed three additional characteristics of such binary mixtures (Hui et al., 1983). First, the total amount of Ca^{2+} bound in a sample decreased as the total amount of PC in a sample increased, in agreement with previous findings (Duzgunes et al., 1981a, b; Ekerdt and Papahadjopoulos, 1982; Wilschut et al., 1981). Second, the bulk segregation was not absolute; after systems containing 60 mole % PC were exposed to Ca^{2+} the collapsed structures contained ~25-30 % PC while the remaining vesicles contained ~88 mole % PC. Third, at very high PC contents (≥10 % but ≤30 mole % PS) small (~10 nm diameter), PS/Ca^{2+} domains, probably formed by *cis* binding (one Ca^{2+} binding to two PS molecules on the same bilayer), are laterally segregated in the liquid-crystalline PC multilayers. Hui et al. (1983) suggest that this is on a scale too small to be detected by DSC, X-ray diffraction, or other standard techniques. The authors also suggest that macroscopic morphological segregation in these multilamellar systems occurs when such microdomains extend beyond a certain critical size (Hui et al., 1983). However, as these samples were washed and rapidly dried prior to microprobe analysis it is possible that these

microdomains may have formed as a result of a dehydration-induced lateral segregation; in a fully hydrated membrane containing ~70-90 mole % PC there may only be randomly spaced pairs of PS molecules joined by *cis* Ca^{2+} binding, not necessarily microdomains.

In contrast, studies using fluorescent and spin-label probes on PS/PC multilamellar vesicle systems suggest that there is separation of PC-rich liquid-crystalline and $Ca(PS)_2$ domains at any PS/PC ratio in excess Ca^{2+} (Feigenson, 1988; Florine and Feigenson, 1987a; Ohnishi and Ito, 1974); studies that have included protocols to ensure Ca^{2+} equilibration. The work of Hui et al. (1983), Silvius and Gagne (1984a) and that presented here suggests a single collapsed phase up to PC contents of ~30 mole %. The only way to account for these disparate findings is lateral lipid separation where pure (or almost pure) neutral lipid domains coexist with the $Ca(PS)_2$ phase, within the same bilayers (L_1, Fig. 15a and Fig. 16). Feigenson (personal communication) has suggested that laterally segregated neutral lipid domains of a minimum size of ~100 molecules would be sufficient to yield the observed probe molecule behaviour indicative of phase separation, as defined in his system (Florine and Feigenson, 1987a). However, the results of Florine and Feigenson (1987a) and Feigenson (1988) also indicate that these neutral lipid domains are fluid. Considering the sharp X-ray diffraction patterns at low (≤~30 mole %) neutral lipid contents and the insensitivity of such samples to dehydration, our results suggest that even PC must become partially dehydrated during the "catastrophic" collapse process resulting from the intermembrane binding of Ca^{2+} to PS and the formation of the $Ca(PS)_2$ phase. Rand and Parsegian (1988) have calculated that about 50 ergs/cm^2 is derived from the formation of the $Ca(PS)_2$ complex and suggest that this energy is more than sufficient to cause lipid demixing; it now appears that this energy may also contribute to the dehydration of PC.

Considering that $Ca(DOPS)_2$, pure DOPC hydrated in only 22 weight % water (Rand, personal communication), and Ca^{2+}-precipitated DOPS/DOPC REV containing ≤~30 mole %

DOPC (L_1) all form 51.4 Å lamellar phases, the experimental results suggest that the DOPC in these L_1 systems becomes dehydrated to a similar extent. Since this 51.4 Å lamellar repeat is dehydration-insensitive in both the $Ca(DOPS)_2$ and L_1 phases (Table 2 and Appendix Table 2), d_l must increase as the remaining water is removed from L_1 phases. Therefore, at low PC contents (Fig. 15a, section 1) the interbilayer binding of Ca^{2+} to PS(removal of hydration repulsion leading to bilayer collapse) results in neutral lipid dehydration and its accomodation with the $Ca(PS)_2$ phase; the L_1 phase in Figures 15a and 16.

At higher PC levels (>~30 mole %) these systems are less susceptible to Ca^{2+}-induced structural changes such as $Ca(PS)_2$ formation (Fig. 15a, section 2) because hydration repulsion prevents any approach close enough for the $Ca(PS)_2$ binding reaction, and subsequent dehydration and close apposition of the membranes, to occur (Rand and Parsegian, 1988). Removal of water using osmotic stress allows closer bilayer approach and the formation of the $Ca(PS)_2$ complex, resulting in further dehydration, closer bilayer apposition, and the possible fusion and collapse of vesicles. However, osmotic stress also induces bulk phase segregation in these systems (Fig. 15b, section 3). Interestingly, at PC contents >80 mole % the observed L_α phases are not subject to osmotically-induced bulk phase segregation (Figs. 15b and 16).

