Bilayers of Phosphatidylserine: A Deuterium and Phosphorus Nuclear Magnetic Resonance Study†

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ABSTRACT: The structure and motion of the phosphatidylserine molecule in bilayers have been studied with deuterium and phosphorus-31 nuclear magnetic resonance. The phase behavior and the thermodynamic properties of phosphatidylserine were further characterized by means of spin-label electron paramagnetic resonance and differential scanning calorimetry. 1,2-Dipalmitoyl-, 1,2-dimyristoyl-, and 1,2-dioleoyl-*sn*-glycero-3-phosphoserine were selectively deuterated in the fatty acyl chains, in the glycerol backbone, and in both the 2 and 3 segments of the serine moiety. The residual deuterium quadrupole couplings and the phosphorus-31 chemical shielding anisotropy of lipid–water mixtures at pH 7.0 in the absence of divalent cations were measured as a function of temperature and provided quantitative data on segmental motions of the serine head group, the phosphate linkage, the glycerol backbone, and the hydrocarbon region. The NMR data of phosphatidylserine are compared with those previously obtained for phosphatidylcholine and phosphatidylethanolamine. Qualitative and quantitative agreement exists for the ordering of the fatty acyl chains of all these lipids. In particular, in all phospholipids, the *sn*-2 chain is bent at the C-2 segment. The bilayer thickness as calculated from the deuterium NMR data is similar to that of phosphatidylcholine. The deuterium magnetic resonance spectra of the C-3 deuterated glycerol backbone of 1,2-dipalmitoyl- and 1,2-dioleoyl-*sn*-glycero-3-phosphoserine are similar to those of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine, suggesting that the average conformation of the glycerol moiety remains constant in these two phospholipid classes. In contrast to the close structural resemblance in the fatty acyl chains and in the glycerol backbone region, distinct differences are observed when the phosphatidylserine head group is compared with either the phosphatidylcholine or phosphatidylethanolamine head groups. In particular, the phosphorus chemical shielding anisotropy and the deuterium quadrupole couplings of both head group C–D segments were distinctively larger than those of any of the previously studied lipids. In addition, the deuterons at the α position of the head group (P–O–CD_2–CH) have been found to be motionally inequivalent, and two quadrupole splittings were observed. The NMR data indicate a relatively *rigid* structure for the phosphatidylserine head groups. Slight structural differences in the head group were observed between the two diastereomers 1,2-dimyristoyl-*sn*-glycero-3-phospho-L-serine and the corresponding D-serine isomer. Thus, phosphatidylserine is very similar to other phospholipids up to the level of the glycerol C-3 segment, and only the head group segments significantly differ.

Phosphatidylserine is an interesting lipid in biological membranes. Primarily, this phospholipid can bind calcium to its head group, resulting in a broadening and a shift to higher temperatures of the gel to liquid-crystalline phase transition in pure phosphatidylserine membranes (Jacobson & Papahadjopoulos, 1975). This could be important in biological systems because this property would allow for a relatively fast change in the lipid phase at a constant temperature. Work on phosphatidylserine in model systems has been mainly concerned with macroscopic properties of natural brain phosphatidylserine, i.e., bilayer lamellar spacings as studied with X-ray diffraction, electron micrographs of lamellar structures, phase transition temperatures, electrophoretic mobilities, monolayer properties, and extensive studies on ion binding. More microscopic properties such as chain ordering or head group structure and motion have not been studied. Compared with the extensive literature for phosphatidylcholine and phosphatidylethanolamine, there is a lack of physical data on synthetic phosphatidylserines. This deficiency has probably been due to the difficulty of the phosphatidylserine synthesis and the more complicated purification techniques required. Phosphatidylserine is also much less stable than either phosphatidylcholine or phosphatidylethanolamine. This instability is especially noticeable with DPPS;[1] the high transition temperature of 53 °C forces one to measure at temperatures where degradation into phosphatidic acid and lysophosphatidylserine readily occurs.

In view of the interesting biological properties of this lipid and the scarcity of data on pure synthetic preparations of this lipid, we have undertaken a thorough study of several synthetic phosphatidylserines in the absence of divalent cations.

^2H and ^{31}P NMR are techniques already demonstrated to be well-suited to the study of membranes (Seelig, 1977; Mantsch et al., 1977; Stockton et al., 1976, 1977; Oldfield et al., 1978). Because of their nonperturbing nature and their sensitivity to small conformational changes, these methods lend themselves well to studies of phospholipid motion and structure [these methods have been adequately reviewed: Seelig (1977, 1978) and Mantsch et al. (1977)]. Previous ^2H and ^{31}P investigations have studied various components of biological membranes. The phospholipids phosphatidylcholine and phosphatidylethanolamine (Seelig, 1977; Stockton et al., 1976), cholesterol (Stockton & Smith, 1976; Oldfield et al., 1978; Brown & Seelig, 1978), and fatty acyl esters (Valic et al., 1979) as well as cerebrosides (Skarjune & Oldfield, 1979) have been examined.

In this laboratory, the chemical synthesis and properties of phosphatidylserine have been studied, leading to the synthesis

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[1] Abbreviations used: DPPS, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoserine; DMPS, 1,2-dimyristoyl-*sn*-glycero-3-phosphoserine; DOPS, 1,2-dioleoyl-*sn*-glycero-3-phosphoserine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DEPS, 1,2-dielaidoyl-*sn*-glycero-3-phosphoserine; DSC, differential scanning calorimetry.

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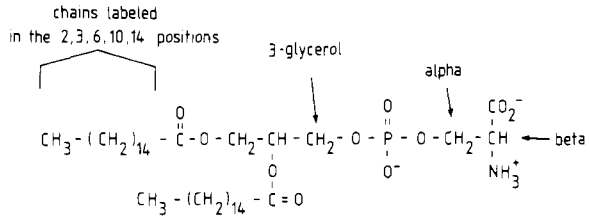

FIGURE 1: Labeled positions in 1,2-dipalmitoyl-*sn*-glycero-3-phosphoserine. The dipalmitoyl derivative is shown; however, head group labeled compounds were only made with either dimyristoyl or dioleoyl chains. Chain-labeled compounds were labeled in *both* chains.

of some eleven different specifically deuterated derivatives (Browning & Seelig, 1979). A comparison of the phosphatidyl fatty acyl chain ^2H NMR data with data from several phosphatidylcholine and phosphatidylethanolamine molecules and a natural system has been published (Seelig & Browning, 1978). Here the data were normalized to account for the various phase transition temperatures of the lipids. It was shown that a characteristic order profile for fatty chains exists in all of the phospholipid systems studied to date (including phosphatidylserine) and that the data agree even quantitatively. Additionally, a bend exists in the *sn*-2 chain, such that the first segments are oriented parallel to the membrane surface. This characteristic order profile and the bend in the *sn*-2 chain appear to be general features of phospholipid bilayers.

