DEUTERIUM MAGNETIC RESONANCE STUDY OF THE GEL AND LIQUID CRYSTALLINE PHASES OF DIPALMITOYL PHOSPHATIDYLCHOLINE

JAMES H. DAVIS, *Department of Physics, University of British Columbia, Vancouver, Canada V6T 1W5*

ABSTRACT Deuterium magnetic resonance is applied to the study of the liquid crystalline and gel phases, and of the phase transition, of a multilamellar dispersion of chain perdeuterated (d_{62})-dipalmitoyl phosphatidylcholine/H_2O. Analysis of the deuterium spectra in terms of the moments of the spectra allows one to make quantitative statements concerning the distribution of quadrupolar splittings even in complicated situations, e.g., when using perdeuterated samples or when there are mixed phases. This analysis indicates that d_{62}-dipalmitoyl phosphatidylcholine in excess H_2O undergoes a sharp phase transition (with a width of $<1°C$) at $\sim 37°C$ and that there appears to be hysteresis in the phase transition of $\sim 1°C$. In the lamellar liquid crystalline phase above 37°C the spectra show a number of well-resolved features whose quadrupolar splittings can be followed as the temperature is varied. The gel phase near 20°C possesses a very broad, almost featureless spectrum that does not seem to support a model of the gel phase wherein the hydrocarbon chains are fully extended in the all-*trans* conformation. At temperatures near 0°C the spectra clearly indicate that a large fraction of the lipid molecules cease the rotation about their long axes, giving a spectrum more characteristic of a rigid or solid sample. These results give a picture of the gel phase as a phase characterized by considerable hydrocarbon chain disorder near 20°C and becoming a more solid-like phase near 0°C. The spin-lattice relaxation time, T_1, has been measured at 20°C in the gel phase, and at 37 and 45°C in the liquid crystalline phase. The values of T_1 obtained for each of the resolvable peaks in the spectrum at 37°C are compared to the values (for each peak) of T_{2e}, the decay time of the quadrupolar echo, obtained at the same temperature. These results are discussed in terms of a simple two-motion model.

INTRODUCTION

A detailed deuterium magnetic resonance (DMR) investigation of acyl chain order in the liquid crystalline (Lα) phase of multilamellar dispersions of specifically deuterium-labeled dipalmitoyl phosphatidylcholine (DPPC) was reported some time ago (Seelig and Seelig, 1974, 1975). The order parameter profile obtained described the flexibility gradient of the bilayer and exhibited the characteristic "plateau" often observed in multilamellar dispersions (Seelig and Seelig, 1974, 1975; Mely et al., 1975; Davis and Jeffrey, 1977). A recent DMR study of *Acholeplasma laidlawii* membranes (Stockton et al., 1977), where the cells were grown on specifically deuterated palmitic acid, gave the order parameter profile for a biological membrane system above the gel-to-liquid crystalline phase transition and found a close correspondence with that obtained earlier for the DPPC model system. This correspondence demonstrated that DPPC (or more generally, phospholipid) multilamellar dispersions are a useful model membrane system in that the physical state of the phospholipid bilayer in the liquid crystalline phase closely resembles that of the natural membrane above the phase

transition. It is found, however, that at the growth temperature the natural membranes are in a mixed phase, containing regions of gel as well as liquid crystalline lipid, so that the spectra are more complex than those obtained for a pure lipid-water system (Stockton et al., 1977).

Although the detailed information obtained from the study of specifically labeled membranes has been crucial in determining the physical state of the bilayer, it is clearly desirable to obtain as much information as possible using a single sample where the phospholipid acyl chains have been completely (per-) deuterated (Davis et al., 1979*a*,*b*). This information, together with what has already been learned about the bilayer structure from the specific label studies, may allow a comparison of different model and biological systems. It is already obvious that such a comparison can be made on a qualitative level; what remains to be seen is to what extent we can make a quantitative comparison.

It has been demonstrated in the case of soaps (e.g., potassium laurate or palmitate (Mely et al., 1975; Davis and Jeffrey, 1977) that the order parameter profile can be easily obtained using perdeuterated samples although specific label studies are needed for the unambiguous assignment of the peaks (Stockton et al., 1976). In these single chain systems many of the individual peaks in the spectrum can be resolved even for powder samples where the spectrum consists of a number of overlapping I = 1 quadrupolar powder patterns (Davis and Jeffrey, 1977). In perdeuterated soaps at temperatures just above the gel-to-liquid crystal phase transition, the plateau shows up as an increase in intensity at the edge of the spectrum where several peaks overlap because they have roughly the same quadrupolar splittings or order parameters (these are for the positions near the polar head). In principle the spectra for chain perdeuterated phospholipids (with saturated chains) can be interpreted in the same manner.

There is of course a fundamental difference between these two model membrane systems. The soaps, having only one hydrocarbon chain, have simpler spectra in which individual peaks can be identified and studied independently. For example, with d_{31}-potassium palmitate, as the temperature is raised the plateau rapidly vanishes and 14 out of the 15 possible peaks in the spectrum can be identified (the 3 and 4 positions give peaks which seem never to be resolvable; Davis and Jeffrey, 1977). Perdeuterated phospholipids, because they have two fatty acid chains that are not equivalent (Büldt et al., 1978; Seelig and Seelig, 1975; Hitchcock et al., 1974), will give a far more complex spectrum. In this situation we cannot expect to resolve many of the individual peaks. Although we cannot unambiguously determine the quadrupolar splitting associated with specific positions on the chains from such spectra, we can make some important general statements about the order parameter profile in the bilayer (Bloom et al., 1978*b*).

This paper presents the results of a study of the temperature dependence of the deuterium spectrum of d_{62}-DPPC in excess water. These results are compared to those reported on selectively deuterated DPPC (Seelig and Seelig, 1974, 1975). A moment analysis, to be discussed more completely in another article,[1] provides a powerful means of studying the gel-to-liquid crystalline phase transition and, more generally, systems consisting of mixed phases such as natural membranes. The nature of the gel phase of DPPC is discussed in terms of the moments of the spectra and, finally, some T_1 and T_{2e} deuterium relaxation measurements are briefly discussed in terms of a two-motion model.

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[1]Bloom, M., J. H. Davis, and F. W. Dahlquist. Unpublished results.

EXPERIMENTAL CONSIDERATIONS

Chain-deuterated L-α-phosphatidylcholine, β,γ-di-d_{31}-palmitoyl (~98% deuterated) was obtained from Lipid Specialties, Boston, Mass. Analysis by thin layer chromatography (using 2 mg of d_{62}-DPPC) indicated that it was pure before the experiment was begun. The sample consisted of a multilamellar dispersion of d_{62}-DPPC in excess H_2O (60% by weight, deionized). The two components of the sample were placed in a 25-mm long, 7.5-mm-diam sample tube and mixed thoroughly at room temperature. The sample was mixed until it appeared visually homogeneous and until further mixing resulted in no change in the fluid phase deuterium spectrum. The experiments were run continuously over a period of 9 d after which the sample was again analyzed by thin layer chromatography (using 2 mg of d_{62}-DPPC) which showed that it now contained a barely detectable trace of lyso-PC. Previous experiments in our laboratory had indicated that samples that appear pure on thin layer chromatography (TLC) may degrade into lyso-PC over the period of many days, especially at temperatures above room temperature, and that this degradation can result in a considerable broadening of the phase transition. The deuterium NMR of the present samples suggests that at most there may have been a slight broadening in the phase transition occurring between the beginning and the end of these experiments. It is extremely important to demonstrate the purity of the sample before and after the experiments have been performed because small amounts (a few percent) of lyso-PC significantly alter the physical state of the bilayer as seen by deuterium NMR.

