Tetraoleoylpyrophosphatidic Acid: A Four Acyl-Chain Lipid Which Forms a Hexagonal II Phase with High Curvature

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ABSTRACT Tetraoleoylpyrophosphatidic acid (bis phosphatidic acid), when hydrated in aqueous buffer, was shown to form an inverted hexagonal phase using ^{31}P NMR. Low-angle x-ray diffraction provided verification of the formation of this phase in dilute aqueous buffer and in 2 M NaCl and permitted comparison of the tube diameter with that of cardiolipin in 2 M NaCl. By using the water cylinder diameters for tetraoleoylpyrophosphatidic acid, bovine cardiolipin, chloroplast monogalactosyldiglyceride, and dioleoyl phosphatidylethanolamine as a means of estimating the spontaneous curvatures, tetraoleoylpyrophosphatidic acid was shown to exhibit the greatest curvature of any of the above lipids, equaled only by the calcium salt of cardiolipin. Inverted micelles of hydrated tetraoleoylpyrophosphatidic acid and of cardiolipin in tetradecane were approximately the diameter of the inverted hexagonal tubes. A rationale is given for the differences.

INTRODUCTION

Phospholipids with two acyl chains comprise the most common components of biological membranes: the phosphatidylcholines and the phosphatidylethanolamines, for example. Of the phospholipids containing more than two acyl chains, cardiolipin, containing four acyl chains, occurs most frequently. With two phosphatidyl groups linked as phosphodiesters to glycerol, the presence of four esterified fatty acids provides interesting possibilities for polymorphism. For example, cardiolipin itself readily hydrates, forming a lamellar phase. However, in the presence of monovalent or polyvalent salts, this lipid forms the nonlamellar inverted hexagonal phase (Rand and Sengupta, 1972; Seddon et al., 1983). Addition of a fifth acyl chain provided a lipid that formed a hexagonal phase in the absence of salt, and removal of two acyl chains provided a water-soluble derivative (Powell and Marsh, 1985).

A number of different phospholipids that occur naturally, including cardiolipin, phosphatidylethanolamine, and monogalactosyldiglyceride, can form polymorphic phases. The former two lipids are found within the inner mitochondrial membrane (Daum, 1985) and the latter within the chloroplast (Dorne et al., 1990). Cardiolipin strongly associates with cytochrome *c* oxidase, the terminal electron transport component, and activates that enzyme, at least partially because of the presence of the two negative charges on the polar headgroup (Abramovitch et al., 1990). Monogalactosyldiglyceride is found in greatest concentration within the thylakoids, where the lipid enhances energy transfer between the different chlorophyll-protein complexes (Siefermann-Harmes et al., 1982) and stimulates the ATP synthase (Pick et al., 1984). Other non-bilayer-forming lipids may influence the activity of receptors (Epand et al., 1995; McCallum and Epand, 1995). The presence of these lipids in energy-transducing organelles suggests that such behavior has important biological consequences. However, attempts to demonstrate the presence of nonbilayer phases in natural membranes under physiological conditions have been unsuccessful (De Kruijff et al., 1982).

Understanding how varying the number of acyl chains, the acyl chain length, and degree of unsaturation or, at the polar head group, subtly influencing the charge, hydration, and area permits the experimenter to alter that balance between the headgroup and acyl chains to obtain readily observable phase separations. This balance has been described in terms of shape or excluded volume (Lee et al., 1993).

Another useful concept for characterizing non-bilayer-forming lipids is by spontaneous curvature. Lipids that form inverted hexagonal phases do so by forming tubes with the polar headgroups directed inward, interacting with water. These tubes are lipid monolayers rolled around a water cylinder much longer than the tube diameter. Gruner and co-workers evaluate the tube diameter s from the hexagonal phase lattice constant. The water cylinder diameter $2R_w$ is obtained by subtracting the length of the acyl chains from s; the spontaneous curvature C_o is approximated by the reciprocal of the diameter of the water cylinder $1/R_w$. The concept was initially derived by Helfrich (1974) for structures that exist under stress but not at minimum energy. Strategies to relieve this stress provided structures with "spontaneous" curvatures (Rand et al., 1990). Lipids that form bilayers will have smaller curvatures; those that form only inverted hexagonal phases are expected to possess large spontaneous curvatures, i.e., the water tubes have small radii.

