

Cation-Dependent Segregation Phenomena and Phase Behavior in Model Membrane Systems Containing Phosphatidylserine: Influence of Cholesterol and Acyl Chain Composition[†]

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ABSTRACT: The addition of Ca²⁺ to model membrane systems containing phosphatidylserine (PS) can have remarkable effects on the distribution of PS and the overall polymorphic phase [bilayer or hexagonal (H_{II})] assumed by the lipid mixture. In this study, we examine the influence of Ca²⁺ on lipid mixtures composed of well-defined (synthetic) species of PS, phosphatidylethanolamine (PE), and phosphatidylcholine (PC) in the presence and absence of cholesterol by employing ³¹P and ²H NMR, freeze-fracture, and X-ray techniques. It is shown that whereas Ca²⁺ can segregate PS into crystalline cochleate domains in equimolar mixtures of dioleoyl-PE and dioleoyl-PS (DOPS), such effects are not observed for mixtures containing more unsaturated (dilinoleoyl) species of PS. The addition of cholesterol to these PE-PS systems inhibits Ca²⁺-induced segregation of DOPS and facilitates Ca²⁺-triggered hexagonal (H_{II}) phase formation for both the PE

and the PS components. In contrast, in equimolar mixtures of DOPS with dioleoyl-PC, Ca²⁺-induced segregation of phospholipid is not affected by the presence of up to 33 mol % cholesterol. These and related effects suggest that, in multicomponent biomembrane systems containing both PE and cholesterol, phase segregation of PS by Ca²⁺ may not be readily achievable. These results are discussed with regard to the reliability of ³¹P NMR phase identifications of phospholipid structure in model and biological membranes and demonstrate that in mixed lipid systems the influence of divalent cations on lipid distribution and structure can be exquisitely sensitive to details of the local lipid composition. In addition, the behavior of individual lipid species such as PS in pure and simple binary mixtures cannot be readily employed to predict the properties of more complex systems.

The possibility of lateral segregation of particular membrane lipid components in response to modulation of factors such as divalent cation concentrations, pH, or local integral or peripheral protein composition provides an attractive means of regulation of membrane-mediated phenomena. Among other possibilities, for example, membrane fluidity, permeability or the tendency to undergo fusion can be markedly sensitive to lipid composition, and factors modulating this local composition could modulate an associated membrane-mediated property. Many studies have been performed to characterize such segregation phenomena, with particular attention paid to cation-induced segregation processes in mixed lipid systems containing acidic (negatively charged) phospholipids. These include Ca²⁺-induced segregation of phosphatidylserine (PS) in PS-phosphatidylcholine (PC) model membranes (Ohnishi & Ito, 1974; Jacobson & Papahadjopoulos, 1975; van Dijk et al., 1978), of phosphatidic acid (PA) in PA-PC model systems (Ohnishi & Ito, 1974; Galla & Sackmann, 1975), and of PS in phosphatidylethanolamine (PE)-PS and PE-PC-PS systems (Tokutomi et al., 1981; Tilcock & Cullis, 1981). These studies have led to proposals for roles of Ca²⁺-dependent lateral segregation of acidic phospholipids in membrane fusion events (Portis et al., 1979), although it remains to be demonstrated that such segregation phenomena can occur in the more complex lipid mixtures found in biological systems.

The addition of Ca²⁺ to mixed lipid systems containing PS can also have marked effects on the polymorphic phase preference observed. In PE-PS dispersions, for example, Ca²⁺

can trigger H_{II} phase formation (Tilcock & Cullis, 1981) as well as in model erythrocyte "inner monolayer" systems where PE and PS are major components (Hope & Cullis, 1979). These observations have been correlated with an ability of Ca²⁺ to induce nonbilayer structures required as transitory intermediates in fusion events (Cullis et al., 1983).

In this work, we have examined in detail the ability of Ca²⁺ or Mg²⁺ to segregate PS in, and modulate the phase preferences of, mixtures of PS with PE and PC and have characterized the influence of cholesterol and acyl chain composition on such abilities by employing ³¹P and ²H NMR, freeze-fracture, and X-ray techniques. It is shown that in PE-PS systems the ability of Ca²⁺ to segregate PS is markedly sensitive to the unsaturation of the PS species and that such segregation may not be possible for more unsaturated varieties. The addition of cholesterol further mitigates against Ca²⁺-induced segregation events and induces a marked predilection for hexagonal (H_{II}) phase structure for both PE and PS components when Ca²⁺ is introduced. In contrast, in PC-PS systems, the ability of Ca²⁺ to segregate phospholipid appears relatively insensitive to the PS acyl chain composition and the presence of cholesterol. These results are discussed with respect to the reliability of ³¹P NMR identifications of lipid phase organization, the possibilities for segregation of PS in complex lipid mixtures found in biological membranes, and the mechanisms whereby Ca²⁺ and other divalent cations can induce H_{II} phase organization for multicomponent lipid mixtures containing cholesterol and PS.

Materials and Methods

Materials. Chromatographically pure oleic and linoleic acids, dimethyl sulfoxide (Me₂SO), 1,1'-carbonyldiimidazole, serine, and ethanolamine were obtained from Sigma. Tetra-butylammonium hydroxide was purchased from Eastman-Kodak and ionophore A23187 from Calbiochem. Miscellaneous reagents were of analytical grade. Chloroform and methanol were distilled immediately before use. 11,11-Di-

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deuterioleic acid ([11,11-²H₂]oleic acid) was synthesized by a modification of the procedure of Tucker et al. (1971) as indicated elsewhere (Farren et al., 1984).

Lipid Synthesis. The fatty acids employed were more than 99% pure on the basis of gas chromatography of their methyl esters. Serine was lyophilized from an aqueous solution before use. Dioleoylphosphatidylcholine (DOPC) and dilinoleoylphosphatidylcholine (DLPC) were synthesized and purified as previously described (Tilcock & Cullis, 1982). Dioleoylphosphatidylethanolamine (DOPE), dioleoylphosphatidylserine (DOPS), [11,11-²H₂]DOPS, and dilinoleoylphosphatidylserine (DLPS) were derived from the respective phosphatidylcholines by employing the base-exchange capacity of phospholipase D (Comfurius & Zwaal, 1977). DOPE was purified by preparative liquid chromatography on silica using CHCl₃-MeOH-H₂O-NH₃ (60:30:1:1 v/v) as the mobile phase. DOPS, [11,11-²H₂]DOPS, and DLPS were purified by employing carboxymethylcellulose column chromatography and converted to their sodium salts as previously described (Hope & Cullis, 1980). All lipids were shown to be 1,2-diacyl-*sn*-glycero-3-phospho conformers on the basis of ¹H NMR (Lammers et al., 1978), were greater than 99% pure with respect to lipid phosphorus as determined by phosphorus analysis following two-dimensional thin-layer chromatography, and were more than 99% pure with respect to their designated fatty acid as determined by gas chromatography and argentation thin-layer chromatography.

