Calorimetric and Spectroscopic Studies of the Thermotropic Phase Behavior of Lipid Bilayer Model Membranes Composed of a Homologous Series of Linear Saturated Phosphatidylserines

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ABSTRACT The thermotropic phase behavior of lipid bilayer model membranes composed of the even-numbered, N-saturated 1,2-diacyl phosphatidylserines was studied by differential scanning calorimetry and by Fourier-transform infrared and ^{31}P-nuclear magnetic resonance spectroscopy. At pH 7.0, 0.1 M NaCl and in the absence of divalent cations, aqueous dispersions of these lipids, which have not been incubated at low temperature, exhibit a single calorimetrically detectable phase transition that is fully reversible, highly cooperative, and relatively energetic, and the transition temperatures and enthalpies increase progressively with increases in hydrocarbon chain length. Our spectroscopic observations confirm that this thermal event is a lamellar gel (L_β)-to-lamellar liquid crystalline (L_α) phase transition. However, after low temperature incubation, the L_β/L_α phase transition of dilauroyl phosphatidylserine is replaced by a higher temperature, more enthalpic, and less cooperative phase transition, and an additional lower temperature, less enthalpic, and less cooperative phase transition appears in the longer chain phosphatidylserines. Our spectroscopic results indicate that this change in thermotropic phase behavior when incubated at low temperatures results from the conversion of the L_β phase to a highly ordered lamellar crystalline (L_c) phase. Upon heating, the L_c phase of dilauroyl phosphatidylserine converts directly to the L_α phase at a temperature slightly higher than that of its original L_β/L_α phase transition. Calorimetrically, this process is manifested by a less cooperative but considerably more energetic, higher-temperature phase transition, which replaces the weaker L_β/L_α phase transition alluded to above. However, with the longer chain compounds, the L_c phase first converts to the L_β phase at temperatures some 10–25°C below that at which the L_β phase converts to the L_α phase. Our results also suggest that shorter chain homologues form L_c phases that are structurally related to, but more ordered than, those formed by the longer chain homologues, but that these L_c phases are less ordered than those formed by other phospholipids. These studies also suggest that polar/apolar interfaces of the phosphatidylserine bilayers are more hydrated than those of other glycerolipid bilayers, possibly because of interactions between the polar headgroup and carbonyl groups of the fatty acyl chains.

INTRODUCTION

Although zwitterionic lipids are the major lipid components of eukaryotic cell membranes, anionic phospholipids are also invariably present in considerable quantities and are known to be essential structural and functional components of such membranes (Gennis, 1989; Lentz, 1999; Buckland and Wilton, 2000). In eukaryotic membranes, phosphatidylserine (PS) is the major anionic phospholipid present, and one of its primary functions seems to be to impart a negative charge to the inner surface of the membrane lipid bilayer. This negative surface charge is required for the binding and activation of various peripheral membrane proteins, including various phospholipases (Buckland and Wilton, 2000), myristoylated proteins (McLaughlin and Aderem, 1995), and components of the blood coagulation process (Lentz, 1999). As well, PS is required for the activation of a variety of integral transmembrane proteins such as protein kinase C (Newton, 1995) and various ion-transporting adenosine triphosphatases (Gennis, 1989). Clearly, a detailed molecular level understanding of the mechanism of the binding and activation of various membrane-associated proteins by PS requires that the organization and dynamics of PS in lipid bilayer membranes be well understood.

Although there has been considerable interest in the physical properties of PS bilayers, most studies have been directed primarily at characterizing the sensitivity of the thermotropic phase behavior of PS model membranes to the effects of variations in pH and ionic strength, and to the presence of divalent cations. Such studies have shown that the gel/liquid crystalline phase transition temperature (T_m) of PS bilayers is very sensitive to pH over pH ranges corresponding to ionization of the phosphate (pH $\sim$ 1–2), carboxylate (pH $\sim$ 3.5–4.5), and amino (pH $\sim$ 9.5–10.5) moieties of the polar headgroup, with the highest phase transition being found for the zwitterionic form of PS existing below pH $\sim$ 2, where electrostatic PS repulsion and hydration of the polar headgroup is minimal (Macdonald et al., 1976; Cevc et al., 1981; Cevc and Marsh, 1987). Other studies have demonstrated that the T_m of PS bilayers increases significantly with increases in the ionic strength of the medium due to salt-mediated screening of the various charged groups on the surface of the PS membrane, and with dehydration of the polar headgroup (Cevc et al., 1981; Cevc and Marsh, 1987).

The effect of metal ion binding on PS polymorphic phase behavior has been the subject of a sizeable portion of

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currently available data on PS bilayers. Such studies have shown that the binding of most divalent cations and strongly polarizing monovalent cations such as lithium can induce the formation of one or more high-melting lamellar gel or crystalline phases (van Dijck et al., 1978; Hauser and Shipley, 1981, 1983, 1984, 1985; Casal et al., 1987a,b,c; 1989; Mattai et al., 1989), the properties of which are comparable to those observed with fully hydrated PS bilayers at low pH or with anhydrous or poorly hydrated PS bilayers (Hauser and Shipley, 1981, 1985; Hauser et al., 1982; Casal et al., 1987c). Such behavior has been attributed to the capacity of those cations to promote the dehydration of the headgroup and polar/apolar interfacial regions of PS bilayers, as well as to the fact that the binding of those cations effectively neutralizes the negatively charged moieties at the surface of the PS bilayer (Hauser and Shipley, 1981, 1985; Casal et al., 1987b).

The effect of membrane surface charge on the conformation, orientation, and dynamics of the lipid polar headgroup at the surfaces of PS bilayers has also been studied (Browning, 1981; Roux and Neuman, 1986; Roux et al., 1988, 1989). Such studies suggest that the polar headgroups of PS are less flexible than those of phosphatidylethanolamine (PE) and phosphatidylcholine (PC; Browning, 1981) and that the orientation and dynamics of the PS headgroups are largely determined by the sign and magnitude of the surface charge of the bilayer, which, in turn, can be affected by the binding of soluble cationic peptides to the membrane surface and by the incorporation of a transmembrane peptide with cationic termini. These results can be rationalized by assuming that the orientation of the P-N dipole of the polar headgroup is dependent on the magnitude and location of charges near the bilayer surface (Seelig et al., 1987; Scherer and Seelig, 1989; MacDonald et al., 1991).

PS bilayers are known to exhibit their gel/liquid-crystalline phase transitions at temperatures that are higher than those of the corresponding PC and phosphatidylglycerol (PG) bilayers but lower than those exhibited by the corresponding PE bilayers at neutral pH and moderate ionic strength and in the absence of divalent cations (Marsh, 1990). These findings have been rationalized on the basis of the relative capacities of the polar headgroups of these phospholipids for intermolecular electrostatic and hydrogen-bonding interactions (Boggs, 1980, 1986, 1987; Cevc and Marsh, 1987). However, there is little information on the gel state polymorphic phase behavior of PS bilayers under these conditions and, in particular, the existence of a lamellar crystalline (L_c) phase has never been reported. This may be due to the fact that most studies of the thermotropic phase behavior of PS bilayers were performed before the it was generally appreciated that phospho- and glycoglycerolipids can form an array of highly condensed L_c phases (Lewis and McElhaney, 1990, 1993; Lewis et al., 1990; Mannock et al., 1988, 1990; Zhang et al., 1997), even in the presence of significant amounts of cholesterol and transmembrane peptides (Zhang et al., 1995; McMullen et al., 1999), and that such phases can also form in biological membranes (Seguin et al., 1987). Moreover, the unrecognized induction of L_c phases can confound the interpretation of data generated in studies of lipid-sterol and lipid-protein interactions. The relative shortage of data on PS thermotropic phase behavior generally is further compounded by the fact that there is considerable variation in the data reported in the literature, probably due to variations in the nature of the salt forms examined and to the sensitivity of PS thermotropic phase behavior to variations in the pH and ionic strength of the medium. Thus, in order to provide a consistent set of reference data for further studies of the interactions of sterols and transmembrane peptides with PS bilayers, we have undertaken a thorough study of the thermotropic phase behavior of a homologous series of N-saturated 1,2-diacylphosphatidylserines, using a combination of differential scanning calorimetry (DSC), and FTIR and ^{31}P-nuclear magnetic resonance (NMR) spectroscopy, under conditions of physiologically relevant pH and ionic strength and in the absence of divalent cations. We also use the sodium salt forms of these PSs obtained from a single commercial source that produces materials of very high purity and excellent batch-to-batch reproducibility.