The spectra were taken at 34.44 MHz in a high resolution superconducting solenoid supplied by Nalorac, Inc., Concord, Calif., on a Bruker SXP 4-100 spectrometer (Bruker Spectrospin Canada Ltd., Mississauga, Ontario). The transient digitization was accomplished with a Nicolet 1090 AR digital oscilloscope (Nicolet Instrument Corp., Madison, Wisc.) interfaced to an Intel 8080A microprocessor (Intel Corp., Santa Clara, Calif.) based data acquisition system. The Fourier transforms and moment analysis were done on a BNC-12 minicomputer (Nicolet Instrument Corp.).

All spectra were obtained using the quadrupolar echo technique with a pulse separation of 75 μs (Davis et al., 1976) accumulating ~1,000 scans with a spectral width of 100 kHz in the liquid crystalline phase, and, for temperatures below the phase transition, ~4,000 scans with a spectral width of 250 kHz. As usual, the experiments were done on resonance so that negative frequencies of the symmetric spectrum are folded over on top of the positive frequencies on transformation, resulting in $\sqrt{2}$ improvement in signal:noise ratio and a decrease in the necessary spectral width by a factor of 2. No filters were used in the acquisition and no phase corrections were made to the transforms. It is emphasized that the lineshape must be faithfully obtained to do the moment analysis presented here.[1]

When dealing with very broad lines it is difficult to obtain a 90° pulse short enough to rotate all parts of the spectrum through the same angle. The choice of experimental conditions and the resulting distortion will be discussed in more detail in the section on the gel phase.

The temperature of the sample was measured and controlled in a cylindrical copper oven enclosing the sample and radio frequency coil. The temperature gradient across the sample volume is undetectable and is estimated to be <0.25°C. After a 1°C change in temperature the sample was allowed 30 min to come to equilibrium. This seems to have been sufficient on the warming half of the cycle, but the dependence of the moments on the thermal history of the sample (within the gel phase) suggests that, on cooling the sample, longer waiting periods may be required.

RESULTS AND DISCUSSION

The Fluid (Lα) Phase Spectroscopy

In the liquid crystalline phase the deuterium spectrum of d_{62}-DPPC exhibits what can be called the typical phospholipid bilayer lineshape, looking very similar to that seen in the d_{31}-palmitate grown *A. laidlawii* membranes (Stockton et al., 1977). Fig. 1 shows three spectra at different temperatures within the liquid crystalline phase (for multilamellar dispersions of d_{62}-DPPC the transition occurs at ~37°C compared to 41°C for protiated

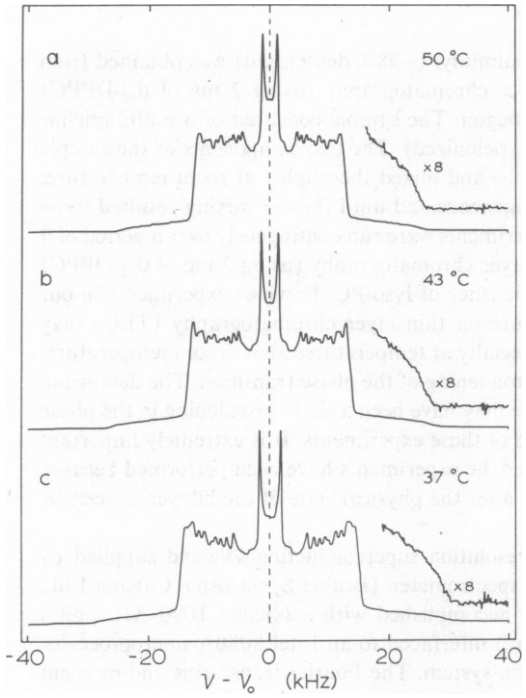

FIGURE 1

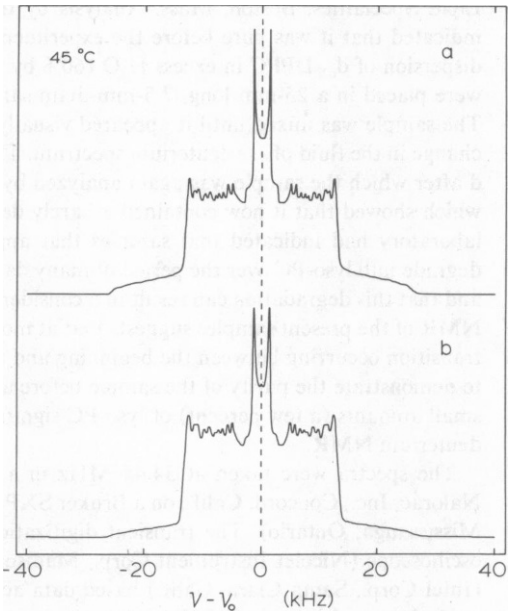

FIGURE 2

FIGURE 1 Deuterium NMR spectra in the liquid crystalline phase of d_{62}-dipalmitoyl phosphatidylcholine, at ν_0 = 34.44 MHz, after accumulating 1,000 scans using the quadrupolar echo, t_w = 4.5 μs. (*a*) T = 50°C; (*b*) T = 43°C; (*c*) T = 37°C. The dashed line through the center of the spectra indicates that these spectra, and all others shown, have been reflected about the central resonance frequency, ν_0. The vertical scale is increased by a factor of eight on the right hand side of the figure.

FIGURE 2 (*a*) Computer simulation of spectrum in (*b*) taken at 45°C, using a Lorentzian lineshape (parameters of simulation are shown in Table I).

DPPC [Peterson et al., 1975]). The expansions of the vertical scale on the right side of these spectra show the clearly defined 0° shoulders of the spectra and the well-behaved base lines. It is clear from these spectra that we can never hope to resolve and identify any more than a few individual peaks, and we can only confidently identify any of the peaks (with the exception of the methyl group peak) by comparison with the studies of selectively deuterated samples. However, we can immediately say upon inspection of the spectrum that its overall square shape and its sharply defined edge are clear indications of a well-defined order parameter plateau. The quadrupolar splitting of the plateau, $\delta\nu$, given by the frequency of the sharp edge of the spectrum, defines the order parameter of the carbon-deuterium bond vector (S_{CD}) for positions that contribute to the plateau. At 37°C, $|S_{CD}|_{\text{plateau}} = \delta\nu/(3e^2qQ/4h) = 0.23$, where $(e^2qQ/h) = 167$ kHz is the quadrupolar coupling constant (Davis and Jeffrey, 1977).

