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^{31}P NMR of Phospholipid Membranes: Effects of Chemical Shift Anisotropy at High Magnetic Field Strengths

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The ^{31}P NMR spectrum of sonicated dipalmitoyl lecithin vesicles consists of two chemically shifted resonances, separated by ~0.15 ppm, which arise from phosphate groups in phospholipid molecules on the inside and the outside of the spherical bilayer vesicles. The widths of the resonances are remarkably sensitive to the crystalline-liquid crystalline phase transition, the magnetic field-strength, and the viscosity of the surrounding aqueous medium. The results are interpreted in terms of two phosphorus relaxation mechanisms: modulation of the anisotropic phosphorus chemical shift and dipolar interaction with protons on adjacent methylene groups. At high magnetic fields (7.5 T) the modulation of the phosphorus chemical shift anisotropy by the Brownian rotation of the intact spherical vesicles dominates the linewidth.

The chemical shift anisotropy in the sonicated vesicles is compared with that in the unsonicated dispersions. It is concluded that the structure around the phosphate group is not significantly disrupted by the sonication process. The order parameter for the internal motion of the phosphate group is estimated, and a tentative model for the motion of the phosphate group in the membrane is proposed.

INTRODUCTION

Sonication of an aqueous phospholipid dispersion results in a relatively homogeneous population of spherical vesicles (~250 Å in diameter) which consist of a continuous bilayer membrane surrounding an inner aqueous phase (*1–5*). The bilayer membrane is composed of two layers of amphipathic phospholipid molecules; the nonpolar fatty acid chains of each layer being apposed to each other, forming a hydrocarbon interior (*6*). The polar headgroups of each layer form the interfaces with the inner and outer aqueous phases.

There is now considerable evidence for the occurrence of lipid bilayer regions within the mosaic structure of biological membranes (*7, 8*). Consequently, vesicular phospholipid bilayer systems have been widely used as models to study the interaction of proteins (*9, 10, 11*) and cholesterol (*12*) with membranes, and also to study ion transport

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(*13*, *14*). Vesicles are also very attractive for use in NMR studies as they give "high resolution" NMR spectra (*15–19*) as compared to the broad, relatively featureless, NMR spectra that are characteristic of unsonicated phospholipid dispersions (*20–24*). Phosphorus magnetic resonance (^{31}P NMR) of phospholipid vesicles is particularly interesting as the spectra are relatively simple. Since different classes of phospholipids have different phosphorus chemical shifts, ^{31}P NMR is an inherently powerful technique for studying the localization of different classes of phospholipids in the membrane (*18*, *19*, *25*), and for studying the specificity of the interaction of different phospholipids with metal ions and proteins.

^{31}P NMR can also provide structural information on an important region of the membrane—the polar headgroups at the aqueous interface. The linewidths of ^{31}P NMR resonances in vesicles may be determined by two different kinds of motion—the rapid, but restricted, internal motion in the polar head-group region, and the overall Brownian rotation of the spherical vesicles. Although it may be assumed that proton relaxation is purely dipolar in origin (*26*, *27*), phosphorus relaxation may occur through two mechanisms—the anisotropy of the phosphorus chemical shift (*28*), and the dipolar interaction with protons on adjacent methylene groups. The present communication describes the magnetic field dependence and the viscosity dependence of ^{31}P NMR resonances from sonicated dipalmitoyl lecithin vesicles. The results indicate that at high magnetic fields the phosphorus linewidths are dominated by the modulation of the anisotropic chemical shift by the Brownian rotation of the intact vesicles. The consequent field-dependence of the linewidths implies that the resolution of chemically-shifted resonances from different classes of phospholipids in cosonicated vesicles will significantly decrease at high magnetic field strengths.

The motion of spin-labeled lipids in phospholipid membranes has been characterized in terms of an order parameter (*29*). This order parameter, which is calculated from the observed average values of the components of the hyperfine coupling tensor, gives information on the amount of restricted internal motion available to the spin-labeled moiety in the membrane. In a completely analogous manner, an order parameter for the phosphate group motion may be defined in terms of the average values of the components of the chemical-shift anisotropy tensor. The order parameters for the phosphate group motion in the sonicated vesicles and unsonicated dispersions are estimated and compared, and a tentative model for the phosphate group motion in the membrane is proposed.

MATERIALS AND METHODS

Synthetic β-γ-dipalmitoyl-L-(3)-lecithin (DPL) was obtained from Koch-Light Laboratories Ltd. (Colnbrook, Bucks., U.K.) and used without further purification. Egg lecithin was obtained from Lipid Products, South Nutfield, England, and neodymium nitrate was obtained from Koch-Light. All other reagents were analar grade and were used without further purification.

Vesicles were produced by a 30 minutes sonication of a 50 mg/ml dispersion of DPL in a D_2O solution containing 25 m*M* tris(hydroxymethyl)aminomethane (Tris) pH 7.2, and 1 m*M* ethylenediaminetetraacetic acid (EDTA). The Dawes sonication system employed was operated at power setting 3 with the titanium soniprobe immersed in the

sample, which was contained in a glass vial. This vial was in turn immersed in a water bath which was held at room temperature (~22°C). After sonication the suspension was centrifuged at 60,000 *g* for 20 minutes to remove traces of titanium. The small flocculent layer at the surface was then removed, and the remaining translucent supernatant withdrawn. Vesicle aggregation that occurred during the course of subsequent experiments was evidenced by an increasing cloudiness of the solution, which could be eliminated by a brief (~1 minute) resonication. Control experiments revealed that the vesicles did not break up and reform to any measurable extent during such resonication. Unsonicated dispersions of DPL in D_2O (200 mg/ml) were prepared according to the method of Sheetz and Chan (*30*). Experiments were also performed on dry DPL powder. No special precautions were employed to remove vestigial traces of water from the powder, which most probably then exists as at least the monohydrate (*6*).

Vesicle preparations with higher solvent viscosities were obtained by the addition of measured amounts of glycerol. Mixing was ensured by gentle agitation of the sample or by a very brief resonication (~30 seconds). Control experiments with vesicles that had been sonicated (30 minutes) in the presence of glycerol indicated that the linewidths were identical to preparations in which glycerol had only been added to the external aqueous medium. The viscosity of the resulting solution relative to that of pure water was calculated using standard tables (*31*).

Two ^{31}P NMR spectrometers were employed, both of which were interfaced with Nicolet BNC-12 computers and operated in the Fourier Transform Mode, with the field stabilized with a deuterium "lock". The lower frequency (36.4 MHz) instrument was a Bruker WH-90, equipped with proton decoupling facilities. The higher frequency (129 MHz) spectrometer was constructed in this laboratory. The sensitivity of the latter instrument was enhanced by a quadrature detection modification. Interpulse times between 2 and 4 seconds and $\pi/4 \leqslant \theta \leqslant \pi/2$ radio-frequency pulses were used at both frequencies. In some cases the accumulated free induction decay was multiplied by an exponentially decaying function to enhance signal-to-noise. The number of scans collected varied between 200 and 1000.

