Monolayer Characteristics and Thermal Behavior of Natural and Synthetic Phosphatidylserines†

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ABSTRACT: The monolayer properties and thermal behavior of different phosphatidylserines are presented. At neutral pH and 22 °C, saturated phosphatidylserines form condensed monolayers while unsaturated phosphatidylserines form liquid-expanded films. Under similar conditions, dimyristoylphosphatidylserine undergoes a transition from the liquid-expanded to the condensed state. At pH 4 and 22 °C, the surface pressure–area isotherms are shifted to smaller areas relative to the monolayers recorded at neutral pH. The condensation observed at pH 4 is close to that produced at pH 7.4 by the addition of 10 mM $CaCl_2$. As regards the molecular packing in monolayers and the thermal behavior, 1,2-dipalmitoyl-*sn*-glycero-3-phospho-L-serine (DPPS) and its ether analogue are similar, albeit not identical. Below 30 mN/m, monolayers of the ether analogue are even more condensed than those of DPPS. The order–disorder transition of the ether analogue occurs usually at higher temperatures than that of the diacyl compound. Sonicated phosphatidylserine dispersions consisting of small unilamellar vesicles show anomalous thermal properties compared to sonicated phosphatidylcholine dispersions. They exhibit sharp order–disorder transitions at similar or even slightly elevated temperatures compared to unsonicated phosphatidylserine dispersions. This anomaly is explained in terms of a pH gradient across the bilayer membrane of the small unilamellar phosphatidylserine vesicle. The internal surface pH is more acidic than the external pH, leading to some protonation of phosphatidylserine molecules. This in turn leads to a condensation of phosphatidylserine molecules on the inner bilayer surface. Such a gradient is proposed to be responsible for the thermodynamic stability of highly curved negatively charged bilayer vesicles.

Phosphatidylserines are important constituents of the plasma membrane of eucaryotic cells (Boggs, 1980), and as such they have been the subject of extensive studies in the past. Diacylphosphatidylserines with long hydrocarbon chains (more than 12 C atoms) aggregate spontaneously in aqueous dispersions, and the predominant phase formed has been shown to be smectic (lamellar) (Hauser & Phillips, 1979; Hauser et al., 1982; Hauser, 1984). This is true both for naturally occurring and also for many synthetic phosphatidylserines. Not only the physicochemical properties of phosphatidylserine bilayers in aqueous dispersions have been widely studied in the past but also the interaction of these bilayers with cations (Papahadjopoulos & Miller, 1967; Newton et al., 1978; Eisenberg et al., 1979; Hauser & Phillips, 1979; Hope & Cullis, 1980; Cevc et al., 1981; Holwerda et al., 1981; Ohki & Kurland, 1981; McLaughlin, 1982; Dluhy et al., 1983; Hauser & Shipley, 1983, 1984, 1985) and small charged and uncharged molecules such as, for instance, local anesthetics (Papahadjopoulos et al., 1975; Boulanger et al., 1981), peptides, and proteins (Marsh, 1985). Despite this relatively large research effort on phosphatidylserines, information concerning their surface properties is still scarce. Here, we present a monolayer study of natural and synthetic phosphatidylserines and relate the molecular packing derived from monolayer work to their thermotropic behavior determined by differential scanning calorimetry.

Materials and Methods

1,2-Dimyristoyl-*sn*-glycero-3-phospho-L-serine (DMPS), 1,2-dipalmitoyl-*sn*-glycero-3-phospho-L-serine (DPPS), 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine (DOPS), and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine (POPS) were synthesized as described (Hermetter et al., 1982). *rac*-1,2-Dihexadecylglycero-3-phospho-L-serine (dihexadecyl-PS) was synthesized by R. Berchtold (Biochemisches Labor, Bern, Switzerland. 1,2-Dielaidoyl-*sn*-glycero-3-phospho-L-serine (dielaidoyl-PS) was synthesized according to Comfurius and Zwaal (1977). Beef brain phosphatidylserine was purchased from Lipid Products (Surrey, U.K.) and used without further purification. The phospholipids used in this work were pure by thin-layer chromatography (TLC) standard using several solvent systems (Hauser et al., 1982) and by C, H, N, P microanalysis. The acid form of phosphatidylserine was converted to the monoammonium salt as described before (Hauser et al., 1982). Alternatively, the conversion of the acid form to different salts, and vice versa, was carried out according to Bligh and Dyer (1959).

Sample Preparation. Hydrated samples for differential scanning calorimetry (DSC) were prepared by weighing the solid phospholipid (2–5 mg) into the DSC pan and adding the appropriate amount of buffer, usually 50 μL, with a Hamilton syringe or gravimetrically. The DSC pan was sealed immediately and transferred to the calorimeter. In case of beef brain phosphatidylserine, a $CHCl_3/CH_3OH$ solution (2:1 v/v) containing the appropriate amount of lipid was injected into the DSC pan, and after evaporation of the organic solvent, the lipid was dried to constant weight. The buffer used to disperse the phospholipid in the DSC pan was either 5 mM phosphate or tris(hydroxymethyl)aminomethane (Tris) buffer