MATERIALS AND METHODS

The PSs used in this study were obtained as their sodium salts from Avanti Polar Lipids Inc. (Alabaster, AL) and were used without further purification. For DSC studies, 4 to 6 mg of dried lipid were dispersed in 600 μl of a buffer containing 50 mM Tris, 150 mM NaCl, 5 mM EDTA, and 2 mM NaN_3 (pH 7.4) by vigorous vortexing at temperatures well above the gel/liquid-crystalline phase transition temperature of the lipid concerned. The sample was subsequently subjected to three cycles of cooling and heating between −20°C and the high temperatures initially used for sample dispersion and, after recooling the sample to room temperature, a 500-μl aliquot was transferred to the hastelloy ampoules of the calorimeter. Data were acquired with a high-sensitivity, multi-cell differential scanning calorimeter (Calorimetry Sciences Corporation, Provo, UT) operating at scan rates of 10°C per hour. The DSC data acquisition protocol was as follows. After sample preparation as described above, three heating and cooling scans were performed between −7°C and temperatures some 20°C above the gel/liquid-crystalline phase transition of the lipid sample. Subsequently, the samples were subjected to a low-temperature incubation protocol to induce the formation of the lamellar crystalline (L_c) phase. First, the hydrated PS preparation was slowly cooled (~10°C/h) to temperatures near −30°C and incubated at that temperature for 12 h. Next the sample was slowly reheated to 4–6°C and incubated at the latter temperature for a length off time appropriate for the complete formation of the L_c phase (see below). Preliminary experiments using FTIR spectroscopy indicated that the incubation period near −30°C served primarily as a nucleation step, since the formation of the L_c phase actually took place during the later incubation at temperatures near 4–6°C. With the shorter chain lipids such as 1,2-dilauroyl-sn-glycero-3-phosphoryl-L-serine (DLPS), L_c phase formation was essentially complete within 2 h of incubation at temperatures near 4–6°C, whereas with longer chain homologues such as 1,2-distearoyl-sn-glycero-3-phosphoryl-L-serine (DSPS), the process took significantly longer (~1–2 days). In this study we routinely employed two cycles of this low-temperature incubation protocol to ensure complete conversion to the L_c phase. Finally, a single heating and cooling scan was performed be-

tween −7°C and temperatures some 20°C above the gel/liquid-crystalline phase transition temperature of the lipid. Preliminary experiments showed that the low-temperature incubation protocol outlined above was sufficient to rapidly induce the formation of the L_c phases of these lipids, and that the L_c phases formed under such conditions are spectroscopically similar to those formed when samples are incubated at 0−4°C for periods ranging from several days to several weeks. The data acquired were analyzed and plotted using the Origin software package (Microcal Software Inc., Northampton, MA).

For the Fourier-transform infrared (FTIR) spectroscopic experiments, samples were prepared by dispersing 1 to 2 mg of the lipid sample in 50 to 75 μl of a buffer composed of 50 mM sodium phosphate, 150 mM NaCl, and 1 mM NaN_3 (pH 7.4) at temperatures well above the gel liquid-crystalline phase transition temperature of the lipid sample. D_2O was used as the buffer solvent in most experiments and H_2O was used as the buffer solvent only when it necessary to examine the O-P-O asymmetrical stretching region of the infrared spectrum. After heating and cooling as described above for the DSC sample, the dispersion was squeezed between the CaF_2 windows of a heatable, demountable liquid cell (NSG Precision Cells, Farmingdale, NY) equipped with a 10 μM teflon spacer. Once mounted in the sample holder of the spectrometer, the sample could be heated between −20°C and 90°C by an external, computer-controlled water bath. Infrared spectra were acquired as a function of temperature with a Digilab FTS-40 Fourier-transform spectrometer (Biorad, Digilab Division, Cambridge, MA) using data acquisition parameters similar to those described by Mantsch et al. (1985). The experiment involved a sequential series of 2°C temperature ramps with a 20-min inter-ramp delay for thermal equilibration, and was equivalent to a scanning rate of 4°C per hour. The data obtained were analyzed using computer programs obtained from the instrument manufacturer and from the National Research Council of Canada, and plotted with the Origin software package. In cases where absorption bands appeared to be a summation of components, a combination of Fourier deconvolution and curve fitting procedures were used to obtain estimates of the position of the component bands and to reconstruct the contours of the original band envelope.

For the ^{31}P-NMR spectroscopic measurements, 10 to 15 mg of dried lipid were dispersed in 0.6 ml of the buffer used for DSC experiments using the same procedures as used for the preparation of the DSC samples. Spectra were acquired as a function of temperature with a Varian Unity-300 spectrometer operating at 121.42 MHz for ^{31}P. Spectra were acquired using the single-pulse data acquisition techniques and other data acquisition parameters described by Lewis et al. (1988) and plotted with the Origin software package.

RESULTS

Differential scanning calorimetric studies

Illustrated in Fig. 1 are DSC thermograms that exemplify the thermotropic phase behavior exhibited by fully hydrated samples of the N-saturated 1,2-diacyl PSs studied. With all of the lipids examined, freshly prepared samples that have not been subject to regimes of low-temperature incubation exhibit a single highly cooperative phase transition, which is also fully reversible and relatively energetic. The temperature and enthalpy changes associated with these processes increase smoothly and progressively with increases in hydrocarbon chain length (see Fig. 2). An examination of the FTIR and ^{31}P-NMR spectroscopic changes that accompany these phase transitions(see below) have enabled an essentially unambiguous assignment of these processes to interconversions between lamellar gel (L_β) and lamellar liquid-crystalline (L_α) phases of these lipids. The thermodynamic characteristics of these phase transitions are listed in Table 1.

Our data also show that the thermotropic phase behavior of these lipid dispersions changes after prolonged periods of low-temperature incubation. With DLPS, the low-temperature incubation protocol outlined above (see Materials and Methods) results in the complete suppression of the L_β/L_α

