Phase Behavior and Physical-Chemical Properties of N-Methylated Phosphatidylserine[†,1]

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Abstract: The phase behavior of the *N*-methyl-substituted 1,2-dipalmitoyl-*sn*-glycero-3-phospho-L-serine (*N*-methyl-DPPS) has been studied by surface chemical methods, differential scanning calorimetry, X-ray diffraction, and infrared and ^{31}P NMR spectroscopy. The gel-to-liquid crystal transition temperature of *N*-methyl-DPPS is at 48 °C; compared to the parent compound, 1,2-dipalmitoyl-*sn*-glycero-3-phospho-L-serine (DPPS), this transition temperature is reduced by 8 °C. The main transition is preceded by a pretransition at 39–43 °C the enthalpy of which varies with thermal history. The pretransition is assigned to a change in the hydrocarbon chain packing mode; the hydrocarbon chain lattice changes from a highly ordered, probably orthorhombic packing mode to a hexagonal lattice. Cooling of liquid-crystalline *N*-methyl-DPPS dispersions below the pretransition temperature produces a lamellar gel phase with hexagonally packed hydrocarbon chains. This gel is metastable and converts with time into the most stable gel. In the resulting minimum free energy phase the *N*-methyl-DPPS bilayer crystallizes, forming a tightly packed, highly ordered hydrocarbon chain lattice. The polar group is also tightly packed and practically immobilized although it remains hydrated. Parent DPPS and other phosphatidylserines with saturated hydrocarbon chains do not form such a tightly packed gel phase. The tight packing of *N*-methyl-DPPS in the most stable gel phase is surprising considering that the molecular area of 42 Å^2 per molecule is larger than that of DPPS (40.5 Å^2 per molecule). The phase behavior and some physical chemical properties of *N*-methyl-DPPS are discussed in comparison with those of the parent compound DPPS.

Membrane lipids self-assemble into supramolecular structures, the bimolecular lipid bilayer being the most widely studied. The interplay of three interactions (hydrogen bonding, hydrophobic attraction, and electrostatics) is responsible for the structures adopted by lipids. In all cases, the properties of these structures depend upon "packing", which in turn depends upon molecular structure.[6,7] It is now well established that the length and degree of unsaturation of the hydrocarbon chains are crucial parameters determining the phase behavior of lipids. These parameters, among others, determine the molecular packing in lipid aggregates, i.e., the area occupied by one lipid molecule. A simple method of determining molecular areas is the monolayer technique: the lipid is spread at the air–water interface and the surface pressure–area curve is measured. With this technique it can be shown that introduction of a single cis double bond in the *sn*-2 fatty acyl chain of synthetic diacylphosphatidylserines produces a 44% increase in molecular area: at 22 °C and at a surface pressure of 30 mN m^{-1} the area per molecule is 44.2 Å^2 for 1,2-dipalmitoyl-*sn*-glycero-3-phospho-L-serine (DPPS); under identical conditions the area per molecule is 63.7 Å^2 for the monounsaturated 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine.[8] The presence of substituents in the hydrocarbon chains of diacylphosphatidylcholines also produces increases in the molecular area; systematic studies[9,10] reveal that the substituent's size as well as its location on the chains play an important role in determining molecular areas.

In a series of recent publications[11-13] it was shown that the interaction of phosphatidylserine bilayers with metal ions depends sensitively on the molecular packing expressed as area per molecule, which is inversely proportional to the surface charge density. The molecular packing apparently determines the intermolecular distances between ligand groups involved in the complexation of metal ions such as the alkaline-earth metal ions.

We report here the synthesis of 1,2-dipalmitoyl-*sn*-glycero-3-phospho-*N*-methyl-L-serine (*N*-methyl-DPPS) and the results of a detailed investigation of the physical–chemical properties of its aqueous dispersions. The effect on molecular area of introducing a single methyl group into the lipid polar group is expected to be negligible or at least small compared to that of introducing a double bond or substituents into the hydrocarbon chains. Spectroscopic methods (infrared and ^{31}P NMR) are used to obtain structural and dynamical information. X-ray diffraction measurements reveal that the bilayer is the structure adopted, while pressure–area isotherms of monomolecular films are used to determine the area of *N*-methyl-DPPS. The thermal phase behavior is studied by differential scanning calorimetry. In all cases, the experimental results obtained are compared with those obtained with DPPS, i.e., the nonmethylated parent compound. We find that both the physical–chemical properties and the thermal phase behavior of *N*-methyl-DPPS differ from those of DPPS to a surprisingly large extent. That such a small chemical modification as introducing a methyl group is responsible for so pronounced physical–chemical changes is a clear manifestation of the delicate balance between the forces responsible for the bilayer structure. Methylation of the serine amino group has primarily a steric effect. The methyl residue interferes sterically with the approach of the amino group to adjacent head groups, thereby affecting electrostatic interactions between charged polar groups. Hydrogen-bonding interactions are also affected since the methyl residue

[†] List of abbreviations. *N*-methyl-DPPS, 1,2-dipalmitoyl-*sn*-glycero-3-phospho-*N*-methyl-L-serine; DPPS, 1,2-dipalmitoyl-*sn*-glycero-3-phospho-L-serine; TLC, thin-layer chromatography; EDTA, ethylenediaminetetraacetic acid; His, histidine; Tris, tris(hydroxymethyl)aminomethane; Tes, 2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethanesulfonic acid; DSC, differential scanning calorimetry.

(1) Issued as NRCC publication No. 30774.
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0002-7863/90/1512-3887$02.50/0

Table I. Chemical Shifts of ^1H NMR Spectra of the NH_4^+ Salt of 1,2-Dipalmitoyl-*sn*-glycero-3-phospho-*N*-methyl-L-serine (*N*-Methyl-DPPS)

signal	chemical shifts,[a] ppm	
	C^2HCl_3 soln	dispersn in ^2H_2O[b]
terminal CH_3	0.88	0.87
$(CH_2)_n$ hydrocarbon chains	1.26	1.28
CH_2-C-CO	1.58	1.59
	1.60	
CH_2-CO	2.29	2.32
	2.31	2.38
N-CH_3	2.72	2.77
CH-N	3.70	3.90
CH_2-P (glycerol)	3.95	4.02
CH_2-O-CO	4.15	4.25
	4.38	4.46
$POCH_2$ (serine)	4.24	4.33
CH-O-CO	5.22	5.31

[a] Chemical shifts are expressed in ppm relative to tetramethylsilane and sodium 3-(trimethylsilyl)propanesulfonate used as internal references in C^2HCl_3 and ^2H_2O, respectively. Chemical shifts are accurate to ±0.01 ppm. Two chemical shift values are presented for CH_2 groups whose protons are chemically inequivalent. [b] Dispersions of *N*-methyl-DPPS in ^2H_2O were prepared as described in the Experimental Section.

replaces a hydrogen-bonding proton. The major effect found is in the packing of the fatty acyl chains. The fatty acyl chains of *N*-methyl-DPPS adopt tight packing with short interchain distances.

Experimental Section

Synthesis and Sample Preparation. 1,2-Dimyristoyl-*sn*-glycero-3-phospho-L-serine and 1,2-dipalmitoyl-*sn*-glycero-3-phospho-L-serine were synthesized as described before.[14] 1,2-Dipalmitoyl-*sn*-glycero-3-phospho-*N*-monomethyl-L-serine was prepared in a six-step synthesis essentially as reported previously.[14] In the early stages of this work, *N*-methyl-L-serine was synthesized according to the method of Quitt et al.;[15] later on, it was purchased from Serva (Heidelberg, FRG). In a two-step synthesis the protected serine derivative *N*-(*tert*-butoxycarbonyl)-L-serine benzhydryl ester was prepared as described previously.[14] 1,2-Dipalmitoyl-*sn*-phosphatidic acid and the racemic analogue were synthesized from the corresponding 1,2-dipalmitoylphosphatidylcholines by the transphosphatidylation reaction in the presence of phospholipase D according to Comfurius and Zwaal.[16] The pyridinium salt of 1,2-dipalmitoylphosphatidic acid and the protected *N*-methyl-L-serine were both dried thoroughly with P_2O_5 under high vacuum overnight, and solutions of the lipid and the protected *N*-methyl-L-serine in anhydrous pyridine were incubated in the presence of 2,4,6-triisopropylbenzenesulfonyl chloride, which catalyses the condensation to 1,2-dipalmitoyl-*sn*-glycero-3-phospho-*N*-*tert*-butoxycarbonyl-L-serine benzhydryl ester.[14] The removal of the protective groups on the *N*-methyl-L-serine residue was accomplished with dry HCl in $CHCl_3$ at 0 °C as described before.[14] The yield was 60%.

