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BILAYERS OF PHOSPHATIDYLDIACYLGLYCEROL AND PHOSPHATIDYLCHOLESTEROL GIVE ^{31}P-NMR SPECTRA CHARACTERISTIC FOR HEXAGONAL AND ISOTROPIC PHASES

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Aqueous dispersions of phosphatidyldiacylglycerol and phosphatidylcholesterol are shown to form bilayers by differential scanning calorimetry, diphenylhexatriene fluorescence polarization, and electron microscopy; however, ^{31}P-nuclear magnetic resonance spectra of these dispersions are characteristic of the 'hexagonal' and 'isotropic' phases, respectively. The theoretical relationship between the conformation of phospholipid molecules in bilayers and the shape of the ^{31}P-NMR line is examined. It is shown that differences in the various characteristic spectral line shapes can result from differences in the phospholipid headgroup conformation without significant changes in the organization of the acyl chains in the bilayer phase.

Introduction

^{31}P-NMR spectroscopy has been used as a diagnostic tool to ascertain the organization of phospholipids in aqueous dispersions [1,2]. Thus, the bilayer, micellar, and hexagonal II phase in aqueous dispersions of phospholipids have been assigned characteristic line shapes which reflect the motional and conformational states of phospholipid molecules and therefore, the orientation of the phosphorus shielding tensors [3]. The 'bent' conformation of diacylphospholipids in an averaged isotropic environment of the micellar phase could give rise to symmetrical lines. In contrast, the non-zero anisotropy of phosphorus tensors in slow tumbling bilayers or hexagonal II phases produces asymmetric lines with characteristic residual chemical shift anisotropies.

We have studied properties of the aqueous dispersions of several synthetic phospholipids designed to restrict them orientationally and conformationally in bilayers [4]. In this paper we demonstrate that while phosphatidylcholesterol and phosphatidyldiacylglycerol form bilayers in aqueous dispersions, they do not exhibit ^{31}P-NMR spectral lines considered to be characteristic of bilayers. However, the observed line shapes can be adequately accounted for in terms of residual chemical shielding anisotropies resulting from the probable molecular conformation of these phospholipids in bilayers [5].

Materials and Methods

The salts of *O*-(1,2-diacyl-*sn*-glycerol-3-phosphoryl)cholesterol, abbreviated as phosphatidylcholesterol or DRPCh, R-acyl chain, were synthesized as previously described [6,7]. Analytical data for the new compound are as follows:

DLPCh · 0.5 Ca: $C_{54}H_{96}O_8P \cdot 0.5$ Ca

Calcd.:	C, 70.2;	H, 10.5;	Ca, 2.2
Found:	C, 70.1;	H, 10.7;	Ca, 2.1

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DMPCh · 0.5 Ca: $C_{58}H_{104}O_8P \cdot 0.5\ Ca \cdot H_2O$

Calcd.:	C, 70.3;	H, 10.8;	Ca, 2.0
Found:	C, 70.5;	H, 10.4;	Ca, 1.9

DSPCh · 0.5 Ca: $C_{66}H_{120}O_8P \cdot 0.5\ Ca \cdot H_2O$

Calcd.:	C, 71,4;	H, 11.1;	Ca, 1.8
Found:	C, 71.7;	H, 10.9;	Ca, 1.7

where L = lauroyl, M = myristoyl and S = stearoyl.

The salts of 1',2'-di-*O*-acyl-3'-*O*-(1,2-di-*O*-acyl-*sn*-glycerol-3-*O*-phosphoryl)-*sn*-glycerol, abbreviated as phosphatidyldiacylglycerol or 4R-bis-PA, R = acyl chain, bis-PA = 'bis-phosphatidic acid', were sythesized as previously described [8,9].

The purity of the phospholipid samples was established by thin-layer chromatography [6–9] and elemental analysis. The 1,2-diacyl-*sn*-glycerols employed in the synthesis of phosphatidylcholesterol and phosphatidyldiacylglycerol were prepared from freshly distilled acyl chlorides shown to be 99.9% and 99.0% pure, respectively, by gas chromatographic analysis of the corresponding methyl esters [7].