The work of Tokutomi et al. (1981) suggested that PE also undergoes lateral phase separation from $Ca(PS)_2$ domains. Interestingly, these authors noted a higher degree of phase separation for PE compared to PC and a much lower Ca^{2+} threshold (an order of magnitude) for phase separation in PS/PE compared to PS/PC membranes. In equimolar DOPS/DOPE multilamellar systems ^{31}P-NMR, freeze-fracture electron microscopy, and X-ray diffraction data indicate a Ca^{2+}-induced bulk separation of DOPE H_{II} and $Ca(DOPS)_2$ phases (Tilcock et al., 1984). Kachar et al. (1986) obtained similar X-ray and freeze-fracture data at DOPE concentrations ≥50 mole %. However, systems using more unsaturated PS species did not

undergo Ca^{2+}-induced phase separation (Tilcock et al., 1984, 1988). Our findings suggest bulk separation of two phases in an equimolar DOPS/DOPE sample. Why our results indicated the presence of only small amounts of H_{II} phase is not readily apparent since our REV samples and those of Kachar et al. (1986) were prepared using identical procedures; the only differences being our use of twice their Ca^{2+} concentrations and 12 hour incubation times compared with their 1 hour. Tilcock et al. (1984) similarly used lower Ca^{2+} concentrations and shorter incubation times in their multilamellar systems suggesting that our samples may have been more thoroughly equilibrated.

In a study identical to that done with PC, Silvius and Gagne (1984b) extensively characterized the behaviour of PS/PE dispersions in excess Ca^{2+}. This work demonstrated that ~20 mole % PE would be accomodated in the $Ca(PS)_2$ phase while 15-20 mole % PS would saturate the liquid-crystalline PE phase. Whether Ca^{2+}/PS microdomains exist at these low concentrations of PS in PE has not been established. The coexistence of segregated $Ca(PS)_2$ and PE-rich liquid-crystalline phases was observed between these boundaries although the calorimetry technique used could not distinguish whether these represented bulk or laterally segregated phases. For the unsaturated DEPS/DEPE systems these boundaries were defined at temperatures above 43°C; below 39°C these systems exhibited strong tendencies to form PE- and PS-rich hydrated gel phases and the $Ca(PS)_2$ phase in varying proportions, dependent upon the lipid ratios used. DMPS/DMPE systems exhibited comparable behaviour. Similarly, our results established that ~25-30 mole % PE could be accomodated with $Ca(DOPS)_2$ in an L_1 phase, at 23°C. With PE contents above ~30 mole % at least one hydrated gel phase (PE- or PS-rich) exists in the DOPS/POPE systems, while a second bulk-segregated phase (probably a PE-rich H_{II} phase) coexists with the 51.4 Å collapsed phase at 50 mole % DOPE in DOPS. The strong tendency for DOPE to assume the H_{II} phase (T_h=10°C) compared to POPE (T_h=69°C) may explain the bulk phase segregation (without osmotic stress) observed with DOPE (50 mole %) but not

with POPE.

Overall, the data from the present study indicate that in mixtures with PS, PC and PE differ significantly in their abilities to promote aggregation and fusion/collapse of these REV systems. According to the DSC and fusion assay studies of Silvius and Gagne (1984a) fusion/collapse only occurs at PS levels above ~60 mole %; the limited number of samples containing high PC levels in our studies suggest an estimate of ~20-100 mole % DOPS for this fusion/collapse range. The studies by Silvius and Gagne (1984a) also demonstrated that the Ca^{2+} threshold for fusion increased with increasing PC content, particularly above ~25 mole % PC; as with our data, these results suggest a significant modification of Ca^{2+}-induced bilayer interactions beginning at PC levels above those that can be accomodated with the $Ca(PS)_2$ phase. In comparison, PS/PE systems containing <10 mole % PS (Appendix Table 10) appear to be completely susceptible to Ca^{2+}-induced fusion and collapse, with Ca^{2+} thresholds much lower than those required for pure PS REV (Silvius and Gagne, 1984b).

