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STRUCTURAL PROPERTIES OF A Ca^{2+}-PHOSPHATIDIC ACID COMPLEX

SMALL ANGLE X-RAY SCATTERING AND CALORIMETRIC RESULTS

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The structures of Ca^{2+} and Cd^{2+} complexes with dipalmitoyl- and dimyristoylphosphatidic acids have been investigated by differential scanning calorimetry and small angle X-ray diffraction. The lipids are found to form complexes with Ca^{2+} which exhibit no thermal phase transitions between temperatures of 25 and 90°C. Transition enthalpies of residual uncomplexed lipid extrapolate to zero near a 1 : 1 lipid to cation ratio indicating a 1 : 1 complex stoichiometry. Ca^{2+} and Cd^{2+} complexes appear isomorphous in the X-ray data and show lamellar phases with short repeat distances (51.5 Å (5.15 nm) and 58.0 Å (5.80 nm) for dimyristoyl and dipalmitoyl homologs, respectively). The data are discussed in terms of structures in which ions bridge phosphates on adjacent bilayers.

Introduction

Anionic lipids are minor but essential constituents of virtually all biological membranes. Interactions of divalent cations with these lipids are also of potential importance. Variations of divalent cation levels, Ca^{2+} in particular, are known to correlate with a number of membrane centered phenomena such as neurotransmitter release and myoblast fusion [1,2]. The specific role of anionic lipid-Ca^{2+} complexes in membrane centered processes has not been elucidated in detail. They may simply act in regulation of ion surface concentration, be involved in protein association or even play a more direct role in membrane transformation. Nevertheless, detailed studies of anionic lipid-cation complexes in well defined model systems can form the basis for future understanding of more complex phenomena.

A system of particular interest has been the phosphatidic acid-phosphatidylcholine system. Like a number of other anionic lipid systems [2–5], vesicles prepared from mixtures of these two lipids fuse to form larger unilamellar vesicles on addition of Ca^{2+}. The system had seemed somewhat unusual in that internally confined solutes (sucrose and tetraethylammonium bromide) did not leak extensively to the external solution during fusion [6]. However, early stages of fusion in phosphatidylserine vesicles have recently been shown to also proceed with minimal leakage [7]. Leakage is retarded, in the phosphatidic acid-phosphatidylcholine system, in spite of the fact that the initial bilayer must break and that this break provides a path for transbilayer movement of divalent ions [8].

It is possible that these properties are inherent characteristics of transition state phases such as the micellar and hexagonal phases proposed by Cullis et al. [9]. It is also possible that there is rather extensive intervesicular bridging of bilayers before rupture and fusion and that this bridging provides

Abbreviations: DMPA, dimyristoylphosphatidic acid; DPPA, dipalmitoylphosphatidic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid.

geometric restraints on solute leakage. Transbilayer complexes have been proposed and studied for the phosphatidylserine system where they have been assigned a dominant role in the distinction between Ca^{2+} and Mg^{2+} in stimulating fusion [10].

We present here a study of inter bilayer complexes of phosphatidic acid with Ca^{2+} and Cd^{2+} using low angle X-ray scattering of unoriented preparations. The intention is to comment on the probable role of such complexes in stimulating fusion and in impeding solute leakage during the fusion process.

Low angle X-ray scattering has provided a great deal of useful information on the structure of lipid systems [11–13]. At a minimum, spacing of reflections can be used to characterize the type of periodic order present in the lipid phase and give fundamental repeat distances. Interbilayer distances have been useful in characterizing the degree of hydration in the phosphatidylserine system, for example [10]. At a maximum, intensity of reflections can be used to deduce electron density profiles for the entire bilayer.

Here we are primarily interested in ion distribution and location. Ca^{2+}, however, has a relatively low electron density and thus does not give rise to an easily identifiable portion of the electron density profile.

For the phosphatidic acid-phosphatidylcholine system we have demonstrated that two ions, Ca^{2+} and Cd^{2+} stimulate vesicle fusion with equal efficiency [14]. These ions also have identical charge and nearly identical ionic radii (0.95 and 0.92 Å). There is every reason to believe that an isomorphic substitution would be possible. A difference Patterson map could be obtained and easily interpreted in terms of ion distribution.