Nuclear Magnetic Resonance. ²H NMR and ³¹P NMR spectra were obtained by using a Bruker WP-200 spectrometer operating at 30.7 MHz for ²H and at 81 MHz for ³¹P. Phospholipid mixtures (35–100 μmol of total phospholipid) containing nondeuterated lipid were dispersed by vortex mixing in 0.8 mL of buffer [10 mM tris(hydroxymethyl)amino-methane hydrochloride (Tris-HCl), 100 mM NaCl, and 10% v/v ²H₂O, pH 7] to which was added the calcium ionophore A23187 (10 μL of a 2 mg/mL solution dissolved in Me₂SO) to ensure equilibration of added Ca²⁺. Mixtures containing deuterated lipid were dispersed in 0.8 mL of buffer (as described above, prepared in deuterium-depleted water) which also contained the calcium ionophore. Titrations with Ca²⁺ or Mg²⁺ were performed by adding aliquots of 100 mM stock solutions of their chloride salts, followed by three cycles of freeze-thawing of the samples to ensure equilibration. Where signal intensities were measured (Figure 2), triphenyl phosphite (10% v/v in CHCl₃) was used as an external standard and was located in a central insert in the NMR tube. For ³¹P NMR, spectra were accumulated for up to 1000 transients by employing a 15-μs 90° pulse, a 20-KHz sweep width, and a 0.8-s interpulse delay in the presence of broad-band proton decoupling. For ²H NMR, spectra were accumulated for up to 40 000 transients by employing a 15-μs 90° pulse, a 30-KHz sweep width, and a 0.04-s interpulse delay. All spectra were collected at 30 °C. Following the NMR studies, thin-layer chromatography and phosphorus analysis showed no evidence of lysophospholipid or fatty acid degradation products.

Freeze-Fracture Electron Microscopy. Aliquots of samples were mixed with 25% (v/v) glycerol to prevent freeze damage. Samples were quenched from 30 °C and replicas prepared according to standard procedures employing a Balzers BAF 301 apparatus. Micrographs were obtained by using a Phillips 400 electron microscope.

X-ray Diffraction. Nickel-filtered Cu Kα (λ = 1.54 Å) X-rays were generated on a Rigaku RU-200 microfocussing rotating anode generator. X-rays were collimated and focused by using either single or double Franks mirror optics and slits

(Gruner, 1977; Milch, 1983). Diffraction patterns were recorded on either of two image-intensified, slow-scan, two-dimensional X-ray detectors (Reynolds et al., 1978; Gruner et al., 1982a,b) at 30 °C. Details of data correction, reduction, and analysis are described elsewhere (Gruner et al., 1982a,b). The X-ray diffraction patterns were recorded as two-dimensional arrays of X-ray intensities in each of 256 × 256 adjacent areas. The patterns consisted of concentric rings of diffracted orders centered about the origin of diffraction. The incident beam consisted of a narrow line (about 0.2 × 1 mm): consequently, the patterns were reduced to graphs of intensity vs. diffraction angle by integrating the data within ±10° of a line perpendicular to the long dimension of the incident beam [see Gruner et al. (1982a,b) for more details]. Integrations to either side of the origin are displayed separately.

Lipids for X-ray studies were dispersed to approximately 30% w/v in buffer (10 mM Tris-HCl-100 mM NaCl, pH 7) and equilibrated at 4 °C for several hours. These lipid dispersions were then loaded into acid-cleaned, 1.5-mm glass X-ray capillaries which were then sealed with epoxy plugs. The capillaries were inserted into a thermostated copper block which had a small hole drilled in it to allow for the passage of the X-ray beam. The copper block was thermoelectrically heated, under computer control, to the desired temperature (±1 °C). Diffraction patterns were typically acquired in less than 2 min of X-ray exposure. Differences between successive diffraction patterns were used to establish when the specimens had come to equilibrium.

Lattice spacings were derived by a least-squares fit to the peak positions of the diffracted orders. Peak positions were taken as the center of a parabolic least-squares fit to the local peak maxima. The accuracy of the lattice spacing thus determined was better than ±0.5 Å.

Results

In previous studies (Tilcock & Cullis, 1981) of PE and PS derived from natural sources (egg yolk and soybean), ³¹P NMR results suggest that Ca²⁺ is able to sequester PS in mixed PE-PS systems, leaving the PE free to adopt the hexagonal (H_{II}) phase it assumes in isolation. At the initial stage of this investigation, the influence of Ca²⁺ on aqueous dispersions of mixtures of well-defined synthetic varieties of PE and PS was examined. As shown in Figure 1, disparate effects are observed, depending on the unsaturation of the PS species. In equimolar mixtures of dioleoyl-PE (DOPE) with dioleoyl-PS (DOPS), the ³¹P NMR line shape obtained (Figure 1A) is transformed on addition of Ca²⁺ from the asymmetric form with a low-field shoulder and high-field peak (characteristic of the bilayer organization) to the narrower form with reversed asymmetry which is characteristic of the hexagonal (H_{II}) phase [for a discussion of ³¹P NMR line shapes and phase properties of phospholipids, see Cullis & de Kruijff (1979)]. This behavior contrasts with that observed for equimolar mixtures of DOPE with dilinoleoyl-PS (DLPS) (Figure 1B) where the lipid dispersion gives rise to "bilayer" ³¹P NMR spectra in both the presence and absence of Ca²⁺.