(E) Relevance to Membrane Structure and Function

The present study was concerned with modeling PS-containing domains within membranes that are exposed to Ca^{2+}. The results suggest that neutral lipid concentrations of less than 30 mole % can be dehydrated by the catastrophic collapse process inherent in the formation of the $Ca(PS)_2$ phase. Modification of such complete Ca^{2+}-induced bilayer interactions seems to occur at neutral lipid contents above ~30 mole %, and these systems are generally susceptible to osmotically-induced bulk segregation. In DOPS/DOPC systems containing >80 mole % PC such osmotically-induced bulk segregation does not occur. However, more extensive and systematic studies are required with systems of high neutral lipid content. Microprobe X-ray analysis suggests that laterally segregated Ca^{2+}/PS microdomains form at PS contents of ~10-30 mole % in PS/PC multibilayer systems (Hui et al., 1983), although such structures have not been identified in hydrated unilamellar systems or in systems containing PE.

As a whole, these results suggest that in excess Ca^{2+}, low concentrations (<30 mole %) of even a strongly hydrated neutral lipid such as PC can exist in laterally segregated domains within interacting bilayer vesicles, and does not interfere with the ability of these bilayers to adhere and collapse. *In vivo*, such an interaction might promote the contact between two membranes bound with receptor and ligand molecules respectively and may thus play a role in mediating any number of membrane-related functions. At contents above ~30 mole % PC (or DG), the neutral lipid domains seem to be large enough that their inherent hydration repulsion tends to inhibit membrane-membrane contact; the adhesion energy of such systems is sufficient only for stable aggregation (Kachar et al., 1986). At PC levels above ~80 mole %, PS and Ca^{2+} levels appear to be too low to facilitate interbilayer Ca^{2+} binding even when the hydration barrier is removed by osmotic stress. However, if a less hydrated neutral lipid such as PE is used, Ca^{2+}-induced membrane-membrane contact does not appear to be affected; because of their low hydration barrier such systems have adhesion energies at least an order of magnitude larger than the systems containing PC (Kachar et al., 1986).

The ability of PE to promote fusion competency has also been noted in other systems (Duzgunes et al., 1981b; Sundler et al., 1981). Therefore, at high PS contents and excess Ca^{2+} levels PS enhances the rate of PS/PE REV fusion/collapse probably by increasing the number of interbilayer Ca^{2+} contacts that promote close bilayer apposition and may serve as point defects involved in the fusion process. At high PE contents, the addition of PS will increase the surface charge and thus increase the amount of bound Ca^{2+} required to neutralize the electrostatic repulsion and allow for close apposition of the vesicles. Therefore, at the relatively low physiological levels of PS and Ca^{2+} the localized presence of PE and membrane components to specify and promote contact with other membranes may be essential for the fusion process *in vivo* . Reconsidering the original objective of this study, the results, including the data from the initial DOPS/DG multilamellar systems (Table 1), appear to support the concept that ~30 mole

% DG can be directly associated with the collapsed $Ca(PS)_2$ structure.

(F) Proposed Further Experimentation

The systematic studies using a combination of X-ray diffraction, thin-layer chromatography, density gradient centrifugation and freeze-fracture electron microscopy, in conjunction with an osmotic stress technique, have yielded data that correlate well with previous studies. But it would be of interest to perform a systematic study of the DOPS/DOPC system in Ca^{2+} using the gradient centrifugation method in conjunction with osmotic stress and X-ray diffraction; progressive increases in osmotic stress from low initial levels could be used to gently induce vesicle aggregation and collapse, thus possibly identifying the thresholds for dehydration-induced bulk phase segregation in these systems. As noted earlier, more extensive and systematic studies of systems containing PE and DG are also required to more fully characterize the effects of these neutral lipids. Using the gradient technique we could now confirm the presence of DG in the samples, thus solving the problem of identifying bulk segregated DG encountered when using only X-ray diffraction. Such studies could (i) confirm the presence of low DG levels in the collapsed (L_1) phase; (ii) determine the level of DG that can be accomodated with $Ca(PS)_2$ in the L_1 phase; and (iii) examine the behaviour at higher DG concentrations.

Feigenson (personal communication) has suggested that Ca^{2+} concentrations may influence the amount of neutral lipid that can be associated with the $Ca(PS)_2$ phase, ie. that the 30 mole % DOPC boundary identified in Figure 15a may increase with increasing Ca^{2+} concentrations. A series of systematic studies involving various DOPS/neutral lipid systems exposed to varying concentrations of Ca^{2+} could be done using the gradient centrifugation method in conjunction with osmotic stress and X-ray diffraction. Such a study would thus provide information on the effects of both Ca^{2+} concentrations and the different neutral lipid species.

