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^{31}P NMR Studies of Oriented Phospholipid Multilayers

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The motional properties of various well-defined species of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylglycerol, as well as phospholipids from natural sources, were investigated in oriented multibilayer systems employing ^{31}P NMR. Particular attention was paid to the motional properties of phospholipids above and below the hydrocarbon phase transition temperature T_c. The effective chemical shift anisotropy observed for hydrated phospholipids is relatively insensitive to the occurrence of gel–liquid-crystalline phase transitions, whereas the dipolar broadening experienced by the phosphate phosphorus in the nonproton decoupled spectra increases dramatically below T_c. It is shown that the basic features of the results obtained may be explained by a simple model where rapid axial rotation ($\tau_r \ll 10^{-5}$ sec) of the entire phospholipid molecule and rapid lateral diffusion occur above T_c, whereas below T_c the axial rotation remains, but the lateral diffusion is restricted. A theory is presented that accounts for the angular dependence of the linewidths of the ^{31}P NMR spectra of the oriented lipid systems in the gel and liquid-crystalline phase, using a second-moment formalism.

A great amount of effort has been devoted to the study of the motion and structure of lipids in bilayer membranes in recent years. Such studies are important to the understanding of biological membranes, as it is now generally agreed that a phospholipid bilayer is a basic structural element of many biological membranes (*1*), and that the motional characteristics of these lipids, (as reflected, for example, by the two-dimensional fluidity of the bilayer), may directly affect or dictate membrane function.

Among the various magnetic resonance techniques which are appropriate for the study of motional and structural characteristics of membrane phospholipids ^{31}P NMR has particular advantages. This is because the phosphate phosphorus of phospholipids in natural abundance provides a well-defined intrinsic probe of motion and structure. Thus it has been shown that ^{31}P NMR spectra of model (*2–9*) and biological (*9–12*) membranes reflect restricted, anisotropic motion consistent with bilayer structure and are sensitive to the occurrence of gel–liquid-crystalline hydrocarbon phase transitions (for a review see Refs. (*13*, *14*)).

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In the present work ^{31}P NMR is applied to hydrated phospholipids in the lamellar phase in a model membrane system consisting of many bilayers of phospholipid oriented between glass plates. A brief preliminary report of some of this work has been made previously (*9*). The results obtained here are shown to provide a firm basis for the understanding of ^{31}P NMR spectra obtained from nonoriented membranes such as liposomes and biological membranes. Particular attention has been paid to the effects of hydrocarbon phase transitions on the motions of the phospholipid molecules, as well as to the effects of different types of polar headgroup structure and fatty acid composition.

MATERIALS AND METHODS

The glass disks (~8-mm diam), employed to orient the phospholipids, were cut from microscope cover slips, (obtained from Chance Propper Ltd., Smethwick, Warley, England). These disks were cleaned by immersion of chromic acid (for 2 days) and then were subsequently washed with distilled water, methanol, and chloroform, in that order. Synthetic 1,2 dimyristoyl-sn-glycero-3-phosphorylcholine (14:0/14:0-phosphatidylcholine), synthetic 1,2 dipalmitoyl-sn-glycero-3-phosphorylcholine (16:0/16:0-phosphatidylcholine), and cholesterol were obtained from Koch–Light Laboratories Ltd. (Colnbrook, Bucks., England), whereas synthetic 1,2 dilauroyl-sn-glycero-3-phosphorylethanolamine (12:0/12:0-phosphatidylethanolamine), 1,2 dimyristoyl-sn-glycero-3-phosphorylglycerol (14:0/14:0-phosphatidylglycerol) and egg phosphatidylglycerol were the kind gift of Dr. B. de Kruijff. Phosphatidylinositol (wheat germ), phosphatidylethanolamine (egg yolk) and phosphatidylserine (bovine spinal cord) were obtained from Lipid Products, South Nutfield, England.

Oriented multilayers were prepared from a chloroform solution of the lipids which was dried under a stream of nitrogen and subsequently placed in a high vacuum for at least 1 hr. The lipids were then hydrated in atmospheres of 75% RH (i.e., over a saturated NaCl solution), except phosphatidylinositol, which was hydrated at 100% RH. In the case of phosphatidylcholines the lipid takes up approximately 14% by weight water (*15*). During hydration the temperature was maintained at least 10°C higher than the phase transition temperature of the lipid. The oriented samples were then prepared as described previously (*15*, *16*). Briefly, a small amount of the lipid was squeezed between the glass disks which were then placed for a short time ($\lesssim$30 sec) on a heating stage at a temperature above the transition temperature of the lipid. Additional pressure was applied to the sample during this time. Subsequently, any excess lipid was removed, and the orientation of the lipid was checked using a polarizing microscope equipped with a $\lambda/4$ plate. The oriented samples were then placed in an environment of the appropriate relative humidity during the course of subsequent experiments. All samples used were at least 95% oriented as indicated by the red color observed when viewed through the polarizing microscope. The orientation of the glass plate–lipid complex in the spectrometer was achieved by placing a Teflon former of the appropriate dimensions in the bottom of a cylindrical sample tube.

The NMR experiments were carried out on a homebuilt 129-MHz ^{31}P NMR

spectrometer of the Department of Biochemistry of the University of Oxford, which has been described elsewhere (*17*). The spectrometer was interfaced with a Nicolet B-NC 12 computer and was operated in the Fourier transform mode. Facilities available included temperature control and quadrature detection. No field-frequency lock was employed, which resulted in a maximum broadening of 10 Hz attributable to drift of the main magnetic field. This is insignificant in comparison to the observed linewidths. Free-induction decays were accumulated from up to 100,000 transients, employing a 60° rf pulse and 0.5 sec interpulse time. All ^{31}P NMR spectra were obtained without ^{1}H decoupling.