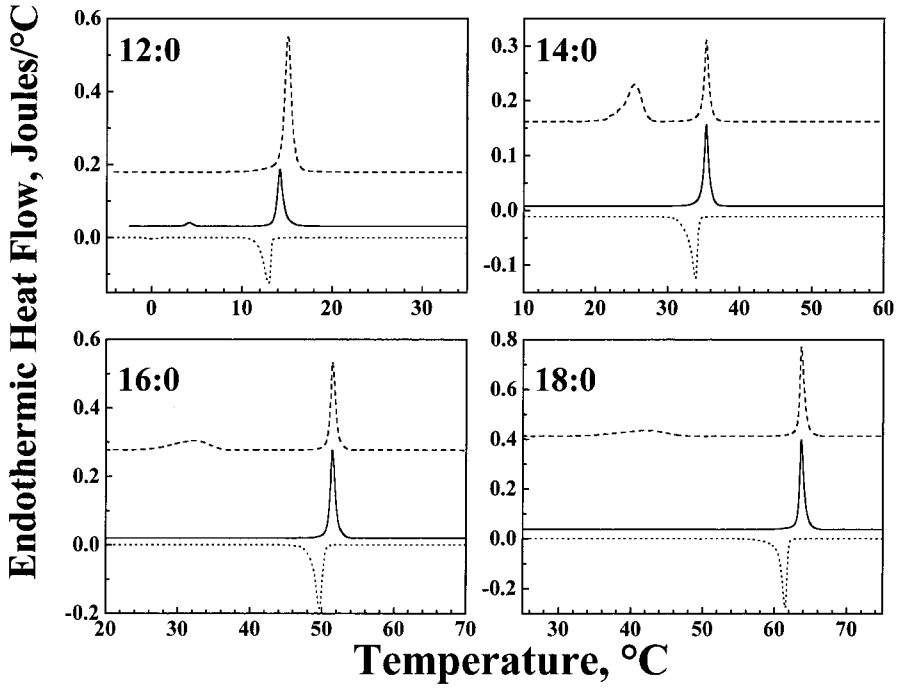

FIGURE 1 DSC heating and cooling thermograms illustrating the thermotropic phase behavior of the N-saturated 1,2-diacyl phosphatidylserines. The data shown represent heating thermograms obtained after extensive low-temperature incubation (*dashed line*), heating thermograms obtained before low-temperature incubation (*solid line*), and cooling thermograms (*dotted line*).

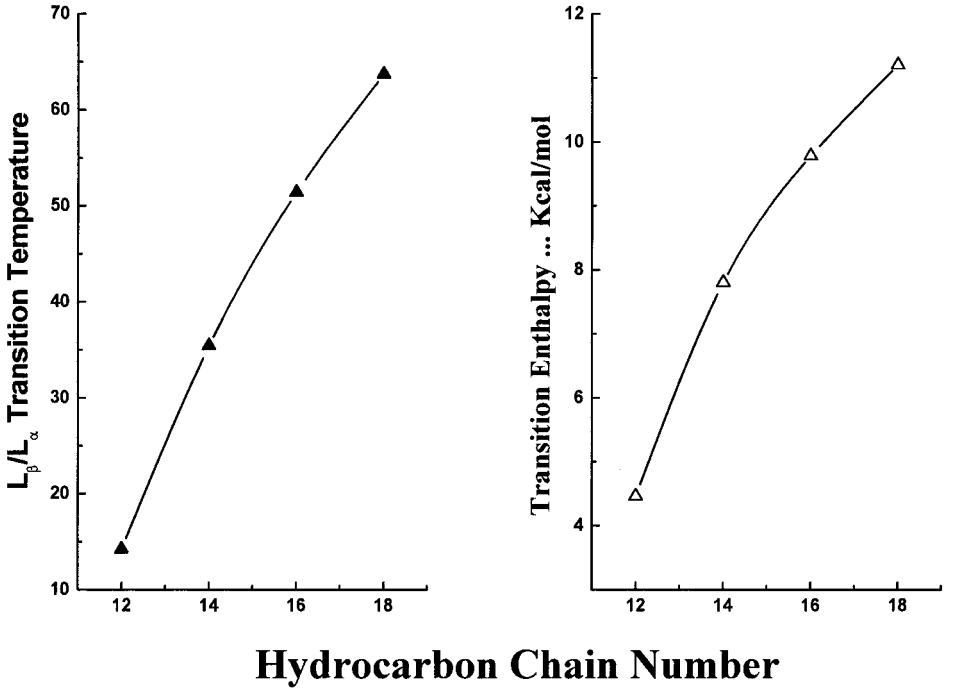

FIGURE 2 Effect of hydrocarbon chain length on the peak temperatures (*left*) and the enthalpy changes (*right*) of the phase gel/liquid-crystalline phase transitions exhibited by the N-saturated 1,2-diacyl phosphatidylserines.

phase transition near 14°C and its replacement by a slightly less cooperative but considerably more energetic phase transition centered near 15°C (for its thermodynamic characteristics, see Table 1). Our spectroscopic data indicate that the latter transition is the result of a direct conversion of the L_c phase to the L_α phase. With the other lipids, however, the same low-temperature incubation protocol results in the appearance of an additional transition at temperatures some 10–20°C below the L_β/L_α phase transition alluded to above (see Fig. 1). Our spectroscopic data indicate that this additional transition represents a conversion of the L_c phase to the L_β phase of these lipids. It is also noteworthy that the enthalpy values estimated for the L_c/L_β phase transitions of DLPS and 1,2-dimyristoyl-sn-glycero-3-phosphoryl-L-serine (DMPS; ~9–11 Kcal/mol) are significantly higher than those observed with the longer chain compounds (~6 Kcal/mol). Our FTIR spectroscopic studies suggest that this can be attributed to the formation of more ordered L_c phases by the shorter chain homologues (see below). The thermodynamic parameters associated with the conversion of the L_c phases of these lipids to either their L_α or L_β phases are also listed in Table 1.

TABLE 1 Thermodynamic characterization of the thermotropic phase transitions exhibited by the N-saturated, 1,2-diacylphosphatidylserines

Hydrocarbon chain number	Transition temperatures (°C)			Enthalpy Change (Kcal/mol)		
	L_c/L_α	L_c/L_β	L_β/L_α	L_c/L_α	L_c/L_β	L_β/L_α
12:0	15.1	n.o.	14.2	14.0	9.5*	4.5
14:0	n.o.	25.4	35.4	n.o.	11.1	7.8
16:0	n.o.	32.2	51.4	n.o.	6.4	9.8
18:0	n.o.	42.2	63.7	n.o.	6.5	11.2

n.o., not observed.
*Estimated from the difference between the transition enthalpies of the L_c/L_α and L_β/L_α phase transitions.

^{31}P-NMR spectroscopic studies

^{31}P-NMR spectra of aqueous dispersions of these PSs were acquired as a function of temperature both before and after low-temperature incubation. The spectra shown in Fig. 3 were acquired with DMPS and are typical of data acquired at temperatures bracketing the L_β/L_α phase transition of these lipids. At temperatures below the T_m, PS samples that have not been extensively incubated at low temperature exhibit ^{31}P-NMR powder patterns characterized by an upfield peak, a downfield shoulder, and basal linewidths of 90 to 100 ppm (Fig. 3, left panel). The overall shape of the powder pattern is characteristic of phospholipid bilayers in which phosphate headgroup motions are essentially symmetrical about the axis of reorientation, the bilayer normal, and the broad features indicate that the rates of axially symmetrical reorientation are slow on the ^{31}P-NMR time scale, as is typical of the L_β-like gel phases of phospholipid bilayers (Seelig, 1978; Campbell et al., 1979). Upon heating, small progressive decreases in basal linewidth are observed while the lipid is in the gel phase, changes attributable to thermally induced increases in the rates of phosphate headgroup reorientation in this phase. At the L_β/L_α phase

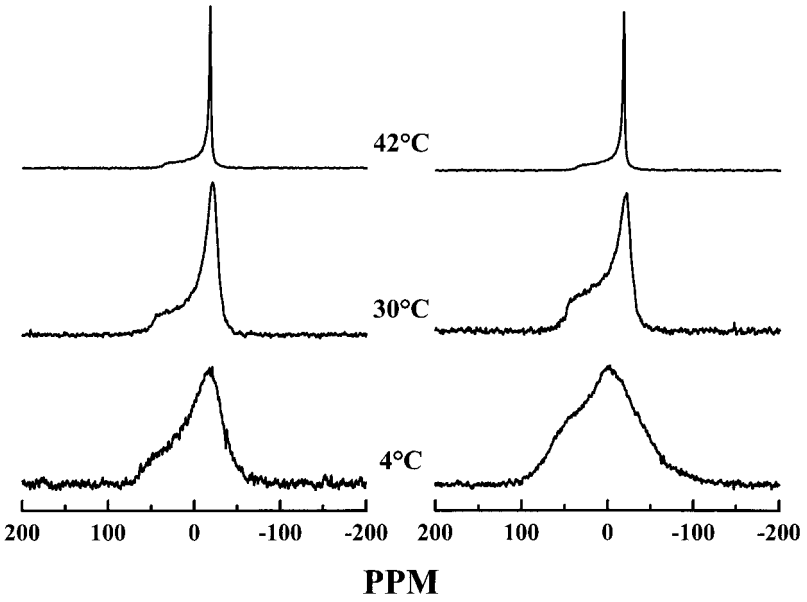

FIGURE 3 Proton-decoupled ^{31}P-NMR spectra exhibited by a sample of DMPS before (*left panel*) and after (*right panel*) application of the low-temperature incubation protocol outlined in Materials and Methods. The data shown were acquired in the heating mode at the temperatures indicated.