This paper extends this previous work on the fatty acyl chains in that the data are more fully presented and discussed. The bulk of the paper, however, concerns the comparison of phosphatidylserine with other phospholipids at the glycerol backbone, phosphorus, and head group positions. The deuterium and phosphorus-31 NMR data presented here represent the first such data on a *charged* phospholipid head group and allow the comparison of various phospholipid head groups on a structural and motional basis.

Experimental Section

Figure 1 diagrams the structure of phosphatidylserine and indicates the deuterated positions discussed in this paper.

*Syntheses. (1) Chain-Deuterated 1,2-Dipalmitoyl-sn-glycero-3-phospho-*L*-serine.* The acid chlorides of fatty acids separately deuterated in the 2, 3, 6, 10, and 14 positions [made by Kolbe electrolysis (Nguyên-Dinh-Nguyên, 1968)] were prepared with a thionyl chloride–dimethylformamide method and used to acylate 3-*O*-benzyl-*sn*-glycerol as described (Sowden & Fischer, 1941). The compound was debenzylated and phosphorylated to yield the corresponding phosphatidic acid (Baer, 1951). Coupling of phosphatidic acid to *N*-carbobenzoxy-*O*-benzyl-L-serine using triisopropylsulfonyl chloride was accomplished according to Aneja et al. (1970). Deprotection and purification over oxalated silica gel and DEAE-cellulose (Rouser et al., 1969) yielded pure DPPS (mp 155–158 °C; free acid). IR spectra were identical with those of authentic DPPS plus the addition of the two C–D bands at 2100 and 2180 cm^{-1}.

*(2) [2-^2H]Serine and [3-$^2H_2$]Serine Derivatives of 1,2-Dimyristoyl- and 1,2-Dioleoyl-sn-glycero-3-phospho-*DL*-serine.* Syntheses have been described (Browning & Seelig, 1979). Several improvements have been incorporated. The protected–coupled DMPS derivative could be quantitatively extracted with diethyl ether by first dissolving the coupling reaction mixture in a small amount of $CHCl_3$, followed by precipitation of triisopropylsulfonic acid with ether. The precipitate was redissolved in $CHCl_3$ and again precipitated with ether. The ether extracts were combined and concentrated. The derivative was then chromatographed as described. The *tert*-butoxycarbonyl protective group was removed by dissolving the thoroughly dried phosphatidylserine–*N*-*tert*-butoxycarbonyl barium salt in dry $CHCl_3$, followed by the addition of an equal volume of trifluoroacetic acid. After 10 min the reaction mixture was diluted with ether and extracted 3 times (2 volumes) with iced water. The phosphatidylserine in the ether phase was concentrated and purified. DMPS was previously purified by CM-cellulose chromatography; however, due to the large volumes of solvents required, this compound was purified by preparative thin-layer chromatography in $CHCl_3$–methanol–acetic acid–H_2O (90:40:12:2). The DMPS band was removed and extracted 3 times with a mixture of 100 mL of $CHCl_3$–methanol–H_2O (65:25:4) and 10 mL of 1 M HCl. The combined organic phases were filtered through $NaHCO_3$ and concentrated. DMPS thus obtained was treated with Dowex and the sodium salt was formed (as described in the previous report), followed by precipitation from $CHCl_3$–methanol with acetone. Gas chromatographic analysis of the fatty chains of the various DOPS derivatives indicated an oleic acid content of over 96% in the final products.

*(3) 1,2-Dipalmitoyl-sn-rac-[3-^2H]glycero-3-phospho-*L*-serine.* 1,2-Isopropylidene-*rac*-[3-^{2}H]*sn*-glycerol was synthesized as described (Browning & Seelig, 1979) and converted to 1,2-dipalmitoyl-*sn*-glycero-3-phosphoric acid and then to DPPS as described above.

*(4) 1,2-[9,10-$^2H_2$]Dioleoyl-sn-glycero-3-phospho-*L*-serine.* 1,2-[9,10-$^2H_2$]Dioleoyl-*sn*-glycero-3-phosphocholine was synthesized by acylation of $CdCl_2$–glycero-3-phosphocholine as described by Gupta et al. (1977) [synthesis of [9,10-$^2H_2$]oleic acid: Seelig & Waespe-Šarčevič (1978)]. Exchange of choline for L-serine using phospholipase D as described (Confurius & Zwaal, 1977) yielded the product. The optical rotation of DOPS has been measured previously [Browning & Seelig, (1979): first entry of Table I of this reference; due to an error this entry is labeled as DMPS].

*(5) 1,2-Dimyristoyl-sn-glycero-3-phospho[3-^2H]-*L*-serine.* [3-$^2H_2$]DL-Serine [3 g, prepared as described by Browning & Seelig, (1979)] was reacted with chloroacetyl chloride as described (Greenstein, 1957). Following reaction, the solution was titrated to pH 7.0 and the entire solution was concentrated under reduced pressure to a thick oil. The oil was extracted twice with 100 mL of hot 2-propanol. The combined 2-propanol extracts were separated from various salts by filtration and concentrated to an oil. The oil was extracted twice with hot ethyl acetate (total 100 mL), and the extracts were pooled and concentrated. The resulting oil slowly crystallized at room temperature. The crystalline mass was stirred with diethyl ether and the crystals were collected: yield 2.9 g (57%) of *N*-(2-chloroacetyl)-DL-serine. Optical resolution of 5.1 g of acetylated DL-serine was achieved with amino-acid acylase (Greenstein, 1957). After reaction with the enzyme, the solution was treated with charcoal, 20 mL of 1 N HCl was added, and the solution was concentrated under reduced pressure. The oily mixture was dried by repeatedly azeotroping with benzene and extracted with 95% ethanol (100 mL). The ethanol extract was separated from salts and concentrated. Addition of 200 mL of acetone resulted in a precipitate which was collected and directly converted to *N*-(*tert*-butoxycarbonyl)-L-serine dicyclohexylamine salt: yield 2.5 g (46%). Conversion to *N*-(*tert*-butoxycarbonyl)-L-[3-$^2H_2$]serine *O*-phthalimidomethyl ester and coupling to 1,2-dimyristoyl-*sn*-glycero-3-phosphoric acid (Browning & Seelig, 1979) yielded the product. The protected serine derivative contained at least 86% L-serine: $[\alpha]^{22}_D$ +6.2° (*c* 7, $CHCl_3$) (lit. $[\alpha]^{22}_D$ +8.5°) (Browning & Seelig, 1979). The final product has the optical

