# Deuterium nuclear magnetic resonance study of the effects of palmitic acid on dipalmitoylphosphatidylcholine bilayers

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D-aldehyde. This suggests that the D enantiomer cannot form the hemiacetal complex. Indeed, if the aromatic side chain of the D enantiomer is placed in the hydrophobic-specificity pocket and the $\alpha$-H is the only group sterically able to fit the $\alpha$-H-specificity locus, then the $\alpha$-C(O)H group must be placed into the *N*-acylamino binding pocket and the *N*-acylamino group placed into the catalytic locus proximate to $O_\gamma$ of Ser-195 (Gammon et al., 1972). With this binding arrangement, the D-aldehyde is incapable of forming the hemiacetal since the aldehyde carbon is too far from the $O_\gamma$ of Ser-195.

**Registry No.** Cht, 9004-07-3; DL-AcFPheal, 82657-49-6; L-AcFPheal, 82691-34-7; D-AcFPheal, 87638-54-8; L-AcFPhe, 330-81-4; D-AcFPhe methyl ester, 87586-95-6; L-AcFPhe methyl ester, 87586-96-7; DL-AcFPhe methyl ester, 87586-97-8; L-AcPheal, 35593-55-6.

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# Deuterium Nuclear Magnetic Resonance Study of the Effects of Palmitic Acid on Dipalmitoylphosphatidylcholine Bilayers†

K. Peter Pauls,* Alex L. MacKay, and Myer Bloom

ABSTRACT: The physical effects of 20 mol % palmitic acid on bilayers of dipalmitoylphosphatidylcholine were examined by deuterium nuclear magnetic resonance, and the fidelity of deuterated fatty acids as membrane probes was examined by comparing samples in which either the free fatty acid or the fatty acyl chains of the phospholipid were perdeuterated. Addition of palmitic acid increased the temperature of the phase transition onset by 2 °C and broadened the coexistence region of gel and liquid-crystalline lipid to span 7–10 °C, depending on which sample was examined. Average order parameters for samples containing free fatty acid were approximately 10% higher than those observed for pure DPPC. Order parameter profiles estimated by an empirical method indicated that the ordering effect of palmitic acid was felt down the whole length of the phospholipid acyl chains. Measurements of the quadrupolar echo relaxation rate suggested that addition of fatty acid inhibits slow motions of the phospholipid side chains. In general, the results indicate that free fatty acid probes perturb the structure of membranes into which they are incorporated but that they are accurate reporters of the order in the perturbed system.

Free fatty acids occur as components of biological membranes, albeit in small quantities (Ray et al., 1969; Mead & Mertin, 1978). Exogenous additions of free fatty acids have been shown to alter a variety of membrane-mediated cellular functions including membrane-bound enzyme activity (Orly & Schramm, 1975; Glass et al., 1977; Rhoads et al., 1982; Schmalzing & Kutschera, 1982), platelet aggregation (Hoak et al., 1970), cell permeability (Shramm et al., 1967), cell fusion (Ahkong et al., 1973; Kantor & Prestegard, 1975), and lymphocyte mitogenesis (Mead & Mertin, 1978). Some of these effects are thought to be the result of fatty acid induced changes in membrane structure.

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A variety of techniques have been used to investigate the effects of free fatty acids on model membrane structure including calorimetry (Eliasz et al., 1976; Mabrey & Sturtevant, 1977; Kantor & Prestegard, 1978; Jain & Wu, 1977; Schullery et al., 1981), fluorescence techniques (Usher et al., 1978; Klausner et al., 1980), electron spin resonance (Muranushi et al., 1981), nuclear magnetic resonance (Podo & Blasie, 1976; Beckman et al., 1980; Marsh & Seddon, 1982), light scattering (Kremer & Wiersema, 1977), electrophoresis (Hauser et al., 1979), and scanning densitometry (Fodor & Epand, 1981). These studies have shown that long-chain saturated fatty acids increase the gel to liquid-crystalline phase transition temperature of phospholipid bilayers, whereas short-chain or cis-unsaturated fatty acids decrease it, and that exogenous additions of any type of fatty acid increase the range over which the transition occurs.