Furthermore, considering that (i) more unsaturated lipid species appear to pack less easily

into the $Ca(PS)_2$ phase (Tilcock and Cullis, 1981; Tilcock et al., 1988); and (ii) DG from PI has longer and more unsaturated hydrocarbon chains than does the species used in this study (Das and Rand, 1986), it would be an interesting comparison to repeat the studies using DOPS with DG derived from PI. A parallel study using this DG and BBPS would provide an even more realistic model. The effect of cholesterol on the behaviour of such systems would also be of interest as indicated by a recent study (Tilcock et al., 1988) which suggests that the presence of this sterol promotes an L-H transition of all the lipids present. Freeze-fracture examination of DOPC REV before and after Ca^{2+} incubation would also be useful in confirming the collapse of these structures as suggested by the present study.

CONCLUSIONS

The present study concerns lyotropic model systems used to mimic local domains found within natural membranes. Using synthetic dioleoyl lipid species as REV suspensions exposed to excess Ca^{2+}, a combination of X-ray diffraction, thin-layer chromatography, osmotic stress, density gradient centrifugation and freeze-fracture electron microscopy has shown that up to 30 mole % DOPC can be accomodated in a single multilamellar phase with the $Ca(DOPS)_2$ complex. Modification of such Ca^{2+}-induced bilayer interactions at PC concentrations >~30 mole % results in reduced REV fusion and collapse, producing single disordered L phases susceptible to osmotically-induced bulk phase segregation. Above ~80 mole % PC there may exist a single, PC-rich liquid-crystalline lamellar phase that is not subject to such osmotically -induced bulk phase segregation. Results consistent with these were also obtained using BBPS/DOPC systems, in spite of the fact that the hydrocarbon chains were more ordered in this system. Similarly, preliminary studies with POPE and DOPE also suggest that 25-30 mole % levels of neutral lipid can coexist with $Ca(PS)_2$ in a collapsed phase. Unlike the PC-containing systems, PS/PE REV containing up to 90 mole % PE interacted strongly in the presence of Ca^{2+}, suggesting a possible role for PE at local sites of membrane fusion *in vivo*. We attribute this to the lower hydration barrier (higher adhesion energy) of PE compared to PC. Comparing this PS/neutral lipid data with that obtained from the initial trial experiment using DOPS/DG multilamellar systems strongly suggests that DG can also exist within a collapsed phase with $Ca(DOPS)_2$.

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APPENDIX TABLE 1:
FULLY HYDRATED DOPS/DOPC MULTILAMELLAR SYSTEMS EXPOSED TO Ca^{2+}

Mole % PC	Temp. (°C)	log P (dynes/cm^2)	Lipid phase(s)	d Spacing(s) (Å)	Comments
0	23	-----	L	51.4	
9	23	-----	L	51.3	
33	23	-----	L	51.7	slightly diffuse reflections
	23	6.84	L	51.6	slightly diffuse reflections
50	23	-----	L	51.3	diffuse reflections
	23	6.84	LL	51.0 60.3	diffuse reflections
75	23	-----	L	56.8	diffuse reflections & central scattering

- All samples (except the final one) hydrated overnight in 2 ml of 2 mM TES (pH 7.3), & exposed to excess $CaCl_2$ (10:1 Ca^{2+}/phospholipid) for 1-2 hours.
- All samples (except the final one) centrifuged at 35K for 1 hour.
- Final sample prepared gravimetrically & hydrated for 3 days.
- L: lamellar phase; LL: coexistence of two lamellar phases.
- Lipid phase and d spacing values were determined from measurements of each diffraction pattern.
- Each phase symbol represents an individual X-ray experiment.

APPENDIX TABLE 2: DOPS/DOPC REV EXPOSED TO Ca^{2+}: INITIAL STUDY USING A SUSPENSION OF 1.0 μm VESICLES

Mole % PC	Temp. (°C)	log P (dynes/cm²)	Lipid phase(s)	d Spacing(s) (Å)	Comments
0	23	-----	L	51.6	
	23	6.72	L	51.5	
	0	6.72	L	51.6	
33	23	-----	L	51.5	slightly diffuse reflections
	23	6.78	L	51.5	slightly diffuse reflections
33	23	-----	L	51.4	exposed to 500mM Ca^{2+}-TES; both of the L reflections obtained were slightly diffuse
	23	8.87	L	51.4	
50	23	-----	L	51.5	diffuse reflections
	23	6.74	L	51.6	diffuse reflections
83	23	-----	?	-----	central scattering.
	23	6.74	LL ?	51.4 57.5	only n=1 reflection for each; central scattering
	23	6.74	L	51.9	1 month later; a slightly diffuse n=1 reflection & central scattering