The acid form of phosphatidylserine was converted into the ammonium salt as described before,[17] or alternatively, the conversion of the acid form into the NH_4 salt and vice versa was carried out according to Bligh and Dyer.[18] Phosphatidylserines were purified by column chromatography on silica gel or by medium-pressure liquid chromatography.[14] Phosphatidylserines were pure by TLC, ^1H NMR spectroscopy, and elemental (C, H, N, P) analysis. The elemental analysis of the NH_4^+ salt of *N*-methyl-DPPS was consistent within experimental error with the formula $C_{39}H_{79}O_{10}N_2P$ (M_r = 767). Solutions of *N*-methyl-DPPS in C^2HCl_3 give well-resolved, high-resolution ^1H NMR spectra at 360 MHz (not shown). The assignment of the resonances carried out by using standard methods is summarized in Table I, together with the observed chemical shifts. The *N*-methyl signal appears as a singlet at 2.72 ppm. *N*-methyl-DPPS is readily dispersed in H_2O at temperatures slightly above 50 °C. The ^1H NMR spectrum of unsonicated dispersions in ^2H_2O consists of a broad signal with some relatively sharp lines superimposed (not shown). The clearly discernible signals of these lines can be assigned to the terminal CH_3 group (0.85 ppm), the $(CH_2)_n$ group of the hydrocarbon chains (1.26 ppm), and the $N(CH_3)$ group (2.72 ppm). Sonicated dispersions in ^2H_2O give relatively good high-resolution ^1H NMR spectra. The chemical shifts of these spectra are included in Table I. Line width and line shape are consistent with the presence of small unilamellar vesicles. Gel filtration on Sepharose 4B supports this interpretation. The Stokes radius of the particles present in sonicated dispersions was determined by gel filtration on a Sepharose 4B column calibrated with various markers[19] (dimension, 33 cm × 2.5 cm; elution buffer, 0.01 M Tris, pH 8.6, 1 mM EDTA, 0.02% NaN_3). The value of the Stokes radius thus obtained is ~150 Å. Gel filtration also shows that $^{22}NaCl$ present in the buffer is entrapped, indicating that these particles are vesicles surrounded by a single ion-impermeable phospholipid bilayer.

Methods. Monomolecular Films. Surface pressure–area isotherms were measured at the air–water interface at 21 °C by use of a Teflon trough (32.2 cm × 17.2 cm), which was placed within a thermostated box. The NH_4^+ salt of DPPS or the *N*-methyl analogue (50 nmol) was dissolved in $CHCl_3/CH_3OH$ (4:1 v/v) and carefully spread at the air–water interface from an Agla microsyringe. The surface tension was measured with a recording Cahn 2000 electrobalance; surface pressure–area curves were recorded at a compression rate of 0.258 $nm^2\ mol^{-1}\ min^{-1}$. The reproducibility was better than 1 $Å^2$ per molecule. Monolayers were spread on the following buffers: 0.066 M phosphate buffer (Na_2HPO_4, KH_2PO_4), pH 7.4, and 0.2 M sodium acetate buffer, pH 4.0.

Differential Scanning Calorimetry (DSC). For DSC measurements, *N*-methyl-DPPS dispersions in water or buffer were prepared by weighing the solid phospholipid (1–5 mg) into the DSC pan and adding 50 μL of water or buffer (5 mM sodium phosphate, pH 7.0, 0.1 M NaCl, 0.1 mM EDTA). Alternatively, the phospholipid dispersions were made as described for the ^{31}P NMR samples, and 50 μL was injected into the DSC pan with a Hamilton syringe. The pan was immediately sealed and transferred to the calorimeter.

DSC measurements were carried out with a Perkin-Elmer DSC-2 differential scanning calorimeter. Each sample was heated and cooled repeatedly at a rate of 5 °C/min unless otherwise stated. The peak in the excess apparent heat capacity vs temperature plot was taken as the transition temperature T_c. Transition temperatures and enthalpies, ΔH, were determined by using a Perkin-Elmer Model 3600 data station.

Infrared Spectroscopy. Samples for infrared spectroscopy were prepared by following procedures described elsewhere.[20] The buffer was prepared with either H_2O or ^2H_2O depending on the spectral region under study and had the following composition: 100 mM NaCl, 2 mM Tes, 2 mM His, and 0.1 mM EDTA, pH 7.0. The concentration of lipid used was always 0.1 M (ca. 14% wt/vol). Hydration was accomplished by adding the buffer solution to the dry lipid, followed by vortexing, heating to 60 °C for 2 min, vortexing while warm, and cooling; this cycle was repeated three times. In this way, a homogeneous dispersion was obtained readily. The samples were held in cells of 12-μm path length fitted with CaF_2 windows. Infrared spectra at 2-cm^{-1} resolution were recorded on a Digilab FTS-60 Fourier transform infrared spectrometer. Band narrowing was achieved by using the procedure of Fourier self-deconvolution.[21]

^{31}P NMR Spectroscopy. Samples for ^{31}P NMR measurements were prepared by evaporating the phospholipid solutions in $CHCl_3/CH_3OH$ (2:1 v/v) in a round-bottom flask and drying the phospholipid in vacuo. The phospholipid was dispersed in 5 mM Tris buffer, pH 7.0, containing 0.05% NaN_3 or in H_2O and adjusting the pH to neutrality. Proton-decoupled ^{31}P powder NMR spectra were recorded on a Bruker CXP 300 Fourier transform spectrometer operating at 121.46 MHz. Axially symmetric ^{31}P powder spectra were recorded by using simple 90° pulses. The spectra of crystalline samples giving rise to axially asymmetric powder spectra were recorded by using the cross-polarization, double-resonance technique.[22] Chemical shielding was referenced to external 85% orthophosphoric acid. The error in determining the width of axially asymmetric powder patterns is 3–5 ppm; the error in the spectral width of symmetric powder patterns is 1–2 ppm.

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Table II. Thermal Behavior of Fully Hydrated Dispersions of 1,2-Dipalmitoyl-*sn*-glycero-3-phosphoserine (DPPS) and 1,2-Dipalmitoyl-*sn*-glycero-3-phospho-*N*-methyl-L-serine (*N*-Methyl-DPPS)[a]

	heating run						
	pretransition		main transition		cooling run		
sample	T_c, °C	ΔH, J/g	T_c, °C	ΔH, J/g	T_c, °C	ΔH, J/g	medium
DPPS			56 ± 1	50.5 ± 1 (9.1 ± 0.2)	49 ± 0.5	49 ± 0.5 (8.8 ± 0.1)	5 mM sodium phosphate buffer, pH 7, 5 mM EDTA, 0.05% NaN_3
N-methyl-DPPS	39 ± 1.5	22 ± 11 (4 ± 2)	48 ± 0.2	50 ± 4 (9.2 ± 0.8)	42.8 ± 0.2	50 ± 2 (9.2 ± 0.3)	5 mM sodium phosphate buffer, pH 7, 0.1 mM EDTA, 0.1 M NaCl
N-methyl-DPPS	40.5 ± 1	28 ± 2 (5.1 ± 0.3)	48 ± 0.2	52.7 ± 4 (9.7 ± 0.8)	42.5 ± 0.2	51 ± 2 (9.3 ± 0.3)	5 mM sodium phosphate buffer, pH 7, 0.1 mM EDTA
N-methyl-DPPS	40 ± 2	26 (4.8)	48 ± 0.5	66 (12.1)	43 ± 0.3	63 ± 4 (11.5 ± 0.8)	H_2O, pH 6.5–7.0
N-methyl-DPPS after incub at 4 °C for 3 days	42.6	46 ± 1.6 (8.4 ± 0.3)	48 ± 0.2	49 ± 5 (9.0 ± 0.9)			5 mM sodium phosphate buffer, pH 7, 0.1 M NaCl, 0.1 mM EDTA

[a] Enthalpies are expressed as J/g; the values in parentheses are kcal/mol calculated by using a value of 767 for the molecular weight of *N*-methyl-DPPS.