In addition to the interest in fatty acid–membrane interactions which arises from the physiological activity of these compounds, the effects of free fatty acids on membrane structure are of some practical interest because fatty acids labeled in various ways have been utilized as probes in spectroscopic studies of membranes. Several investigations have measured the order of membranes by examining the NMR spectrum of intercalated perdeuterated or selectively deuterated fatty acids (Saito et al., 1973; Seelig & Niederberger, 1974; Stockton et al., 1974, 1976; Stockton & Smith, 1976; Davis et al., 1979).

In the present study, deuterium nuclear magnetic resonance ($^2$H NMR)[1] was used to compare multilamellar samples of chain-perdeuterated dipalmitoylphosphatidylcholine (DPPC-$d_{62}$), a mixture of DPPC-$d_{62}$ plus palmitic acid (20 mol %), and a mixture of protiated DPPC plus 20 mol % perdeuterated palmitic acid (PA-$d_{31}$). The purpose was to examine the effects of long-chain free fatty acids on membrane structure and to determine whether perdeuterated free fatty acids serve as reliable probes of membrane structure.

## Materials and Methods

Chain-perdeuterated dipalmitoylphosphatidylcholine (99.8% deuterated) was obtained from Lipid Specialties, Boston, MA. Perdeuterated palmitic acid (99.1% deuterated) was prepared from palmitic acid (Calbiochem, A grade, 99.5% analysis) by a method reported by Dinh-Nguyen & Stenhogen (1967). L-$\alpha$-Dipalmitoylphosphatidylcholine (A grade) was obtained from Calbiochem. All lipids gave only one spot when checked for purity by TLC (65:25:4 $CHCl_3$:MeOH:$H_2O$) before using.

Lipid mixtures were prepared by codissolving the components in chloroform, followed by removal of the solvent with a stream of nitrogen and leaving the sample under vacuum for an extended period of time (8 h). The lipid samples were hydrated with excess (at least 50% by weight) 0.05 M Tris–acetate buffer, pH 7.0, containing 100 mM NaCl. The mixtures were heated to a temperature just above their respective transition temperature and mixed until homogeneous. In addition, they were subjected to several freeze–thaw cycles. The prepared samples were sealed under nitrogen.

$^2$H NMR spectra were obtained with a Bruker SXP4-100 spectrometer operating at 35.32 MHz as described in detail elsewhere (Davis et al., 1976; Davis, 1979). All spectra were taken on resonance by using the quadrupolar echo technique (Davis et al., 1976) using a cycle consisting of four pairs of pulses [$90_0$–$\tau$–$90_{90}$ (add); $90_{180}$–$\tau$–$90_{90}$ (subtract); $90_0$–$\tau$–$90_{270}$ (add); $90_{180}$–$\tau$–$90_{270}$ (subtract)]. Echos arising from alternate pairs of pulses were added to and subtracted from the computer memory as indicated. Quadrature detection was used, and the spectrometer was adjusted so that the signal occurred in only one channel. The out of phase channel, which contained only noise, was zeroed. This procedure results in perfectly symmetrical spectra on Fourier transformation and increases the signal to noise ratio by $2^{1/2}$ (Davis et al., 1976; Davis, 1983). No filters were used, and no phase corrections were required. For the samples containing DPPC-$d_{62}$, 45° instead of 90° pulses were used in the gel phase to minimize the spectral distortion of these broad spectra (Bloom et al., 1980). The separation between the echo-forming pulses was 40 $\mu$s, the length of the 90° pulse was $\sim$4 $\mu$s, and the echo sequence was repeated every 0.5 s in the gel phase and every 1 s in the liquid-crystalline phase. A total of 2000–8000 transients were collected for each spectrum.

The temperature of the sample was regulated by an oven enclosing the sample and the radio-frequency coil. Sample temperatures were measured with a thermocouple inserted into the oven casing. With this arrangement, the temperature gradient across the sample is much less than 0.5 °C. At least 30 min was allowed between each temperature change. In any case, enough time was allowed for the dielectric properties of the sample (as detected by the tuning bridge for the coil) to come to equilibrium. Because the measurements for each sample took several days to complete, they were interrupted by several overnight periods in which the samples were stored at −20 °C. These interruptions of the heating regime had no noticeable effect on the temperature plots of the moments, indicating that the NMR experiments were indeed equilibrium measurements.

