Phosphorus-31 Nuclear Magnetic Resonance Spectra Characteristic of Hexagonal and Isotropic Phospholipid Phases Generated from Phosphatidylethanolamine in the Bilayer Phase†

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ABSTRACT: ^{31}P nuclear magnetic resonance (NMR) spectroscopy is recognized as a technique which yields information concerning both the dynamics and organization of phospholipid molecules in biological membranes and phospholipid dispersions. In this theoretical paper, we examine the relationship between the conformation of the phospholipid molecule and the shape of the predicted ^{31}P NMR spectrum. Using a simple model of rotation of the phospholipid molecule about its long axis, we show that it is possible to generate spectra previously considered typical of the bilayer ($\sigma_\parallel < \sigma_\perp$), isotropic ($\sigma_\parallel \simeq \sigma_\perp$), and hexagonal II ($\sigma_\parallel > \sigma_\perp$) packing arrangements by simply changing the phospholipid head-group conformation while *retaining the molecules in the bilayer phase.*

In recent years, there has been a tremendous growth in the use of ^{31}P nuclear magnetic resonance (NMR) spectroscopy as a means of studying phospholipids in both model systems and biological membranes (Seelig, 1978; Cullis & DeKruijff, 1979). Due to the high natural abundance of the ^{31}P isotope and the few chemically inequivalent sites which exist for phosphorus in membranes, ^{31}P NMR spectra are relatively easy to acquire and interpret. ^{31}P NMR spectroscopy gives a dynamic picture of the state of the membrane, in contrast with the static picture derived from electron microscopy and X-ray crystallography, and does so without the introduction of the perturbing probe molecules often needed for fluorescence and electron spin resonance studies.

The phospholipids of membrane and unsonicated model membrane systems are in a state of anisotropic motion, intermediate between the isotropic motion characteristic of molecules in solution and the static behavior common for molecules in the solid state. Once the effects of dipolar broadening are removed by the appropriate decoupling techniques, ^{31}P NMR spectra of phospholipids reflect the motional state of the molecules. The spectra typically exhibit an asymmetric line shape caused by the residual partially averaged ^{31}P chemical shielding anisotropy. Both the line width and line shape have been shown to be qualitatively sensitive to changes of fluidity and the presence of perturbants in the phospholipid environment (Kohler & Klein, 1977a; Seelig, 1978; Cullis & DeKruijff, 1979).

On a more quantitative level, the ^{31}P NMR line shape from phospholipids in bilayers has been understood in terms of the orientation of the phospholipid head group and the motions necessary to produce the observed residual chemical shielding anisotropy. Early studies showed the line shape observed for phospholipid bilayers to be compatible with the head group assuming a bent orientation while the entire molecule undergoes a series of rotational motions about molecular axes roughly parallel to the bilayer normal (Kohler & Klein, 1977a; Seelig & Gally, 1976; Griffin et al., 1978; Van et al., 1974).

Further experimental studies have shown that ^{31}P NMR spectra of phospholipids known to be in the hexagonal II phase appear exactly as predicted from bilayer results if one assumes the molecular conformation to be unchanged (Cullis & DeKruijff, 1979). These spectra of the hexagonal II phase have half the width and opposite sense of the anisotropy when compared to their bilayer counterparts, as illustrated in Figure 1.

These early successes in understanding the ^{31}P NMR spectra of phospholipids have led to increasingly widespread use of the technique to study phospholipid systems. However, in some instances, spectra characteristic of hexagonal or isotropic phospholipid phases have been observed for systems where the presence of these phases has not been confirmed by other techniques such as freeze–fracture electron microscopy and X-ray crystallography. Hui et al. (1981), for example, have studied a mixed phosphatidylethanolamine/phosphatidylcholine system which produces hexagonal phase NMR spectra but for which careful electron microscopy and X-ray studies failed to produce any corroborating evidence of the hexagonal phase. In addition, we have examined the membranes of *Dictyostelium discoideum* both in the native cells and as a plasma membrane extract and have observed characteristic hexagonal phase spectra in both instances (A. M. Thayer and S. J. Kohler, unpublished results). If one assumes the fluid mosaic model of membrane structure is valid (Singer & Nicholson, 1972), it is difficult to understand how the phospholipids in these biological membranes could exist predominantly in the hexagonal phase. Therefore, we question the spectroscopic interpretation.

