Biochimica et Biophysica Acta, 774 (1984) 227–236
Elsevier

BBA 72133

STRUCTURE OF DIVALENT CATION-PHOSPHATIDIC ACID COMPLEXES AS DETERMINED BY ^{31}P-NMR

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(Received November 7th, 1983)
(Revised manuscript received March 12th, 1984)

Key words: Phase transition; Phosphatidic acid; X-ray scattering; ^{31}P-NMR; Ion (Ca^{2+}, Cd^{2+}, Mg^{2+})-lipid complex; Divalent cation; (Model membrane)

A comparative study of the structure of various phosphatidic acid-divalent cation complexes has been completed using ^{31}P solids NMR methods. These complexes had been implicated as important intermediates in the fusion of phospholipid vesicles and several pieces of evidence had suggested that differences in activity of various ions may stem from structural differences among the complexes. Solids NMR studies using spin one-half nuclei reflect structural properties of molecules through partial averaging of chemical shift tensors. We have found significant differences in the chemical shift tensors observed for Mg, Ca and Cd complexes. Low angle X-ray scattering data were used to assure comparison of similar phases. At low temperatures Ca and Cd complexes exhibit unique phases prohibiting comparison with Mg complexes. At higher temperatures, all complexes exhibit a hexagonal phase, and ^{31}P tensors of the complexes in the hexagonal phase can be interpreted using headgroup geometries similar to those found in crystal structures of phospholipids and assuming motional averaging by simple axial motions. Tensors of Ca and Cd complexes are very similar to one another but are significantly broader than those observed for Mg complexes suggesting a more erect headgroup structure. The differences parallel the fusion activities of the ions for which Ca and Cd are similar and significantly enhanced over that of Mg, supporting a structural link to fusion activity.

Introduction

Complexes of divalent ions with anionic lipids are believed to be important intermediates in fusion of several model and biological membrane systems. While the details of fusion mechanisms in these systems are not fully understood, there are a number of pieces of evidence suggesting a strong correlation of fusion activity with the promotion of certain lipid phases and with lipid structural characteristics. For example, correlations have been observed between fusion and appearance of hexagonal phase and lipidic particles on addition of Ca to vesicles containing anionic lipids [1,2]. Papahadjopoulos and co-workers have correlated Ca-stimulated fusion of vesicles containing anionic lipids with phase separation and phase transition of the anionic lipid to the gel state [3,4]. It is significant that different ions have different abilities to promote these phases and that ion activities in fusion also vary substantially within the divalent ion series [5,6].

Most structural data on phases formed with divalent ions have resulted from low-angle X-ray scattering experiments [7,8] and freeze-fracture electron microscopy [9–11]. Significant differences in long-range order for phases formed with different divalent ions do exist, but correlation with structural differences at a molecular level is miss-

Abbreviations: DCC, dicyclohexylcarbodiimide; DMAP, *N*,*N*-dimethylaminopyridine.

ing. One intriguing possibility is that preferred coordination geometries for various ions observed in simple model systems may dictate changes in long-range order of lipid complexes. Recent single-crystal X-ray studies [12], for example, have allowed comparison of structures of diethylphosphate complexes with Mg, Cd and Ba. The three structures are qualitatively similar, all consisting of a chain of cations bridged by phosphates arranged on the surface of a hypothetical cylinder surrounding the ions. There are substantial differences in coordination of ions and density of packing of ligands. Area on the hypothetical cylinder per phosphate is, for example, 22, 27 and 34 $Å^2$ for Cd, Ba and Mg, respectively. A similar trend is seen for hexagonal phases formed by cardolipin complexed with Ca, Mg and Ba when studied by low-angle X-ray scattering [8]. Here lipid headgroups point toward a long cation containing channel forming cylinders which pack into a hexagonal array. Area per phosphate for the cardiolipin series is calculated to be 29, 38 and 38 $Å^2$ for the series Ca, Mg and Ba. The analogy, assuming Ca and Cd to substitute for one another, is suggestive, but experimental verification of differences in molecular conformation or coordination in lipid phases would strengthen the case for preferred coordination being a factor in determining lipid phase properties.

