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Phospholipids Chiral at Phosphorus. Properties of Small Unilamellar Vesicles of Chiral Thiophosphatidylcholine†

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ABSTRACT: The recent observation of the differences in the biophysical properties between 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and the R_P, S_P, and $R_P + S_P$ isomers of 1,2-dipalmitoyl-*sn*-glycero-3-thiophosphocholine (DPPsC) in the multilamellar phase [Tsai, M.-D., Jiang, R.-T., & Bruzik, K. (1983) *J. Am. Chem. Soc. 105*, 2478–2480] prompted us to investigate the biophysical properties of the small unilamellar vesicles (SUV) of the above phospholipids. It was found that DPPC and DPPsC isomers showed approximately the same critical micelle concentrations and formed spherical SUV upon injection of their ethanolic solutions into an aqueous solution. However, the average sizes of the SUV of DPPsC isomers were significantly greater than that of DPPC prepared under the same conditions, as shown by their electron micrographs. The results of both ^{31}P NMR line widths and the ratios of the entrapped solute to the total phospholipids further supported the following order in the average radius of the SUV: (S_P)-DPPsC > $(R_P + S_P)$-DPPsC > (R_P)-DPPsC > DPPC. Complete lysis of the SUV by melittin was demonstrated in all four cases. The DPPsC isomers showed gel–liquid-crystal transition temperatures of 43.8 ± 0.1 °C, which are considerably higher than that of DPPC (37.9 °C) under the same conditions. In the SUV of an equimolar mixture of DPPC and $(R_P + S_P)$-DPPsC, DPPsC preferred to stay in the inner layer on the basis of ^{31}P NMR studies by use of a shift reagent $PrCl_3$. The results indicate that the SUV of thiophospholipids bear most physical and biochemical properties of that of natural phospholipids, but there are considerable differences between the properties of DPPC and DPPsC isomers. This supports the theory that the structure and configuration of the phosphate head group of phospholipids are important in the structure and properties of bilayer membranes.

Thiophospholipids, in which one of the nonbridging phosphoryl oxygen atoms of phospholipids is substituted by a sulfur atom, were first synthesized by Nifant'ev et al. (1978) and Chupin et al. (1979). Taking advantage of the 50–60 ppm downfield shift in ^{31}P NMR of thiophospholipids, Vasilenko et al. (1982) and Hui et al. (1983) have used thiophospholipids to study the phase changes of lipids in a mixture of phospholipids and thiophospholipids. However, the physical and biochemical properties of thiophospholipids have not been well characterized. Furthermore, the above authors have ignored the fact that thiophospholipids are chiral at phosphorus and have used thiophospholipids as a mixture of diastereomers that have potentially different properties.

On the basis of stereospecific hydrolysis by phospholipases A_2 and C (Bruzik et al., 1982; Orr et al., 1982), we have prepared and characterized separate diastereomers of 1,2-dipalmitoyl-*sn*-glycero-3-thiophosphocholine (DPPsC)[1] (Figure 1) (Bruzik et al., 1982, 1983) and 1,2-dipalmitoyl-*sn*-glycero-3-thiophosphoethanolamine (DPPsE) (Jiang et al., 1984). In addition, the absolute configurations of DPPsC and DPPsE have been elucidated (Jiang et al., 1984) by correlating with the configuration of chiral [^{18}O]phosphatidylethanolamines (Bruzik & Tsai, 1984).

Although DPPC and DPPsC isomers show similar conformational properties in solution on the basis of ^1H NMR, ^{13}C NMR, and ^{31}P NMR studies (Bruzik et al., 1983), their properties in the multilamellar phase are significantly different. Both the chemical shift anisotropy in ^{31}P NMR and the quadrupolar splitting in ^{14}N NMR follow the order DPPC > (S_P)-DPPsC > (R_P)-DPPsC (Tsai et al., 1983). These results suggested the potential significance of the phosphate structure and configuration on the biophysical properties of phospholipid membranes and prompted us to investigate the properties of the small unilamellar vesicles (SUV) of DPPC and DPPsC isomers.

In this paper we compare the following properties of DPPC and DPPsC isomers: critical micelle concentration (cmc), preparation, properties and relative sizes of SUV, lysis of SUV by melittin, gel–liquid-crystal transition temperature (T_m) of SUV, and the inside/outside distribution of the SUV from an equimolar mixture of DPPC and DPPsC. The significance and implication of the results are discussed.