X-ray Diffraction. Samples for X-ray diffraction were prepared by weighing the desired amount of phospholipid in a glass tube with a narrow constriction near its center. Tris buffer (5 mM, pH 7.0) containing 0.1 M NaCl and 0.1 mM EDTA was added to give lipid dispersions with a water content of 50%. The tube was sealed and heated to 60 °C for 1 min, and the phospholipid dispersion was centrifuged at 4000 rpm through the narrow constriction. The centrifugation through the constriction was repeated until the dispersion was homogeneous. The lipid dispersion was filled into thin-walled (0.01-mm) glass capillaries of 2-cm length and internal diameter of 1 mm (Mark capillaries from Glas, West Berlin). The glass capillary was sealed, inserted into a thermostated sample holder, and exposed to the X-ray beam for 2–8 h. An Elliot GX-6 rotating anode generator (Elliot Automation, Borehamwood, U.K.) served as the source for nickel-filtered Cu Kα X-radiation. A Franks-type camera was used to collimate the X-ray beam into a point source. X-ray diffraction patterns were recorded on a CEA Reflex 25 film (CEA, Mülheim, FRG).

Results

Monomolecular Films. The chemical structure of *N*-methyl-DPPS is shown in Figure 1A (inset) together with the force–area curves of its NH_4^+ salt spread on 0.066 M phosphate buffer, pH 7.4, at different temperatures. Comparison with the parent compound DPPS shows that the monolayer of the *N*-methyl analogue is more expanded at all pressures and temperatures. Furthermore, at 21 °C the *N*-methyl analogue undergoes a two-dimensional phase transition from the liquid-expanded to the condensed state at 3 mN m^{-1} and 94 Å^2 per molecule. As expected, with increasing temperature the range of the liquid-expanded phase increases: at 37 °C the onset of the phase transition is at 18 mN m^{-1} corresponding to an area of 70 Å^2 per molecule. The *N*-methyl analogue occupies a larger molecular area even in the condensed state: at collapse pressure the limiting area observed for DPPS is 40.5 Å^2 per molecule while that of the *N*-methyl analogue is 42 Å^2. Figure 1B shows the force–area curves of *N*-methyl-DPPS and its parent compound spread on 0.2 M sodium acetate buffer, pH 4.0. At acidic pH both isotherms are condensed. Similarly to the force–area curves obtained on phosphate buffer, pH 7.4, the molecular area of the *N*-methyl analogue is larger than that of the parent compound except at high pressures. At collapse pressure the limiting molecular area is 40 Å^2, approaching that of DPPS.

Differential Scanning Calorimetry. DSC scans of the NH_4^+ salt of *N*-methyl-DPPS dispersed in 5 mM phosphate buffer, pH 7.0, containing 0.1 M NaCl and 0.1 mM EDTA are shown in Figure 2. When the dispersion is heated from 0 °C a pretransition and a main transition are observed reproducibly. The main transition occurs at 48 ± 0.2 °C with an enthalpy ΔH = 50 ± 4 J/g (9.2 ± 0.8 kcal/mol) (Figure 2A, Table II). Both the temperature and the enthalpy of the pretransition are variable, depending on the history of the sample. With freshly prepared samples (Figure 2A) the temperature of the pretransition is at 39 °C with an enthalpy ΔH = 33.5 ± 6 J/g (6 ± 1 kcal/mol). After the first heating run the sample shown in Figure 2A was subjected to repeated cooling–heating cycles. A representative thermogram of the heating curve obtained after the sample was taken repeatedly through cooling–heating cycles is shown in Figure 2B. While the main transition is unchanged, the pretransition occurs as a broad peak at 38.5–41 °C and its enthalpy is reduced, e.g., upon heating the dispersion for the third time the enthalpy is ΔH = 15.9 J/g (2.9 kcal/mol). When the *N*-methyl-DPPS dispersion is cooled, a single, sharp but asymmetric exotherm is observed reversibly with a peak temperature at 42.8 ± 0.2 °C and an enthalpy ΔH = 50 ± 2 J/g (9.2 ± 0.3 kcal/mol). The transition temperature is depressed by ~5 °C compared to the transition temperature observed upon heating; the enthalpy of the

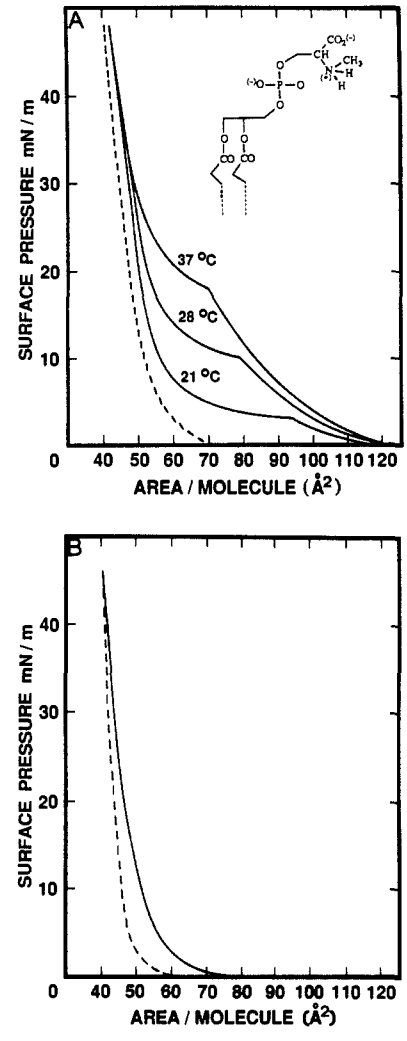

Figure 1. Surface pressure (mN m^{-1})–molecular area (Å^2/molecule) curves of *N*-methyl-DPPS (solid traces) and of the parent compound DPPS (dashed trace recorded at 21 °C). Monolayers were spread on 0.066 M phosphate buffer, pH 7.4 (A) and on 0.2 M sodium acetate, pH 4.0 (B). The chemical structure of *N*-methyl-DPPS is shown in the inset.

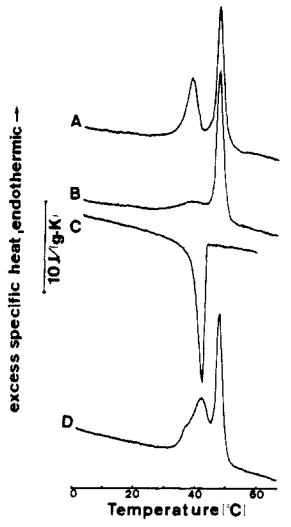

Figure 2. DSC heating and cooling curves of the NH_4^+ salt of *N*-methyl-DPPS (48 mg/mL, 0.063 M) dispersed in 5 mM sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl and 0.1 mM EDTA. Heating and cooling curves were recorded at 5 °C/min. (A) A freshly prepared dispersion of *N*-methyl-DPPS was cooled to 0 °C and then the heating curve shown was recorded; (B) after the first heating run the sample was subjected to repeated cooling–heating cycles and the thermogram shown is the third heating run; (C) representative cooling curve of the same sample; (D) the same dispersion described above was incubated at 4 °C for 24 h and the thermogram shown is the first heating run recorded after incubation.

exotherm (Figure 2C) is, within experimental error, identical with that of the main transition observed upon heating. The thermal behavior of aqueous *N*-methyl-DPPS dispersions is summarized in Table II. For comparison, the thermal behavior of the parent DPPS is included in this table. Dispersions of *N*-methyl-DPPS in H_2O (pH 6.5–7) behave similarly to dispersions in buffer except that the enthalpy of the main transition is somewhat larger (Table II).

