Interactions of Monovalent Cations with Phosphatidylserine Bilayer Membranes†

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ABSTRACT: Following the recent description of the structure and thermotropic properties of hydrated diacylphosphatidylserines [Hauser, H., Paltauf, F., & Shipley, G. G. (1982) *Biochemistry 21*, 1061–1067], the effect of monovalent cations on the behavior of the same homologous series of phosphatidylserine (PS) has now been examined. With the utilization of differential scanning calorimetry and X-ray diffraction, the ammonium salts of diacylphosphatidylserines [di-C_{12} (DLPS), di-C_{14} (DMPS), di-C_{16} (DPPS), and di-C_{18} (DSPS)] were shown to form hydrated bilayer membrane structures exhibiting chain length dependent gel → liquid-crystal transitions. Addition of up to 1 M concentrations of NaCl, KCl, RbCl, and CsCl produces relatively minor changes in DMPS bilayer structure of stability. For example, NH_4^+-dimyristoyl-PS (NH_4^+-DMPS) dispersed in NaCl or KCl of increasing molarity, 0–1.5 M, shows only a small, sigmoidal increase in transition temperature from 39 to 42 or 43 °C, with little or no change in transition enthalpy ($\Delta H \simeq 7.4$ kcal/mol). X-ray diffraction of 50 wt % aqueous dispersions of NH_4^+-DMPS shows a lamellar bilayer repeat distance $d = 107$ Å and a sharp reflection at 1/4.2 (Å^{-1}) characteristic of the "hexagonal gel" hydrocarbon chain packing. In the presence of increasing NaCl molarity, the bilayer periodicity is progressively reduced as Na^+ shields the negatively charged bilayer surface. A limiting value of 62 Å is reached at ~0.5 M NaCl. No changes occur to the 1/4.2 (Å^{-1}) reflection, showing that no significant change occurs in the hydrocarbon chain packing. Similar behavior is demonstrated for DLPS, DPPS, and DSPS. In marked contrast, addition of LiCl produces progressive "crystallization" of DLPS, DMPS, and DPPS bilayers as revealed by the presence of a number of X-ray diffraction lines in the wide-angle region, and the bilayer periodicity is reduced. For example, at 1 M LiCl, the bilayer periodicity of NH_4^+-DMPS is reduced to 51 Å, and the Li^+–PS complexes all exhibit transitions from a crystalline bilayer form to liquid-crystal phase at much higher temperatures ($\Delta T = 40$–50 °C), and the enthalpy change associated with the transition approximately doubles compared to Li^+-free PS. Thus, in contrast to the other monovalent cations, Li^+ induces an isothermal crystallization of PS bilayers, the hydrocarbon chains adopting a more ordered packing mode than the hexagonal arrangement of the usual hydrated gel state. The structural changes produced in PS bilayers by Li^+ are similar to those produced by Mg^{2+} and Ca^{2+}, an observation perhaps pertinent to the pharmacological use of Li^+ in the treatment of manic-depressive illness.

Although phosphatidylserine (PS)[1] is a major anionic phospholipid in mammalian cell membranes, being particularly prevalent in peripheral and central nervous system myelin, erythrocytes, and platelets (White, 1973), in comparison with the zwitterionic lipids phosphatidylcholine and phosphatidylethanolamine, relatively little is known about its structure and intermolecular interactions. Much of the early work was performed with phosphatidylserine isolated from bovine brain. It was shown that bovine brain PS formed lamellar bilayer structures and that, due to their negatively charged surface, PS bilayers exhibit "continuous swelling", i.e., can incorporate large amounts of water between adjacent bilayers (Papahadjopoulos & Miller, 1967; Atkinson et al., 1974). The hydration of bovine brain PS bilayers was shown to be sensitive to monovalent and divalent cations (Papahadjopoulos & Miller, 1967; Hauser & Phillips, 1973; Atkinson et al., 1974; Papahadjopoulos et al., 1977) with Ca^{2+}–PS or Mg^{2+}–PS interactions perhaps playing a role in membrane fusion processes [for a review, see Papahadjopoulos (1978)].

More recently, the availability of synthetic PS of controlled fatty acyl chain length and unsaturation has led to more detailed studies of the structure and properties of PS bilayers. For example, DPPS has been shown to undergo a thermotropic transition at approximately 53 °C, utilizing differential scanning calorimetry (MacDonald et al., 1976; van Dijck et al., 1978; Browning & Seelig, 1980; Cevc et al., 1981), spin-label partitioning (Luna & McConnell, 1977; Cevc et al., 1981), and ^{31}P and ^2H nuclear magnetic resonance (NMR) (Browning & Seelig, 1980). Our own recent study (Hauser et al., 1982) examined the structure and thermotropic properties of a homologous series of synthetic diacylphosphatidylserines (di-$C_{10:0}$, di-$C_{12:0}$, di-$C_{14:0}$, di-$C_{16:0}$, di-$C_{18:0}$) in their acidic and NH_4^+ salt forms, utilizing X-ray diffraction and scanning calorimetry. With the exception of DSPS, which forms a hexagonal (type II) liquid-crystal phase [for phase nomenclature, see Luzzati (1968) and Shipley (1973)] above its gel–liquid-crystal transition, hydrated NH_4^+-PS undergo chain length dependent transitions between bilayer gel and bilayer liquid-crystal phases, both of which take up water continuously. A detailed study of the hydration of NH_4^+-DMPS at pH 6.8 has provided the temperature–composition phase diagram of NH_4^+-DMPS, and an analysis of the electron

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[1] Abbreviations: PS, phosphatidylserine; DDPS, didecanoylphosphatidylserine; DLPS, dilauroylphosphatidylserine; DMPS, dimyristoylphosphatidylserine; DPPS, dipalmitoylphosphatidylserine; DSPS, distearoylphosphatidylserine; DL-DLPS, dilauroylphosphatidylserine with glycerol C-2 in the racemic form; DL-DPPS, dipalmitoylphosphatidylserine with glycerol C-2 in the racemic form; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; DSC, differential scanning calorimetry; TLC, thin-layer chromatography. Unless stated otherwise, the glycerol of PS is in the native D configuration and the serine in the native L form.

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density profiles derived from low-angle X-ray diffraction data has provided accurate structural parameters of both the gel and liquid-crystal bilayers (Hauser et al., 1982). Hydrated acidic PS at pH 1–2 show higher gel → liquid-crystal transitions than the corresponding NH_4^+ salts but do not exhibit extensive hydration (Hauser et al., 1982).