The ability of Ca²⁺ to "trigger" H_{II} phase formation as detected by ³¹P NMR in the DOPE-DOPS system is similar to behavior observed in mixtures of PE and PS derived from natural sources (Cullis & Verkleij, 1979; Tilcock & Cullis, 1981) and is consistent with an ability of Ca²⁺ to sequester the DOPS into crystalline "cochleate" domains. In order to place this interpretation on a firm basis, additional techniques were employed. First, as initially shown for pure egg PS systems (Hope & Cullis, 1980), the addition of Ca²⁺ results in precipitation of PS and formation of crystalline cochleate

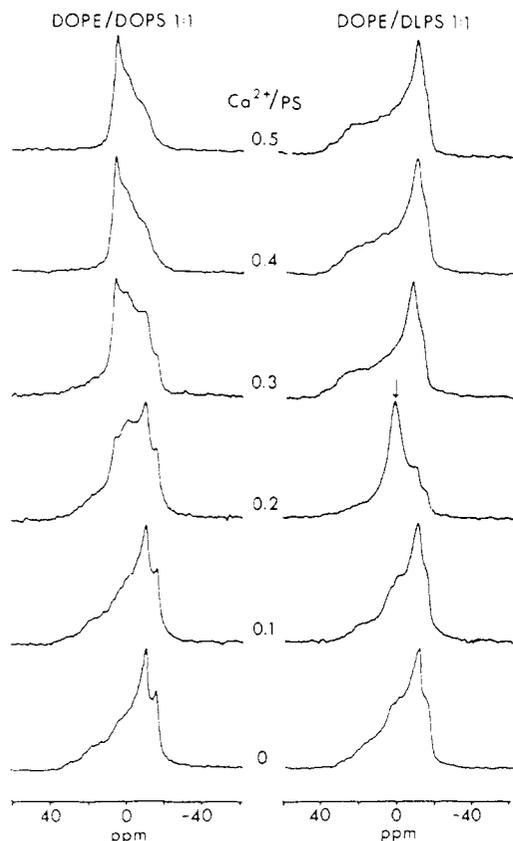


FIGURE 1: 81.0-MHz ^{31}P NMR spectra obtained at 30 °C from aqueous DOPE–DOPS (1:1) and DOPE–DLPS (1:1) dispersions in the presence of varying amounts of Ca^{2+} . The Ca^{2+}/PS ratio refers to the molar ratio of Ca^{2+} to phosphatidylserine. For details, see Materials and Methods. Note that all graphs have been scaled to the same peak height. In fact, the integrated signal intensity is a function of the Ca^{2+}/PS ratio (see Figure 3). The arrow indicates a narrow resonance arising from phospholipid experiencing isotropic motional averaging which likely arises from small vesicular structures [see Nayar et al. (1982)].

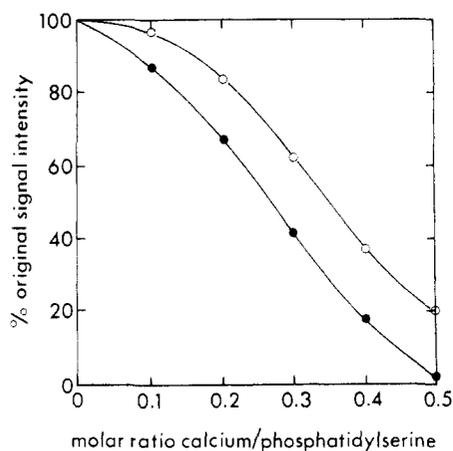


FIGURE 2: Integrated ^{31}P NMR signal intensities for DOPS (●) and DLPS (○) as a function of added Ca^{2+} concentration. For details of the protocol, see Nuclear Magnetic Resonance under Materials and Methods.

structures (Papahadjopoulos et al., 1975) which display much broader "rigid-lattice" ^{31}P NMR spectra and much longer spin-lattice relaxation times. As discussed elsewhere (Tilcock & Cullis, 1981), under our experimental conditions, this results in the disappearance of the bilayer ^{31}P NMR signal. This also occurs for DOPS and DLPS as illustrated in Figure 2. In the case of DOPS, the ^{31}P NMR signal is virtually eliminated at a Ca^{2+}/PS ratio of 0.5, whereas for DLPS approximately 20% of the original signal intensity remains at a Ca^{2+}/PS ratio

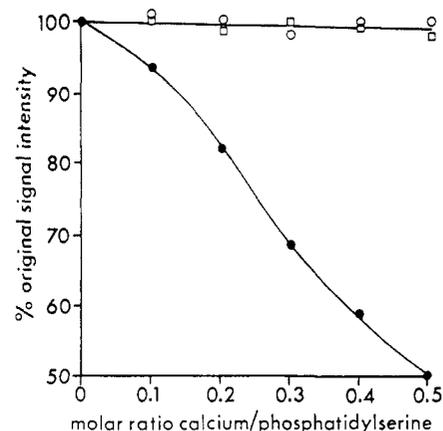


FIGURE 3: Integrated ^{31}P NMR signal intensities for DOPE–DOPS (1:1) (●), DOPE–DLPS (1:1) (○), and DOPE–DOPS–cholesterol (CHOL) (1:1:1) (□) mixtures as a function of added Ca^{2+} concentration.

of 0.5. The results for DOPS are consistent with previous findings indicating a 1 to 2 Ca^{2+} to PS stoichiometry in the condensed cochleate precipitate (Portis et al., 1979; Eckerdt & Papahadjopoulos, 1982). The inability of Ca^{2+} to decrease the signal intensity of DLPS to the same extent as for DOPS is presumably a consequence of the greater unsaturation of DLPS and is consistent with previous results for soy PS (Tilcock & Cullis, 1981). At sufficiently high $\text{Ca}^{2+}/\text{DLPS}$ ratios (>3), the DLPS bilayer signal could be entirely eliminated. These systems gave rise to the very broad rigid-lattice ^{31}P NMR spectrum observed previously for the Ca^{2+} –egg PS system (Hope & Cullis, 1980).

If Ca^{2+} -induced segregation of DOPS into cochleate domains occurs in the DOPE–DOPS system, it would therefore be expected that the ^{31}P NMR signal intensity should be reduced by 50% at a Ca^{2+} to DOPS molar ratio of 0.5. Figure 3 shows that this is the case. The results of Figure 1A and Figure 3 thus strongly indicate the presence of hexagonal (H_{II}) phase DOPE and segregated Ca^{2+} –PS cochleate structures in the DOPE–DOPS– Ca^{2+} system. Graphic support for such an interpretation is provided by the freeze–fracture studies of DOPE–DOPS (1:1) in the presence of Ca^{2+} presented in Figure 4. Extended areas of the striated pattern indicative of hexagonal phase lipid are clearly visible (Figure 4a) as are regions (Figure 4b) with the Swiss roll appearance characteristic of condensed cochleate structures (Papahadjopoulos et al., 1975).