Materials and Methods

Materials. (R_P)-DPPsC, (S_P)-DPPsC, and $(R_P + S_P)$-DPPsC were prepared as described previously (Bruzik et al., 1983; Jiang et al., 1984). The diastereomeric purity of (R_P)- and (S_P)-DPPsC was >97% as determined by ^{31}P NMR (CH_3OD, 121.5 MHz). The following biochemicals were obtained from Sigma: ascorbic acid, DPPC (synthetic, >98%

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[1] Abbreviations: AMP, adenosine 5′-monophosphate; cmc, critical micelle concentration; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPPE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; DPPsC, 1,2-dipalmitoyl-*sn*-glycero-3-thiophosphocholine; DPPsE, 1,2-dipalmitoyl-*sn*-glycero-3-thiophosphoethanolamine; DSC, differential scanning calorimetry; EDTA, ethylenediaminetetraacetate; EM, electron microscopy; HPLC, high-pressure liquid chromatography; Mops, 3-(*N*-morpholino)propanesulfonic acid; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; P_i, inorganic phosphate; $R_{o/i}$, outside/inside ratio of small unilamellar vesicles; SUV, small unilamellar vesicles; T_1, spin–lattice relaxation time; TLC, thin-layer chromatography; T_m, gel to liquid-crystal transition temperature; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

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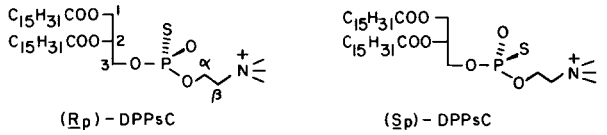

FIGURE 1: Structures of chiral thiophosphatidylcholine.

purity), AMP (sodium salt, >99% purity), and Sephadex G-50 (for gel filtration, bead size 50–150 μm). The YM-2 and XM-100A membranes were obtained from Amicon. $PrCl_3$ (99.9%) was purchased from Alfa. Other chemicals were of reagent grade or the highest purity available.

The mellitin from bee venom was obtained from Sigma and further purified (Quay & Condie, 1983) by passing through a Sephadex G-50 column (eluted with 0.02 M sodium phosphate buffer, pH 7.2). The contaminating phospholipase A_2 was eluted off first. The melittin peak was pooled and lyophilized. The final product contained <0.15 unit of phospholipase A_2 activity/mg of melittin, as assayed by the spectrophotometric method (Kupferberg et al., 1981; Bruzik et al., 1983).

Determination of cmc. The procedure of Smith & Tanford (1972) was followed, with only minor modifications. The phospholipid (DPPC or DPPsC, 7.5 mg) was first dissolved in 18 mL of methanol (HPLC grade, Fisher). The solution was mixed with 2 mL of H_2O, incubated at 20 ± 1 °C for 2 days, and filtered through an Amicon YM-2 ultrafiltration membrane (cutoff M_r 1000) under a pressure of 20 psi. The first 2 mL of the filtrate was collected, five 0.2-mL aliquots of which were used to determine the phosphate content according to the procedure of Ames (1966): the sample was first mixed with 30 μL of 10% $Mg(NO_3)_2 \cdot 6H_2O$ in 95% ethanol, dried and ashed, cooled, added with 0.3 mL of 0.5 N HCl, heated at 100 °C for 15 min, cooled, mixed with 0.7 mL of a 1/6 mixture of 10% ascorbic acid/0.42% ammonium molybdate·$4H_2O$ in 1 N H_2SO_4, incubated at 45 °C for 20 min, and quantified by measuring the absorbance at 820 nm.

Electron Microscopy of SUV. Four micromoles of DPPC or DPPsC was dissolved in 0.2 mL of absolute ethanol. The solution was injected, by a 10-μL Hamilton syringe, into 4 mL of 0.15 M NaCl, which was being stirred vigorously at 50 °C, during a period of 20 min. A small drop of the resultant SUV solution was applied to a Parlodion carbon-coated copper grid (200 mesh, sprayed with a thin film of carbon). After 1–2 min the excess solution was removed with a blotter, and the vesicles were stained with a drop of 1% phosphotungstic acid solution (pH 7.2). After another 1–2 min the grids were blotted to remove excess staining agent and allowed to dry (Sheetz & Chan, 1972). The grids were observed on a Hitachi HU-12 electron microscope and recorded at 70000× magnification.

Differential Scanning Calorimetry. DSC curves of SUV samples were measured on a MC-1 ultrasensitive scanning calorimeter (MicroCal, Inc., Amherst, MA). The samples were prepared by injecting a 20 mM ethanolic solution of lipids (1 mL) into 20 mL of 0.15 M NaCl in H_2O as described above. The larger DPPC vesicles (Figure 3b,c) were prepared by injecting more concentrated ethanolic solutions (80 mM, 0.25 mL, for Figure 3b and 160 mM, 0.125 mL, for Figure 3c) into 5 and 2.5 mL, respectively, of 0.15 M NaCl. To obtain DSC curves, 0.7 mL of the SUV solution was placed in the sample cell and 0.7 mL of a blank solution (containing the same solvents and solutes except vesicles) was placed in the reference cell. Only heating scans were obtained, from 25 to 55 °C, with a rate of 20 deg/h. The DPPC sample was used without further purification. The DPPsC samples used were extensively purified to >98% purity.