The examination of molecular level structures in membrane phases is not an easy task. Such systems are not sufficiently ordered to allow the use of single-crystal diffraction, and at the same time, are too ordered to allow the use of methods such as high-resolution NMR. Recently developed solids NMR methods are capable of providing some structural data on these partially ordered systems. ^{31}P and ^{13}C solids NMR have, for example, been used in studies of lipid headgroup conformation and dynamics [13–15]. Solids spectra of these spin $\frac{1}{2}$ nuclei, obtained using proton decoupling, are dominated by dependence of the chemical shift on average orientation of functional groups in the applied magnetic field [15]. For lipid dispersions, order exists only in local domains which are themselves randomly oriented. This results in powder spectra which have shapes and widths dependent on molecular conformation and averaging within each domain. To a first approximation, high temperature lipid phases exhibit rapid ($> 10^4$ Hz) axially symmetric motion about one or more director axes. This leads to observation of axially symmetric powder patterns, reduced in width from the static pattern by an amount dependent on the orientation of functional groups relative to the director axes. In cases where the static chemical shift anisotropy and its molecular orientation are known, the set of allowed molecular conformations consistent with the data can be drastically reduced through observation of partially averaged chemical shift anisotropy powder patterns.

We will present here ^{31}P-NMR data on Ca, Cd, and Mg complexes with phosphatidic acids. Structural information obtained will be compared to structural differences observed in crystalline model compounds and used to correlate results of previous fusion studies with conformational preferences of various ion complexes.

Experimental

Dilauroylphosphatidic acid and dioleoylphosphatidic acid were synthesized from glycerophosphate by the method of Khorana and co-workers [16], slightly modified. The use of lipids with two different fatty acid types, one saturated ond one unsaturated, allowed different phases of interest to be studied at readily accessible temperatures. The Khorana procedure calls for formation of phosphatidic acid by reacting the glycerophosphate with the anhydride of the appropriate fatty acid, using *N,N*-dimethylaminopyridine (DMAP) as a catalyst for the condensation reaction. The anhydride is first formed by a condensation reaction with dicyclohexylcarbodiimide (DCC) and isolated. Instead, we applied a method developed by Ziegler and Berger [17] to generate the anhydride in situ, and the entire reaction was carried out in one pot. Racemic glycerophosphate, (Sigma, St. Louis, MO) rather than the naturally occurring L-isomer, was used because of its much lower cost. Arnett and Gold [18] recently performed an exhaustive comparison of the physical properties of racemic and D- and L-isomers of phosphatidylcholine and were unable to detect differences. We are therefore confident that this does not pose a problem in our study. The di-

sodium salt of racemic glycerophosphate was converted to the pyridinium salt by passage through a pyridinium Dowex-50 ion exchange column and then dried overnight under vacuum. The fatty acids used were lauric acid (Eastman, Rochester, NY), used without further purification, and oleic acid (Fisher, Fair Lawn, NJ; lab grade) partially purified by recrystallization from ethanol. The glycerophosphate and fatty acid, along with excess DCC and DMAP, were dissolved in freshly distilled methylene chloride. The reaction was allowed to stir for a few days and then the product isolated using a silicic acid column eluted with a stepwise gradient of CH_3OH in $CHCl_3$. Fractions containing product were identified by TLC. Plates were developed in a $CHCl_3/CH_3OH/7$ M NH_4OH mixture (50:25:6, by vol.) [19]. Fatty acid is eluted first, followed by product. Early fractions containing product appeared impure by TLC and required a second column purification. Overall yields were typically 30% relative to glycerophosphate.

The fatty acid composition of the synthesized phosphatidic acids was analyzed by gas chromatography of methyl esters of the fatty acids. The dilauroylphosphatidic acid sample resulted in a single peak at a retention time appropriate for laurate. The dioleoylphosphatidic acid samples showed, in addition to oleate, other methyl ester impurities constituting approximately 20–25% of the total. While this level of impurity is far from ideal, the samples represent a better defined system than phosphatidic acids from natural sources and were used as prepared.

The Mg, Cd and Ca salts of glycerophosphate were prepared as models for structural comparison to phospholipid. They were formed by mixing aqueous or H_2O/CH_3OH solutions of the disodium salt of racemic glycerophosphate and the chloride of the appropriate cation. The M^{2+}-glycerophosphate which precipitated was filtered, washed and dried.

Lipid samples for NMR were prepared by dispersing the lipid in an aqueous buffer and slowly adding a solution of the chloride salt of the appropriate cation to obtain a lipid-cation ratio of 1:1. The lipid-cation complex which precipitated was centrifuged to form a wet pellet and NMR experiments performed on the wet solid. The buffer used was 0.1 M sodium acetate adjusted to pH 5.5 ± 0.2.