The availability of a physical technique that can be used to estimate spontaneous curvature without the necessity of collecting and analyzing x-ray diffraction patterns would be useful. Sen and Hui (1994) introduced a new technique in

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which the diameters of inverted micelles, formed from hydrated lipids in tetradecane, could be measured rapidly using quasi-elastic light scattering. They showed that the diameters of inverted micelles of several diacyl phosphatides approximate those obtained from x-ray diffraction. We have used this method to compare the size of inverted micelles of cardiolipin and tetraoleoylpyrophosphatidic acid with those obtained from x-ray diffraction. This comparison provided a critical evaluation of the generality of this method as well as further characterization of these lipids.

In the present work, tetraoleoylpyrophosphatidic acid (Ptd_2), a synthetic lipid, is shown to form an inverted hexagonal phase. Analogous to cardiolipin, both lipids contain two phosphatidyl groups with a total of four acyl chains per lipid molecule, but the two phosphodiesters, instead of being separated by a glycerol as they are in cardiolipin, are directly connected in Ptd_2 as a pyrophosphate. This change results in a polar headgroup that is small relative to the volume of the acyl chains, a change expected to favor the formation of a nonbilayer phase for the hydrated lipid (Israelashvili et al., 1980). ^{31}P NMR spectroscopy was used to demonstrate that this lipid assumed an inverted hexagonal phase. Quasi-elastic light scattering was used to compare the diameters of the inverted micelles of hydrated tetraoleoylpyrophosphatidic acid and of cardiolipin in tetradecane with their respective hexagonal tube diameters from x-ray diffraction. X-ray diffraction was used to further characterize the inverted hexagonal phase for Ptd_2 and to estimate the spontaneous curvature for this lipid. By comparing the spontaneous curvatures for several lipids including cardiolipin, we found that the curvature of Ptd_2 was the largest that has so far been observed. This great curvature recommends the use of Ptd_2 in reconstitution and for functional studies designed for evaluating the effects of curvature in model systems.

MATERIALS AND METHODS

The disodium salt of tetraoleoylpyrophosphatidic acid was a gift from Avanti Polar Lipids (Alabaster, AL), and bovine cardiolipin as the disodium salt was purchased from Avanti Polar Lipids. Samples were prepared from lipid dried from chloroform under nitrogen and held under vacuum for at least an hour before hydrating in 2.5 mM HEPES (x-ray diffraction) (pH 7.0) with or without 2 M NaCl. NMR samples (50 mg) were dried overnight and hydrated in 2 ml 20 mM HEPES in D_2O (pD = 7.0). Proton-decoupled ^{31}P NMR spectroscopy was done at 81.015 MHz with a Bruker AC-F200 spectrometer operating in the Fourier transform mode. A 30° pulse with a repetition rate of 0.1 s and decoupling power of 9 W was used. Spectra were collected at 20°C using a spectral window of 50,000 Hz, with phosphoric acid as the reference (0 ppm).

Low-angle x-ray diffraction was carried out on 2-mg samples hydrated in excess 2.5 mM HEPES (pH 7.0) contained in thin-walled glass x-ray capillaries in a temperature-controlled holder, using a Frank camera mounted on a Rigaku (Denvers, MA) rotating-anode x-ray generator. The diffraction patterns were recorded with a TEC (Knoxville, TN) position-sensitive detector.

Quasi-elastic light scattering was carried out using the Nicomp (Santa Barbara, CA) submicron particle sizer model 370. The measurements of the small, inverted micelles were carried out using the Innova 70 argon ion laser (Coherent Laser Products, Palo Alto, CA), which provided high-intensity 488-nm light. Inverted micelles were prepared by dissolving in 1 ml tetradecane, 10 mg Ptd_2 dried from chloroform under nitrogen gas and held in a vacuum for 1 h. One milliliter 2.5 mM HEPES (pH 7.0) with or without 2 M NaCl was vortexed for 30 s with the tetradecane-Ptd2 and separated by centrifugation at 6,000 × *g* for 5 min. The supernatants were transferred to cuvettes for size determination. The inverted micelles were treated as solid particles for size estimation.