THEORY

The phosphorus spin Hamiltonian for the phospholipid phosphate in the rigid lattice situation (i.e., no motion) may be written as (*18*)

$$\mathscr{H} = \mathscr{H}_{CSA} + \mathscr{H}_{DIP}\,, \qquad [1]$$

where

$$\mathscr{H}_{CSA} = \gamma_P \hbar \mathbf{B}\cdot(\mathbf{1} - \sigma)\cdot\mathbf{S} \qquad [2]$$

and

$$\mathscr{H}_{DIP} = (\mu_0/4\pi)\gamma_P\gamma_H\hbar^2 S_z \sum_i (1 - 3\cos^2\vartheta_i) I_{iz}/r_i^3\,. \qquad [3]$$

The value **B** is the magnetic field, γ_P and γ_H are the phosphorus and proton gyromagnetic ratios, and μ_0 is the permeability of space. The values of **S** and **I** refer to the phosphorus and proton spin angular momentum. The chemical shift tensor is denoted by σ, which is in the principal coordinate system given by

$$\sigma = \begin{pmatrix} \sigma_{11} & 0 & 0 \\ 0 & \sigma_{22} & 0 \\ 0 & 0 & \sigma_{33} \end{pmatrix} \qquad [4]$$

and **1** is the unit matrix. The phosphorus–proton internuclear vector $\mathbf{r}_i$ makes an angle ϑ_i with **B**. It should be noted that $\mathscr{H}_{DIP}$ contributes to Eq. [1] only in the absence of proton decoupling.

We consider the simplest case of anisotropic motion, in which the phosphate group experiences a fast rotation about a fixed axis, such that $\Delta\omega_{RL}\tau_r \ll 1$, where $\Delta\omega_{RL}$ is the rigid lattice chemical shift anisotropy and τ_r is the correlation time of the rotational motion. It may be calculated that for motional narrowing $\tau_r \ll 10^{-5}$ sec (*7*). For phospholipid molecules in a bilayer this axis of rotation will by symmetry coincide with the normal to the bilayers (i.e., the director or optical axis). It is then straightforward to show that the rigid lattice chemical shift tensor is replaced by an effective time-averaged tensor σ_{EFF}, which is axially symmetric (*7*),

$$\sigma_{EFF} = \begin{pmatrix} \sigma_\perp & 0 & 0 \\ 0 & \sigma_\perp & 0 \\ 0 & 0 & \sigma_\parallel \end{pmatrix}, \qquad [5]$$

where $\sigma_\parallel$ and $\sigma_\perp$ are the components parallel and perpendicular to the optical axis.

Equation [5] may be written as

$$\sigma_{EFF} = \sigma_i \mathbf{1} + (1/3)\Delta\sigma_{EFF} \begin{pmatrix} -1 & 0 & 0 \\ 0 & -1 & 0 \\ 0 & 0 & 2 \end{pmatrix}. \quad [6]$$

The effective chemical shift anisotropy $\Delta\sigma_{EFF}$ is given by (7)

$$\Delta\sigma_{EFF} = \sigma_{\parallel} - \sigma_{\perp} = (1/2)(3\cos^2\beta - 1)\{\sigma_{33} - (1/2)(\sigma_{11} + \sigma_{22})\} + (3/4)(\sigma_{11} - \sigma_{22})\sin^2\beta\cos 2\alpha, \quad [7]$$

where α and β are the Euler angles between the rotation axis (optical axis) and the principal axes of the rigid lattice chemical shift tensor. The isotropic chemical shift value σ_i is given by

$$\sigma_i = (1/3)(\sigma_{11} + \sigma_{22} + \sigma_{33}) = (1/3)(2\sigma_{\perp} + \sigma_{\parallel}). \quad [8]$$

Thus in the case of fast anisotropic motion the chemical shift anisotropy part of the spin Hamiltonian (Eq. [2]) becomes

$$\mathscr{H}_{CSA} = \gamma_P \hbar \mathbf{B} \cdot (\mathbf{1} - \sigma_{EFF}) \cdot \mathbf{S},$$

yielding the resonance frequency

$$\nu(\theta) = \nu_0\{1 - \sigma_i - (2/3)\Delta\sigma_{EFF}P_2(\cos\theta)\}, \quad [9]$$

where $\nu_0 = (\gamma_P/2\pi)B$ and $P_2(\cos\theta)$ is a Legendre polynomial, given by

$$P_2(\cos\theta) = (1/2)(3\cos^2\theta - 1) \quad [10]$$

and θ is the angle between the optical axis and **B**. The chemical shift of the resonance $\sigma(\theta)$ with respect to the isotropic position (the "magic angle" ($\theta = 54.7°$) position) is given by

$$\sigma(\theta) = \{\nu_0 - \nu(\theta)\}/\nu_0 - \sigma_i = (2/3)\Delta\sigma_{EFF}P_2(\cos\theta). \quad [11]$$

From Eq. [11] it is observed that the chemical shift of the phosphorus NMR signal is a sensitive function of the angle θ through the $P_2(\cos\theta)$ term.

The dipolar terms of the spin Hamiltonian (Eq. [3]) are most easily treated by using the second-moment formalism (*18*, *19*). For the rigid lattice case, the second moment S_{RL} is given by

$$S_{RL} = (1/3)(\mu_0/4\pi)^2\gamma_P^2\gamma_H^2\hbar^2 I(I+1)\sum_i (3\cos^2\vartheta_i - 1)^2/r_i^6, \quad [12]$$

where I is the spin quantum number of the proton. For a rigid bilayer there is still rotation symmetry around the optical axis, making it possible to express the second moment in terms of the angle θ between the optical axis and **B**. For the $\cos\vartheta_i$ terms then the following relation holds,

$$\cos\vartheta_i = \cos\Delta_i\cos\theta + \cos\phi\sin\Delta_i\sin\theta, \quad [13]$$

where Δ_i is the angle between $\mathbf{r}_i$ and the optical axis and ϕ is the aximuthal angle. The angular dependence of the second moment follows from an integration over ϕ, yielding.