Table I: Phase Properties of Phosphatidylserines

PS	phase transition T_c (°C)	phase transition ΔH (kcal/mol)	conditions	bilayer structure	techniques	ref
DPPS	53[a]	9.0 ± 0.5	0.1 M NaCl; buffer, pH 7.0; EDTA	lamellar	DSC, ^{2}H-^{31}P NMR, Tempo partitioning	*d*
DPPS	51		0.2 M NaCl; buffer, pH 7.2; EDTA		Tempo partitioning	*e*
DPPS	53	3.0[b]	1 equiv of NaOH; preheating to 90 °C		DSC, turbidity	*f*
DPPS			anhydrous	lamellar to hexagonal II phase transition	X-ray diffraction	*g*
DMPS	36[a]	7.0 ± 0.5	0.1 M NaCl; buffer, pH 7.0; EDTA	lamellar	DSC, ^{2}H-^{31}P NMR, Tempo partitioning	*d*
DMPS	36	8.0 ± 0.5	0.1 M NaCl; buffer, pH 6.0; EDTA		DSC	*h*, *i*
DEPS	22				DSC	*j*
DOPS	−11[a]	8.8 ± 0.5	0.1 M NaCl; buffer, pH 7.0; EDTA	lamellar	DSC, ^{2}H-^{31}P NMR	*d*
DOPS	−7	7.5	0.15 M NaCl; buffer, pH 9.5; EDTA		DSC	*k*
brain[c] PS	7	4.5 ± 0.5	0.1 M NaCl; buffer, pH 7.4; EDTA		DSC	*l*
brain PS			hydrated NA salt	lamellar	X-ray diffraction	*m*, *n*

[a] DSC data; midpoint of integrated transition. [b] Very broad transition; sample probably decomposed due to preheating to 90 °C. [c] Fatty acid composition: 49% 18:0; 37% 18:1; 14% higher unsaturated. [d] This paper. [e] Luna & McConnell (1977). [f] MacDonald et al. (1976). [g] Williams & Chapman (1970). [h] van Dijck et al. (1978). [i] Mombers et al. (1979). [j] Confurius & Zwaal (1977). [k] van Dijck (1979). [l] Portis et al. (1979). [m] Papahadjopoulos & Miller (1967). [n] Hauser et al. (1977).

rotation of $[\alpha]^{22}_D$ −6.8 to −7.4° (*c* 1, $CHCl_3$) [lit. $[\alpha]^{22}_D$ −6.0° (*c* 2, $CHCl_3$)], also indicating that the synthesized diastereomer was optically rather pure.

Sample Preparation. Mono- and disodium salts of phosphatidylserine were prepared as described (Abramson et al., 1964), and IR spectra confirmed the presence of the proper salt forms. Dispersions of phosphatidylserine were prepared by mixing with an excess of buffer: 0.05 M *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid sodium salt–Tris, pH 7.0, 0.1 M NaCl, and 1.0 mM Na_2EDTA. This buffer is used because it is free of phosphorus and therefore does not interfere with the phosphate group in ^{31}P NMR measurements. No deviation in the gel to liquid-crystal transition temperature was observed with Hepes–Tris buffer over other systems. Typically, 10 mL of buffer was added to 50 mg of lipid coated on the surface of a round bottom flask, and the mixture was vigorously vortexed at a temperature above the phase transition. When the sample was well dispersed, the lipid was centrifuged to form a pellet (DOPS required $10^5 g$ for 10 min to pellet the lipid). Only the pellet was measured. In all cases the pH was unaltered in the pellet and the lipid was in the presence of excess water. The pH in the pellet was controlled originally by the shift in the ^{31}P chemical shift of a trace of added PO_4^{2-}. This shift is pH dependent and can be calibrated. Later, plastic pH indicator sticks were used to directly measure pH in the pellet. The average pellet contained about 10 wt % lipid. The sodium salts of phosphatidylserine dispersed much more easily than the free acid [as reported by Ladbrooke & Chapman (1969)]. DPPS was dispersed as the disodium salt while DOPS and DMPS were dispersed as monosodium salts. Since the buffer (used in excess) contained 0.1 M NaCl, the salt form did not alter the experiment. It should be noted that while DPPS was used for measurements on the fatty chains, the compound is very unstable at the temperatures required for the fluid phase (60–80 °C). Two hours at this temperature was usually sufficient to cause conversion of 5–20% of the sample into mainly phosphatidic acid even at pH 7.0 under well-buffered conditions.

Measurements. Differential scanning calorimetric analyses were obtained with two different instruments: a differential thermal analysis with a Mettler TA 2000 and an adiabatic differential scanning calorimeter (Blume et al., 1976). ^{31}P NMR spectra (^{31}P frequency 36.4 MHz) were recorded on a Bruker HX-90 FT instrument with 5–10 W of broad-band proton decoupling power applied. ^{2}H NMR spectra were obtained on a Bruker WH-400-FT (^{2}H frequency 61.4 MHz) instrument operated in a quadrature detection mode. Recycle times were 0.1 (^{2}H) and 0.3 s (^{31}P).

Results

Phase Properties of Phosphatidylserine. The phase behavior of phosphatidylserine was studied by several techniques. Partitioning of the nitroxide spin-label Tempo is a relatively quick method for the determination of phase transition temperatures (Shimshick & McConnell, 1973). Figure 2 plots the *f* parameter (related to the concentration of probe in the membrane) as a function of temperature. Both of the saturated phosphatidylserine derivatives, DPPS and DMPS, exhibited sharp phase transitions very similar to published data for DPPS (Luna & McConnell, 1977), while the unsaturated DOPS showed a very broad transition despite reasonably pure (>95%) dioleoyl chains. In contrast, our calorimetric studies show that the phase transition for all three phosphatidylserine compounds including DOPS is relatively sharp (Figure 2). The phase transition temperatures of DPPS and DMPS are in agreement with values previously reported (see Table I). The reason for the rather broad Tempo partioning curve in DOPS remains unclear but a similarly broad transition curve has been observed for Tempo partitioning in unsaturated phosphatidylcholine (Schreier et al., 1978).