RESULTS

The structure of tetraoleoylpyrophosphatidic acid (Ptd_2) is compared with bovine cardiolipin (diphosphatidylglycerol) in Fig. 1. The pyrophosphate is the smallest polar headgroup that can be obtained from two phosphatidyl groups, smaller than the naturally occurring cardiolipin, which contains two phosphodiester groups, also bearing two negative charges separated by a glycerol (Fig. 1). From the presence of four oleoyl chains associated with a small polar headgroup, the formation of a nonlamellar phase was expected. The ^{31}P NMR spectrum observed for Ptd_2 hydrated in dilute aqueous buffer is shown in Fig. 2. The observed powder pattern has a positive chemical shift anisotropy of +12 ppm, which is characteristic of an inverted hexagonal phase (Seelig, 1978; Tilcock et al., 1986). No isotropic component was observed, and freezing and thawing had no effect on the

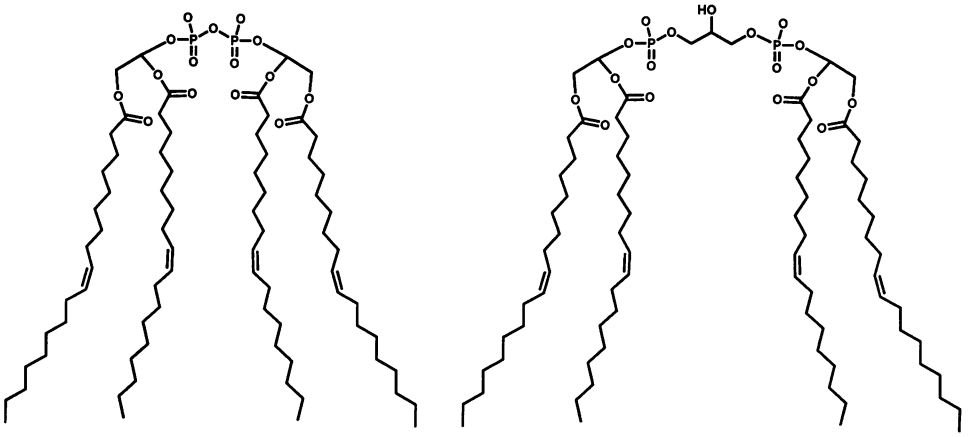

FIGURE 1 Structures of tetraoleoylpyrophosphatidic acid (*left*) and cardiolipin (*right*).