transition, the basal linewidth of the powder pattern decreases abruptly to values near 50 ppm (Fig. 3, left panel), and, aside from very slight narrowing of the basal linewidth, further heating does not result in significant changes in the overall features of the powder pattern observed. This observation is consistent with the formation of lamellar liquid-crystalline phospholipid bilayers in which axially symmetrical reorientational motions of the phosphate headgroup are fast on the ^{31}P-NMR timescale (Seelig, 1978; Campbell et al., 1979). These spectroscopic changes can thus be attributed to a phase state-induced increase in the rate of phosphate headgroup reorientation due to the overall decrease in conformational, packing, and rotational order of the lipid molecules that occurs when lipid hydrocarbon chains melt, indicating that the single phase transition observed before low-temperature incubation of these PS bilayers is an L_β/L_α phase transition.

As noted above, the thermotropic phase behavior of these lipids changes after the low-temperature incubation protocol is applied. In the ^{31}P-NMR spectroscopic experiment, this change is reflected by the appearance of ^{31}P-NMR powder patterns with very broad resonance envelopes (basal linewidths ~160–180 ppm; see Fig. 3, right panel). These broad powder patterns are observed at all temperatures below the onset of the L_c/L_α (DLPS) or L_c/L_β (DMPS, DPPS, DSPS) phase transitions; upon heating, they convert to the axially symmetrical powder patterns typical of the either the L_α phase (DLPS) or the L_β phase (DMPS, DPPS, DSPS). The observed low-temperature powder patterns are significantly narrower than those observed when PS bilayers interact with divalent cations and lithium (Casal et al., 1987c, 1989; Mattai et al., 1989) or those of solid phospholipids, which usually exhibit resonance envelopes with basal line widths approaching 230 ppm (Seelig, 1978; Campbell et al., 1979). However, these spectra are considerably broader than those observed in L_β phospholipid phases and contain some of the features that typify the ^{31}P-NMR spectra of solid phospholipids. These observations are consistent with the existence of a structure in which there is some motional averaging of the ^{31}P tensor elements about the axis or reorientation (the bilayer normal), albeit with rates and amplitudes that are fairly low. We therefore conclude that after low-temperature incubation, the L_β phases of these lipids convert to a structure in which the phosphate headgroup motions are slow but not completely immobilized on the ^{31}P-NMR timescale. Such behavior has been observed upon formation of the lamellar crystalline phases of various phospholipid bilayers (Lewis et al., 1984; Mantsch et al., 1985; Lewis et al., 1988; Lewis and McElhaney, 1993; Zhang et al., 1997).

FTIR spectroscopic studies: thermotropic phase behavior

Illustrated in Fig. 4 are plots of the temperature dependence of the frequencies of selected marker bands observed in various regions of the infrared spectrum of DMPS that has been subjected to the low-temperature incubation protocol described above. These data clearly show that spectroscopic markers emanating from infrared-active groups present in the hydrophobic, interfacial, and headgroup domains of the lipid bilayer are each differentially sensitive to the two thermotropic phase transitions observed by calorimetry. Thus, the spectroscopic marker examined in panel I of Fig. 4 ($\nu_s CH_2$, a hydrocarbon chain conformation marker; Lewis and McElhaney, 1996) shows a major discontinuity

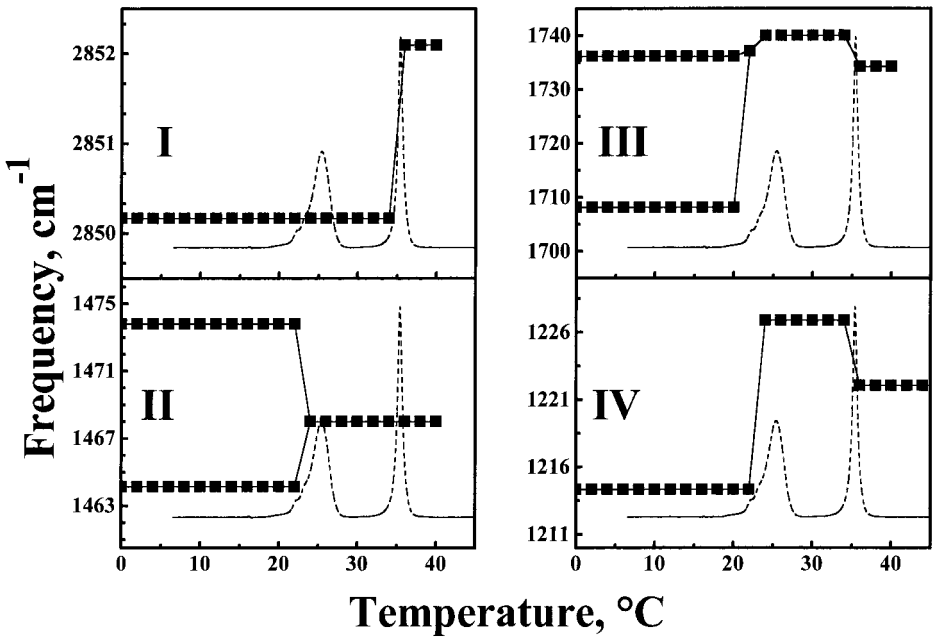

FIGURE 4 Temperature dependence of the frequencies of diagnostic marker bands in the hydrophobic (I and II), interfacial (III), and headgroup (IV) region of DMPS. The data shown were obtained upon heating a DMPS sample that was extensively incubated at low temperature. To effect a comparison between the spectroscopic data (*filled symbols*) and the calorimetric results, the DSC thermogram exhibited by the sample is shown as a dashed line superimposed over the spectroscopic data. Data are presented for (*A*) the CH_2 symmetrical stretching absorption band; (*B*) the CH_2 scissoring absorption band; (*C*) the C = O stretching absorption band; and (*D*) the O-P-O asymmetrical absorption band.

at the L_β/L_α phase transition but is relatively insensitive to the occurrence of the L_c/L_β phase transition, whereas the reverse is true of the spectroscopic marker shown in panel II of Fig. 4 ($_\chi CH_2$, a hydrocarbon chain packing marker; Lewis and McElhaney, 1996). In contrast, the spectroscopic markers emanating from both the interfacial (C = O stretching band) and the headgroup ($_\delta$O-P-O stretching band) regions of the lipid bilayer show major discontinuities at the L_c/L_β phase transition and relatively smaller discontinuities at the L_β/L_α phase transition (Fig. 4, III and IV). With DLPS, however, samples that have been incubated at low temperature exhibit a single phase transition when heated to temperatures near 15°C (Fig. 1), and plots of the temperature dependence of the spectroscopic markers used in Fig. 4 all show evidence of a single discontinuity near 15°C (data not shown). This latter result indicates that the nature and magnitude of the changes in these spectroscopic markers accompanying that single phase transition are equivalent to a summation of all of the spectroscopic changes observed at the two individual thermotropic transitions shown in Fig. 4, thus suggesting that the physical processes occurring are equivalent to the two phase transitions exhibited by the longer chain homologues.