NMR spectra of these samples were obtained on a Bruker CXP-200 spectrometer operating at 81 MHz for ^{31}P. The ^{31}P probe contains a 5 mm horizontal solenoid coil, double-tuned for observation and proton irradiation and equipped for variable temperature operation (± 2 K). 90° pulse times were typically 5 μs. For low temperature lipid samples and for the glycerophosphate salts, spectra were obtained using cross-polarization for sensitivity enhancement. In the cross-polarization experiments, a 4 μs proton pulse was followed by a 5 ms contact time during which proton and ^{31}P rf (radio frequency) fields were present at levels to achieve a Hartman-Hahn match. A 4 s recycle delay was used. For the more fluid lipid phases, a single-pulse experiment was used with approximately 60° pulses and a 1 s recycle time. Proton decoupling at approx. 12 gauss was employed during acquisition in all cases. Typical cross-polarization spectra required 0.5 h for the glycerophosphate samples and 1–2 h for lipid samples. Typcial single pulse spectra required 0.5 h. Shielding tensor elements were extracted from spectra using computer simulation of the line shapes, which assumed a random distribution of phosphate orientations and a constant Lorentzian linewidth independent of orientation [15]. Reported chemical shift anisotropies represent best fits within these restrictions.

Low angle X-ray scattering was used to confirm the identity of various lipid phases. The same samples used in NMR experiments were used for low-angle X-ray scattering experiments, which were performed as described previously [20]. X-rays produced by a Philips 1.4 kW 0.4×8 mm^2 fine focus copper X-ray tube were nickel filtered and focused by a bent glass mirror. The sample was positioned in a thermostated holder mid-way between the mirror and the detector. Temperature was controlled with a Lauda circulating water bath with a deviation of ± 2 K. The measurements were made using a Tennelec PSD 100 one-dimensional position sensitive detector, with 29.047 channels/cm. Sample-to-detector distance was 63.8 cm with most of the beam path maintained under vacuum to reduce scattering by air. Data were accumulated for 2–14 h, depending on the sample, and stored in a Canberra multichannel analyzer. The data are

displayed as number of counts versus channel number. The repeat spacing, d, was calculated using Bragg's law, $2d \sin \theta = n\lambda$ where 2θ is the scattering angle and λ is the wavelength (15.4 nm ≡ 1.54 Å).

Results

Fig. 1 shows ^{31}P spectra of Cd, Ca and Mg salts of glycerophosphate. All three show very similar chemical shift anisotropies with slight deviations from axial symmetry. To extract chemical shift tensor components, computer simulations were attempted. The experimental spectra deviate slightly from theoretical lineshapes, so simulated spectra did not yield ideal fits. The distortions may result from orientation-dependent contributions to linewidth or from non-uniform cross-polarization. Best fit chemical shift tensor values are reported in Table I.

Fig. 2 shows ^{31}P spectra of a Mg-dilauroylphosphatidic acid sample prepared as described above, at temperatures from 253 K to 343 K. The lowest temperature spectrum (Fig. 2a) shows a chemical shift anisotropy of 85 ppm. At this temperature, the lipid is expected to be in the

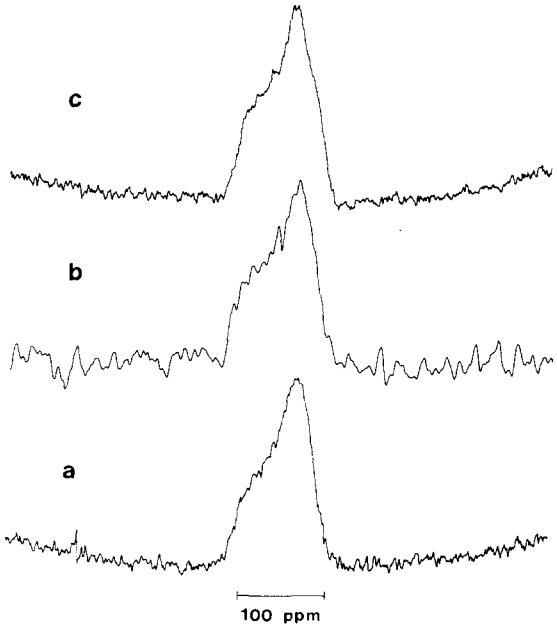

Fig. 1. ^{31}P-NMR spectra of glycerophosphate salts. (a) Mg, (b) Cd, and (c) Ca.