In a related scanning calorimetry study, van Dijck et al. (1978) showed that the transition temperature of DMPS decreased on titrating the carboxylate-group proton ($pK_a \simeq 4.4$) from ~54 (pH <3.8) to ~37 °C (pH ~6.8) [see also MacDonald et al. (1976)]. A more detailed study by Cevc et al. (1981) utilizing electron spin resonance (ESR) spin-labeling and DSC confirms this pH dependence of the transition behavior for both DMPS and DPPS and, in addition, identifies a second pK_a at $\simeq$11.5 corresponding to deprotonation of the amino group of the serine, with (a) a decrease in the transition temperature to 7 °C for DMPS and (b) the appearance of a "pretransition". In general, there is good agreement concerning the thermotropic properties of hydrated diacyl-PS [see, for example, MacDonald et al. (1976), van Dijck et al. (1978), Cevc et al. (1981), and Hauser et al. (1982)].

Of obvious importance is the effect of monovalent and divalent cations on PS structure and properties, since ion–lipid interactions could act as "triggers" to alter membrane-surface behavior. For example, it has been shown that Ca^{2+} produces hydrocarbon chain "crystallization" in bovine brain PS (Newton et al., 1978; Hauser et al., 1977) and, presumably through a related mechanism, induces lateral phase separation in mixed phosphatidylserine–phosphatidylcholine bilayers (Ohnishi & Ito, 1974; van Dijck et al., 1978). Again, most studies of the effect of monovalent cations have utilized bovine brain PS monolayers and bilayers (Papahadjopoulos & Miller, 1967; Papahadjopoulos, 1968; Atkinson et al., 1974; Nir et al., 1978; Kurland et al., 1979; Eisenberg et al., 1979; Puskin, 1977; Abramson et al., 1961; Hauser et al., 1975), but few conclusions can be derived concerning the ion dependence of either the PS bilayer structure or its thermotropic behavior. However, the recent study of DMPS and DPPS by Cevc et al. (1981) does address the problem of Na^+ effects on the thermotropic behavior of DMPS (see Discussion).

This study documents the effects of Li^+, Na^+, K^+, Rb^+, and Cs^+ on a homologous series of synthetic diacyl-PS. Alterations in PS structure and thermotropic properties induced by these ions have been monitored by X-ray diffraction and DSC. The effects of Li^+ are particularly interesting, and we have reported briefly on the Li^+–DMPS system (Hauser & Shipley, 1981). Here, we explore in more detail the interaction of monovalent cations with the homologous series of PS. The corresponding study of the effect of divalent cations will be reported later (H. Hauser and G. G. Shipley, unpublished observations).

Materials and Methods

Materials. A homologous series of 2,3-diacyl-D-glycero-1-phospho-L-serines [for a discussion of this chemical nomenclature, see Hauser et al. (1981)] was synthesized as described elsewhere (Hermetter et al., 1982). DMPS, DPPS, and DSPS were also synthesized by R. Berchtold (Biochemisches Labor, Bern, Switzerland) essentially according to the method of Aneja et al. (1970). The amino acid serine was always in the native L form and unless stated otherwise, the optically active form of glycerol was used with the C-2 atom in the native D configuration. The lipids were purified, and their purity was monitored as described earlier (Hauser et al., 1982). The interconversion of the acid form and the ammonium salt of the above lipids was also described before (Hauser et al., 1982). NH_4Cl and alkali metal chlorides (Puriss grade) were purchased from Merck (Rahway, NJ). All other chemicals used were analytical grade.

Sample Preparation. Hydrated samples for scanning calorimetry (DCS) were prepared by weighing the solid (0.5–5 mg) into the DSC pan and adding the appropriate amount of aqueous solvent gravimetrically. The DSC pan was immediately sealed and transferred to the calorimeter. The aqueous solvent consisted usually of 5 mM ammonium phosphate buffer, pH 5.8–7.0, to which NH_4Cl or alkali metal chlorides had been added. Hydrated samples for X-ray diffraction were prepared by weighing the lipid into a glass tube with a narrow constriction in the center. After addition of the appropriate salt solution in H_2O or buffer, the glass tube was immediately sealed and the lipid dispersion homogenized by centrifuging it through the constriction 6–12 times at a temperature above the chain melting temperature.

DSC was performed at heating and cooling rates of 5 °C/min in a Perkin-Elmer DSC-2 scanning calorimeter (Norwalk, CT). Transition temperatures were determined from the peak maximum, and the transition enthalpy was determined from the area under the peak as measured by planimetry and compared with the known enthalpy of the standards gallium and indium.

For X-ray diffraction studies, nickel-filtered Cu Kα X radiation from an Elliott GX-6 rotating anode generator (Elliott Automation, Borehamwood, England) was used. The X-rays were focused with a camera having toroidal optics (Elliott, 1965), and the X-ray diffraction patterns were recorded from samples maintained at different temperatures in a variable-temperature specimen holder. X-ray diffraction intensities were derived by using a Joyce-Loebl Model III-CS microdensitometer. For more details of the method, see Janiak et al. (1976).

Results

Differential Scanning Calorimetry. Scanning calorimetry of NH_4^+-DMPS dispersed in 0.025 M ammonium phosphate, pH 6.8, shows a sharp endothermic order–disorder transition, reproducibly at 39 °C (ΔH = 7.4 kcal/mol of DMPS) (Figure 1, top curve). Single, sharp transitions were also observed on cooling, but the transition temperature was depressed by 4–5 °C. Adding the disodium salt of EDTA (up to 15 mM) to the solvent had no effect on the thermal behavior of NH_4-DMPS; the transition temperature as well as the transition enthalpy remained unchanged. The effect of 0.5 M NH_4^+ and alkali metal chlorides (Li^+, Na^+, K^+, Rb^+, Cs^+) on the thermal behavior of NH_4^+-DMPS bilayers is illustrated in Figure 1. With the exception of Li^+, all monovalent cations up to 0.5 M concentrations produced only small changes in the transition temperature T_c and transition enthalpy (see Figure 1 and legend). The effect of NaCl and KCl over the concentration range 0–1.5 M on the endothermic gel → liquid-crystalline transition of NH_4^+-DMPS is shown in Figure 2. Over the range 0–1.5 M, the reversible gel → liquid-crystalline transition temperature increases sigmoidally from 39 °C and apparently levels off at ~43 °C for Na^+ and ~42 °C for K^+. Although the differences in the transition temperature data are small (see Figure 2), they do suggest a slightly higher binding constant for Na^+ compared to K^+, consistent with previously published binding-constant data [see Eisenberg et al. (1979) and Puskin (1977)].