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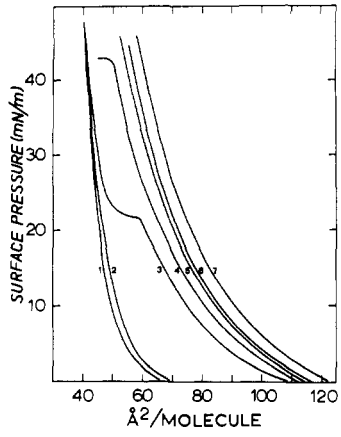

FIGURE 1: Surface pressure (millinewtons per meter)–molecular area (angstroms squared per molecule) curves of different phosphatidylserines spread on 0.066 M phosphate buffer, pH 7.4, at 22 °C: (1) dihexadecyl-PS; (2) DPPS; (3) DMPS; (4) dielaidoyl-PS; (5) beef brain PS; (6) POPS; (7) DOPS. The phospholipids were spread as the Na^+ salt except for POPS which was used as the NH_4^+ salt. The same surface pressure–area isotherm was obtained for DMPS regardless of whether the Na^+ salt or the acid form of DMPS was spread on the phosphate buffer.

of neutral pH containing 5 mM disodium ethylenediaminetetraacetate (Na_2EDTA) and 0.05% NaN_3. Sonicated dispersions of the NH_4^+ salts of dihexadecyl-PS and DPPS of appropriate concentration were prepared as described previously (Hauser & Phillips, 1973); 50 μL of the sonicated dispersion was pipetted into the DSC pan with a Hamilton syringe.

Force–Area Curves. Force–area curves were measured at the air–water interface at 22 °C using a Teflon trough of dimensions 32.2 cm long × 17.2 cm wide. The surface tension was determined with a recording Beckman LM 500 electrobalance. The trough and all the measuring devices were housed in a thermostated box. The phospholipid (50 nmol) dissolved in $CHCl_3/CH_3OH$ (4:1 v/v) was carefully spread at the air–water interface from an Agla microsyringe. Force–area curves were recorded at 22 ± 1 °C using a compression rate of 0.258 $nm^2\ mol^{-1}\ min^{-1}$ with a reproducibility better than 1 Å²/molecule. The following buffers were used: 66 mM phosphate (Na_2HPO_4, KH_2PO_4) buffer, pH 7.4, 0.2 M sodium acetate buffer, pH 4.0, 0.2 M borate (H_3BO_3, $Na_2B_4O_7$) buffer, pH 7.4, all dissolved in triple-distilled water.

Differential Scanning Calorimetry. Differential scanning calorimetry (DSC) was carried out with a Perkin-Elmer DSC-2 differential scanning calorimeter. Each sample was subjected to repeated heating and cooling cycles; unless otherwise stated, the heating and cooling rates were 5 °C/min. The DSC curves of excess apparent heat capacity vs temperature were evaluated in terms of transition temperature (T_c) and enthalpy (ΔH) using a Perkin-Elmer Model 3600 data station. The maximum in the excess apparent heat capacity vs temperature plot was taken as the transition temperature.

RESULTS

Monolayers. Figure 1 depicts the force–area curves of different phosphatidylserines spread on phosphate buffer, pH 7.4. DPPS and dihexadecyl-PS give similar condensed force–area curves with a limiting area of 40.5 Å²/molecule (curves 1 and 2; Figure 1). Below 30 mN/m, the monolayer of dihexadecyl-PS is even more condensed than that of DPPS. In contrast to these phosphatidylserines with 16 C atoms in the fatty acyl chains, the force–area curve of DMPS exhibits a two-dimensional phase transition from the liquid-expanded to the condensed state at 21.6 mN/m and at a molecular area

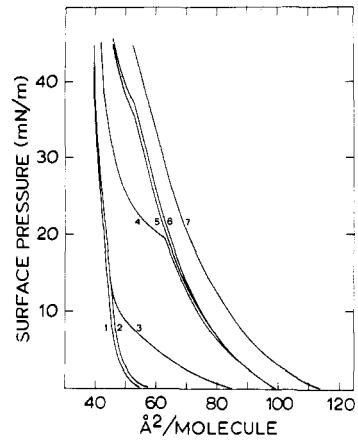

FIGURE 2: Surface pressure (millinewtons per meter)–molecular area (angstroms squared per molecule) curves of different phoshatidylserines spread on 0.2 M sodium acetate buffer, pH 4.0, at 22 °C: (1) dihexadecyl-PS; (2) DPPS; (3) DMPS; (4) dielaidoyl-PS; (5) beef brain PS; (6) POPS; (7) DOPS. The acid form of the different phosphatidylserines was spread on the acetate buffer.