- All samples in a solution of 2 mM TES (pH 7.3) and excess $CaCl_2$ (10:1 Ca^{2+}/phospholipid) for 12 hours (33% PC sample in 500mM = 500:1 Ca^{2+}/phosphlipid).
- All samples centrifuged at 35K for 1 hour.
- Examination of each sample by TLC before REV preparation, and after final X-ray analysis qualitatively confirms the presence and proportions of DOPS and DOPC.
- L: lamellar phase; LL: coexistence of two lamellar phases.
- Lipid phase and d spacing values were determined from measurements of each diffraction pattern.
- Each phase symbol represents an individual X-ray experiment.

APPENDIX TABLE 3:
DOPS/DOPC REV EXPOSED TO Ca^{2+}: RESULTS OF CENTRIFUGING ON A CONTINUOUS SUCROSE DENSITY GRADIENT

Mole % PC	Temp. (°C)	log P (dynes/cm^2)	Lipid phase(s)	d Spacing(s) (Å)	Comments
0	23	-----	L	51.4	frozen chains
	23	6.87	L	51.4	frozen chains
17	23	-----	L	51.7	faint indications of frozen chains.
	23	6.89	L	51.7	faint indications of frozen chains.
33	23	-----	L	51.5	slightly diffuse reflections.
	23	6.85	L	51.5	slightly diffuse reflections.
50	23	-----	L	51.5	diffuse reflections & central scattering.
	23	6.82	LL	51.5	diffuse reflections & a second L phase of ~57.6 Å.
100	23	-----	L	58.4	diffuse reflections & central scattering.
	23	6.85	L	56.7	slightly diffuse reflections
f1	23	-----	L	51.5	slightly diffuse reflections
	23	6.88	L	51.3	slightly diffuse reflections
f2	23	-----	L	51.7	
	23	6.83	L	51.7	
f3	23	-----	L	51.2	frozen chains
	23	6.85	L	51.2	faint indications of frozen chains.

- All samples in a solution of 2mM TES (pH 7.3) and excess $CaCl_2$ (10:1 Ca^{2+}/phospholipid) for 12 hours.
- All samples centrifuged at 35K for 1 hour.
- All samples washed twice in the Ca^{2+}-TES solution after exposure to a continuous gradient of 6 wt.% to 20 wt.% sucrose.
- Examination of each sample by TLC before REV preparation, and after final X-ray analysis qualitatively confirms the presence and proportions of DOPS and DOPC.
- L: lamellar phase; f1, f2, f3: fractions 1, 2, & 3 from a pooled sample of the 0, 17, & 33 % PC REV.
- Lipid phase and d spacing values were determined from measurements of each diffraction pattern.
- Each phase symbol represents an individual X-ray experiment.

APPENDIX TABLE 4:
DOPS/DOPC REV EXPOSED TO Ca^{2+}: PROBING SYSTEMS OF HIGH PC CONTENT USING A CONTINUOUS SUCROSE DENSITY GRADIENT

Mole % PC	Temp. (°C)	log P (dynes/cm^2)	Lipid phase(s)	d Spacing(s) (Å)	Comments
50	23	-----	L	51.5	diffuse reflections & central scattering.
75	23	-----	L	55.9	diffuse reflections & central scattering.
83	23	-----	L	56.3	diffuse reflections & central scattering.
89	23	-----	L	59.3	diffuse reflections & central scattering.
100	23	-----	L	59.3	diffuse reflections & central scattering.
	23	4.02	L	60.6	diffuse reflections & central scattering.
	23	6.80	L	56.6	diffuse reflections.
Control - not exposed to sucrose gradient					
100	23	-----	L	60.3	sharp reflections & central scattering.
	23	4.02	L	61.0	diffuse reflections & central scattering.
	23	6.84	L	55.0	sharp reflections & central scattering.

- All samples in a solution of 2 mM TES (pH 7.3) and excess $CaCl_2$ (10:1 Ca^{2+}/phospholipid) for 12 hours.
- All samples centrifuged at 35K for 1 hour.
- All samples washed twice in the Ca^{2+}-TES solution after exposure to a continuous gradient of 0 wt. % to 11 wt. % sucrose.
- Examination of each sample by TLC before REV preparation, and after final X-ray analysis qualitatively confirms the presence and proportions of DOPS and DOPC.
- L: lamellar phase.
- Lipid phase and d spacing values were determined from measurements of each diffraction pattern.
- Each phase symbol represents an individual X-ray experiment.