FTIR spectroscopic studies: structural interpretation

Illustrated in Fig. 5 are infrared absorption bands exhibited by the L_c, L_β, and L_α phases of DPMS. These three polymorphic phases each exhibit a distinctive pattern of infrared absorption. A structural interpretation of these infrared spectra and temperature-induced changes therein is presented below.

The carbon-hydrogen stretching region (2800–3000 cm^{-1})

The major features of this region of the infrared spectra of these lipids are the symmetrical and asymmetrical CH_2 stretching bands near ~2850 cm^{-1} and 2920 cm^{-1}, respectively, and the methyl asymmetrical stretching band centered near 2955 cm^{-1}. The frequencies of these bands (especially the CH_2 stretching bands) are sensitive to the conformation of the hydrocarbon chains (Lewis and McElhaney, 1996). At temperatures above the calorimetrically detected main phase transition, these absorption bands are all fairly broad, and the symmetrical and asymmetrical stretching bands exhibit their maxima near 2852 and 2922 cm^{-1}, respectively, indicating that the lipid hydrocarbon chains are mobile and conformationally disordered. Fig. 5 *I* also shows that the contours of the C-H stretching absorption bands observed in the L_β and L_c phases of these lipids are narrower than the corresponding bands in the L_α phase and that the maxima of the symmetrical and asymmetrical CH_2 stretching bands decrease in frequency to 2849 cm^{-1} and 2916 cm^{-1}, respectively, values consistent with the presence of predominantly all-*trans* polymethylene hydrocarbon chains. Moreover, aside from the occurrence of slightly narrower bands in the L_c phase, the C-H stretching absorption bands exhibited by the L_β and L_c phases of these lipids are very similar, indicating that the conformations of the hydrocarbon chains are also very similar, though those

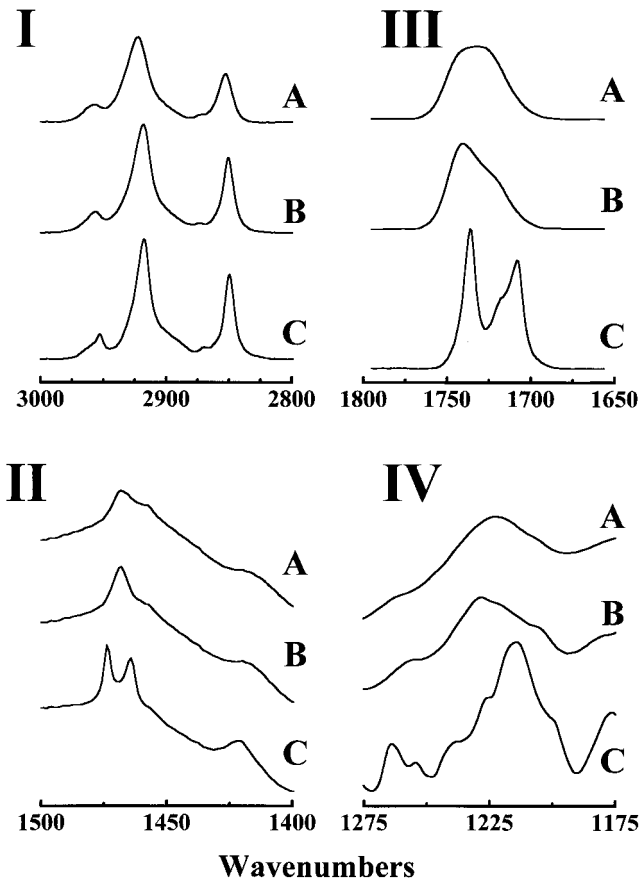

FIGURE 5 Infrared spectra showing the contours of the C-H stretching (*I*), the CH_2 bending (*II*), the C = O stretching (*III*), and O-P-O asymmetrical stretching (*IV*) regions of the infrared spectra observed upon heating a DMPS sample which was extensively incubated at low-temperature. The data shown were obtained at temperatures which bracket the calorimetrically resolved thermotropic transitions thus: (*A*) the L_α phase, 42°C; (*B*) the L_β phase, 28°C; and (*C*) the L_c phase, 0°C.

of the L_c phase are less mobile and/or undergo conformational fluctuations of lower amplitude.

The carbon-hydrogen deformation region (1400–1500 cm^{-1})

The most structurally relevant absorption bands in this region of the infrared spectra occur near 1418 cm^{-1} and 1468 cm^{-1} and arise from the scissoring (or bending) vibrations of the α-CH_2 groups of the hydrocarbon chains and from the scissoring vibrations of the remaining chain CH_2 groups, respectively. The properties of the latter are very sensitive to lateral interactions between the hydrocarbon chains (Lewis and McElhaney, 1996). As illustrated in Fig. 5 *II*, the absorption bands are broad in the L_α phase, a feature consistent with the high mobility of all vibrating groups and with the high amplitude fluctuations which are known to occur in liquid-crystalline lipid phases. These absorption bands become narrower in the L_β phase and their bandwidths decrease even further upon formation of the L_c phase, consistent with a progressive immobilization of the vibrating groups. However, the most striking and structurally relevant feature of the data shown in Fig. 5 *II* is the phase-state induced change in the properties of the main CH_2 scissoring band near 1468 cm^{-1}. At temperatures above T_m, its contours are fairly broad and centered near 1468 cm^{-1}, and upon conversion to the L_β phase, it sharpens considerably without a significant change in frequency. Upon conversion to L_c phase, however, the main CH_2 scissoring absorption band splints into two very sharp components which are centered near 1466 cm^{-1} and 1472 cm^{-1} (Fig. 5 *II*). These components remain very sharp at all temperatures at which the L_c phase is stable and are relatively insensitive to temperature change until warmed to temperatures just below the onset of its thermal decomposition. With DLPS this occurs at its L_c/L_α phase transition near 15°C, whereas with the longer chain compounds this process occurs at the L_c/L_β phase transition of the lipid concerned (for an example, see Fig. 4 II). These results indicate that in both the L_β and L_α phases, the PS hydrocarbon chains are organized in a rotationally disordered hexagonal array, in which the rates and amplitudes of hydrocarbon chain reorientational fluctuations in the L_α phase are fairly high on the bond vibrational time scale but are reduced in the L_β phase. However, this picture of the L_β phase is only applicable at temperatures relatively close to T_m, as illustrated by the behavior of DSPC at temperatures well below T_m (Fig. 6). Upon cooling, the single CH_2

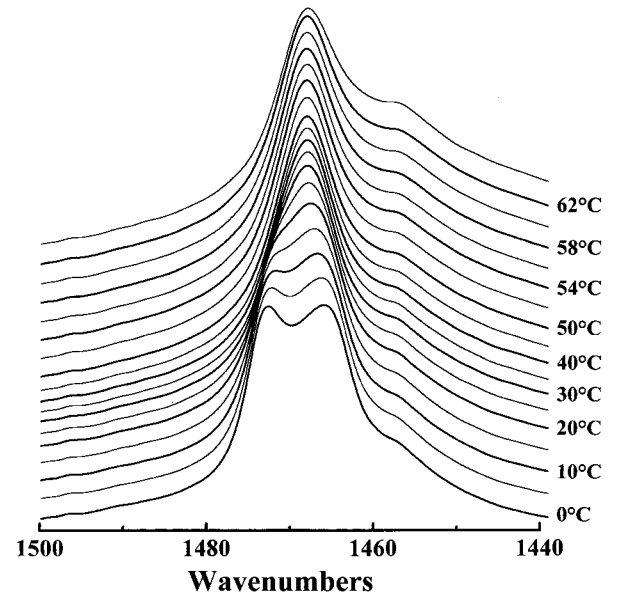

FIGURE 6 Stacked plot showing the temperature-dependent changes in the contours of the main CH_2 scissoring band of DSPS observed upon cooling its L_β phase. The absorbance spectra were acquired at the temperatures indicated.