This rather small effect on T_c is in marked contrast to the effects produced by LiCl. At 0.5 M concentration, Li^+ produced a 50 °C increase in the transition temperature from 39 to 89 °C (Figure 1g). The transition enthalpy ΔH = 16.0 ±

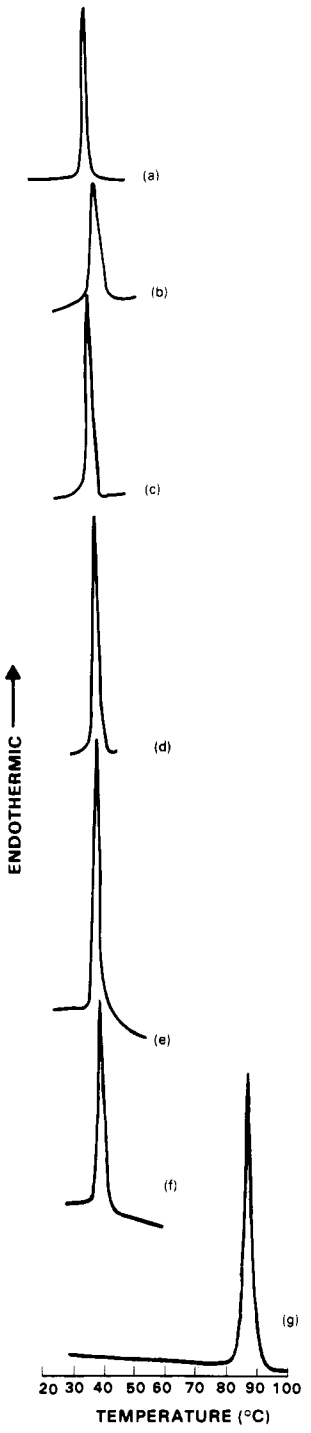

FIGURE 1: DSC heating curves showing effect of different monovalent cations on transition temperature, T_c, of NH_4^+-DMPS. Solid lipid (a single batch of NH_4^+-DMPS was used) was weighed into the DSC pan and the appropriate amount of aqueous solvent added to the pan to give 1–1.5 wt % lipid dispersions: (a) NH_4^+-DMPS dispersed in 0.025 M ammonium phosphate, pH 6.8 (I = 0.05). NH_4^+-DMPS dispersed in 5 mM ammonium phosphate, pH 5.8–6.3, containing 0.5 M concentrations of the following salts: (b) NH_4Cl, (c) NaCl, (d) KCl, (e) RbCl, (f) CsCl, (g) LiCl. Heating curves were recorded at a heating rate of 5 °C/min. Single, sharp transitions were also observed upon cooling (5 °C/min); the transition temperature was usually depressed by 4–5 °C (compared to the transition temperature on heating), except for the experiment with LiCl. With LiCl, a much larger hysteresis effect was observed, and the transition temperature was depressed by 16–20 °C. The transition temperatures (transition enthalpies, ΔH) were as follows: (a) 39.0 °C (7.4 ± 0.5 kcal); (b) NH_4Cl, 39.0 °C (7.5 ± 0.5 kcal); (c) NaCl, 39.0 °C (7.2 ± 0.5 kcal); (d) KCl, 38.5 °C (7.5 ± 0.7 kcal); (e) RbCl, 38.0 °C (7.3 ± 1 kcal); (f) CsCl, 39.0 °C (7.3 ± 1 kcal); (g) LiCl, 89.0 °C (16.0 ± 0.4 kcal).

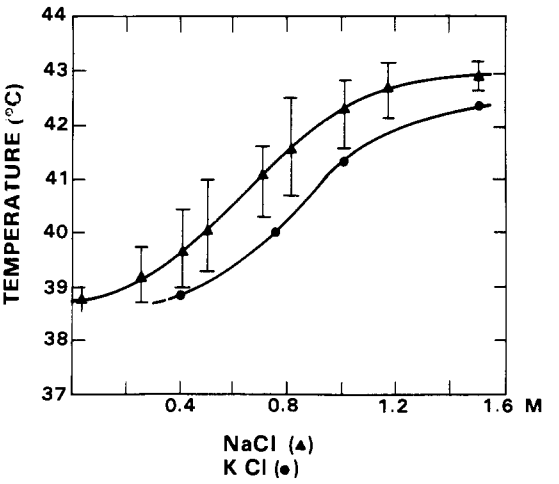

FIGURE 2: Effect of increasing concentrations of NaCl (▲) and KCl (●) on transition temperature of NH_4^+-DMPS. The temperature of the peak of the endotherm recorded at a heating rate of 5 °C/min is plotted as a function of the alkali metal chloride concentration. The bars represent the range of values obtained with different batches of NH_4^+-DMPS (three experiments per point). The experiments with KCl were carried out with a single NH_4^+-DMPS batch, resulting in a smoother curve.

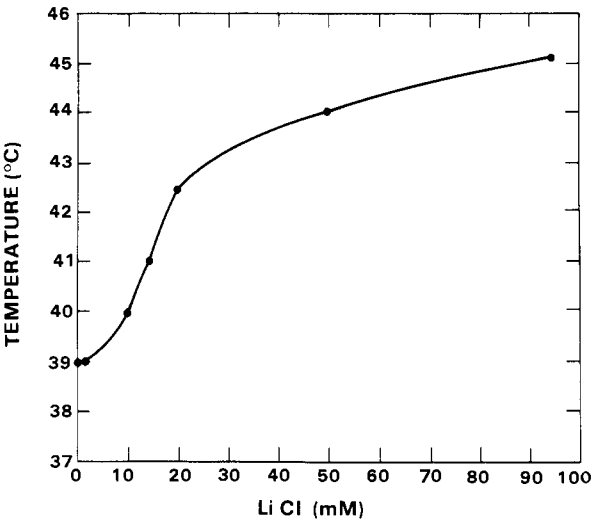

FIGURE 3: Effect of LiCl (0–100 mM) on transition temperature of NH_4^+-DMPS. The lipid was dispersed in 5 mM ammonium phosphate buffer, pH 6.7, containing the appropriate amount of LiCl. Similar curves were observed on cooling except that the transition temperatures were lowered.

0.4 kcal/mol DMPS measured at 0.5 M LiCl was more than twice that of the other ions, indicating that, in contrast to the other alkali metal ions, Li^+ forms a stable complex with DMPS. The overall effect of increasing LiCl concentrations on the thermal behavior of DMPS bilayers was the subject of an earlier report [see Hauser & Shipley (1981)]. Briefly, at about 10 mM LiCl, a second transition appeared at higher temperatures, and with increasing LiCl concentration the enthalpy associated with the high-temperature transition increased at the expense of that observed at low temperatures. At 0.5 M LiCl, the low-temperature transition had almost disappeared and had been replaced by a sharp transition at 89 °C with ΔH = 16.0 kcal/mol (see also Figure 1g). In the concentration range 0.01–0.5 M LiCl, both transitions were observed as rather broad endotherms. We now show that in the range 0–100 mM LiCl a sigmoidal increase in the low transition temperature is observed, reaching a limiting value of ~45 °C (Figure 3). In addition, we show that other