^{31}P and ^{2}H NMR techniques also can be used to measure the phase transition temperature. In the case of ^{31}P NMR, decreased motional freedom in the gel state results in a larger chemical shielding anisotropy, i.e., a broadening of the spectra. Increases in the chemical shielding anisotropy of 10–20 ppm were observed with phosphatidylserine at the phase transition (Figure 3). Likewise, decreased motional freedom of deuterium labels in the gel phase results in a very large broadening of the deuterium signal, the broadening appearing sharply at the phase transition. The temperatures where the ^{2}H or ^{31}P

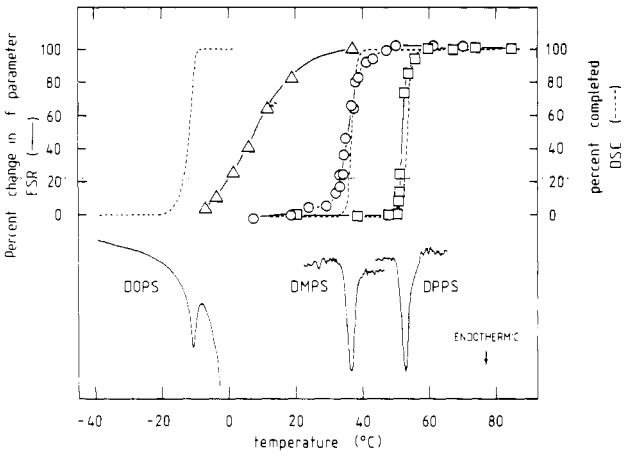

FIGURE 2: Results of Tempo partitioning and DSC experiments on phosphatidylserine bilayers. Plotted at the top is the percent change in the *f* parameter [an indicator of percent Tempo in the membrane; see Shimshick & McConnell (1973) for a discussion] as a function of temperature for three phosphatidylserine compounds (solid line): (△) DOPS; (○) DMPS; (□) DPPS. The dashed line shows percent completion of the phase transition as determined by integration of the DSC for the same three compounds. Below are shown DSC tracings for these three derivatives. DOPS was analyzed with a Mettler TA 2000 instrument with a 2 °C/min scan rate. The rapidly changing base line is due to the ice peak. DMPS and DPPS were measured with a home-built instrument (Dr. Klump, Freiburg, Germany) with a 0.23 °C/min scan rate.

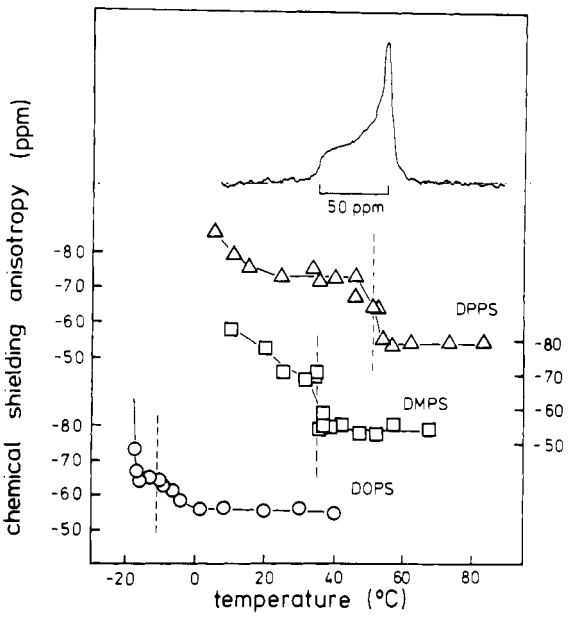

FIGURE 3: Variation of the ^{31}P chemical shielding anisotropy (in parts per million) with temperature for three different phosphatidylserines. Insert shows a typical DOPS ^{31}P NMR proton-decoupled spectrum at −5 °C (fluid state, 17 000 scans). Dotted lines indicate the gel to liquid phase transition temperatures of the various phosphatidylserines. Chemical shift anisotropies were measured by taking the line width at half the foot height (left side of the signal) and are accurate to ±1 ppm.

NMR signal changed for all labeled compounds were the same as the phase transition temperatures determined with DSC.

^{31}P NMR is a very good technique for studying phospholipid phase properties [for a review, see Seelig (1978)]. A lamellar phase is characterized by phosphorus spectra with a negative chemical shielding anisotropy, the shielding anisotropy being obtained from the separation of the spectral edges. Alternatively, with a hexagonal phase, a positive chemical shift anisotropy is observed, and spectra of this phase are readily distinguishable from lamellar phases. ^{31}P NMR spectra of bovine brain phosphatidylserine have been recorded by Kohler & Klein (1977) and Cullis & Verkleij (1979). Kohler and Klein observed a lamellar phase for their phosphatidylserine preparation with a small component of the isotropic (micellar) or hexagonal phase (maximum 10–20%), while Cullis and Verkleij also found basically a lamellar phase, but a much larger percentage (30–50%) of the second phase was present. A typical phosphatidylserine ^{31}P NMR spectrum is shown in Figure 3 and represents the lamellar phase. Such spectra were observed for all three compounds DPPS, DMPS, and DOPS at all temperatures. Unsaturated phosphatidylethanolamine compounds have a tendency to form a hexagonal phase (Cullis & de Kruijff, 1978), yet with DOPS this is not the case. The second phase present in the spectra of Cullis & de Kruijff could be due to micelles formed as a result of lysophosphatidylserine impurities. We have noticed that as little as 5–10% lysophosphatidylserine leads to the appearance of an isotropic or micellar component in the ^{31}P NMR spectra.

In Table I are collected all transition temperatures and transition enthalpies available for pure phosphatidylserine. The transition temperatures of DMPS and DPPS are 11–13 °C higher than those of the corresponding phosphatidylcholine derivatives and 11–13 °C lower than those of the corresponding phosphatidylethanolamines (Chapman, 1975). Thus, although there is variation in the absolute values of the transition temperatures of these phospholipids, the difference in T_c between the 14- and 16-carbon derivatives of phosphatidylserine (ΔT_c = 17 °C) is very similar to the differences in the corresponding phosphatidylcholines (ΔT_c = 18 °C) and phosphatidylethanolamines (ΔT_c = 14 °C). DOPS behaves somewhat differently in that its transition temperature lies 11 and 5 °C above both 1,2-dioleoyl-*sn*-glycero-3-phosphocholine and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine, respectively.