Spectra were analyzed by using the method of moments which has been described in detail previously (Bloom et al., 1978; Nichol et al., 1980; Davis, 1979). For $^2$H NMR spectra, which are symmetric about the Larmor frequency, the moments of the half-spectra are related to the moments of the distribution of order parameters ($S_{CD}$) so that the first two moments ($M_1$ and $M_2$) give the mean orientational order parameter ($\langle S_{CD}\rangle$) and its mean squared value ($\langle S^2\rangle$). The fractional mean squared width of the distribution of order parameters is given by

$$\Delta_2 = \frac{\langle S_{CD}{}^2\rangle - \langle S_{CD}\rangle^2}{\langle S_{CD}\rangle^2} = \frac{M_2}{1.35{M_1}^2} - 1$$

It should be noted that $\langle S_{CD}\rangle$ and $\langle S_{CD}{}^2\rangle$ represent averages over all deuterons in the sample (Davis, 1979).

Liquid-crystal spectra were "de-Paked" to give the oriented $^2$H NMR spectra (Bloom et al., 1981). Areas under the peaks were used to determine the number of deuterons which contribute to each peak and also to construct order parameter profiles for the samples.

The time constant for the decay of the quadrupolar echo amplitude as a function of twice the separation of the two pulses was measured at two temperatures for each sample to determine average values of $1/T_{2e}$ values for the deuterons along the acyl chains. Relaxation rates ($1/T_{2e}$) for each of the sharp peaks in the liquid-crystalline spectra were determined as well (Davis, 1979).

## Results

Figure 1 shows spectra obtained at various temperatures from multilamellar liposomes prepared from pure chain-perdeuterated dipalmitoylphosphatidylcholine (DPPC-$d_{62}$), from

[1] Abbreviations: $^2$H NMR, deuterium nuclear magnetic resonance; DPPC-$d_{62}$, chain-perdeuterated dipalmitoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; PA-$d_{31}$, perdeuterated palmitic acid; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)aminomethane.