of 59 Å²/molecule (curve 3, Figure 1). The limiting area of the DMPS monolayer is identical with that of the saturated long-chain analogues. The mixed fatty acyl chain derivative POPS gives a liquid-expanded monolayer over the total pressure range measured (curve 6, Figure 1). At 30 mN/m, the molecular area of POPS is increased by 19.5 Å²/molecule compared to DPPS. For comparison, DOPS with two cis double bonds per molecule gives only a small additional increase in molecular area of 3.8 Å²/molecule at the same pressure of 30 mN/m (curve 7, Figure 1). The force–area curve of beef brain phosphatidylserine (curve 5) is close to that of POPS. Dielaidoyl-PS (curve 4, Figure 1) with two trans double bonds per molecule exhibits a force–area curve which is intermediate between those of DPPS and DOPS; it gives a sharp collapse pressure of 43 mN/m at a limiting molecular area of 50.7 Å². At low surface pressures, the difference in the force–area curve between dielaidoyl-PS and DOPS is smaller, but this difference appears to increase with increasing surface pressure. Similar force–area curves to those shown in Figure 1 were obtained when the different phosphatidylserines were spread on borate buffer, pH 7.4, or on Tris buffer, pH 7.4 (data not shown). The only exception was DMPS. When DMPS was spread on 5 mM Tris buffer, pH 7.4, it gave a liquid-expanded monolayer at all pressures. In the presence of Tris ions, the two-dimensional phase transition observed with DMPS spread on phosphate buffer was absent. However, other features of the force–area curve such as the lift-off area of 105 Å²/molecule and the limiting area of 41 Å²/molecule were in good agreement with the monolayer characteristics shown in Figure 1.

Force–area curves of different phosphatidylserines spread on acetate buffer, pH 4, are presented in Figure 2. The isotherms of the saturated, long-chain phosphatidylserines, DPPS and dihexadecyl-PS, are further condensed compared to the force–area curves obtained at pH 7.4 (curves 1 and 2, cf. Figures 1 and 2). The most dramatic effect is observed with DMPS (curve 3, Figure 2): the two-dimensional phase transition is absent. Above surface pressures of about 10 mN/m, the interfacial behavior of DMPS is identical with that of DPPS and dihexadecyl-PS. With all saturated phosphatidylserines, the limiting area observed at surface pressures above ~30 mN/m is 40.5 Å²/molecule. Below 30 mN/m, the dihexadecyl-PS monolayer is apparently even more condensed than that of the ester analogue DPPS. The force–area curves of unsaturated phosphatidylserines spread on acetate

Table I: Molecular Areas at 30 mN/m of Phosphatidylserine Monolayers Spread on Buffers Differing in pH and Ionic Content[a]

phosphatidylserine	area/molecule (Å²)		
	pH 7.4	pH 7.4, 10 mM $CaCl_2$	pH 4.0
DMPS	45.5	40.2	40.7
DPPS	44.2	40.2	40.7
dihexadecyl-PS	43.5	40.2	40.5
beef brain PS	62.0	54.7	56.1
POPS	63.7	56.6	57.2
dielaidoyl-PS	57.2	48.0	46.0
DOPS	67.5	62.5	62.1

[a] Phosphatidylserine monolayers were spread on 5 mM Tris buffer, pH 7.4, with and without 10 mM $CaCl_2$ and on 0.2 M acetate buffer, pH 4.0.

buffer, pH 4.0, are also shifted to lower molecular areas compared to those spread on phosphate buffer, pH 7.4. For instance, at pH 4 and 30 mN/m, beef brain phosphatidylserine (curve 5, Figure 2), POPS (curve 6), and DOPS (curve 7) have molecular areas of 56, 57, and 62 Å²/molecule, respectively (Table I). This is a decrease in the area per molecule of 5.5–6.5 Å² compared to the molecular areas of the same compounds at pH 7.4. Like at pH 7.4, POPS and beef brain phosphatidylserine give similar force–area curves (curves 5 and 6, Figure 2). Dielaidoyl-PS gives a force–area curve very similar to beef brain PS and POPS up to 19.6 mN/m (curve 4, Figure 2). At this pressure, dielaidoyl-PS undergoes a two-dimensional phase transition from the liquid-expanded to the condensed state with a limiting surface area of 43.1 Å²/molecule at 40 mN/m. POPS and beef brain phosphatidylserine also undergo a two-dimensional transition to a slightly more condensed state at a surface pressure of about 36 mN/m. Interestingly, the force–area curves of both phosphatidylserines exhibit very similar transitions at pH 7.4 in the presence of 10 mM $CaCl_2$ (data not shown). The force–area curves of phosphatidylserines spread on 5 mM Tris-HCl buffer, pH 7.4, in the presence of 10 mM $CaCl_2$ are similar to those at pH 4 shown in Figure 2, indicating that the condensing effect of 10 mM Ca^{2+} is similar to that of reducing the pH to 4 (Table I). The agreement is particularly good at higher surface pressures. The results summarized in Table I document the condensing effect which a reduction in the pH to 4 has on the molecular packing of phosphatidylserine monolayers and stress the similarity in the condensing effect of reducing the pH from 7.4 to 4 and adding 10 mM Ca^{2+} to the Tris-HCl buffer, pH 7.4.

Differential Scanning Calorimetry (DSC). Differential scanning calorimetry of the NH_4^+ salt of dihexadecyl-PS dispersed in excess 5 mM phosphate buffer, pH 7, gives reproducible sharp order–disorder transitions on both heating and cooling (Figure 3A). Upon heating, a single sharp reversible gel to liquid-crystal transition is observed at 59 °C (ΔH = 55 J/g or 9.6 kcal/mol; see Table II). Upon cooling, an exothermic sharp transition is observed reproducibly at 57 °C (Figure 3A). Changing the heating rate from 1.25 to 5 °C/min or leaving out Na_2EDTA in the buffer has no effect on the transition temperature and enthalpy. For comparison, the thermal behavior of the NH_4^+ salt of DPPS is shown in Figure 3B. From a comparison of panels A and B of Figure 3 and from an inspection of the data in Table II, it is clear that although the thermal behavior of DPPS and its ether analogue is similar there are noticeable differences.