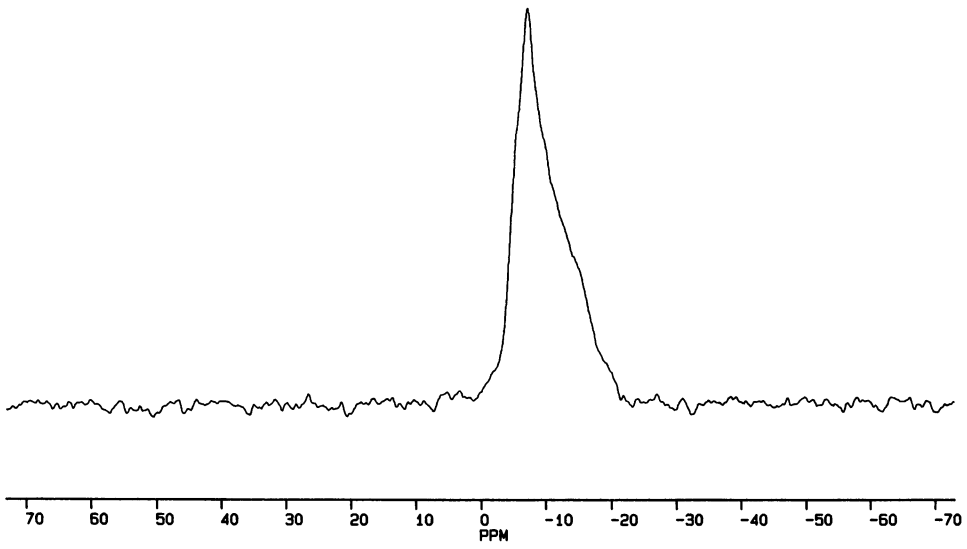

FIGURE 2 Proton dipolar-decoupled 81 MHZ ^{31}P NMR spectrum of tetraoleoyl pyrophosphatidic acid. The spectrum was obtained from 50 mg Ptd_2 in 2 ml 20 mM HEPES (pD 7.0) in D_2O at 20°C. The spectrum was from 3,000 transients with a spectral window of 50,000 Hz. Zero ppm was the location of the resonance line for 85% phosphoric acid. Sodium pyrophosphate (pD 7.0) exhibits a single, narrow line −6.9 ppm from that for phosphoric acid under these conditions.

appearance of the spectrum. When the dried Ptd_2 was hydrated for spectroscopy, little swelling was apparent, further distinguishing the gross physical characteristics of this lipid from lipids like cardiolipin, which readily disperse in dilute aqueous buffers forming a lamellar phase. High-salt (>1.5 M NaCl) was required to induce the formation of an inverted hexagonal phase in cardiolipin (Seddon et al., 1983; Powell and Marsh, 1985).

Low-angle x-ray diffraction for Ptd_2 in dilute aqueous buffer gave the repeat spacings shown in Table 1. The (100) reflection for the inverted hexagonal phase for this lipid was almost immediately apparent after initiating data collection, but higher order reflections were much weaker and required several hours of collection to resolve them from background. The spacings satisfied the relations for a hexagonal lattice. Samples in 2 M NaCl also provided a strong (100) reflection and weak higher order reflections (not shown). The smaller spacing in the presence of 2 M NaCl (Table 1) is consistent with some dehydration in the presence of increased salt. Freezing and thawing the sample had no effect on the intensities or the spacings, suggesting the absence of additional nonlamellar phases in these samples. When the sample was hydrated and examined at 4°C, the same spacings were observed at this temperature at temperatures up to ambient. Over this limited temperature range, the hexagonal phase was the only phase observed for Ptd_2. Higher temperatures are expected only to increase the probability of this lipid remaining in a nonbilayer phase.

TABLE 1 X-ray diffraction repeat spacings for dispersions of tetraoleoyl pyrophosphatidic acid in 2.5 mM HEPES (pH 7.0) at 15°C in the presence and absence of 2 M NaCl

	d(hkl) (nm)				
NaCl (M)	(100)	(110)	(200)	(210)	(300)
0	4.02	2.29	1.88	1.55	1.33
2	3.60				

The spacings shown (in nanometers) were obtained after 3 h of data collection followed by an equal length of time subtraction of background (i.e, just water in the capillary).

To reduce the hydrocarbon stress, which tends to alter the hexagonal tube radius from the spontaneous radius (Rand et al., 1990), 10% and 20% (w/w) tetradecane was added to some of the lipid samples. The hydrated samples containing tetradecane showed insignificant alteration of diffraction spacings (<3% change) (data not shown) when compared with those observed for Ptd_2 without added hydrocarbon. The water cylinder radius was calculated from the tube radius by the method outlined in the footnote of Table 3. The spontaneous radius was defined to be the radius of the pivotal circle (Rand et al., 1990), which was very close to the water cylinder radius. Thus the spontaneous radius could be approximated by the radius of the water cylinder under the "stress-free" condition.

Inverted micelles were formed by taking up phospholipids in tetradecane and then equilibrating with aqueous buffer (see Materials and Methods). The small, inverted micelles remain in the hydrocarbon phase after centrifugal separation of excess aqueous buffer, and the diameters of the inverted micelles can be estimated using quasi-elastic light scattering. The diameters of the inverted micelles formed in this way have been related to the size of the structures observed by x-ray diffraction (Sen and Hui, 1994). The inverted micelles of Ptd_2 in the clear hydrocarbon supernatant had about the same diameters as those formed by bovine cardiolipin (Table 2). In the presence of salt, the inverted micelles of both Ptd_2 and cardiolipin were smaller than in its absence; the cardiolipin micelles may be slightly larger than those formed from Ptd_2. These micellar structures were all similar in size to the hexagonal tubes observed by x-ray diffraction (Table 3).