The actual systems to be studied are 1 : 1 Ca^{2+} and Cd^{2+} complexes of dimyristoylphosphatidic acid and dipalmitoylphosphatidic acid. Anionic lipids such as phosphatidylserine in mixed lipid systems have been shown to phase segregate into pure anionic lipid regions in the presence of Ca^{2+} and to exist in a well ordered gel-lamellar phase [2]. The dependence of extent of fusion on the potential amount of phosphatidic acid-cation complex in a mixed vesicle suggests that the same is true for the phosphatidic acid system. Saturation of fusion at 1 : 1 anionic lipid to cation ratio also suggests that 1 : 1 is the appropriate stoichiometry. Differential scanning calorimetric data are presented to show that dimyristoyl- and dipalmitoylphosphatidic acids form 1 : 1 complexes with Ca^{2+} and that the complexes show no transition to a liquid crystalline phase at temperatures accessible in aqueous solution. They thus provide a well characterized model for a potential vesicle fusion intermediate.

Materials and Methods

Materials. Dipalmitoylphosphatidic acid was purchased from Serdary Research Laboratories (London Ontario, Canada) and was used without further purification. Dimyristoylphosphatidic acid was synthesized by the action of partially purified phospholipase D from cabbage on an diethyl ether/water dispersion of dimyristoylphosphatidylcholine. The dimyristoylphosphatidylcholine was purchased from Sigma (St. Louis, MO). The digestion and purification procedures which are derived from the works of Papahadjopoulos and Miller have been described in detail elsewhere [6]. 2-(N-Morpholino)ethanesulfonic acid (Mes) used as a buffering agent was purchased from Calbiochem. (San Diego, CA). All other chemicals were of reagent grade and were used without further analysis.

Differential scanning calorimetry. Samples for calorimetry were prepared at a concentration of 0.2% (w/v) in total lipid in pH 6.0 ± 0.1 buffer solution containing 10 mM Mes, 100 mM NaCl and 10% ^2H_2O. Weighed amounts of lipid were suspended in buffer solution at a temperature 10°C higher than the phase transition temperature of the lipid. An equal volume of Ca^{2+} solution at concentrations which would approach Ca^{2+} : phosphatidic acid ratios from 0.25 to 1 was added to the dispersion and the mixtures were incubated at 80°C for at least 30 min to aid the homogeneous distribution of Ca^{2+}. pH was rechecked and adjusted to be 6.0 ± 0.1. Examination of phosphatidic acid after 1 h at 80°C by TLC showed less than 5% degradation. The solutions were then stored at 4°C until use.

The differential scanning calorimeter used for these experiments was developed by Privalov et al. [15]. The cells constructed of gold with platinum capillary filling tubes are of 'pillbox' shape and have nearly equal volumes of 1 ml. In a typical experiment, the calorimetric scan was performed with a

scan rate of 1 K/min. At this scan rate, the instrument has a sensitivity of approximately 20 μcal/K. The calorimeter cells filled with samples were equilibrated at 20°C before scanning. For some experiments, the cells were heated to 80°C for 30 min and then cooled to the starting point in order to achieve satisfactory reproducibility. In most cases, the scan was repeated without emptying and refilling the cells. The enthalpy of transition was evaluated from the area under the calorimetric curve. The integration of this area was accomplished using a planimeter or by 'cutting and weighing'.

Small angle X-ray scattering. Samples for small angle scattering were prepared as wet pellets obtained from the mixtures of lipid and cation solutions. Weighed amounts of lipid, approx. 10 mg, were dispersed in 1 ml pH 6.0 ± 0.1 buffer containing 10 mM Mes, 100 mM NaCl and 10% ^2H_2O. For these experiments, the suspensions were sonicated at a temperature above the phase transition before gradual addition of an equal volume of cation solution at a concentration sufficient to achieve the desired Ca^{2+} or Cd^{2+} : phosphatidic acid ratio. However, omission of the sonication step on samples made with phosphatidic acid derived from egg was shown to make no difference in the X-ray results. To obtain homogeneous samples, all mixtures were incubated in a water bath above the phase transition temperature for 1 h. The flocculent precipitates were then separated from the supernatant using a hand-driven centrifuge and stored under N_2 at 4°C until use. A 1-mm i.d. glass capillary was used as a sample cell. The wet pellets were packed into cells by centrifugation until a sample approx. 1.5 cm high was obtained.