The transition enthalpies for the various phosphatidylserines appear to be slightly larger than those observed for other phospholipids with similar chain lengths. The values of 7.0 and 9.0 kcal/mol for DMPS and DPPS, respectively, are larger than the 6.6 and 8.6 kcal/mol for the corresponding phosphatidylcholines and 6.5 and 8.6 kcal/mol for the corresponding phosphatidylethanolamines (Chapman, 1975). The value of 8.0 kcal/mol previously obtained for DMPS is also larger than that of DMPC (Mombers et al., 1979). In all three cases an incremental change in the transition enthalpy of about 0.5 kcal/mol per CH_2 segment is observed. The value of 8.8 kcal/mol obtained for DOPS is in good agreement with the 8.3 kcal/mol observed with 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (Chapman, 1975) but not with a distinctly smaller value of 4.5 kcal/mol given for 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (van Dijck et al., 1976). In general, the thermodynamic properties of negatively charged phosphatidylserines are not appreciably different from those observed for the zwitterionic phosphatidylcholines or -ethanolamines.

Fatty Acyl Chain Region. The ^2H NMR spectrum of a selectively deuterated saturated fatty chain consists of a doublet, the separation of the two lines being the quadrupole splitting or quadrupole coupling. A typical spectrum for a DPPS molecule deuterated at the C-10 position in both chains is shown in Figure 4. Quadrupole couplings for various positions and temperatures are presented in Table II. The observed quadrupole splitting is related to the C–D bond order parameter according to $S_{CD} = {}^4/_3\Delta\nu(e^2qQ/h)^{-1}$ where $\Delta\nu$ is the observed quadrupole splitting and e^2qQ/h is the static deuteron coupling constant (170 kHz). The "ordering" is found to decrease along the chain, corresponding to the normal

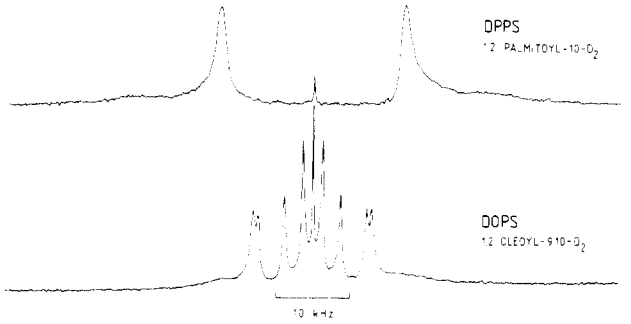

FIGURE 4: Deuterium NMR spectra of unsonicated multilayers of chain-deuterated phosphatidylserines. The center signal is due to the natural abundance of deuterium in water. The C-10-DPPS spectrum was recorded at 59 °C with 10015 scans, while the [9,10-$^2H_2$]DOPS spectrum was recorded at 11 °C with 37419 scans.

Table II: Residual Quadrupole Splittings, $\Delta\nu_Q$ (kHz), for Various Positions in Phosphatidylserine (Powder Spectra)

position[a]	DOPS −5 °C	DOPS 30 °C	DMPS 40 °C	DMPS 60 °C	DPPS 55 °C	DPPS 70 °C
serine head group						
β-^{2}H	15.3	13.2	15.5	13.0		
α-$^2H_2$: ^2H_a[b]	1.1	0.9	4.1	3.4		
α-$^2H_2$: ^2H_b	17.1	12.3	13.7	10.9		
glycerol backbone						
rac-3-^{2}H[c]	26.5	25.0			27.6	26.7
fatty acyl chains						
2-$^2H_2$ chain 1[d]					28.2	25.4
2-$^2H_2$ chain 2: ^2H_a					13.8	13.3
^2H_b					19.0	17.0
3-$^2H_2$					28.1	24.4
6-$^2H_2$					28.6	25.2
10-$^2H_2$					26.1	20.7
9,10-$^2H_2$ chain 1: 9[e]	16.6	14.5				
10	8.1	6.5				
9,10-$^2H_2$ chain 2: 9	15.2	13.4				
10	2.8	2.4				
14-$^2H_2$					17.9	13.1

[a] Cf. Figure 1. [b] Assignment of the two deuterons is arbitrary; data are presented for the L,L diastereomer except for ^2H_a DOPS, where the average is given. [c] Average of two closely spaced signals. [d] For the assignment to chain 1 or 2, see Seelig & Seelig (1975). Assignment as ^2H_a or ^2H_b is arbitrary. [e] Assignment to the two chains and to the 9 and 10 positions is explained in the text.

order profile observed in all phospholipids studied to date. This comparison has been previously published (Seelig & Browning, 1978). The temperature dependence of the order parameter, S_{CD}, is shown in Figure 5 and to a first approximation is similar to that of DPPC (Seelig & Seelig, 1974). The ^{2}H NMR spectrum of DPPS labeled in the 2 position of both chains exhibits three signals due to the inequivalence of the two chains. This observation has been previously discussed and is a result of a bend in the *sn*-2 chain (Seelig & Seelig, 1975; Seelig & Browning, 1978).

A spectrum of DOPS labeled at the cis double bond (9,10 position) of both chains is shown in Figure 4. Normally, the two deuterons of a CD_2 group are equivalent and only one quadrupole splitting is observed, but at the 9,10 position, the average angle between the axis of motional averaging and the C–D bond vectors is different for the 9 and 10 deuterons. Because of this orientational inequivalence, the two deuterons are observed as separate signals (Seelig & Waespe-Šarčevič, 1978). Additionally, at this position, differences between the two chains are observed giving rise to four separate signals. The signals in Table I for this position have been assigned to

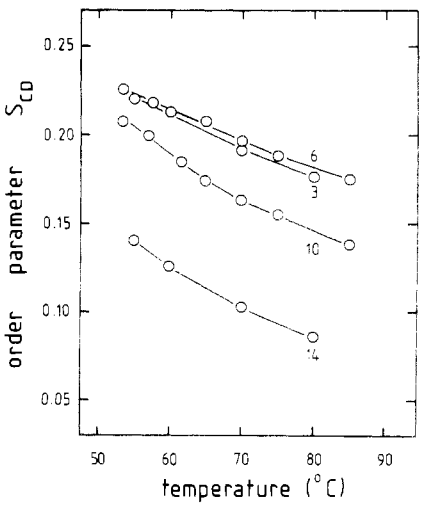

FIGURE 5: Temperature dependence of the order parameter $|S_{CD}|$ of DPPS bilayers labeled at the 3-, 6-, 10-, and 14-carbon atoms of both palmitoyl chains.

the *sn*-1 and *sn*-2 chain by comparison with 1-palmitoyl-2-oleoylphosphatidylcholine where a [9,10-$^2H_2$]oleic acid labeled phosphatidylcholine exhibited quadrupole splittings of 15.8 (9) and 3.6 (10) kHz for the two deuterons at 0 °C.