In light of these difficulties, and in view of the growing number of phospholipid studies which rely on ^{31}P NMR alone, we undertook this theoretical investigation to examine in detail the predicted chemical shielding anisotropies and the resultant spectral line shapes for phosphatidylethanolamine in a variety of molecular conformations. We use a simple model of fast rotation of the phospholipid about a fixed molecular axis, in which case the predicted spectrum will depend on the orientation of the rotation axis with respect to the principal axis frame of the chemical shielding tensor. While retaining the bilayer packing arrangement, we assume rotation of the

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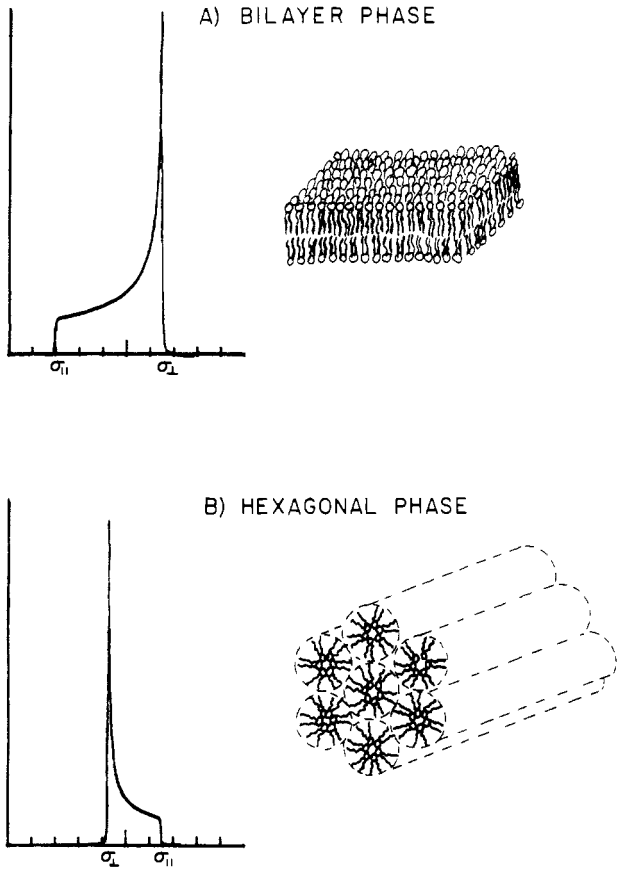

FIGURE 1: Expected ^{31}P NMR spectra for two different phospholipid packing arrangements. The spectrum shown for the bilayer phase is a computer-simulated spectrum which closely resembles experimental spectra of phospholipid bilayers. If the molecular conformation remains unchanged, the spectrum shown in (B) would be expected for phospholipids in the hexagonal II phase. Note that the hexagonal phase spectrum has half the width and opposite sense to the anisotropy when compared to the bilayer spectrum.

phospholipid about the bilayer normal and explore the relationship between head-group orientation and predicted chemical shielding anisotropy.

Experimental Procedures

The chemical shielding tensors of phospholipids are known to be nonaxial, having three distinct principal elements. Fast rotation of the molecule about a fixed axis will average such a shielding tensor to one which is axially symmetric and has the components

$$\sigma_{\perp} = \frac{\sigma_{11}}{2}(\cos^2\theta\cos^2\psi + \sin^2\psi) + \frac{\sigma_{22}}{2}(\cos^2\theta\sin^2\psi + \cos^2\psi) + \sigma_{33}\sin^2\theta$$

$$\sigma_{\parallel} = \sigma_{11}\sin^2\theta\cos^2\psi + \sigma_{22}\sin^2\theta\sin^2\psi + \sigma_{33}\cos^2\theta$$

where σ_{11}, σ_{22}, and σ_{33} are the principal elements of the shielding tensor and θ and ψ are the Euler angles of the rotation axis with respect to the principal axis frame of the shielding tensor (Mehring et al., 1971).

The principal elements of the shielding tensor for phosphatidylethanolamine used in this study are summarized in Table I. Use of similar values from other work (Herzfeld et al., 1978) did not significantly change the results of the calculations. Principal elements of shielding tensors are readily determined from spectra of powders, but spectra from single crystals are needed to determine the orientation of the shielding tensor with respect to the molecular reference frame (Mehring, 1976). Such data for phospholipids are not available in the literature, and therefore the orientation of the tensor on the phosphatidylethanolamine molecule had to be inferred from the available tensor data for organophosphate mono- and diesters (Kohler & Klein, 1977b; Herzfeld et al., 1978). We assumed a tensor orientation in which the principal axis for σ_{33} is along a vector from one nonesterified phosphate oxygen to the other [O(14)–O(13), using the notation of Hitchcock et al. (1974)], and the axis for σ_{11} is in the plane defined by the σ_{33} direction and a vector between the two remaining esterified phosphate oxygens [O(11)–O(12)].