SUV Containing Entrapped AMP. The 20 mM ethanol solution of phospholipids (1.33 mL) was injected into 26.7 mL of 0.1 M AMP (disodium salt, containing 0.1 mM EDTA, pH 7.0, vigorously stirred at 50–52 °C) by a 10-μL Hamilton syringe during a period of 45 min. The resultant solution of SUV was concentrated to 1.5 mL by filtering through an Amicon XM-100A ultrafiltration membrane (cutoff M_r 100 000), loaded onto a Sephadex G-50 column (1.3 cm diameter × 30 cm height), and eluted with 0.15 M NaCl containing 0.1 mM EDTA. The elution of vesicles was monitored by UV absorption at 260 nm. The elution profiles showed a small peak within the void volume due to AMP entrapped in vesicles, followed by a large peak due to external AMP. The fractions containing vesicles were pooled together (ca. 3.5 mL), added with 1.0 mL of 0.15 M NaCl in D_2O, and transferred to a 10-mm NMR tube. All of the above procedures were performed at 45–50 °C, above the transition temperature of SUV. ^{31}P NMR spectra were taken immediately, at 121.5 MHz, 45 °C, on a Bruker WM-300 NMR spectrometer equipped with a multinuclear probe.

SUV samples for the determination of the [AMP]/[phospholipids] ratio were prepared as above but on a smaller scale (7.4 mg of DPPC or 7.5 mg of DPPsC in 0.5 mL of ethanol, injected to 10 mL of 0.1 M AMP). The fractions of the first peak were pooled together, lyophilized, and then extracted with 10 mL of $CHCl_3$ and 10 mL of H_2O. The water layer was further washed with 5 mL of $CHCl_3$. The aqueous layer contained AMP, which was quantified by A_{260}. The chloroform extract contained phospholipids, which was evaporated to dryness and redissolved in 10 mL of EtOH. Aliquots of 20 μL were used to determine the phosphate content as described above. The AMP content cannot be directly quantitated from the elution profiles because the UV light was partially scattered by SUV at low wavelengths. However, the elution profiles seem to reflect the order of vesicle sizes as described under Results: DPPC vesicles were eluted off the latest, with the smallest peak, whereas (S_P)-DPPsC vesicles were eluted off the earliest, with the largest peak.

^{31}P NMR of Mixed SUV. The vesicles were prepared in 0.15 M NaCl solution (containing 0.1 mM EDTA) as described above, except in a larger scale: 0.1 mmol each of DPPC and (R_P + S_P)-DPPsC in 10 mL of ethanol was injected into 200 mL of NaCl solution. The resulting SUV solution was concentrated to 3 mL through an XM-100A membrane, added with 1 mL of 0.15 M NaCl in D_2O, and subjected to ^{31}P NMR measurements of NOE and T_1 at 81.0 MHz, 45 °C. The NOE was determined by comparing the intensities of ^{31}P resonances under broad-band ^1H decoupling (decoupler power 2 W) and under inverse-gated ^1H decoupling, with 18 s of acquisition delay. T_1 was measured by the inversion–recovery technique.

The sample was then titrated with a 10 mM $PrCl_3$ solution (prepared by dissolving 6.174 g of $PrCl_3$ in 250 mL of H_2O, stirring vigorously, and filtering to remove residual insoluble material) until the resonances due to inner and outer layers were fully separated. The ratio $R_{o/i}$ was then measured under inverse-gated ^1H decoupling, with 17 s of acquisition delay. The final concentration of phospholipids in the NMR tube was determined by the phosphate test (Ames, 1966).

The SUV containing entrapped $PrCl_3$ were prepared from 0.08 mmol each of DPPC and (R_P + S_P)-DPPsC and 160 mL of a 0.13 M NaCl solution containing 0.01 M $PrCl_3$ (pH 6.8) by the same procedure as the preparation of SUV containing entrapped AMP. The relative instability of these SUV

Table I: Summary of Physical Properties of DPPC and DPPsC Isomers

	DPPC	(R_P)-DPPsC	(S_P)-DPPsC	(R_P + S_P)-DPPsC
cmc[a] (in 9/1 MeOH/H_2O) ($M \times 10^5$)	8.3 ± 0.7	6.9 ± 1.4	8.1 ± 0.1	7.0 ± 0.8
outer radius (Å)[b] (from EM of SUV)	115 ± 30	210 ± 70	240 ± 75	
AMP-containing SUV				
[AMP]/[phospholipids][c]	0.041 ± 0.005	0.071 ± 0.009	0.108 ± 0.025	0.082 ± 0.004
line width in ^{31}P NMR (Hz)	24	35	50	48
^{31}P chemical shift (ppm)	0.11	57.26	57.20	57.20

[a] Average of two independent sets of experiments. Each experiment involves three determinations of phosphates of a particular phospholipid. [b] The ± values represent the range of distribution in size. [c] Average of two independent sets of experiments.