TABLE I

CHEMICAL SHIELDING TENSORS OF GLYCEROL PHOSPHATE COMPLEXES WITH VARIOUS IONS

Larger values indicate upfield chemical shifts (ppm from 85% H_3PO_4).

Complex with	σ_{11}	σ_{22}	σ_{33}
Mg	−52	18	43
Ca	−54	12	46
Cd	−64	16	41

L_β gel state or other highly ordered state, with motions too slow to result in averaging of the chemical shift anisotropy. This spectrum agrees both in symmetry and in total anisotropy with the spectrum of crystalline Mg-glycerophosphate, sup-

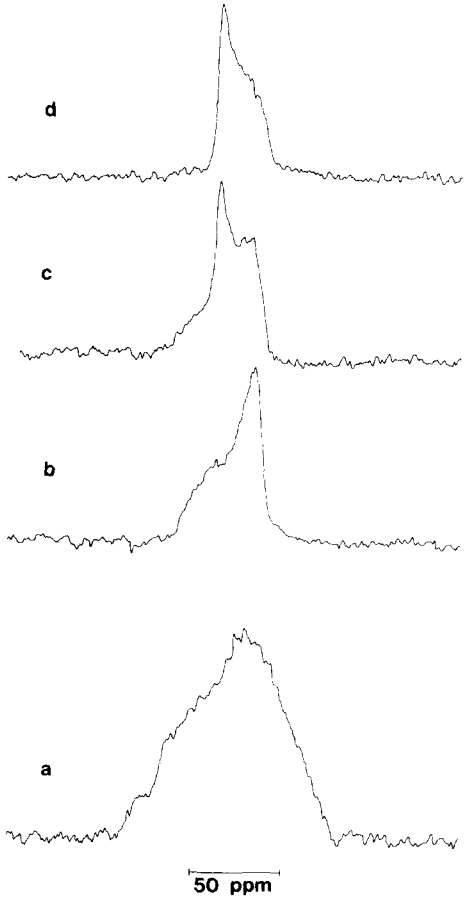

Fig. 2. ^{31}P-NMR spectra of Mg-dilauroylphosphatidic acid (1 : 1) at temperatures (a) 253 K; (b) 298 K, (c) 333 K, and (d) 343 K.

porting the idea that the lipid is immobilized. The distortion from ideal line shape may result from slow motions other than rotational diffusion, from orientation-dependent contributions to linewidth, or from non-uniform cross-polarization by neighboring protons. At 298 K, the spectrum of Mg-dilauroylphosphatidic acid is axially symmetric, but the chemical shift anisotropy is reduced to 46 ppm. Reduction in the chemical shift anisotropy commonly occurs on transition to the more fluid liquid crystal L_α phase, in which rapid rotation of lipids about the bilayer normal is allowed. The spectrum at 343 K shows a chemical shift anisotropy of -24 ppm, reduced further by a factor of 1.9 and reversed in symmetry compared to the 298 K spectrum. This is typical of changes observed on transition from a lamellar to a hexagonal phase. Spectra between these two temperatures appear to be a mixture of the two types of spectra. The spectrum of a Mg-dioleoylphosphatidic acid complex (not shown) is typical of a hexagonal phase at 298 K and lower temperature (283 K), with the same chemical shift anisotropy of -24 ppm.

Low-angle X-ray scattering experiments were performed on the Mg-dilauroylphosphatidic acid sample to support the identification of the phases given above. Table II lists the reflections observed at 298 and 343 K. For a lamellar phase, reflections are expected at $1/d$, $2/d$, ... where d is the distance between lamellae. At 298 K, the large amplitude reflection at $1/48$ Å^{-1} and the small one at $1/24$ Å^{-1} are consistent with a lamellar phase with a spacing of 48.0 Å. For a hexagonal phase, two sets of reflections are expected, at $1/d$, $2/d$, ... and at $1/\sqrt{3}\,d$, $2/\sqrt{3}\,d$, ... where d and $\sqrt{3}\,d$ correspond to the spacings of the two different sets of planes that describe the packing of the rows of cylinders that comprise the hexagonal phase. One set of planes contains axes of nearest neighbor cylinders so that successive planes are separated by $\sqrt{3}$-times the cylinder radius. The other contains axes of next nearest neighbor cylinders so that successive planes are separated by the cylinder radius. Hence, d can be associated with the radius of a lipid cylinder in the hexagonal phase [21]. A 298 K, the small peaks at $1/43$ Å^{-1} and $1/25$ Å^{-1}, in a ratio of $1/\sqrt{3}:1$, support the presence of a small fraction of hexagonal phase with nearest neighbor cylinder spacing of 50 Å. This small fraction was not apparent until slightly higher temperatures in the NMR experiments and may occur here because of differences in samples composition or temperature calibration. At 343 K, these small reflections in the X-ray experiments show increased intensity while those due to the