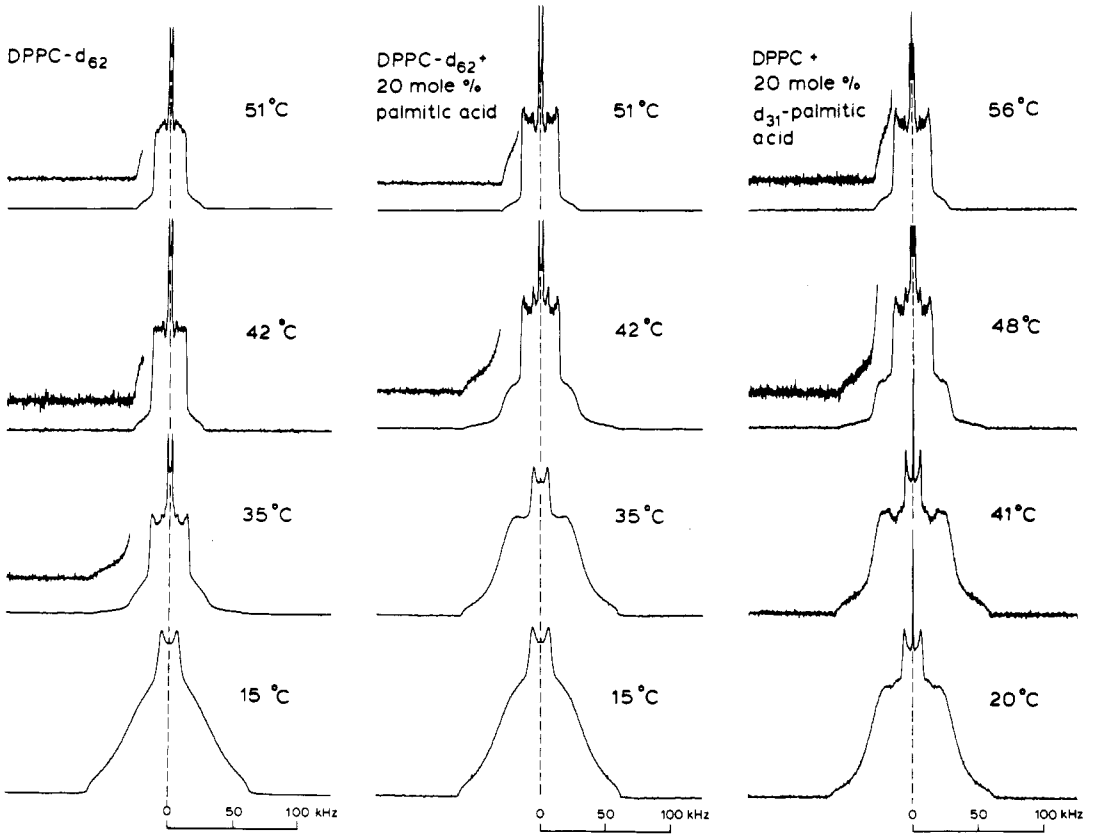


FIGURE 1: $^2$H NMR spectra of DPPC-$d_{62}$, DPPC-$d_{62}$ + 20 mol % palmitic acid, and DPPC + 20 mol % PA-$d_{31}$ multilamellar preparations at various temperatures. The vertical scale is amplified by a factor of 4 on the left-hand side of some spectra.

a mixture of DPPC-$d_{62}$ plus 20 mol % palmitic acid, and from a mixture of protiated DPPC plus 20 mol % perdeuterated palmitic acid, all in excess water. The spectra obtained at the lowest temperatures from all of the samples are typical of perdeuterated chains in the gel state in that they show a well-resolved splitting for the terminal methyl deuterons sitting on a broad featureless methylene spectrum probably arising from nonaxially symmetric motion of the acyl side chains in the gel state (Westerman et al., 1982; Wittebort et al., 1982).

At 35 °C, pure DPPC-$d_{62}$ bilayers are a mixture of gel-phase and liquid-crystalline lipids. The vertically amplified inset shows the broad gel-phase spectrum underlying the liquid-crystalline spectrum (Figure 1). Above this temperature, the sample was completely liquid crystalline, and the recorded spectra have sharp edges. The sharp edges are associated with a plateau in the variation of the deuterium quadrupolar splitting values with position along the acyl chain (Seelig & Seelig, 1974; Davis, 1979).

The addition of 20 mol % palmitic acid to DPPC-$d_{62}$ caused the gel phase to persist to higher temperatures in the resulting mixed bilayer as compared with the pure system. Differences between the liquid-crystalline phase of pure DPPC-$d_{62}$ and the phospholipid–fatty acid mixture are evident from a comparison of the spectra obtained from these samples at 51 °C. These spectra are shown in more detail in Figure 2. In spite of differences in the overall shape, both spectra have the same number of clearly resolvable peaks. These have been labeled with letters starting from the terminal methyl resonance line.

Spectra obtained from perdeuterated palmitic acid incorporated into protiated DPPC at a concentration of 20 mol % are similar to those observed for the DPPC-$d_{62}$ sample containing 20 mol % protiated palmitic acid. In Figure 1, the representative spectra shown for the former sample have been chosen to be several degrees higher than corresponding spectra from the other two because protiated DPPC has a transition temperature approximately 5 °C higher than chain-perdeuterated DPPC (Petersen et al., 1975). A liquid-crystalline spectrum from this sample is also shown in Figure 2. It is evident that, although the number and position of the sharp peaks in this spectrum differ from those observed for the DPPC-$d_{62}$ plus 20 mol % palmitic acid mixture, the spectral shape and the range of quadrupolar splitting values are very similar for the two samples containing palmitic acid.

