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# Thermotropic properties of mixtures of negatively charged phospholipids with cholesterol in the presence and absence of $Li^+$ or $Ca^{2+}$ ions

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**Mixtures of cholesterol with dipalmitoylphosphatidylserine or phosphatidic acid were investigated by differential scanning calorimetry. As in mixtures of natural phosphatidylserine with cholesterol (Bach, D. (1984) Chem. Phys. Lipids 35, 385–392), also here phase separation of cholesterol at molar ratios of 2:1 (phospholipid:cholesterol) and below is observed. The limited solubility of cholesterol in negatively charged phospholipids is found to be independent of the nature of the acyl chain residues, and independent of whether the negative charge resides on both $COO^-$ and $PO^-$ groups (as in phosphatidylserine) or on $PO^-$ only (as in phosphatidic acid). The separate cholesterol phase is also seen by DSC in mixtures of natural phosphatidylserine or phosphatidic acid with cholesterol in the presence of $Ca^{2+}$; and in phosphatidylserine/cholesterol mixtures in the presence of $Li^+$, by DSC and X-ray diffraction.**

## Introduction

Cholesterol and negatively charged phospholipids are major constituents of biological membranes. Despite this fact, the thermodynamic and structural properties of mixtures of these lipids have not been thoroughly investigated. Recently we have studied mixtures of natural PS with cholesterol, employing DSC and X-ray diffraction [1,2]. Unlike zwitterionic phosphatidylcholines, where phase separation is not observed until about 1:1 molar ratio is reached [3–6], we found that at about 2:1 molar ratio of PS:cholesterol and below, separation into a crystalline cholesterol phase and a PS/cholesterol phase takes place. It was also reported that the PS-cholesterol phase at initial molar ratio 1:1 still undergoes melting, indicating that the cholesterol has only a small effect on the cooperativity of melting of the phospholipid. Such complex thermotropic behavior requires an explanation in terms of the stereochemical and electrostatic properties of the anionic phospholipids and their interaction with the sterol.

In the present work we attempt to determine whether the limited solubility of cholesterol in the negatively charged bilayers is influenced by three structural features of the phospholipids: (1) the type of the acyl chains; (2) the type of the charged headgroup; and (3) the binding of certain cations ($Li^+$ and $Ca^{2+}$). $Ca^{2+}$ has been found to participate in many biological processes and $Li^+$ is used as a therapeutic agent in mental disorders [7]. The binding of $Ca^{2+}$ or $Li^+$ has been found to result in chain crystallization of natural and synthetic negatively charged phospholipids, inducing an isothermal phase transition [8–11]. Cholesterol interacting with zwitterionic phospholipids lowers the cohesive forces between hydrocarbon chains, preventing their crystallization and increasing the fluidity of the gel phase. It therefore appears that cholesterol, on the one hand, and $Ca^{2+}$ or $Li^+$ on the other, have opposing effects on the structure of phospholipids. With regard to (1), we have examined mixtures of cholesterol with DPPS, a synthetic, disaturated phospholipid; and with regard to (2) and (3), mixtures of cholesterol with PS or PA in the presence (or absence) of varying amounts of $Ca^{2+}$ or $Li^+$. In PA, the charge is contributed by $PO^-$ only, whereas in PS (at neutral pH) the negative charge is due to $PO^-$ and $COO^-$ groups and the positive one to $-NH_3$, resulting in one negative charge.

Abbreviations: PS, phosphatidylserine (natural); DPPS, dipalmitoylphosphatidylserine; PA, egg phosphatidic acid; DPPA, dipalmitoylphosphatidic acid; PE, phosphatidylethanolamine; DSC, differential scanning calorimetry.

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## Materials and Methods

L-α-Phosphatidic acid dipalmitoyl sodium salt and L-α-phosphatidic acid from egg yolk (sodium salt) were purchased from Sigma (St. Louis, MO). Dipalmitoylphosphatidylserine (acid form) was purchased from R. Bertchold Lab (Bern, Switzerland). The phospholipid was converted to the sodium salt by shaking in chloroform/methanol/sodium borate buffer (pH 9). The lipid was recrystallized from acetone and lyophilized [12]. PS from bovine spinal cord Grade I monosodium salt (in 2:1 chloroform/methanol) was purchased from Lipid Products (South Nutfield, U.K.). Cholesterol extra pure was from Merck (Darmstadt, F.R.G.); it was recrystallized twice from ethanol and stored in the dark at 4°C. A single spot on TLC plates was obtained. All other reagents were of analytical grade. The lipids were dissolved in chloroform/methanol (2:1) and the interaction products were prepared by mixing the solutions of the phospholipids with appropriate volumes of the cholesterol solution. The solvents were driven off by a stream of nitrogen and the samples were kept under high vacuum for 3 h. Subsequently, the dry lipids or lipid mixtures were treated in two ways:

(1) The dry lipids or lipid mixtures (0.5–1.5 mg) were weighed directly into the aluminum pans of the instrument either on a Cahn Balance (Cahn Ventron, Paramount, CA) or on a Mettler Balance (Mettler, Switzerland) and an excess (5–15-fold) of a solution of $1.5 \cdot 10^{-1}$ M NaCl in $2 \cdot 10^{-2}$ M Tris-HCl buffer (pH 7.5) was added. In the experiments with $Li^+$ or $Ca^{2+}$ the salt solution contained $1.5 \cdot 10^{-1}$ M NaCl in buffer plus the appropriate concentration of LiCl or $CaCl_2$. In some experiments only the concentration of NaCl was varied. All the samples were shaken on a vortex mixer and incubated for 1 h at (°C): 75 (DPPS) or 90 (DPPS in the presence of $Li^+$), 80 (DPPA), 70 (PS) and 45 (egg PA). These temperatures of incubation were used to assure that under the experimental conditions the lipid is about 15°C above the phase-transition temperature.