Samples for ^{31}P-NMR were prepared by hydrating solid phospholipids with the buffer containing 200 mM Tris in deuterated water at pH 8.0 (corrected). The hydrated samples were equilibrated for 60 min with vigorous shaking at 70°C. Thin-layer chromatographic analysis demonstrated that the phospholipids are not degraded during preparation of samples or during the NMR runs. The 101.27 MHz, high power proton noise-decoupled ^{31}P-NMR measurements were conducted on a Brucker WM-250 instrument.

Samples for differential scanning calorimetry were prepared as described elsewhere by hydrating solid samples in aluminum sample pans [4]. Liposomes for fluorescence polarization were prepared by dispersing phospholipids (10 mg/ml) by sonication for about 3 min in buffer containing 20 mM Tris at pH 8.0. Stable aqueous dispersions were diluted to 20 μM phospholipid concentration for fluorescence polarization measurements on Elsinct fluorescence polarization instrument (model MV1A). The lipid to diphenylhexatriene ratio was 90 to 1 for all measurements. This instrument directly gives the fluorescence polarization ratio from the values of fluorescence intensity.

For standard electron microscopy [10] phospholipid dispersions were prepared in buffers containing 25% glycerol to prevent the formation of ice crystals. Samples were frozen by quenching from 20°C in a slush of solid and liquid nitrogen. In some cases, the ultrarapid jet-freezing technique [11] was also employed. With this technique, there is no ice crystallization, and therefore glycerol is omitted from the buffer.

Results

Phase transition of phosphatidyldiacylglycerol and phosphatidylcholesterol by fluorescence polarization

Diphenylhexatriene partitioned into phospholipid dispersions exhibits a fluorescence enhancement, and the fluorescence polarization ratio undergoes a sharp change at the phase transition temperature of bilayers. As shown in Fig. 1a and b the transition temperature obtained by polarization measurements compares favorably with the cooperative thermotropic phase transition temperature obtained by differential scanning calorimetry. The polarization ratio of 0.4 for bilayers of both lipids is observed below the phase transition

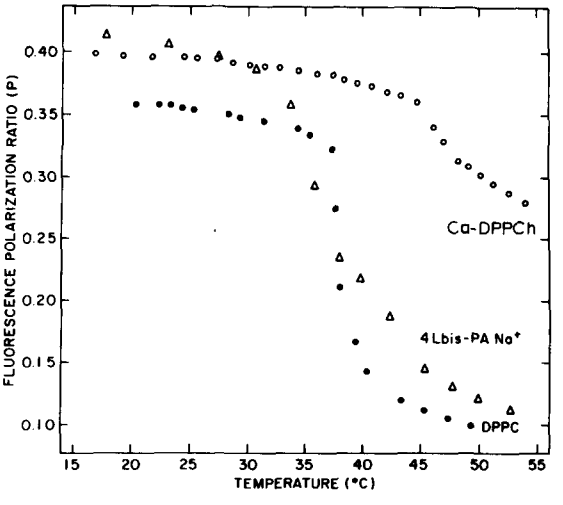


Fig. 1. Fluorescence polarization ratios of (a) dilauroylphosphatidyldilauroylglycerol (4L bis-PA), and (b) calcium salt of dipalmitoylphosphatidylcholesterol (DPPCh) dispersions as a function of temperature. Buffer (pH 8.0) Tris. The polarization curve for dipalmitoylphosphatidylcholine (DPPC) is shown for comparison. Phase transition for NH_4·DPPCh could not be detected.

temperature, and the same value is observed for the gel phase of other phospholipid bilayers. The polarization ratio of about 0.1 is observed for bilayers of phosphatidyldiacylglycerol above the phase transition temperature, and indeed comparable values are observed for other phospholipids in the liquid crystalline phase. Furthermore, the polarization ratio for phosphatidylcholesterol bilayers above the phase transition temperature is 0.25, and this somewhat higher value could be due to higher 'microviscosity' in the cholesterol containing acyl chain region. These observations demonstrate that the thermotropic phase transition in phosphatidylcholesterol bilayers and in phosphatidyldiacylglycerol bilayers brings about an abrupt change in the motional freedom of the diphenylhexatriene probe, that is localized in the acyl chain region.