scissoring band initially observed at frequencies near 1468 cm^{-1} begins to split into two components which are centered near 1470–2 cm^{-1} and 1466–68 cm^{-1}, and upon further cooling a progressive decrease in the widths of these components and an increase in the separation of their maxima occur. Moreover, as long as DSPS is not incubated for long periods of time at low temperature, these spectroscopic changes are fully reversible when the samples are rewarmed to temperatures just below T_m. However, unlike the situation in the L_c phase, the appearance of this splitting is not the result of the formation of a new lipid phase, it is not correlated with any resolvable thermotropic event, and its appearance is not correlated with any other spectroscopic indication of a lipid phase change. This band splitting (also known as crystal field splitting, correlation-field splitting, or factor-group splitting) is generally observed with crystalline or quasi-crystalline under conditions where their all-*trans* polymethylene chains pack into subcells with their zigzag planes perpendicular to each other (Lewis and McElhaney, 1996). This spectroscopic phenomenon is usually associated with the assembly of the hydrocarbon chains into orthorhombic $\perp$ subcells (Snyder, 1961, 1967, 1979) though, in principle, it should also be observed upon formation of orthorhombic $\perp'$ subcells. Nevertheless, because this form of band splitting has an absolute requirement for perpendicular packing of all-*trans* polymethylene chains, its appearance can also be taken as evidence for the formation of tightly packed hydrocarbon chain domains in which rotational motions and/or high-amplitude reorientational fluctuations are severely damped.

In the case of the L_c phases of these lipids, the basis of the observed band splitting is also the formation of hydrocarbon subcells (most probably orthorhombic $\perp$ or orthorhombic $\perp'$ subcells) in which the zigzag planes of the lipid molecules are perpendicular to each other. Unlike the situation observed upon cooling the L_β phase, however, the emergence of this splitting is attributable to the formation of a structurally different lipid phase and, as illustrated in Fig. 4 (panel II), the collapse of the splitting on heating coincides with a bona fide thermotropic event. Also, the fact that the two components of the CH_2 scissoring band are very sharp and remain relatively temperature-insensitive below the onset of the L_c/L_β (or L_c/L_α) phase transition also suggests that the hydrocarbon chains are organized in a highly ordered array within which thermally induced reorientational fluctuations are of very low amplitude. This situation differs markedly from what is observed in the cooled L_β phase, which exhibits marked thermally induced changes in the bandwidth and splitting in lieu of a thermotropic phase change (see above).

The carbonyl stretching region (1600–1800 cm^{-1})

The carbonyl stretching region of the infrared spectra of these lipids contains two major bands centered near 1620 cm^{-1} and 1730 cm^{-1}, which are attributable to the stretching vibrations of the headgroup carboxylate and the hydrocarbon chain ester carbonyl groups, respectively. The median frequency of the carboxylate absorption band occurs near 1623 cm^{-1} in both the L_α and L_β phases but, upon formation of the L_c phase, decreases to 1617 cm^{-1} (data not shown). From this we can conclude that the local environments of the headgroup carboxylate groups in the L_α and L_β phases are very similar. The downward shift in the headgroup carboxylate frequency observed upon formation of the L_c phase suggests that the formation of this phase is accompanied by a small increase in the degree of ionization of the carboxylate group (Holloway and Mantsch, 1988; Dunach et al., 1989; Wright et al., 1997). Most probably this is the result of a subtle change in the local environment of the headgroup carboxylate moieties, possibly because of phase state-induced changes in headgroup conformation and/or orientation. Aside from the above, the properties of this headgroup carboxylate band are essentially insensitive to the thermotropic phase changes observed in these studies and will not be discussed further.

The data presented in Figs. 4 and 5 show that the thermotropic phase changes exhibited by these phospholipids are accompanied by very striking changes in the contours of the ester C = O stretching band. The properties of this absorption band are sensitive to the conformation, hydration state, and the degree and nature of hydrogen-bonding interactions in the polar/apolar interfaces of glycerolipid bilayers (Lewis and McElhaney, 1996). In the L_α phase, the C = O stretching absorption band is very broad and centered near 1733 cm^{-1} (spectrum *A* in Fig. 5 *III*). As noted above, the broad features of the absorption band are consistent with the high mobility of the vibrating groups and with the high amplitude fluctuations that are known to occur in liquid-crystalline lipid phases. An analysis of the substructure of this absorption band suggests that it consists of a relatively small higher frequency component centered near 1743 cm^{-1} and considerably larger lower frequency component centered near 1728 cm^{-1} (see Fig. 7 *A*). Currently available data indicate that in fully hydrated glycerolipid bilayers, these components are attributable to subpopulations of free and hydrogen-bonded ester carbonyl groups, respectively (Blume et al., 1988; Lewis et al., 1994), suggesting that hydrogen-bonded ester carbonyl groups are the predominant ester carbonyl species in the polar/apolar interfacial regions of liquid-crystalline PS bilayers. This observation markedly differentiates the L_α phases of these PS bilayers form those of most of other 1,2-diacyl glycerolipids studied so far, where a much larger proportion of non-hydrogen-bonded ester carbonyl groups are present (Blume et al., 1988; Lewis and McElhaney, 1990, 1993; Lewis et al., 1994, 1996; Zhang et al., 1997).

In the L_β phase, the ester C = O stretching band is slightly narrower and appears to be a composite of a relatively sharp higher frequency peak and a broader lower

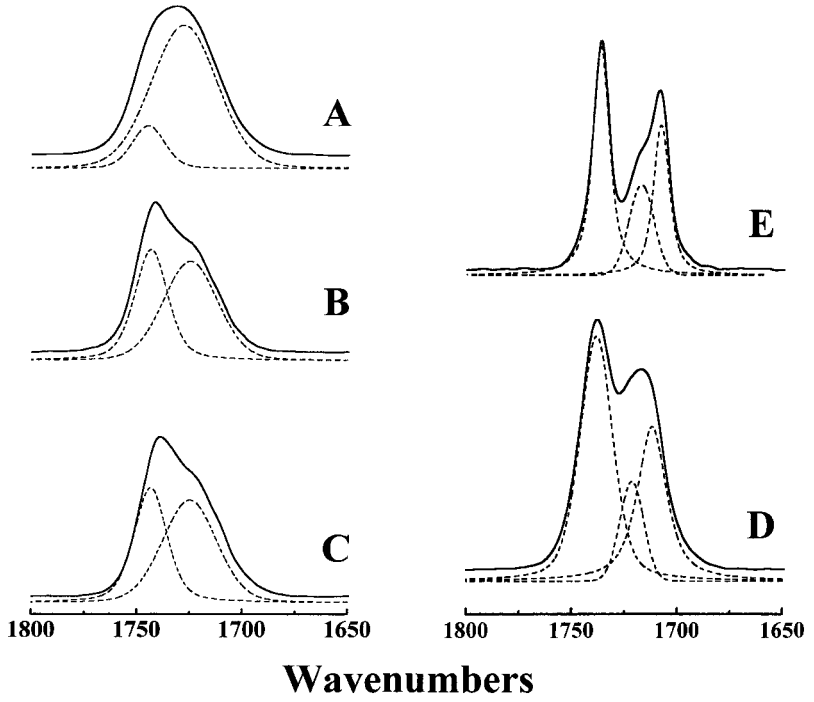

FIGURE 7 The contours of the C = O stretching absorption bands exhibited by the polymorphic phases exhibited by the n-saturated 1,2-diacyl phosphatidylserines. The data shown are representative of spectra exhibited by following: (*A*) the L_α phase (all lipids) $T > T_m$; (*B*) the L_β phase (all lipids) $T \cong T_m$-10°C; (*C*) the L_β phase (all lipids) $T \cong T_m$-50°C; (*D*) the L_c phase (DPPS and DSPS) T = 0°C; and (*E*) the L_c phase (DLPS and DMPS) T = 0°C. The absorbance spectra as presented with the solid lines representing the contours of the spectra actually observed and the dashed lines representing our estimates of the contours of underlying components.

temperature shoulder (Fig. 5 *III*). Our analyses of the substructure of this band indicates that, as observed with the L_α phase, this band is also a composite of a sharp higher frequency component near 1743 cm^{-1} and a broad lower frequency component centered near 1728 cm^{-1}. In this phase, however, the higher-frequency component makes a considerably greater contribution (~42%) to the overall integrated intensity of the absorption band than in the L_α phase (~13%). The observed decrease in the relative size of the hydrogen-bonded population upon formation of the L_β phase could be the result of a phase state-induced decrease in the hydration of the bilayer polar/apolar interface (see Discussion). We also note that the relative sizes of the free and hydrogen-bonded ester carbonyl populations present in the L_β phase do not change appreciably when samples are cooled to temperatures well below T_m (Fig. 7, *B* and *C*).