The values of the transition temperature and enthalpy of the ether analogue exceed those measured for DPPS. This seems to be generally true when comparing the thermal behavior of diacylglycerophospholipids with their ether analogues (Paltauf, 1983). Furthermore, the transition of dihexadecyl-PS is significantly sharper; i.e., the peak width at half-height is smaller than that of DPPS. The difference in peak width of the order–disorder transition reflects differences in the cooperativity of the transition. From a comparison of the van't Hoff enthalpy with the enthalpy ΔH, determined calorimetrically, the cooperativity of the transition can be calculated according to Mabrey and Sturtevant (1978). The cooperativity of the dihexadecyl-PS transition is about a factor of 2 greater than that of DPPS. The comparison of the thermal behavior of DPPS and its ether analogue is extended in Table III. Both the crystalline, anhydrous samples (data not shown) and also the fully hydrated dispersions exhibit single sharp order–disorder transitions upon heating and cooling. The exothermic transitions observed upon cooling occur at temperatures usually

Table II: Transition Temperatures (T_c) and Enthalpies (ΔH) of Fully Hydrated Diacylphosphatidylserines[a]

phosphatidylserine	T_c (°C)	ΔH, J/g (kcal/mol)	experimental conditions	ref
DMPS	40 ± 1	45 ± 1 (7.5 ± 0.2)		*b*
DMPS	41 ± 1	24 ± 3 (4.0 ± 0.5)	sonicated	
DPPS	56 ± 1	50.5 ± 1 (9.1 ± 0.2)		*b*
DPPS	57.5 ± 0.5	35 ± 1 (6.3 ± 0.2)	sonicated	
dihexadecyl-PS	59 ± 0.5	55 ± 1 (9.6 ± 0.2)		
dihexadecyl-PS	58.8 ± 0.5	41 ± 1 (7.2 ± 0.2)	sonicated	
beef brain PS (Na^+ salt)	14.5 ± 0.5	26 ± 3 (5.2 ± 0.6)		
POPS	13 ± 1	23 ± 3 (4.4 ± 0.6)	5 mM Tris, pH 7	
POPS	14 ± 0.5	24 ± 3 (4.5 ± 0.5)	5 mM Tris, 0.5 M NaCl	
DOPS (Na^+ salt)	−11 ± 1	45 ± 2 (8.7 ± 0.3)		
dielaidoyl-PS (Na^+ salt)	25 ± 1			*c*

[a] Unless otherwise stated, unsonicated dispersions of the NH_4^+ salts of the different phosphatidylserines in 5 mM phosphate buffer, pH 7, containing 5 mM Na_2EDTA and 0.05% NaN_3 were used. [b] Hauser et al. (1982). [c] Li Xin Zhion, W. Jordi, and B. de Kruijff, unpublished results.

Table III: Comparison of the Thermal Behavior of DPPS and Dihexadecyl-PS (Ether Analogue)

sample	DPPS		dihexadecyl-PS	
	T_c (°C)	ΔH, J/g (kcal/mol)	T_c (°C)	ΔH, J/g (kcal/mol)
(1) crystalline, anhydrous, protonated form	68	64 (11.3)	67	74 (12.8)
(2) crystalline, anhydrous NH_4^+ salt	91	39 (7.0)	87	39 (6.8)
(3) aqueous,l unsonicated dispersion				
in 0.01 M HCl	67	46 (8.1)	76.5	54 (9.4)
in 0.001 M CH_3COONa			77	54 (9.4)
(4) aqueous unsonicated dispersion of NH_4^+ salt in 5 mM phosphate buffer, pH 7	56	51 (9.2)	59	57 (9.6)