The heating curve of an *N*-methyl-DPPS dispersion after incubation at 4 °C for 3 days is shown in Figure 2D. Both the temperature and the enthalpy of the pretransition are increased compared to the first (Figure 2A) and subsequent heating runs of freshly made dispersions (Figure 2B). The pretransition is at 42.6 °C, its enthalpy is increased to $\Delta H = 46 \pm 1.6$ J/g (8.4 ± 0.3 kcal/mol), and it has a conspicuous shoulder on the low-temperature side (Figure 2D). The characteristics of the main transition are hardly changed: its temperature is at 48 ± 0.2 °C and its enthalpy is $\Delta H = 49 \pm 5$ J/g (9.0 ± 0.9 kcal/mol) (Table II). If the sample shown in Figure 2D is cooled to 0 °C and immediately reheated, the enthalpy of the pretransition is reduced to $\Delta H = 14.6$ J/g (2.7 kcal/mol). Subsequently, this sample was incubated at 4 °C for increasing periods of time, and heating runs were recorded after timed intervals. The enthalpies measured for the pretransition after incubation at 4 °C for 0.5, 1, and 3 h and 1 day were $\Delta H = 29$ J/g (5.4 kcal/mol), $\Delta H = 37$ J/g (6.7 kcal/mol), $\Delta H = 46$ J/g (8.5 kcal/mol), and $\Delta H = 46$ J/g (8.5 kcal/mol), respectively. The enthalpy of this transition increased with incubation time, reaching a plateau of ~46 J/g (8.5 kcal/mol) after ~3 h.

Infrared Spectroscopy. The thermal phase behavior of *N*-methyl-DPPS was also studied by infrared spectroscopy. In Figure 3 we show the temperature dependence of the methylene symmetric stretching vibration, $\nu_s(CH_2)$, arising from the fatty acyl chains.[20] The behavior of a freshly prepared sample (Figure 3A) is compared with that of a sample incubated at 6 °C for 66 h (Figure 3B). In the case of the freshly prepared sample there are two discontinuities in the frequency vs temperature plot. They are at 38 and 48 °C and correspond well with the pretransition and main transition observed in the DSC experiment (Figure 2A). At 38 °C there is an increase of 0.3 cm^{-1} in the frequency and an increase of 2.5 cm^{-1} at the main transition (48 °C). Similarly, there are two discontinuities in the plot of frequency vs temperature

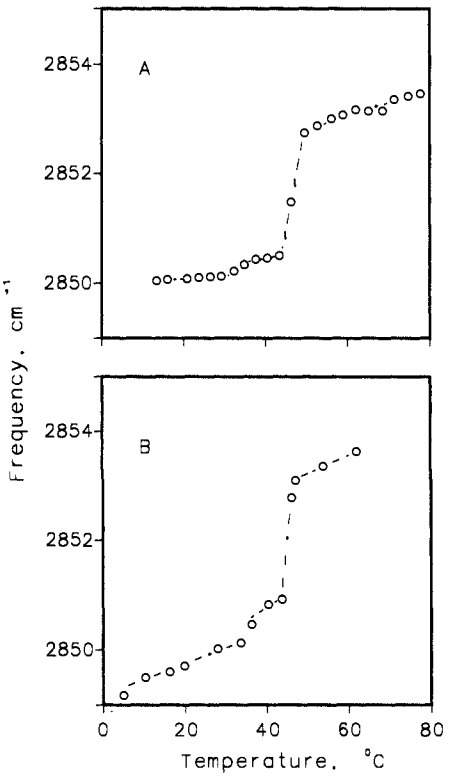

Figure 3. Temperature dependence of the frequency of the symmetric CH_2 stretching vibrational mode of *N*-methyl-DPPS (0.1 M) dispersions in buffer prepared in ^2H_2O (2 mM Tes, 2 mM His, pH 7.0, 0.1 M NaCl, 0.1 mM EDTA). (A) Freshly prepared sample; (B) sample incubated at 6 °C for 66 h.

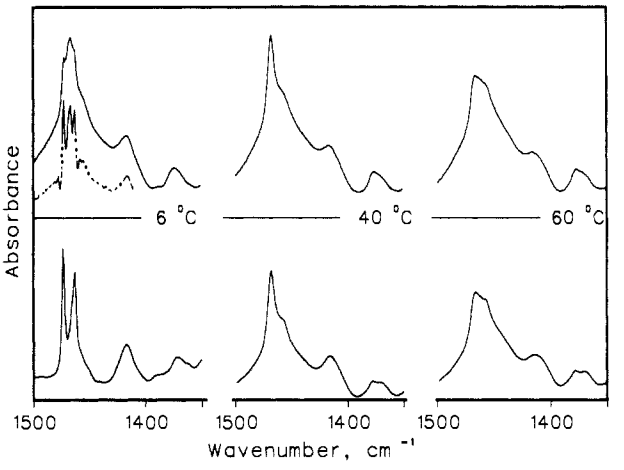

Figure 4. Infrared spectra (1500–1350 cm^{-1}) of *N*-methyl-DPPS (0.1 M in buffer pH 7.0; see legend to Figure 3) recorded at 6, 40, and 60 °C. (Top) Freshly prepared sample and (bottom) sample incubated at 6 °C for 66 h. The dotted line below the 6 °C spectrum (top) is the result of deconvolution with a Lorentzian line shape of 5-cm^{-1} width.[21]

obtained from the spectra of the incubated sample (Figure 3B); they are at 48 °C (main transition) and 39 °C (pretransition). The shift observed at the main transition (48 °C) for the incubated sample is the same, 2.5 cm^{-1}, as in the case of the freshly prepared sample. The shift observed at the pretransition (39 °C) is larger for the incubated sample (0.7 cm^{-1}) than for the freshly prepared sample (0.3 cm^{-1}). The frequency values observed in the temperature range 0–35 °C are lower for the incubated sample; this is due to the fact that upon incubation the fatty acyl chains become well ordered conformationally and tightly packed (see below).

The infrared spectra of freshly prepared *N*-methyl-DPPS aqueous dispersions are compared to those of incubated dispersions in Figures 4 and 5. In the top part of Figure 4 we show the 1500–1350-cm^{-1} spectral region for a freshly prepared sample (dispersed in pH 7.0 buffer prepared in ^2H_2O), while the corre-

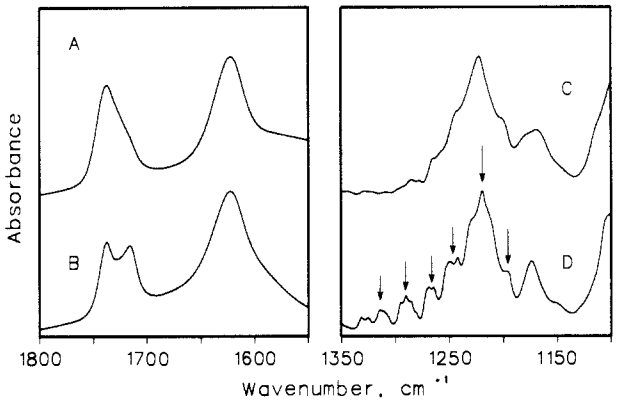

Figure 5. Infrared spectra at 20 °C of 0.1 M dispersions of *N*-methyl-DPPS. (A) 1800–1550-cm^{-1} region of the spectra of a freshly prepared sample dispersed in ^2H_2O buffer, pH 7.0 (see legend to Figure 3); (B) same sample as under A after incubation at 6 °C for 66 h; (C) 1350–1100-cm^{-1} region of the spectra of a freshly prepared sample dispersed in H_2O buffer, pH 7.0; (D) same sample as under C after incubation at 6 °C for 70 h.

sponding spectra of a sample incubated at 6 °C for 66 h are shown in the bottom part. The spectra shown were recorded at 6, 40, and 60 °C. In this spectral region the relevant bands are those arising from the CH_2 deformation (or scissoring) vibrational mode, $\delta(CH_2)$, which appear between 1475 and 1460 cm^{-1}. These bands yield information about the packing of the fatty acyl chains.[23] In the spectrum of the freshly prepared sample (Figure 4, top) recorded at 6 °C there are three bands due to $\delta(CH_2)$ at 1473, 1467, and 1463 cm^{-1} (see deconvolved spectrum, dotted line). After incubation at 6 °C for 66 h there are only two $\delta(CH_2)$ bands at 1473 and 1463 cm^{-1} (Figure 4, bottom). We note that in the spectrum of the incubated sample these $\delta(CH_2)$ bands are narrower than those of the freshly prepared sample. Thus, incubation of *N*-methyl-DPPS dispersions results in the disappearance of the $\delta(CH_2)$ band at 1467 cm^{-1} and pronounced narrowing of the remaining bands. The width of the band at 1473 cm^{-1} is $\Delta\nu_{1/2}$ = 8 cm^{-1} before incubation and $\Delta\nu_{1/2}$ = 3 cm^{-1} after incubation. The spectra recorded at 40 °C (above the pretransition) and at 60 °C (above the main transition) are practically the same for the freshly prepared sample and for the incubated sample (compare Figure 4 top and bottom). This shows that the molecular events taking place at these two transitions are comparable regardless of the thermal history of the sample. In the spectra recorded at 40 °C there is a single $\delta(CH_2)$ band at 1468 cm^{-1} (Figure 4, 40 °C). Heating to temperatures above the main transition does not induce frequency shifts but induces broadening and a decrease in intensity of the $\delta(CH_2)$ band. This effect on the $\delta(CH_2)$ band is characteristic of the melting of polymethylene systems and has been observed in the spectra of a number of lipids[20] at their main transitions.