Further verification of the phase behavior of the DOPE–DOPS system was obtained via low-angle X-ray diffraction. In this regard, the indexing of X-ray reflections observed in phase-separated lipid systems is often ambiguous because only a few reflections arising from a given lattice may be apparent. However, such diffraction patterns may be reliably indexed by noting the different behavior of the separate lattice components as the temperature of the specimen is varied. For example, Figure 5 illustrates the thermotropic behavior of the DOPE–DOPS– Ca^{2+} system. At 30 °C, the specimen is separated into well-defined hexagonal and lamellar lattices with basis vectors of 68 and 51 Å, respectively. When the temperature is lowered to 10 °C, the hexagonal component diminishes and is completely absent at –10 °C. If the temperature scan is run from low to high temperature, the sequences of patterns are reversed, given a few degrees of hysteresis (data not shown). Such hysteresis is often observed for lamellar–hexagonal transitions. Note that the repeat spacing of the lamellar component is independent of temperature.

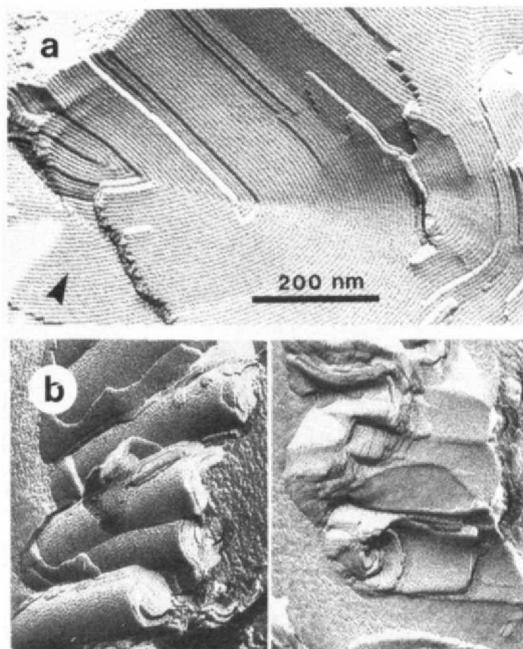


FIGURE 4: Freeze-fracture micrographs of domains within a DOPE-DOPS (1:1) mixture in the presence of Ca^{2+} ($\text{Ca}^{2+}/\text{PS} = 0.5$). Figure 4a shows H_{II} structure and Figure 4b (both bottom panels) cochleate structures. The black bar corresponds to 200 nm, and the direction of shadowing is indicated by the arrowhead.

In the absence of Ca^{2+} , the diffraction pattern of DOPS-DOPE (1:1) is weaker, much slower to come to equilibrium, and much more dependent on the thermal history of the specimen. Typical behavior is shown in Figure 6 at 50 °C, where the specimen gives rise to a well-defined hexagonal lattice (with a repeat of 73 Å) and an ill-defined lamellar component (repeat roughly 50–53 Å). On cooling to 30 °C, the hexagonal component has disappeared. Note that the lamellar repeat (Figure 6, 20 and 30 °C) varies with temperature.

The X-ray diffraction conclusively demonstrates that the high-temperature behavior of the DOPE-DOPS system consists of coexisting lamellar and hexagonal lattices. In the presence of Ca^{2+} , a lamellar component is observed with a repeat spacing similar to the 53-Å repeat found by Papahadjopoulos et al. (1975) for the Ca^{2+} -PS cochleate structure. The repeat spacing of the Ca^{2+} -induced lamellar lattice does not change with temperature, in marked contrast to the lamellar component in the absence of Ca^{2+} , which suggests that the lattice is especially rigid.

To summarize the results for the DOPE-DOPS (1:1) system, therefore, the three independent techniques of ^{31}P NMR, freeze-fracture, and X-ray are entirely consistent with lamellar structure in the absence of Ca^{2+} , and the addition of Ca^{2+} results in segregation of the DOPS component into rigid cochleate domains. This allows the DOPE to revert to the hexagonal (H_{II}) organization preferred by this lipid species in isolation.

The behavior of the DOPE-DOPS (1:1) mixtures on addition of Ca^{2+} is in marked contrast to that observed for the DOPE-DLPS (1:1) system (Figure 1), where a bilayer ^{31}P NMR spectrum is maintained at a $\text{Ca}^{2+}/\text{DLPS}$ ratio of 0.5. No decrease in the ^{31}P NMR signal intensity was observed at such Ca^{2+} levels (Figure 3), and the ^{31}P NMR line shape maintained the lamellar form even at $\text{Ca}^{2+}/\text{DLPS}$ ratios in excess of 5 (results not shown). These results indicated that Ca^{2+} cannot induce lateral segregation of DLPS in these systems.

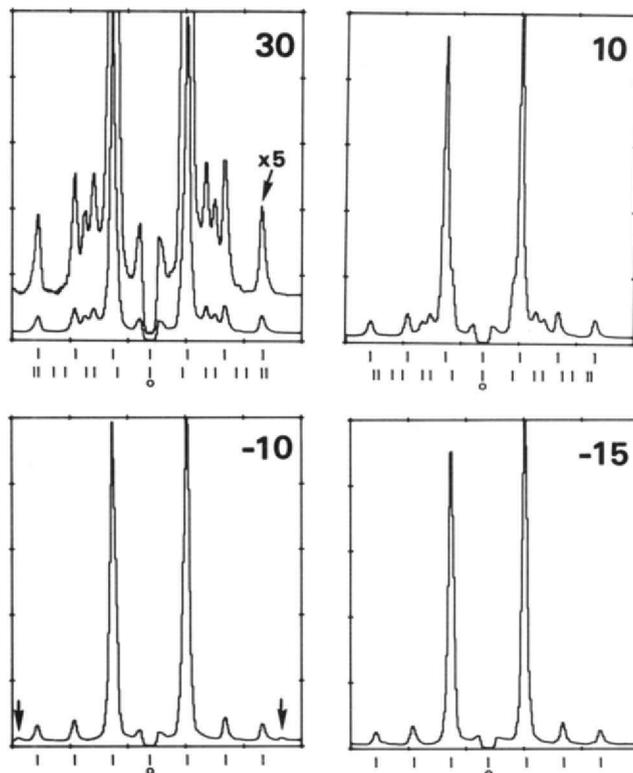


FIGURE 5: Graphs of the diffracted X-ray intensity (arbitrary units) vs. scattering angle for a DOPE- ^{2}H DOPS (1:1) mixture in the presence of Ca^{2+} ($\text{Ca}^{2+}/\text{PS} = 0.5$) at temperatures of 30, 10, -10, and -15 °C. For all the X-ray data presented (Figures 5, 6, and 9), the equidistant tic marks represent the expected peak positions of a lamellar lattice which fits the data; nonequidistant tic marks are for fits to the lower angle peaks from a hexagonal lattice. Only lattices which fit the data to within experimental error are represented. The origin of diffraction, behind the beam stop shadow, is indicated by an "o" below the zero order. For data at -10 °C, arrows indicate a weak reflection with a 15-Å repeat of unknown origin. Basis vectors for the lattices are the following: 30 °C, lamellar = 51 Å, hexagonal = 68 Å; 10 °C, lamellar = 50 Å, hexagonal = 72 Å; -10 °C, lamellar = 50 Å; -15 °C, lamellar = 50 Å.