The distribution of intensity in spectra such as those shown in Fig. 1 can be determined by a computer simulation where the simulated spectrum is made up of a superposition of a number of overlapping I = 1 quadrupolar powder patterns (Davis et al., 1976). Fig. 2 *b* is the spectrum obtained at 45°C, and Fig. 2 *a* is the simulation using 18 Lorentzian powder patterns. The fact that the simulated spectrum shows too much intensity in the region of the 0° shoulder is felt to

TABLE I
SIMULATION PARAMETERS FOR SPECTRUM AT 45°C

Peak*	$\delta\nu^{exp}$*	Deuterons in peak‡	$\delta\nu^{sim}$§	Chain position (tentative assignment)¶
	kHz	n	kHz	
a	2.68	6	2.78	16 (methyl)
b	9.96	2	10.06	15*a*
c	11.57	3	11.77	15*b*, $2b_2$
d	13.43	2	13.48	14*a*
e	14.80	2	14.90	14*b*
f	15.97	2	16.07	13*a*
g	17.97	4 (2,2)	17.87, 18.37	13*b*, 12*a*
h	19.73	4 (2,2)	19.62, 20.13	12*b*, 11*a*
i	21.92	5 (2,3)	21.72, 22.12	11*b*, 10*a*, $2b_1$
j	22.95	6 (2,4)	22.92, 23.42	10*b*, 9*a*,*b*
k	24.95	26 (4,8,8,6)	24.75, 25.15 25.65, 26.25	8–3, 2*a*

*See Fig. 3. Assignment is based on results for specifically labeled DPPC (Seelig and Seelig, 1974, 1975).
‡For simulation, numbers in parentheses are for peaks whose widths require more than one splitting in simulation.
§Multiple entries are for those peaks requiring more than one splitting.
¶Letters a and b are to indicate that the two chains give different splittings. $2b_1$, $2b_2$ refer to the two inequivalent deuterons on the chain at position 2.

be a result of the use of a Lorentzian lineshape (which has a large intensity in the wings) rather than due to any distortion of the experimental spectrum. The key parameters in the simulation are listed in Table I. It should be noted that the Lorentzian linewidths ($\delta = 1/T_2^*$) assumed in this simulation were chosen to match the appearance of the spectrum and do not reflect the behavior of the quadrupolar echo relaxation time (T_{2e}) as a function of chain position (see below).

By comparison of these spectra with the studies of specifically labeled samples, the resolvable peaks in them can be tentatively assigned to specific chain positions. In particular the intensities of the peaks require that at least some positions on the two chains are inequivalent, as has been found by Seelig and Seelig (1974, 1975). The assignment here has been chosen to agree as closely as possible with their results. Such an assignment scheme is shown in Table I. The positions of these peaks can be followed as the temperature is varied, and Fig. 3 shows the temperature dependence of the quadrupolar splittings in the liquid crystalline phase. Above 37°C there is a smooth gradual decrease in all of these splittings as the temperature is increased.

The values of the splittings obtained from the specifically deuterated studies at 50°C (Seelig and Seelig, 1974, 1975) are plotted in this figure at 46°C (because the d_{62}-DPPC gel-to-liquid crystalline phase transition is ~4°C lower than that of DPPC [Peterson et al., 1975]). Near the methyl end of the chains the two sets of values agree rather well. However, in the plateau region the values for the splittings of the specifically deuterated samples are significantly larger. This difference may be due to sample differences (water content, impurities, etc.), or it may be that the chains of d_{62}-DPPC are more disordered (i.e., an isotope effect).

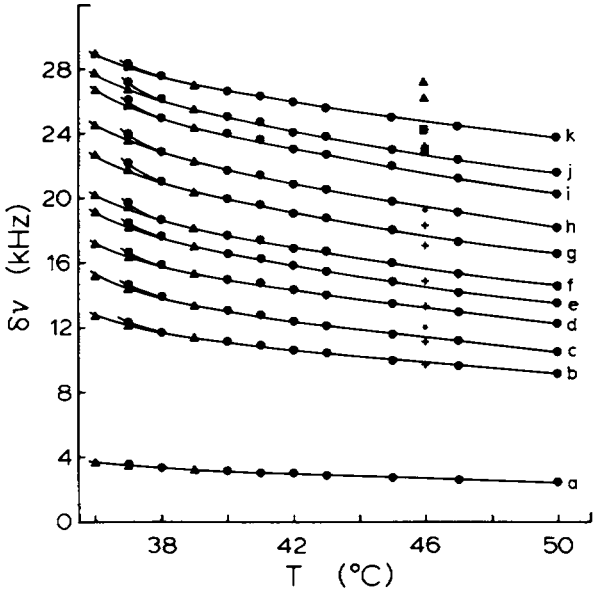

FIGURE 3 The quadrupolar splittings vs. temperature, measured from each of the sharp peaks in the spectra of the liquid crystalline phase. The letters *a–k* are to identify each of the peaks giving these splittings. The symbols shown for 46°C are the reported values for selectively labeled dipalmitoyl phosphatidylcholine (Seelig and Seelig, 1974, 1975). The different symbols indicate the number of deuterons with a given splitting: ▲, six deuterons; ■, four deuterons; +, two deuterons; ●, one deuteron.

An important distinction between the two types of model membrane systems (soaps and phospholipids) is clearly illustrated by the spectra shown in Fig. 1. In the lamellar, liquid crystalline phase of DPPC the spectrum retains its overall shape (characteristic of the plateau) as the temperature is raised and simply becomes narrower due to increased mobility at the higher temperatures. This is in sharp contrast to the behavior observed in potassium palmitate multilamellar dispersions where increasing the temperature by only 10°C above the gel-to-liquid crystal phase transition substantially destroys the plateau (Davis and Jeffrey, 1977). It seems clear that the interactions between the two acyl chains on a given molecule of DPPC provides enough hindrance to their motion to maintain the plateau over a wide temperature range. Previous experiments in this laboratory indicate that the spectrum still exhibits the plateau at temperatures as high as 85°C.

This persistence of the plateau for the double-chained phospholipids may have biological significance—given that the plateau in the bilayer flexibility gradient is a biologically "desirable" characteristic of bilayer structure—because the single chain molecules seem to be incapable of maintaining the plateau in a changing environment. One effect of adding cholesterol to the membrane of *A. laidlawii*, for example, is to smear out the normally sharply defined order parameter plateau (Stockton et al., 1977). Cholesterol as well as phospholipid chain heterogeneity have been proposed to cause a decreased phospholipid chain-chain interaction (Baldassare et al., 1976). The variation of the strength of this interaction may be a mechanism for controlling the flexibility gradient of the bilayer.

Moment Analysis

The general shape of the spectrum of a perdeuterated sample allows one to infer, for example, that the sample is in a liquid crystalline phase and that the order parameter variation takes the form of a plateau. As discussed more fully elsewhere (Bloom et al., 1978*b*)[1] we can obtain a more quantitative description of the state of the bilayer from deuterium spectra obtained from a perdeuterated sample by evaluating the moments of the spectra (Davis et al., 1979*a*,*b*).

The nth moment of the spectrum with lineshape $g(\Omega - \Omega_0)$, where ω_0 is the central or Larmor frequency in angular frequency units, is defined as (in terms of the half-spectrum, $\omega \geq \omega_0$, we may define the odd moments):

$$M_n \equiv \int_{-\infty}^{\infty} d\omega(\omega - \omega_0)^n g(\omega - \omega_0) \Big/ \int_{-\infty}^{\infty} d\omega g(\omega - \omega_0)$$

$$= \int_0^{\infty} dx x^n g(x) \Big/ \int_0^{\infty} dx g(x), \tag{1}$$

since for a first-order quadrupolar powder pattern $g(|\omega - \omega_0|) = g(-|\omega - \omega_0|)$ (Abragam, 1961). For a single I = 1 quadrupolar powder pattern neglecting broadening, i.e., when the linewidth is much smaller than the quadrupolar splitting, the moments of the spectrum are functions only of the quadrupolar splitting $\delta\omega = 2\pi\delta\nu$ (Bloom et al., 1978*b*), and the measurement of any one of the moments is equivalent to a measurement of the quadrupolar splitting.

For a sample labeled in more than one position or having more than one environment for the deuterons, the moments of the spectrum can be used to determine the distribution of quadrupolar splittings. In fact, the moments of the spectrum are directly and simply related to the moments of the distribution of splittings.[1]

$$M_n = A_n(2\pi)^n \langle(\delta\nu)^n\rangle = A_n(2\pi\nu_Q)^n \langle S_{CD}^n\rangle, \tag{2}$$

where, for example,

$$A_1 = (2/3\sqrt{3}), \qquad A_2 = (1/5), \text{ etc.},$$

and S_{CD} is the C—D bond order parameter defined as $S_{CD} = \frac{1}{2}\overline{(3\cos^2\theta_{CD} - 1)}$, where θ_{CD} is the angle between the C—D bond vector and the bilayer normal, and the average is over motions that take place in a time shorter than the inverse of the quadrupolar interaction.