Thermotropic phase transition properties of phosphatidylcholesterol and phosphatidyldiacylglycerol as a function of acyl chain length

The temperature and enthalpy of the main cooperative transition of aqueous dispersions of diacylphospholipids depend on acyl chain length. The phase transition temperatures for aqueous dispersions of phosphatidylcholesterol and phosphatidyldiacylglycerol of varying chain length also exhibit such a dependence. As shown in Table I, the transition temperature increased with chain length. Typically, the change in the transition temperature is about 10 K per $-CH_2-CH_2-$ change in the acyl chain length. This observation suggests that the molecular parameters governing the conformational disorder of acyl chains give rise to the observed transitions. One of the important differences between tetraacyl- and diacylphospholipids and diacylphosphatidylcholesterol transitions is observed in the half height widths. For phosphatidyldiacylglycerol for example, the width of the transition does not change appreciably with chain length; it is about 1.5 K for the palmitoyl and stearoyl derivatives. Only the calcium salts of diacylphosphatidylcholesterols exhibit phase transition and T_m increases with increasing chain length, being 298, 311, 320, and 324 K for lauroyl-, myristoyl-, palmitoyl-, and stearoyl-phosphatidylcholesterol analogs. This could mean that the factors governing the phase transition, such as the conformation of acyl chains, are partially disrupted or they are less prominent in short chain analogs of phosphatidylcholesterol.

TABLE I

PHASE TRANSITION CHARACTERISTICS OF THE AQUEOUS DISPERSIONS OF PHOSPHATIDYLDIACYLGLYCEROLS (SODIUM-SALTS) AND PHOSPHATIDYLCHOLESTEROLS (CALCIUM-SALTS) OF VARYING ACYL CHAIN LENGTHS [a]

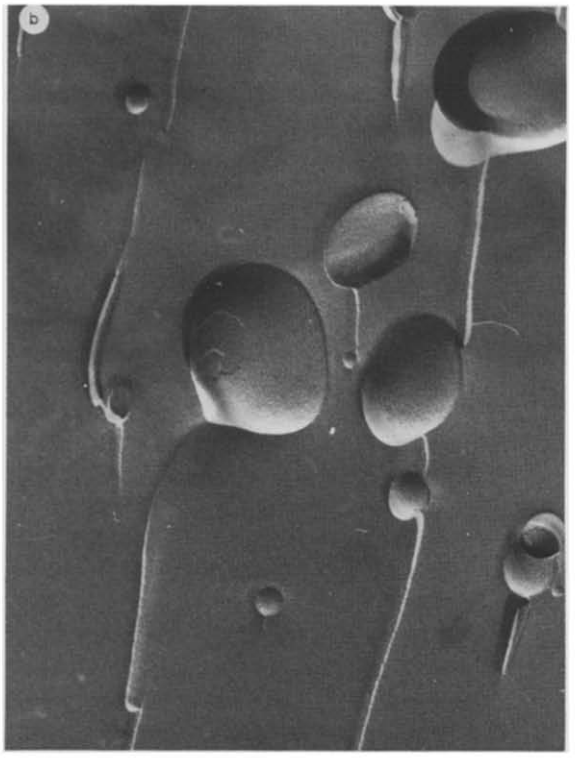
Acyl chain	T_m (K)	
	Phosphatidyl-diacylglycerol	Phosphatidyl-cholesterol
Dodecanoyl-	313	298
Tetradecanoyl-	330	311
Hexadecanoyl-	348	320
Octadecanoyl-	–	324

[a] Analytical samples of the appropriate salts were dispersed in distilled water in the sample pan for DSC.

Freeze-fracture electron microscopy

Data reported elsewhere [7], the present results (Fig. 2b), and additional experiments not shown here, demonstrate that the aqueous dispersions of phosphatidylcholesterol contain typical multilayered liposomal structures of 0.1–2 μm diameter. In general, the morphology of the lipid dispersions is identical using both freezing techniques.

Phosphatidyldiacylglycerol dispersions also exhibit a bilayer type of organization (Fig. 2a). The sodium salts appear to be organized as highly curved bilayers or as stacked liposomes. In the jet-frozen preparations we observed elevated structures in the fracture plane. Similar structures have been described in codispersions of dipalmitoylphosphatidylcholine and cholesterol using the jet-freezing technique [11]. The dispersions of the calcium salt of phosphatidyldiacylglycerol show more densely stacked and longer extended layers by using both types of freezing procedures. The appearance of such features is reasonable since the phase transition temperature of dipalmitoylphosphatidyldipalmitoylglycerol is high ($\sim 75°C$), and it is possible that these bilayers are partially dehydrated and more closely stacked in the gel phase at room temperature.