Previous ^{31}P NMR studies (Cullis & de Kruijff, 1978; Tilcock et al., 1982) have indicated that cholesterol can destabilize bilayer structure in mixed (unsaturated) PE-PC systems, inducing the hexagonal (H_{II}) arrangement for all component lipids. As shown in Figure 7, the presence of cholesterol in DOPE-DOPS and DOPE-DLPS systems also has strong effects. The amount of Ca^{2+} required to obtain an entirely " H_{II} " ^{31}P NMR line shape in the DOPE-DOPS-cholesterol (1:1:1) system is reduced in comparison to the situation when cholesterol is absent (Figure 1A). More strikingly, Ca^{2+} can induce H_{II} ^{31}P NMR lineshapes in the DOPE-DLPS-containing system (compare Figure 7B to Figure 1B). Further, measurement of the integrated ^{31}P NMR signal intensities on addition of Ca^{2+} revealed no decrease for either the DOPE-DOPS-cholesterol (1:1:1) (Figure 3) or the DOPE-DLPS-cholesterol (1:1:1) system (data not shown), suggesting that the DOPS and DLPS adopt H_{II} phase organization in concert with the DOPE and are not segregated into separate cochleate domains.

This interpretation has been subjected to rigorous examination for the DOPE-DOPS-cholesterol (1:1:1) system by employing ^2H NMR and X-ray procedures. First, as indicated elsewhere (Gally et al., 1980; Tilcock et al., 1982), specifically ^2H -labeled phospholipids can reflect bilayer to hexagonal (H_{II}) transitions in a manner analogous to ^{31}P NMR in that the quadrupolar splitting, ΔQ , observed for the ^2H NMR signal

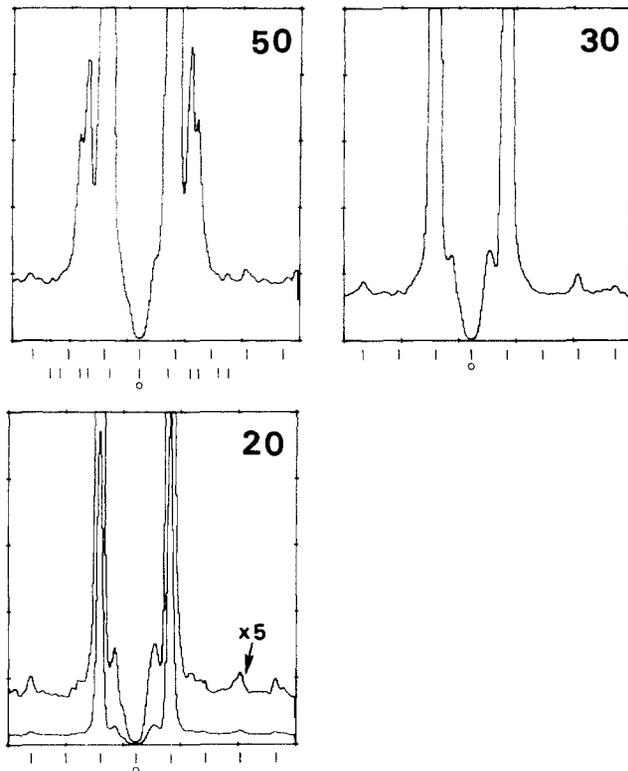


FIGURE 6: Diffraction from DOPE- ^2H]DOPS (1:1) in the absence of Ca^{2+} at 50, 30, and 20 °C. The diffraction is shown full-scale and $\times 5$ for the 20 °C data; the 30 and 50 °C data are only shown on an expanded vertical scale to emphasize weak orders. Note that the lattice is purely lamellar at 30 °C (Compare to Figure 5). Basis vectors are the following: 50 °C, lamellar = 52 Å, hexagonal = 73 Å; 30 °C, lamellar = 52 Å; 20 °C, lamellar = 54 Å.

decreases by more than a factor of 2 on progressing from the lamellar organization to the H_{II} arrangement [see Figure 1 of Tilcock et al. (1982)]. Thus, ^2H NMR studies of DOPE-DOPS-cholesterol systems in which the DOPS is ^2H labeled should show a marked decrease in ΔQ on addition of Ca^{2+} (to a value less than that observed in the absence of Ca^{2+}) if the DOPS enters the H_{II} phase. Alternatively, segregation of the DOPS into cochleate domains should reveal a marked increase in ΔQ due to the crystalline nature of this structure. We therefore synthesized DOPS which is ^2H labeled at the 11-position of the oleic acid acyl groups (11,11- $^2\text{H}_2$]DOPS), and the ^2H NMR results obtained for a DOPE-[11,11- $^2\text{H}_2$]DOPS-cholesterol (1:1:1) system in the presence and absence of Ca^{2+} are illustrated in Figure 8. A reduction of ΔQ (measured as the frequency separation between the two major peaks) from ~ 12 to ~ 5 kHz is observed on addition of Ca^{2+} to achieve a Ca^{2+} to DOPS molar ratio of 0.5, which is direct evidence that the DOPS enters the H_{II} phase matrix along with the DOPE component. ^{31}P NMR studies on the same sample revealed an H_{II} -type line shape in the presence of Ca^{2+} (results not shown).