As shown in the results section, the ^{31}P NMR spectrum of the sonicated vesicles may be regarded as a superposition of two resonances, separated by approximately 0.15 ppm, that arise from phospholipids on the inside and outside of the bilayer vesicle, respectively. In order to estimate the inside and outside linewidths, a curve-fitting technique was employed, with the assumption that the individual resonances had Lorentzian shape and equal linewidth. Since there were experimental indications in the 36.4 MHz results that the inside resonance was up to 20% narrower than the outside resonance for temperatures above the phase transition, the calculated individual linewidths should therefore be regarded as an average of the outside and inside linewidths. A further correction was made to the 129 MHz linewidths in order to compensate for the lack of proton decoupling at this frequency. In order to obtain this correction, a knowledge of the coupling constant between the phosphorus and the four (almost) equivalent protons on the nearest neighbor methylene groups in the polar headgroup moiety was required. This was obtained from a nondecoupled spectra of egg lecithin in chloroform-methanol, and from the characteristic 1 : 4 : 6 : 4 : 1 spectral pattern a coupling constant of 6.1 Hz was derived. A curve-fitting technique could then be employed to calculate the decoupled linewidths at 129 MHz.

RESULTS

High resolution phosphorus magnetic resonance spectra of dipalmitoyl lecithin vesicles were obtained throughout the temperature range 20°C to 55°C. Above the transition temperature (~41°C) the 36.4 MHz spectra consist of two distinct resonances with a chemical shift difference between them of 0.15 ppm (see Fig. 1). Below the transition temperature the linewidths are substantially broadened so that the two resonances can no longer be resolved. Both the apparent linewidth and the computed

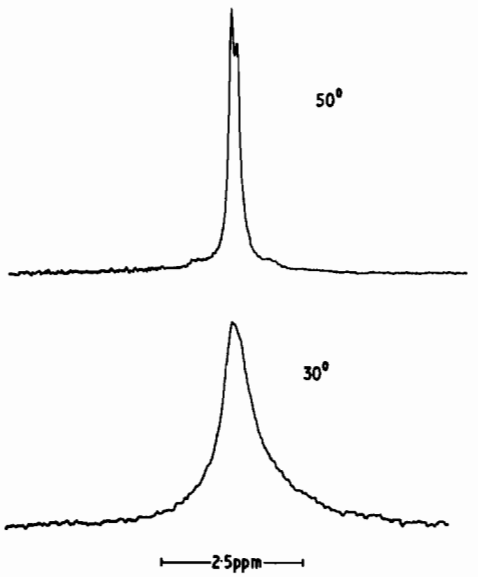

FIG. 1. 36.4 MHz ^{31}P NMR spectra of sonicated DPL vesicles (50 mg/ml) at 50° and 30°. The sonication was carried out in the presence of 25 m*M* Tris buffer (pH 7.2) and 2 m*M* EDTA. The magnetic field scale increases to the right.

linewidth of the two individual resonances (after correction for the chemical shift difference between them (see Methods)) are plotted in Fig. 2. The linewidths decrease sharply as the temperature is raised from 20°C up to the transition temperature, with an apparent activation energy of 8.0 kcal/mole. Above the transition temperature the linewidths are less temperature-dependent, with an apparent activation energy of only 3.9 kcal/mole.

At all temperatures employed the linewidths are approximately a factor of five broader at 129 MHz than at 36.4 MHz. As at 36.4 MHz, the temperature dependence of the linewidth at 129 MHz changes markedly at the phase transition temperature. Figure 3 illustrates the temperature dependences of the composite linewidth (upper curve) and the linewidth of the individual components of the composite spectra (lower curve) after correction for the chemical shift difference and the lack of proton decoupling at 129 MHz (see Methods). The apparent activation energies of the (individual) linewidths are 7.8 kcal/mole and 4.3 kcal/mole below and above the phase transition temperature, respectively.

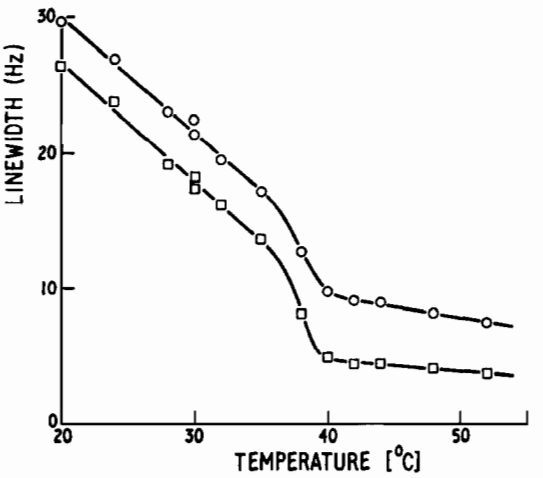

FIG. 2. 36.4 MHz ^{31}P NMR linewidth of DPL vesicles as a function of temperature. The upper curve describes the uncorrected experimental linewidths observed for the composite spectra. The lower curve shows the temperature dependence of the individual "inside" or "outside" linewidths after correction for the inside–outside chemical shift difference (see Methods). Experimental conditions were the same as described in Fig. 1.

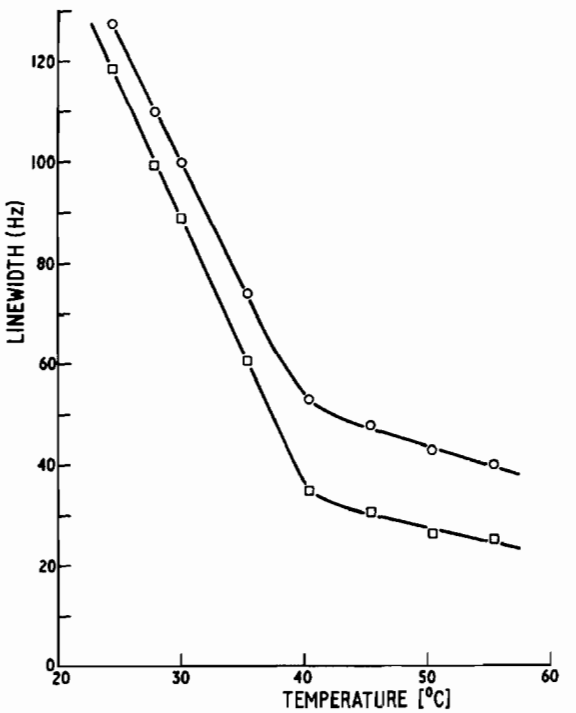

FIG. 3. 129 MHz ^{31}P NMR linewidths of DPL vesicles as a function of temperature. The upper curve gives the uncorrected experimental linewidths of the composite spectra. The lower curve shows the linewidths of the individual inside or outside resonances after correction for the inside–outside chemical shift difference and the absence of proton decoupling (see Methods). Other experimental conditions are as described in Fig. 1.