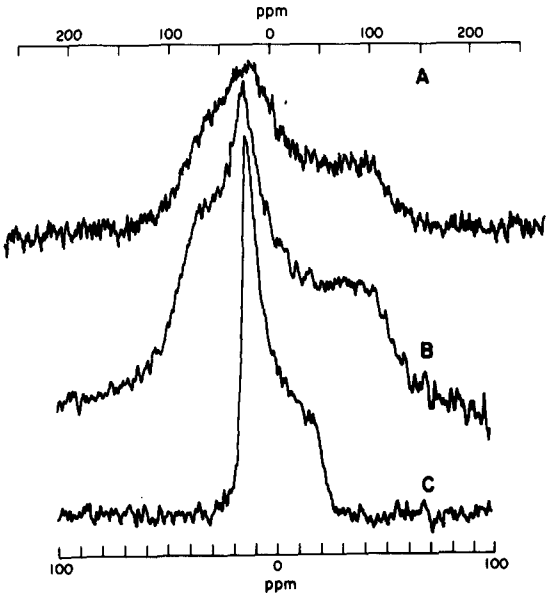


Fig. 3. ^{31}P-NMR spectra of sodium salt of didodecanoylphosphatidyldidodecanoylglycerol (A) in powder form; (B) as aqueous despersions below the phase transition temperature, (C) as aqueous dispersions above the phase transition temperature.

^{31}P-NMR studies

The ^{31}P-NMR spectra for the powder and for the aqueous dispersions of dilauroylphosphatidyldilauroylglycerol are shown in Fig. 3. The spectra for dipalmitoylphosphatidyldipalmitoylglycerol are essentially identical (not shown here) except for the higher phase transition temperature exhibited by the aqueous dispersions. The spectrum for the solid sample is typically non axial, similar to that exhibited by solid phospholipids (compare data in Table II and Ref. 1). The spectra of the aqueous dispersions exhibit a strong temperature dependence. The transition temperature obtained from ^{31}P-NMR data is similar to that obtained by differential scanning calorimetry and fluorescence polarization. Below the phase transition temperature, the spectrum is non-axial and narrower than the spectrum in the dry sample. However, above

Fig. 2. Freeze-fracture electron micrographs of dispersions of: (a) dipalmitoylphosphatidyldipalmitoylglycerol sodium salt, and (b) dipalmitoylphosphatidylcholesterol calcium salt, quenched from room temperature. Magnification: ×45000.

TABLE II

^{31}P-NMR CHARACTERISTICS OF SOME PHOSPHOLIPIDS IN THE SOLID STATE

Phospholipid Phase	σ_{11}	σ_{22}	σ_{33}	δ	A
4L-bis-PA Na, dry	−100	−30	130	0	0.30
4P-bis-PA Ca/2, dry	−90	−30	120	0	0.28
4L-bis-PA Na, aq, (below T_m)	−67	−15	84	1	0.34

the phase transition temperature the spectrum has a narrower axial line.

The ^{31}P-NMR spectra for dispersions of the calcium salt of diacylphosphatidylcholesterol give a broad isotropic line (Fig. 4), which is not significantly altered in the absence of calcium, or by heating above or cooling below the phase transition temperature. An interpretation of these spectra is developed below.

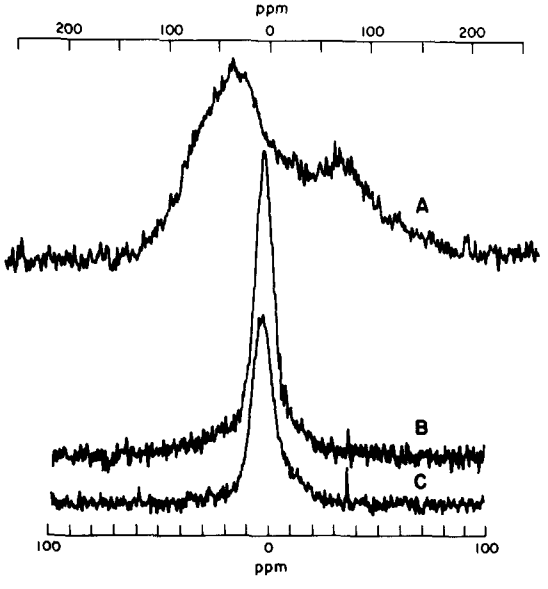


Fig. 4. ^{31}P-NMR spectra of dipalmitoylphosphatidylcholesterol calcium salt, (A) powder (B) dispersions below, and (C) above the phase transition temperature. The spectra for the NH_4^+ salt are similar.