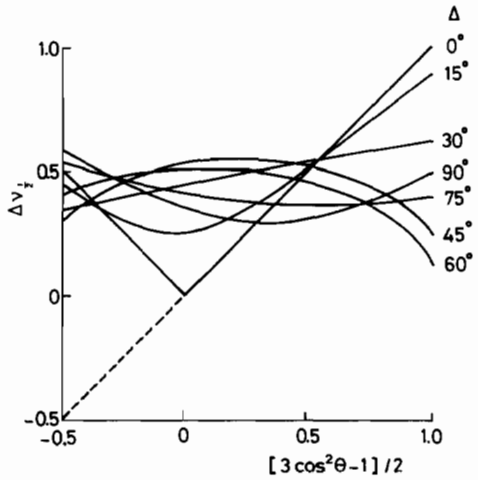

FIG. 1. Theoretical linewidths at half-height $\Delta\nu_{1/2}$ in the rigid limit case, calculated from Eqs. [14] and [18] for a single proton as a function of $P_2(\cos\theta)$ for various values of Δ. The vertical scale is in arbitrary units. The dotted line for $\Delta = 0°$ gives $-\Delta\nu_{1/2}$ vs $P_2(\cos\theta)$.

$$S_{RL}(\theta) = (1/3)(\mu_0/4\pi)^2\gamma_P^2\gamma_H^2\hbar^2 I(I+1)\sum_i F(\Delta_i, \theta)/r_i^6\,, \qquad [14]$$

with the function $F(\Delta_i, \theta)$ given by (*20*)

$$F(\Delta_i, \theta) = (1/512)\{(315\cos 4\Delta_i + 180\cos 2\Delta_i + 81)\cos 4\theta + (180\cos 4\Delta_i + 432\cos 2\Delta_i + 156)\cos 2\theta + (81\cos 4\Delta_i + 156\cos 2\Delta_i + 467)\}. \qquad [15]$$

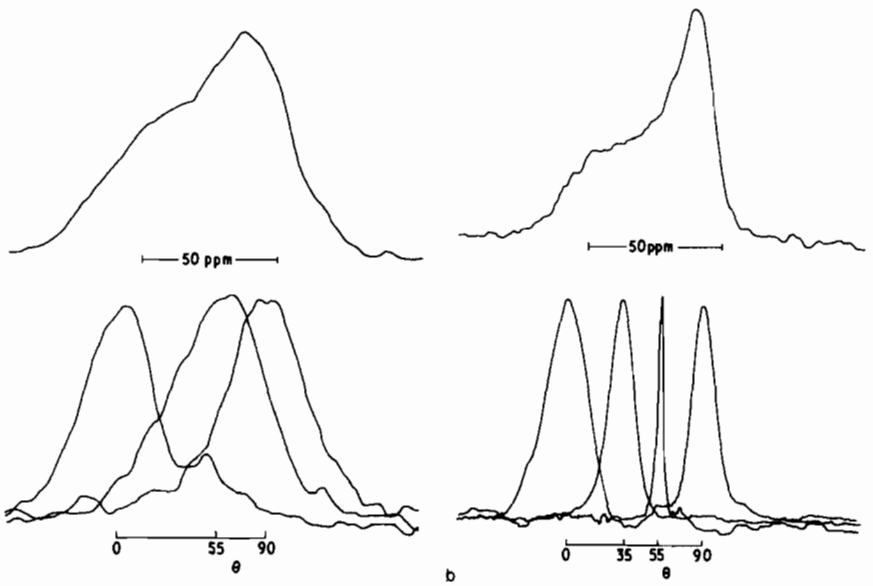

FIG. 2. 129-MHz ^{31}P NMR spectra of oriented and nonoriented 14:0/14:0-phosphatidylcholine above and below the transition temperature: (a) T = 10°C; (b) T = 30°C.

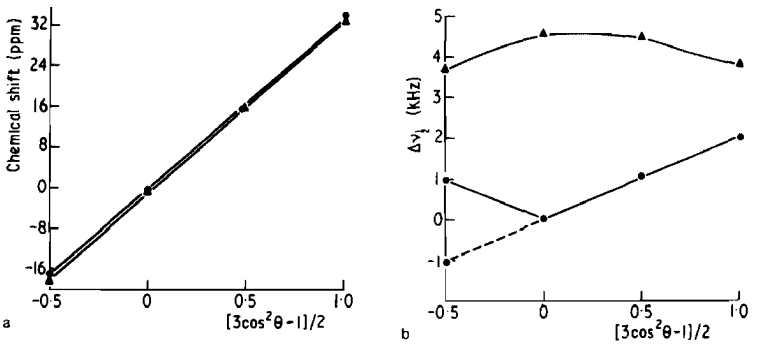

FIG. 3. (a) Chemical shift $\sigma(\theta)$ of the spectra obtained from oriented 14:0/14:0-phosphatidylcholine as a function of $P_2(\cos\theta)$ at 10°C (▲) and 30°C (●). (b) Linewidths at half-height $\Delta\nu_{1/2}$ of the spectra from oriented 14:0/14:0-phosphatidylcholine as a function of $P_2(\cos\theta)$ at 10°C (▲) and 30°C (●). The dotted line gives $-\Delta\nu_{1/2}$ vs $P_2(\cos\theta)$.

In the case of rapid anisotropic motion around the optical axis, the time average of $3\cos^2\vartheta_i - 1$ should be taken, prior to squaring this term in Eq. [12] (*18*). From Eq. [13] it may be calculated that

$$\overline{3\cos^2\vartheta_i - 1} = P_2(\cos\theta)(3\cos^2\Delta_i - 1). \qquad [16]$$

The angular dependence of the time-averaged second moment $S_{\mathrm{EFF}}(\theta)$ is then

$$S_{\mathrm{EFF}}(\theta) = (1/3)(\mu_0/4\pi)^2\gamma_{\mathrm{P}}^2\gamma_{\mathrm{H}}^2\hbar^2 I(I+1)P_2^2(\cos\theta)\sum_i (3\cos^2\Delta_i - 1)^2/r_i^6. \qquad [17]$$

At this stage it is important to distinguish between intra- and intermolecular contributions of the dipolar interactions, since their averaging process is different. In the case of rapid anisotropic motion of the phosphate group of a phospholipid molecule around an axis perpendicular to the bilayer, the intramolecular dipolar interactions are averaged and give an effective second moment as described by Eq. [17]. The intermolecular part is not, or only partly averaged and results in a rigid limit second moment given by Eq. [14]. Complete averaging of the intermolecular dipolar interactions is obtained, if the phospholipid molecules are in rapid motion with respect to each other (*16*), i.e., perform a rapid lateral diffusion in the plane of the bilayer.