Under conditions where the two individual components of the vesicle spectra are resolved (above the transition-temperature at 36.4 MHz) the addition of neodymium nitrate (3 m*M*) clearly shifts one of the two resonances to lower field (Fig. 4). This result indicates that the two resonances arise from phospholipid molecules on the inside and outside of the spherical bilayer vesicle, respectively. Further experiments with cobalt (not shown) indicate that the downfield resonance arises from phospholipid molecules on the outside of the spherical bilayer vesicles. The chemical shift difference between the

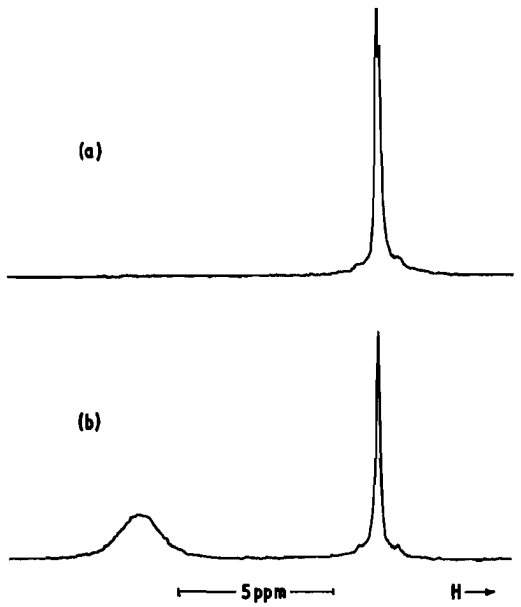

FIG. 4. 36.4 MHz ^{31}P NMR spectra of sonicated DPL vesicles at 50° (a) before the addition of Nd^{3+} and (b) after the addition of 2.5 m*M* Nd^{3+} in excess of EDTA. Other conditions were the same as in Fig. 1.

inside and outside phosphate groups decreases slightly as the temperature is raised (Fig. 3). Furthermore it was found that the ionic strength of the aqueous medium also affected the chemical shifts of the inside and outside resonances. The addition of high concentrations (2*N*) of sodium chloride shifts the outside phospholipid peak to lower field by ~5 Hz (at 36 MHz), increasing the separation of the inside and outside resonances. The resolution of the two peaks is not substantially improved, however, as the linewidths of both the inside and outside resonances also increase when high salt concentrations are employed. If sodium chloride is introduced into the internal aqueous medium of the vesicle (by sonication in the presence of salt) an equivalent shift of both the inside and outside resonances is observed, and the individual lines assume the same widths as in the absence of salt.

Phosphorus NMR spectra of unsonicated DPL dispersions in D_2O were obtained both at 30°C and 50°C at 129 MHz, and are illustrated in Fig. 5. As indicated, these "solid state" spectra allow an estimate of the contribution of the chemical shift aniso-

tropy, $\sigma_{\parallel} - \sigma_{\perp}$ (see Discussion), to the unsonicated linewidth. At 30°C $\sigma_{\parallel} - \sigma$ is estimated to be about 62 ppm, whereas at 50°C, $\sigma_{\parallel} - \sigma_{\perp}$ is estimated to be approximately 44 ppm. Although the chemical shift tensor in these "solid state" spectra appears to be axially symmetric, the experimental spectra were not simulated with the usual axially symmetric lineshape, as preliminary experiments with oriented multilayers indicate that the linewidth is strongly dependent on the angle of the axis of the chemical shift tensor with respect to the external magnetic field.

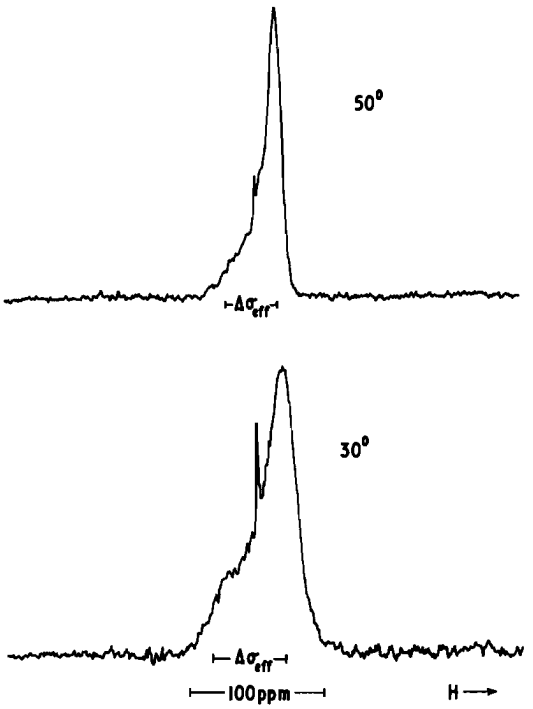

FIG. 5. 129 MHz ^{31}P NMR spectra of unsonicated DPL liposomes (200 mg/ml) at 50° and 30°. The preparation was made in the presence of 25 m*M* Tris buffer (pH 7.2) and 2 m*M* EDTA.

The "solid state" spectra at 20° of the DPL powder (see Materials and Methods) and unsonicated aqueous DPL dispersions are compared in Fig. 6. It is interesting to observe that the sign of the effective chemical shift anisotropy is reversed in the aqueous dispersions as compared with the powder.

The viscosity of the aqueous medium external to the vesicles was varied by the addition of up to 45% w/w of glycerol. As shown in Fig. 7, the ^{31}P NMR linewidths of the sonicated vesicles were found to be linearly dependent on the viscosity of the medium At 36.4 MHz a twofold change in the viscosity of the aqueous medium causes about 40% increase in linewidth at 50°C, and approximately 65% increase in linewidth at 30°C. At 129 MHz, however, a twofold change in viscosity causes about 75% increase in the linewidth at both 30° and 50°.

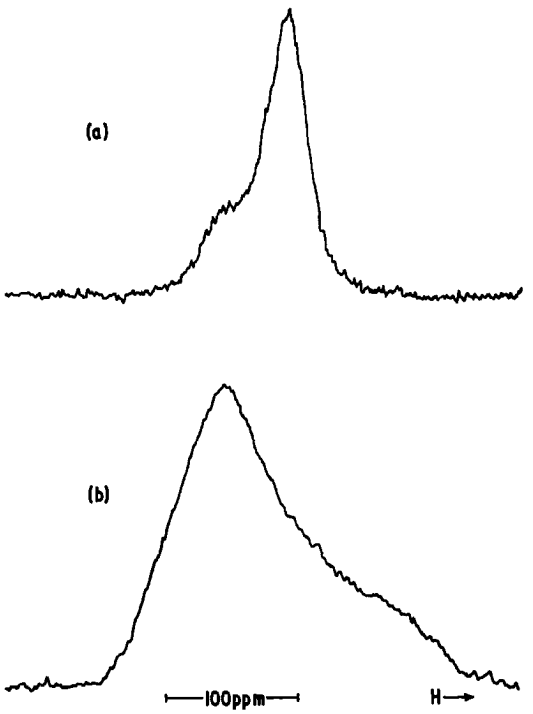

FIG. 6. 129 MHz ^{31}P NMR spectra of DPL at 20° (a) unsonicated DPL liposomes (200 mg/ml) prepared in the same manner as for Fig. 5, and (b) anhydrous DPL powder.