Theory of ^{31}P-NMR lineshapes with special reference to phospholipids

A general NMR shielding tensor (with principal shielding tensor components (σ_{11}, σ_{22}, σ_{33})) averaged by a rapid axial rotation about an axis with Eulerian angle (α, β) in the pricipal axis system of the shielding tensor will give an axial pattern with an effective shielding tensor ($\sigma_{//}$, $\sigma_{\perp}$) [3]. Since the isotropic shielding constant

$$\bar{\sigma} = (\sigma_{11} + \sigma_{22} + \sigma_{33})/3 \tag{1}$$

is generally assumed to be unaffected by rotation, there is a good bit of redundancy in these relationships. For this reason, and for purposes which will become apparent soon, it is convenient to define several unitless parameters. The first is defined as an asymmetry parameter.

$$A = \frac{\sigma_{22} - \sigma_{11}}{\sigma_{33} - \sigma_{11}} \tag{2}$$

This definition is a convenient one for phospholipid NMR for several reasons. First, it is independent of any sign convention. Shielding tensor components are assigned $\sigma_{11} \rightarrow \sigma_{33}$ from low to high field with respect to external 85% H_3PO_4 in water. The ASTM convention for chemical shifts would require the value of σ_{33} to be negative since it is normally upfield from the reference. Nearly all the data in phospholipid NMR field use the opposite convention so $\sigma_{33} > \sigma_{22} > \sigma_{11}$; Eqn. 2 cannot be confused so long as reported results are consistent. A more important fact is that the value of A appears to be characteristic for all the phosphodiester ^{31}P-NMR data which we have examined. The data summary given by Seelig [1] for 7 phospholipids shows $\bar{A}$ values that are from 0.27 to 0.32 with the variation among results in a given compound being generally greater than the variation of results of the same investigator. Excluding only two disparate points (both with $A = -0.41$) we can average 15 measurements on 6 phospholipids to get $\bar{A} = 0.31$ with standard deviation 0.02.

To characterize the axially averaged pattern we have a choice of two parameters of varying convenience. One is

$$E = \frac{\sigma_{\parallel} - \sigma_{11}}{\sigma_{33} - \sigma_{11}} \tag{3}$$

This quantity, related to the eccentricity of an ellipse, is also an experimental quantity. Sometimes it is more convenient to deal with another quantity:

$$S = \frac{\Delta\sigma}{\sigma_{33} - \sigma_{11}} \tag{4}$$

Since the symbol S is often used as meaning an order parameter, we must emphasize that S as defined here *is not an order* parameter. Seelig [1] has made the point emphatically that two order parameters are needed to characterize headgroup motion; we shall make the same point but from a different perspective. However, S is similar to an order parameter and might be termed and 'effective order parameter'. The advantage of S (and A and E as well) is that they are immediately calculable from observed spectra without any ad hoc assumptions concerning the nature of the motion. The quantities S and E are, of course, related.

$$S = (3E/2) - (A/2) - (1/2) \tag{5}$$

With these definitions the equations of Kohler and Klein can be written as

$$E = (1 - \sin^2\beta) + A\sin^2\alpha\sin^2\beta \tag{6}$$

From Eqn. 6 two important deductions are apparent. (1) The system is undetermined; we have one equation with two unknowns (α, β). (2) A given axial pattern can be produced by rotation about more than one axis. The amount of information about headgroup motion which can be derived form ^{31}P-NMR is inherently limited. For this reason the ^{2}H-NMR method of Seelig is certainly preferable. Phosphorous NMR still has two formidable advantages: (1) high sensitivity, (2) isotopic substitution is unnecessary so it can always be used. Our thesis is that ^{31}P-NMR can give some useful information if properly interpreted.