(2) Dry phospholipids or phospholipid/cholesterol mixtures were dispersed in appropriate salt solutions at a concentration of 4–10 mg/ml and incubated for 0.5–1 h at 80°C with frequent vortexing. In several experiments with $Na^+$ and $Li^+$, and in all experiments with $Ca^{2+}$, the incubation was followed by three cycles of freezing-thawing. The results were similar in the two treatments. To ensure equilibration and binding of $Ca^{2+}$, $Ca^{2+}$ solutions at various concentrations in $1.5 \cdot 10^{-1}$ M NaCl in $2 \cdot 10^{-2}$ M Tris-HCl buffer were added to dry phospholipid (approx. 4 mg PS) or to phospholipid/cholesterol mixtures. After the incubation the mixtures were centrifuged in an Eppendorf centrifuge for 10 min and the wet pellets were weighed into the aluminum pans of the instrument. After completion of the experiments the pans were punched, dried at approx. 90°C to constant weight, and the weight of the phospholipid was calculated.

In the second method of preparation, higher ratios of ion-to-phospholipid were obtained; however, at the same molar ratios of ions-to-phospholipid the results were similar, indicating that equilibration with ions and water was achieved in both methods of preparation.

DSC experiments were performed on a Du Pont 990 calorimeter equipped with cell base II. Scanning rate was usually 5 C°/min and the sensitivities employed are indicated in legends to figures.

For checking the purity of the phosphatidylserines and to verify that the treatments do not cause degradation, TLC of the phospholipids was performed. 40 μg of the phospholipids in chloroform:methanol were applied to the TLC plate. The TLC was performed on PS and DPPS (stock solutions); on PS and DPPS that had been incubated for 70–80°C for 1 h; and on PS and DPPS after DSC experiments. In the last two cases the aqueous solutions were evaporated with $N_2$ and the dry lipid redissolved in chloroform/methanol (2:1) at appropriate concentrations. The plate was developed in chloroform/methanol/ammonia (65:25:5) and the spots were detected after ninhydrin spraying. PS (natural) before and after the treatment gave only one single spot, whereas DPPS without and with treatment gave a major spot and a second, very faint one. These results indicate that the treatments do not induce degradation.

Low-angle X-ray diffraction experiments were performed on a Philips sealed-tube fine-focus generator operated at 40 kV and 34 mA producing copper radiation. Monochromatization was provided by a nickel filter and one Franks mirror and the beam was collimated to 4 mm height and 350 μm width in the plane of the specimen. The specimen to detector distance was 460 nm. The diffraction pattern was recorded by a linear position-sensitive electronic detector of the delay-line type [13]. Exposure times were generally 10–15 min. During acquisition, the data were histogrammed into 256 channels and stored in a Z-80 based microprocessor unit. Following completion of the experiment the data were transferred to an IBM 3081 computer for further analysis. The temperature of the specimen was controlled by coolant flowing through the brass block capillary support. Approx. 5 min equilibration time was allowed at each temperature.

## Results and Discussion

### *Interaction of cholesterol with DPPS*

Mixtures of cholesterol and natural PS (from spinal cord) were investigated previously by DSC [1]. At high PS:cholesterol ratios, only one peak was seen in the thermograms. At a ratio of about 2:1, another peak, at around 38°C, appeared, which is due to the transition of the almost pure cholesterol phase. Anhydrous

cholesterol undergoes a polymorphic crystalline transition at approx. 38°C with an enthalpy change of 0.81 ± 0.21 kcal/mol [14,1]. Based on X-ray powder diffraction studies, it has been concluded that the crystal structures of the two polymorphic forms are similar [14]. No evidence for increased disorder in the high temperature polymorph is indicated. Thus, a cholesterol phase and a PS/cholesterol phase coexist at this and lower ratios. The composition of the PS/cholesterol phase is not constant. It is a function of the molar fraction of cholesterol added, as revealed by the continuous decrease of the enthalpy of melting of the phospholipid. However, even at about 0.6 : 1 (initial PS : cholesterol) ratio, the melting peak of PS is still seen in the thermograms, indicating that the effect of cholesterol on the cooperativity of melting of PS chains is quite weak.

The spinal cord PS [1] is a natural product, heterogeneous with respect to the acyl residues. To ascertain whether the nature of acyl residues of PS contribute to the complex thermotropic behavior, mixtures of synthetic disaturated PS-DPPS with cholesterol were investigated.