(presumably caused by an osmotic pressure due to different concentrations of electrolytes inside and outside vesicles) limited the duration of ^{31}P NMR measurements and the signal/noise ratio of the resultant spectra.

Results

Critical Micelle Concentration. cmc values of amphipathic compounds depend somewhat on the methods used for their determination (Fendler, 1982). For DPPC, a value as low as 4.7×10^{-10} M has been reported (Smith & Tanford, 1972). Such a low concentration of DPPC is very difficult to measure accurately. Because our main interest is in the comparison between DPPC and DPPsC isomers, we chose to measure the cmc in a mixture of methanol and water (9/1 v/v) by ultrafiltration (Smith & Tanford, 1972; Reynolds et al., 1977). The result is summarized in Table I (first row). The cmc of DPPC is consistent with that reported by Smith & Tanford (1972) (1×10^{-4} M) under the same conditions. The DPPsC isomers seem to have the same cmc values as DPPC within the limit of experimental accuracy. Thus, DPPsC isomers show the same tendency to form micelles as the natural DPPC.

Preparation and Size of Small Unilamellar Vesicles (SUV). Single bilayer vesicles can be prepared by several different methods, as reviewed by Fendler (1982). Two widely used ones are the sonication method (Huang, 1969) and the injection method (Batzri & Korn, 1973). Because the P–S bond of thiophospholipids is a potential site of oxidation during high-energy sonciation, and because the injection method is known to produce vesicles with more homogeneous sizes (Fendler, 1982), we elected to prepare vesicles by injecting an ethanol solution of thiophospholipids (20 mM) into a well-stirred 0.15 M NaCl solution, thermostated at 50 °C.

The electron micrographs of the SUV of DPPC and DPPsC isomers show that DPPsC isomers are capable of forming spherical vesicles, but the size of DPPsC vesicles is larger than that of DPPC vesicles. The average outer radii measured from the electron micrographs are also summarized in Table I (second row). The ± values indicate the range of distribution in the size of SUV. Although (R_P)- and (S_P)-DPPsC cover approximately the same range, the average radius of S_P vesicles is larger than that of R_P vesicles. Thus, DPPsC isomers are capable of forming SUV, but the sulfur substitution and the configuration at phosphorus seem to have a significant effect on the size of the vesicles.

SUV Containing Entrapped AMP. To further investigate the properties and the relative size of the SUV of DPPC and DPPsC isomers, we prepared SUV containing entrapped AMP as described under Materials and Methods. The ^{31}P NMR spectra of these SUV samples (Figure 2a–d) show different line widths and different [AMP]/[phospholipids] ratios. Because the line width of ^{31}P NMR signals of SUV is proportional to the size of vesicles (De Kruijff et al., 1976), the observed line widths (Table I) suggest that the size of SUV falls into the order (S_P)-DPPsC > (R_P + S_P)-DPPsC > (R_P)-DPPsC > DPPC.