X-ray diffraction studies revealed that in the presence of Ca^{2+} , the diffraction pattern at 20 °C and higher arises from a hexagonal lattice; a substantial lamellar component does not appear until relatively low temperatures (Figure 9). In contrast to the DOPE-DOPS system without cholesterol, there is no evidence for coexisting lamellar and hexagonal lattices at 30 °C. In the absence of Ca^{2+} , however, the diffraction from the DOPE-DOPS-cholesterol system was marked by an experimentally frustrating variability of results dependent upon the thermal history of the sample. At 30 °C, the diffraction patterns observed had a peculiar shape with sharp peaks on a broad background. Such diffraction characteristics could

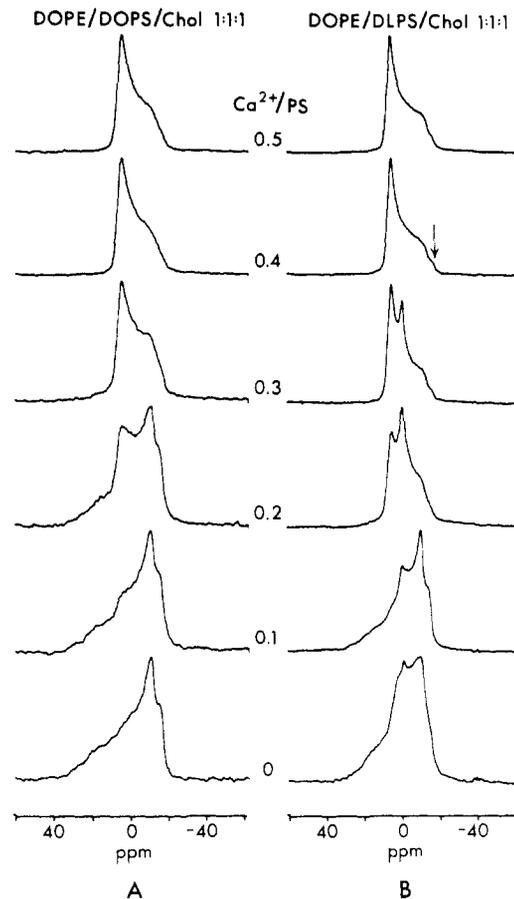


FIGURE 7: 81.0-MHz ^{31}P NMR spectra obtained at 30 °C from aqueous DOPE-DOPS-CHOL (1:1:1) and DOPE-DLPS-CHOL (1:1:1) dispersions in the presence of varying amounts of Ca^{2+} . The ratio Ca^{2+}/PS refers to the molar ratio of Ca^{2+} to phosphatidylserine. The arrow indicates the dual high-field shoulder, which suggests that both PS and PE contributed to the H_{II} line shape (see text).

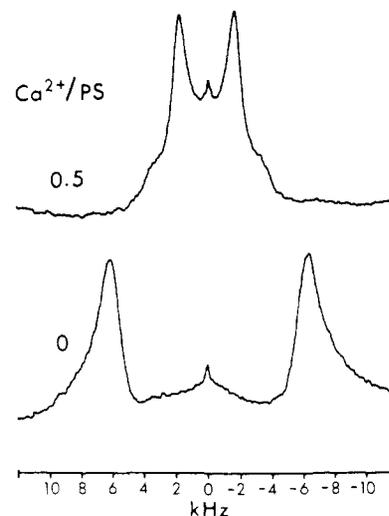


FIGURE 8: 30.7-MHz ^2H NMR spectra obtained at 30 °C for a DOPE-[11,11- $^2\text{H}_2$]DOPS-CHOL (1:1:1) mixture in the absence of Ca^{2+} and in the presence of Ca^{2+} ($\text{Ca}^{2+}/\text{DOPS}$ molar ratio = 0.5).

be modeled by a continuous distribution of lamellar lattices; however, too few details were present to reliably test such models. Consequently, the X-ray diffraction could not be used to test the ^{31}P NMR phase assignment.

In summary, the ^{31}P NMR, ^2H NMR, and X-ray results unequivocally demonstrate that the addition of Ca^{2+} to the DOPE-DOPS-cholesterol (1:1:1) system does not result in phase separation of the DOPS but rather direct incorporation

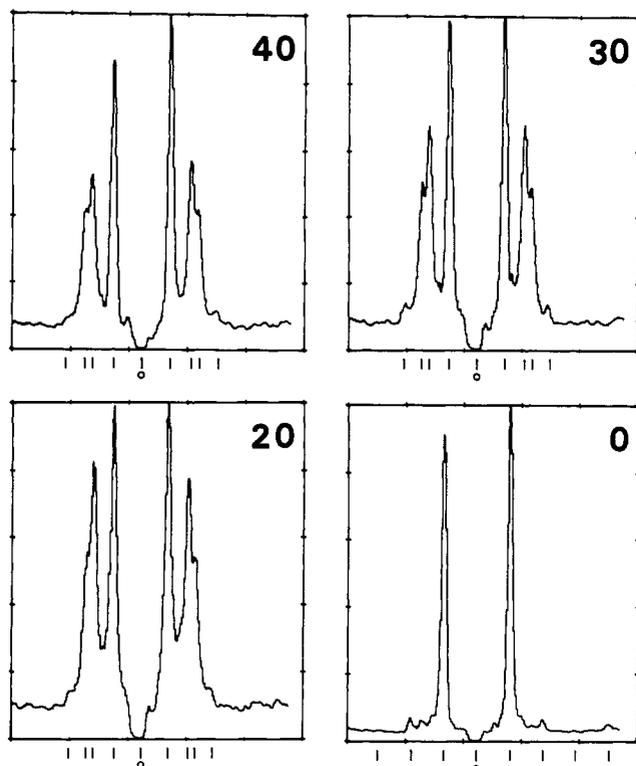


FIGURE 9: Diffraction from DOPE- $[^2\text{H}]$ DOPS-cholesterol (1:1:1) in the presence of Ca^{2+} ($\text{Ca}^{2+}/\text{DOPS} = 0.5$) at 40, 30, 20, and 0 °C. As in Figure 5, Ca^{2+} favors the presence of a hexagonal component at lower temperatures. Basis vectors are the following: 40 °C, hexagonal = 77 Å; 30 °C, hexagonal = 79 Å; 20 °C, hexagonal = 81 Å; 0 °C, lamellar = 57 Å.

of the DOPE and DOPS into a hexagonal lattice.

To return to the influence of cholesterol on the DOPE-DLPS (1:1) system, the results of Figure 7B and the absence of a loss of signal intensity on adding Ca^{2+} suggest that the DOPE-DLPS-cholesterol (1:1:1) system behaves in a manner similar to the DOPE-DOPS-cholesterol system. This is also suggested by the observation of two high-field shoulders for the spectra in the presence of Ca^{2+} (Ca^{2+}/PS molar ratio of 0.4 or 0.5). As indicated elsewhere (Hope & Cullis, 1980), PS has a somewhat larger (absolute) chemical shift anisotropy than PE (~ -52 ppm as opposed to ~ -40 ppm), and thus the shoulder to high field may be assigned to DLPS in the H_{II} phase and the other assigned to H_{II} phase DOPE.