In a complex case, such as for a perdeuterated sample, a complete determination of the distribution of quadrupolar splittings (or C—D bond order parameters) requires the evaluation of a large number of moments. How many moments can be accurately evaluated depends not so much on the signal:noise ratio of the spectrum but rather on the fidelity of the spectrum (eventually, of course, the signal:noise ratio limits one's confidence in the fidelity of the spectrum). If the spectra are obtained using the quadrupolar echo technique where no information is lost due to receiver recovery time and where no phase errors are introduced or phase corrections required, one can confidently evaluate a large number of moments.[1] For the fluid phase spectra presented here the first eight moments are calculated. The eighth moment is reproducible to within ±3%.

For a general superposition of powder patterns,

$$M_1 = \frac{4\pi}{3\sqrt{3}} <\delta\nu> = \frac{4\pi}{3\sqrt{3}} \nu_Q <S_{CD}>$$

$$M_2 = \frac{4\pi^2}{5} <(\delta\nu)^2> = \frac{4\pi^2}{5} \nu_Q^2 <S_{CD}^2>. \quad (3)$$

For comparison, from the studies of specifically deuterated DPPC (Seelig and Seelig, 1974, 1975), averaging over all measured values of S_{CD} and interpolated values for those positions not measured, we find that $<S_{CD}> \approx 0.164$ and $<S_{CD}^2>^{1/2} \approx 0.171$ at 50°C, whereas the spectrum of d_{62}-DPPC at the approximately corresponding temperature of 45°C (recall that the phase transition is ~4°C lower here) gives $<S_{CD}> = 0.151$ and $<S_{CD}^2>^{1/2} = 0.159$. At 41°C the selectively deuterated values gives $<S_{CD}> \approx 0.192$ and $<S_{CD}^2>^{1/2} \approx 0.197$, whereas d_{62}-DPPC at 37°C has $<S_{CD}> = 0.175$ and $<S_{CD}^2>^{1/2} = 0.183$. The larger values obtained for the specifically deuterated samples simply reflect the fact that in the plateau region the splittings obtained for these samples are considerably larger than those obtained for d_{62}-DPPC.

Just as the first moment of the spectrum gives the mean C—D bond order parameter, or the mean of the distribution of order parameters, the second moment of the spectrum together with the first moment gives the mean square deviation from the mean of the distribution of order parameters. Then the relative mean square deviation Δ_2 is defined by:

$$\Delta_2 = \frac{<S_{CD}^2> - <S_{CD}>^2}{<S_{CD}>^2} = \frac{M_2}{1.35\, M_1^2} - 1. \quad (4)$$

Δ_2 is a very useful parameter characterizing the width of the distribution of quadrupolar splittings. For the simple case of a single powder pattern $\Delta_2 = 0$, because we are neglecting broadening, which in the L_α phase is completely negligible. Higher moments of the distribution of order parameters can be readily obtained from higher moments of the spectrum. Indeed, even the shape of the order parameter profile (the variation of order parameter with chain position) can be obtained, at least in principle, from the moments (Bloom et al., 1978*b*).

The temperature dependence of the first two moments is shown in Fig. 4. In the liquid crystalline phase (37°C and above) these moments, M_2 in Fig. 4 *a* and M_1 in Fig. 4 *b*, decrease continuously as the temperature is increased, reflecting an overall decrease in the chain order due to increased motional averaging of the quadrupolar interaction. The parameter, Δ_2, shown in Fig. 5, increases with increasing temperature, indicating that the distribution of quadrupolar splittings is getting broader even though the average splitting, $<\delta\nu>$, is decreasing. This occurs because the smaller splittings (those for positions nearer the terminal methyl group) are decreasing relatively more rapidly than those in or near the plateau.

The moments provide a powerful means of obtaining quantitative information on the distribution of quadrupolar splittings. This technique is most useful when the usual spectroscopic technique of measuring the splittings of sharp spectral features cannot be used. The gel phase, mixed-phase regions, perdeuterated systems, and labeled biological membranes are examples of situations where the moment method is most effective (Davis et al., 1979*a*, *b*).

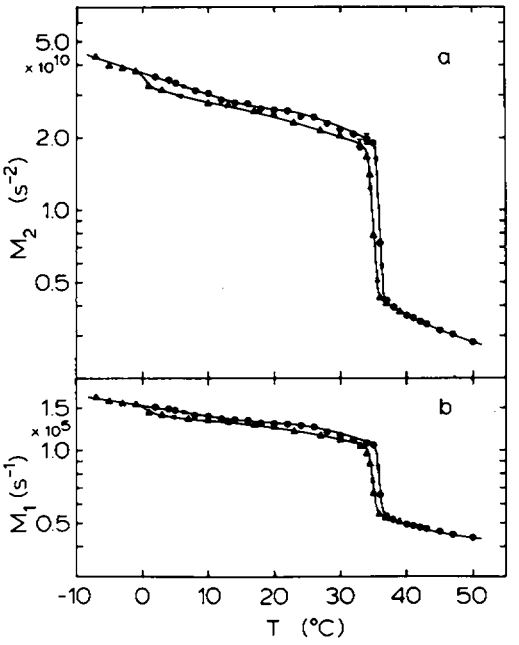

FIGURE 4 (*a*) The second moment and (*b*) the first moment of the deuterium spectrum vs. temperature. The small arrows on the solid line indicate the direction of the temperature increments. Random errors in M_1 and M_2 in the fluid phase are <1%; in the gel phase these errors are ~ ± 3% for M_2 and ~ ± 1% for M_1. The systematic errors due to finite pulse length become important at low temperatures.

The Transition Region

The gel-to-liquid crystalline phase transition in aqueous dispersions of phospholipids has been extensively studied by a variety of techniques (Lee, 1977; Hinz and Sturtevant, 1972; Chapman, 1975; Chapman et al., 1977; Rand et al., 1975; Janiak et al., 1976; Levine et al., 1972; Tardieu et al., 1973; Albon and Sturtevant, 1978). For DPPC the transition temperature (hydrocarbon chain melting temperature) is at 41–42°C; however, for d_{62}-DPPC the transition temperature occurs 4°C lower. In fully hydrated DPPC, and in a number of other lecithins (Tardieu et al., 1973) the main gel-to-liquid crystalline transition is preceded by a so-called "pretransition" occurring for DPPC at ~34°C. There is disagreement in the literature on the nature of the pretransition in DPPC and on the state of the bilayer in the pretransition region between 34 and 41°C. Janiak et al. (1976) using x-ray diffraction, have concluded that the structure of DPPC in this temperature interval is that of a rippled bilayer, the $P_{\beta'}$ structure (notation credited to Luzzati, 1968). On the other hand, Rand et al. (1975), also using x-ray diffraction, have indicated that in this region DPPC has a planar structure where the chains are parallel to the bilayer normal, the L_β structure. These researchers agree that at lower temperatures the gel phase has the $L_{\beta'}$ structure characterized by a planar bilayer with the hydrocarbon chains tilted with respect to the bilayer normal (see Discussion below). Further, it had earlier been suggested that changes in head-group conformation were associated with the pretransition (Levine et al., 1972). However, recent NMR (Seelig et al.,