Table I: Effect of Excess 1 M LiCl on Thermal Behavior of Ammonium Salts of Diacylphosphatidylserines[a]

lipid	sample	transition temp, T_c (°C)	transition enthalpy, ΔH (kcal/mol)
NH_4^+-DL-DLPS	14 wt % NH_4^+-DL-DLPS in 0.025 M ammonium phosphate buffer, pH 6.8	17.0	4.9
NH_4^+-DL-DLPS	2.3 wt % NH_4^+-DL-DLPS in 1 M LiCl in 5 mM ammonium phosphate, pH 6.0	68.0	8.7
NH_4^+-DMPS	21 wt % NH_4^+-DMPS in 0.025 M ammonium phosphate, pH 6.8	39.0	7.4
NH_4^+-DMPS	3.2 wt % NH_4^+-DMPS in 1 M LiCl in 5 mM ammonium phosphate, pH 6.0	92.0	17.0
NH_4^+-DPPS	14 wt % NH_4^+-DPPS in 0.025 M ammonium phosphate, pH 6.8	54.0	9.1
NH_4^+-DPPS	5.1 wt % NH_4^+-DPPS in 1 M LiCl in 5 mM ammonium phosphate, pH 6.0	98.0	18.4

[a] The NH_4^+ salt of PS was mixed with an excess of 1 M LiCl in the DSC pan, which was then immediately sealed hermetically.

saturated diacylphosphatidylserines form similar complexes with LiCl (Table I). Similar to DMPS, the reversible gel → liquid-crystalline transition temperatures of DLPS and DPPS were also markedly increased in the presence of excess 1 M LiCl, and also, the transition enthalpies increased by a factor of 1.8 and 2, respectively, under these conditions. DSPS is not included in Table I because excess LiCl had only a small effect. With DSPS, LiCl concentrations of 0.5–1 M produced an increase in T_c from about 70 (Hauser et al., 1982) to 75–83 °C; the transition enthalpy remained essentially unchanged.

The effects of alkali metal ions on the thermal behavior of phosphatidylserines can be used to derive *estimates* for the apparent binding constants (Figure 2). For Na^+ and K^+, the sigmoidal curves shown in Figure 2 were used.[2] Approximate values derived under these conditions were K_{app} = 1–1.5 M^{-1} for both Na^+ and K^+ binding to DMPS. Considering the assumptions involved, the value for Na^+ is in reasonable agreement with values derived from NMR, K_{app} = 1.2 M^{-1} (Kurland et al., 1979), and monolayer experiments, K_{app} = 6.7 M^{-1} (Hauser et al., 1976), utilizing bovine brain PS.

[2] The sigmoidal curves (Figure 2) are used to derive order of magnitude estimates for the binding of Na^+ and K^+ to DMPS. The following assumptions are made: (i) All binding sites are accessible to the cation. (ii) Equilibrium is attained for the reaction cation + DMPS ⇌ cation–DMPS. (iii) According to this reaction scheme, a 1:1 stoichiometry applies and

$$K_{app} = [\text{DMPS–cation}]/([\text{DMPS}]_{free}[\text{cation}]_{free})$$

(iv) The binding of Na^+ or K^+ to DMPS is weak so that $[Na]_{total} \gg [Na]_{bound}$, and therefore, the approximation $[Na]_{free} \simeq [Na]_{total}$ holds. That this is a valid assumption for all monovalent ions except Li^+ is shown by the following considerations: in the concentration range where binding of Na^+ and K^+ occurs, e.g., at concentration > 0.6 M, there is a 50–100-fold excess of cations, and even if all the DMPS binding sites are occupied by cations, the concentration of the remaining free cations would approximate the total cation concentration. (v) The "high-melting" form in Figure 2 is due to the DMPS–cation complex. (vi) The binding of cations to DMPS does not induce a conformational change in the DMPS molecule, thus modifying its binding properties. These assumptions appear to be reasonable for the system DMPS plus Na^+ or K^+; for the midpoint (inflection point) in Figure 2, we have therefore: $[DMPS]_{free} = [DMPS–cation] = [DMPS]_{total}/2$, and the above equation for K_{app} reduces to $K_{app} = 1/[cation]_{free} \simeq 1/[cation]_{total}$. In contrast to Na^+ and K^+, the errors involved in estimating the Li^+ binding constant from the calorimetric data are substantial. For this reason, the calorimetric and X-ray diffraction data in Figures 3 and 5 can only be presented as a function of the molarity of the LiCl solution in which they were dispersed and *not* as a function of the molarity of the LiCl solution with which the Li^+-free and Li^+-bound DMPS are in equilibrium. Although potentially a similar problem exists for the Na^+ and K^+ data presented in Figures 2 and 5, for the reasons discussed above, the molarity plotted on the abscissa more closely approximates that of the salt solution in equilibrium with the DMPS bilayers.

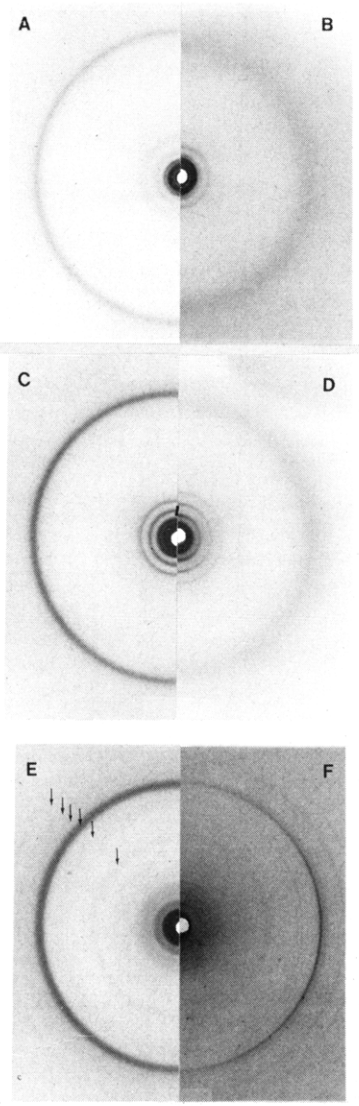

FIGURE 4: X-ray diffraction patterns of 50 wt % dispersions of NH_4^+-DMPS in 5 mM ammonium phosphate buffer, pH 6.7. (A) NH_4^+-DMPS, 20 °C; (B) NH_4^+-DMPS, 50 °C; (C) NH_4^+-DMPS in the presence of 0.5 M NaCl, 20 °C; (D) NH_4^+-DMPS in the presence of 0.5 M NaCl, 50 °C; (E) NH_4^+-DMPS in the presence of 0.5 M LiCl, 20 °C; (F) NH_4^+-DMPS in the presence of 1.0 M LiCl, 20 °C.