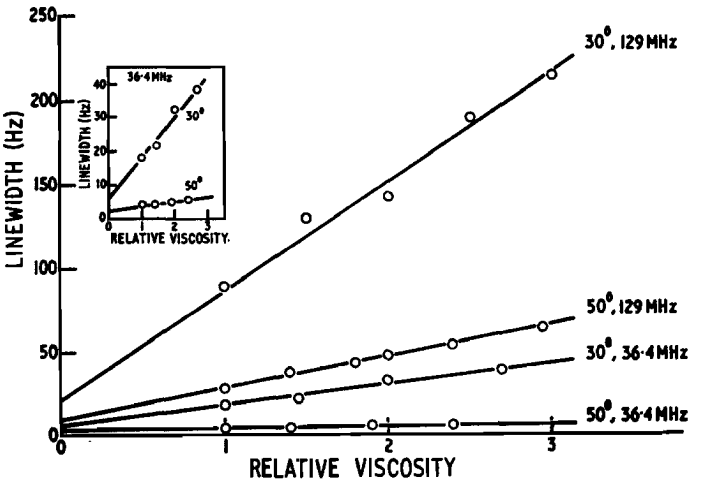

FIG. 7. 36.4 and 129 MHz ^{31}P NMR linewidths of sonicated DPL vesicles, after correction for the inside–outside chemical shift difference and proton decoupling (see Methods), as a function of the relative viscosity of the aqueous medium. The viscosity of the aqueous medium was varied by the addition of measured amounts of glycerol. Other conditions were identical to those for Fig. 1.

DISCUSSION

The ^{31}P NMR spectrum of dipalmitoyl lecithin vesicles consists of two resonances, separated by 0.15 ppm (at 50°), which arise from phosphate groups in phospholipid molecules on the inside and the outside layers of the spherical bilayer vesicles. Somewhat larger chemical shifts (~0.5 ppm) between inside and outside phospholipid ^{31}P NMR resonances have also been inferred for vesicles constituted of negatively charged egg-yolk phosphatidyl glycerol (*18*). Kostelnik and Castellano (*32*) have also observed chemical shift differences (~0.015 ppm) between inside and outside proton resonances arising from the choline trimethylammonium region of the polar headgroup. The origin of these inside-outside chemical shift differences is not clear. Such an effect could arise from the difference in bulk susceptibility between the aqueous and lipid phases, producing a slightly different magnetic field in the inner aqueous phase than in the external aqueous phase (*32*). The order of magnitude difference between the ^{1}H and ^{31}P inside-outside chemical shift differences does not, however, support this conjecture. A more feasible explanation would be that differences in the packing of the phospholipid molecules in the inner and outer layers of the bilayer membrane (produced by the high curvature of the spherical vesicles (*30*)) slightly alter the electrostatic interactions in the headgroup region. The altered electrostatic interactions will thus affect the chemical shift. This explanation would be consistent with the observed effects of high salt concentrations on the phosphorus chemical shifts.

Under most experimental conditions the linewidths of the inside and the outside resonances are of the order of, or larger than, the chemical shift difference between them. In order to analyze the composite lineshape a curve-fitting procedure is therefore necessary (see Methods). Within experimental error (±20%) the linewidths of the inside and outside resonances are equal, and the following discussion refers to the corrected values of the individual linewidths of the two resonances.

As shown in Figs. 2 and 3, the ^{31}P NMR linewidths are sensitive to the crystalline-liquid crystalline phase transition of the hydrocarbon chains. As is shown more explicitly in Fig. 8, the linewidth of the phosphorus resonances shows an apparent phase transition occurring at 36.5° (±1°) as compared with the hydrocarbon phase transition which occurs at 41° in unsonicated dipalmitoyl lecithin[1] (*6*). However, it must be noted that a sharp change in the magnetic resonance linewidth as a function of temperature does not necessarily reflect a (first-order) phase transition, but may simply reflect the thermal activation of an internal motion (*33*). If the rate of the restricted internal motion is increased through the "motional narrowing" region for that specific interaction, the linewidth will decrease abruptly, and then become constant as a function of temperature (*33*). Also, even if the abrupt change in the linewidth does reflect a first-order phase transition, the apparent transition-temperature (determined from the temperature-dependence of the linewidth) may not give the true phase-transition temperature (determined from calorimetric studies). This is because the rate of the internal motion may increase through the "motional narrowing" region before the actual cooperative phase-transition occurs. It is therefore difficult to determine if the apparent phase-transition observed in Fig. 8 implies that the phosphate region of the head group under-

[1] It is interesting in this regard that the sonicated vesicles have the same hydrocarbon phase transition temperature and the same enthalpy of transition as the unsonicated preparations (Dr. B. de Kruyff, personal communication).

goes a first-order phase transition, and if so, what the precise transition-temperature is. The change in the phosphorus linewidth could simply be reflecting motional changes in the phosphate region which are induced by the increased mobility in the lipid region as the hydrocarbon phase-transition is approached. However, the presence of a small pretransitional peak in calorimetric studies (*6*) suggests that the headgroup region may have a phase-transition slightly below the bulk hydrocarbon region.

The strong frequency dependence of the phosphorus linewidth is rather surprising. One possible explanation is that the phospholipid headgroups exist in different environments in the plane of the membrane, with each environment exhibiting a different

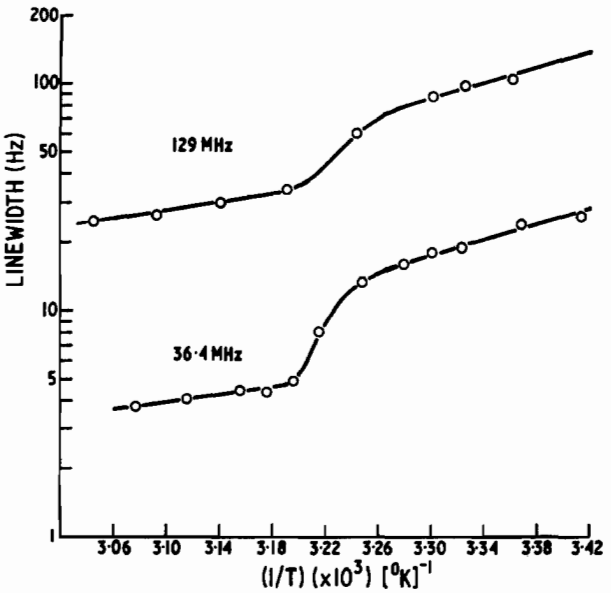

FIG. 8. 36.4 and 129 MHz ^{31}P NMR linewidths (after correction for the inside–outside splitting and proton decoupling—see Methods) of sonicated DPL vesicles as a function of inverse temperature.

chemical shift. However, the phospholipid molecules perform rapid lateral diffusion in the plane of the membrane; for a lateral diffusion constant of 3×10^{-8} cm^2/sec (*24*) and a phospholipid area of 40 Å (*35*), the effective exchange time for adjacent phospholipid molecules will be 10^{-7} seconds. This rapid lateral exchange will average any reasonable chemical shift difference (less than about 1 kHz) arising from intermolecular interactions among the headgroups to less than one Hz. If the different headgroup environments were the result of intramolecular interactions (i.e., interactions between the positively charged choline moiety and the negatively charged phosphate group), these interactions might have longer effective "exchange" times. However, in this case the linewidths would not be expected to be very sensitive to the viscosity of the medium, whereas at 129 MHz the linewidths are almost directly proportional to the viscosity (see Fig. 7). Also, if any static chemical shift differences existed because of intramolecular interactions, the field-dependence of the linewidth would be at most linear, whereas increasing the field by a factor of 3.6 increases the observed linewidths by a factor of at least 5.