APPENDIX TABLE 5:
DOPS/DOPC REV EXPOSED TO Ca^{2+}: COMPARING THREE PROTOCOLS

	Ca^{2+} Precipitate		+ Freeze-thaw		+ Sucrose Gradient	
Mole % PC	Lipid phase	d Spacing (Å)	Lipid phase	d Spacing (Å)	Lipid phase	d Spacing (Å)
0	L	51.4 fc	L	51.5 fc	L	51.4 fc
17	L	51.8	L	51.4 fc	L	51.4 fc
33	L	51.5 fc º	L	51.4 fc º	L	51.5 fc º
50	L	51.4 +*	L	51.3 +*	L(f2)	51.8 +*
					L(f1)	53.7 º
83	L	52.9 +*	L	53.4 +*	L	53.8 +*
100	L	60.5	L	60.3 +º	L	59.3

- REV stocks of each specific PS/PC ratio were exposed to a solution of 2mM TES (pH 7.3) and excess $CaCl_2$ (10:1 Ca^{2+}/phospholipid) for 12 hours; after centrifuging at 35K for 1 hour, a sample was taken from each stock.
- Lipid stocks were then freeze-thawed in fresh Ca^{2+}-TES (10 cyles); after centrifuging at 35K for 1 hour an aliquot of each stock was taken.
- Each lipid stock was then layered onto a separate continuous density gradient of 1 wt.% to 22 wt. % sucrose, centrifuged at 10K for 1 hour, recovered and washed (centrifuged at 35K for 1 hour in fresh Ca^{2+} solution; repeated twice).
- Examination of each sample by TLC before REV preparation, and after final X-ray analysis qualitatively confirms the presence and proportions of DOPS and DOPC.
- L: lamellar phase; f1, f2: fractions 1 and 2 recovered from the sucrose density gradient; fc: frozen chains; +: central scattering; º: slightly diffuse reflections; *: diffuse reflections.
- Lipid phase and d spacing values were determined from measurements of each diffraction pattern.
- Each phase symbol represents an individual X-ray experiment at 23°C.

APPENDIX TABLE 6:
DOPS/DOPC REV EXPOSED TO Ca^{2+}: CUMULATIVE RESULTS OF ANALYSES INVOLVING CONTINUOUS SUCROSE DENSITY GRADIENTS

	Ca^{2+} Precipitate (Appendix Table 3)			Ca^{2+} Precipitate (Appendix Table 4)			Ca^{2+} ppt. + Freeze-thaw (Appendix Table 5)		
Mole % PC	Lipid phase	d Spacing (Å)	Density (g/ml)	Lipid phase	d Spacing (Å)	Density (g/ml)	Lipid phase	d Spacing (Å)	Density (g/ml)
0	L	51.4 fc	1.080	-----	-----	-----	L	51.4 fc	1.077
17	L	51.7 fc	1.075	-----	-----	-----	L	51.4 fc	1.069
33	L	51.5 º	1.050	-----	-----	-----	L	51.5 fc º	1.053
50	L	51.5 +*	1.040	L	51.5 +*	1.034	L f2	51.8 +*	1.044
							L f1	53.7 º	1.034
75	-----	-----	-----	L	55.9 +*	1.017	-----	-----	-----
83	-----	-----	-----	L	56.3 +*	1.013	L	53.8 +*	1.006
89	-----	-----	-----	L	59.3 +*	1.010	-----	-----	-----
100	L	58.4 +*	-----	L	59.3 +	1.007	L	59.3	-----
Pooled:	L f1	51.5 º	1.052	-----	-----	-----	-----	-----	-----
0, 17, &	L f2	51.7	1.076	-----	-----	-----	-----	-----	-----
33%	L f3	51.2 fc	1.080	-----	-----	-----	-----	-----	-----

- All samples in a solution of 2 mM TES (pH 7.3) and excess $CaCl_2$ (10:1 Ca^{2+}/phospholipid) for 12 hours.
- All samples centrifuged at 35K for 1 hour; at 10K for 1 hour; and again at 35K for 2 hours.
- All samples washed twice in the Ca^{2+}-TES solution after exposure to sucrose.
- Examination of each sample by TLC before REV preparation, and after final X-ray analysis qualitatively confirms the presence and proportions of DOPS and DOPC.
- L: lamellar phase; f1, f2: fractions 1 and 2 recovered from the sucrose density gradient; fc: frozen chains; +: central scattering; º: slightly diffuse reflections; *: diffuse reflections.
- Lipid phase and d spacing values were determined from measurements of each diffraction pattern.
- Each phase symbol represents an individual X-ray experiment at 23°C.