For a Gaussian lineshape the second moment S is related to the linewidth at half-height $\Delta\nu_{1/2}$ according to

$$\Delta\nu_{1/2} = [(2\ln 2)^{1/2}/\pi]S^{1/2}. \qquad [18]$$

To get a feeling for the order of magnitude of the linewidths expected for a particular orientation, Eqs. [17] and [18] may be evaluated for a single proton at a distance of 0.28 nm in a phosphate ester (*21*). Assuming for simplicity $\Delta_i = 0$, the following relation is obtained:

$$\Delta\nu_{1/2} = 5.8|P_2(\cos\theta)|\mathrm{kHz}. \qquad [19]$$

Thus at orientations where $\theta = 0$, linewidths of the order of 6 kHz may be expected. This may be compared with the spectral width of a 129-MHz ^{31}P NMR spectrum

of randomly oriented phospholipid molecules, which is in the order of 7 to 9 kHz (*3*, *9*).

The angular dependence of the linewidth $\Delta\nu_{1/2}$ in the rigid limit case may be calculated from Eqs. [14] and [18]. As shown in Fig. 1, a quite different behavior is found, which is very sensitive to Δ_i, in contrast to the linewidths in the fast motional case, given by Eq. [19].

RESULTS

As shown in the previous section, if a phospholipid experiences rapid axial rotation ($\tau_r \ll 10^{-5}$ sec) about an axis perpendicular to the plane of the bilayer, the chemical shift of the observed spectra depends on the orientation of the bilayer with the magnetic field **B**, through the $P_2(\cos\theta)$ relation (see Eq. (11)). As indicated in the oriented spectra of 14:0/14:0-phosphatidylcholine illustrated in Figs. 2a and b, the chemical shift of the spectra is a sensitive function of θ, and, as is more precisely illustrated in Fig. 3a, this dependence is very accurately described by the $P_2(\cos\theta)$ relation both above and below the hydrocarbon phase transition temperature T_c, which occurs at 23°C (*22*, *23*). Thus the observed orientation dependence of the chemical shift may be interpreted to indicate rapid rotation in the phosphate region about an axis perpendicular to the bilayer in both gel and liquid-crystalline states, in agreement with other work (*2–12*).

The orientation dependence of the dipolar broadening experienced by the phospholipid phosphorus, however, indicates a basic difference between the molecular motions in the gel and liquid-crystalline states. As indicated in Fig. 2a, below the phase transition the linewidths $\Delta\nu_{1/2}$ are nearly independent of θ; indeed, the linewidths at the magic angle ($\theta = 54.7°$) are somewhat broader than at other orientations (see Fig. 3b). However, above the phase transition (see Figs. 2b and 3b) the linewidths depend on θ through the $P_2(\cos\theta)$ relation. The linewidths of the spectra from oriented 14:0/14:0-phosphatidylcholine are consistent with rapid axial rotation about an axis perpendicular to the bilayer above and below the phase transition. However, as noted in the previous section, such a rotation only averages

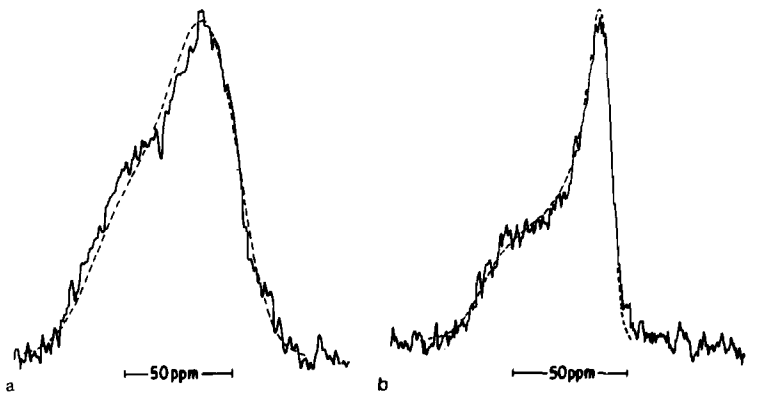

FIG. 4. Comparison of simulated (dashed line) and experimental (solid line) 129-MHz ^{31}P NMR spectra of unoriented 14:0/14:0-phosphatidylcholine: (a) $T = 10°C$; (b) $T = 30°C$.

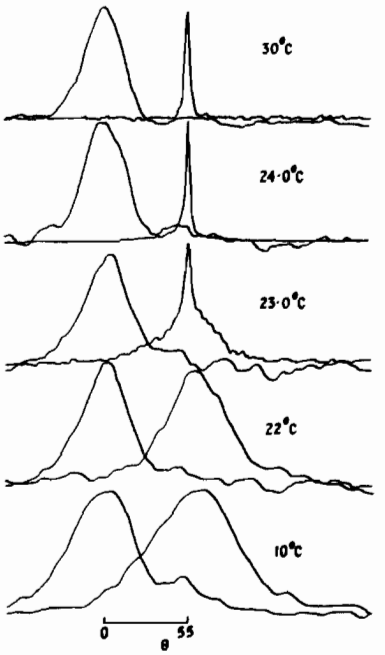

FIG. 5. Temperature dependence of the 129-MHz ^{31}P NMR spectra of 14:0/14:0-phosphatidylcholine oriented at $\theta = 0°$ and $\theta = 55°$.

the intramolecular dipolar interactions and is not sufficient to average the intermolecular terms. For complete averaging of the intermolecular terms a rapid lateral diffusion process should be invoked, which results in a $P_2(\cos\theta)$ function (see Eq. [17]) for the linewidth above T_c. In the gel state lateral diffusion is inhibited, resulting in a linewidth that is a complicated function of θ (see Eqs. [14] and [17], and Fig. 1). This effect is more fully discussed in the next section.