Small angle scattering measurements were performed using a modification of the system previously described by McDonald et al. [16]. 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, resulting in nearly monochromatic CuK_α radiation. The beam width as measured by the detector was 140 μm full width at half maximum, and the beam height as measured by vertical displacement of the detector was 0.8 cm between points of 80% of maximal intensity.

The sample was positioned in a thermostated holder midway between the mirror and the detector. Temperature was controlled with a Lauda circulating water bath with a deviation of ±2°C. The measurements were made using a Tennelec PSD 100 one-dimensional position-sensitive detector. Specimen-to-detector distance was 33 cm with most of the beam path maintained under vacuum to reduce scattering by air.

Typically, data from the position sensitive detector were accumulated for 5 000 s and stored in a Canberra multichannel analyzer. The accuracy of reciprocal spacing from the instrument itself is 0.0357 cm^{-1} per channel. Small variations in detector properties were compensated by averaging the scattering curves on either side of the beam stop.

The raw data, displayed as the number of counts versus the channel number, were digitized and transferred to a PDP 11/45 computer. They were corrected for the buffer and camera background by subtracting the buffer scattering from intensity, followed by converting the data points to $I\sin\theta$, (Lorenz correction) where I is the number of counts in a channel and θ is calculated from the 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 (1.54 Å = 0.154 nm). The difference Patterson maps were obtained by Fourier transformation of the differences between intensities for Cd^{2+} and Ca^{2+} samples.

Results

X-ray data are most easily analyzed if we are dealing with a single structural form. Since anionic lipid-cation complexes are believed to phase segregate, a less than full complement of cation could lead to a mixture of two structurally different phases and a complex scattering curve. There is unfortunately disagreement in the literature as to the precise Ca^{2+}-phosphatidic acid stoichiometry. Both 2 : 1 and 1 : 1 complexes have been reported [17–20]. Uncomplexed dimyristoylphosphatidic acid at pH 6 has a well-defined gel to liquid crystalline phase transtransition at approx. 50°C. Addition of divalent ions to anionic lipids is expected to shift the transition temperature to higher values, and in some cases to experimentally inaccessible values [21–25]. Disappearance of this transition in a series of differential scanning calorimetry curves in a series of samples of