Infrared spectra of the gel phases of freshly prepared and incubated dispersions of *N*-methyl-DPPS are compared in Figure 5. The spectra shown in Figure A and B contain the ester C=O stretching bands (1750–1700 cm^{-1}) and the antisymmetric stretching mode of the serine carboxylate group, $\nu_a(CO_2^-)$. The frequency of the $\nu_a(CO_2^-)$ band is 1622 cm^{-1} in the spectrum of the freshly prepared sample; this value is characteristic of the gel phase of phosphatidylserine bilayers with hydrated carboxylate groups.[24] Incubation at 6 °C for 66 h (Figure 5B) does not induce changes in the $\nu_a(CO_2^-)$ band: the frequency observed after incubation is also 1622 cm^{-1} and there are no changes in the width of this band. Incubation does induce changes in the C=O stretching bands of the ester groups linking the fatty acyl chains to the glycerol backbone. Before incubation (Figure 5A) there are two C=O stretching bands at 1738 and 1723 cm^{-1}; after 66 h at 6 °C (Figure 5B) there are also two main bands at 1738 and 1716 cm^{-1} but they are narrower, e.g., the half-width of the low-frequency band is estimated to change from $\Delta\nu_{1/2}$ = 28 cm^{-1} to $\Delta\nu_{1/2}$ = 20 cm^{-1} as a result of incubation. The intensities (as integrated areas) of the ester C=O stretching bands were determined by curve fitting the observed spectra; the relative intensity of the low-frequency C=O band increases upon incubation from 50% to 60% of the combined C=O intensity.

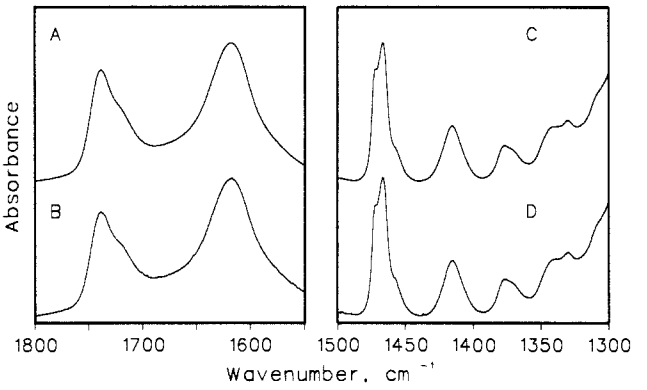

Figure 6. Infrared spectra at 6 °C of DPPS, 0.1 M dispersions in pH 7.0 buffer (see legend to Figure 3) prepared in ^2H_2O. (A) 1800–1550-cm^{-1} region of the spectrum of a freshly prepared sample; (B) same as A after incubation at 6 °C for 40 h; (C) 1500–1300-cm^{-1} region of the spectrum of a freshly prepared sample; (D) same as C after incubation at 6 °C for 40 h.

The spectra shown in Figure 5 C and D contain the antisymmetric PO_2 stretching mode and superimposed on it the components of the CH_2 wagging band progression.[23] The PO_2 stretching mode is at the same frequency, 1223 cm^{-1}, before and after incubation. The components of the CH_2 wagging band progression, observed at 1200, 1220, 1245, 1265, 1290, and 1310 cm^{-1}, are labeled with ↓; their intensity is markedly increased in the spectrum of the incubated sample[25] (Figure 5D).

For comparison, we show in Figure 6 spectra of the NH_4^+ salt of DPPS as a freshly prepared sample and a sample incubated at 6 °C for 40 h; clearly, incubation does not induce detectable changes in the spectra. In both spectra (Figure 6C and D) there is a $\delta(CH_2)$ band at 1466 cm^{-1} with a shoulder at 1472 cm^{-1}. There are also no detectable changes in the ester C=O stretching bands and $\nu_a(CO_2^-)$ mode. These results show that the gel phase of the NH_4^+ salt of DPPS is stable and does not undergo changes with incubation at low temperatures. The ester C=O stretching bands in the spectra of DPPS are at 1740 and 1726 cm^{-1}; the low-frequency band accounts for 70% of the combined C=O intensity.

^{31}P NMR. Figure 7A shows the proton-decoupled ^{31}P NMR powder spectrum of crystalline samples of DPPS and the *N*-methyl analogue (both as the NH_4^+ salts). The two spectra are practically superimposable. The spectral width of ~191 ppm and the values of the principal components of the chemical shielding tensor were derived from computer simulations of the spectra shown in Figure 7A: σ_{11} = −80.5 ppm, σ_{22} = −22 ppm, and σ_{33} = 110 ppm. These values are characteristic of the alkali salts of synthetic phosphatidylserines such as dilauroyl- and dimyristoylphosphatidylserine and DPPS. They are also consistent with those of barium diethyl phosphate, which has been used as a model for the ^{31}P chemical shielding tensor of the phosphodiester group.[26]

Figure 7B shows the ^{31}P powder NMR spectrum of an aqueous dispersion of the NH_4^+ salt of *N*-methyl-DPPS at 25 °C. For comparison, the ^{31}P powder NMR spectrum of the NH_4^+ salt of the parent compound DPPS is included (Figure 7C). DPPS gives an axially symmetric powder spectrum with a chemical shielding anisotropy of ~80 ppm as is characteristic for phosphatidylserine

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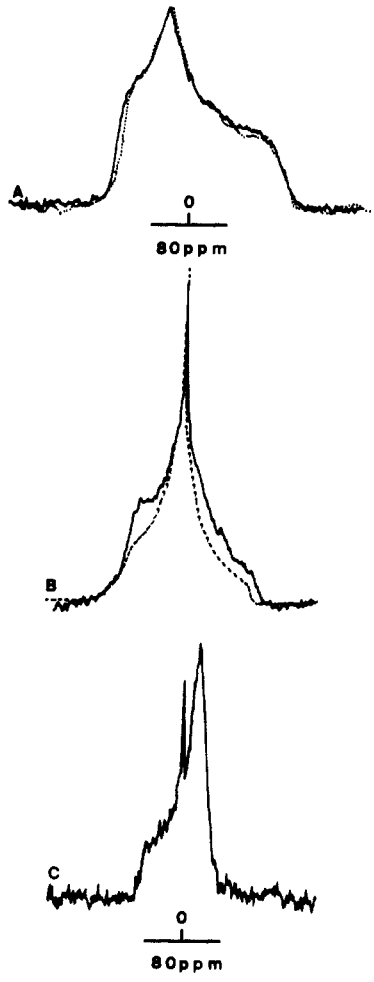

Figure 7. Proton-decoupled ^{31}P powder NMR spectra of synthetic phosphatidylserines recorded at 121.46 MHz and at room temperature. (A) Crystal powder of the NH_4^+ salt of *N*-methyl-DPPS (solid line) and crystal powder of the NH_4^+ salt of DPPS (dotted line); (B) dispersion of the NH_4^+ salt of *N*-methyl-DPPS in water apparent pH ~8, 87 mg/mL, 0.113 M (solid line), and the same dispersion after heating it to 45 °C and incubating the sample at 4 °C for 1 day (dotted line); (C) dispersion of the NH_4^+ salt of DPPS in water apparent pH 6.9, ~100 mg/mL, 0.133 M.