Table I: ^{31}P Chemical Shielding Tensor of Phosphatidylethanolamine[a]

Static Tensor				
σ_{11}		σ_{22}		σ_{33}
−81		−20		105
Averaged Tensors				
torsion angle[b]	θ	ψ	$\sigma_{\perp}$	$\sigma_{\parallel}$
−211	58.6	−43.0	7	−10
−200	64.0	−32.1	18	−31
−190	69.6	−23.0	27	−50
−180	75.6	−14.6	35	−66
−170	81.9	−6.7	40	−76
−160	88.3	0.9	42	−81
−150	−84.9	8.6	41	−78
−140	−79.5	16.4	37	−70
−130	−72.4	24.4	29	−54
−120	−66.5	33.1	20	−36
−110	−61.2	42.5	10	−16
−100	−56.5	56.2	−0.4	5
−90	−52.9	64.3	−7	18

[a] The static tensor values are taken from spectra of powder samples (Kohler & Klein, 1977a). The values of $\sigma_{\perp}$ and $\sigma_{\parallel}$ for the averaged tensors were calculated for a range of phospholipid conformations, assuming fast rotation of the phospholipid molecule about the bilayer normal, as explained in the text. θ and ψ are the Euler angles in degrees between the rotation axis and principal axis frame of the tensor. All tensor values are in parts per million relative to 85% H_3PO_4. [b] The torsion angle is expressed in degrees and is about the C(1)–O(11) bond. The initial angle of −211° is that corresponding to the original X-ray crystallographic value (Hitchcock et al., 1974).

The initial conformation of the phosphatidylethanolamine in the bilayer was taken to be identical with that determined for dilauroylphosphatidylethanolamine by X-ray crystallographic methods (Hitchcock et al., 1974). Assuming the axis of rotation to be parallel to the bilayer normal (the crystallographic c axis), we calculated $\sigma_{\perp}$ and $\sigma_{\parallel}$ for a series of molecular conformations generated by pivoting the head group about the C(1)–O(11) bond (shown in Figure 2).

Equations adapted from Siderer & Luz (1979) which convolute the theoretical tensor line shape with a Lorentzian broadening term were used to computer-simulate spectra for given pairs of $\sigma_{\perp}$ and $\sigma_{\parallel}$ values. The plots of molecular conformations were generated by using the ORTEP program.

Results

The initial molecular orientation upon which our calculations were based is that determined by X-ray crystallography for a phosphatidylethanolamine and is fully compatible with the head-group conformation in oriented phosphatidylcholine bilayers as found by Griffin et al. (1978). A simple rotation of a phospholipid in this conformation about the bilayer normal is not sufficient to produce a spectrum in accord with what is normally observed for phospholipid bilayers. The sign of the anisotropy is as expected ($\sigma_{\parallel} < \sigma_{\perp}$), but the magnitude

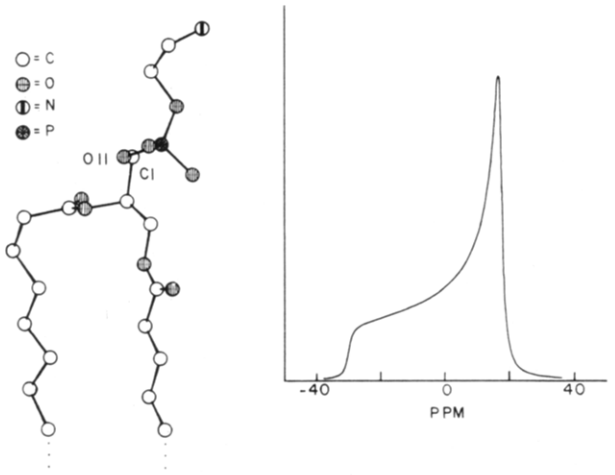

FIGURE 2: Conformation of phosphatidylethanolamine which produces a bilayer type spectrum. The spectrum shown would be expected if a phosphatidylethanolamine molecule experienced fast rotation about the bilayer normal. The C(1)–O(11) torsion angle is −116.9°, $\sigma_\parallel$ = −30.0 ppm, $\sigma_\perp$ = 17.0 ppm, and 40-Hz line broadening was used. The scale is in parts per million, placing 85% H_3PO_4 at 0 ppm.