DISCUSSION

Hydrated tetraoleoyl pyrophosphatidic acid (Ptd_2) dispersions in excess water form a well-defined inverted hexag-

TABLE 2 Size of inverted micelles formed from hydrated tetaoleoyl pyrophosphatidic acid or cardiolipin in the presence of 2 M NaCl in tetradecane

	Diameter (nm)
Tetraoleoyl phosphatidic acid	
0 M NaCl	7.6 ± 2
2 M NaCl	3.4 ± 0.7
Cardiolipin	
0 M NaCl	8.2 ± 2
2 M NaCl	4.9 ± 0.8

onal phase as shown by ^{31}P NMR. The positive anisotropy of +12 ppm (Fig. 1) compares with the chemical shift anisotropies observed for the inverted hexagonal phases assumed by bovine cardiolipin in 2 M NaCl (+19 ppm) and by acyl cardiolipin, which has five acyl chains, in aqueous buffer (+14.5 ppm) (Powell and Marsh, 1985). The smaller chemical shift anisotropy observed for Ptd_2 could reflect differences in the orientation of the phosphorous tensors in this pyrophospholipid or differences in motional averaging for the cardiolipin and this other four-chain lipid. Additional studies may be able to relate the slightly smaller anisotropy observed to the smaller tube diameter and the degree of completeness of motional averaging. The spacings obtained by low-angle x-ray diffraction satisfy the relationships for a hexagonal phase. Taken together, these data leave little doubt that Pdt_2 forms an inverted hexagonal phase rather than lamellar or cubic phases in dilute aqueous buffer or 2 M NaCl and ambient temperatures.

TABLE 3 Comparison of the cuvatures of cardiolipin analogs

Lipid	Tube diameter (nm)	Water cylinder Diameter (nm)	Water cylinder Curvature (nm^{-1})
Tetraoleoylpyrophosphatidic acid			
0 M Na Cl	4.7 ± 0.1	1.8	1.1
2 M NaCl	4.1 ± 0.1	1.2	1.5
Acyl cardiolipin*			
3 M NaCl	5.03 ± 0.1	2.28	0.88
Monogalactosyldiglyceride‡		3.02	0.67
Bovine cardiolipin			
2 M NaCl*	7.7 ± 0.1	4.9	0.41
$CaCl_2$		1.5	1.3
Dioleoyl phosphatidylethanolamine¶		4.4	0.45

The tube diameter s was calculated from the lattice constant, d_h: $s = (2d_h\bar{3})$. The tube diameter comprises the water core and the surrounding acyl chains. The water cylinder diameter ($2R_w$) was estimated for cardiolipin, acyl cardiolipin, and tetraoleoyl pyrophosphatidic acid by subtracting the contribution of the acyl chains, 2.8 nm (Seddon et al., 1983). Bovine cardiolipin contains 87% linoleate (more than three chains per molecule) and acyl cardiolipin contains the bovine complement of linoleate plus one oleate acyl chain per molecule (Powell and Marsh, 1985). The water cylinder radius R_w was estimated from the tube diameter, $2R_w = s - 2.8$ nm. The curvature was approximated as the reciprocal of the water cylinder radius, $C_o = 1/R_w$.

* Powell and Marsh, 1985.

‡ The water cylinder for monogalalactosyldyceride was for low lipid concentration and 20°C in Shipley et al. (1973). A hydrocarbon chain length of 3.0 nm was used.

§ The value for the water cylinder of calicum cardiolipin was for 20°C in Table II of Rand and Segupta (1972).

¶ The value for the water cylinder of dioleoyl phosphatidylethanolamine was taken from Table I of Rand et al (1990) for excess water and tetradecane and a hydrocarbon chain length of 3.0 nm.

From the x-ray diffraction spacings (d_{hex}), the lattice dimensions of the hexagonally packed tubes and the tube diameter were estimated (Table 3). Because the tube diameter is the diameter of the water core surrounded by acyl chains, the water core diameter can be estimated if the acyl chain length is known. Under the "stress-free" condition, that is in excess water and tetradecane, the diameter of the water core is governed by the spontaneous curvature of the lipid (Rand et al., 1990). The fact that lattice constants obtained for hydrated Ptd_2 were unchanged in the presence of 10% or 20% (w/w) tetradecane attests that the values in Table 1 are consistent with those for excess water and hydrocarbon for the spontaneous curvature measurement. The spacings observed for Ptd_2 were the same when the lipid was hydrated at 4°C as when observed at 15°C, suggesting that the inverted hexagonal phase was the stable phase near ambient temperatures.