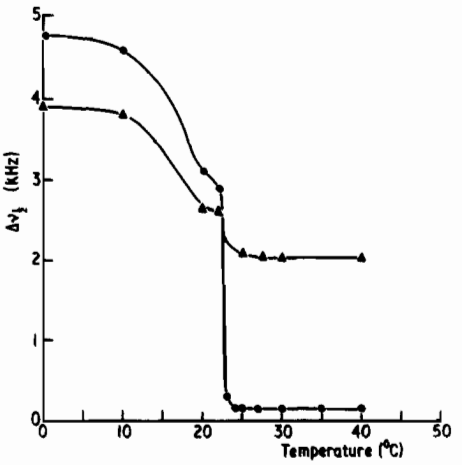

FIG. 6. Temperature dependence of the linewidth at half-height $\Delta\nu_{1/2}$ of the 129-MHz ^{31}P NMR spectra of 14:0/14:0-phosphatidylcholine oriented at $\theta = 0°$ (▲) and $\theta = 55°$C (●).

As noted previously (*3*, *6*) the spectra expected from unoriented hydrated phospholipid systems may be simulated using Eq. [11], if $\Delta\sigma_{EFF}$ and the linewidths and lineshapes at various orientations are known. Such parameters may be obtained from 14:0/14:0-phosphatidylcholine from the oriented spectra of Fig. 2, where at 10°C $\Delta\sigma_{EFF}$ = 52 ppm (as measured from the chemical shift between the oriented spectra obtained at θ = 0 and 90°) and the linewidths $\Delta\nu_{1/2} \approx 4$ kHz (independent of orientation), whereas at 30°C $\Delta\sigma_{EFF}$ = 52 ppm and $\Delta\nu_{1/2} = 2.1|P_2(\cos\theta)|$kHz. It may also be noted that the lineshapes of the oriented spectra are well described by a Gaussian, as is expected for spins experiencing dipolar broadening. This justifies the use of Eq. [18] for the calculation of the linewidth $\Delta\nu_{1/2}$ from the second moment. As indicated in Fig. 4, the spectra simulated on the basis of this information (with no variable parameters) give an excellent fit to the observed spectra of nonoriented 14:0/14:0-phosphatidylcholine both above and below the phase transition.

This conclusively demonstrates that the motion and conformation in the phosphate region of the lipids in the oriented and unoriented model membrane systems is practically identical. Thus a clear understanding of the local motion and structure in the phosphate region of phospholipids obtained in the oriented multibilayer systems gives a firm basis for a more precise interpretation of the ^{31}P NMR spectra obtained from nonoriented phospholipids in other model and biological membranes.

The temperature dependent behavior of the spectra obtained from 14:0/14:0-phosphatidylcholine oriented at θ = 0° and θ = 55° is illustrated in Fig. 5. As the temperature is increased toward the hydrocarbon transition temperature, which occurs at 23°C in similarly hydrated 14:0/14:0-phosphatidylcholine (*22*), the θ = 0° spectra narrow somewhat (see Fig. 6), whereas at the phase transition the magic-angle spectra (θ = 55°) undergo an abrupt and dramatic narrowing. Thus the most striking manifestation of the liquid-crystalline state as detected by ^{31}P NMR is the onset of a rapid lateral diffusion of the phospholipid molecules, which averages the intermolecular dipolar interactions experienced by the phosphorus about an axis perpendicular to the plane of the bilayer.

It is interesting to note that in the region of the phase transition the magic-angle spectrum is clearly composed of two components, (see Fig. 5, T = 23°C), where the narrow component corresponds to lipids in the liquid-crystalline state, and the broad component to lipids in the gel state. Thus lateral phase separation occurs

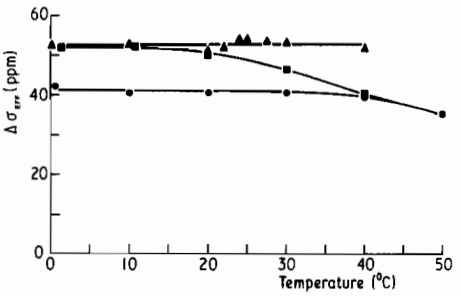

FIG. 7. Temperature dependence of $\Delta\sigma_{EFF}$ for a variety of oriented synthetic phospholipids: ▲, 14:0/14:0-phosphatidylcholine; ■, 12:0/12:0-phosphatidylethanolamine; ●, 14:0/14:0-phosphatidylglycerol.

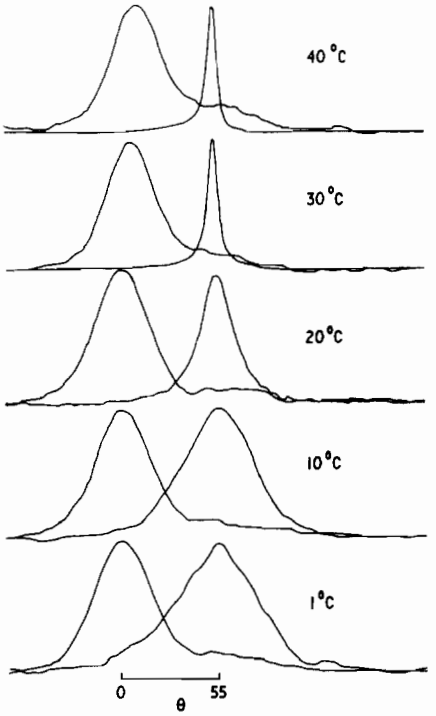

FIG. 8. Temperature dependence of the 129-MHz ^{31}P NMR spectra obtained from 12:0/12:0-phosphatidylethanolamine oriented at $\theta = 0°$ and $\theta = 55°$.

at the phase transition between domains of gel and liquid-crystalline phospholipid of the same molecular species.

As may be inferred from Fig. 5, and is more clearly illustrated in Fig. 7, the observed effective chemical shift anisotropy $\Delta\sigma_{EFF}$ is almost insensitive to the occurrence of the phase transition. This suggests that the type of restricted motion experienced in the phosphate region is similar both above and below the phase transition.