Glycerol Backbone. The quadrupole splittings of DPPS and DOPS labeled at the C-3 segment of the glycerol backbone were in the range of 25–28 kHz (cf. Table I), and the spectra were found to contain considerable fine structure. Very similar spectra and quadrupole splittings were observed previously for DPPC labeled at the same backbone position (Gally et al., 1975). The detailed analysis of these spectra will be subject of a separate manuscript.

Head Group Region. ^{31}P NMR data are useful not only for studying the phase behavior of phospholipids but also for examining the structure and motion of the phospholipid head group. A phosphorus spectrum of DOPS is shown in Figure 3. This spectrum is typical of a phosphorus whose motion is both anisotropic and axially averaged, and the shape of the spectrum is identical with the shape of spectra of saturated chain derivatives of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylglycerol. The phosphorus chemical shielding anisotropies of pure DPPS, DMPS, and DOPS as a function of temperature are presented in Figure 3. The chemical shielding anisotropies below the phase transition are only approximate, because the available proton decoupling power was too small to give a perfect proton-decoupled spectrum. In all cases, a chemical shift anisotropy of −55 ppm was observed for phosphatidylserine in the fluid phase. This is the largest value observed to date for a phospholipid [for comparison: phosphatidylcholine, −45 ppm (Gally et al., 1975); phosphatidylethanolamine, −38 to −40 ppm (Seelig & Gally, 1976); phosphatidylglycerol, −41 ppm (Wohlgemuth et al., 1980); cardiolipin, −30 ppm (Cullis et al., 1978)]. Earlier work with bovine brain phosphatidylserine has also shown a large chemical shift anisotropy of −50 (Kohler & Klein, 1977) and −53 ppm (Cullis & Verkleij, 1979). This difference is quite large as substantial changes in the chemical shielding anisotropy are generally not observed even with large external perturbations. For example, the addition of an equimolar amount of cholesterol has no effect on the chemical shift anisotropy (Brown & Seelig, 1978). Similarly, an equal amount of cytrochrome *c* oxidase decreased the chemical shift anisotropy of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine by only 5 ppm (Seelig & Seelig, 1978). Below the phase transition the head group motion is decreased as indicated by

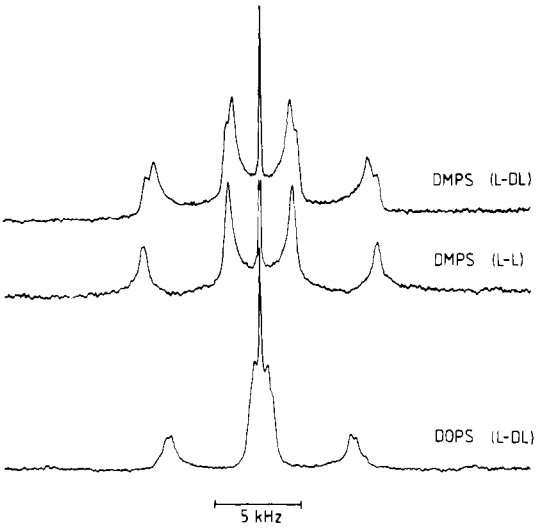

FIGURE 6: Comparison of the deuterium NMR spectra of various phosphatidylserines deuterated at the α position of the head group (P–O–CD_2–CH). The letters in parentheses refer to the diastereomer, i.e., L,DL is 1,2-dimyristoyl-*sn*-glycero-3-phospho-DL-serine. Spectra were recorded at 42 °C and 22900 scans [DMPS (L,DL)], 42 °C and 10000 scans [DMPS (L,L)], and 42 °C and 15800 scans [DOPS (L,DL)].

a sharp increase of 10–20 ppm in the chemical shift anisotropy.

The nomenclature of α and β for the positions in the phosphatidylserine head group (see Figure 1) has been employed to facilitate comparison with earlier studies on phosphatidylcholine and phosphatidylethanolamine and work on phosphatidylglycerol where the designations α, β, and γ are especially convenient. Representative ^2H NMR spectra of α-CD_2-DMPS and α-CD_2-DOPS are shown in Figure 6. All spectra consist of two quadrupole splittings, one with a large separation of 13–17 kHz and the other with a much smaller separation of 1–4 kHz (cf. Table II). For the racemic diastereomer L,DL-DMPS [i.e., 3-*sn*-L-glycerol and DL-serine], each of the two major signals was found to be further subdivided into two closely spaced signals; for racemic DOPS these finer separations were resolved only as shoulders on one of the major peaks. In contrast to the two closely spaced signals observed with the racemic serine labeled compounds, no such splitting was found for the pure diastereomer α-CD_2-L,L-DMPS. Thus, it can be concluded that the fine structure of the α position results from the physical inequivalence of the two diastereomers L,L-and L,D-phosphatidylserine. The two major signals are believed to arise from the inequivalence of the two deuterons as discussed below. The unequal intensity of the two signals is a result of the experimental conditions employed. Due to insufficient radiofrequency power, resonances nearest the pulsing frequency will be most intense, with quadrature detection; these are the resonances closest to the water signal at the center of the spectrum. The α-CD_2-DMPS and α-CD_2-DOPS spectra are similar, only the difference between the two signals is larger with DOPS.

^2H NMR spectra of β-CD_1-L,DL-DMPS and β-CD_1-L,DL-DOPS were similar with only one quadrupole coupling being observed (Figure 7). The presence of only one quadrupole coupling despite the racemic nature of the compound indicates that the two diastereomers are "equivalent" at this position. Figure 8 shows the temperature dependence of the head group deuterium quadrupole couplings for DOPS and DMPS. In both cases a transition from a sharp well-resolved spectrum to a loss of signal occurred exactly at the gel to liquid phase transition temperature. Below this critical temperature, the head group is apparently locked into a rigid conformation

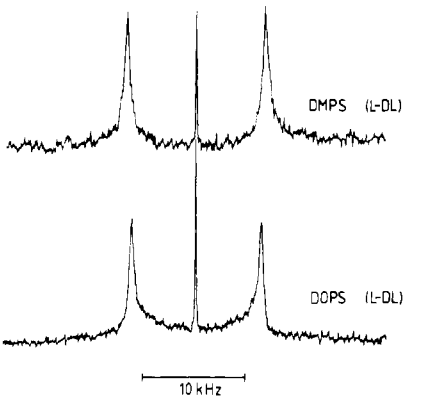

FIGURE 7: Deuterium NMR spectra of phosphatidylserine deuterated at the β position of the head group (P–O–CH_2–*CD*–). Spectra were recorded at 52 °C and 2900 scans (DMPS) and 27 °C and 5700 scans (DOPS).