Fig. 1A presents thermograms of DPPS (sodium salt) alone and in the presence of increasing concentrations of cholesterol. The thermotropic properties of the pure phospholipid are similar to those reported by Cevc et al. [11]. In some batches of DPPS, a very small pretransition (detected only at the highest sensitivity) at around 40°C was seen and this small peak disappeared upon addition of cholesterol. As seen from the figure, interaction with cholesterol causes a downward shift of $T_m$ of DPPS of about 4 C° (at $X_{chol} = 0.2$). However, further addition of cholesterol hardly influenced the $T_m$ to within experimental error. At around $X_{chol} = 0.3$, a second peak in the thermograms is seen; this peak stems from the transition of an almost pure cholesterol phase, as its $T_m$ is similar to that at $X_{chol} = 1$ (Fig. 1A, 1C and Ref. 14). With increase of $X_{chol}$ further incorporation of cholesterol into the DPPS phase takes place as the enthalpy of melting of the phospholipid decreases with increase of the cholesterol content (Fig. 1B). But even at $X_{chol} = 0.57$, a melting peak of DPPS is still discerned, its enthalpy amount to about 15% of the initial value.

The results reported here are similar to those obtained previously for natural PS/cholesterol mixtures

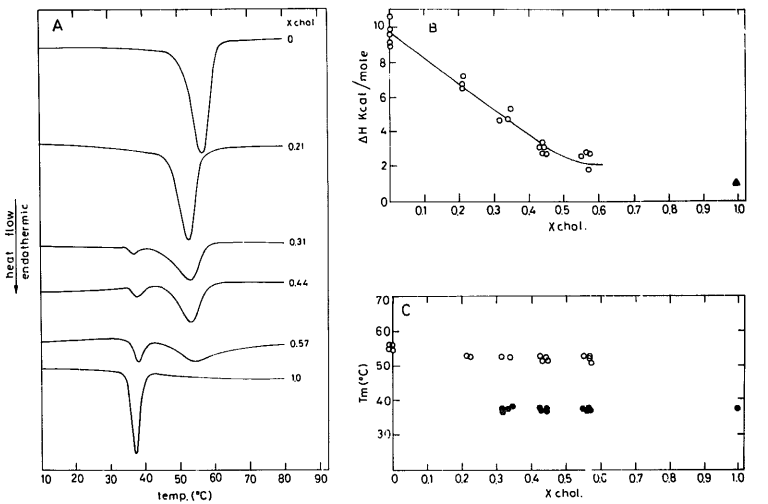


Fig. 1. (A) Thermograms of DPPS/cholesterol mixtures: $X_{chol}$ molar fraction of cholesterol; sensitivity 0.02 mcal/s per inch, except for $X_{chol}$ 0.57–0.01 mcal/s per inch. (B) The enthalpy of melting $\Delta H$ (kcal/mol DPPS) as a function of $X_{chol}$. ○, enthalpy of DPPS; ▲, enthalpy of cholesterol. (C) $T_m$ as a function of $X_{chol}$. ○, DPPS; ●, cholesterol.

[1], the only difference being that in the case of DPPS/cholesterol mixtures the transition of cholesterol precedes the melting of the phospholipid.

*Interaction of cholesterol with PA*

At neutral pH, PS has one negative charge [12] and the bilayer is stabilized by intermolecular hydrogen bonds [15]. To investigate whether the type of negative charge influences the miscibility properties of cholesterol in phospholipids, the thermotropic properties of PA/cholesterol mixtures were studied.

Fig. 2 presents thermograms of egg PA alone and with increasing concentrations of cholesterol. Phase separation at about $X_{chol} \approx 0.3$ is seen, similar to results in PS/cholesterol mixtures. However, the downward shift of $T_m$ of PA caused by addition of cholesterol is larger than in the case of PS/cholesterol mixtures. PA and PS are stabilized by intermolecular hydrogen bonds, as expressed by their high temperatures of melting. Interaction with cholesterol causes partial rupture of these bonds, resulting in lowering of $T_m$. A similar effect of cholesterol on $T_m$ of PE bilayers, which are also stabilized by hydrogen bonds, was reported by Blume [16]. The enthalpy of melting of PA is not presented, since part of the peak is shifted under the ice melting peak, preventing exact calculation. However, it seems that cholesterol causes a significant decrease in the enthalpy of melting of PA. Phase separation of cholesterol was detected also in mixtures of DPPA with cholesterol (data not shown). In this case, the cholesterol transition precedes that of the phospholipid, similar to the case of DPPS/cholesterol mixtures. The fact that the cholesterol transition does not depend on whether the phospholipid is the higher or lower melting component proves that the limited miscibility of cholesterol in these mixtures is independent of the state of the phospholipid, i.e., gel or liquid crystalline, as was also shown by X-ray diffraction [2].