The transition behavior of oriented 12:0/12:0-phosphatidylethanolamine and 14:0/14:0-phosphatidylglycerol as detected by ^{31}P NMR is qualitatively similar to that observed for 14:0/14:0-phosphatidylcholine as shown in Figs. 8 and 9. The transition temperature T_c of the oriented 12:0/12:0-phosphatidylethanolamine may be estimated to be approximately 30°C, in good agreement with previous observations (*4*). However, the T_c that may be inferred for 14:0/14:0-phosphatidylglycerol is in the region of 45°C, which is somewhat higher than previous estimates (*4*). Such behavior may arise because of a low water content, or a low internal pH of the (unbuffered) oriented sample. The chemical shifts of the oriented spectra in Figs. 8 and 9 obey the $P_2(\cos\theta)$ dependence both above and below T_c. Alternatively, the dipolar linewidths only display the $P_2(\cos\theta)$ dependence in the liquid-crystalline state. This indicates rapid axial rotation but restricted lateral diffusion in the gel state. This behavior is in broad agreement with that for 14:0/14:0-phosphatidylcholine.

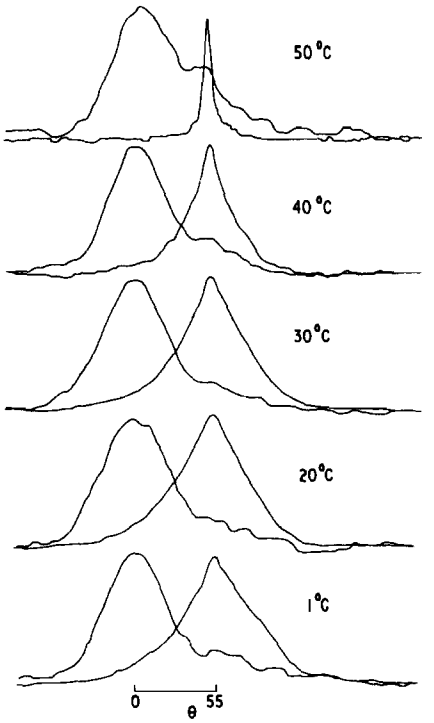

FIG. 9. Temperature dependence of the 129-MHz ^{31}P NMR spectra obtained from 14:0/14:0-phosphatidylglycerol oriented at $\theta = 0°$ and $\theta = 55°$.

The temperature dependence of the linewidths $\Delta\nu_{1/2}$ of the spectra at $\theta = 0$ and 55° is given in Figs. 10 and 11. It may be seen on comparing Figs. 10 and 11 with Fig. 6, that the narrowing of the magic-angle spectra occurs over a much larger

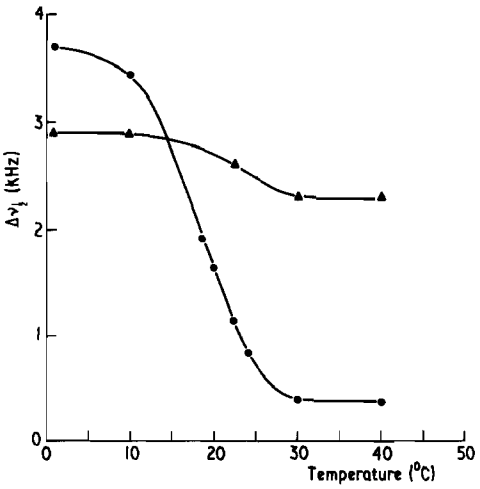

FIG. 10. Temperature dependence of the linewidth at half-height ($\Delta\nu_{1/2}$) of the 129-MHz ^{31}P NMR spectra of 12:0/12:0-phosphatidylethanolamine oriented at $\theta = 0°$ (▲) and $\theta = 55°$ (●).

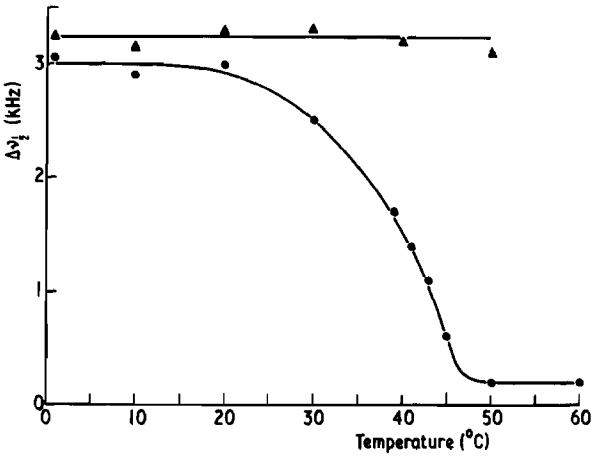

FIG. 11. Temperature dependence of the linewidth at half-height ($\Delta\nu_{1/2}$) of the 129-MHz ^{31}P NMR spectra of 14:0/14:0-phosphatidylglycerol oriented at $\theta = 0°$ (▲) and $\theta = 55°$ (●).

temperature interval for 12:0/12:0-phosphatidylethanolamine and 14:0/14:0-phosphatidylglycerol than for 14:0/14:0-phosphatidylcholine, and does not decrease abruptly at the phase transition. This is in accord with previous results obtained in nonoriented liposome systems (*4*). A further difference is illustrated in Fig. 7, where it is noted that the observed value of $\Delta\sigma_{EFF}$ for 12:0/12:0-phosphatidylethanolamine decreases as the temperature is increased above the phase transition.

The effect of equimolar concentrations of cholesterol on the ^{31}P NMR spectra of phosphatidylcholines below their transition temperature is illustrated in Fig. 12.

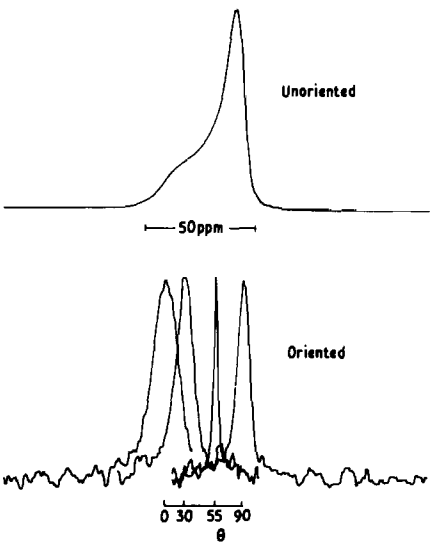

FIG. 12. 129-MHz ^{31}P NMR spectra of a nonoriented and oriented equimolar mixture of 16:0/16:0-phosphatidylcholine and cholesterol ($T = 20°C$).