In making the comparisons between lipids (Table 3), one should keep in mind that the pyrophosphatidic acid contains oleoyl chains, whereas bovine cardiolipin contains 87% linoleoyl chains (more than three of the four chains per molecule are linoleoyl); the acyl cardiolipin described contains the bovine complement of linoleoyl chains plus one additional oleoyl chain (Powell and Marsh, 1985). Whereas large changes in curvature resulting from changes in the number of double bonds would be unexpected for a cardiolipin composed of only oleoyl chains, it would be useful in an additional study to compare the curvatures of bovine cardiolipin and tetraoleoyl cardiolipin, for example, so that the effects of an additional double bond in an 18-carbon chain could be clarified.

The diameters of inverted micelles observed by light scattering were similar in size to the diameters of the structures observed by x-ray diffraction. The diameters of inverted micelles of Ptd_2 and of cardiolipin hydrated with dilute aqueous buffer were larger than with 2 M NaCl (Table 2). The effect of salts in screening the negative charges of the polar headgroups and in partially dehydrating the polar headgroups is well documented, and both effects decrease the water core of inverted micelles. Interestingly, the tube diameter observed for Ptd_2 changed little with salt.

Correspondence between the tube diameters and the diameters of inverted micelles was qualitatively shown for phosphatidylethanolamines (Sen and Hui, 1994), but the exact relationship is not well defined, because of the unknown gaussian modulus k_g in the expression for the bending energy E (Helfrich, 1974):

$$E = (\tfrac{1}{2})k_c(c_1 + c_2 - c_0)^2 + k_g c_1 c_2$$

where c_1, c_2, and c_0 are two principal curvatures and the spontaneous curvature, respectively, and k_c is the bending

modulus. For cylinders, c_2 is zero, and the spontaneous curvature may be obtained directly from curvature of the tube at the minimum of bending energy under stress-free conditions. For spherical inverted micelles, $c_1 = c_2$, and these curvatures do not necessarily equal c_0 at the minimum energy state, because of the unknown value of k_g in the second term in the above equation. Nonetheless, given that the value of k_g is unchanged for the same lipid, one may expect the same trend for curvature change to hold for hexagonal tubes as for inverted micelles. This is just what we observed. Further experimentation will be required before this light scattering method can provide the spontaneous curvature.

Using the values in Table 1 and approximating the spontaneous curvature as the reciprocal of the radius of the water core, one obtains the results shown in Table 3, where the spontaneous curvature of Ptd_2 is compared with other lipids known to form inverted hexagonal phases. The spontaneous curvature for Ptd_2 is extremely large, more than twice that of phosphatidylethanolamine and nearly twice that of bovine cardiolipin in 2 M NaCl. Bovine cardiolipin forms a lamellar phase in the absence of salt (Rand and Sengupta, 1972; Powell and Marsh, 1985), and dioleoylphosphatidylethanolamine is lamellar at ambient and lower temperatures (Rand et al., 1990). By way of comparison, the cristae within mitochondria contain both phosphatidyl ethanolamine and cardiolipin and assume curved lamellar structures with diameters of about 13 nm. These structures are small enough to favor the presence of lipids with large radii of curvature in regions of great curvature. Chloroplast monogalactosyldiglyceride (Shipley et al., 1973) and acyl cardiolipin (Powell and Marsh, 1985), which have larger spontaneous curvatures (Table 3), do not form lamellar phases, even in the absence of salt. The spontaneous curvature of Ptd_2 is still larger; no lamellar phase was found. The curvature of this synthetic lipid is approached only by the calcium salt of cardiolipin (Table 3). Together these two lipids are the most highly curved so far studied. Tetraoleoylpyrophosphatidic acid, with its very large spontaneous curvature, should prove useful in reconstitution studies, for examining how lipids with this character can influence biological function.

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