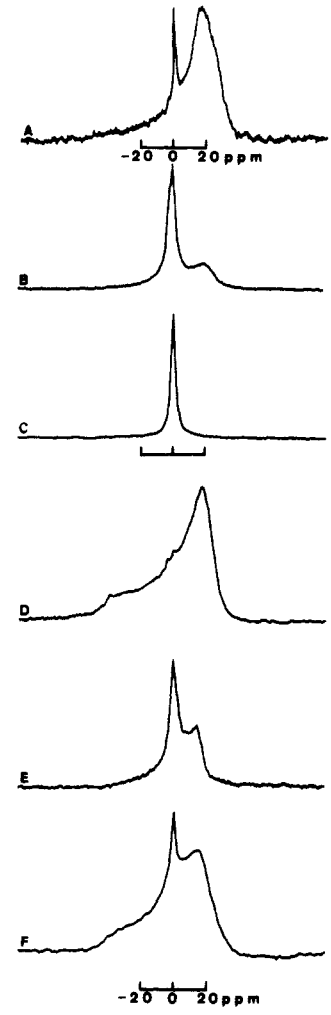

Figure 8. Proton-decoupled ^{31}P powder NMR spectra of the NH_4^+ salt of *N*-methyl-DPPS as a function of temperature. Lipid dispersions (100 mg/mL, 0.133 M) were made in 5 mM Tris buffer, pH 7.0, containing 0.05% sodium azide; (A) ^{31}P powder NMR spectrum recorded at 35 °C; (B) at 41 °C; (C) at 45 °C; (D) of a dispersion of ammonium 1,2-dimyristoylphosphatidylserine (101 mg/mL, 0.144 M) in 5 mM Tris buffer, pH 7.0, 0.05% sodium azide, at 27 °C and (E) at 43 °C; (F) of the same dispersion as described under A–C after cooling the dispersion from 45 °C (C) to 27 °C; the same dispersion as under F after incubation at 4 °C for 1 day is included in Figure 7B (dotted spectrum).

dispersions in the gel state at room temperature. Despite crystalline hydrocarbon chains, the polar group of the phospholipid undergoes motional averaging leading to an axially symmetric powder pattern. The DPPS spectrum is contrasted by that of the *N*-methyl analogue, which under comparable conditions gives an axially asymmetric ^{31}P NMR powder pattern. The spectral width is $|\Delta\sigma|$ ~151 ppm indicating that motional averaging of the phospholipid polar group occurs, leading to a significant reduction of the chemical shielding anisotropy as compared to $|\Delta\sigma| = 191$ ppm measured in the crystalline sample (Figure 7A). Superimposed on the axially asymmetric powder pattern (Figure 7B) is a sharp isotropic signal at 0 ppm indicating that besides the crystalline phospholipid phase some small phospholipid particles are present. These must be such that their Brownian tumbling together with the molecular motion of *N*-methyl-DPPS averages out completely the ^{31}P chemical shielding anisotropy giving rise to an isotropic ^{31}P NMR signal.

The temperature dependence of the ^{31}P NMR spectrum of *N*-methyl-DPPS is shown in Figure 8. Heating the sample to 35 °C produces an axially symmetric powder pattern with a small isotropic component (Figure 8A). When the temperature is raised to 41 °C the isotropic component grows at the expense of the axially symmetric powder pattern (Figure 8B). At even higher temperatures, 45 °C, which is close to the main transition temperature (see DSC and infrared results above), a single, broad isotropic signal is observed (Figure 8C). This indicates that the motion of the polar group of *N*-methyl-DPPS close to the gel-to-liquid crystal transition temperature is almost isotropic. For comparison, the ^{31}P powder NMR spectra of aqueous dispersions of 1,2-dimyristoyl-*sn*-phosphatidylserine are included in this figure. It is seen (Figure 8D) that this phosphatidylserine gives an axially symmetric powder pattern in the gel phase at 27 °C with a chemical shielding anisotropy $|\Delta\sigma| = 80$ ppm. At 43 °C, which is ~3 °C above the gel-to-liquid crystal transition temperature, a composite spectrum is obtained: a broad isotropic signal at ~0 ppm similar to that observed with the *N*-methyl-DPPS at 45 °C is superimposed on an axially symmetric powder pattern with $|\Delta\sigma| = 57$ ppm characteristic of the liquid-crystalline state (Figure 8E). We used here dimyristoyl-*sn*-phosphatidylserine rather than DPPS because phosphatidylserines are heat sensitive; the transition temperature of the former is 40 °C, i.e., significantly lower than that of DPPS, which is 56 °C.[17]

That the broad, isotropic signal of *N*-methyl-DPPS at 45 °C is not due to lipid degradation induced by heating is shown in Figure 7B (dotted line) and Figure 8F. After the sample was cooled to 27 °C, the ^{31}P NMR spectrum in Figure 8F was obtained. It is essentially an axially symmetric powder pattern with $|\Delta\sigma|$ ~75 ppm with a small isotropic component superimposed on the powder spectrum. Incubating the same dispersion at 4 °C for 1 day produced the composite ^{31}P NMR spectrum shown in

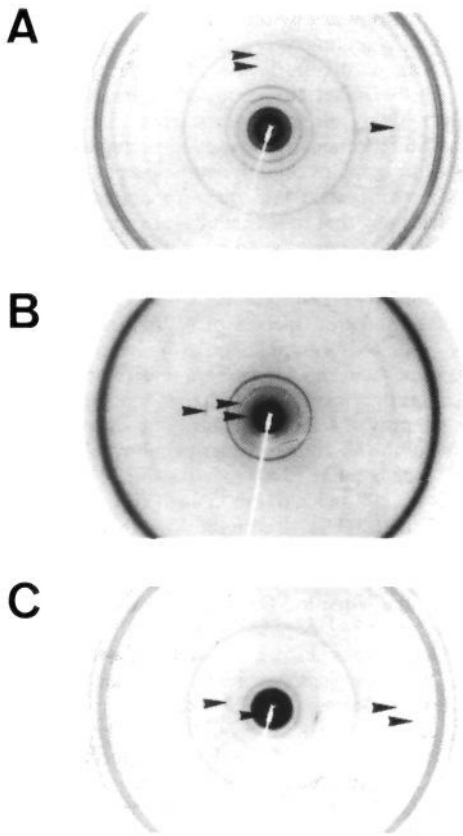

Figure 9. X-ray diffraction patterns of 50% (1.3 M) dispersions of *N*-methyl-DPPS in 5 mM Tris buffer, pH 7.0, containing 0.1 M NaCl and 0.1 mM EDTA. (A) Prior to recording the X-ray diffraction pattern at 4 °C, the *N*-methyl-DPPS dispersion was incubated at 4 °C for 3 days; (B) the same dispersion was heated to 55 °C for 5 min and subsequently it was cooled to 22 °C, at which temperature the diffraction pattern was recorded; (C) the X-ray diffraction pattern of a freshly prepared sample recorded at 22 °C.

Figure 7B (dotted line). Its line shape and width are similar to the original spectrum (solid line) shown in this figure. The main feature is the axially asymmetric component. The two spectra shown in Figure 7B (dotted line) and Figure 8F indicate that the events induced by heating the *N*-methyl-DPPS dispersion from room temperature to ~45 °C are reversed upon cooling the sample to the original temperature.

X-ray Diffraction. As shown before,[17,27,28] phosphatidylserines have a preference for the lamellar phase. This is also true for the *N*-methyl analogue. Figure 9A gives the X-ray diffraction pattern of a 50% dispersion of *N*-methyl-DPPS in buffer after incubation of the dispersion at 4 °C for 3 days. There are six sharp low-angle reflections, four strong ones, which are clearly seen in Figure 9A, and two weak ones marked by arrows. These reflections are in the ratio of $1:{}^1/_2:{}^1/_3:{}^1/_4:{}^1/_6:{}^1/_7$ indicating that the phospholipid forms a smectic (lamellar) phase. The average lamellar repeat distance derived from the low-angle reflections is 62.7 ± 0.9 Å. There are several sharp reflections in the wide-angle region at ${}^1/_{7.04}$, ${}^1/_{4.92}$, ${}^1/_{4.48}$, ${}^1/_{4.19}$, ${}^1/_{4.07}$, and ${}^1/_{3.82}$ Å^{-1} characteristic of highly ordered hydrocarbon chains. When this dispersion was heated to 55 °C for 5 min and subsequently cooled to 22 °C, the X-ray diffraction pattern shown in Figure 9B was obtained. Both the low-angle as well as the wide-angle reflections are changed compared to the original dispersion shown in Figure 9A. There is a series of sharp lamellar reflections in the low-angle region corresponding to a repeat distance of 65 ± 2 Å. Instead of the many sharp reflections in the wide-angle region a single sharp one corresponding to ${}^1/_{4.21}$ Å^{-1} is observed. This reflection is characteristic of the gel phase with hexagonally packed hydrocarbon chains.