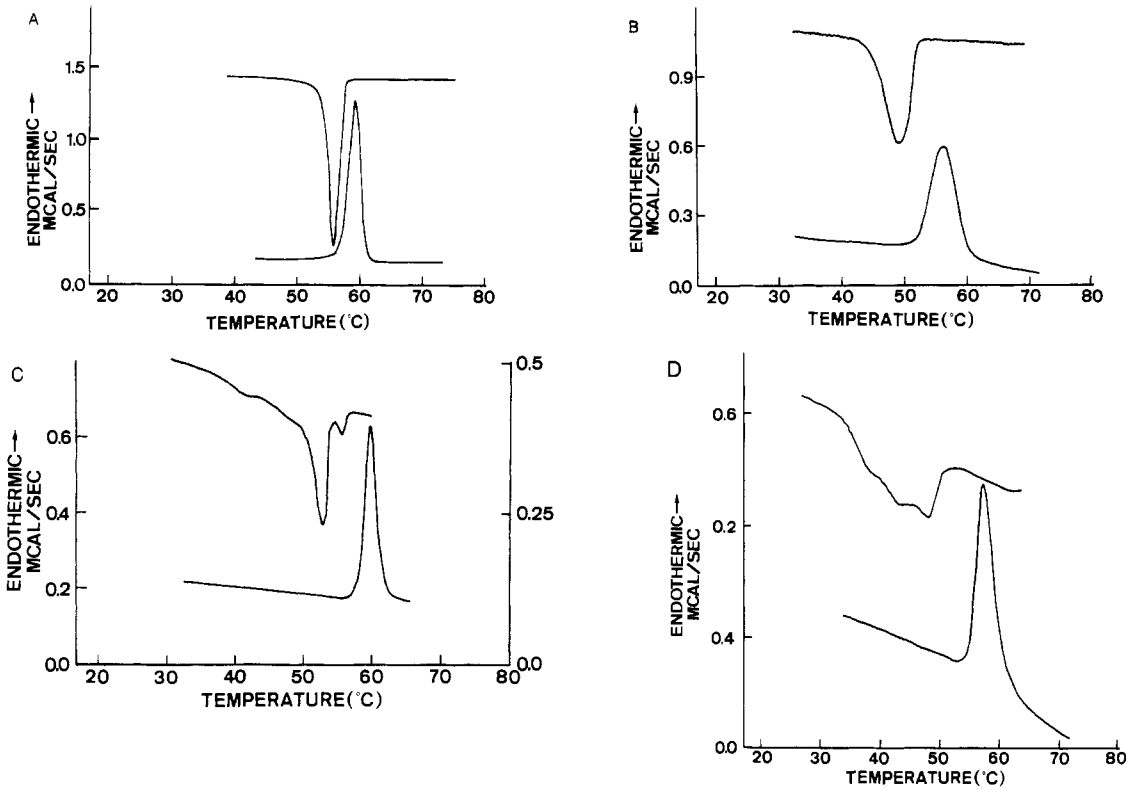

FIGURE 3: Order–disorder transitions of unsonicated and sonicated dispersions of the NH_4^+ salts of dihexadecyl-PS and DPPS. Heating and cooling curves of phosphatidylserine dispersions in 5 mM phosphate buffer, pH 7, 5 mM Na_2 EDTA, and 0.05% NaN_3 were recorded at 5 °C/min. (A) Unsonicated dispersion of dihexadecyl-PS (0.13 M); (B) unsonicated dispersion of DPPS (0.067 M); (C) sonicated dispersion of dihexadecyl-PS (0.033 M); (D) sonicated dispersion of DPPS (0.052 M).

3–6 °C depressed compared to the transitions observed on heating. The crystalline, anhydrous NH_4^+ salts undergo order–disorder transitions at higher temperatures than the corresponding protonated forms. The NH_4^+ salts in the dry state give order–disorder transitions about 20 °C higher than the protonated forms. In the fully hydrated state, the situation is reversed: the NH_4^+ salt of DPPS melts at a temperature 11 °C lower than protonated DPPS, and the NH_4^+ salt of dihexadecyl-PS melts 18 °C lower than its protonated form (Table III). The reproducibility of the dry samples can be limited; on repeated heating–cooling cycles, the transition temperature may change with the concomitant appearance of shoulders, indicating that heating dry phosphatidylserine to relatively high temperatures is probably accompanied by chemical degradation.

The thermal behavior of sonicated dispersions of the NH_4^+ salts of dihexadecyl-PS and DPPS in 5 mM phosphate buffer, pH 7, is included in Figure 3 (panels C and D, respectively). Sonicated phosphatidylserine dispersions apparently give reproducibly sharp gel to liquid-crystal transitions similar to the unsonicated dispersions. The transition temperatures are as high or sometimes even 1–2 °C higher than those of the corresponding unsonicated dispersions (Table II). The transition enthalpy (ΔH) of sonicated phosphatidylserine dispersions is reduced to 70–80% of the values measured for unsonicated dispersions (Table II). The thermal behavior of sonicated phosphatidylserine dispersions is distinctly different from that of sonicated phosphatidylcholine dispersions. The latter have been shown to give broadened gel to liquid-crystal transitions at lower temperatures compared to unsonicated dispersions. As shown in Figure 3C,D the cooling curves of sonicated phosphatidylserine dispersions are complex. Broad exothermic transitions are observed reproducibly consisting of several peaks. The maximum temperatures of the broad envelope obtained upon cooling of dihexadecyl-PS and DPPS dispersions are 53 and 48 °C, respectively. Sonicated dispersions of the NH_4^+ salt of DMPS showed analogous thermal behavior: a single sharp endothermic transition at 41 °C (ΔH = 24 J/g or 4 kcal/mol, Table II), and upon cooling a broad envelope consisting of several transitions spread out over about 20 °C was observed (data not shown). The transition enthalpy amounted to only 53% of the value measured for unsonicated dispersions (Table II).