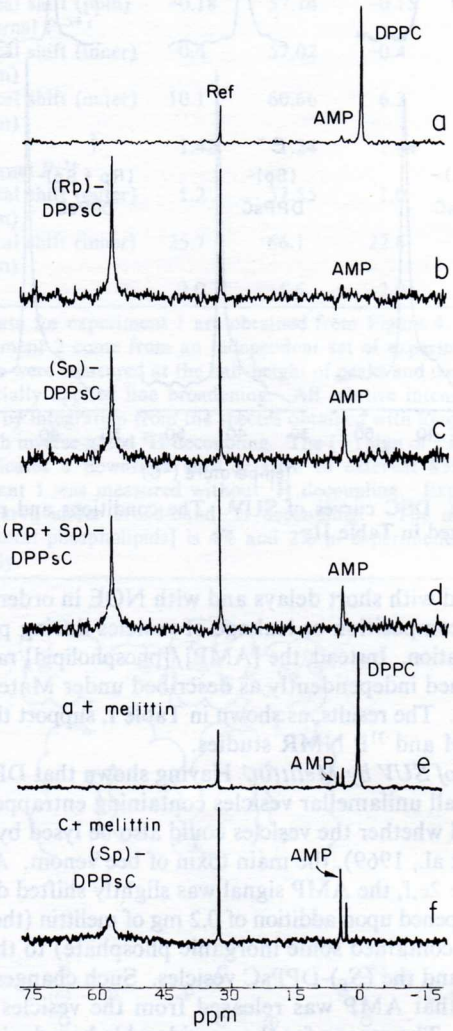

FIGURE 2: ^{31}P NMR spectra (at 121.5 MHz) of SUV containing entrapped AMP: (a) DPPC; (b) (R_P)-DPPsC; (c) (S_P)-DPPsC; (d) (R_P + S_P)-DPPsC; (e) addition of 0.2 mg of melittin to (a); (f) addition of 0.2 mg of melittin to (c). Sample conditions are described under Materials and Methods. Spectral parameters: spectral widths 11.9 KHz; acquisition time 0.69 s; pulse angle 30°; 16K data points; ^2H locked; ^1H decoupled (decoupler power 5 W); number of scans 15 600 (a–d) and 31 200 (e, f); temperature 44 °C; line broadening 15 Hz. The signal at 32.4 ppm is due to triphenylphosphine oxide (Ph_3PO) as a reference in a coaxial inner tube. The extra peak in (e) and (f) is due to a small amount of P_i present in the melittin preparation. The assignment of AMP and P_i in (e) and (f) is based on their titration curves reported by Jaffe & Cohn (1978).

The entrapped volume of SUV is also proprtional to the size of SUV (Hauser et al., 1973). Larger vesicles should have a larger ratio of [AMP]/[phospholipids] because the volume of spherical vesicles is dependent on r^3 whereas the surface area is dependent on r^2 (r is radius). The AMP content, however, cannot be determined from Figure 2 directly. Because the vesicles containing AMP were relatively unstable due to the osmotic pressure, the spectra in Figure 2 were

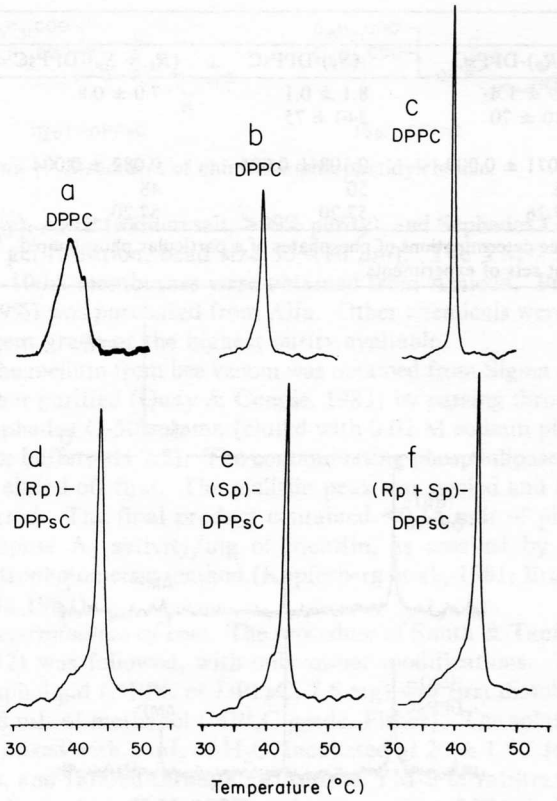

FIGURE 3: DSC curves of SUV. The conditions and results are summarized in Table II.

measured with short delays and with NOE in order to minimize decomposition or leakage of vesicles during prolonged accumulation. Instead, the [AMP]/[phospholipid] ratios were determined independently as described under Materials and Methods. The results, as shown in Table I, support the results from EM and ^{31}P NMR studies.

Lysis of SUV by Melittin. Having shown that DPPsC can form small unilamellar vesicles containing entrapped AMP, we asked whether the vesicles could also be lysed by melittin (Sessa et al., 1969), the main toxin of bee venom. As shown in Figure 2e,f, the AMP signal was slightly shifted downfield and sharpened upon addition of 0.2 mg of melittin (the melittin solution contained some inorganic phosphate) to the DPPC vesicles and the (S_P)-DPPsC vesicles. Such changes seem to suggest that AMP was released from the vesicles into the solution. The reason for the considerable broadening of the DPPsC signal is unclear.

In separate experiments, leakage of vesicles was monitored by quantitating the AMP (based on A_{260}) released from a dialysis tubing. It was found that addition of 0.1 mg of melittin to 10 mg of SUV (at 45 °C) caused complete lysis in <1 h for DPPC and DPPsC isomers. In the absence of melittin <10% of AMP was released spontaneously. However, due to the difference in the size of SUV, the high transition temperature, and the limited stability of DPPsC vesicles containing AMP, detailed kinetic study is difficult at this stage.