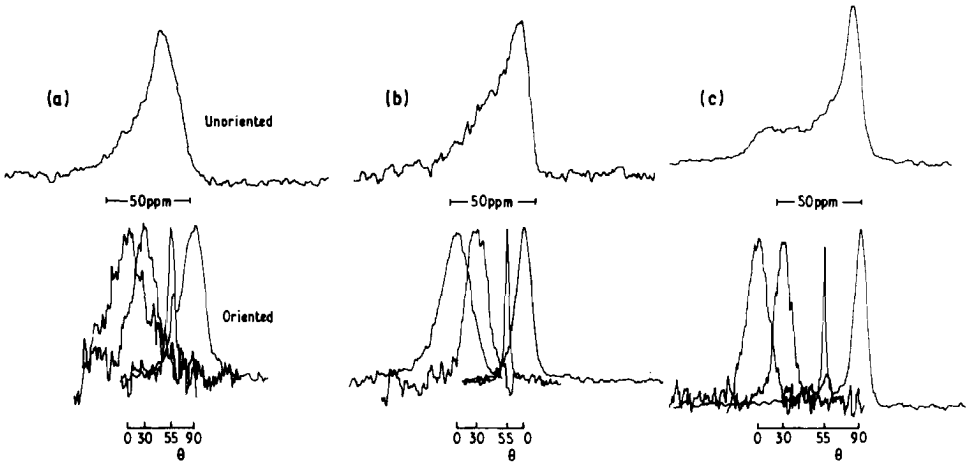

FIG. 13. 129-MHz ^{31}P NMR spectra of nonoriented and oriented phospholipids from natural sources (T = 20°). (a) Egg-yolk phosphatidylethanolamine, (b) Phosphatidylserine (bovine spinal cord), (c) Phosphatidylinositol (wheat germ).

It may be noted that the spectra then display similar characteristics as observed for liquid-crystalline species, but the effective chemical shift anisotropy $\Delta\sigma_{EFF}$ is appreciably reduced (from 52 to 35 ppm in the case of 16:0/16:0-phosphatidylcholine).

Spectra obtained from a variety of liquid-crystalline phospholipids from natural sources are illustrated in Fig. 13. It may be noted that these spectra indicate that the motion in the phosphate region is again consistent with rapid axial rotation and lateral diffusion in the liquid-crystalline state, in full accord with the results obtained from synthetic phospholipids.

DISCUSSION

The results obtained here for the synthetic and naturally occurring oriented liquid-crystalline phospholipids investigated have been shown to be fully consistent with motion in the methylene–phosphate–methylene region which is axially symmetric about an axis perpendicular to the bilayer. Such motion may arise from axial rotation of the entire phospholipid molecule and/or from rapid rotation about C(2)–C(1) bond (see Fig. 14), as it has been suggested that this bond is oriented perpendicular to the plane of the bilayer (*24*). In the presence of lateral diffusion, it is likely that axial rotation of the entire phospholipid occurs. If the lateral diffusion model of Saffman and Delbrück (*25*) is employed it may be calculated that

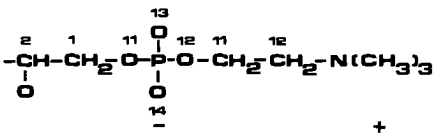

FIG. 14. Structural formula of the polar headgroup region of phosphatidylcholine.

rotational correlation times $\tau_r \lesssim 10^{-7}$ sec would be expected for lipids experiencing lateral diffusion rates $D_t \gtrsim 10^{-12}$ m^2 sec^{-1}. Such fast motion of the lipid molecule would easily suffice to produce the observed effects.

Below the phase transition, however, the results obtained here suggest that lateral diffusion is inhibited ($\tau_r > 10^{-5}$ sec) for the synthetic phospholipids investigated, as the dipolar interactions experienced by the phosphate phosphorus do not reflect the averaging effects of such motion. Such restriction of lateral diffusion may arise from the very viscous nature of gel-state lipids (*26*). It may be calculated that rotational correlation times $\tau_r > 10^{-5}$ sec correspond to membrane viscosities greater than 15.0 Pa · sec, as compared to liquid-crystalline bilayers, which exhibit viscosities on the order of 0.2 Pa · sec (*26*).

As noted for 14:0/14:0-phosphatidylcholine the effective chemical shift tensor has axial symmetry about an axis perpendicular to the plane of the membrane both above and below the phase transition temperature. Similar behavior is exhibited by the saturated species of phosphatidylethanolamine and phosphatidylglycerol. Also, as observed for 14:0/14:0-phosphatidylcholine, situations may arise where there is *no* change in $\Delta\sigma_{EFF}$ on going through the phase transition. It may be noted, in general, that any changes which are observed for various phospholipids (*3*, *4*) are relatively small and do not show a sharp break at the phase transition. It is logical that polar headgroup motion which is possible in the gel state would also occur in the liquid-crystalline state. Thus the motion in the phosphate region which averages the chemical shift tensor must be the same, or be equivalent, both above and below the phase transition. Details of the motion of the polar headgroup of saturated species of phosphatidylcholine and phosphatidylethanolamine have recently been the subject of intensive investigation (*5*, *27*, *28*).

The intra- and intermolecular dipolar interactions experienced by the phosphate phosphorus are averaged about an axis perpendicular to the plane of the membrane by rotational motions and lateral diffusion when the lipids are in the liquid-crystalline state. However, averaging of the intermolecular dipolar interactions does not occur below the phase transition.

Using Eqs. [14] and [17] the predicted second moment in the gel phase and liquid-crystalline phase may be described by

$$S_{gel} = (1/3)(\mu_0/4\pi)^2\gamma_P^2\gamma_H^2\hbar^2 I(I+1) \times \left\{ P_2^2(\cos\theta) \sum_i (3\cos^2\Delta_i - 1)^2/r_i^6 + \sum_j F(\Delta_j, \theta)/r_j^6 \right\} \quad [20]$$

and

$$S_{lc} = (1/3)(\mu_0/4\pi)^2\gamma_P^2\gamma_H^2\hbar^2 I(I+1)P_2^2(\cos\theta) \times \left\{ \sum_i (3\cos^2\Delta_i - 1)^2/r_i^6 + \sum_j (3\cos^2\Delta_j - 1)^2/r_j^6 \right\}, \quad [21]$$

respectively, where i indicates the intramolecular protons and j the protons on neighboring phospholipid molecules.