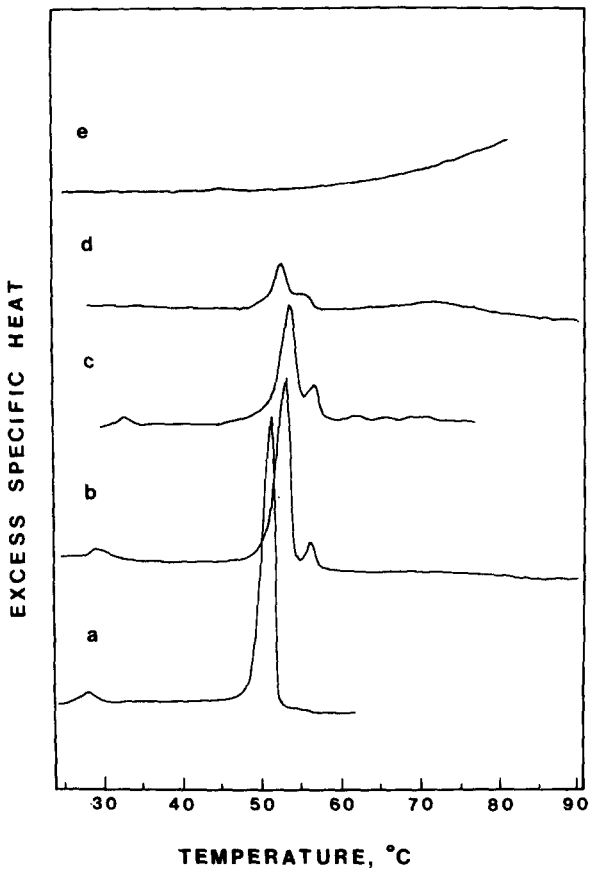

Fig. 1. Transition curves for dimyristoylphosphatidic (DMPA) acid dispersions (a) in the absence of Ca^{2+} and (b–e) in the presence of Ca^{2+} at a ratio of: (b) 0.25 : 1, Ca^{2+} : DMPA; (c) 0.50 : 1, Ca^{2+} : DMPA; (d) 0.75 : 1, Ca^{2+} : DMPA and (e) 1 : 1, Ca^{2+} : DMPA.

different ion to anionic lipid ratios can be used to clarify the stoichiometry question. The data also serve to define temperature regions where the more ordered gel phase exists. X-ray data on this more ordered system will be of higher quality.

The calorimetric scans for samples 0.2% in lipid are presented in Fig. 1 as a function of Ca^{2+} mole ratio. The most significant change in the scans is the change in area under the gel to liquid crystal transition of the uncomplexed phosphatidic acid initially at 51°C. The area under the narrow peak clearly decreases as the Ca^{2+} mole ratio increases. At a mole ratio of 1 : 1, the narrow peak has disappeared and no other transition can be observed in the 50°C region.

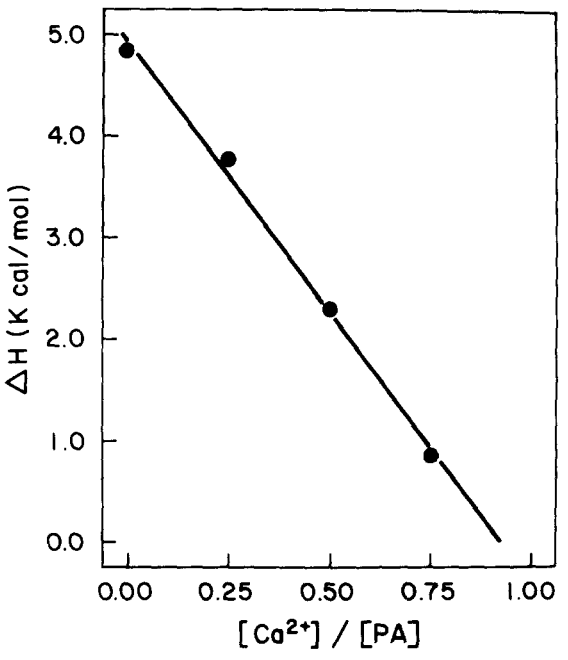

Fig. 2. Transition enthalpy versus calcium to phosphatidic acid mole ratio. Data points are derived from calorimetric scans in Fig. 1.

The near constancy of T_m for the 50°C transition is suggestive of a nearly pure uncomplexed phosphatidic acid phase. Amounts of this phase should be linearly related to excess specific heat. Enthalpies of the 50°C transitions were determined by integrating the excess specific heat under the main transition profile and these values are presented in Fig. 2. Extrapolation of specific heats to zero near a 1 : 1 Ca^{2+} to phosphatidic acid ratio strongly supports the existence of a well defined ion complex of 1 : 1 stoichiometry.

It is perhaps surprising that phosphatidic acid, which carries a single negative charge at pH 6, can bind more Ca^{2+} than what is estimated from the charge neutralization point of view. It has, however, been suggested that phosphatidic acid releases one proton when binding with Ca^{2+} [18–20]. Measurement of pH changes on addition of Ca^{2+} to unbuffered phosphatidic acid solution confirms this trend. It also argues against the participation of Cl^- anion in the complex.