Gel–Liquid-Crystal Transition Temperature, T_m. Although it has been well established that DPPC in the multilamellar phase undergoes a pretransition at 35.3 °C and a main transition at 41.4 °C (Mabrey & Sturtevant, 1978), the thermotropic properties of SUV are less well-defined. Figure 3a shows the DSC curve of the SUV of DPPC prepared under the present conditions (containing NaCl and no AMP). The low main transition (37.9 °C) and the large range of transition are consistent with those reported by Lentz et al. (1976). Upon

Table II: Summary of DSC Results from Figure 3

curve	compd	concn of the ethanolic solution (mM)	T_m (°C)[a]
a	DPPC	20	37.9
b	DPPC	80	39.5
c	DPPC	160	39.9, 40.1
d	(R_P)-DPPsC	20	43.7, 43.9
e	(S_P)-DPPsC	20	43.8, 43.9
f	(R_P + S_P)-DPPsC	20	43.75, 43.75

[a] The multiple data reported were from independent experiments by use of further purified samples.

increasing the size of the SUV by increasing the concentration of DPPC in the ethanolic solution (Kremer et al., 1977), the T_m of DPPC increased and the peak narrowed (Figure 3b,c), which agreed with the result on the SUV of DMPC (Van Dijck et al., 1978). The SUV of DPPsC isomers, prepared under the same conditions as that of Figure 3a, showed sharp transitions at 43.8 °C, as shown in Figure 3d–f. The results are summarized in Table II. The higher T_m of DPPsC isomers relative to the unilamellar vesicles of DPPC (and the multilamellar DPPC) suggests that the packing of alkyl chains is more rigid in DPPsC than in DPPC (Fendler, 1982; Tanford, 1980). The narrower transitions of DPPsC isomers support the above result that DPPsC isomers form larger SUV than DPPC under the same conditions. The broader bases in the DSC curves of DPPsC isomers could be due to the broader distribution in the size of the SUV of DPPsC, as also suggested by the EM results. Because the estimated error in T_m is ±0.1 °C, the difference between the T_m of DPPsC isomers seems too small to be detected under the present conditions.

SUV of the Mixture of DPPC and DPPsC. Direct comparison of the spectral properties of the SUV of DPPC and DPPsC is difficult because such properties are sensitive to the size of SUV. However, the comparison can be made in the SUV of the mixed phospholipids. Figure 4a shows the ^{31}P NMR spectrum of the SUV prepared from a 1/1 mixture of DPPC and (R_P + S_P)-DPPsC. The NOE and T_1 were also measured, and the results (summarized in Table III) are generally consistent with those reported for the vesicles of DPPC (Viti & Minetti, 1981) and egg lecithin (Yeagle et al., 1975, 1976a; Burns et al., 1983) under various conditions. The smaller T_1 value of DPPsC relative to DPPC could be a result of different motional freedom or a different intrinsic relaxation mechanism.

The most interesting observation is that DPPsC has a smaller outside/inside ratio ($R_{o/i}$) than DPPC, as shown by the Pr^{3+}-shifted spectrum (Figure 4b) and the results in Table III. The 15–20% difference between the $R_{o/i}$ values of DPPC and DPPsC was reproducible in two independent sets of experiments, with different concentrations of Pr^{3+}. A separate control experiment by use of DPPC vesicles indicated that $R_{o/i}$ (2.0) is independent of [Pr^{3+}] as long as the inner and outer ^{31}P resonances are fully separated. The smaller $R_{o/i}$ values for both DPPC and DPPsC in the mixed SUV, relative to the value of 2.1 ± 0.1 reported for the SUV of egg lecithin (prepared by sonication) (Hutton et al., 1977) and the value of 2.0 observed for the SUV of pure DPPC vesicles prepared under the present conditions, reflect the larger size of the mixed SUV relative to the SUV of pure egg lecithin or DPPC (Hauser et al., 1983). The asymmetric distribution of DPPC and DPPsC in the mixed SUV was further confirmed by use of the mixed SUV containing entrapped $PrCl_3$ (Figure 4c). Because these SUV were prepared under different conditions, the $R_{o/i}$ values (which are sensitive to the size of vesicles) were