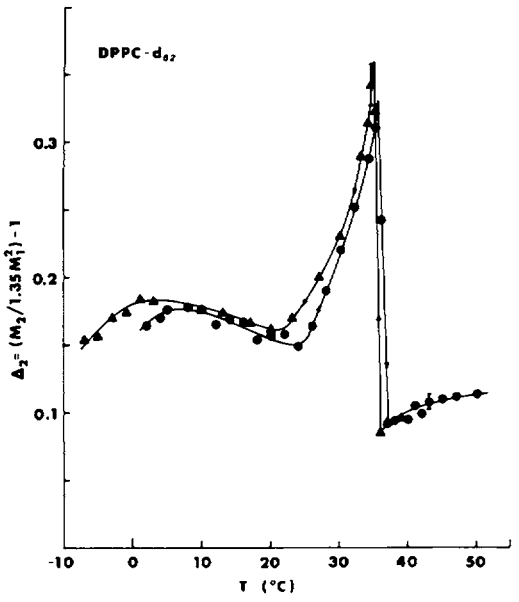

FIGURE 5

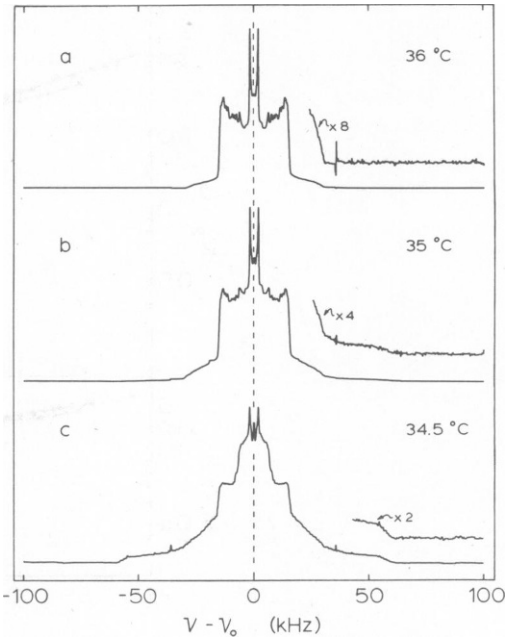

FIGURE 6

FIGURE 5 The parameter Δ_2 vs. temperature. Δ_2 gives the relative width of the distribution of quadrupolar splittings. The small arrows on the solid line indicate the direction of the temperature increments. The error bars represent the estimated random error in Δ_2; ~ ± 2% in fluid phase, ~ ± 4% in gel phase. The systemic error due to finite pulse length is not shown here but becomes significant at low temperatures.

FIGURE 6 Deuterium NMR spectra in the vicinity of the phase transition (decreasing temperature increments). (*a*) T = 36°C, 1,000 scans, t_w = 4.5 μs (*b*) T = 35°C, 1,000 scans, t_w = 4.5 μs; (*c*) T = 34.5°C, 2,000 scans, t_w = 2.25 μs. The vertical scale is increased by a factor of eight on the right hand side of the figure to illustrate the growth of the broad component.

1977) and neutron diffraction (Büldt et al., 1978) studies have shown that the head group retains essentially the same conformation it has in the liquid crystalline phase.

Because ~90% of the entropy change occurring at the main phase transition can be attributed to disordering (melting) of the acyl chains (Philips, 1972), and because deuterium NMR can provide a sensitive measure of acyl chain order, it seems appropriate to study the phase transition using DMR on chain-deuterated DPPC.

Fig. 6 shows three spectra taken in the region of the phase transition. After decreasing the temperature in the liquid crystalline phase to 36°C, the spectrum in Fig. 6 *a* was obtained. The flat base line and sharp cutoff of signal intensity indicate that at this temperature the sample is still in the liquid crystalline phase. At 35°C, the spectrum in Fig. 6 *b*, the expansion of the vertical scale very clearly shows signal intensity out to ~ ±63 kHz. This broad component is due to the formation of regions of gel phase. It is only the high quality of the spectrum and its base line that allows us to identify this spectrum as not being characteristic of the liquid crystalline phase. Lowering the temperature by another 0.5°C to 34.5°C we obtain the spectrum shown in Fig. 6*c*. This is also clearly in the mixed-phase region and the intensity of the broad component has increased substantially. To determine whether the

sample was in thermal equilibrium in this transition region, spectra were repeated several times at one temperature (35°C of the increasing temperature scan) and the spectra were nearly identical (the first 4 moments agreeing to within 1%). No such check was made for the decreasing temperature scan and it is possible that when cooling, 30 min is not sufficient to attain thermal equilibrium.

The plots of first and second moments of the spectrum vs. temperature in Fig. 4 show an apparent hysteresis in the phase transition. (The small arrows on the solid lines in this figure and in Fig. 5 show the direction of temperature variation). The transition occurs at a temperature ~1°C lower when cooling from the liquid crystalline phase than when warming from the gel phase. Also, in the region below the main transition, at least between 0°C and the transition temperature T_c, the values of the moments are systematically smaller when the temperature is decreasing. The behavior at 0°C is not expected to be due to the freezing of water because the small water peak in the low temperature spectra persists until −7°C. Above T_c, in the liquid crystalline phase, the spectra and their moments are identical on cycling the temperature. Unfortunately, due to concern over the stability of the sample against degradation into lyso-PC, the sample was cycled only once through the complete temperature range, starting at 2°C, raising the temperature in steps to 50°C, and then lowering in steps to −7°C.

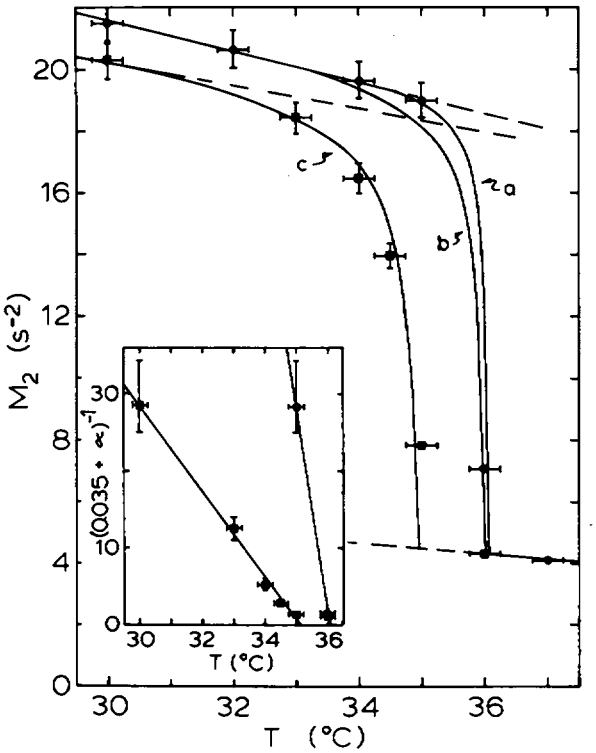

FIGURE 7 The phase transition. Circles are data taken after increasing temperature to specified value; squares are data taken after decreasing temperature to specified value. Dashed lines are extrapolations of M_2^{gel} and M_2^{fluid} through the transition temperature region. Curves *a*, *b*, and *c* are calculated assuming only impurity broadening of the phase transition using the impurity equilibrium constant $K = 0.034$. Curve *a* uses molar impurity concentration $X_2 = 0.0025$, curve *b* uses $X_2 = 0.005$, and curve *c* uses $X_2 = 0.0084$.

The main transition was cycled through a total of three times, and the spectra taken at 37°C were always identical. This indicated that the phase transition had not been broadened or shifted substantially due to degradation.