X-ray scattering results

All samples for X-ray scattering were prepared to be 1 : 1 cation to phosphatidic acid at pH 6 so that all phosphatidic acid binding sites would be uniformly saturated by cation. We first show the data on the dipalmitoylphosphatidic acid-Ca system. Fig. 3 presents the scattering intensities versus reciprocal space for a sample at 25°C. At low angles, the channel number is approximately linearly related to the reciprocal spacing. It clearly shows that besides the strong band which corresponds to a spacing of 58 ± 0.5 Å, several submultiple bands of regular spacing are seen. The pattern fits a D, $D/2$, $D/3$, $D/4$ ... series with $D = 58$ Å, suggesting a lamellar phase [13]. The large number of lamellar reflections detectable, indicates a high degree of order, consistent with the gel phase. A periodicity of 58 Å is much shorter than that for phosphatidic acids in the absence of cations [26]. Anionic lipids usually swell more than neutral lipids, incorporating large amounts of water in the head group region. The short spacing in the presence of cation, can be interpreted as a high degree of cation-induced dehydration. The peak in the high angle region of Fig. 3 is in a position expected for the diffraction from hydrocarbon chains. The presence of a sharp reflection at $(4.2)^{-1}$ Å rather than a diffuse band centered near $(4.6)^{-1}$ Å is also consistent with a gel phase in which chains are packed in a hexagonal array.

A similar diffraction spectrum with an identical

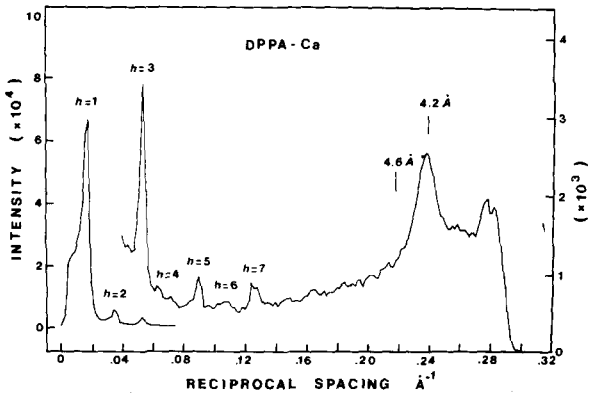

Fig. 3. Low angle X-ray diffraction spectrum for a 1 : 1 Ca^{2+} : dipalmitoylphosphatidic acid complex (DPPA-Ca).

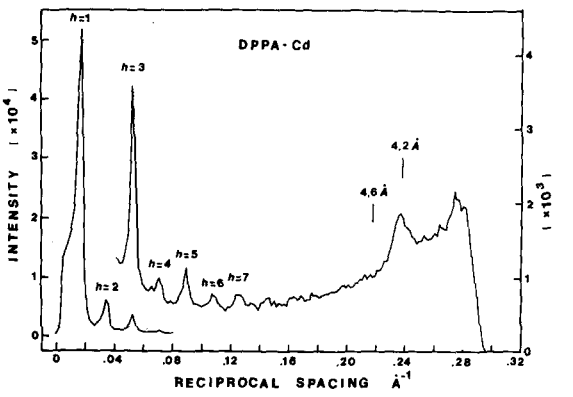

Fig. 4. Low angle X-ray diffraction spectrum for a 1 : 1 Cd^{2+} : dipalmitoylphosphatidic acid complex (DPPA-Cd).

repeat spacing was obtained for a Cd^{2+}-dipalmitoylphosphatidic acid sample (Fig. 4). This supports our assumed isomorphic substitution. Since Cd^{2+} has 28 more electrons than Ca^{2+}, more intense scattering and better resolved data were obtained. For our purpose, the cation distribution is of more interest. Comparison of Figs. 3 and 4 provides a convenient way to investigate the location of cations. This was done by consideration of a difference Patterson map. The significance of the difference Patterson map is that many features common to both the Ca and the Cd derivatives cancel out so that the information is more directly related to the difference between the two structures, namely the presence of the heavy metal.