The results presented here and in Ref. 1 suggest that the presence of negative charge: $COO^-$ and $PO^-$ (as in PS) or $PO^-$ (as in PA), as well as the additional effect of hydrogen bonding, might determine the limited solubility of cholesterol in negatively charged bilayers. Indeed, it was reported by Browning and Seelig [17] that PS has a more rigid headgroup than that of phosphatidylcholine or PE. In the case of the interaction of cholesterol with zwitterionic phosphatidylcholines it was suggested that the $\beta$-OH of cholesterol resides at the polar interface, whereas the steroid ring and aliphatic chain are embedded in the hydrophobic core of the bilayer. Based on theoretical considerations it was proposed that the hydroxyl group of cholesterol forms a hydrogen bond with the carboxyl oxygen of the phospholipids [18]. However, some more recent studies on the influence of esterified cholesterol on phosphatidylcholine bilayers have shown that the hydrogen bonding is not a prerequisite for the interaction. The presence of a polar group in cholesterol is required only for anchoring the molecule at the polar region of the bilayer [19]. The high rigidity of the headgroup of PS and probably also of PA might create unfavorable steric conditions for the accomodation of the polar cholesterol moiety, resulting in limited solubility of cholesterol in these bilayers.

*Interaction of $Ca^{2+}$ with PA / cholesterol mixtures*

The effect of $Ca^{2+}$ on the thermotropic properties of PA/cholesterol mixtures was investigated. The experiments were performed at $X_{chol} = 0.33$ and $X_{chol} = 0.40$ and in an excess of $Ca^{2+}$ (10-fold molar ratio). The results are presented in Fig. 2B. As seen from the figure, phase separation of cholesterol from $Ca^{2+}$-PA is also

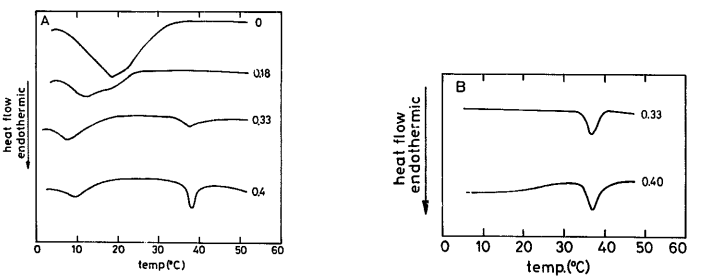


Fig. 2. Thermograms of PA/cholesterol mixtures; numbers indicate molar fraction of cholesterol. (A) In the presence of NaCl buffer only; (B) in the presence of $Ca^{2+}$. $X_{chol} = 0.33$: $7 \cdot 10^{-2}$ M $CaCl_2$ ($Ca^{2+}$ : PS = 9.9 : 1); $X_{chol} = 0.40$: $7 \cdot 10^{-2}$ M $CaCl_2$ ($Ca^{2+}$ : PS = 9.3 : 1).

obtained. The peak of melting of PA/cholesterol-$Ca^{2+}$ is shifted upwards to about 70 °C.

*Interaction of PS / cholesterol mixtures with cations*

It is known that cholesterol 'fluidizes' zwitterionic phospholipid bilayers in the gel state [20]. This is also true for cholesterol-PS mixtures (Ref. 1 and Fig. 1B) as judged by the decrease of the enthalpy of melting of the phospholipids. This effect of cholesterol has been explained by its intercalation between the acyl chains of phospholipids preventing their crystallization. It was also shown that the polar moiety of cholesterol is required for the interaction [19]. On the other hand, biologically important ions such as $Ca^{2+}$, $Li^+$ bind to PS headgroups, inducing isothermal chain crsytallization [8–11]. Since cholesterol is a major constituent of biological membranes, it was of interest to investigate the effect of these ions on the thermotropic properties of PS/cholesterol mixtures.

*Effect of $Li^+$ on PS and on PS / cholesterol mixtures*

The effect of $Li^+$ has been investigated only in synthetic-disaturated phosphatidylserines [9–11]. As no data on the effect of $Li^+$ on natural PS have been published, we present these thermograms first. Fig. 3 shows the thermograms of PS dispersed in $1.5 \cdot 10^{-1}$ M NaCl in $2 \cdot 10^{-2}$ M Tris-HCl buffer alone and with increasing concentrations of LiCl added (indicated in the legends to figures). In the absence of $Li^+$, the $T_m$ of PS is 13.5 °C and its enthalpy of melting is about 5 mcal/mg (part of the peak is unaccounted for as the melting begins below 0 °C). Addition of low concentrations of $Li^+$ causes a small upward shift of $T_m$ of about 7 C° and the enthalpy of melting stays constant within experimental error (Fig. 3, trace 1). This shift of $T_m$ is larger than the one obtained by addition of similar concentrations of $Na^+$ (Ref. 12 and Bach, D., unpublished results). A similar small shift of $T_m$ was observed when $Li^+$ interacts with synthetic phosphatidylserines [11]. At higher concentrations of LiCl (0.7 M or higher) a larger upward shift of $T_m$ of about 45 C° is seen, and the enthalpy of melting increases to 10 mcal/mg. If the samples at high concentrations of $Li^+$ are cooled quickly after the first scan and rescanned immediately at 5 C°/min the profiles have a different shape (Fig. 3, traces 4, 6). Three peaks in the thermograms are seen: an endothermic peak at 25 °C, an exothermic one at about 30 °C and a second endothermic peak at the same temperature as in the first scan (60 °C). When the sample is rescanned at a lower scan rate (2 C°/min), the $T_m$ of the first endothermic peak shifts downward, its size and that of the exothermic peak decrease, whereas the $T_m$ of the second endothermic peak stays almost the same. If the sample is left overnight at 4 °C, the first endothermic and the exothermic peaks disappear completely, giving the same picture as in the initial scan.