A freshly prepared 50% dispersion of *N*-methyl-DPPS in buffer at 21 °C gave the X-ray diffraction pattern shown in Figure 9C. The series of lamellar reflections at low angles gives an average repeat distance of 73 ± 0.6 Å. The wide-angle reflection pattern resembles that of the incubated sample (Figure 9A) with reflections at ${}^1/_{7.29}$, ${}^1/_{4.99}$, ${}^1/_{4.53}$, ${}^1/_{4.24}$, ${}^1/_{4.12}$, and ${}^1/_{3.90}$ Å^{-1}.

Discussion

Taken together the results from several different physical measurements demonstrate some unique properties of *N*-methyl-DPPS; they illustrate clearly the dramatic effects brought about by a slight chemical modification in the lipid polar group. The introduction of a methyl residue into the serine amino group results in a slight increase of ~5% in cross-sectional area of the molecule as deduced from surface pressure–area measurements (Figure 1A). This is small compared to the 44% increase observed when a palmitoyl chain of DPPS is replaced by an oleoyl chain.[8] The increase observed for *N*-methyl-DPPS is also small when compared to that observed upon introduction of methyl substituents in the hydrocarbon chains of lipids.[9] For example, the molecular area (at collapse pressure) of 1,2-distearoyl-*sn*-glycero-3-phosphocholine increases by 13% (from 38 to 43 Å^2) upon introduction of a methyl at position 16 of the *sn*-2 chain.

The introduction of a single methyl into the polar group of phosphatidylserine produces significant changes in the phase behavior and physical–chemical properties. The calorimetric and infrared spectroscopic data show that the temperature of the main gel-to-liquid crystal phase transition of *N*-methyl-DPPS is 48 °C, i.e., 8° lower than the corresponding transition temperature of the parent DPPS, which is 56 °C; see Table II. *N*-Methyl-DPPS bilayers undergo a pretransition between 39 and 43 °C (cf. Figure 2). The existence of this pretransition is due to the presence of the *N*-methyl residue since bilayers of DPPS and other diacylphosphatidylserine molecules with saturated fatty acyl chains do not exhibit thermal events at temperatures below their gel-to-liquid crystal transition. A priori, the lowering of the gel-to-liquid crystal transition temperature may be taken to indicate that a larger head group induces a larger separation between lipid molecules; this in turn destabilizes the bilayers and consequently a lower transition temperature results. However, as mentioned above, the steric effects are accompanied by alterations of electrostatic and hydrogen-bonding interactions. Hydrogen bonding plays an important role in determining lipid-phase behavior; reduced hydrogen-bonding capacity results in lower gel-to-liquid crystal transition temperatures.[29] Methylation of the serine amino group replaces a hydrogen-bonding proton and therefore the probability of hydrogen-bonding interactions decreases. It has also been proposed that weakening of hydrogen bonds in phosphatidylserine is related to the existence of pretransitions.[30] Even though the results presented here do not give explicit information on intermolecular hydrogen bonding, the differences in phase behavior between *N*-methyl-DPPS and DPPS could be rationalized in terms of a reduced hydrogen-bonding capacity of *N*-methyl-DPPS. Differences in phase behavior induced by introduction of a methyl residue into the polar group of phosphatidylethanolamines[31-33] have also been rationalized in terms of changes in hydrogen-bonding interactions.[29,34]

The calorimetric experiments (Figure 2) reveal that the characteristics of the pretransition of *N*-methyl-DPPS depend on the thermal history of the sample. The enthalpy of the pretransition varies depending on the time of incubation of the gel phase.

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A maximum value for the enthalpy of the pretransition ΔH = 46 J/g (8.5 kcal/mol) is measured after incubation of the *N*-methyl-DPPS dispersion at 4 °C for 3 h or longer. The pretransition is not observed on cooling. This behavior indicates that the gel phase obtained immediately after cooling is metastable. The characteristics of the $\delta(CH_2)$ mode in the infrared spectra (cf. Figure 4) are compatible with the coexistence of two types of acyl chain packing in the freshly obtained gel phase, and furthermore, these characteristics change with time. Therefore, they are compatible with gel-phase metastability. This conclusion derived from DSC and infrared evidence is also supported by X-ray diffraction data (Figure 9). An *N*-methyl-DPPS dispersion cooled from above the gel-to-liquid crystal transition temperature gives a wide-angle diffraction pattern that is significantly different from that of a dispersion equilibrated at 4 °C. Instead of a series of sharp reflections characteristic of highly ordered hydrocarbon chains in the incubated dispersion, the rapidly cooled dispersion gives a single reflection at wide angles characteristic of hexagonally packed hydrocarbon chains. Apparently, the hydrocarbon chains have not snapped into their tightest packing of minimum energy. The changes observed in the infrared spectra at the pretransition indicate that there is a change in acyl chain packing. Below the main transition and above the pretransition the packing is hexagonal, and below the pretransition the packing is orthorhombic-like. Therefore, the pretransition represents a change in the hydrocarbon chain packing mode. However, the incubation experiments demonstrate clearly that adoption of the preferred acyl chain packing is not immediate upon cooling from the liquid-crystalline phase. This explains why the pretransition is not observed on cooling scans or why it is reduced in the second heating run performed immediately after cooling (Figure 2). The interpretation of the pretransition based on infrared evidence is entirely consistent with X-ray data. The effect of heating the *N*-methyl-DPPS dispersion above the pretransition temperature is essentially a change in the wide-angle reflections. The series of sharp wide-angle reflections is replaced by a single sharp reflection at $^1/_{4.21}$ Å^{-1}, indicating that the hydrocarbon chain packing changes from a highly ordered to the less ordered, hexagonal chain packing mode (Figure 9B).

Information concerning the motion of the phosphate and in turn of the polar group of *N*-methyl-DPPS is derived from ^{31}P NMR. In the gel phase freshly prepared by cooling an *N*-methyl-DPPS dispersion from ~45 to 27 °C (Figure 8F), the motional characteristics of the lipid polar group are similar to those of the gel phase of dimyristoylphosphatidylserine and DPPS (cf. Figure 7C and Figure 8D and E). Even though it is clear from X-ray diffraction and infrared studies that the bilayers are in the gel state with crystalline hydrocarbon chains, there is motion in the lipid polar group. This motion apparently averages the ^{31}P chemical shielding tensor so that axially symmetric powder spectra are obtained (Figures 7C and 8D and E). We note that these motions must be restricted to bond rotations in the lipid polar group; molecular rotation, i.e., rotation of the lipid molecule as a whole about its long axis can be safely ruled out on the basis of X-ray diffraction and infrared evidence. Both methods indicate the presence of a crystalline chain lattice. ^{31}P NMR shows that, upon heating *N*-methyl-DPPS dispersions to temperatures close to the order–disorder transition (Figure 8C), the lipid polar group is significantly more mobile compared to the parent compound DPPS. This is a consequence of the introduction of a methyl into the phosphatidylserine polar group making the polar group less polar and probably weakening electrostatic interactions within the polar group lattice.

We now discuss in some detail the properties of *N*-methyl-DPPS dispersions incubated at temperatures below the pretransition. As shown by DSC, incubation under these conditions, e.g., at 4 °C for more than 3 h, produces the most stable bilayer structure observed with *N*-methyl-DPPS. We note that DPPS and other saturated phosphatidylserines form gel phases, the properties of which do not change with time, at least not on the time scale of days. Ordinary phosphatidylserines do not form this type of tightly packed gel phase obtained with the *N*-methyl analogue after prolonged incubation at low temperatures.