TABLE II

DILAUROYLPHOSPHATIDIC ACID-Mg LOW-ANGLE X-RAY SCATTERING

	Channel No. [a]	Intensity [b]	Reciprocal spacing (Å^{-1})
$T = 298$ K [c]	69	68000	$(48.4)^{-1}$
	76	12100	$(43.3)^{-1}$
	130	200	$(23.8)^{-1}$
	145	400	$(21.1)^{-1}$
$T = 343$ K [d]	76	126000	$(43.3)^{-1}$
	123	1000	$(25.3)^{-1}$
	145	3000	$(21.2)^{-1}$

[a] Detector at channel No. 10.
[b] Total counts for each reflection.
[c] Total time of experiment was 1.9 h.
[d] Total time of experiment was 5.5 h.

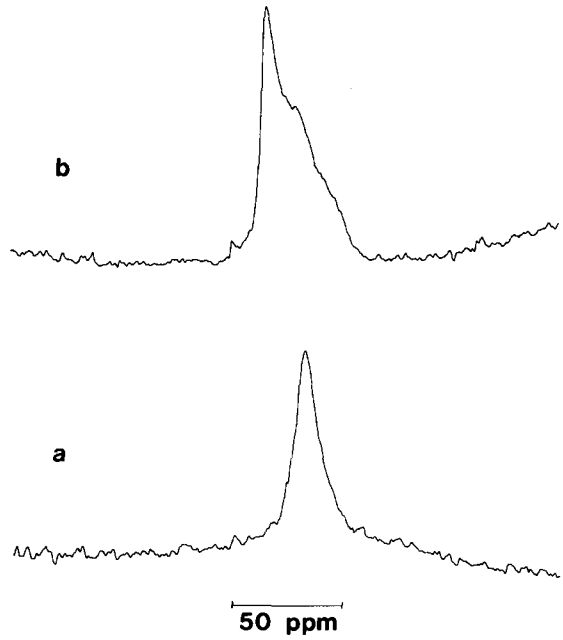

Fig. 3. ^{31}P-NMR spectra of Cd-dioleoylphosphatidic acid (1 : 1) at temperatures (a) 298 K and (b) 353 K.

lamellar phase have disappeared in complete parallel with the NMR trends. Hence, the X-ray data support the interpretation of changes in NMR spectra given above.

Fig. 3 and 4 show ^{31}P-NMR spectra of Ca- and Cd-dioleoylphosphatidic acid samples at 298 and 253 K. The higher temperature spectra (Fig. 3b and 4b) show the symmetry characteristic of hexagonal phase, with chemical shift anisotropies of -40 and -35 ppm, respectively. The similarity suggests very nearly equivalent geometries for these isosteric ions. The high temperature spectra of Ca or Cd complexes are on the other hand, quite different from those of Mg complexes. The primary difference is in chemical shift anisotropy -40 and -35 ppm vs. -24 ppm. This difference could arise either from different motional averaging due to conformational differences or from electronic structure differences among the complexes. Dilauroylphosphatidic acid complexes with all four ions were therefore examined at low temperature (253 K) where motion is presumed to be in the slow regime. All chemical shift anisotropies were approx. 85 ppm, and very similar to the glycerophosphate spectra. Ion substitution is therefore seen to have minimal effect on the static chemical shift tensor. Hence, differences in chemical shift anisotropy observed at higher temperature must result from differences in motionally accessible orientations of the phosphate group relative to the director axis or from the existence of fundamentally different phases at temperatures where comparisons are being made.

Low angle scattering experiments were performed on the Ca-dioleoylphosphatidic acid sample in an effort to confirm that comparisons were being made on similar phases. Table III lists the observed reflections at 353 K, showing reflections at $1/35$ Å^{-1} and $1/20$ Å^{-1}. Their positions at $1/\sqrt{3}\,d$ and $1/d$ confirm the presence of hexagonal phase with a cylinder spacing of $2d = 40$ Å. This suggests that the differences in chemical shift anisotropy among various ion complexes arise from differences in accessible conformations and not differences in long-range phase structure.