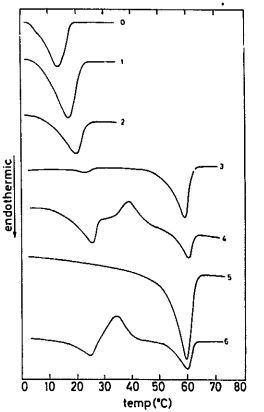


Fig. 3. Thermograms of PS in the presence of $Li^+$. 0, PS only; 1, $10^{-1}$ M $Li^+$ (molar ratio $Li^+$ : PS = 7); 2, $3 \cdot 10^{-1}$ M $Li^+$ ($Li^+$ : PS = 25); 3, $7 \cdot 10^{-1}$ M $Li^+$ ($Li^+$ : PS = 54) first scan; 4, second scan of 3; 5, 1 M $Li^+$ ($Li^+$ : PS = 94) first scan; 6, second scan of 5. Sensitivities (mcal/s per inch): 0.04 (1,2); 0.02 (3); 0.1 (4, 5, 6).

Such behavior indicates that the Li-PS complex exists in two gel states: a stable one obtained only after slow cooling and a metastable one. The metastable form transforms into a stable one as expressed by an exothermic peak and this crystalline form undergoes the gel-to-liquid transition (i.e., melting of the hydrocarbon chains) at about 60 °C. X-ray diffraction data suggested that $Li^+$ binding to synthetic PS causes dehydration of the phospholipid [11,21] and that $Li^+$-dihexadecyl-PS complexes exist in two crystalline forms, but no exothermic peaks in DSC measurements were reported [11]. Metastable behavior was seen previously in the case of dimyristoylphosphatidylglycerol interacting with $Mg^{2+}$ [22] and in the phase transitions of cerebrosides [23,24].

*PS / cholesterol mixtures in the presence of $Li^+$*

The effect of $Li^+$ on PS/cholesterol mixtures was studied at three molar fractions of cholesterol: 0.2, 0.33 and 0.4. Fig. 4 presents the thermograms of the mixtures at $X_{chol} = 0.33$ with various concentrations of $Li^+$ added. The main features of the thermograms are: (i) the cholesterol peak (38 °C) is seen at low concentrations of $Li^+$; (ii) a small upward shift of the $T_m$ of the PS melting peak at low $Li^+$ concentrations, and a shift of about 45 C° at concentrations of $Li^+$ 0.7 M and higher; (iii) appearance of an exothermic peak in the

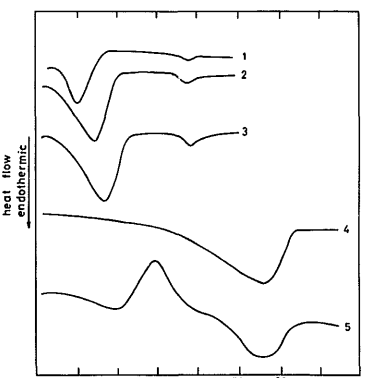


Fig. 4. Thermograms of PS/cholesterol mixtures ($X_{chol} = 0.33$) in the presence of $Li^+$. 1, PS/cholesterol mixture only; 2, $10^{-1}$ M $Li^+$ (molar ratio $Li^+$ : PS = 9); 3, $3 \cdot 10^{-1}$ M $Li^+$ ($Li^+$ : PS = 25); 4, 1 M $Li^+$ ($Li^+$ : PS = 107) first scan; 5, second scan of 4. Sensitivities (mcal/s per inch): 0.02 (1, 2, 3); 0.04 (4, 5).

second scan of mixtures with $Li^+$ at concentrations of 0.7 M and higher. Similar results were obtained for $X_{chol} = 0.4$.

The upward shift of $T_m$ is similar to the one induced by $Li^+$ in the absence of cholesterol; however, the absolute values of temperatures are lower than those found in the absence of cholesterol (Fig. 3), indicating that the small downward shift of $T_m$ caused by addition of cholesterol (Ref. 1 and Fig. 1) is seen here also. The presence of the cholesterol transition indicates that phase separation takes place at low $Li^+$ concentrations. At concentrations of $Li^+$ of at least 0.7 M the cholesterol transition is not visible, as it is probably masked by the main peak, which is very broad. The enthalpy of melting increases from approx. 1.6 mcal/mg at $10^{-1}$ $Li^+$ (part of the peak is unaccounted for, due to the interference of water) up to 5 mcal/mg at 1 M LiCl. Also here the exothermic peak is seen in the second scan – indicating that cholesterol/PS mixtures exist in two crystalline forms at high concentrations of $Li^+$, similar to pure PS.