We have shown previously (Tilcock & Cullis, 1981) that the ability of Ca^{2+} to induce the cochleate structure for PS from natural sources and to segregate PS in PE-PS mixtures appears relatively specific in that Mg^{2+} is not able to induce similar effects. This is supported by the observation that Mg^{2+} did not influence the ^{31}P NMR behavior of the DOPE-DOPS (1:1) or the DOPE-DLPS (1:1) systems at Mg^{2+} to PS ratios up to 0.5 (results not shown). However, as illustrated in Figure 10, the addition of Mg^{2+} to such systems when cholesterol is present results in ^{31}P NMR spectra characteristic of the H_{II} phase. Further, as indicated by the dual high-field shoulders exhibited by the DLPS-containing system (arrow, Figure 10), the PS component appears to be incorporated into the H_{II} phase matrix. It may thus be concluded that both Mg^{2+} and Ca^{2+} have a very similar influence when cholesterol is present, implying that their effects result from nonspecific charge neutralization phenomena, in contrast to the segregation processes specific to Ca^{2+} when cholesterol is absent.

The preceding results establish that the ability of Ca^{2+} to segregate PS in PE-PS systems is markedly sensitive to the acyl chain unsaturation and the presence of cholesterol where

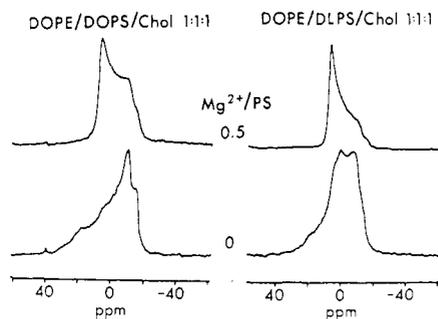


FIGURE 10: 81.0-MHz ^{31}P NMR spectra at 30 °C of DOPE-DOPS-CHOL (1:1:1) and DOPS-DLPS-CHOL (1:1:1) mixtures in the absence and presence of Mg^{2+} (Mg^{2+}/PS ratio = 0.5).

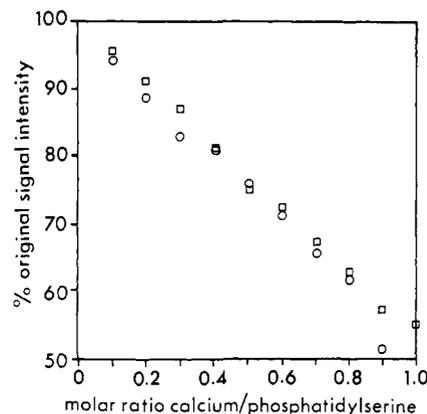


FIGURE 11: Integrated ^{31}P NMR signal intensities for DOPC-DOPS (1:1) (○) and DOPC-DOPS-CHOL (1:1:1) (□) mixtures as a function of added Ca^{2+} concentration.

both increased unsaturation and increased cholesterol content mitigate against Ca^{2+} -induced segregation phenomena. It is of interest to extend such studies to mixtures of PS with other phospholipid species. The properties of DOPC-DOPS systems were therefore investigated in the presence and absence of cholesterol. As may be expected, there was little or no change in the bilayer ^{31}P NMR line shape on addition of Ca^{2+} to either DOPC-DOPS (1:1) or DOPC-DOPS-cholesterol (1:1:1) systems (results not shown), as the DOPC will remain in a bilayer organization even if the DOPS is sequestered by Ca^{2+} . However, as shown in Figure 11, the presence of Ca^{2+} did result in a marked reduction in the ^{31}P NMR signal intensity for both systems, indicating that Ca^{2+} is able to sequester lipid into a rigid phase in the DOPC-DOPS system irrespective of the presence of cholesterol. We believe this is likely to be due to a Ca^{2+} sequestering of DOPS. This interpretation was supported by freeze-fracture studies, as characteristic cochleate structures were visible in both the DOPC-DOPS (1:1) and DOPC-DOPS-cholesterol (1:1:1) systems at Ca^{2+} to DOPS molar ratios of 1.0 (data not shown). It may be noted that a Ca^{2+} to DOPS ratio of 1 was required to reduce the ^{31}P NMR signal intensity to 50% (Figure 11), which is twice that required to produce similar effects for the DOPE-DOPS system (Figure 3). This is consistent with previous observations that a 1:2 Ca^{2+} to PS stoichiometry is not attainable in PC-PS (1:1) systems (Ekerdt & Papahadjopoulos, 1982).

The results of Figure 11 in conjunction with the PE-PS results presented here establish that the influence of Ca^{2+} on the distribution of lipid in mixed phospholipid systems is also sensitive to the phospholipid species contained therein, in addition to acyl chain composition and cholesterol content. This dependence on such a variety of factors entails some difficulty for making a priori predictions regarding the influence of Ca^{2+} on a given membrane system. By way of

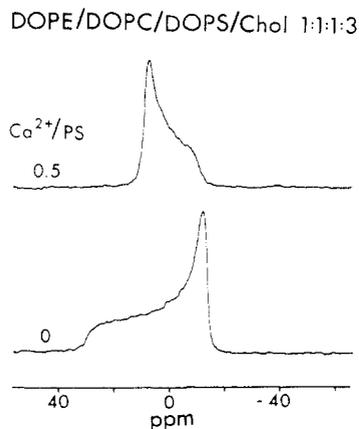


FIGURE 12: 81.0-MHz ^{31}P NMR spectra at 30 °C of a DOPE-DOPC-DOPS-cholesterol (1:1:1:3) mixture in the absence and presence of Ca^{2+} to achieve a Ca^{2+} to PS ratio of 0.5.

example, we have investigated the influence of Ca^{2+} on a DOPC-DOPE-DOPS-cholesterol (1:1:1:3) system by employing ^{31}P NMR techniques as illustrated in Figure 12. In the absence of Ca^{2+} , a bilayer line shape is observed, whereas in the presence of Ca^{2+} ($\text{Ca}^{2+}/\text{DOPS}$ ratio of 0.5), an H_{II} phase line shape is apparent. No reduction of ^{31}P NMR signal intensity was observed, and it would therefore appear that Ca^{2+} is unable to induce segregation of lipid into a rigid lattice and that all the component lipids adopt the H_{II} organization in the presence of Ca^{2+} .