In order to obtain the difference Patterson map, the raw data in Figs. 3 and 4 were treated by means of Fourier Transformation as described in the methods section. The scattering intensities for Ca^{2+} and Cd^{2+} samples were normalized by amounts of lipids estimated from the areas under the peaks in the high angle region which are assumed to correspond to diffractions from hydrocarbon chains. Because of the signal to noise problem, data were truncated at the 7th order reflection. Fig. 5 shows the transformed difference Patterson map with five repeating units. The narrow but tall bands are dominated by cation-cation spacings and have a half-width of 7 Å. A large fraction (5 Å) of this width results from truncation at seven reflections. Based on this fact and the fact that widths in the Patterson map usually are larger than distribution widths, the

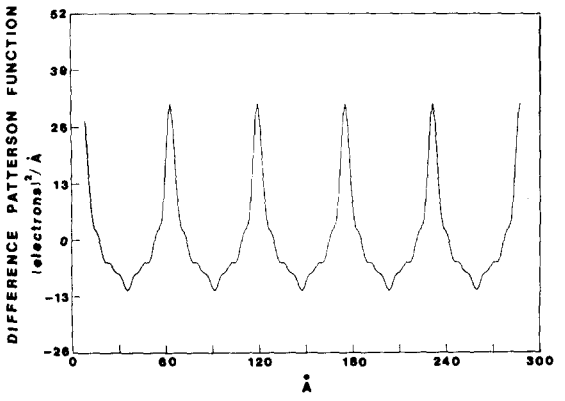

Fig. 5. The difference Patterson map between Cd^{2+}-DPPA and Ca^{2+}-DPPA diffractions.

profile in Fig. 5 suggests a very well-defined cation distribution. Despite this lamellar diffraction, no well-defined Ca^{2+}-Ca^{2+} spacing was observed.

It would be very useful to gain some information about the structural relationship of the ion to the phospholipid head group. The relatively short lamellar repeat distances observed do suggest a low level of hydration but without a detailed comparison of scattering curves and electron density profiles, allocation of the lamellar repeat distance to hydrocarbon and headgroup regions is not usually possible. Comparison of data in which the hydrocarbon chain length is systematically varied provides an alternate means of accomplishing an allocation. The scattering of a dimyristoylphosphatidic acid-Cd^{2+} sample was therefore examined. At 25°C, the repeat distance is found to be 51.5 ± 0.5 Å. This is 6.4 Å shorter than that for the dipalmitoylphosphatidic acid sample at the same temperature. Spacings do vary slightly as a function of temperature. For the dimyristoylphosphatidic acid-Cd complex, distance decreases to 49.5 in going from 25 to 75°C. It has become customary to compare properties of homologous lipids at equal temperature displacements from their gel to liquid crystalline phase transition. If we assume a 20°C variation between dimyristoylphosphatidic acid and dipalmitoylphosphatidic acid, and if we assume a linear increase in spacing with temperature, the relevant repeat distance for dimyristoylphosphatidic acid would be 52.5 Å. The reduction of 5.5 Å is approximately what one would expect on removing four methylene groups from the hydrocarbon layer if all chains were trans and parallel to the bilayer normal (5.0 Å). This information, along with that on ion distribution can be used to reach some preliminary conclusions about ion complex structures.

Discussion

Several general statements can be made about the Cd^{2+} and Ca^{2+} phosphatidic acid complexes of myristic and palmitic acid analogs. First, the complexes have a 1 : 1 cation ion to phosphatidic acid stoichiometry with no evidence of additional counterions participating in the complex. Second, the complexes are arranged in a lamellar phase with the ions located in a single well-defined plane presumably centered in the aqueous space between bilayers. And third, the bilayer-bilayer spacing is quite short, 58 Å for dipalmitoylphosphatidic acid compared to 64 Å for fully hydrated dipalmitoylphosphatidylcholine. Since the hydrocarbon chains appear to be all *trans* with axes perpendicular to the bilayer surface; this leaves a very small aqueous space. Both the repeat distance and the perpendicular chains are characteristic of low levels of hydration. A low level of hydration has been cited in the phosphatidylserine system as contributing factor to facile bilayer transformations [10].