At lower temperature (298 K), both Cd and Ca complexes show a relatively narrow chemical shift anisotropy pattern which is nearly symmetrical (Figs. 3a and 4a). This is in marked contrast to the data on Mg-dioleoylphosphatidic acid which show an axially symmetric pattern indicative of hexagonal phase at low temperatures or data on Mg-dilauroylphosphatidic acid which at lower temperatures show a wide chemical shift anisotropy pattern typical of the liquid crystal L_α phase. Again, X-ray data were acquired to eliminate the possibility of comparisons being made on dissimilar phases. X-ray data on Ca-dioleoylphosphatidic

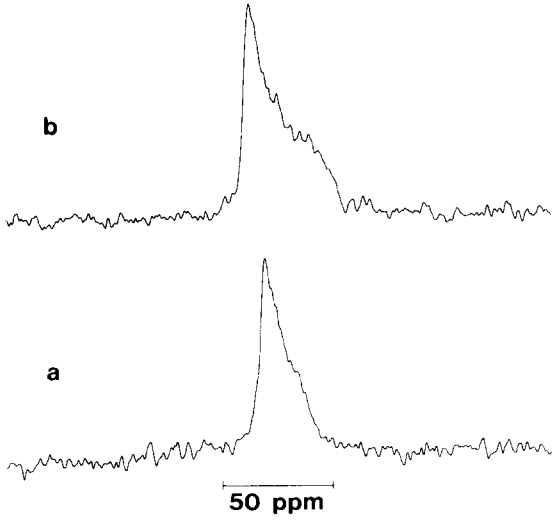

Fig. 4. ^{31}P-NMR spectra of Ca-dioleoylphosphatidic acid (1 : 1) at temperatures (a) 298 K and (b) 353 K.

TABLE III

DIOLEOYLPHOSPHATIDIC ACID-Ca LOW-ANGLE X-RAY SCATTERING

	Channel No. [a]	Intensity [b]	Reciprocal spacing (Å^{-1})
$T = 283$ K [c]	62	16000	$(55)^{-1}$
	75	27000	$(44)^{-1}$
$T = 298$ K [d]	64	13000	$(53)^{-1}$
	74	27000	$(45)^{-1}$
$T = 353$ K [e]	92	21000	$(34.8)^{-1}$
	154	700	$(19.8)^{-1}$

[a] Detector at channel No. 10.
[b] Approximate total counts for each reflection.
[c] Total time for experiment was 13.9 h.
[d] Total time for experiment was 6.9 h.
[e] Total time for experiment was 12.2 h.

acid at 298 and 283 K show only two broad reflections, which do not change in relative intensity with temperature. These occur at $1/54$ Å^{-1} and $1/44$ Å^{-1}. No higher order reflections were observed, indicating a low degree of order. Unfortunately, the absence of higher order reflections prohibits a definitive characterization of the phase present. However, the width of the reflections and absence of high-order reflections indicate a lack of regular periodicity and argue against a simple lamellar phase, as is the case for the Mg-dilauroylphosphatidic acid complex, and the spacing is not in the ratio $1/\sqrt{3}:1$ as expected for a hexagonal phase. Therefore, comparison of chemical shift anisotropy tensor values of Ca, Cd and Mg complexes at lower temperature cannot be used to extract conformational differences.

Discussion

Of the data presented above, the Mg-dilauroylphosphatidic acid data can be interpreted in the most striaghtforward manner. In a static powder sample, all orientations of the molecule are equally represented, giving rise to a powder pattern. For totally non-protonated phosphate monoesters, the static powder pattern consists of a low amplitude extremum at σ_{11}, the unique axial value, and a high amplitude extremum at $\sigma_{22} = \sigma_{33}$, the nearly equivalent values. At low temperature, the similarity of the observed chemical shift anisotropy powder pattern to crystalline glycerophosphate complexes suggests a very low level of molecular motion for the lipid complexes. Insensitivity of these patterns to ion substitution further suggests that elements of the static shift tensor are dictated by the local electronic structure of the doubly ionized phosphate group. This group has a unique 3-fold symmetry axis along the esterified O-P bond. The glycerophosphate spectra actually deviate slightly from axial symmetry (see Table I), showing that the ions perturb the phosphate electron structure to some extent. Ideally, single-crystal data would allow experimental determination of tensor orientation [22]. In the absence of these data, we have assumed an axially symmetric tensor ($\Delta\sigma = 85$ ppm), oriented with its unique axis (σ_{11}) along the esterified O-P bond. This allows evaluation of conformational differences using the simple model for motional averaging which follows.