The sharp order–disorder transitions of the saturated phosphatidylserines (cf. Figure 3A,B) are contrasted by the thermal behavior of the Na^+ salt of beef brain PS in unsonicated dispersions in excess phosphate buffer, pH 7. It exhibits broad asymmetric transitions on both heating and cooling which are reversible (Figure 4A). Upon heating, the gel to liquid-crystal transition occurs reproducibly at 14.5 °C (ΔH = 26 J/g or 5.2 kcal/mol; Table II), and upon cooling, the exothermic transition is depressed by 6 °C. Sonicated beef brain PS dispersed in the same buffer gave no distinct endothermic transition. Upon cooling the sonicated dispersion, a broad exothermic transition was observed consisting of two to three maxima spread out between 0 and 20 °C (data not shown). The absence of an observable endothermic transition is probably due to the gel to liquid-crystal transition being spread out over a considerable temperature range so that it is lost in the noise. The thermal behavior of the NH_4^+ salt of the mixed-chain POPS is similar to that of beef brain PS. Reversible transitions are observed upon heating and cooling. The endothermic transition is at 13 °C with a transition enthalpy ΔH = 23 J/g or 4.4 kcal/mol (Figure 4B, Table II). Upon cooling, an exothermic transition occurs at 10 °C. In contrast to the naturally occurring beef brain PS, the transitions of the synthetic POPS with well-defined fatty acyl chains are much sharper; the width is approximately one-third of that of beef brain PS (cf. Figure 4A,B). Adding NaCl up to 0.5 M concentrations to the dispersion buffer had little effect

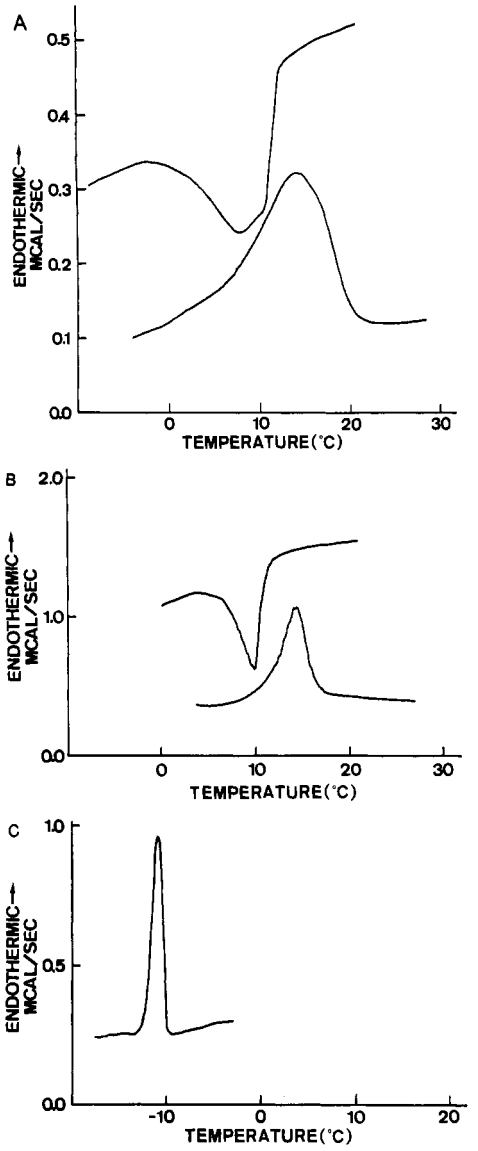

FIGURE 4: Order–disorder transitions of unsonicated dispersions of the Na^+ or NH_4^+ salts of unsaturated phosphatidylserines. Heating and cooling curves of phosphatidylserine dispersions in 5 mM phosphate buffer, pH 7, 5 mM Na_2EDTA, and 0.05% NaN_3 were recorded at 5 °C/min. (A) Beef brain PS (Na^+ salt) at 0.094 M; (B) POPS (NH_4^+ salt) at 0.055 M; (C) DOPS (Na^+ salt) at 0.086 M.

on the thermal behavior of POPS (Table II).

The order–disorder transition of the Na^+ salt of DOPS dispersed in 5 mM phosphate buffer, pH 7, is shown in Figure 4C. The ice melting peak has to be minimized by carefully balancing the amount of buffer in the reference cell; otherwise, the endothermic transition of DOPS is superimposed on a greatly sloping base line. As a result, the accuracy of the measurement would suffer. A sharp endothermic transition occurs at −11 °C with a transition enthalpy of ΔH = 45 J/g or 8.7 kcal/mol (Table II). This is in good agreement with the work of Browning and Seelig (1980).