While the physiologically important ions Na^+ and K^+ had only minor effects on the thermal behavior of NH_4^+-DMPS bilayer, organic cations such as those present in the widely used Tris buffer had a marked effect. In Tris buffer at 10 mM

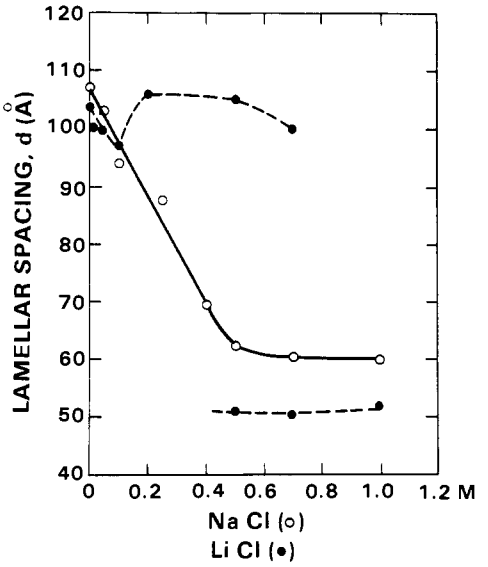

FIGURE 5: X-ray diffraction long spacings, d, of 50 wt % aqueous dispersions of NH_4^+-DMPS as a function of NaCl (○) and LiCl (●) concentration. The lipid was dispersed in water or 5 mM ammonium phosphate buffer, pH 6.7, containing the appropriate amount of either NaCl or LiCl as described under Materials and Methods. X-ray diffraction patterns were recorded at 20 ± 2 °C.

concentrations, pH 7.3, the transition temperature of DMPS was depressed by ~3 °C (T_c = 36 °C on heating, T_c = 31 °C on cooling), and at 100 mM (pH 7.35), it was depressed by nearly 10 °C (T_c = 29.5 °C on heating, T_c = 25.0 °C on cooling).

X-ray Diffraction. The different effects of NaCl and LiCl on the structure of DMPS bilayers are clearly demonstrated by X-ray diffraction experiments (Figure 4). In the absence of alkali metal chloride, 50 wt % dispersions of NH_4^+-DMPS in 5 mM ammonium phosphate buffer, pH 6.8, at 20 °C gave a series of rather broad lamellar reflections in the low-angle region corresponding to a repeat distance, d (lipid bilayer + intercalated water), of 107 ± 3 Å (Figure 4A). The wide-angle region shows a single sharp reflection at 1/4.2 ($Å^{-1}$) characteristic of the "hexagonal" hydrocarbon chain packing associated with the "gel" state of phospholipids. At 50 °C, above the gel → liquid-crystal transition, lamellar reflections of periodicity d = 77.0 Å are observed, together with a broad reflection at 1/4.5 ($Å^{-1}$) characteristic of the "melted" liquid-crystalline state (Figure 4B). As described previously for bovine brain phosphatidylserine (Atkinson et al., 1974), increasing amounts of Na^+ shield the negatively charged bilayer surface, and a [Na^+]-dependent reduction in the lamellar periodicity occurs as water is excluded from the interbilayer space. Diffraction patterns of NH_4^+-DMPS dispersed in 0.5 M NaCl at temperatures below (20 °C) and above (50 °C) the gel → liquid-crystal transition are shown in panels C and D of Figure 4, respectively. The dependence of the bilayer periodicity on [NaCl] at 20 °C is shown in Figure 5. The lamellar repeat distance decreased linearly to [NaCl] ≃ 0.5 M and then leveled off. At 0.5 M NaCl the lamellar periodicity was reduced to about 60% of its original value, i.e., from 107 to 62 ± 0.5 Å (mean of six measurements ± SD). However, over the whole concentration range studied, no change occurred in the wide-angle region, and only the 1/4.2 ($Å^{-1}$) reflection (Figure 4C) was observed. This indicates that there is no significant change in the hydrocarbon chain packing of DMPS as NaCl is added. A similar pattern of behavior is observed in the liquid-crystalline state of DMPS at 50 °C, where again the lamellar periodicity decreases with increasing

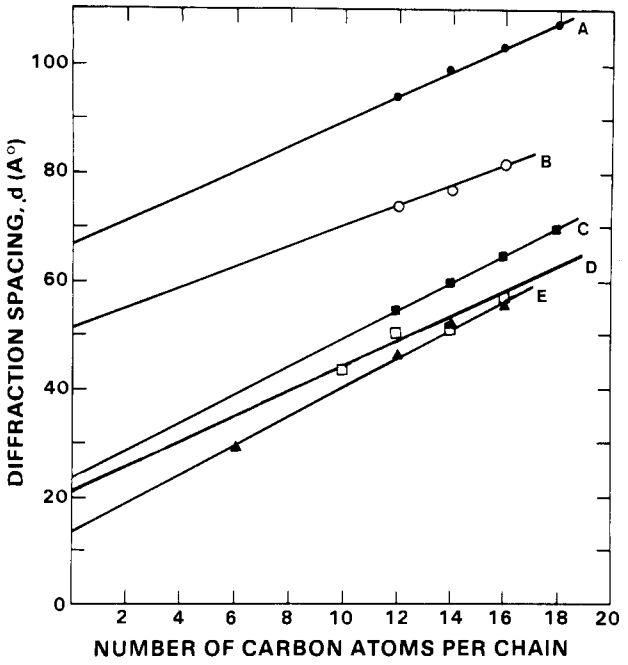

FIGURE 6: X-ray diffraction long spacings, d, of diacylphosphatidylserines dispersed in aqueous buffer in the absence (A and B) and presence (C and D) of 0.5 NaCl, as a function of hydrocarbon chain length. 50% dispersions of the phospholipid in 5 mM ammonium phosphate buffer, pH 6.6, in the absence and presence of 0.5 M NaCl were prepared as described under Materials and Methods. X-ray diffraction data in the absence of added ions in the gel (●) and liquid-crystalline (○) states are taken from Figure 5 of Hauser et al. (1982). X-ray diffraction patterns in the presence of 0.5 M NaCl were recorded at 20 °C, i.e., below the gel to liquid-crystal transition temperature (T_c) and in the liquid-crystalline state at temperatures ~13 °C above the transition temperature. For the latter, DDPS was measured at 20 °C, DLPS at 37 °C, DMPS at 53 °C, DPPS at 69 °C, and DSPS at 84 °C. The solid lines are least-squares fit with r^2 = 1.0 and r^2 = 0.92 for the gel (■) and the liquid-crystalline (□) state, respectively. Lamellar repeat distances of saturated diacyl-PS phosphatidylserines in the presence of 1 M LiCl are also plotted as a function of the hydrocarbon chain length (▲). 50 wt % dispersions of the lipids in H_2O, pH 6.0, containing 1 M LiCl were prepared as described under Materials and Methods. No difference in the X-ray diffraction pattern was observed when 5 mM ammonium phosphate buffer, pH 6.9, was used instead of water as the solvent. X-ray diffraction patterns were recorded at 20 ± 2 °C. With the short-chain compounds dihexanoyl-PS and DL-DLPS, only the first-order reflections were observed. The solid line is a least-squares fit, with a linear regression analysis, $y = 13.2 + 2.7x$, with r^2 = 0.99. For reasons discussed under Results, the data obtained with DSPS were not included.