The contours of the ester carbonyl stretching bands observed in the L_c phase differ markedly from those observed in the L_α and L_β phases in ways which are structurally significant. First, two clearly resolved ester C = O band maxima are observed in the L_c phase (Fig. 5 *III-C* and Fig. 7, *D* and *E*). This observation can be attributed to the fact that the bandwidths of all the component bands are considerably smaller than observed in the L_α and L_β phases. The narrower bandwidths are consistent with the formation of a highly ordered structure in which the mobility of the various populations of ester carbonyl groups has been drastically reduced. Second, the contours of the observed band envelope varies with the length of the lipid hydrocarbon chain, suggesting that the shorter- and longer-chain compounds may be forming structurally different types of L_c phases. With the shorter chain compounds (DLPS and DMPS), the ester C = O absorption band envelope contains two resolved peak maxima centered near 1735 cm^{-1} and 1708 cm^{-1}, with a shoulder near 1717 cm^{-1} on the higher-frequency wing of the 1708 cm^{-1} band (Fig. 5 *III-C* and Fig. 7 *E*). Our analyses indicate that the band envelope is probably a summation of two very sharp bands centered near 1735 cm^{-1} and 1708 cm^{-1} and a broader band centered near 1717 cm^{-1}. Also, the integrated intensity of higher frequency component near 1735 cm^{-1} is comparable to the combined integrated intensities of the two lower frequency bands. We therefore conclude that three spectroscopically distinct populations of ester carbonyl groups are present. One of these populations, the 1735 cm^{-1} population, probably resides in a less polar environment than the others (note its higher frequency) and seems to be highly ordered (note its narrow bandwidth). The other two populations reside in more polar environments (note their lower frequencies) and may well be hydrogen-bonded to moieties resident in the polar regions of the lipid bilayer (see Discussion).

With the L_c phases of both 1,2-dipalmitoyl-sn-glycero-3-phosphoryl-L-serine (DPPS) and DSPS, the ester C = O band envelope contains two clearly resolved bands with maxima at frequencies near 1735 cm^{-1} and 1717 cm^{-1} (see Fig. 7 *D*). These bands are not as well resolved as those observed with the shorter chain compounds, probably because the underlying bands are broader (see below). Upon further analysis, these ester C = O band envelopes also appear to be summations of three underlying components though, in this case, they seem to be centered near 1735

cm^{-1}, 1721 cm^{-1}, and 1710 cm^{-1} (see Fig. 7 *E*). These bands are somewhat broader than those observed with the L_c phases of the shorter chain compounds, but, as observed with the shorter chain compounds, the integrated intensity of the higher frequency component centered near 1735 cm^{-1} is comparable to the combined integrated intensities of the two lower frequency bands. These observations indicate that aside from small differences in bandwidth and frequency, the underlying component structures are the ester C = O bands observed in the L_c phases of the shorter and longer chain PSs are similar. However, the lower bandwidths and lower frequencies observed with the shorter chain compounds suggest that their ester carbonyl groups are more ordered and that they may reside in more polar environments and/or may be more strongly hydrogen-bonded.

The O-P-O asymmetrical stretching region (1200–1300 cm^{-1})

The dominant absorption band in this region of the infrared spectrum is the O-P-O asymmetrical stretching band which, in hydrated phospholipid bilayers, usually exhibits is absorption maximum near 1230 cm^{-1}. In the L_α phase, these PS bilayers exhibit a broad and seemingly featureless O-P-O absorption band centered near 1222 cm^{-1} (Fig. 5 *IV-A*). In the absence of water, potential sources of hydrogen-bonding donor groups, and polar solvents, the O-P-O asymmetrical stretching band of phosphodiester moieties is usually observed at frequencies near 1260 cm^{-1} (Wong and Mantsch, 1988), and this frequency tends to decrease with increases in the overall polarity of the local environment, with increases in the degree of phosphate headgroup hydration, or upon its interaction with hydrogen-bonding donor groups (Lewis and McElhaney, 1996). Thus, the relatively low frequency of the O-P-O asymmetrical stretching band observed in the L_α phase suggests that the phosphate headgroups are located in a fairly polar environment, are well hydrated, and/or undergo hydrogen-bonding interactions with one or more donor groups. Also, the broad contours of this band suggests a mobile polar headgroup, a feature expected in the L_α phase. In the L_β gel phase, the O-P-O asymmetrical band becomes slightly narrower, its maximum shifts upward to frequencies near 1227 cm^{-1} (Fig. 5 *IV-B*) and, aside from further narrowing, the contours remain essentially unchanged upon further cooling to temperatures well below the T_m. We conclude, therefore, that the conversion to the L_β phase is accompanied by a decrease in the overall rates and amplitudes of phosphate headgroup motions and by a slight decrease in the overall polarity of the local environment of the phosphate headgroup. The latter could be indicative of a slight decrease in phosphate headgroup hydration at the L_α/L_β phase transition. Finally, with the formation of the L_c phases, a significant narrowing of the O-P-O asymmetrical stretching band occurs and its maximum shifts downward to frequencies near 1215 cm^{-1} (Fig. 5 *IV-C*). This change is also accompanied by the appearance of a several weak bands superimposed over the underlying O-P-O asymmetrical stretching band (Fig. 5 *IV-C*). These additional weaker bands are a manifestation of the CH_2 wagging band progressions, a common feature of the infrared spectra of compounds containing long, all-*trans* polymethylene chains (Lewis and McElhaney (1996) and references cited therein). We also find that apart from slight differences in band width and in the position of the individual peaks of the CH_2 waging band progression, the O-P-O asymmetrical stretching regions observed in the infrared spectra of the L_c phases of all of the compounds studied are essentially similar. We therefore conclude that the formation of the L_c phases of all of these PSs is accompanied by a drastic decline in the rates and amplitudes of the motions phosphate headgroups and, possibly, the formation of fairly strong hydrogen bonds between the phosphate group and a hydrogen-bond donor.