The temperature dependence of the quadrupolar splitting values measured for each of the sharp peaks in the liquid-crystalline phase spectra obtained from the three preparations is shown in Figure 3. The splittings for pure DPPC-$d_{62}$ decrease smoothly from 36 to 70 °C. For samples containing palmitic acid, however, the splittings first rise and then fall with increasing temperature. This feature is associated with the extended mixed-phase regions in the temperature profiles of the fatty acid–phospholipid mixtures, as will be explained under Discussion. The highest quadrupolar splitting values for both samples were found at temperatures just above the mixed-phase region. Subsequent increments of temperature resulted in a monotonic decrease in the quadrupolar splitting values. Figure 3 also shows that the samples containing palmitic acid have a greater range of values for the quadrupolar splittings than the pure DPPC-$d_{62}$ sample.

The average orientational order in the samples was examined quantitatively by using the spectral moments. Because the gel to liquid-crystalline phase transition is associated with a large decrease in chain packing order, phase changes in the samples can easily be identified in plots of the first or second moment vs. temperature. This transition occurred over a narrow temperature range for DPPC-$d_{62}$ bilayers, and it was centered around 35 °C (Figure 4). The first and second moments decrease respectively from $1.05 \times 10^5$ to $5.30 \times 10^4$ $s^{-1}$ and from $1.71 \times 10^{10}$ to $4.00 \times 10^9$ $s^{-2}$ during the phase transition. A smaller change (10%) centered about 25 °C is

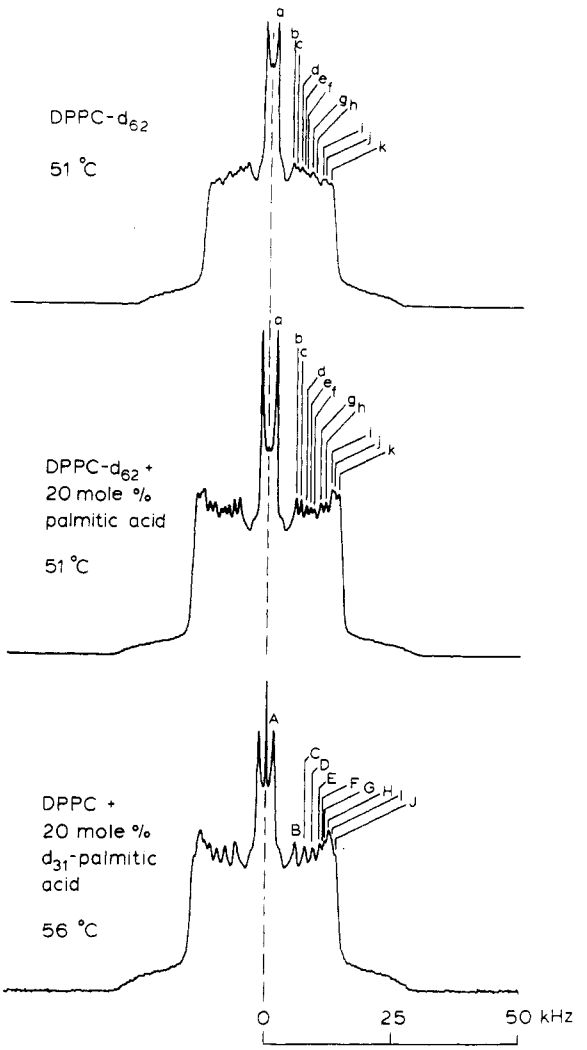


FIGURE 2: Comparison of liquid-crystalline phase $^2H$ NMR spectra from DPPC-$d_{62}$, DPPC-$d_{62}$ + 20 mol % PA, and DPPC + 20 mol % PA-$d_{31}$. Resolved peaks in the spectra are labeled with letters. The temperatures at which the spectra were taken are indicated.

also seen in the temperature plots of these two parameters (Figure 4). The latter feature is probably associated with the pretransition observed for multilamellar dispersions of DPPC in excess water (Janiak et al., 1976).

The first and second moments changed much more gradually with temperature for the samples containing 20 mol % palmitic acid (Figure 4). The coexistence of spectral components from the gel and liquid-crystal phases spanned 10 °C (from 36 to 46 °C) for the DPPC-$d_{62}$ plus 20 mol % palmitic acid mixture (Figure 4) and 7 °C (from 43 to 50 °C) for the DPPC plus 20 mol % perdeuterated palmitic acid mixture (Figure 4). No evidence of a pretransition can be seen in these samples (Figure 4). Both preparations have values for the first and second moments which are approximately 25% higher than those obtained for the pure DPPC-$d_{62}$ sample in the liquid-crystalline phase. However, in the gel phase, the $M_1$ values recorded for the samples containing palmitic acid are similar to those recorded for the pure DPPC-$d_{62}$ dispersion.