NaCl concentration (see below and Figures 4D and 6).

Similar to DMPS dispersions, a reduction of the lamellar repeat distance d was also observed for other diacylphosphatidylserines (di-C_{12}, di-C_{16}, di-C_{18}) in the presence of NaCl. This is exemplified by 50 wt % dispersions of diacylphosphatidylserines in 0.5 M NaCl, both in the gel and in the liquid-crystalline state. In the presence of 0.5 M NaCl all samples give low-angle diffraction lines in the ratio 1: $^1/_2$:$^1/_3$:$^1/_4$..., indicative of a lamellar bilayer phase. Below the transition temperature a single, sharp reflection at 1/4.2 ($Å^{-1}$) is observed, characteristic of a hydrated gel phase. Above the transition phase a single broad reflection at 1/4.5 ($Å^{-1}$) indicative of melted hydrocarbon chains is observed (for DMPS examples, see Figure 4C,D). In both the gel and the liquid-crystalline states, NaCl produced a significant reduction in the lamellar repeat distance d. For 50 wt % dispersions of diacyl-PS in 0.5 M NaCl, the lamellar repeat distance d shows a linear relationship with PS hydrocarbon chain length (Figure 6, lines C and D). In the bilayer gel state the data were

recorded at a fixed temperature (20 °C) and in the bilayer liquid-crystal state at temperatures ~13 °C above the gel → liquid-crystal transition temperature. A linear regression analysis was applied to the data to derive the repeat distance d at zero chain length. In the gel and liquid-crystalline state (lines C and D, respectively) values of 23.5 and 21.0 Å are obtained, respectively. These extrapolated values correspond to the thickness of two polar group layers plus the intercalated water at 50 wt % dispersions. The lamellar repeat distances d of 50 wt % diacylphosphatidylserines of different chain length dispersed in 0.025 M ammonium phosphate buffer without Na^+, below and above the transition temperature, are plotted for comparison [lines A and B, respectively, in Figure 6; data taken from Hauser et al. (1982)]. This comparison clearly shows that, for all hydrated PS bilayers, the addition of [NaCl] = 0.5 M produced a significant reduction in the lamellar repeat distance d in both the bilayer gel and liquid-crystal states (cf. line A with C and line B with D in Figure 6, respectively).

The pattern of behavior with added LiCl is quite different. First, in 0–0.5 M LiCl there appears to be a small reduction in the lamellar periodicity (0–0.1 M LiCl) (Figure 5). However, over this concentration range, significant changes do occur in the wide-angle region (Figure 4E). Several reflections are now visible in the wide-angle region [note the reflections at 1/6.55, 1/4.75, 1/4.18, 1/3.93, 1/3.70, and 1/3.55 ($Å^{-1}$), arrowed in Figure 4E], characteristic of a more ordered hydrocarbon chain packing mode. In the range 0.5–0.7 M LiCl, diffraction patterns from two phases are evident, one characterized by the original lamellar periodicity ($d \simeq 105$ Å) and another one with a reduced lamellar repeat $d = 51.8 \pm 0.5$ Å (Figure 5). In 1 M LiCl, only one phase is present with $d = 51.8 \pm 0.4$ Å (Figure 4F and 5). The wide-angle reflections of this sample and of other samples in 0.5–1 M LiCl are as described above (cf. Figure 4E,F). Thus, in contrast to Na^+, Li^+ binding to DMPS induces "crystallization" of the hydrocarbon chains, and a more ordered bilayer structure is produced.

This behavior was demonstrated for different diacylphosphatidylserines. DLPS, DMPS, and DPPS were dispersed in 50% H_2O, pH 6.0, and LiCl (0.7–1 M) was added. X-ray diffraction patterns were recorded below the transition temperature at 20 ± 2 °C. When the lamellar repeat distance, d, was plotted as a function of hydrocarbon chain length, a linear relationship was obtained (Figure 6, line E), and extrapolation to zero chain length gives a periodicity corresponding to the thickness of two polar groups and any intercalated water. In fact, both line E and the extrapolated value (13.5 Å) are in close agreement with the data derived from crystal form I of *anhydrous* acidic PS or NH_4^+-PS plotted in a similar fashion [see Figure 1 in Hauser et al. (1982)].

Discussion

Our calorimetric data (Figures 1 and 2) show that with the exception of LiCl, the alkali metal chlorides produce only a relatively small change in the thermotropic behavior of DMPS. Over the concentration range 0–1.5 M, NaCl (KCl) produces a sigmoidal increase in the bilayer gel → liquid-crystal transition temperature from 39 to 43 (42.5) °C, with little or no change in the transition enthalpy. In this concentration range, these effects are similar to those observed by Cevc et al. (1981) for Na^+ on DMPS, utilizing an ESR spin-labeling approach. In addition, these authors (Cevc et al., 1981) showed that at higher NaCl concentrations, in the range 2–6 M, a second and somewhat larger increase in transition temperature occurred. At pH 6.5–7, each PS molecule has a single net negative charge. An increase in ionic strength by added salt (NaCl or KCl) effectively screens the surface potential of negatively charged bilayers (Eisenberg et al., 1979), and an increase in transition temperature is predicted [see Träuble & Eibl (1974)]. Cevc et al. (1981) have examined the electrostatic contribution to the transition temperature of DMPS and have shown that the screening at high-salt concentrations is greater than that predicted by the Gouy–Chapman theory. It is clear from the X-ray diffraction data (Figure 4) that over this range of salt concentrations (0–1.5 M) no major change in lipid packing within a PS bilayer occurs. X-ray diffraction shows an identical reflection at 1/4.2 ($Å^{-1}$) in the presence or absence of salt. The major effect of added NaCl, as shown in Figure 5, is the pronounced decrease in the lamellar periodicity due to salt-induced exclusion of aqueous buffer from the *inter*bilayer space. For 50 wt % DMPS dispersions at 20 °C ($d \simeq 107$ Å), this condensing effect is maximal over the [NaCl] range 0–0.4 M, reaching a limiting value of 62 Å at 0.5–0.6 M NaCl. Thus, screening of the bilayer-surface potential produces only a small alteration in lipid packing and thermodynamic properties but a major change in the *inter*bilayer interactions.[3] At 50 °C, hydrated DMPS in the liquid-crystalline state exhibits similar behavior; in this case, the lamellar periodicity decreases from 77 to 51.5 Å. As indicated in Figure 6, similar effects on interbilayer forces are observed for all the PS studied in both the gel and liquid-crystalline states.