DISCUSSION

Several structurally interesting aspects of the phase behavior and organization of PS bilayers have emerged from these studies. First, it is clear that before low-temperature incubation, the thermotropic phase behavior of these PS bilayers under physiologically relevant conditions of pH and ionic strength, and in the absence of divalent cations, is relatively simple. As is typical of most N-saturated 1,2-diacyl glycerolipids, these lipids exhibit highly cooperative phase transitions between a L_β phase and the L_α phase, and the transition temperatures and transition enthalpy values reported here are all compatible with those of previously reported work using a similar salt form of the lipid and similar conditions (for examples, see Caffrey, 1993). As noted previously (Marsh, 1990), these transitions occur at significantly higher temperatures than do those of corresponding PC and PG bilayers, and at lower temperatures than occurs with the corresponding PE bilayers. Such observations have been rationalized by considerations of the relative capacities of the given phospholipid headgroups to engage in intermolecular electrostatic and hydrogen-bonding interactions (Boggs, 1987; Cevc and Marsh, 1987). Interestingly, however, the observed L_β/L_α transition enthalpy values are 1 to 2 Kcal/mol higher than those observed corresponding PC, PG, and PE bilayers (Lewis et al., 1987; Zhang et al., 1997; Lewis and McElhaney, 1993). Although the physical basis of this observation is unclear, our FTIR spectroscopic studies suggest that the polar/apolar interfaces of liquid-crystalline PS bilayers may be better hydrated than those of the other phospholipids and that the change in interfacial hydration occurring at the L_β/L_α phase transitions of these PS bilayers may be significantly greater than with other phospholipids. We suggest, therefore, that the higher enthalpy values observed at the L_β/L_α phase transi-

tion of PS bilayers may be at least partly attributable to greater differences in the phase state-induced changes in the hydration of and/or hydrogen-bonding to moieties in their polar/apolar interfacial regions. The idea that anionic PS bilayers may possess hydration characteristics that differ significantly from those of other phospholipid bilayers is particularly interesting, because it suggests that they may have the capacity to impart to lipid bilayers other characteristics that are unrelated to its negative charge per se. Similar behavior has been noted in previous studies of anionic phosphatidylglycerol bilayers (Zhang et al., 1997).

Second, we demonstrate here for the first time that fully hydrated PS model membranes are capable of forming highly ordered L_c phases in the absence of divalent and/or other exotic cations. The capacity to form such structures seems to be an intrinsic property of most of the naturally-occurring classes of 1,2-diacyl phospho- and glycolipids that have been examined so far (Lewis and McElhaney, 1990, 1993; Mannock et al., 1988, 1990; Zhang et al., 1997, and references cited therein). Moreover, the formation of the L_c phases of these compounds is a relatively slow process that gets progressively slower as lipid hydrocarbon chain length increases. Comparable patterns of kinetic behavior have been observed during the formation of the L_c phases of other 1,2-diacylglycerolipid bilayers. Our FTIR spectroscopic data also suggest that the L_c phases formed by the longer chain homologues may be less ordered than those formed by their shorter chain counterparts, a suggestion compatible with the results of our calorimetric measurements, which show that the enthalpy changes observed at the L_c/L_β phase transitions of the longer chain compounds are 3–5 Kcal/mol smaller than observed with the shorter chain compounds. Such structural differences could be a manifestation of a chain length-dependent change in the balance between the hydrophobic and hydrophilic contributions to the overall free energy of the lipid assembly (Lewis and McElhaney, 1990). Interestingly, however, our results also show that aside from the differences in the contours of the ester C = O stretching band, the spectroscopic features of the L_c phases formed by shorter-chain PSs are otherwise indistinguishable from those exhibited by their longer-chain counterparts. Given this observation, it seems unlikely that the spectroscopic differences noted above can be attributed to major structural differences between the L_c phases of the shorter and longer chain compounds.

Our spectroscopic studies, in combination with previously published work, provide a fairly detailed structural picture of the lamellar phases which these lipids can form under physiologically relevant conditions. The data support the existence of an L_α phase in which there is a significant content of gauche conformers in the hydrocarbon chains and a relatively high degree of conformational and orientational disorder in the lipid assembly as a whole, features which are typical of the liquid-crystalline phases of all glycerolipid bilayers studied so far. However, our studies also suggest the relative size of the population(s) of hydrated and/or hydrogen-bonded ester carbonyl groups in the polar/apolar interfaces of the L_α phases of these PS bilayers is considerably higher than normally observed with other 1,2-diacyl phospho- and glycoglycerolipid bilayers (Blume et al., 1988; Lewis and McElhaney, 1990, 1993; Lewis et al., 1994, 1996; Zhang et al., 1997). It is not clear why this aspect of the organization of PS bilayers should be so different from the other glycerolipid bilayers that have been studied. However, unlike these other glycerolipids, the PS headgroup contains three charged moieties that are potentially available for direct and/or indirect interaction with moieties in the bilayer polar/apolar interface. Moreover, because of the proximity of these charged groups and their associated hydration shells, the interfacial regions of PS bilayers would probably be more polar than those most other glycerolipid bilayers and, as a result, there would be a greater tendency for water to penetrate into the bilayer polar/apolar interface. Such suggestions can provide a feasible rationale for our experimental observations and have been implied in previous studies showing that the orientation of the PS headgroup at the bilayer surface is different from that of other common phospholipids (Browning, 1981; Seelig et al., 1987; Scherer and Seelig, 1989).

Our spectroscopic studies have also enabled the construction of a low resolution structural picture of the L_c phases of these lipids. Our results suggest that both the shorter and longer chain analogues form L_c phases in which hydrocarbon chains form a compact array of orthorhombic $\perp$ subcells with strong lateral interactions between the hydrocarbon chains. In the polar/apolar interfacial region, there is evidence for the presence of three vibrationally inequivalent populations of ester carbonyl groups, one of which is relatively immobilized and is not involved in hydrogen-bonding interactions. The other two populations are probably hydrogen-bonded to donor groups, which may be located on the polar headgroup or in the solvent. Our data also indicate that increases in hydrocarbon chain length are associated with an increase in the mobility of these hydrogen-bonded ester carbonyl groups as well as a decrease in the strength of their interactions with the hydrogen-bond donors. Finally, the data indicate that the rates and amplitudes of the reorientation of the phosphate headgroups are fairly slow and suggest that the phosphate moiety may be strongly hydrogen-bonded to a donor group. Although the structural picture outlined above is not sufficiently detailed to enable one to identify the hydrogen bond donor groups involved, the predominant sources of hydrogen bond donors present under our experimental conditions are probably water and the α-amino groups of the phosphoserine moiety. Given this, and our evidence that there are two vibrationally inequivalent populations of hydrogen-bonded ester carbonyl groups in the L_c phases of these lipids, these populations may be attributable to hydrogen-bonding interactions between the ester carbonyl groups and either water or the polar head-

group. However, we caution that our data do not enable any assignment of the possible location of these carbonyl groups (i.e., whether on the *sn*1 or *sn*2 fatty acyl chain) and that our observations could also be attributed to inequivalent conformations of the two populations within the unit cell and, possibly, to differences in the hydration of microcrystalline domains of these lipids. The resolution of this issue and others will require more detailed x-ray diffraction and spectroscopic studies using uniaxially oriented preparations and, possibly, specifically isotopically labeled materials.

Finally, we note that the structural picture that emerges from these studies differs significantly from that provided by previous studies of the crystal-like polymorphs formed in the presence of lithium ions and various divalent cations. Previous studies of such cation-induced crystalline phases indicate that there is considerable heterogeneity in the organization of the hydrophobic and interfacial domains of the bilayer structures formed (Casal et al., 1987a,b,c; Hubner et al., 1994) and suggest that the phosphate headgroups are essentially immobilized on the ^{31}P-NMR timescale, presumably because they are substantially dehydrated (Casal et al., 1987c, 1989; Mattai et al., 1989). The properties of these cation-induced L_c phases are almost certainly attributable to the binding of the lithium and divalent metal ions to the phosphate moieties of the lipid and suggest that the primary driving force favoring the formation of such crystalline phases is probably the low aqueous solubility of the phosphate salts of lithium and the divalent metals involved. We conclude, therefore, that these cation-induced crystalline structures are structurally distinct from the lamellar phases formed under our conditions. Thus, although the structures of these cation-induced crystalline phases may provide insight into the capacity of PS bilayers to bind such cations, they may not reflect the intermolecular interactions which occur at the surfaces of PS bilayers in the absence of such ions.

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