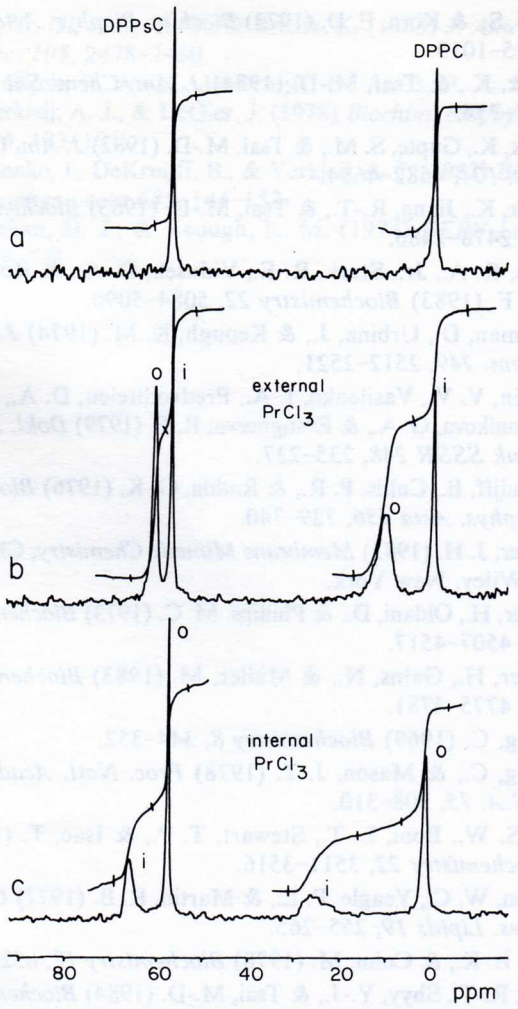

FIGURE 4: ^{31}P NMR study of the outer/inner distribution of mixed SUV (81.0 MHz, 45 °C). (a) SUV prepared from a mixture of 0.1 mmol each of DPPC and (R_P + S_P)-DPPsC. (b) Addition of 0.8 mL of 10 mM $PrCl_3$ to the above sample. Final concentrations: 83.7 mM total phospholipids and 3.4 mM $PrCl_3$. (c) Mixed SUV containing entrapped $PrCl_3$, prepared from a mixture of 0.08 mmol each of DPPC and (R_P + S_P)-DPPsC. The final concentrations were not determined. Spectral parameters: spectral width 8928 Hz; acquisition time 0.918 s; 90° pulse; acquisition delay 17 s; inverse-gated ^1H decoupling (no NOE); line broadening 20 Hz; number of transients 60 (a), 1500 (b), and 2800 (c). The letters o and i represent peaks due to outer and inner layers, respectively.

different from the values with external $PrCl_3$. However, the $R_{o/i}$ of DPPC was reproducibly greater than that of DPPsC within the limit of experimental accuracy as shown in Table III. It is therefore concluded that DPPsC prefers to locate in the inner layer in the SUV of an equimolar mixture of DPPC and DPPsC.

Discussion

Significance of the Results. Chiral thiophospholipids provide an excellent probe to study the biological roles of the phosphate group in phospholipids. The high stereospecificity of phospholipase A_2 toward the R_P isomer and phospholipases C and D toward the S_P isomer has enhanced our understanding in the mechanism of these enzymes (Bruzik et al., 1982, 1983; Jiang et al., 1984). The results of this paper suggest that DPPsC isomers have approximately the same cmc as DPPC and that they all form small unilamellar vesicles that can entrap electrolytes and can be lysed by melittin. Thus, chiral thiophospholipids can also be used as a model of phospholipids in the unilamellar phase.

Table III: ^{31}P NMR Results of SUV of Equimolar Mixture of DPPC and (R_P + S_P)-DPPsC[a]

	experiment 1		experiment 2	
	DPPC	(R_P + S_P)-DPPsC	DPPC	(R_P + S_P)-DPPsC
without Pr^{3+}				
line width (Hz)	22	16	21	15
relative intensity	1.0	0.97	1.0	0.95
NOE	1.3	1.2	1.3	1.2
T_1 (s)[b]	2.4	1.6	2.5	1.7
chemical shift (ppm)	−0.18	57.14	−0.15	57.16
with external Pr^{3+} [c]				
chemical shift (inner) (ppm)	−0.4	57.02	−0.4	57.04
chemical shift (outer) (ppm)	10.1	60.66	6.3	59.11
$R_{o/i}$	1.42	1.24	1.44	1.21
with internal Pr^{3+}				
chemical shift (outer) (ppm)	1.2	57.55	1.0	57.67
chemical shift (inner) (ppm)	25.7	66.1	22.6	65.4
$R_{o/i}$	2.0	1.6	2.0	1.5

[a] The data for experiment 1 are obtained from Figure 4. The data for experiment 2 come from an independent set of experiments. All line widths were measured at the half-height of peaks and corrected for the artificially applied line broadening. All relative intensities were measured by integration from the spectra obtained with long delay (17 s) and with inverse-gated ^1H decoupling. The (+) sign of ^{31}P chemical shifts indicates a downfield shift relative to external 85% H_3PO_4. [b] Experiment 1 was measured without ^1H decoupling. Experiment 2 was measured under broad-band ^1H decoupling. [c] The molar ratio [$PrCl_3$]/[total phospholipids] is 4% and 2% in experiments 1 and 2, respectively.