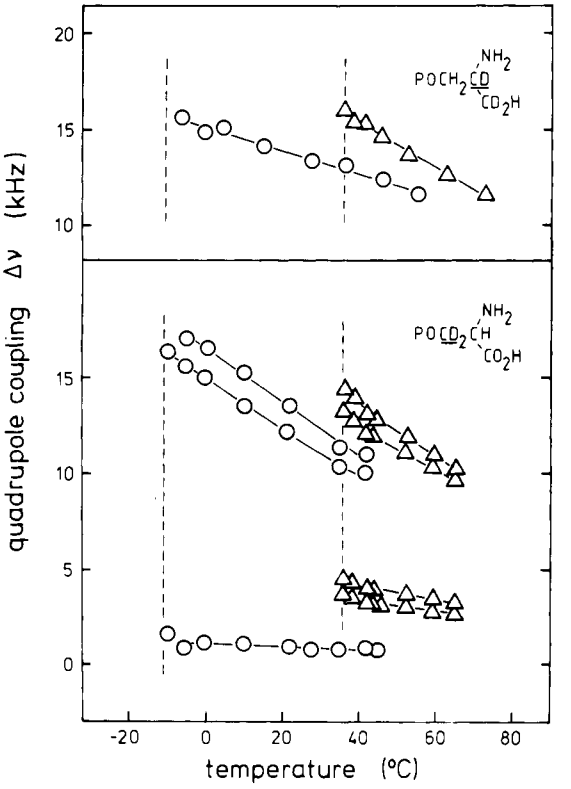

FIGURE 8: Variation of the deuterium quadrupole coupling of head group deuterated DMPS (Δ) and DOPS (O) with temperature. Gel to liquid transition temperatures are indicated with dotted lines. Data are given for both diastereomers (L,L and L,D) except for the smaller DOPS signal where adequate resolution was not obtained and the average quadrupole splitting is plotted. Comparison with Figure 7 shows that the signals with the larger quadrupole couplings are associated with the L,L diastereomer.

which coupled with a decrease in the reorientation rate leads to very broad line widths and the signal cannot be detected under our measuring conditions. This is contrasted with DPPC, where the head group deuterium signals can be observed below the phase transition (Gally et al., 1975).

Discussion

The differences in the phase transition temperatures of the various phospholipids, while relatively large on a physiological scale, are thermodynamically quite small. The phase transition temperature, T_c, is given by $T_c = \Delta H/\Delta S$, where ΔH and ΔS are the transition enthalpy and entropy, respectively. It can

be readily seen that a 20 °C change in T_c at physiological temperatures can be the result of only a 5% change in either ΔH or ΔS. In the case of phosphatidylserine, ΔH values were found to be about 0.5 kcal/mol larger than those of the corresponding phosphatidylcholine.

From the deuterium order parameters, it can be concluded that phosphatidylserine in the fatty acyl chain region is quantitatively similar to phosphatidylcholine. The chain order profiles allow a calculation of the bilayer thickness (Seelig & Seelig, 1974). This calculation is based upon determination of the probabilities for gauche or trans conformations at each segment from the order parameters. Bilayer thicknesses for the hydrocarbon region of 34.8, 34.3, and 33.1 Å at 55, 60, and 80 °C, respectively, were calculated[2] by using the data in Figure 5 and extrapolating to those segments where no data are available. The all-trans state has a thickness of 45.9 Å (measured from the ester bonds), thus the difference of these two values is the expected transbilayer contraction of a DPPS bilayer undergoing a gel to liquid-crystal transition. Extrapolation of the above bilayer thicknesses back to the temperature of the phase transition yields a value of 10.25 Å for the amount of transbilayer expansion at the phase transition. This value is identical with that obtained for DPPC. Comparison with recent neutron diffraction studies on DPPC (Zaccai et al., 1979) shows that this calculation, while exceptionally simple, is nevertheless in excellent agreement with the scattering data. On the basis of the reduction in bilayer thickness with increasing temperature, a linear thermal expansion coefficient can be defined (Seelig & Seelig, 1974). A value of -2×10^{-3} K^{-1} was found for DPPS, which is slightly smaller than that of -2.5×10^{-3} K^{-1} calculated for DPPC.

The similarity of the order parameter profile, the bend in the *sn*-2 chain, the state of the cis double bond in the oleic acid containing derivative, the calculated bilayer thickness, the transbilayer expansion coefficient, and the phase transition enthalpies all strongly indicate that the hydrocarbon region of phosphatidylserine bilayers is similar to those of the zwitterionic phosphatidylcholine analogues. Thus, it appears that the charged head group alone does not exert a strong influence on the properties of the hydrocarbon region in the fluid state.

A similar conclusion can be reached for the glycerol backbone, at least as far as the *sn*-3 segment is concerned. The deuterium NMR spectra of DMPS and DOPS deuterated at this segment are almost identical with those of DPPC (Gally et al., 1975). The large quadrupole splitting of about 27 kHz at this position is indicative of a rather rigid glycerol backbone, which has been further substantiated by the observation of relatively short T_1 relaxation times. A T_1 relaxation time of 13 ms at 51 °C indicates that the motion of the glycerol *sn*-3 segment is reduced by almost a factor of 3 compared to the adjacent fatty acyl chain segments [$T_1 \simeq 33$ ms; cf. Brown & Seelig (1979)] even though the ordering (as measured by the quadrupole splitting) is practically identical. The spectra contain an additional complexity due to some motional inequivalence of the two deuterons. This will be discussed in a separate publication.

Let us now turn to a discussion of the ^{31}P and ^{2}H NMR head group data. The major feature of the phosphatidylserine phosphorus NMR spectra is the large chemical shielding anisotropy, which is independent of the chemical nature of the fatty acyl chains. Since the static shielding tensor of phosphatidylserine is not significantly different from that of phosphatidylcholine or phosphatidylethanolamine (Kohler & Klein, 1977), this difference indicates that the phosphatidylserine phosphate segment is conformationally or motionally different from the other phospholipids. ^{31}P T_1 relaxation times have been measured for phosphatidylserine (Browning and Seelig, unpublished experiments) and have been found to be dramatically shorter than those found for the other phospholipids. This suggests that the phosphatidylserine head group is more rigid than that of the other phospholipids, which would be in agreement with a larger chemical shielding anisotropy.