The Eulerian angle of the rotation axis (α, β) is defined with respect to the principal axis of the phosphorous shielding tensor; this can be determined only by single crystal NMR. Since single crystals of phospholipids are hard to obtain, it is generally assumed (Ref. 3) that the principal axis σ_{11} is parallel to a vector between the two ester oxygens, σ_{22} is parallel to a vector between the two non-bonded oxygens, σ_{33} is perpendicular to these two. Another important feature is the orientation of the axis (α, β) with respect to the phospholipid molecule. For bilayer, it is usually thought to be the surface normal; for hexagonal phase this may not be true [2]. It is possible that the headgroup is a composite of several bond rotations [3], but it may be a motion of the phospholipid molecule as a whole. But (and this is a point often misunderstood) a composite rotation can be represented as a single rotation about some single axis. So long as all of the contributing rotations are fast on a NMR time scale, a single rotation (α, β) can be defined. Eqn. 5 is valid as long as the observed NMR pattern is truely axial, independent of the detailed nature of the motion or motions which produce axial averaging.

The calculated results can be summarized as follows. For a given set of experimental parameters (A, E) or (A, S) a group of rotation axes (α, β) can be defined. These will form a series of curves on the surface of a sphere. Fig. 5 shows these curves for $A = 0.30$ and values of S from -0.60 to 0.80. Several features may be noted. These curves are two families of ellipses on the surface of a sphere, one centered about the x axis (i.e., the σ_{11} axis) and the other centered about the z (σ_{33}) axis. At some value ($S = -0.2$ if $A = 0.3$) these two families merge to become two circumferential circles passing through the y (σ_{22}) axis. These ellipses degenerate into a point for rotation about the z axis where $\sigma_{\|} = (\sigma_{11} + \sigma_{22})/2$ ($E = 1$, $S = 0.85$ if $A = 0.3$). They also become a point for rotation about the x axis when $\sigma_{\|} = (\sigma_{22} + \sigma_{33})/2$ ($E = 0$, $S = -0.65$ if $A = 0.3$). Rotation about the y axis is, however, unique; an effective shielding $\sigma_{\|} = (\sigma_{11} + \sigma_{33})/2$ ($S = -0.2$ when $A = 0.3$) may imply rotation about the y axis, but not necessarily. Fig. 5 shows that there are many axes which can produce this pattern. Diacylphospholipids such as phosphatidylethanolamine or -choline have negative anisotropies (some investigators rather carelessly report them as positive), but we will show examples of 'reversed' patterns for which the anisotropy (i.e., S) is positive. A value of $S = 0$ is quite possible, this gives an 'isotropic' line shape. For this reason, the observation of an isotropic line shape does not necessarily imply isotropic motion.

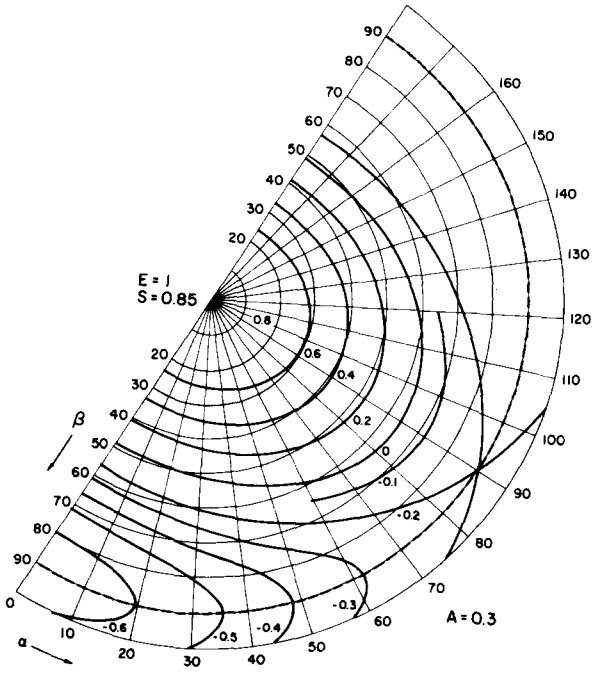


Fig. 5. Calculated relationships between effective order parameter (S) and eccentricity (E) when asymmetry parameter $A = 0.3$.