Since at concentrations of $Li^+$ higher than 0.7 M a transition peak of cholesterol would be masked by the main peak of PS, we measured the low-angle X-ray diffraction from PS/cholesterol mixtures in the presence of 0.7 and 1.0 M LiCl. If a fraction of the cholesterol molecules were sequestered in a crystalline phase, then characteristic cholesterol reflections would be observed. Fig. 5 shows typical low-angle X-ray diffraction patterns for natural PS in 0.15 M NaCl/1.0 M LiCl, in the absence of (A) and in the presence of cholesterol, $X_{chol} = 0.34$ (B). Diffraction peaks at 34 Å and 17 Å, typical of cholesterol crystals, are observed for the cholesterol-containing specimen, in addition to the three orders of the phospholipid bilayer spacing ($d$ spacings) [2]. The value of $d$ for the first-order reflection gives the thickness of the bilayer plus the thickness of the interbilayer water. The temperature dependence of the $d$ spacings of these reflections is presented in Fig. 6A, B. Only lamellar phases were observed at all the temperatures investigated, in spite of the fact that in the $Li^+$-dihexadecyl-PS complex at high $Li^+$ concentration in the liquid crystalline state most of the lipids were in the $H_\alpha$ form [11]. PS in 1.0 M LiCl obviously forms ordered bilayer structures with interbilayer distance approx. 60 Å in the $L_\beta$ phase, approx. 10 Å smaller than that observed for PS in 0.5 M NaCl [2]. The presence of $Li^+$ shifts the midpoint of the hydrocarbon chain melt-

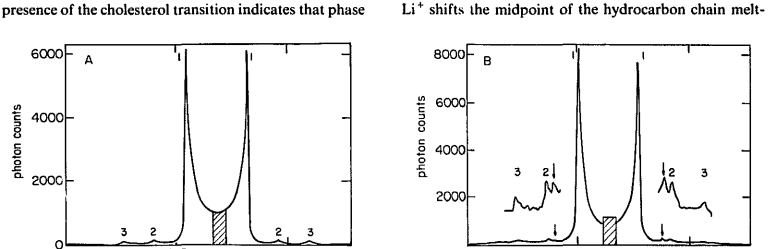


Fig. 5. Low-angle X-ray diffraction patterns of bovine spinal cord phosphatidylserine in 0.15 M NaCl: (A) $0.2 \cdot 10^{-2}$ M Tris-HCl buffer (pH 7.5) at 23°C in the presence of 1.0 M LiCl and (B) at 19°C for $X_{chol} = 0.34$ and in the presence of 1.0 M LiCl. The data were obtained as described in Materials and Methods section. Numbers 1–3 indicate the first three orders of the bilayer spacing and the arrows mark reflections from crystalline cholesterol. The central shaded region indicates the position of the beam stop.

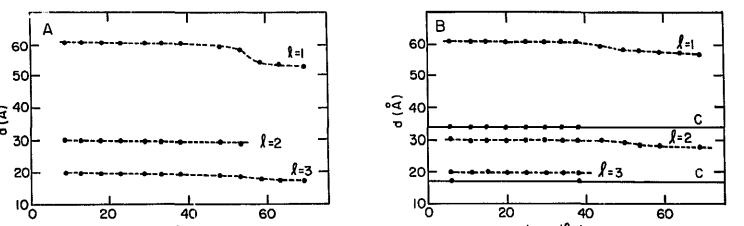


Fig. 6. The temperature dependence of the $d$ spacings of the low-angle X-ray reflections from bovine spinal cord phosphatidylserine in 0.15 M NaCl (A) in $2 \cdot 10^{-2}$ M Tris-HCl (pH 7.5) in the presence of 1.0 M LiCl and (B) for $X_{chol} = 0.34$ and in the presence of 1.0 M LiCl. The orders of the bilayer spacing are noted and C marks the theoretical positions of the first two reflections of crystalline cholesterol.

ing transition upward markedly from 19 °C (in the presence of $Na^+$ only) to 56 °C. Addition of cholesterol reduces the cooperativity of the transition ($L_\beta \rightarrow L_\alpha$ phase) as seen from the X-ray data, rendering the determination of the transition temperature very difficult. Nevertheless, a downward shift is clearly observed. This is in agreement with the DSC results already presented, where the transition is also very broad. Above the transition region, the bilayer spacing in the presence of cholesterol is several ångstroms larger than in its absence. Diffraction peaks due to the separate crystalline phase persist until close to 40 °C. Then they become very difficult to detect, for reasons which are not yet clear. Such a weakening of the intensity of the cholesterol reflections with temperature was not observed in PS/cholesterol mixtures in 0.5 M NaCl [2]. In that case, the cholesterol reflections were of similar intensity in both the gel and liquid crystalline states up to 40 °C, the highest temperature investigated. Similar results were obtained for natural PS in 0.7 M LiCl/0.15 M NaCl, at $X_{chol} = 0.34$. Attempts to observe phase separation of cholesterol for $X_{chol} = 0.15$ in the presence of 1.0 M LiCl were unsuccessful.

Preliminary DSC experiments were also performed on the effect of $Li^+$ on DPPS/cholesterol mixtures at low concentrations of $Li^+$. The results are similar to those of PS/cholesterol mixtures (not shown).

The experimental findings presented in this section show that when cholesterol and $Li^+$ are present together, the effects of both are seen. This may indicate that the interaction of PS with cholesterol is weak and the cholesterol molecules, acting as spacers for the headgroups, do not interfere with $Li^+$ binding.