At the gel to liquid-crystalline phase transition, additional motional freedom is introduced. At a minimum, molecular rotation about the bilayer normal occurs. This axis in many bilayer systems is also nearly parallel to the acyl chains. If rotation about this axis were the only motion, the chemical shift anisotropy would be modified by a factor of $1/2(3\cos^2\theta - 1)$, where θ is the angle between the director axis (the bilayer normal) and the esterified O-P bond, yielding a spectrum with partially averaged tensor values σ'_{11}, σ'_{22}, and σ'_{33}. Within the limitation that the $\cos^2$ function yields two solutions, θ can be calculated from the observed chemical shift anisotropy and a knowledge of the magnitude and orientation of the static tensor. For dilauroylphosphatidic acid-Mg, $\theta = 46°$ or 134°. 46° is in reasonable agreement with the glycerol O-P orientation found in crystal structures of phospholipids (30° for dilauroylphosphatidylethanolamine [23]; 33° for dimyristoylphosphatidylcholine, conformation A; and 36° for dimyristoylphosphatidylcholine conformation B [24]. Values were obtained by assuing the 1-chain to be parallel to the bilayer normal.)

Occurrence of a hexagonal phase at higher temperature allows, at a minimum, additional averaging about the long axes of hexagonally packed rods. Assuming molecular conformation remained the same and axial averaging about a vector parallel to the hydrocarbon chains continued, this would average σ'_{11} with σ'_{22} and make σ'_{33} the unique axis. This reduces the chemical shift anisotropy by a factor of 2 and reverses its symemtry. In the spectrum of Mg-dilauroylphosphatidic acid at 343 K, the symmetry is reversed and the chemical shift anisotropy is reduced by a factor of 1.9. All of this suggests a rather classical behavior of Mg-dilauroylphosphatidic acid with results in accord with studies on other phospholipids [1].

Comparison of other ion complexes with the Mg complexes is most straightforward at high temperatures where all show hexagonal phase. Comparison of glycerophosphate spectra and very low temperature lipid spectra show that changes in ion have minimal effect on the static chemical shift tensor. Also, motional averaging inherent in the hexagonal structure would be assumed the same. Yet, Cd and Ca complexes give notably broader

hexagonal phase powder patterns than do the Mg complexes. If we retain our assumption of two rapid axial rotations about orthogonal axes, we can again solve for θ, the angle between the esterified O-P bond and the bilayer axis, giving $\theta = 28°$ or 152° for Cd and $\theta = 16°$ or 154° for Ca. $\theta = 28°$ and 16° for Cd and Ca, respectively, offers the smalelst departure from known crystal structure conformations and a reasonable structural interpretation. Of course, it is always possible that motions other than the axial ones described contribute to tensor averaging.

At lower temperatures, Cd and Ca complexes of dioleoylphosphatidic acid show pronounced departure from Mg complexes known to be in a liquid-crystalline lamellar L_α phase or a hexagonal phase. The spectra are very narrow and nearly symmetric. This could result from an angle θ near 54° or it could result from pseudo-isotropic motion. Certain phases, such as the cubic phase, allow such motions. However, we could not obtain sufficient low angle scattering data to confirm or reject the possible existence of such a phase.

Very narrow ^{31}P spectra have been observed in several lipid systems [1,25,26], but these are much narrower than what we have observed. These spectra are often associated with 'lipidic particles' seen in electron microscopy, which have been proposed to be inverted micelles sandwiched within the bilayer [25] or local conical distortions of the bilayer, suggested to be intermembrane attachment sites [26]. These particles are small enough (100–1000 Å) to result in complete averaging of the chemical shift anisotropy. While it is possible that the narrow spectra we observe could arise from similar lipid structures, these have not occurred as dominant structural forms in other lipid systems.

Other studies have been conducted on phosphatidic acid complexes with divalent ions, using freeze-fracture electron microscopy and ^{31}P- and ^{2}H-NMR. A molecular-level conformational analysis was not attempted and variations in conditions such as ion-lipid ratio were not exactly the same, but the information on long-range organization presented in these studies compliments our observations. In particular, an ill-defined, non-bilayer phase has been observed for Ca complexes of phosphatidic acid. At pH 6, Cullis and co-workers [27] observed a somewhat narrow ^{31}P powder spectrum in complexes with Ca, which they were not able to identify. This was not observed for Mg. For the Ca sample, they simultaneously observed a broad and featureless ^{2}H spectrum using dioleoylphosphatidic acid labeled at the C-11 position, which was different from that observed for lamellar or hexagonal phases. They also performed freeze-fracture studies on a 1 : 1 dioleoylphosphatidic acid-Ca sample, which showed regions of short hexagonal-like cylinders, but the long-range structure was very disorganized. As with our data, ^{31}P-NMR spectra of 1 : 1 complexes at pH 6 could not be equated with a specific phase.