The relaxation mechanism which determines the phosphorus linewidths in sonicated vesicles must therefore be field-dependent. The only relaxation mechanism which provides significant field dependence involves the modulation of the chemical shift anisotropy (*36*). In small molecules exhibiting isotropic motion the contribution of the chemical shift anisotropy to the linewidth is straightforward (*36*). However, in sonicated phospholipid vesicles the phosphate group would be expected to experience two distinct classes of motion: rapid internal motion through a restricted solid angle, due to the local motions of the phosphate group in the vesicle, and slow isotropic motion, duc to the Brownian rotation of the vesicles as a whole. The rapid internal motion will average part of the chemical shift anisotropy, while the isotropic tumbling of the vesicle will average any remaining part. In principle, both processes can contribute to the linewidth. The situation is further complicated by the contribution to the linewidth from dipolar interactions with protons on adjacent methylene groups.

Under these circumstances, it is difficult to analyze the linewidth data to obtain information on the specific details of the internal motion of the phosphate group in the membrane. However, as a first approximation, we will assume that the internal correlation times are much shorter than the Brownian rotation time of the intact vesicle. No explicit estimates of the correlation times for the internal motions are as yet available and this assumption therefore remains to be verified by further work. However, as the Brownian rotational time of the vesicle can be calculated from Stokes' Law to be 2×10^{-6} seconds, the approximation appears reasonable. With this assumption it is possible to separate the contributions from the anisotropic chemical shift and the dipolar relaxation mechanisms. It is also possible to separate the respective contributions to both of these mechanisms from the rapid restricted motions and the vesicle tumbling.

It is useful to construct a simple but relatively general model for the restricted internal motions. First, we consider a coordinate frame fixed at the surface of the phospholipid vesicle, with the z' axis in the average direction of the axis of symmetry of the chemical shift tensor. The z' axis of this membrane-fixed system has a polar angle θ with respect to the applied magnetic field H_0. The instantaneous direction of the axis of symmetry of the chemical shift tensor has axial and azimuthal angles Δ and ψ in this membrane-fixed system. The restricted internal motion of the phosphate group may then be expressed as restricted motion of Δ and ψ, with respective correlation times τ_Δ and τ_ψ. The isotropic tumbling of the intact vesicle may be expressed as a modulation of θ, with a correlation time τ_R. Using the adiabatic approximation the contribution to the linewidth from the modulation of the chemical shift anisotropy may then be written as (see Appendix)

$$1/T_2 = (4/45)\gamma_I^2 H_0^2 \langle \Delta\sigma_{EFF} \rangle^2 \tau_R + G(\tau_\Delta, \tau_\psi) H_0^2 \qquad [1]$$

where $\Delta\sigma_{EFF} \equiv (\sigma_\parallel - \sigma_\perp) \langle (3 \cos^2 \Delta - 1)/2 \rangle$, $(\sigma_\parallel - \sigma_\perp)$ is the chemical shift anisotropy in the rigid lattice and $\langle (3 \cos^2 \Delta - 1)/2 \rangle$ is averaged over all angles allowed by the restricted motion. $G(\tau_\Delta, \tau_\psi)$ $(\equiv (1/9)\ (\sigma_\parallel - \sigma_\perp)^2\ \gamma_I^2\ g(\tau_\Delta, \tau_\psi)$ (see Appendix) is a complicated function of the correlation times for internal motion and the averages of second-order spherical harmonics over the restricted solid angles. It is assumed that the chemical shift tensor is axially symmetric.

With the assumption that the internal correlation times are much shorter than the

vesicle tumbling time, the dipolar contribution to the linewidth may also be split into two terms:

$$1/T_2 = F\tau_R + D,$$

where F is proportional to the averaged value of the dipolar interaction over the restricted internal motion, and D is an involved function of the internal correlation times and the averages of spherical harmonics over the restricted internal motion. For the dipolar interaction the angular functions now refer to the directions of the phosphorus-proton vectors, and a sum must be taken over the four adjacent protons. The total linewidth may then be written as

$$1/T_2 = [(4/45)\gamma_I^2 H_0^2 \langle \Delta\sigma_{EFF}\rangle^2 + F]\tau_R + [GH_0^2 + D], \quad [2]$$

where the second term is independent of the vesicle tumbling time, τ_R. Since the vesicle tumbling time τ_R depends linearly on the viscosity of the medium (through Stokes' Law) there are two independently variable parameters in Eq. [2]; the applied magnetic field H_0, and τ_R. The dependence of the observed linewidths on the viscosity of the external medium at different fields strengths (see Fig. 4) then allows the four different terms in Eq. [2] to be estimated.

The values obtained for the different contributions to the linewidths are given in Table 1. At 129 MHz the modulation of the anisotropic chemical shift dominates the

TABLE 1

RESOLUTION OF ^{31}P NMR LINEWIDTHS INTO COMPONENTS DUE TO DIPOLAR AND CHEMICAL SHIFT ANISOTROPY MECHANISMS[a]

		Chemical shift anisotropy		Dipolar	
		$(4/45)\gamma_I^2 H_0^2\langle\Delta\sigma_{EFF}\rangle^2\tau_R$	$G(\tau_\Delta, \tau_\psi)H_0^2$	$F\tau_R$	D
$T = 30°$	36 MHz	4.65[b]	1.2	7.3	4.8
	129 MHz	57.8	14.9	7.3	4.8
$T = 50°$	36 MHz	1.53	0.60	0	1.4
	129 MHz	19.0	7.5	0	1.4

[a] See Equations [1] and [2] in the text.
[b] Contributions to the linewidth (full height at half width) are given in Hz.

phosphorus linewidth, contributing ~85% and ~95% of the total linewidth below and above the phase transition, respectively. The contribution from the modulation of the chemical shift anisotropy by the restricted internal motion is always less than that due to the modulation of the chemical shift anisotropy by the Brownian tumbling of the vesicles.