*Interaction of $Ca^{2+}$ with PS in the presence and absence of cholesterol*

The effect of $Ca^{2+}$ on the thermotropic properties of phosphatidylserines has been investigated extensively (e.g., Ref. 8). It was claimed that $Ca^{2+}$ binding to PS induces an isothermal phase transition, shifting the transition peak of the phospholipid to temperatures higher than 80 °C at molar ratios of 1 : 1.

Recently it was reported by Dluhy et al. [25] that in infrared measurements on a Ca-PS complex (in an excess of $Ca^{2+}$) structural changes taking place in the 30–40 °C temperature range were detected. This transition was described as a solid–solid phase transition similar to the one occurring in alkanes or fatty acids. In the present work we have investigated the possibility of phase separation of cholesterol in the presence of $Ca^{2+}$. As a prerequisite to this investigation, thermograms of pure PS as a function of $Ca^{2+}$ were recorded. Samples were prepared as described in Materials and Methods. The highest sensitivities of the DSC instrument (0.02 or 0.01 mcal/s per inch) were employed. The effect of $Ca^{2+}$ on pure PS was investigated in a range of molar ratios $Ca^{2+}$ : PS 0.2–15. Thermograms of PS as a function of $[Ca^{2+}]$ are presented in Fig. 7A. Trace 1 presents the melting profile of PS alone. $T_m$ is 13 °C and $\Delta H \approx 4.5$ mcal/mg. Addition of $10^{-3}$ M $CaCl_2$ to the phospholipid (molar ratio $Ca^{2+}$ : PS = 0.2) causes an appearance of a second peak with $T_m$ of approx. 42.5 °C and $\Delta H \approx 1.1$ mcal/mg and a decrease of the enthalpy of melting of the lower temperature peak to about 2.3 mcal/mg (trace 2). At a molar ratio $Ca^{2+}$ : PS ≈ 1, the lower temperature peak disappears, but the second peak is still seen. This peak is seen at all the ratios of $Ca^{2+}$ investigated, even in large excess of $Ca^{2+}$ at molar ratio $Ca^{2+}$ : PS = 15, showing that it stems from the transition of the $Ca^{2+}$-PS complex. Up to 80 °C, no other peaks due to melting of the hydrocarbon chains of the phospholipid are discerned. These results agree with the findings of Dluhy et al. [25], which indicate that the 43 °C transition is due to a gel-gel transition from a more ordered to a less ordered form. Probably, in the early DSC experiments of PS in the presence of $Ca^{2+}$, the concentrations used were too low, so that no small broad peak at around 43 °C was seen. Such a transition

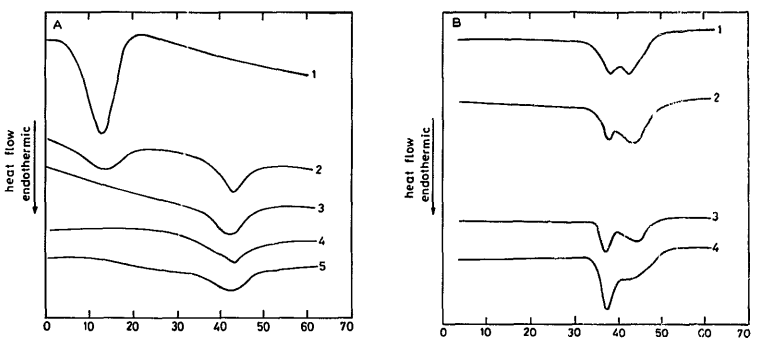


Fig. 7. Thermograms of PS in the presence of $Ca^{2+}$. (A) PS only. Traces: 1, without $Ca^{2+}$; 2, $10^{-3}$ M $CaCl_2$ ($Ca^{2+}$ : PS = 0.2); 3, $5 \cdot 10^{-3}$ M $CaCl_2$ ($Ca^{2+}$ : PS = 1.2); 4, $10^{-2}$ M $CaCl_2$ ($Ca^{2+}$ : PS = 2.2); 5, $6.6 \cdot 10^{-2}$ M $CaCl_2$ ($Ca^{2+}$ : PS = 15.1). (B) PS/cholesterol mixtures. 1, $X_{chol} = 0.33$; $10^{-2}$ M $CaCl_2$ ($Ca^{2+}$ : PS = 2); 2, $X_{chol} = 0.33$; $6.6 \cdot 10^{-2}$ M $CaCl_2$ ($Ca^{2+}$ : PS = 13); 3, $X_{chol} = 0.4$; $10^{-2}$ M $CaCl_2$ ($Ca^{2+}$ : PS = 2); 4, $X_{chol} = 0.4$; $7 \cdot 10^{-2}$ M $CaCl_2$ ($Ca^{2+}$ : PS = 10).

is not seen in PA-$Ca^{2+}$ mixtures, where the only melting peak of the phospholipid is above 70 °C.