APPENDIX TABLE 7: BBPS/DOPC REV EXPOSED TO Ca^{2+}

Mole % PC	Temp. (°C)	log P (dynes/cm^2)	Lipid phase(s)	d Spacing(s) (Å)	Comments
0	23	-----	L	51.3	frozen chains
	37	-----	L	51.0	frozen chains
	23	8.06	L	51.1	frozen chains
	23	dessicated	L	51.1	frozen chains
17	23	-----	L	51.7	frozen chains & slightly diffuse reflections.
	37	-----	L	51.7	frozen chains & slightly diffuse reflections.
	23	8.06	L	51.7	frozen chains
33	23	-----	L	52.5	frozen chains & slightly diffuse reflections.
	37	-----	L	52.5	frozen chains & slightly diffuse reflections.
	23	8.06	L	52.9	frozen chains & diffuse reflections.
	23	dessicated	LL ?	46.6 52.5	frozen chains; 46.6 Å phase only an n=1 reflection
50	23	-----	L	52.4	diffuse reflections & central scattering.
	37	-----	L	52.0	diffuse reflections & central scattering.
	23	8.06	LL	49.8 53.0	frozen chains & diffuse reflections.
	23	dessicated	L	51.5	frozen chains & diffuse reflections.
83	23	-----	L	51.3	diffuse reflections & central scattering.
	37	-----	L	50.6	diffuse reflections & central scattering.
	23	8.06	L	51.2	sharp reflections

- All samples in a solution of 2 mM TES (pH 7.3) and excess $CaCl_2$ (10:1 Ca^{2+}/phospholipid) for 12 hours.
- All samples centrifuged at 35K for 1 hour.
- Examination of each sample by TLC before REV preparation, and after final X-ray analysis qualitatively confirms the presence and proportions of BBPS and DOPC.
- L: lamellar phase; LL: coexistence of two lamellar phases.
- Lipid phase and d spacing values were determined from measurements of each diffraction pattern.
- Each phase symbol represents an individual X-ray experiment.

APPENDIX TABLE 8: FULLY HYDRATED DOPC, POPE, AND DOPE: CHARACTERIZING NEUTRAL LIPIDS EXPOSED TO Ca^{2+} OR Mg^{2+}

Lipid	Divalent cation	Ratio divalent:PL	Temp. (°C)	log P (dynes/cm^2)	Lipid phase(s)	d Spacing(s) (Å)	Comments
DOPC	Ca	10	23	-----	L	58.8	central scattering.
			37	-----	L	57.9	central scattering.
DOPC (REVs)	Ca	500	23	-----	LL	53.1 61.2	slightly diffuse reflections
				6.89	L	55.6	clean, sharp reflections
DOPC	Mg	10	23	-----	L	58.3	
			37	-----	L	57.6	
POPE	Ca	10	23	-----	LL	53.6 61.0	frozen chains; the 61 Å phase is a strong reflection
POPE	Ca	10	23	-----	L	61.2	frozen chains
			30	-----	L	52.9	
DOPE	Ca	10	23	-----	H	63.5	
			37	-----	H	61.4	

- Unless noted otherwise, all samples were multilamellar systems hydrated overnight in 2 mM TES and exposed to excess Ca^{2+} or Mg^{2+} for 12 hours.
- All samples centrifuged at 35K for 1 hour.
- L: lamellar phase; LL: coexistence of two lamellar phases; H: inverse hexagonal (H_{II}) phase.
- Lipid phase and d spacing values were determined from measurements of each diffraction pattern.
- Each phase symbol represents an individual X-ray experiment.

APPENDIX TABLE 9:
FULLY HYDRATED DOPS/POPE MULTILAMELLAR SYSTEMS: GRAVIMETRIC SAMPLES TO CHARACTERIZE THE LIPID MIXTURES

Mole % PE	Temp. (°C)	Weight % total PL	Lipid phase(s)	d Spacing(s) (Å)	d_l Spacing (Å)	Comments
25	23	43.0	L	88.1	37.9	fluid chains
33	23	39.9	L	94.7	37.8	fluid chains
50	23	38.1	L	99.5	37.9	fluid chains
100	23	41.6	LL	53.5 61.0		frozen chains
	31	41.6	L	52.5	21.8	

- All samples equilibrated for three days in 2 mM TES.
- L: lamellar phase; LL: coexistence of two lamellar phases.
- Lipid phase and d spacing values were determined from measurements of each diffraction pattern.
- Each phase symbol represents an individual X-ray experiment.