Fig. 7 is an expansion of the horizontal and vertical scales of Fig. 4*a* in the region of the phase transition. The error bars represent an uncertainty of ±3% in the second moments of gel-phase spectra. The transition observed for the increasing temperature run appears significantly sharper than that observed in the decreasing temperature run. Because the data for increasing temperature was taken before that for decreasing temperatures, the increase in the width of the phase transition is consistent with (but does not prove) some slight degradation of the sample at higher temperatures.

The broadening of the phase transition due to a single impurity can be easily calculated if one knows the mole fraction, $\alpha(T)$, of fluid phase present as a function of temperature through the transition region (Mastrangelo and Dornte, 1955). If it is assumed that the spectrum in the transition region is a simple superposition of a fluid-phase spectrum and a gel-phase spectrum, the fraction, α, of fluid component can be calculated from the total second moment of the composite spectrum. From the equation (Mastrangelo and Dornte, 1955; Albon and Sturtevant, 1978),

$$(T - T_c)_{\text{impurity}} = \frac{RT_c^2}{\Delta H_f} X_2 \frac{1}{\frac{k}{1-k} + \alpha}, \tag{5}$$

where R = 1.9872 cal/°K mole is the gas constant; ΔH_f = 8,470 cal mol^{-1} is the enthalpy of fusion (Mabrey and Sturtevant, 1976), assuming that the ΔH_f of d_{62}-DPPC is not very different from that of protiated DPPC; and T_c is the phase transition temperature, we can determine graphically both the impurity mole fraction, X_2, and the equilibrium constant k for the distribution of the impurity between the phases.

The values of $((k/C1 - k) + \alpha)^{-1}$ for temperatures between 33 and 35°C (inclusive) fall on a straight line whose slope gives X_2 = 0.0084 and whose x-axis intercept is T_c = 35.1°C. Using Eq. 5 and the above value of impurity concentration, the variation of M_2 is shown by the solid curve labeled *c* in the figure. Because we have assumed that impurity broadening is the only contribution to the width of the phase transition, this value of X_2 should be considered an upper limit inasmuch as there are other factors that may broaden the transition.

For the increasing temperature run the smaller value of X_2, curve *a*, is consistent with the data, whereas the larger value of X_2, curve b, clearly overestimates the width of the transition.

We estimate that at the beginning of this series of experiments the impurity concentration of our sample was no more than approximately four times that of Albon and Sturtevant (1978) who took extraordinary precautions to ensure the purity of their sample. Although the increased width of the transition observed on cooling may be due to increased sample impurity, the fact that <24 h elapsed between the heating and cooling scans through the transition, and the fact that even after 9 d there was no observable change in the spectrum at 37°C suggest that the increased width may be due primarily to difficulty in maintaining thermal equilibrium while cooling through the transition. Nevertheless, because evidence of sample degradation was found by TLC after only 9 d, an important conclusion we must draw

is that, if only the usual (heretofore the accepted) precautions are taken, one cannot be confident of the stability of the sample against degradation into lyso-PC plus fatty acid beyond a few days. It is therefore imperative to check the sample for degradation after experiments taking times of this order or longer.

The origin of the apparent hysteresis in the phase transition is unclear. In view of the maximum impurity concentrations estimated above for the transition in both directions and the weak dependence of the phase transition on small concentrations of lyso-PC (Klopfenstein et al., 1974), it seems unlikely that any slight degradation of the sample occurring between the two sets of measurements is responsible for this hysteresis.

The parameter, Δ_2, which is the relative width of the distribution of quadrupolar splittings, is sensitive to sample inhomogeneities such as the coexistence of phases. As shown in Fig. 5 there is a sudden dramatic increase in Δ_2 upon entering the transition region from the high-temperature side. A change in temperature from 36 to 35°C (corresponding to the spectra in Fig. 6 *a,b*) results in a change of Δ_2 by almost a factor of four. On further decreasing the temperature, Δ_2 reaches a maximum at ~34°C and falls off much more slowly to reach a local minimum at ~23°C. Again, like M_1 and M_2, Δ_2 shows a dependence on the thermal history of the sample at the phase transition and below.

Although these measurements cannot determine the nature of the pretransition region, it is clear from the behavior of Δ_2 that in this temperature range the sample is more heterogeneous than in either the higher temperature liquid crystalline phase or in the lower temperature gel phase.

The Gel Phase

A model for hydrated lecithins (e.g., DPPC) in the gel phase is that the molecules are in an ordered state, hexagonally packed, with the hydrocarbon chains essentially in the all-*trans* conformation. The orientational disorder observed in x-ray diffraction (Tardieu et al., 1973) and the C—D bond order parameters ($S_{CD} \simeq 0.5$) of the gel-phase deuterium spectra are most easily interpreted as being due to rapid reorientation about the long molecular axis. Bilayer thickness, deviation from hexagonal packing, and broadening of the x-ray diffraction intensity profile near 4.2 Å lead to the conclusion that there is some tilt in the hydrocarbon chains (Tardieu et al., 1973). The magnitude of this tilt angle and its degree of cooperativity have not been accurately determined. Early work with proton NMR in hydrated lecithins (Veksli et al., 1969) and deuterium NMR of chain perdeuterated dimyristoyl PC (Oldfield et al., 1971) indicated that although the degree of motion on the gel phase is much reduced from that in the fluid phase, there apparently still is considerable molecular motion occurring in the gel phase. Finally, it has been demonstrated that lateral diffusion is much slower in the gel phase than in the liquid crystalline phase (Cullis, 1976).

Fig. 8 shows spectra at three different temperatures within the gel phase. Fig. 8*a* is the spectrum for 20°C, just below the pretransition region. This spectrum was taken at the local minimum in the plot of Δ_2 vs. temperature. It is immediately apparent that this is not the spectrum expected for molecules in the all-*trans* conformation rotating about their long axis. For that situation the spectrum would consist of two well-defined powder patterns, one for the methyl groups with a quadrupolar splitting of ~21 kHz (due to effects of rotation about the terminal C—C bond) and another for the methylene groups, all equivalent, with a splitting of

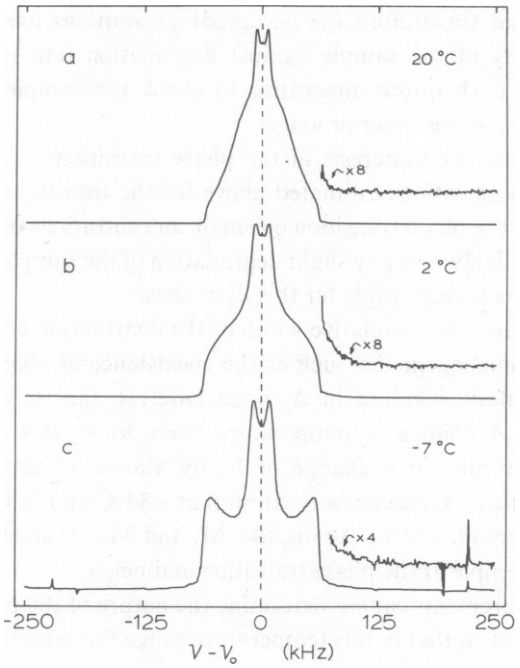

FIGURE 8 Deuterium NMR spectra in the gel phase of d_{62}-dipalmitoyl phosphatidylcholine, $t_w = 2.25$ μs (*a*) $T = 20$°C, 4,000 scans; (*b*) $T = 2$°C, 4,000 scans; (*c*) $T = -7$°C, 4,000 scans. The vertical scale is increased on the right hand side of the figure by the factors shown.