The most striking characteristic of the phosphatidylserine head group ^{2}H NMR spectra is the presence of two widely separated signals in the spectrum of α-CD_2-phosphatidylserine. The unusual feature here is more the large difference in the *size* of the two quadrupole splittings than the fact that *two* splittings are observed. Indeed, two signals at the α-CD_2 head group position are characteristic of all the natural lipids studied to date. For phosphatidylcholine and phosphatidylethanolamine, the splittings are close together and are difficult to resolve. Reexamination of α-CD_2-DPPC and α-CD_2-DPPE at 61.4 MHz showed two closely spaced signals which had not been separated in our earlier studies. With DPPC, proton decoupling sufficiently sharpened the line width to resolve the previously observed signal (quadrupole coupling 6 kHz) into two signals with quadrupole couplings differing by about 0.3 kHz. With DPPE a difference of 0.5–0.6 kHz could be observed between the quadrupole couplings of the two signals without the need for proton decoupling (Akutsu and Seelig, unpublished data). Even better resolution of the two α-CD_2 signals was achieved for phosphatidylglycerol. Spectra of 1,2-dipalmitoyl-*sn*-glycero-3-phospho-3′-glycerol labeled in the α-CD_2 group showed two signals with a difference of 2 kHz. When the same lipid was stereospecifically deuterated with just one deuteron, only one signal was obtained. Thus, here the two quadrupole couplings can be unambiguously shown to arise from the motional inequivalence of the two deuterons (Wohlgemuth et al., 1980). By analogy, we conclude that in the α-CD_2 position of phosphatidylserine the two signals also originate from the two deuterons. Thus, the inequivalence of the two α-CD_2 deuterons is a common characteristic of all four phospholipid classes investigated.

Only one signal is observed at the β position of DOPS and DMPS as is the case in phosphatidylcholine, -ethanolamine, and -glycerol. In an earlier study (Gally et al., 1975), a progression in the DPPC head group quadrupole couplings of 6, 4, and 1 kHz for the α, β, and γ positions, respectively, was found. The quadrupole splittings decreased in a manner crudely analogous to that observed in the fatty chains, and this data was interpreted in terms of increasing flexibility as one progressed from the phosphorus toward the quaternary amine. By comparison, the DMPS quadrupole couplings are 15 kHz at both the α and β positions. In view of the very large coupling at the β position, a model of increasing flexibility along the head group is unlikely for phosphatidylserine.

^{2}H NMR T_1 relaxation measurements of selectively deuterated phospholipid head groups provide direct information on the relative flexibility of the various head groups. These measurements (Browning and Seelig, unpublished experiments) have shown that the deuterons in the phosphatidylserine head group have much shorter T_1 relaxation times than the corresponding deuterons in either the phosphatidylcholine or phosphatidylethanolamine head groups. This is strong evidence

[2] In this calculation a value of 3.83 Å per monolayer is added to the chain length as calculated according to Seelig & Seelig (1974, eq 8). This value arises from the contribution of the O–CO ester linkage, the terminal C–H bond, and the van der Waals radius due to the terminal methyls at the junction of the two bilayer halves.

indicating a rigid head group for phosphatidylserine. A rigid head group structure is probably a result of electrostatic interactions or hydrogen bonding between or within the phosphatidylserine head groups. Experiments with DMPS–DMPC mixtures substantiate this concept and suggest that these interactions are intermolecular. Dilution of DMPS with neutral DMPC results in a qualitatively identical α-CD_2-DMPS spectrum, i.e., two signals as in pure DMPS, except that the two quadrupole couplings have been reduced by the same factor ($\sim$40% at 30:70 DMPS/DMPC). The α-CD_2-DMPS quadrupole couplings decrease to values similar to those observed in phosphatidylcholine and phosphatidylethanolamine head groups. This suggests that dilution of negatively charged DMPS with neutral DMPC removes these interactions and allows for greater freedom of motion of the phosphatidylserine head group.

The inequivalence of the two diastereomers L,L and L,D of DMPS and DOPS represents the first direct demonstration of differing head group structures for two phospholipid diastereomers. The quadrupole couplings of the two phosphatidylserine diastereomers differ by only 0.9 kHz (larger of the two signals). This difference when interpreted with a geometric model [like Seelig et al. (1977)] could be explained by rather small conformational differences. The X-ray crystal structures of DL-serine *O*-phosphate and L-serine *O*-phosphate differ by 10–15° at most of the torsional angles (Sundaralingam & Putkey, 1970; Putkey & Sundaralingam, 1970). The fact that the asymmetric carbon (β-carbon) manifests its chirality at the α-CD_2 position but not at the β-CD_1 position even though the β deuteron resides directly on the asymmetric center is unusual. Several explanations could be proposed, but this phenomenon remains poorly understood at this time.

In conclusion, we feel that the concept of a relatively rigid phosphatidylserine head group is supported by the following points. (a) The quadrupole couplings observed in the α and β positions are large relative to those of phosphatidylcholine and phosphatidylethanolamine. (b) The dramatic inequivalence of the two α-CD_2-phosphatidylserine deuterons requires a relatively rigid structure. (c) Observation of different structures for the L,L and L,D diastereomers indicates that there is insufficient motion to average these similar structures. (d) The ^{31}P and ^{2}H T_1 relaxation times of phosphorus and deuterium in the phosphatidylserine head group are dramatically shorter than in the phosphatidylcholine or phosphatidylethanolamine head groups. ^{2}H quadrupole coupling constants and ^{31}P chemical shift anisotropies are influenced by both motional and geometrical considerations. The DPPC head group has been interpreted with a quantitative model using mainly geometrical considerations (Seelig et al., 1977). Now, following analysis of the phosphatidylcholine, -ethanolamine, -glycerol, and -serine head groups, studies on several simpler model compounds, and reanalysis using more sensitive NMR spectrometers, it is clear that simple geometrical or motional models alone are insufficient to account for the data and that a more complex combination of these two concepts is necessary. A comprehensive examination of ^{31}P T_1 relaxation times as well as ^{2}H T_1 relaxation times of selectively deuterated phospholipid head groups provides a much better understanding of head group motion. For these reasons, a quantitative discussion of the head group data will be presented in combination with the T_1 relaxation time data.

We hope that this basic information on phosphatidylserine will allow us to extend our studies to include interactions with proteins in biological systems. Presently, interactions with divalent cations and local anesthetics are being investigated.

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