Utilizing the chain length dependent data presented in Figure 6, we can calculate structural parameters of PS bilayers and evaluate their modification by the presence of 0.5 M NaCl. First, in the gel state in the absence of NaCl (Figure 6, curve A), the increment is 2.25 Å/CH_2 group; in the presence of 0.5 M NaCl (curve C), the increment is 2.55 Å/CH_2 group. This would suggest that although the two bilayer structures are closely related, a change from a bilayer with hydrocarbon chains slightly tilted with respect to the bilayer normal [curve A, see Discussion in Hauser et al. (1982)] to a bilayer with chains perpendicular to the bilayer (curve C) may be induced by NaCl. Small changes in hydrocarbon chain tilting would not alter significantly the 1/4.2 ($Å^{-1}$) wide-angle diffraction and could explain the slightly increased chain melting transition. If one assumes that the contribution of two glycerophosphoserine polar groups is identical in the presence and absence of NaCl, the change in aqueous layer thickness for *all* PS can be calculated from the difference in lamellar periodicities at zero chain length. A decrease of 43.5 Å in the thickness of the aqueous layer is predicted for gel-state PS in the presence of 0.5 M NaCl.

For the liquid-crystalline state, the increments do not differ significantly (2.0 Å/CH_2 group, Figure 6, curve B; 2.1 Å/CH_2 group, Figure 6, curve D), suggesting very similar bilayer arrangements and chain packing, as would be expected. Again, is one assumes that the contribution of the polar groups is identical in the absence and presence of NaCl, a reduction of 28 Å in the aqueous thickness is predicted. An increase in

[3] It is clear from Figure 5 that negatively charged phosphatidylserine bilayers in H_2O, pH ≃7, containing NaCl come to an equilibrium spacing, d_{eq}. Figure 5 shows that increasing quantities of NaCl produce a decrease in d_{eq} that levels off at $d \simeq 62$ Å when [NaCl] > 0.5 M. Values of d_{eq} can be predicted from the equilibrium of long-range forces of repulsion and attraction. The former are treated as coulombic forces of repulsion, and estimates of these forces can be obtained with a modified Gouy–Chapman theory, allowing for specific cation–PS interactions at the bilayer surface. At equilibrium, these forces are balanced by forces of attraction due to electrodynamic van der Waals interaction between opposing bilayers. Comparison of the calculated equilibrium spacings d_{eq} with the experimental values will be the subject of a separate paper (H. Hauser and G. G. Shipley, unpublished observations).

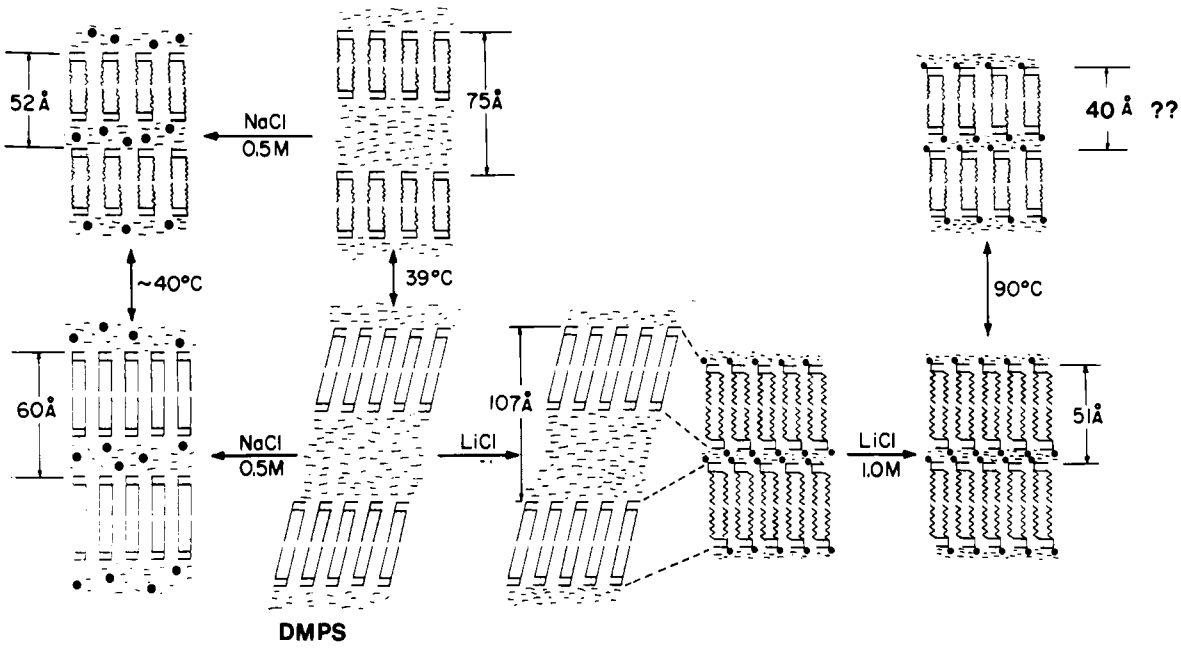

FIGURE 7: Schematic representation of structures of 50 wt % aqueous dispersions of NH_4^+-DMPS and changes induced by the presence of NaCl and LiCl. The center portion shows 50 wt % NH_4^+-DMPS in aqueous buffer in the absence of added alkali metal chlorides. At $T < 39$ °C, the bilayer gel phase of DMPS is shown with all the water between adjacent bilayers (periodicity $\simeq 107$ Å). At $T > 39$ °C, in the bilayer liquid-crystal phase, a reduced periodicity (75 Å) is shown due to both a decrease in bilayer thickness and a decrease in the aqueous thickness (see text for explanation). The left portion shows that in the presence of 0.5 M NaCl, the Na^+ ions (●) screen the DMPS bilayer surface potential, and reductions in the thickness of the aqueous layer in both the gel (bottom) and liquid-crystal (top) phases are observed. It is possible that Na^+ ions induce a small change in molecular tilt, as indicated. The right portion shows the effect of increasing molarity of LiCl. In 50 wt % dispersion, low concentrations of Li^+ (•) induce DMPS crystallization, dehydration, and lateral phase separation from Li^+-free DMPS. At intermediate Li^+ concentrations, the low-angle X-ray diffraction data suggest that complete three-dimensional separation of the two DMPS phases occurs. At 1.0 M Li^+ only the crystalline Li^+–DMPS phase is present. This crystalline Li^+–DMPS bilayer phase is stable, melting to a liquid-crystal phase (periodicity ~40 Å; precise lattice type unknown) at ~90 °C.

area occupied per PS molecule at the lipid–water interface, and the resultant decrease in the surface charge density, accompanying the bilayer gel → liquid-crystal transition, would account for the differences in shrinkage of the aqueous layer. A schematic representation of the structural, hydration, and thermotropic behavior of DMPS induced by NaCl (and presumably KCl, RbCl, and CsCl) is given in Figure 7 (left).