~63 kHz. This ideal spectrum would have $M_1 = 1.43 \times 10^5$ (s^{-1}), $M_2 = 2.86 \times 10^{10}$ (s^{-2}), and $\Delta_2 = 0.043$; compared to the values obtained for the spectrum in Fig. 7 *a*, $M_1 = 1.288 \times 10^5$ (s^{-1}), $M_2 = 2.590 \times 10^{10}$ (s^{-2}), and $\Delta_2 = 0.157$. Instead of a sharply defined powder pattern we observe a broad, smeared-out spectrum with a large distribution of quadrupolar splittings. If the shape of this spectrum was a result of a large dipolar broadening where $M_2^{tot} = M_2^Q + M_2^{dip}$, with M_2^Q the quadrupolar powder pattern second moment and M_2^{dip} the additional contribution due to broadening, the total moment M_2^{tot} would be greater than that of the above ideal spectrum. Also, the values of T_{2e} at low temperatures (see Table II) are not consistent with any large dipolar contribution to the broadening.

There are still at least two possible explanations for the observed spectrum: (*a*) there is still a considerable degree of chain disorder in the gel phase and therefore a variation of quadrupolar splitting along the chain, or (*b*) that rigid rod motions (motions affecting all positions on the chain equally) are responsible for a variation in the molecular order, possible only if different regions of the sample experience different degrees of motion. Raman spectroscopic studies (Gaber and Peticolas, 1977; Gaber et al., 1978; Yellin and Levin, 1977) have clearly shown the presence of gauche conformations below the phase transition implying that we might expect to see some variation in order and quadrupolar splitting with chain position. Studies of specifically deuterated samples in the gel phase should determine if this is the case.

Fig. 8*b* is the spectrum obtained at 2°C where there has been a sizeable build-up of intensity at ±63 kHz that, by −7°C, dominates the spectrum, as shown in Fig. 8 *c*. The

molecules, which at 20°C are essentially all rotating about their long axes, appear to slow down as the temperature is lowered until by −7°C nearly all of them have stopped rotating (at least on the frequency scale of deuterium NMR, i.e., several kilohertz) (see also, Cullis et al., 1976). The sharp edges of the spectrum in Fig. 8 *c* are at ±63 kHz, the maximum quadrupolar splitting for a methylene group (Davis and Jeffrey, 1977), characteristic of the absence of motion. The intensity of the central part of this spectrum (resembling that of the methyl groups) is very large, containing more than simply the methyl group contribution, suggesting that in a substantial part of the sample the molecules are still rotating about their long axes, thereby contributing to the central part of the spectrum. The T_{2e} measurements at 2°C (Table II) indicate that the two types of lipid, rotating and nonrotating, are not in rapid exchange with one another because at very long times only the broader component due to the nonrotating molecules remains.

The plot of Δ_2 in Fig. 5 shows that between 20 and −7°C, Δ_2 goes through a weak, broad maximum. This is to be expected when the spectrum consists of components with very different quadrupolar splittings whose relative intensities vary monotonically with temperature. The large value ($\Delta_2 = 0.154$) at −7°C indicates again that there is still a mixture of rotating and nonrotating lipid at that temperature. There is, however, some distortion of the spectra at very low temperatures.

When the spectra are as broad as those encountered in the gel phase it becomes difficult to satisfy the condition $\omega_Q t_w \ll 1$ for a 90° pulse (ω_Q is the quadrupolar splitting in angular frequency units and t_w is the pulse width). If this inequality is not satisfied the spectrum may be seriously distorted. For this reason all spectra below the main phase transition were

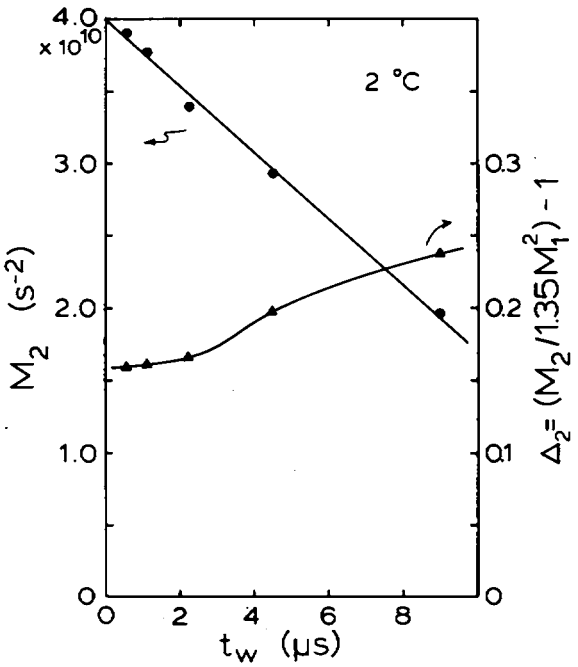

FIGURE 9 The second moment and the parameter Δ_2 vs. pulse length at T = 2°C. The second moment values (•) refer to the vertical scale on the left, the Δ_2 values (▲) refer to the vertical scale on the right.

obtained using two 45° pulses of length $t_w = 2.25$ μs to form the echo. For the longer length pulses there is a strong attenuation of the spectrum's intensity far from resonance. Fig. 9 shows the values of M_2 and Δ_2 obtained at 2°C for different pulse lengths. From this figure it is clear that even with $t_w = 2.25$ μs we are making a systematic error in the values of the moments (~15% error in M_2 for this spectrum). At higher temperatures the error is considerably smaller, in fact, it is only when there is a significant fraction of nonrotating lipid (that with the maximum quadrupolar splitting) that this effect becomes serious. It is possible to make a theoretical correction to the spectra to counteract this systematic distortion.[2]

In summary, the deuterium spectra are in agreement with a model where the gel phase near 20°C consists of an array of molecules most of which are rapidly rotating about their long axes. The spectrum in this region has a large value of Δ_2, i.e., a broad distribution of quadrupolar splittings, suggesting that the molecules may not be in the all-*trans* conformation. At lower temperatures the molecules stop rotating; this can be expected to alter the hexagonal packing because the molecules can no longer be thought of as cylindrical.

Relaxation: T_1 and T_2

The motions occurring in phospholipid model membrane systems are certain to be complex, probably requiring, in a detailed treatment, a consideration of collective modes (particularly near the phase transition) and possibly a broad spectrum of correlation times. How many of these motions are important in a given situation is far from clear. Membranes are very dynamic systems and relaxation studies are an invaluable aid to the understanding of the molecular dynamics of these systems.

There are expected to be two contributions to the relaxation in deuterated systems, quadrupolar and dipolar interactions. Because of the large quadrupolar moment of the deuteron (and therefore large residual quadrupolar interaction) and small nuclear gyromagnetic ratio, the quadrupolar interaction is expected to dominate the relaxation processes. From the value of the proton dipolar second moment in DPPC, M_2^{dip} (proton) = 3.7×10^8 (s^{-2}) (Bloom et al., 1978*a*), and the value of proton and deuteron spin and gyromagnetic ratios, the dipolar relaxation time expected for perdeuterated chains, is $T_2^{dip} \approx 1.3$ ms, considerably longer than the relaxation times observed here. Also, for both soaps and phospholipids the quadrupolar echo envelope is exponential, contrary to what is expected for dipolar dephasing. These points strongly suggest that $1/T_{2e}$ is primarily due to the quadrupolar interaction.