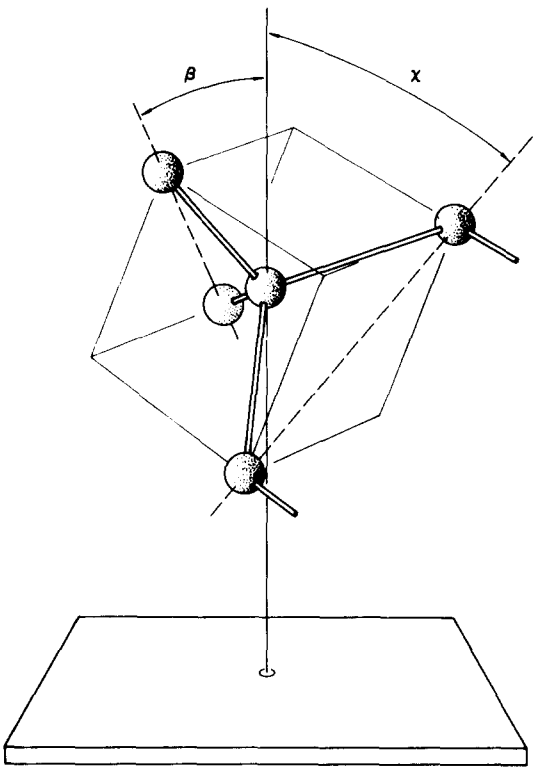


Fig. 6. Two characteristic angles β and χ that describe orientation of phosphate tensors with respect to the bilayer surface normal.

Similar points to these have been made by Seelig [1] and Thayer and Kohler [12] in different terms, but the situation is widely misunderstood in the literature. We believe that the approach outlined here maximizes the utility of the available information. The Eulerian angles are difficult to visualize so we have prepared a chart using another angle, χ. As shown in Fig. 6 the angle β is the angle between the vector between the 'non-bonded' oxygens and the rotation axis (surface normal); the angle χ is the angle of the vector between the ester oxygens and the rotation axis. Fig. 7 shows the S contours for $A = 0.30$ for these angles. This set of angles is not independent (e.g., $\chi = 0$, $\beta = 0$ is impossible) so the function exists only in a limited domain.

The anisotropy is generally found to change at phospholipid phase transition. Typically diacylglycerolphospholipids have $S \sim -0.2$ above T_m and $S \sim -0.3$ below T_m. It should be evident from the preceding that these changes need not imply any change in motion. They could be caused by only a small (approx. 10°) change in either the headgroup orientation or the axis of rotation with respect to each other. Inspection of models will show these values to be quite consistent with probable headgroup orientations. We shall show that much more radical changes are possible.

Interpretation of ^{31}P-NMR of phosphatidyldiacylglycerol

The ^{31}P-NMR pattern of bis-PA dry powder is rather typical of those found in most organic phosphodiesters (Fig. 3). When the sample is hydrated, the ^{31}P-NMR at room temperature (below the $T_c = 313$ K) is still a powder pattern quite similar to that of the dry bis-PA. This is not a typical behavior and demonstrates that the phospholipid molecule is not rotating about its long axis as normal phosholipids do. However the line for the hydrated sample is about 30% narrower than that for the dry sample ($\sigma_{33} \simeq 80$ ppm, $\sigma_{22} \simeq -20$ ppm, $\sigma_{11} \simeq -60$ ppm) indicating some sort of motional averaging other than an axial rotation. This could be due to a flipping of the headgroup among two or more orientations. The conclusion

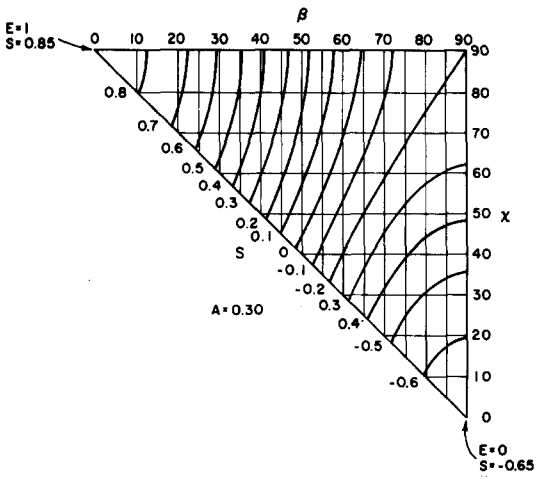


Fig. 7. Relationship between χ and β for the various values of asymmetry parameters, $A = 0.30$.

that the powder pattern for the hydrated sample is due to slow axial rotations with incomplete averaging cannot be totally excluded, but is is unlikely since the pattern does not change noticeably with temperature until T_c.