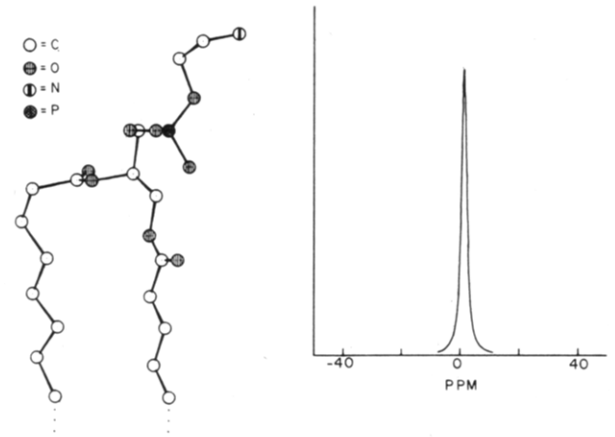

FIGURE 3: Conformation of phosphatidylethanolamine which produces an isotropic type spectrum. The spectrum shown would be predicted if a phosphatidylethanolamine molecule experienced fast rotation about the bilayer normal. The C(1)–O(11) torsion angle is −100.3°, $\sigma_\parallel$ = 2.0 ppm, $\sigma_\perp$ = 1.0 ppm, and 40-Hz line broadening was used. The scale is in parts per million, placing 85% H_3PO_4 at 0 ppm.

of the anisotropy is incorrect. In order to produce the spectrum indicated, further rotations about molecular axes approximately parallel to the bilayer normal must be included (Kohler & Klein, 1977a; Seelig & Gally, 1976).

A variety of phospholipid conformations were generated from the initial molecular orientation by varying the torsion angle about the C(1)–O(11) bond, thus swinging the ethanolamine head group up and away from the rest of the molecule. For each of these conformations, $\sigma_\perp$ and $\sigma_\parallel$ values were calculated by assuming averaging due only to fast rotation of the molecule about the bilayer normal. The results of these calculations are given in Table I and show a gradual evolution from bilayer ($\sigma_\parallel < \sigma_\perp$) through isotropic ($\sigma_\parallel \approx \sigma_\perp$) to hexagonal ($\sigma_\parallel > \sigma_\perp$) type spectra.

In order to get a better insight into the relationship between the molecular conformations and the predicted spectra, a number of examples were plotted. Figure 2 shows the conformation which will lead to a typical bilayer spectrum if rotation only about the bilayer normal is allowed. A change in the torsional angle from −116.9° to −100.3° generates the orientation shown in Figure 3 which would lead to a spectrum indistinguishable from that of lipids in an isotropic phase. A

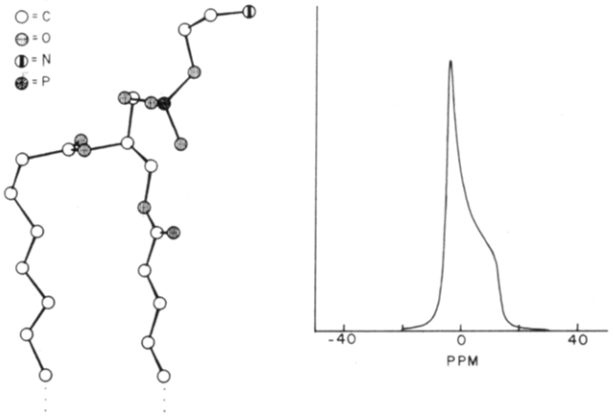

FIGURE 4: Conformation of phosphatidylethanolamine which produces a hexagonal type spectrum. The spectrum shown would be expected if a phosphatidylethanolamine molecule experienced fast rotation about the bilayer normal. The C(1)–O(11) torsion angle is −93.4°, $\sigma_\parallel$ = 13.4 ppm, $\sigma_\perp$ = −4.7 ppm, and 40-Hz line broadening was used. The scale is in parts per million, placing 85% H_3PO_4 at 0 ppm.

further change in the torsional angle to −93.4° creates the conformation shown in Figure 4. This conformation would be expected to give a spectrum with half the width and opposite sense of anisotropy ($\sigma_\parallel > \sigma_\perp$) to that of the typical bilayer spectrum in Figure 2. In other words, this phospholipid conformation in a bilayer phase would result in a spectrum typical of hexagonal phase lipids.

Space-filling models were constructed to investigate the possibility of steric interactions which would prohibit some of the proposed conformations. Manipulations of the models led to the conclusion that steric hindrance was not significant.

Conclusions

The results of these calculations show that it is indeed possible for phospholipid molecules in a bilayer phase to produce ^{31}P NMR spectra which were previously considered to be characteristic of phospholipids in the hexagonal or isotropic phases. A simple reorientation of the phospholipid head group accompanied by a fast rotation of the phospholipid molecule about its long axis in the bilayer is sufficient to produce the full range of line shapes.