The X-ray diffraction pattern of the incubated sample of *N*-methyl-DPPS shows that in this bilayer the fatty acyl chains are very well packed. In fact, the sharp reflections in the wide-angle region reflect highly ordered hydrocarbon chains. A similar pattern of sharp reflections in the wide-angle region was observed in the crystalline complex of dimyristoylphosphatidylserine with Li^+. The X-ray diffraction data are corroborated by the infrared spectra, which show that in the incubated sample the fatty acyl chains are very well packed and very well ordered conformationally. The two $\delta(CH_2)$ bands at 1473 and 1463 cm^{-1} (Figure 4, bottom) are sharp (~3 cm^{-1}) and practically identical with those observed in the infrared spectra of crystalline odd-numbered *n*-alkanes.[35] The conformational ordering induced during incubation is evident from the growth in intensity of bands characteristic of all-antiperiplanar acyl chains and the simultaneous decrease in intensity of "gauche" bands. The CH_2 wagging bands characteristic of the all-antiperiplanar palmitoyl chains[23] increase in intensity. The band at 1340 cm^{-1} due to end-gauche group[36] and the bands at 1300 and 1367 cm^{-1} due to kink defects[36] decrease in intensity. Thus, it is clear that in the *N*-methyl-DPPS gel phase the interchain distances are shorter than those in the gel phase of parent DPPS and other saturated phosphatidylserines. This is so in spite of the *N*-methyl-DPPS molecule exhibiting a larger cross-sectional area (42 Å^2) than that of DPPS (40.5 Å^2).

The ^{31}P NMR results (Figures 7 and 8) show that in incubated *N*-methyl-DPPS dispersions, i.e., in bilayers in the stable gel state, the molecules of *N*-methyl-DPPS are practically immobilized (Figure 7B) compared to bilayers of DPPS in the gel state. This conclusion derived from ^{31}P NMR is supported by infrared spectroscopy. In the spectrum of incubated *N*-methyl-DPPS dispersions the C=O stretching bands, observed at 1738 and 1716 cm^{-1}, are very narrow (cf. Figure 5B). In fact, the frequency and width of these two bands are the same as those observed in the corresponding bands of the infrared spectrum of dimyristoylphosphatidylserine recorded at −75 °C.[24] These features of the C=O stretching bands indicate immobilization. This is compatible with the observation that $\delta(CH_2)$ and wagging bands are also very narrow (~3 cm^{-1}; Figure 4), indicating that the reorientational fluctuations of the acyl chains in *N*-methyl-DPPS are damped compared to those in DPPS (Figure 6).

The characteristics of the C=O stretching bands also indicate that these groups are engaged in hydrogen bonding; the band at ~1720 cm^{-1} may be assigned to C=O groups forming one hydrogen bond to one molecule of water.[37] The C=O stretching band characteristic of hydrogen-bonded C=O groups increases in intensity (from 50% to 60%) upon incubation; thus, incubation does not induce dehydration of the ester carbonyl groups. However, the extent of hydrogen bonding to the carbonyl groups of *N*-methyl-DPPS is less than in the case of the parent DPPS; in the latter case the relative intensity of the hydrogen-bonded C=O group is ca. 70%. The rest of the head group is also hydrated in these *N*-methyl-DPPS bilayers incubated at 6 °C for 66 h. The $\nu_a(PO_2)$ band observed at 1223 cm^{-1} (Figure 5D) and the $\nu_a(CO_2^-)$ band at 1622 cm^{-1} (Figure 5B) are characteristic of hydrated phosphate and serine carboxylate groups, respectively. The lamellar repeat distance of 62.7 Å found for the incubated sample is also indicative of hydrated bilayers. A unique feature of this compound is that, even though well hydrated, it gives a ^{31}P powder NMR spectrum characteristic of an axially asymmetric chemical shielding tensor. The line shape and the value of the chemical shielding anisotropy indicate that the phosphate group is almost immobilized.

In summary, the most striking difference between DPPS and its *N*-methyl analogue is the ability of the latter compound to form a characteristic gel phase upon incubation at temperatures lower

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than the pretransition temperature. This gel phase is the most stable bilayer structure of *N*-methyl-DPPS, representing a pronounced energy minimum. Ordinary saturated phosphatidylserines like the parent compound DPPS do not form such a stable gel phase. The driving force for the formation of this stable gel phase seems to be the hydrocarbon chains adopting their preferred, minimum energy conformation and packing. In doing so they force the polar group to also become rigidly packed. It seems that in this case the forces responsible for the chains adopting their packing prevail over repulsive electrostatic forces between the charged polar groups. The fact that this is so in the case of *N*-methyl-DPPS and not in DPPS leads us to speculate that the extra methyl residue serves as a charge separator or shield between like charges of adjacent phospholipid molecules. The extra methyl group apparently reduces the polarity of the polar group sufficiently so that the formation of the stable gel phase is facilitated. As to the molecular packing in the stable gel phase of *N*-methyl-DPPS, the slightly larger molecular area of 42 $Å^2$ per molecule as compared to that of 40.5 $Å^2$ per DPPS molecule is probably overcompensated for by the decrease in the polarity of the polar group and in turn by the decrease in intermolecular electrostatic repulsion (cf. ref 41).

The unique features of *N*-methyl-DPPS compared to its nonmethylated parent compound DPPS are obviously due to the presence of the extra methyl residue. Previous work[31-33] on phosphatidylethanolamines has focused on the effect of "extra" methyl residues in the polar group. Methyl substitution in the case of phosphatidylethanolamines induces a decrease in the gel-to-liquid crystal transition temperature, e.g., with dipalmitoyl- and dimyristoylphosphatidylethanolamine. This is similar to the present case of methyl substitution in DPPS.

The metastability of the gel phase is not unique to *N*-methyl-DPPS. For example, Mulukutla and Shipley[33] studied the phase behavior of phosphatidylethanolamines and *N*-methyl derivatives. These authors found that the bilayer gel phase of these compounds is metastable: the original gel phase with rotationally disordered hydrocarbon chains packed in a hexagonal lattice converts (upon incubation at −4 °C for weeks) to a low-temperature "crystalline" phase in which the hydrocarbon chains adopt a specific packing mode. Extensive studies of phosphatidylcholines[38-40] have been reported with particular emphasis on the effects of prolonged incubation at low temperatures. In all cases, it has been found that upon prolonged incubation the gel phases revert into other, less hydrated, and better ordered states. As mentioned, incubation of *N*-methyl-DPPS at low temperatures does induce chain crystallization and immobilization. However, there are qualitative differences between incubation of *N*-methyl-DPPS and these other lipids. In the case of *N*-methyl-DPPS, incubation does not induce dehydration of the polar group and there are no thermal events corresponding to the subtransition of dipalmitoylphosphatidylcholine.

As pointed out in the introduction, the structures adopted by polar lipids are determined by the interplay of three interactions: hydrogen bonding, hydrophobic attraction, and electrostatics. In the present work, by introducing a methyl residue in the polar group of phosphatidylserine we have modified only two of these interactions: hydrogen bonding and electrostatics. The capability for hydrogen bonding is reduced by the presence of the *N*-methyl residue and so is the electrostatic repulsion between the negatively charged polar groups. In this case, reducing both hydrogen bonding and electrostatic repulsion results in the hydrophobic attraction prevailing and forcing the fatty acyl chains to adopt very tight packing in a pronounced energy minimum. However, while this occurs the molecular area of the N-methylated derivative is larger reflecting steric effects. The present study thus constitutes a unique example of the balance of forces determining bilayer structure.[41] *N*-Methyl-DPPS is also unusual in its interaction with metal ions. The ion-binding properties of *N*-methyl-DPPS will be the subject of a future publication.

Acknowledgment. We acknowledge the contribution of Dr. W. Leslauer, who built the X-ray camera used in this study. We thank Dr. J.-D. Smit for help with the installation of the X-ray equipment. We are indebted to Professors G. G. Shipley and R. N. McElhaney for reading the manuscript and for valuable discussions and suggestions. This research was supported by a grant from the Swiss National Science Foundation (31-25719.88).

Registry No. *N*-Methyl-DPPS, 125761-39-9; *N*-methyl-DPPS ammonium salt, 125761-40-2.

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