Above the phase transition temperature, T_c, a definite axial pattern is observed indicating a definite onset of rotation at or near T_c. The anisotropy is $\Delta\sigma = +27$ ppm and it does not change with temperature above T_c. The sign of anisotropy is opposite to that observed in diacylphospholipids such as phosphatidylcholine. This anisotropy can be interpreted with the theory outlined earlier. We use parameters $S = 0.12$, $A = 0.30$, and Fig. 7 to determine that the limits are: $45° < \beta < 58°$, and $45° < \chi < 90°$. (The value of χ really goes to 135°, because of symmetry of $F(\chi)$ about $\chi = 90°$.)

The angle $\chi = 90°$ is certainly a possibility since this places the vector between the ester oxygens parallel to the lipid surface, that is perpendicular to the rotation axis. If $\chi = 90°$, then we must have $\beta \simeq 60°$, that is, the headgroup is tipped with respect to the normal. (In any case β cannot be less than 45° to give $S \simeq 0.1$.) There are of course two equivalent tipped positions (that is $\pm 60°$ with respect to the normal) and flipping between these positions could be responsible for the non-axial motional averaging which we may see below T_c.

^{31}P-NMR spectra of dipalmitoylphosphatidylcholesterol (DPPCh)

The ^{31}P-NMR spectra of Ca DPPCh (Fig. 4) in dry powder state is as expected. When hydrated the resulting line is symmetrical with a width of about 13 ppm; a slight asymmetry in the experimental data could be real, or it could also be due to incomplete phase correction. The same symmetrical pattern for DPPCh is observed from 300 to 360 K for the ammonium salt (data not shown). There are two possible interpretations for this observation.

(1) Some sort of isotropic or pseudoisotropic rapid rotation which averages the shielding tensor and 'pre-empts' averaging by axial rotation (if any occurs). While a headgroup flip, as may occur in bis-PA, is possible, it seems unlikely this would give an isotropic line.

(2) Based on the calculations shown in Fig. 7, axial rotational averaging with $S = 0$, is a definite possibility for angles β and χ not much different than we found in bis-PA. However, if this is the case, the axial rotation is not affected by the phase transition.

A combination of reasons 1 and 2 is the likely correct explanation but cannot be proven by NMR data alone. Whatever the motion responsible for averaging, it is the same above and below T_c.

Discussion

By freeze-fracture electron microscopy it is shown that the aqueous dispersions of mono- and divalent ion salts of diacylphosphatidylcholesterol and phosphatidyldiacylglycerol form multilamellar bilayer structures. The phase transition in these bilayers is detected by fluorescence polarization of diphenylhexatriene. However, by differential scanning calorimetry a phase transition is seen with all these dispersions except the monovalent cation salts of phosphatidylcholesterol. The transitions are endothermic and the transition temperature decreases with decreasing chain length. These thermotropic transitions are, therefore, similar to the gel-to-liquid crystalline phase transitions detected in aqueous dispersions of other phospholipids where they organize as bilayers.

The line shape of ^{31}P-NMR of aqueous dispersions of phospholipids has been used as a diagnostic method for inferring presence of bilayer, micellar or isotropic, and hexagonal II phases [2]. Our ^{31}P-NMR studies suggest that such conclusions

may have only a limited validity. The theoretical relationships shown in Fig. 7 demonstrate that the ^{31}P-NMR line shapes are very sensitive to the angles β and χ that describe orientation of phosphate tensors with respect to the bilayer surface normal as defined in Fig. 6. Thus, depending upon the relative orientation of the phosphate tensor one can generate a wide range of line shapes.

The factors influencing orientation of phosphate tensors are not established, however, geometrical constraints arising from packing and organization of phospholipids are certainly expected to contribute significantly. In diacylphospholipids only one of the substituents would be buried in the acyl chain region of the bilayer. In contrast, diacylphosphatidylcholesterol and phosphatidyldiacylglycerol have a second large hydrophobic substituent, cholesterol and diacylglycerol moieties, respectively. These hydrophobic residues would also be buried into acyl chain regions of bilayers, presumably next to and parallel to the other two acyl chains in the same molecule. This is consistent with the freeze-fracture and the physicochemical data presented in this paper. Thus, based on the ^{33}P-NMR line shapes, the calculated values of β and χ are consistent with this interpretation. These predictions may ultimately be confirmed by X-ray crystallographic data.

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