A very simple model of motion was used for this study, and it is recognized that other more complicated models of motion might also be applicable to this problem. More than one axis of rotation might be included, and the phospholipid might be allowed to wobble, as was done with success in the original treatments of motion in a bilayer. Alternatively, a slow jump model of rotation might be employed (Campbell et al., 1979). This latter approach is not felt to be applicable to the problem at hand, however, due to the lack of characteristic discontinuities in the experimental spectra which we seek to simulate. The slow jump approach might be more suitable for phospholipids at low temperatures or in the presence of appropriate perturbants (C. Goulon-Ginet and S. J. Kohler, unpublished results). The goal of this work was to demonstrate the range of line shapes possible for phospholipids within the limitations of a bilayer packing arrangement. The model employed was sufficient for this task, and therefore, the use of a more complicated model is not warranted.[1]

[1] The use of more than one axis of rotation would change the conformations necessary to produce hexagonal or isotropic spectra, but it still would be possible to find such conformations. Calculations could also be performed for other classes of phospholipid molecules and would give similar results.

The question of actual head-group orientation for phospholipids in bilayers is one that needs more experimental investigation. Our results show that the line shape will be highly dependent on this orientation and that unambiguous interpretation of ^{31}P NMR results for these systems is possible only if the head-group orientation can be determined. The orientation has been determined for phosphatidylcholine in bilayers where the molecule has been shown to assume a bent conformation (Griffin et al., 1978). Presumably, electrostatic interactions between neighboring molecules are at least partially responsible for this preferred orientation. It seems reasonable that changes in pH, temperature, or ionic strength could affect the electrostatic interactions and head-group orientations. In a similar fashion, electrostatic binding of proteins to the membrane surface or intercalation of molecules within the bilayer might also allow head-group reorientation. All these factors have been postulated, on the basis of NMR evidence alone, to induce bilayer to hexagonal phase changes in some systems. It is our feeling that simple head-group reorientation is an equally plausible explanation of the observed spectra in most instances and cannot be ruled out without further experimental investigation.

It is obvious that ^{31}P NMR spectroscopy is a sensitive probe of phospholipid orientation and mobility. It is a powerful technique when used in conjunction with other methods such as electron microscopy and X-ray crystallography and is also useful when the phospholipid head-group conformation is constrained, as it is in the cardiolipin molecule. However, the results of this theoretical study show that caution is in order when interpreting ^{31}P NMR results alone.

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On the Mechanism of Action of Phenylalanine Hydroxylase†

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ABSTRACT: The oxidation of 6-methyltetrahydropterin and tetrahydrobiopterin coupled to the formation of tyrosine by phenylalanine hydroxylase generates a precursor species to the quinonoid product that is tenatively identified as a 4a-hydroxy adduct based on its spectral similarity to the 4a-hydroxy-6-methyl-5-deazatetrahydropterin. The rate of appearance of this intermediate and that of tyrosine are equal and hydroxylase catalyzed in accord with the completion of the hydroxylation event. This observation, which confirms and extends an earlier one by Kaufman [Kaufman, S. (1975) in *Chemistry and Biology of Pteridines* (Pfleiderer, W., Ed.) p 291, Walter de Gruyter, Berlin], serves to link the reaction courses followed by pterin and pyrimidine cofactor analogues and supports the hypothesis that the 4a position is a site of O_2 attachment. Thus, as expected, no prereduction of the enzyme was observed in anaerobic experiments utilizing stoichiometric amounts of enzyme and tetrahydropterin in the presence or absence of 1 mM phenylalanine. Activation of the hydroxylase by 1 mM lysolecithin leads to oxidation of the tetrahydropterin in the absence of phenylalanine. A ring-opened pyrimidine analogue of the tetrahydropterin, 2,5-diamino-4-[(*meso*-1-methyl-2-aminopropyl)amino]-6-hydroxypyrimidine, was studied to examine the possibility of tetrahydropterin ring opening in the enzymatic reaction prior to 4a-hydroxy adduct formation. However, no hydroxylase-catalyzed ring closure was observed.

L-Phenylalanine hydroxylase (phenylalanine 4-monooxygenase, EC 1.14.16.1), an essential enzyme of mammalian metabolism, catalyzes the formation of L-tyrosine from L-phenylalanine and molecular oxygen by utilizing tetrahydrobiopterin as the natural cofactor (Kaufman & Fisher, 1974). In the course of the reaction the tetrahydropterin cofactor is oxidized to the unstable quinonoid dihydropterin which rearranges in a buffer-catalyzed reaction to 7,8-dihydropterin or can be recycled back to the tetrahydro species by using either dithiothreitol (Bublitz, 1977) or NADH and dihydropteridine reductase (Craine et al., 1972; Kaufman, 1957) to regenerate the cofactor.

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