There is a pronounced maximum in plots of $\Delta_2$ vs. temperature for all three preparations in their respective transition regions (Figure 5). For DPPC-$d_{62}$ + 20 mol % palmitic acid, the variation of $\Delta_2$ is symmetrical about the maximum, while for the other two samples the variation is more gradual on the low-temperature side of the maximum than on the high-temperature side. In the liquid-crystalline phase, the $\Delta_2$ values were consistently smaller for bilayers containing fatty acid than

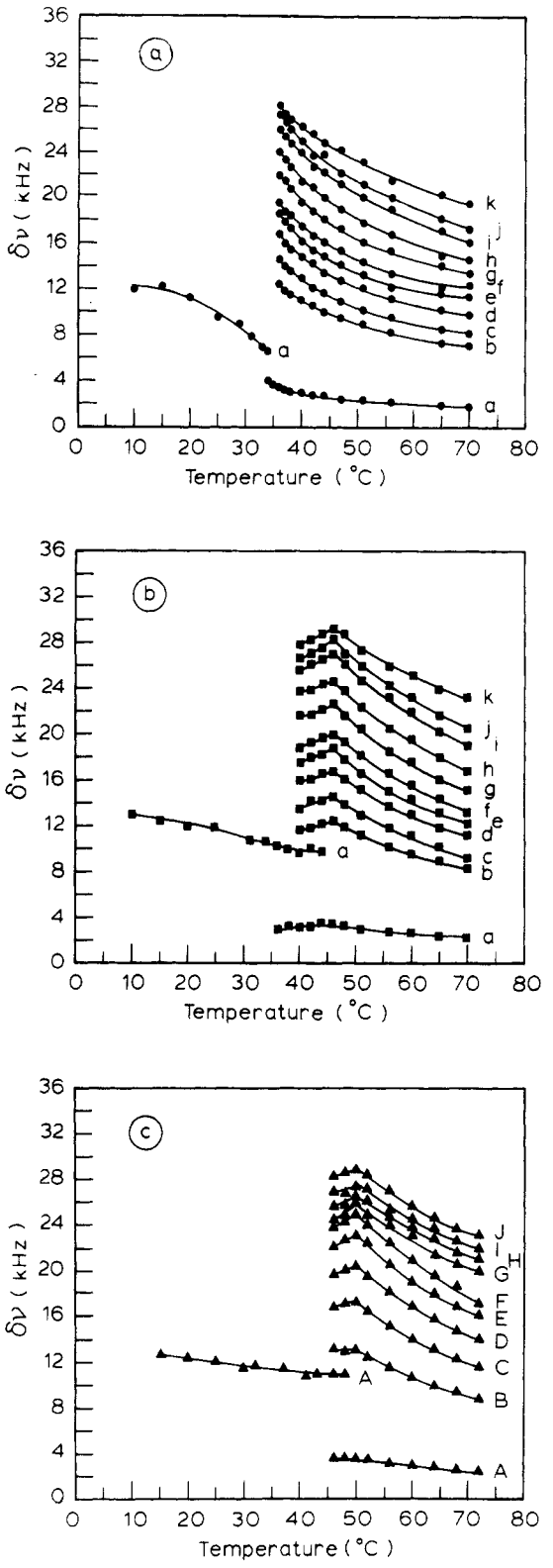


FIGURE 3: Plots of the quadrupolar splittings vs. temperature, measured for each of the resolved peaks in the spectra of (a) DPPC-$d_{62}$, (b) DPPC-$d_{62}$ + 20 mol % palmitic acid, and (c) DPPC-$d_{62}$ + 20 mol % PA-$d_{31}$. Letters refer to peaks labeled in Figure 2.

those measured for pure DPPC-$d_{62}$.