Two interesting conclusions emerge from these equations. First, it is seen that $S_{gel} = S_{lc}$ for $\theta = 0°$, so that the predicted linewidth at this orientations should be independent of temperature on going through the phase transition. This behavior

is observed for 14:0/14:0-phosphatidylglycerol (see Fig. 11) and somewhat less for 12:0/12:0-phosphatidylethanolamine (see Fig. 10). However, 14:0/14:0-phosphatidylcholine displays quite different behavior. As shown in Fig. 6 the 0° linewidth is reduced by about 50% above T_c. This suggests that for 14:0/14:0-phosphatidylcholine above T_c additional motions in the headgroup region appear that average the intra- and intermolecular dipolar interactions. For each fast additional rotation about a fixed axis, the linewidth is reduced by a factor of $(1/2)(3\cos^2\xi - 1)$, where ξ is the angle between the rotation axis and the phosphorus–proton vector. Since these rotations should not affect the averaging of the chemical shift tensor, they should be located in the choline part of the headgroup. The appearance of additional motions in the choline headgroup is also consistent with the large increase in the area per lipid molecule at the phase transition (from 0.44 to 0.56 nm^2 (*29*)) with consequently reduced steric hindrance to rotation. These rotations may be absent in the synthetic phosphatidylethanolamines and phosphatidylglycerol and it is therefore likely that rotations of the *N*-methyl region are responsible for this effect in phosphatidylcholine.

Second, it is seen from Eq. [20] that in the gel state at $\theta = 55°$ (the magic angle) the intramolecular contributions to the second moment vanish. Thus the linewidth in the gel state at $\theta = 55°$ arises *completely* from the intermolecular dipolar interactions, making it possible to derive information about the location of the phospholipid molecules with respect to each other. For 14:0/14:0-phosphatidylcholine the linewidth at $\theta = 55°$ is 4.8 kHz, whereas a value of 3.7 kHz is obtained for 12:0/12:0-phosphatidylethanolamine. The main difference between the two phospholipids is the nine *N*-methyl protons in the phosphatidylcholine, suggesting that these protons of neighboring phospholipid molecules are in close proximity to the phosphate phosphorus. This is consistent with the choline group aligned parallel to the plane of the bilayer, in agreement with other work on phosphatidylcholines in the gel and liquid-crystalline phase (*28*, *30*, *31*). Another indication for a parallel orientation of the choline group may be found from a comparison of the angular dependence of the linewidth in Fig. 3b and Fig. 1. It may be estimated that most intermolecular phosphorus–proton vectors make an angle of about 60° with the normal to the bilayers. From model building it is found that such an angle is impossible, if the choline group would be fully extended. Thus from an analysis of the angular dependence of the linewidth in the gel state, it may also be suggested that the choline head group of 14:0/14:0-phosphatidylcholine is oriented parallel to the plane of the bilayer. It is, however, not possible to derive such a conclusion for 12:0/12:0-phosphatidylethanolamine and 14:0/14:0-phosphatidylglycerol.

Several other aspects of the phase transition behavior of the saturated species of phospholipid are of interest.

It has been shown (*32*) that in the hydrocarbon chain region of lipids in the gel state (the $L\beta'$ phase), there is a significant tilt (up to 30°) of the acyl chains with respect to the plane of the bilayer. If such a tilt is carried over into the phosphate region of the polar headgroup, it might be suggested that the broad, orientation independent spectra observed for the oriented multilayers below the transition temperature arise from a distribution of chemical shifts from various domains of lipids which have different local orientations with respect to the magnetic field. There

are two strong arguments which indicate that this is not the case. First, below the phase transition an excellent simulation of the nonoriented spectra of 14:0/14:0-phosphatidylcholine is given assuming dipolar linewidths which are independent of orientation. It may be noted that the inclusion of $P_2(\cos\theta)$ dependent dipolar linewidths causes a marked change in the ^{31}P NMR lineshape obtained from nonoriented systems (compare the unoriented spectra of Fig. 2). Second, if a distribution of chemical shifts did cause significant broadening in the oriented gel-state phospholipid spectra, a marked narrowing and possibly also a marked change in the chemical shift of the spectra obtained at the 0° orientation would be expected on going through the phase transition, where the lipids leave the tilted $L\beta'$ structure and enter the $L\beta$ liquid-crystalline phase where the acyl chains are perpendicular to the plane of the bilayer (*32*). As indicated in Fig. 5, and more precisely in Fig. 6, the 0° orientation spectra linewidths are only reduced by <10% on proceeding through the phase transition, indicating that the contribution of a static distribution of chemical shifts attributable to a distribution of orientation to the observed gel-state linewidths is a maximum of 10%. It may be noted that the latter point suggests that any tilt of the hydrocarbon chains in the gel state does not affect the orientation of the phosphate group region with respect to the plane of the bilayer.

The influence of equimolar concentrations of cholesterol on 16:0/16:0-phosphatidylcholine below the phase transition, as illustrated in Fig. 12, shows that the phospholipid molecules are able to undergo rapid lateral diffusion as a result of the "liquifying" role of cholesterol below the phase transition. It may be noted that *both* the $\Delta\sigma_{EFF}$ and the linewidth at the 0° orientation are reduced over those normally observed in the liquid-crystalline state, and thus it would appear that the presence of cholesterol increases the allowed motion in the polar headgroup region both below *and above* the hydrocarbon phase transition. Such effects are in agreement with other observations (*3*) and suggest that in the phosphatidylcholine–cholesterol system there are less steric hindrances to motion in the headgroup attributable to a "spacing" effect of cholesterol.

The results obtained from the various species of phospholipid obtained from natural sources show the typical liquid-crystalline behavior that may be expected. Again, the results indicate a remarkable similarity in the phosphate group motion and conformation between the various phospholipids.

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