Discussion

Figures 1 and 2 show that all monolayer states are possible with phosphatidylserines differing in the nature of the hydrocarbon chains. The Na^+, K^+, and NH_4^+ salts of saturated phosphatidylserines at neutral pH form condensed monolayers while those of the unsaturated ones give liquid-expanded films. As noted previously (Phillips & Chapman, 1968), the two monolayer states are well-defined, and at any particular temperature, there is only one compound out of a homologous series that exhibits the transition between the liquid-expanded and condensed states. With the homologous series of saturated diacylphosphatidylserines, it is DMPS that undergoes the transition at ambient temperature (Figure 1). In this respect, phosphatidylserines behave like saturated 1,2-diacylphosphatidylethanolamines. In contrast, with the homologous series of saturated 1,2-diacylphosphatidylcholines, it is dipalmitoylphosphatidylcholine that exhibits the transition state at room temperature (Phillips & Chapman, 1968). The limiting area per molecule in condensed monolayers of the NH_4^+ salts of saturated phosphatidylserines is 40 $Å^2$; this value is also very similar to that measured for saturated 1,2-diacylphosphatidylethanolamines. It is considerably smaller than the limiting value of 45 $Å^2$ of a condensed phosphatidylcholine monolayer (Phillips & Chapman, 1968). This result indicates that the phosphatidylserine molecules in condensed phosphatidylserine monolayers are more closely packed than those in comparable phosphatidylcholine films. This is so despite the electrostatic repulsion between phosphatidylserine molecules which, at neutral pH, have been shown to bear a net negative charge. The charge is due to the −COOH group of serine being practically fully ionized at neutral pH (Hauser et al., 1976; Seimiya & Ohki, 1973). We can conclude that the polar group of phosphatidylserine in the fully charged state at neutral pH allows tighter packing than the bulkier glycerophosphocholine group of phosphatidylcholine. Comparison of Figures 1 and 2 indicates that phosphatidylserines undergo a significant condensation when spread on acid subsolutions which is due to changes in free energy mediated by the protonation of the $-COO^-$ group and hence the conversion of the negatively charged salt form to the isoelectric protonated form (Boggs, 1980). It is interesting to note that the condensation of phosphatidylserine monolayers induced by acidification to pH 4 (cf. Figures 1 and 2, Table I) is similar to that produced by adding 10 mM $CaCl_2$ to the Tris buffer, pH 7.4. The condensing effect of Ca^{2+} on phosphatidylserine monolayers was observed before (Papahadjopoulos & Miller, 1967). Inspection of Figures 1 and 2 also reveals that below 30 mN/m the force–area curves of the ether analogue are more condensed than those of DPPs. This indicates that below 30 mN/m the molecules of the diether compound, i.e., dihexadecyl-PS with alkyl chains linked to the glycerol group via ether bonds, are packed more tightly than those of the corresponding diester compound, DPPS.

It was observed with the homologous series of phosphatidylcholines and phosphatidylethanolamines (Chapman et al., 1967; Ladbrooke & Chapman, 1969) that the transition temperature (T_c) of the order–disorder transition increases with increasing chain length. Given a certain chain length, the T_c temperature decreases with increasing unsaturation in the hydrocarbon chains. Phosphatidylserines certainly follow these general trends (cf. Table II). Moreover, for the NH_4^+ or Na^+ salts of phosphatidylserines, there appears to be an inverse correlation between the molecular packing (area per molecule) measured in monolayers and the transition temperature T_c determined calorimetrically as demonstrated in Figure 5.

It was also noted before that the gel to liquid-crystal transition temperature of saturated diacylphosphatidylethanolamines is always considerably higher than that of the corresponding saturated diacylphosphatidylcholines (Ladbrooke & Chapman, 1969; McElhaney, 1982; Mabrey & Sturtevant, 1978). The transition temperatures (T_c) of the dipalmitoyl derivatives of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine in the fully hydrated state are 41.5 °C

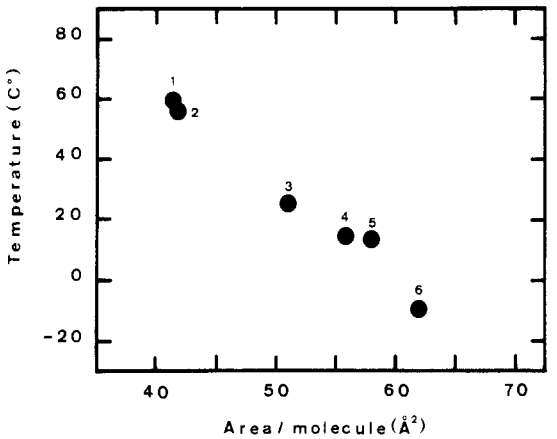

FIGURE 5: Relationship between the temperature (T_c) of the order–disorder transition and the molecular packing at 40 mN/m of different phosphatidylserines. The transition temperature T_c was determined calorimetrically, and the molecular areas (in angstroms squared per molecule) were derived from the surface pressure–molecular area curves shown in Figure 1: (1) dihexadecyl-PS; (2) DPPS; (3) dielaidoyl-PS; (4) beef brain phosphatidylserine; (5) POPS; (6) DOPS.

(Chapman et al., 1967; Mabrey & Sturtevant, 1978), 63.8 °C (Mabrey & Sturtevant, 1978; McElhaney, 1982), and 57 °C (this work), respectively. These values are all significantly greater than the melting point of the corresponding hydrocarbon, *n*-hexadecane, which is 16.8 °C. The extent to which the value for the pure hydrocarbon is exceeded may be taken as a rough measure of the strength of the intermolecular interaction between the phospholipid polar groups. According to this criterion, the polar group interaction in phosphatidylserine bilayers would be in between that of phosphatidylcholine and phosphatidylethanolamine.

The thermal behavior of aqueous unsonicated dispersions of the NH_4^+ or Na^+ salts of dihexadecyl-PS is similar to, albeit not identical with that of the corresponding salts of DPPS (Table II). This points to similarities in the molecular packing of dihexadecyl-PS and DPPS. Similar thermal behavior and physicochemical properties were also reported for diacylphosphatidylcholines and their ether analogues (Paltauf et al., 1971; Schwarz et al., 1976; Schwarz & Paltauf, 1977; Hauser et al., 1981; Smaby et al., 1983; Ruocco et al., 1985).