The effects on PS structure and thermotropic properties produced by Li^+ are markedly different from those produced by Na^+ and the other monovalent cations studied. As shown in Figure 1g [see also, Hauser & Shipley (1981)], addition of Li^+ induces the formation of Li^+–DMPS complex, which *alone* melts at >95 °C with an enthalpy change $\Delta H \simeq 16$–17 kcal/mol DMPS, as shown in Table I, a value more than twice that of NH_4^+-PS (or other alkali metal ion-PS) (ΔH = 7–8 kcal/mol DMPS). The conversion to Li^+–DMPS appears to be complete at ~0.7 M LiCl. Between 0 and 0.7 M LiCl, the calorimetric data show two peaks on heating (Hauser & Shipley, 1981), clearly indicating the presence of two phases. The low-melting phase shows a small, but significant, increase in transition temperature with increasing [LiCl], leveling off at approximately 45 °C (Figure 3), but only small amounts of this phase are present at 0.5 M LiCl (Figure 1g). At low [Li^+], the high-melting transition has an endothermic peak maximum at ~70 °C, which increases sharply up to ~0.05 M LiCl after which only a gradual increase in transition temperature occurs with added LiCl (Hauser & Shipley, 1981)). At intermediate LiCl concentrations, this behavior clearly indicates lateral phase separation of clusters of Li^+–DMPS complexes from Li^+-free DMPS, as shown schematically in Figure 7.

This interpretation of lateral and eventually three-dimensional phase separation of DMPS induced by Li^+ is clearly supported by the X-ray diffraction data (Figures 4 and 5). At low (<0.1 M) LiCl concentrations several sharp reflections appear in the wide-angle region, indicative of formation of an ordered hydrocarbon chain lattice, without major changes in the low-angle region. This suggests lateral phase separation of clusters of Li^+–DMPS from Li^+-free DMPS as shown schematically in Figure 7 (near right) without complete three-dimensional separation. At higher LiCl concentrations. e.g., 0.5 (see Figure 4E) and 1.0 M (Figure 4F), the wide-angle region is progressively dominated by these reflections. The changes in lamellar periodicity with increased [LiCl] are rather more difficult to interpret. At 20 °C, in the presence of 1 M LiCl, complete conversion to the Li^+–DMPS complex results in a highly ordered structure with a bilayer periodicity of 51 Å, reduced from the original hydrated NH_4^+-DMPS system ($d \simeq 107$ Å). The bilayer periodicity (51 Å) of the Li^+–DMPS complex is similar to the bilayer periodicity of *anhydrous* DMPS [crystal form 1, see Hauser et al. (1982)] and strongly suggests that Li^+–DMPS is completely dehydrated. At intermediate [LiCl], the low-angle diffraction data are complicated due to the presence of coexisting, and probably three-dimensionally separated, bilayer phases, the Li^+–DMPS complex and Li^+-free DMPS. In some cases (see Figure 5) we can see evidence of coexistence of both types of phases: (i) hydrated DMPS, bilayer periodicity ~100 Å, and (ii) anhydrous Li^+–DMPS, bilayer periodicity ~51 Å. Clearly, at intermediate LiCl concentrations, DMPS bilayers will show significant bilayer packing defects at the interfacial regions between the two laterally phase-separated areas, one of which dehydrates (Li^+–DMPS) and the other (Li^+-free DMPS) which can hydrate even further if more aqueous buffer is released from the "dehydrated" regions (see Figure 7). It seems probable that at low Li^+ concentrations lateral PS

crystallization occurs in bilayer patches. At higher Li^+ concentrations both DMPS crystallization and dehydration occur to produce a highly ordered "three-dimensional" complex (Figure 7, right).

From the X-ray diffraction data summarized in Figure 6 (line E), it is clear that Li^+ induces a similar "crystallization" of DLPS, DPPS, and also short-chain dihexanoyl-PS (H. Hauser and G. G. Shipley, unpublished observations). The bilayer periodicities for the series of Li^+–PS complexes are in close agreement with those of the anhydrous acidic PS or NH_4^+-PS (Hauser et al., 1982), adding support to our view that a consequence of Li^+ binding is dehydration. It is interesting to note that DSPS did not appear to interact with Li^+ in the same way as the lower PS homologues, at least as judged by its thermotropic behavior. Thus it appears that subtle differences in the molecular arrangement of the PS bilayer surface alter its Li^+ binding capacity.

Because of the markedly different behavior of Li^+ at PS interfaces, we have raised the possibility that Li^+-induced initiation of crystallization of anionic lipids and lateral phase-separation phenomena in circulatory and/or neuronal membranes could be related to the pharmacological use of Li^+ in treating manic-depressive illness (Hauser & Shipley, 1981). Although the concentrations of Li^+ required to produce the effects of PS described here are significantly higher than the pharmacological dose and, in addition, there appears to be some specificity in terms of the characteristics of the PS surface (see results for DSPS), further studies of membrane effects induced by Li^+ could contribute to a better understanding of its therapeutic and/or toxic effects.

Finally, there are marked similarities in the effects of interaction of Li^+ with PS bilayers and the effects induced by the divalent cations, particularly Mg^{2+} and Ca^{2+}. It is well recognized that Li^+ shows anomalous properties compared to other group I alkali metals, due to its small ionic radius and large hydration shell, and on this basis, the diagonal relationship in the periodic table links Li^+ with Mg^{2+}. As we will discuss in more detail in a later paper, the divalent cations Mg^{2+} and Ca^{2+} also induce chain crystallization, lateral phase separation, and high melting ($\sim$95 °C for Mg^{2+}, $\sim$155 °C for Ca^{2+}) M^{2+}–PS complexes (H. Hauser and G. G. Shipley, unpublished observations). If one assumes that this lipid binding property is but one example of Li^+ in some way mimicking the biologically important divalent cations, the potential perturbation of Mg^{2+}- and Ca^{2+}-dependent processes by Li^+ merit investigation (Williams, 1973; Frausto da Silva & Williams, 1976).

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Registry No. NH_4^+-DL-DLPS, 80629-07-8; NH_4^+-DMPS, 80581-65-3; NH_4^+-DPPS, 80538-62-1; DDPS, 80581-67-5; DLPS, 76260-76-9; DMPS, 64023-32-1; DPPS, 40290-42-4; DSPS, 51446-62-9; dihexanoyl-PS, 84773-42-2; DL-DLPS, 2954-46-3; Li, 7439-93-2; Na, 7440-23-5; K, 7440-09-7; Rb, 7440-17-7; Cs, 7440-46-2; NH_4^+, 14798-03-9.

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