The effect of $Ca^{2+}$ on the thermotropic properties of PS-cholesterol mixtures at $X_{chol} = 0.33$ and 0.4 was studied. The experiments were performed in an excess of $Ca^{2+}$ (2 and ≈ 10-fold molar ratio) and the results are presented in Fig. 7B. As seen from the figure, phase separation of cholesterol takes place from the $Ca^{2+}$-PS complex, as indicated by a shoulder at 38 °C on the PS-$Ca^{2+}$ melting peak. The shoulder becomes more pronounced as the cholesterol content increases (traces 3 and 4), where $X_{chol} = 0.4$. This behavior is similar to that observed for PA/cholesterol mixtures in the presence of $Ca^{2+}$ (Fig. 2B).

It is known that $Ca^{2+}$ induces phase separation of PS in mixtures with zwitterionic phospholipids. Based on our findings here we may speculate that addition of $Ca^{2+}$ to biological membranes might produce PS-rich, PS/cholesterol and cholesterol patches that undergo transitions at temperatures encompassing the physiological range of temperature. Recently it has been reported that Ca-PS patches in the presence and absence of cholesterol were seen in erythrocyte membranes [26].

## Conclusions

In the present paper, we have extended earlier investigations [1,2], in which it was shown that in mixtures of negatively charged phospholipids with cholesterol, phase separation of the cholesterol takes place at molar ratios of phospholipid : cholesterol of approx. 2 : 1 and below. This limited solubility of cholesterol is now observed to be a property of natural PS, DPPS, DPPA and natural PA. It is not found for the zwitterionic phosphatidylcholines and is therefore probably determined by the negatively charged headgroups and possibly by intermolecular hydrogen bonding. From studies on zwitterionic phospholipids, it is known that a prerequisite for interaction of cholesterol with phospholipids is the presence of the polar group in cholesterol ($\beta$-OH, either free or esterified). This group is needed for anchoring the cholesterol at the polar interface. The rigid headgroup of negatively charged phospholipids may prevent the attachment of the polar group of cholesterol to the interface, resulting in limited solubility and the resulting cholesterol phase separation. Addition of $Li^+$ or $Ca^{2+}$, which bind to the headgroup, probably stiffen it further, thereby maintaining the phase separation. In the presence of $Ca^{2+}$, PS is found to organize in a second gel phase which is observed around 43 °C. Its presence near physiological temperatures may be significant for the functioning of $Ca^{2+}$ on biological membranes.

## References

1 Bach, D. (1984) Chem. Phys. Lipids 35, 385–392.
2 Wachtel, E. and Bach, D. (1987) Biochim. Biophys. Acta 922, 234–238.
3 Mabrey, S., Mateo, P.L. and Sturtevant, J.M. (1978) Biochemistry 17, 2464–2468.
4 Estep, T.N., Mountcastle, D.B., Biltonen, R.L. and Thompson, T.E. (1978) Biochemistry 17, 1984–1989.

5 Davis, P.J. and Keough, K.M.W. (1983) Biochemistry 22, 6334–6340.
6 Finean, J.B. and Hutchinson, A.L. (1988) Chem. Phys. Lip. 46, 63–71.
7 Ehrlich, B.E. and Diamond, J.M. (1980) J. Membr. Biol. 52, 187–200.
8 Jacobson, K. and Papahadjopoulos, D. (1975) Biochemistry 14, 152–161.
9 Hauser, H. and Shipley, G.G. (1981) J. Biol. Chem. 256, 11377–11380.
10 Hauser, H. and Shipley, G.G. (1983) Biochemistry 22, 2171–2178.
11 Cevc, G., Seddon, J.M. and Marsh, D. (1985) Biochim. Biophys. Acta 814, 141–150.
12 Cevc, G., Watts, A. and Marsh, D. (1981) Biochemistry 20, 4955–4965.
13 Reich, M.H., Kam, Z. and Eisenberg, H. (1982) Biochemistry 21, 5189–5195.
14 Loomis, C.R., Shipley, G.G. and Small, D.M. (1979) Lipid Res. 20, 525–535.
15 Buggs, J.M. (1980) Can. J. Biochem. 58, 755–770.
16 Blume, A. (1980) Biochemistry 19, 4908–4913.
17 Browning, J.L. and Seelig, J. (1980) Biochemistry 19, 1262–1270.
18 Huang, Ch.-H. (1977) Lipids 12, 348–355.
19 Demel, R.A., Parish, E.J., Chitrakorn, S. and Smith, I.P. (1984) Biochim. Biophys. Acta 771, 142–150.
20 McElhaney, R.N. (1982) Chem. Phys. Lip. 30, 229–359.
21 Hauser, H. and Shipley, G.G. (1985) Biochim. Biophys. Acta 813, 343–346.
22 Van Dijck, P.W.M., Ververgaert, P.H.J.Th., Verkleij, A.J., Van Deenen, L.L.M. and De Gier, J. (1975) J. Biochim. Biophys. Acta 406, 465–478.
23 Freire, E., Bach, D., Correa-Freire, M., Miller, I. and Barenholz, Y. (1980) Biochemistry 19, 3662–3665.
24 Curatolo, W. (1982) Biochemistry 21, 1761–1764.
25 Dluhy, R.A., Cameron, D.G., Mantsch, H.H. and Mendelsohn, R. (1983) Biochemistry 22, 6318–6326.
26 Haverstick, D.M. and Glaser, M. (1987) Proc. Natl. Acad. Sci. USA 84, 4475–4479.