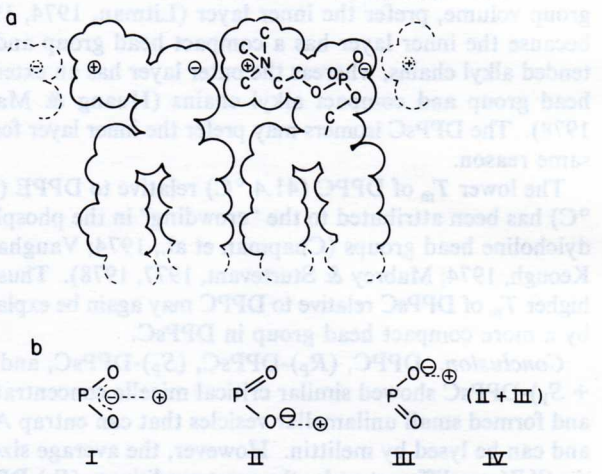

FIGURE 5: (a) Interactions between the head groups of phosphatidylcholines in membranes [redrawn from Yeagle (1978)]. (b) Possible forms of the phosphate group of phospholipids in a chiral environment.

The current understanding of the head-group conformation of phospholipid bilayers is limited to that shown in Figure 5a, which was formulated on the basis of a variety of physical studies reviewed by Yeagle (1978). The close proximity between the phosphate group and the quaternary ammonium group is well supported by ^{31}P NOE studies (Yeagle et al., 1975, 1976a). Although a direct ionic interaction between the phosphate anion and the positive charge at choline has not been identified unequivocally, the phosphate group may interact with neighboring choline, with positive residues from proteins, or with other small electrolytes or water in biological membranes. Since the phosphorus atom is a prochiral center, the phosphate group can in principle exist in four possible forms in a chiral environment: achiral (I), chiral (II), chiral (III), and racemic (IV) (as a mixture of II and III) (Figure 5b).

It will be very difficult to determine which state the phosphate group exists in natural membranes (macroscopically or microscopically). However, the R_P, S_P, and $R_P + S_P$ isomers of DPPsC can serve as models for forms II, III, and IV, respectively. The results in this paper, in conjunction with the previous report on the differences in the ^{31}P and ^{14}N NMR properties of DPPC and DPPsC isomers in the multilamellar phase (Tsai et al., 1983), indicate that forms II, III, and IV can all exist as bilayer membranes, but they have different properties. Thus, the structure, configuration, and conformation of the phosphate group of phospholipids could be important in the structure and function of biological membranes. Although our result has not answered the question as to which is the predominant form for natural membranes, it suggests that one should not presume that natural membranes exist only in form I.

Differences between DPPsC and DPPC. Although we are still unable to explain the differences between R_P, S_P, and $R_P + S_P$ isomers of DPPsC, the differences between DPPsC and DPPC may suggest that DPPsC bilayers have a "more compact head group" than DPPC (although the molecular basis of this property is totally unclear). Thus, DPPsC forms "larger vesicles" because small vesicles would require an "extended head group" in the outer layer.

The so-called "transmembrane asymmetry" is a common phenomenon in biological membranes as well as in small unilamellar vesicles containing more than one component of phospholipids (Op den Kamp, 1979; Yeagle et al., 1976b). In a number of model membrane systems, the asymmetry has been attributed to the head-group volume or structure. For example, phosphatidylethanolamines, with a smaller head-group volume, prefer the inner layer (Litman, 1974, 1975) because the inner layer has a compact head group and extended alkyl chains, whereas the outer layer has an extended head group and compact alkyl chains (Huang & Mason, 1978). The DPPsC isomers may prefer the inner layer for the same reason.

The lower T_m of DPPC (41.4 °C) relative to DPPE (63.8 °C) has been attributed to the "crowding" in the phosphatidylcholine head groups (Chapman et al., 1974; Vaughan & Keough, 1974; Mabrey & Sturtevant, 1977, 1978). Thus, the higher T_m of DPPsC relative to DPPC may again be explained by a more compact head group in DPPsC.

Conclusion. DPPC, (R_P)-DPPsC, (S_P)-DPPsC, and ($R_P + S_P$)-DPPsC showed similar critical micelle concentrations and formed small unilamellar vesicles that can entrap AMP and can be lysed by melittin. However, the average sizes of the SUV are different under the same conditions: (S_P)-DPPsC > ($R_P + S_P$)-DPPsC > (R_P)-DPPsC > DPPC, as suggested by the results of EM, ^{31}P NMR, and the entrapped volume. The gel–liquid-crystal transition temperatures of the SUV of DPPsC isomers are significantly higher than that of DPPC. In the SUV of an equimolar mixture of DPPC and ($R_P + S_P$)-DPPsC, DPPsC preferred to stay in the inner layer. The results, in conjunction with the previous report on the properties of multilamellar phase of DPPC and DPPsC isomers (Tsai et al., 1983), suggest the potential significance of the phosphate group on the properties of biological membranes and warrant further investigation on the molecular basis of these effects.

Registry No. DPPC, 63-89-8; (R_P)-DPPsC, 82482-77-7; (S_P)-DPPsC, 82482-78-8; (±)-DPPsC, 78599-45-8; AMP, 61-19-8; melittin (bee venom), 20449-79-0.

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