It is interesting to compare the values of the effective chemical shift anisotropy $\langle\Delta\sigma_{EFF}\rangle$ in the sonicated vesicles with that in the unsonicated aqueous dispersions. For the unsonicated dispersions the overall tumbling rate of the large aggregates is much too slow to average the effective chemical shift anisotropy, and a "solid-state" spectrum is observed (see Fig. 6), with the width of the spectrum being $\langle\Delta\sigma_{EFF}\rangle$ (*37*). Although the chemical-shift tensors in these "solid-state" spectra appear to be axially symmetric,

the experimental spectra were not simulated with the usual axially symmetric lineshape, as preliminary experiments with oriented multilayers indicated that the linewidth is strongly dependent on the orientation of the membrane with respect to the magnetic field. This effect presumably arises because of restricted internal motion of the phosphate group. The values of $\langle \Delta\sigma_{EFF} \rangle$ are thus estimated as shown in Fig. 6. The values of $\langle \Delta\sigma_{EFF} \rangle$ for the unsonicated dispersion at 129 MHz are tabulated in Table 2. These values are consistent with those obtained by Sheetz and Chan (*30*) for unsonicated dispersions at 89 MHz and are also similar to the calculated values of $\langle \Delta\sigma_{EFF} \rangle$ for the sonicated vesicles (see Table 2).

TABLE 2

CHEMICAL SHIFT ANISOTROPY $\langle \Delta\sigma_{EFF} \rangle$ FOR SONICATED DIPALMITOYL LECITHIN VESICLES AND UNSONICATED DISPERSIONS

	$\langle \Delta\sigma_{EFF} \rangle$ (ppm)	
	30°	50°
Sonicated vesicles	45[a]	32[a]
Unsonicated dispersions[b] ($\nu = 89$ MHz)	56 ± 10[c]	45 ± 10[c]
Unsonicated dispersion[d] ($\nu = 129$ MHz)	62	44

[a] Estimated from Eq. [2] and Table 1.

[b] Taken from Sheetz and Chan (*30*).

[c] Exact temperature not specified, temperatures are given as "above" and "below" the phase transition (42°), respectively.

[d] Estimated from Fig. 5.

The mobility of spin-labeled lipids in phospholipid membranes has been characterized in terms of an order parameter, S_{ESR} (*29*). The ESR order parameter is a measure of the amount of restricted internal motion available to the spin-labeled moiety in the membrane, and is given by the expression (*29*)

$$S_{ESR} = \langle (3\cos^2 \delta - 1)/2 \rangle,$$

where δ is the angle between the axis of symmetry of the hyperfine coupling tensor and its average value, and the angular brackets denote the average value over all allowed internal restricted motion. The ESR order parameter is calculated from a comparison of the average values of the hyperfine coupling tensor with the rigid lattice values. In phosphorus NMR a completely analogous order parameter S_{NMR}, may be defined as

$$S_{NMR} \equiv \langle (3\cos^2 \Delta - 1)/2 \rangle,$$

where Δ is the angle between the axis of symmetry of the phosphorous chemical shift tensor and its average value. The ^{31}P NMR order parameter thus gives information on the amount of restricted internal motion available to the phosphate group in the membrane. The order parameter ranges from a value of zero, for isotopic internal motion, to a value of one for no internal motion. The phosphorus NMR order parameter can be calculated from a comparison of the average values of the chemical shift tensor with the rigid lattice values. In the case of axial symmetry, S_{NMR} is simply given as (*37*)

$$S_{NMR} \equiv \left\langle \frac{3\cos^2 \Delta - 1}{2} \right\rangle = \frac{\langle \Delta\sigma_{EFF} \rangle}{(\sigma_{\parallel} - \sigma_{\perp})}$$

and is thus just the ratio of the averaged value of the chemical shift anisotropy to the rigid-lattice value. Since the effective values of the chemical shift anisotropy are very similar for the sonicated vesicles and the unsonicated dispersions, the order parameters for the two systems will likewise be similar. The close agreement between the values found for the sonicated vesicles and the unsonicated dispersions implies that the sonication process has not significantly modified the structure of the phosphate headgroup region. This is in contrast to the hydrocarbon chain region which has been shown to be significantly disrupted by the sonication process (*38*), presumably because of the high curvature of the spherical vesicles.

The value for the effective chemical shift anisotropy, $\langle \Delta\sigma_{\mathrm{EFF}} \rangle$, could be employed to give an estimate of the order parameter for the phosphate group in hydrated phospholipid systems if the rigid lattice value of the chemical shift anisotropy ($\sigma_{\parallel} - \sigma_{\perp}$) were known. Although the rigid lattice value of $\sigma_{\parallel} - \sigma_{\perp}$ has not yet been reported for the diesterified phosphate group, it would not be expected to be much larger than that observed in pyrophosphate ($\approx$200 ppm (*39*)). This is consistent with the ^{31}P NMR spectrum of DPL powder (see Fig. 6) which gives an estimate of $\sigma_{\parallel} - \sigma_{\perp}$ of 170 $\pm$ 20 ppm at 20°. It is not known, however, if there are residual motions in the powder at 20°, which would reduce the width of the observed spectrum from the true rigid-lattice value. It is also not clear that the observed chemical shift tensor is completely axially symmetric. The observed spectrum was not simulated with the theoretical axially symmetric lineshape because of the possibility of orientation-dependent linewidths, as discussed earlier. Since this phenomenon presumably arises because of internal motions in the aqueous dispersions, it is not clear if it would be a factor in the powder at 20°. Further experiments to clarify the point are in progress.

If the value of 170 ppm is used as a first approximation to the rigid-lattice value, the the order parameter for the vesicles is calculated to be 0.26 below the phase transition (30°). This order parameter may be interpreted in terms of the allowed motion of the phosphate group by either of two simple models. The first model assumes that the axis of symmetry of the chemical shift tensor rotates about an average direction, but always maintains a fixed angle with Δ_0 respect to this direction. It may then be noted that the observed chemical shift anisotropy will change sign as the angle Δ_0 is increased through the magic angle (54° 44′). However, as the respective signs of $\langle \Delta\sigma_{\mathrm{EFF}} \rangle$ and $(\sigma_{\parallel} - \sigma_{\perp})$ are not known, the sign of the order parameter is also unknown. The order parameter of $\pm$0.26 thus corresponds to $\Delta_0 = 44°$ and $\Delta_0 = 66°$ for the positive and negative signs, respectively.

The second simple motional model assumes that the axis of symmetry of the chemical shift tensor rotates around its average direction, but that it can take any angular position within a cone defined by the polar angle Δ_0. In this model it may be assumed that the order parameter is positive, as it will not change sign until $\Delta_0 > 90°$, which is considered unrealistic for phosphate group motion in the bilayer membrane. The angle Δ_0 is then calculated to be 68° below the phase transition.