Sonicated phosphatidylserine dispersions have been shown to consist of small unilamellar vesicles (Hauser & Phillips, 1973) very similar to those of sonicated phosphatidylcholine dispersions. Despite this morphological similarity, the thermal behavior of sonicated phosphatidylserine dispersions is markedly different from that of sonicated phosphatidylcholine dispersions. The order–disorder transition of dimyristoyl- and dipalmitoylphosphatidylcholine vesicles present in sonicated dispersions occurs at lower temperatures compared to the corresponding unsonicated dispersions (Suurkuusk et al., 1976; Mabrey & Sturtevant, 1978). The pretransition observed with unsonicated phosphatidylcholine dispersions is absent in sonicated dispersions. The main transition is significantly broadened, and the transition enthalpy is greatly reduced. In contrast, sonicated phosphatidylserine dispersions give sharp gel to liquid-crystal transitions at the same or slightly elevated temperatures (Table II). The slightly increased transition temperature observed with sonicated phosphatidylserine vesicles reflects a stabilization of the small unilamellar phosphatidylserine vesicles. The reason for this behavior and the mechanism of the stabilization remain unclear at the moment. It seems, however, to be a general phenomenon of negatively charged small unilamellar phospholipid vesicles since sonicated dispersions of sodium phosphatidate dispersed at neutral pH showed analogous thermal behavior (unpublished observation). A tentative explanation of this phenomenon is as follows. Some phosphatidylserine molecules located on the inner bilayer surface become protonated, and as a result, the degree of ionization and thus the electrostatic repulsion between phosphatidylserine molecules within the inner layer of the bilayer will be reduced. Probably strong attractive forces such as ion–dipole and dipole–dipole interactions become effective instead. Consequently, the internal monolayer of the bilayer will undergo a condensation; this is entirely consistent with the condensation induced in phosphatidylserine monolayers upon lowering the pH of the subphase (cf. Figure 2). In this way, electrostatic repulsion and also steric constraints on the highly curved internal bilayer surface are minimized. The driving force of the protonation of the internal bilayer surface is apparently a significant gain in free energy that leads to the stabilization of the small unilamellar phosphatidylserine bilayer vesicles. A result of the protonation of the internal bilayer surface is the generation of a pH gradient across the phosphatidylserine bilayer with the internal surface pH being lower than the external surface pH.

This explanation could also account for an observation reported previously (Hauser & Phillips, 1973; Hauser & Gains, 1982). It was found that small unilamellar phosphatidylserine vesicles in H_2O produced by sonication are significantly smaller than unilamellar phosphatidylcholine vesicles produced under identical conditions. The small radius of curvature would be difficult to reconcile with phospholipids having greater surface charge densities on the internal monolayer of the bilayer. Such a situation is expected to lead to a greater charge repulsion on the internal vesicle surface and hence to a destabilization of the small vesicle. However, contrary to expectation, small unilamellar vesicles of charged phospholipids were found to be more stable despite the fact that they were smaller in diameter than phosphatidylcholine vesicles. We propose that the stabilizing principle is protonation of the inner surface resulting in a pH gradient inside more acidic than outside. ^{13}C and ^{31}P NMR measurements are currently carried out on small unilamellar vesicles of charged phospholipids in order to verify the proposed pH gradient. Such a pH gradient can also be envisaged to stabilize highly curved charged surfaces of biological membranes.

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The "γ Subunit" of Na,K-ATPase: A Small, Amphiphilic Protein with a Unique Amino Acid Sequence†

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ABSTRACT: The "γ subunit", or "proteolipid", of Na,K-ATPase is a small, membrane-bound protein that copurifies with the α and β subunits of this enzyme. The importance of γ in the function of Na,K-ATPase remains to be established, but some evidence indicates that it may be involved in forming a receptor site for cardiac glycosides. We have previously communicated [Reeves, A. S., Collins, J. H., & Schwartz, A. (1980) *Biochem. Biophys. Res. Commun. 95*, 1591–1598] the purification and amino acid composition of sheep kidney γ, and in this paper we present the first available sequence information on this protein. Although the amino terminus of γ seems to be blocked and it is resistant to proteolytic cleavage, we have determined approximately half of its amino acid sequence. Our results indicate that γ contains a total of 68 amino acid residues, with a calculated M_r of 7675. The sequenced portion appears to be at the carboxyl terminus of the polypeptide chain. The γ sequence is unique, providing strong evidence for its homogeneity and establishing for the first time that it is not a breakdown product of the α or β subunits. γ is not a true proteolipid, but rather it is an amphiphilic protein with two distinct structural domains. The amino-terminal domain (residues 1–49) is very hydrophilic, with many charged amino acid side chains, and must be extracellular. This domain includes a concentrated segment of four aromatic residues which may be involved in glycoside binding. The carboxyl-terminal domain (residues 50–68) is hydrophobic and probably spans the cell membrane.

Na,K-ATPase is the enzyme responsible for the active transport of Na^+ and K^+ across animal cell membranes. The resulting electrochemical gradients are essential for a variety of vital physiological functions [for reviews, see Schwartz and Collins (1982), Jorgensen (1982), Kaplan (1985), Stahl and Harris (1986), and Stahl (1986)]. Na,K-ATPase contains two major protein subunits: the α, or catalytic subunit (M_r ~ 112 000), and the β, or glycoprotein subunit (M_r ~55 000).

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