Verkleij et al. [9] also studied phosphatidic acid-cation complexes by freeze-fracture. For a dioleoylphosphatidic acid-Ca sample at pH 6, made with excess cation, they observed regions of curved hexagonal phase cylinders, along with lamellae and regions which they describe as transitions between the two phases. The electron micrographs are similar to Cullis' data, in that the hexagonal regions appear rather disorganized. However, their interpretation is different in the sense that two or more phases were suggested to be present simultaneously. They also report similar behavior for different ions (Mg and Mn), in contrast to Cullis' data, which show pronounced differences between Ca and Mg. For our samples, we tend to discount the presence of multiple phases. Had our Ca-dioleoylphosphatidic acid sample been simply a mixture of bilayer and hexagonal phases, we would expect the X-ray data to show two separate sets of reflections, as was observed for the dilauroylphosphatidic acid-Mg sample. That we observed instead only two broad reflections, whose ratio was independent of change in temperature, and that we did not observe superimposed ^{31}P-powder patterns, suggests that this sample adopts a fundamentally different phase at intermediate temperatures. Because observation of this unusual phase could be traced to any number of factors including the slight acyl chain heterogeneity in our synthetic dioleoylphosphatidic acid we prefer to emphasize structural differences in the better defined hexagonal phases.

The relationship of the existence of various phases and various molecular conformations to cellular function invites speculation. The hexagonal phase has been proposed as an intermediate in

membrane fusion and membrane transport. In the model phosphatidic acid-divalent ion system, variations in fusion activity of the ions Mg, Ba, Cd and Ca have been noted, with Ca and Cd showing equivalent and maximum activity [6]. The data presented here show Ca, Cd and Mg all to promote hexagonal phase within the temperature range studied, but with conformational differences. Ca and Cd, which have similar fusion activity, show similar chemical shift anisotropy patterns and hence have similar conformations. Mg, which has lower fusion activity, shows a substantially different chemical shift anisotropy and therefore has a different conformation.

The structural basis of these differences may parallel those seen in crystal structures of simple model compounds. Comparison of the Mg, Cd and Ba salts of diethylphosphate show differences in packing, with the Cd structure having the tightest packing of both ions and phosphate groups [12]. The largest spacing of the three is found for Mg, in which just two non-esterified oxygens are involved in the bridging network. In the more tightly packed Cd structure, three phosphate oxygens are involved. In a lamellar system, two oxygens can be made available with a fairly large angle of the esterified O-P bond relative to the bilayer normal. Three oxygens are best made available to ions on the surface with a more vertical R-O-P orientation. Expansion and contraction of membrane dimensions in response to ions has been noted in hexagonal phases of cardiolipin [8,10] and phosphatidic acid [9] and in lamellar phases of phosphatidylserine [28]. In all of these studies, repeat spacing was smallest for Ca. This is in qualitative agreement with our conformational interpretation of NMR data, although the actual values obtained vary somewhat. That these variations in response to ion substitution seem to be consistent over a wide range of complexes and phases suggests that they may result from fundamental coordination requirements.

Whether the hexagonal phase on which we are able to obtain comparative conformational data is itself the relevant phase for fusion activity is open to question. In fact, the data presented here suggest it may not be. Mg, which has lowest fusion activity, promotes hexagonal phase at the lowest temperature. Alternatively, Ca and Cd, which have high fusion activity, are unique in promoting a phase that gives a nearly isotropic chemical shift anisotropy pattern. Regardless of the particular phase which proves most relevant, structural differences among various ion complexes do exist. Stress placed on the normal bilayer structures by alteration of preferred headgroup geometry may in fact be more significant in promoting fusion than the thermodynamically preferred phases observed under particular conditions of pH, ion concentration and temperature.

Acknowledgments

This work was supported by a grant from the National Institute of General Medical Sciences, GM-19035. We would also like to thank Mr. Neocles Leontis for providing low-angle X-ray scattering data.

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