Without further information it is difficult to determine which of the two simple models most closely describes the motion of the phosphate group in the membrane. However, a comparison of the ^{31}P NMR spectra for the DPL powder with the spectrum for aqueous unsonicated DPL suspensions (Fig. 6) indicates that the average value of the chemical shift anisotropy in the aqueous dispersions has a different sign from that in the

rigid lattice. This would suggest that the first model is more correct, i.e., that below the phase transition the axis of symmetry of the phosphate group rotates around its average direction with a fixed polar angle of ~66°. The conclusion must be treated with some caution, as there is another possible explanation for the apparent change of sign of the chemical shift anisotropy between the "anhydrous" powder and the aqueous dispersions. It is conceivable that, in the rigid lattice, the axis of symmetry of the chemical shift tensor lies in a direction normal to the methylene chain axis. The introduction of excess water to the powder may then allow a considerable degree of motion about the methylene chain axis, causing the effective axis of symmetry to shift from normal to parallel to the methylene chain axis.

This possibility is currently being investigated by a study of the rigid lattice ^{31}P NMR spectra of oriented model phosphate compounds, which will give the direction of the principal axes of the chemical shift tensor in the rigid lattice. Even if it is found that in the rigid lattice the axis of symmetry does not lie along the methylene chain axis, the large degree of mobility about the methylene chain axis in the aqueous dispersions (*40*) will be expected to produce an effective axis of symmetry along the methylene chain axis. Thus, the angular dependence of the phosphorus chemical shift in oriented multilayers will provide information on the orientation of the phosphate group with respect to the plane of the membrane. This information will then indicate the direction of the axis of the polar head group with respect to the plane of the membrane, i.e., if the alignment is normal or parallel to the membrane surface. Also, the effect of divalent metal ions (i.e., calcium) on the orientation of the polar headgroup (especially in systems of charged phospholipids) can be studied. These studies are currently in progress.

The field dependence of the ^{31}P NMR linewidths in sonicated phospholipid vesicles has important practical implications. Above the phase transition, the ^{31}P NMR linewidths in sonicated vesicles are narrow enough to resolve chemically shifted resonances from different classes of phospholipids, and also to resolve the resonances from the respective inside and outside phospholipid molecules of each class (*19*). ^{31}P NMR is thus a powerful technique for investigating the transverse and lateral distribution of different classes of phospholipids in the membrane (*25*), and for studying the specificity of the interaction of proteins and metal ions with different phospholipids. Since the differences in chemical shifts among the different classes of phospholipids are of the same order as the linewidths, it is desirable to find conditions which optimize the resolution of the different resonances. The usual technique for increasing the resolution of chemically-shifted resonances—using higher magnetic field strengths—may well be counter-productive. Since the linewidths increase as the field strength is raised, both the resolution and the sensitivity will begin to decrease at field-strengths where the chemical shift anisotropy becomes the dominant term. The optimum frequency occurs when the anisotropic chemical shift terms are roughly equal to the dipolar terms, a condition which may be different for vesicles composed of different classes of phospholipids. For example, for sonicated dipalmitoyl lecithin vesicles the optimum frequency may be estimated to be ≈ 50 MHz.

From the above results one might expect that in general phosphate groups localized in macromolecular complexes in such a way as to retain a large effective chemical shift anisotropy may display field-dependent linewidths. For instance, phosphate groups bound to enzymes, either as enzyme-phosphate or as enzyme-nucleotide complexes, or

phosphate groups in polynucleotide chains such as RNA or DNA, might provide examples.

APPENDIX

CALCULATION OF RELAXATION EFFECTS OF RESTRICTED INTERNAL MOTIONS

For nonoverlapping transitions, the linewidth of the transition from state i to state j is given by Redfield's formulation as (*41*, *42*)

$$\left[\frac{1}{T_2}\right]_{ij} = -R_{ij,\,ij} = \frac{1}{2\hbar^2}\left[-2J_{iijj}(\omega_{ij}) + \sum_k J_{kiki}(\omega_{ki}) + \sum_k J_{kjkj}(\omega_{kj})\right] \qquad [3]$$

where $J_{kiki}(\omega_{ki}) = \int_{-\infty}^{+\infty} \langle k|H_1(t)|i\rangle \langle i|H_1(t-\tau)|k\rangle\, e^{-\omega_{ki}\tau}\, d\tau$. $H_1(t)$ is the time dependent part of the Hamiltonian which is responsible for the relaxation, and is generally written as (*43*)

$$H_1(t) = \sum_q A_q F_q(t), \qquad [4]$$

where the A_q are functions of the spin operators of the system and the $F_q(t)$ are functions of the spatial coordinates, which are functions of time. The secular term in Eq. [4] gives rise to the "adiabatic" contribution to the linewidth while the nonsecular terms give rise to the "nonadiabatic" contributions.

If the correlation times which determine the linewidth satisfy the condition

$$\omega_{ki}\tau_c \gg 1$$

then the "nonadiabatic" contributions to the linewidth may be ignored. In this case only the secular term in Eq. [4] need be considered. The validity of this approximation may be directly verified. Since the "nonadiabatic" terms in the linewidth dominate the longitudinal relaxation rate, the observation that

$$(T_1)_{ij}/(T_2)_{ij} \gg 1$$

is sufficient to ensure that the adiabatic term dominates the linewidth (assuming the absence of significant exchange terms in $(1/T_2)_{ij}$). This condition has been shown to be valid for phosphorus in sonicated phospholipid vesicles (*44*).

If only the secular term in Eq. [4] is considered, and if the chemical shift tensor is assumed to be axially symmetric, Eq. [3] may be rewritten as

$$\left[\frac{1}{T_2}\right]_{ij} = \frac{1}{2\hbar^2}[\langle i|A_{sec}|i\rangle - \langle j|A_{sec}|j\rangle]^2 \int_{-\infty}^{+\infty} \overline{F_{sec}(t)\,F_{sec}(t+\tau)}\, d\tau \qquad [5]$$

where $A_{sec} = \frac{1}{3}(\sigma_\parallel - \sigma_\perp)\, I_z\, \gamma_1$ and $F_{sec}(t) = (3\cos^2\theta_0(t) - 1)$. $\sigma_\parallel - \sigma_\perp$ is the difference between the chemical shift when the magnetic field H_0 is parallel or perpendicular to the axis of symmetry of the chemical shift tensor, respectively. θ_0 is the angle between the axis of symmetry of the chemical shift tensor and the external magnetic field. The rotation about the longitudinal axis of the polar headgroup would be expected to average any components of the chemical shift tensor perpendicular to this axis. It may then be assumed that the axis of symmetry of the chemical shift tensor and the longitudinal axis of the polar headgroup coincide.

For isotropic motion the correlation function is simply given by the expression

$$\overline{F_{sec}(t)\,F_{sec}(t+\tau)} = \overline{F_{sec}(t)^2}e^{-\tau/\tau_c}$$

where τ_c is the correlation time. However, in the presence of restricted internal motions which do not completely average the anisotropic chemical shift, the correlation function becomes a sum of exponentials, instead of a single exponential. In order to calculate the correlation function a motional model must be constructed which explicitly takes into account the two types of motion: the rapid, restricted motion of particular segments of the phospholipid molecules, and the slow isotropic tumbling of the spherical vesicles.