Discussion

The results presented here significantly extend our understanding of the influences of divalent cations both on the distributions of PS in mixed lipid systems and on the overall polymorphic phase preferences expressed by them. Before discussing these properties, however, we emphasize the close agreement between the results obtained from the complementary techniques employed in this work.

The ^{31}P NMR technique has been employed extensively to characterize the bilayer or hexagonal (H_{II}) phase preferences of phospholipids in model and biological membranes [for a recent review, see Cullis et al. (1983)]. The validity of these assignments has been questioned on the basis that a change in head-group orientation could result in a change from bilayer to H_{II} phase line shapes without any change in phase structure (Thayer & Kohler, 1981). We know of no study which demonstrates that such reorientation can occur for naturally occurring phospholipids and note that an extremely close correspondence exists between ^{31}P NMR and X-ray phase identifications of lipid systems. This includes the PC-, PE-, and PS-containing systems investigated here as well as the ^{31}P NMR phase identifications of sphingomyelin (Cullis & Hope, 1980) and cardiolipin (Cullis et al., 1978) which are in full agreement with X-ray characterizations (Shibley et al., 1974; Rand & Sengupta, 1972). Such correspondence is also demonstrated in combined ^{31}P NMR and X-ray studies on other PC and PE species (Ferguson et al., 1982; Marsh & Seddon, 1982). In summary, the broad agreement between ^{31}P NMR and other techniques for phase identification strongly supports the usefulness of the NMR method.

Two other NMR techniques employed in this work merit attention. First, the correlation between the decrease in ^{31}P NMR signal intensity from PS-containing systems in the presence of Ca^{2+} and the appearance of Ca^{2+} -PS coheate aggregates clearly provides a useful method for investigation of Ca^{2+} -induced segregation phenomena in model and biological membranes. Second, the introduction of specifically

^2H -labeled phospholipids into mixed lipid systems followed by ^2H NMR studies provides a direct procedure for monitoring the motional or structural properties of an individual lipid species in a mixed system.

We now discuss the results obtained in this study, which demonstrate that divalent cations exert a plethora of effects on mixed PS-containing lipid systems, depending on the lipid species, acyl chain composition, and cholesterol content. For the purpose of discussion, it is convenient to divide the effects observed into those related to Ca^{2+} -induced segregation of PS and those related to the influence of divalent cations on the polymorphic phase preferences of mixed lipid systems. These two areas are discussed in turn.

The results presented for DOPS- and DLPS-containing systems show clearly that DOPS can be segregated by Ca^{2+} in equimolar mixtures with DOPE. Less direct evidence is presented which indicates that DOPS can also be segregated in equimolar mixtures with DOPC. These results are consistent with previous studies employing mixtures of (spin-labeled) PE, PC, and PS from natural sources (Ohnishi & Ito, 1974; Tokutomi et al., 1981). The subsequent observation that this segregation can be inhibited by cholesterol in the DOPE-DOPS-cholesterol (1:1:1) system but apparently not in the corresponding system containing DOPC may suggest that PS is more readily segregated from more complex mixtures if PE is not present. The addition of DOPE, for example, to the DOPC-DOPS-cholesterol mixture apparently inhibits Ca^{2+} segregation of the PS component. In our opinion, however, there is insufficient evidence for such specific conclusions, and we only note the general trend that, in multicomponent systems containing PE, more unsaturated varieties of PS are less easily segregated by Ca^{2+} . Such an observation is potentially important as PS is normally one of the more unsaturated varieties of lipids found in membranes. In the retinal disks of the rod outer segment, for example, the primary acyl chain substituent of PS is docosahexanoic acid (Miljanich et al., 1981; Areldano & Bazan, 1983), and this membrane also contains 15 mol % cholesterol. The results presented here would suggest that regulation of membrane properties by a cation-induced segregation of PS may not be readily achievable in such systems.

The results presented here and elsewhere (Cullis et al., 1983) illustrate the dramatic influence divalent cations and cholesterol can have on lipid polymorphism. The general point we emphasize is that in mixed PE-containing systems the presence of cholesterol strongly enhances the ability of all component lipids (including nominally bilayer lipids such as PC and PS) to adopt the H_{II} organization on introduction of Ca^{2+} or Mg^{2+} . This is consistent with the well-documented ability of cholesterol to promote H_{II} phase structure in unsaturated PE-PC systems (Cullis & de Kruijff, 1978; Tilcock et al., 1982). The molecular basis of this influence of cholesterol is not understood; however, some rationalization of the behavior of these mixed systems can be achieved through consideration of the geometry or "shape" of component lipids as well as the hydration properties. As discussed elsewhere (Cullis & de Kruijff, 1979; Cullis et al., 1983), substantial support exists for the simplistic hypothesis that a generalized shape property of lipids determines the phase structures adopted. Within this characterization, bilayer lipids are considered to exhibit a cylindrical shape whereas lipids preferring the H_{II} phase are considered to exhibit a "cone" shape compatible with that organization. It should be noted that lipid shape is an inclusive term, reflecting the effects of molecular size and dynamics in polar and apolar regions, head-group hydration and charge,

and hydrogen-bonding processes and the effects of counterions among other possibilities. In the case of the PS-containing systems investigated here for which Ca^{2+} does not induce segregation, it may be expected that the hydration of the PS head group and the electrostatic repulsion between the polar serine moieties will be reduced by the distributed presence of the divalent cation. This may be considered to reduce the effective size of the polar head group (providing a more cone-shaped molecule with similarities to PE) as well as reducing interbilayer separation, both of which are necessary for H_{II} phase formation. Similarly, with respect to cholesterol itself, the distributed presence of this cone-shaped (Cullis & de Kruijff, 1979; Gallay & de Kruijff, 1982) molecule may be suggested to favor H_{II} organization. In addition, the fact that cholesterol hydrates relatively poorly in comparison to phospholipid may facilitate closer interbilayer contact and the H_{II} organization.

In summary, the results presented here illustrate that Ca^{2+} -induced lateral segregation of PS in mixed lipid systems as well as the overall phase preferences of such systems is remarkably sensitive to details of lipid species, acyl chain composition, and cholesterol content. Such observations graphically illustrate the difficulties involved in extrapolating from the behavior of a given lipid species in isolation of its properties in a multicomponent system, such as a biological membrane.

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Registry No. DOPS, 70614-14-1; DOPE, 4004-05-1; DOPC, 4235-95-4; DLPS, 89616-59-1; Ca, 7440-70-2; cholesterol, 57-88-5.

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