Probably the simplest model one can consider in discussing these systems, though obviously an unrealistic one, is the isotropic approximation of quadrupolar relaxation (Davis et al., 1978). In this case,

$$(1/T_1) = \frac{3}{80}\left(\frac{eQ}{\hbar}\frac{\partial^2 V}{\partial z'^2}\right)^2 [J(\omega_0) + 4J(2\omega_0)], \tag{6}$$

where $(eQ\partial^2 V/\partial z'^2)$ is the product of the nuclear electric quadrupole moment and the z-component of the electric field gradient (we assume the asymmetry parameter $\eta = 0$), and $J(\omega)$ is the spectral density at frequency ω. In this same approximation,

[2]Bloom, M., J. H. Davis, and M. Valic. Unpublished results.

$$(1/T_{2e}) = \frac{1}{160}\left(\frac{eQ}{\hbar}\frac{\partial^2 V}{\partial z'^2}\right)^2 [9J(0) + 15J(\omega_0) + 6J(2\omega_0)] \tag{7}$$

Because we are actually dealing with a case in which $<\mathcal{H}_Q> \neq 0$, where $\mathcal{H}_Q$ is the quadrupolar Hamiltonian, the integral of $J(\omega)$ over all frequencies is reduced from $<|H_Q|^2>$, expected for isotropic fluids, to the value $<|H_Q|^2> - <H_Q>^2$ (Davis et al., 1978). Then, for short τ_c

$$J(0) \simeq J(\omega_0) \simeq J(2\omega_0) \simeq (1 - S_n^2)\tilde{J}(\omega_0), \tag{8}$$

where S_n is the order parameter at the nth position and $\tilde{J}(\omega)$ is the reduced spectral density. In this limit, $T_{2e} = T_1$; however, it is found experimentally that $T_{2e} \ll T_1$. If we consider a model consisting of two motions: one fast motion with correlation time $\tau_1 \ll 1/\omega_0$, so that for this motion we are in the short correlation time limit, and another slower motion with a correlation time $\tau_2 \gg 1/\omega_0$, we find that

$$J(\omega_0) \simeq J(2\omega_0) \simeq f(1 - S_n^2)\tilde{J}(\omega_0) \simeq f(1 - S_n^2)2\tau_1$$

and

$$J(0) \simeq (1 - f)(1 - S_n^2)2\tau_2, \tag{9}$$

where $f(1 - S_n^2)$ is the fraction of the total spectral density corresponding to the faster motion and S_n is the order parameter of the nth position. For this simple two-motion model we find that:

$$(1/T_1)_n = \frac{3}{8}\left(\frac{e^2qQ}{\hbar}\right) f(1 - S_n^2)\tau_1 \tag{10}$$

$$(1/T_2e)_n \approx \frac{3}{80}\left(\frac{e^2qQ}{\hbar}\right)(1 - S_n^2)\tau_1\left[3(1 - f)\frac{\tau_2}{\tau_1}\right], \tag{11}$$

for $\tau_1 \ll 1/\omega_0 \ll \tau_2$.

Spin lattice relaxation times were measured at three temperatures in d_{62}-DPPC; $T = 21°C$ (in the gel phase), and 37 and 45°C (in the liquid crystalline phase). T_{2e} was measured at 37°C for comparison with T_1 at the same temperature and at 2°C. The values obtained are given in Table II, listing the relaxation times for each of the sharp peaks in the liquid crystalline spectra and for the methyl and methylene positions in the gel phase spectrum. The values of T_1 in the liquid crystalline phase agree well both in magnitude and in dependence on chain position with the values obtained by Brown, Seelig, and Haeberlen[3] on specifically deuterated samples. $1/T_1$ shows an almost linear dependence on quadrupolar splittings as does $1/T_{2e}$ for positions near the methyl end of the chains. However, midway between the plateau and the methyl end of the chains $1/T_{2e}$ begins to decrease rapidly, attaining its minimum value at the plateau. The simple model discussed above cannot account for this dependence on chain position. Introducing dipole-dipole interactions would have the opposite effect on T_{2e} since deuterons on positions in the plateau region would be on better speaking terms with one another than those near the methyl group.

[3]Brown, M., J. Seelig, and U. Haeberlen. Unpublished results.

TABLE II
RELAXATION TIMES IN d_{62}-DPPC

Peak*	37°C			45°C		20°C		2°C	
	$\delta\nu$	T_1‡	T_{2e}§	$\delta\nu$	T_1	$\delta\nu$	T_1	$\delta\nu$	T_{2e}
	kHz	*ms*	*ms*	kHz	*ms*	kHz	*ms*	kHz	*ms*
a	3.51	265	1.86	2.68	275	12.21	100 ± 20	14.9	0.56 ± 0.05
b	12.35	68	0.66	9.96	58				
c	14.65	57	0.65	11.57	52				
d	16.65	51	0.62	13.43	48				
e	18.41	45	0.62	14.80	44				
f	19.68	39	0.59	15.97	41	62.5	25 ± 10	62.5	0.32 ± 0.03
g	22.12	36	0.61	17.97	38				
h	23.93	29	0.67	19.73	35				
i	26.07	28	0.71	21.92	31				
j	27.15	25	0.74	22.95	29				
k	28.32	24	0.79	24.95	27			120.0	0.50 ± 0.05

*See Fig. 3.
‡T_1 values are ± 5% except for a which is ± 10%.
§Echo decay times are ~ +2%, −5% for b–e, ~ ±2% for f–k, and ±5% for a.

The variation of T_1 and T_{2e} with position on the chain is likely to be due to a competition between two or more motions. A complete study using all available relaxation techniques will be required to unravel this complex behavior. The soaps, being significantly simpler, are probably a more reasonable subject for such a study. However, the different behavior in the two systems suggests that their motions are significantly different.

CONCLUSION

In the liquid crystalline phase of a mixture of d_{62}-DPPC in excess water, the deuterium NMR spectrum contains a number of sharp resolvable features whose frequencies (quadrupolar splittings) and decay or relaxation times can be measured as a function of temperature. Even though not all positions on the chain are resolvable, the spectrum clearly indicates the existence of a "plateau" or a hydrocarbon chain flexibility gradient. Computer simulation of the spectrum permits the determination of the distribution of intensity, and a comparison of the parameters of the simulation with available data on specifically labeled systems allows an assignment of the peaks in the spectrum. The temperature dependence of the splittings obtained from these spectra can be simply interpreted as the result of a decrease in chain order as the temperature is increased. The analysis of deuterium spectra in terms of moments is useful when there are complex overlapping spectra such as are obtained for perdeuterated samples, with mixtures of phases, or in biological membranes.

Because the gel-to-liquid crystalline phase transition of phospholipid dispersions involves the melting of the hydrocarbon chains, deuterium NMR on chain deuterated lipids is a very appropriate technique for studying this phase transition. The pretransition region in DPPC appears from the behavior of Δ_2 to be an inhomogeneous region which, as the temperature is lowered, seems to transform smoothly into the more well-defined gel phase.

The distribution in quadrupolar splittings in the gel phase may be due to a variation in

quadrupolar splitting with chain position, similar to that found in the liquid crystalline phase. At low temperatures (~ -7°C) the spectra exhibit the maximum deuterium quadrupolar splitting, characteristic of a rigid system. The variation in the shape of the spectrum and the variation of the parameter Δ_2 indicate that between -7 and $+20$°C the sample goes from an essentially rigid or solid state to one where the phospholipid molecules are undergoing a rapid reorientation about their long axes. This reorientation results in a reduction of the observed maximum quadrupolar splitting by a factor of two.

Relaxation measurements can in principle determine the intensities and the time scales of the motions causing the fluctuations in the local fields at the nucleus. In the liquid crystalline phase the relaxation times, T_1 and T_{2e}, have been measured for the resolvable peaks in the spectrum. A comparison of T_1 and T_{2e} at 37°C (just above the gel-to-liquid crystalline phase transition) is suggestive of a simple two-motion model. However, the variation in these relaxation times with chain position indicates that such a simple model is inadequate.

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