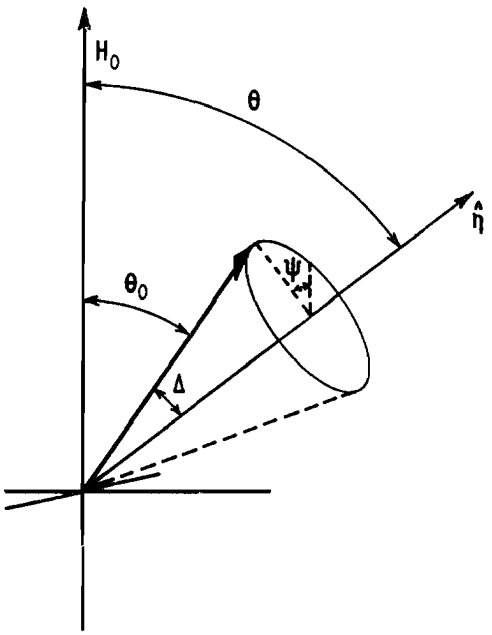

FIG. 9. A geometrical model of the motion of the axis of the axially symmetric chemical shift tensor. The vector η describes the average orientation of the axis of the chemical shift tensor. The instantaneous orientation of the chemical shift tensor with respect to η is described by the polar and azimuthal angles θ and ψ, respectively. The applied magnetic field H_0 defines the z direction and has polar angles θ and θ_0 with respect to the average and instantaneous axis of the chemical shift tensor.

In the bilayer membrane the polar headgroup moiety may be considered to undergo two distinct types of motion: rotation about the longitudinal axis of the polar headgroup and the angular motion of the longitudinal axis of the polar headgroup itself. These motions are assumed to be independent and characterized by different correlation times. A mathematical model which incorporates these two classes of motion is illustrated in Fig. 9. Defined as the average direction of the longitudinal axis of the polar headgroup, $\hat{\eta}$ is a fixed direction in the bilayer membrane and has a polar angle θ with respect to the external magnetic field H_0. The direction of the instantaneous position of the longitudinal axis of the polar headgroup with respect to $\hat{\eta}$ is defined by the polar and azimuthal angles Δ and ψ. The instantaneous direction of the longitudinal axis of the

polar headgroup with respect to the magnetic field θ_0 may be expressed in terms of θ, Δ, and ψ, and it may be shown (*45*) that

$$(3\cos^2\theta_0(t) - 1) = +\tfrac{1}{2}(3\cos^2\Delta(t) - 1)\,(3\cos^2\theta(t) - 1) - 6\sin\Delta(t)\cos\Delta(t)\sin\theta(t)\cos\theta(t)\cos\psi(t) + \tfrac{3}{2}\sin^2\Delta(t)\sin^2\theta(t)\cos 2\psi(t).$$

Thus, the restricted motion around the longitudinal axis of the polar headgroups is represented by motion of ψ, motion of the longitudinal axis of the polar headgroup is represented by motion of Δ, and overall tumbling of the vesicle is represented by motion of θ. These motions are characterized by three different correlation times; τ_ψ, τ_Δ, and τ_R, respectively.

The correlation function of a random function $f(t)$, which has a nonzero average value may be given by the expression

$$\overline{f(t)f(t+\tau)} = \langle f(t)\rangle^2 + \langle \delta f(t)^2\rangle\, e^{-\tau/\tau_c}, \qquad [6]$$

where $\langle f(t)\rangle$ is the average value of $f(t)$, and $\delta f(t)$ is defined by the equation

$$f(t) \equiv \langle f(t)\rangle + \delta f(t).$$

Using Eq. [6] the correlation function for the motion of the longitudinal axis of the polar headgroup may be written as

$$\overline{(3\cos^2\theta_0(t) - 1)\,(3\cos^2\theta_0(t+\tau) - 1)} = \tfrac{1}{4}\langle 3\cos^2\Delta(t) - 1\rangle^2\,\langle(3\cos^2\theta - 1)^2\rangle e^{-\tau/\tau_R} + \sum B_i\, e^{-\tau/\tau_i}, \qquad [7]$$

where the B_i are the average values of spherical harmonics over the allowed restricted motions, and the $^1/\tau_i$ are linear combinations of $^1/\tau_R$, $^1/\tau_\Delta$, and $^1/\tau_\psi$. In the limit that

$$\tau_R \gg \tau_\Delta, \tau_\psi,$$

the second term in Eq. [5] is independent of τ_R.

Inserting Eq. [7] into Eq. [5] results in the expression

$$1/T_2 = (4/45)\,(\langle \Delta\sigma_{EFF}\rangle\,\gamma_I H_0)^2\,\tau_R + (1/9)\,[(\sigma_\parallel - \sigma_\perp)\,\gamma_I H_0]^2\, g(\tau_\Delta, \tau_\psi), \qquad [8]$$

where

$$\langle \Delta\sigma_{EFF}\rangle \equiv (\sigma_\parallel - \sigma_\perp)\left\langle \frac{3\cos^2\Delta - 1}{2}\right\rangle \qquad g(\tau_\Delta, \tau_\psi) = \frac{1}{2h^2}\int_{-\infty}^{+\infty} \sum B_i\, e^{-\tau/\tau_i}\, d\tau.$$

Equation [8] describes the contribution to the linewidth arising from the chemical shift anisotropy. In principle this expression is only valid for nonoverlapping transitions for which the chemical shift anisotropy and other relevant relaxation mechanisms are independent. At first glance, these conditions would not appear to be valid for the phospholipid vesicle system under discussion, as the coupling between the phosphorus and the two adjacent methylene groups gives rise to an overlapping quintet structure (with a coupling constant of ~6 Hz). Also, part of the contributions of the chemical shift anisotropy and dipolar interactions to the linewidth may be governed by the same correlation time, and therefore would not be independent. For instance, the isotropic Brownian rotational motion of the vesicle may modulate both part of the chemical

shift anisotropy and part of the dipolar interactions. This correlation may be shown to result in an expression for the component linewidth which contains cross-terms between the dipolar and chemical shift anisotropy contributions, and the linewidths may then vary through the multiplet (*46*, *47*). In the present situation, however, the effects due to these cross-terms should be very small, as the component linewidths at 129 MHz are much larger than the coupling constant, and at 36 MHz the quintet structure is collapsed by proton decoupling. At both frequencies the resultant superposition of lines can be shown to give a resonance which has a width closely approximating the value expected in the absence of any cross-terms.

The second problem concerns the applicability of the Redfield formulation to overlapping resonances (*42*). If $R_{ij,kl}$ is of the same magnitude as $R_{ij,ij}$ or $R_{kl,kl}$, then if the two resonances arising from the transitions $i \rightarrow j$ and $k \rightarrow l$ overlap, the resulting spectrum is not simply the superposition of the individual components (*42*). However, the $R_{ij,kl}$ terms can be shown to be nonsecular, so in the present case may be neglected with respect to the $R_{ij,ij}$ or $R_{kl,kl}$. Thus, the resulting spectrum may be considered as a simple superposition of the different hyperfine components.

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