APPENDIX TABLE 10: DOPS/POPE REV EXPOSED TO Ca^{2+}

Mole % PE	Temp. (°C)	log P (dynes/cm^2)	Lipid phase(s)	d Spacing(s) (Å)	Comments
25	23	-----	L	51.7	frozen chains.
	23	8.06	L	51.7	frozen chains.
33	23	-----	L	52.3	frozen chains.
	23	8.06	L	52.3	frozen chains
50	23	-----	L	52.2	frozen chains.
	23	8.06	L	52.5	frozen chains & slightly diffuse reflections.
	23	8.87	L +	52.5	frozen chains, sharper reflections & faint second phase of ~ 37 Å (only n=1 reflection).
83	23	-----	L	53.0	frozen chains.
	23	8.06	L	53.0	frozen chains
	23	8.60	L	55.1	frozen chains
91	23	-----	?	?	complicated lipid segregation & frozen chains.
	30	-----	L	52.3	
	23	8.06	LL	56.0 47.7	frozen chains & a faint second L phase of ~47.7 Å.
	30	8.06	L	51.3	frozen chains

- All samples in a solution of 2 mM TES (pH 7.3) and excess $CaCl_2$ (10:1 Ca^{2+}/phospholipid) for 12 hours.
- All samples centrifuged at 35K for 1 hour.
- Examination of each sample by TLC before REV preparation, and after final X-ray analysis qualitatively confirms the presence and proportions of DOPS and POPE.
- L: lamellar phase; LL: coexistence of two lamellar phases.
- Lipid phase and d spacing values were determined from measurements of each diffraction pattern.
- Each phase symbol represents an individual X-ray experiment.

APPENDIX TABLE 11: DOPS/DOPE REV's EXPOSED TO Ca^{2+}

Mole % PE	Temp. (°C)	log P (dynes/cm^2)	Lipid phase(s)	d Spacing(s) (Å)		Comments
25	23	-----	L	51.8		frozen chains.
	23	8.06	L	51.8		frozen chains.
33	23	-----	L	51.4		
	23	8.06	L (L)	51.5		frozen chains & a weak second phase of ~34 Å (only n=1 reflection).
50	23	-----	LL	36.8	51.7	slightly diffuse reflections.
	23	8.06	LL	46.2	51.7	frozen chains & slightly diffuse reflections.

- All samples in a solution of 2 mM TES (pH 7.3) and excess $CaCl_2$ (10:1 Ca^{2+}/phospholipid) for 12 hours.
- All samples centrifuged at 35K for 1 hour.
- Examination of each sample by TLC before REV preparation, and after final X-ray analysis qualitatively confirms the presence and proportions of DOPS and DOPE.
- L: lamellar phase; LL: coexistence of two lamellar phases.
- Lipid phase and d spacing values were determined from measurements of each diffraction pattern.
- Each phase symbol represents an individual X-ray experiment.

APPENDIX TABLE 12: DOPS/DOPC REV EXPOSED TO Mg^{2+}

Mole % PC	Temp. (°C)	log P (dynes/cm²)	Lipid phase(s)	d Spacing(s) (Å)	Comments
33	23	-----	L	50.0	central scattering & diffuse reflections.
	37	-----	L	50.4	central scattering & diffuse reflections.
	23	8.06	L	50.3	clean, sharp reflections.
	37	8.06	L	49.3	clean, sharp reflections.
50	23	-----	L	51.5	central scattering & diffuse reflections.
	37	-----	LL	51.1	central scattering, diffuse reflections & a faint second phase of ~41 Å.
	23	8.06	L	50.6	clean, sharp reflections.
	37	8.06	L	49.0	clean, sharp reflections.

- All samples in a solution of 2 mM TES (pH 7.3) and excess $CaCl_2$ (10:1 Ca^{2+}/phospholipid) for 12 hours.
- All samples centrifuged at 35K for 1 hour.
- Examination of each sample by TLC before REV preparation, and after final X-ray analysis qualitatively confirms the presence and proportions of DOPS and DOPC.
- L: lamellar phase; LL: coexistence of two lamellar phases.
- Lipid phase and d spacing values were determined from measurements of each diffraction pattern.
- Each phase symbol represents an individual X-ray experiment.