A hexagonally packed hydrocarbon lattice, a small aqueous space, and a high density of divalent ions place sufficient restraints on the system to allow speculation about more detailed models for the complex. Two crystal structures involving low hydration phospholipids have been published, one for dilauryl phosphatidylethanolamine [27] and one for dimyristoyl phosphatidylcholine [28]. Moreover a crystal structure for a Cd complex, glycerophosphocholine-$CdCl_2$ also exists [29]. Recent work by Browning and Seelig has shown that, with the exception of the headgroup, phospholipids retain very similar molecular conformation in bilayers suggesting that comparison of these crystal structures to data obtained here might be valid [30]. Dilauryl phosphatidylethanolamine and the A form of dimyristoyl phosphatidylcholine have very similar glycerol backbone and acyl chain conformations. For dilauryl phosphatidylethanolamine hydrocarbon chains are packed in a pseudohexagonal lattice with axes nearly

perpendicular to the bilayer plane. Average chain-chain spacing (4.6 Å) is in fact very close to that observed here. It therefore provides a good estimate of the hydrocarbon contribution to the bilayer repeat distance. The glycerol C_3-C_3 transbilayer distance is 36.0 Å in dilauryl-phosphatidylethanolamine. This should be increased by 5.0 Å for the addition of 4 methylene groups in the dimyristoyl-phosphatidic acid bilayer.

The C_1-C_2-C_3-O dihedral angle in phospholipids and phospholipid analogs appears to be quite variable [28]. There is also NMR evidence that the addition of multivalent cations to phosphatidylcholines changes headgroup conformations to a more extended state [31]. A more extended state in which the C_1-C_2-C_3-O and C_2-C_3-O-P angles are nearly trans, is observed in the B form of dimyristoylphosphatidylcholine and in glycerophosphocholine-$CdCl_2$. In this conformation, an additional 7.6 Å is added to give an outer phosphate oxygen-phosphate oxygen transbilayer spacing of 48.6 Å. Compared with our measured 52.5 Å for dimyristoylphosphatidic acid this would leave approx. 4 Å in which to place a plane of Cd^{2+} ions. It is interesting to note that in glycerophosphocholine-$CdCl_2$, Cd^{2+} bridges rows of phosphates, with the phosphate oxygens at the apices of distorted octahedra. The oxygen-oxygen distance in these complexes is 4.4 Å, within experimental error of our estimate. Thus, the transbilayer observed spacing is appropriate for a bridging complex.

One might also ask if the in plane ion density is appropriate for a bridging complex such as that observed in glycerophosphocholine-$CdCl_2$. If the head group conformation for dimyristoylphosphatidic acid is as suggested, phosphates lie on an extension of the 1-glycerol-acyl chain axis. With packing similar to the dilaurylphosphatidylethanolamine crystal, phosphates would form rows 4.6 Å apart with 7.9 Å between phosphates. The phosphate-phosphate distance in the rows of Cd^{2+} bridges in glycerophosphocholine is 7.4 Å, suggesting that with minor displacements the Cd^{2+}-phosphate portions of the structures of Cd^{2+}-phosphatidic acid and glycerophosphocholine-$CdCl_2$ could be very similar.

It is important to note, however, that despite suitable lateral spacing for formation of bridging complexes, there is no direct evidence for in-plane order. If a structure such as that suggested existed with a high degree of lateral order, one would expect to see Cd^{2+}-Cd^{2+} reflections at 4.6 and 4.0 Å. These are not apparent, suggesting a more disordered structure. Unfortunately, these reflections would also be very close to the 4.2 Å reflection from hexagonally packed hydrocarbon chains and we cannot absolutely exclude their existence.

Whether or not extensive lateral order exists, the suitability of both lateral and normal bilayer dimensions for formation of bridging complexes of Ca^{2+} and Cd^{2+} similar to those found in glycerophosphocholine-$CdCl_2$, may be an important factor in the observed increase in fusion rate when Cd^{2+} or Ca^{2+} is used to stimulate vesicle fusion as opposed to smaller (Mg^{2+}) or larger (Ba^{2+}) ions [14]. The lack of solvent spaces in such structures may also be a factor in the relatively low leakage of vesicle contents in fusion of phosphatidic acid-phosphatidylcholine vesicles.

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