The fractional composition of the membranes in the mixed-phase region was calculated from the moment vs. temperature plots by assuming that the moments of the spectra in the transition region are given by

$$M_n = fM_n^G + (1 - f)M_n^L$$

where $f$ is the fraction in the gel phase and $M_n^G$ and $M_n^L$ are

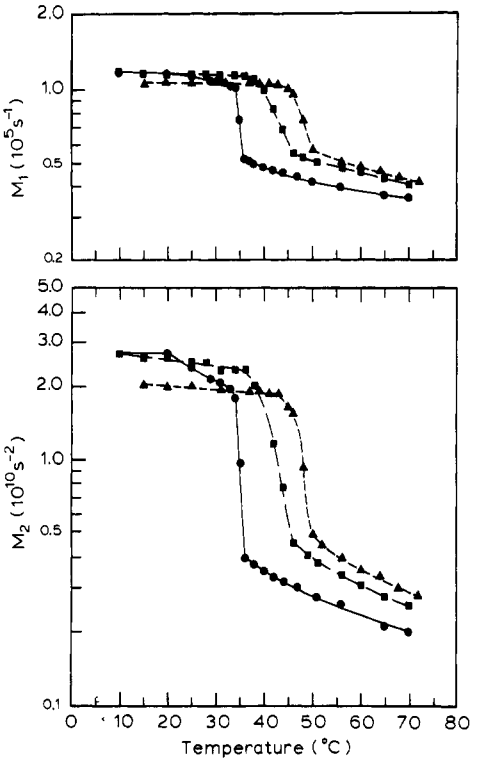


FIGURE 4: Plots of the average quadrupolar splitting ($M_1$) and mean square quadrupolar splitting ($M_2$) in units of angular frequency for (●) pure DPPC-$d_{62}$, (■) DPPC-$d_{62}$ + 20 mol % palmitic acid, and (▲) DPPC + 20 mol % PA-$d_{31}$. Errors in calculating $M_1$ and $M_2$ are less than 1% in the liquid-crystalline phase and ±1% and ±2% for $M_1$ and $M_2$, respectively, below the phase transition.

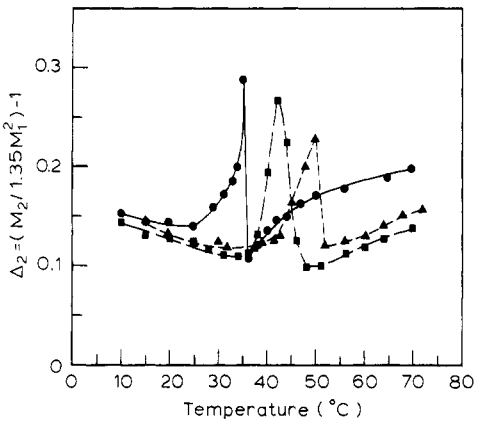


FIGURE 5: Mean squared fractional width of the distribution of quadrupolar splittings as a function of temperature for (●) DPPC-$d_{62}$, (■) DPPC-$d_{62}$ + 20 mol % palmitic acid, and (▲) DPPC + 20 mol % PA-$d_{31}$.

the $n$th moments of the gel and liquid-crystalline components of the spectrum, respectively (Jarrell et al., 1981; Bienvenue et al., 1982). Values for $M_n^G$ and $M_n^L$ were obtained from extrapolations of the monotonically decreasing segments in the gel and liquid-crystalline sections of the first moment vs. temperature curve above and below the mixed-phase region. Figure 6 plots the fraction of DPPC-$d_{62}$ in the gel phase as a function of temperature for bilayers of pure DPPC-$d_{62}$ and DPPC-$d_{62}$ + 20 mol % palmitic acid, as well as the fraction of gel-phase PA-$d_{31}$ in bilayers of DPPC + 20 mol % PA-$d_{31}$. Gel and liquid-crystalline phospholipid coexist only in a very narrow temperature range for pure DPPC-$d_{62}$, but addition of 20 mol % protiated palmitic acid extends the mixed-phase region over 10 °C. The transition region for protiated DPPC + PA-$d_{31}$ as reported by PA-$d_{31}$ is narrower than that observed

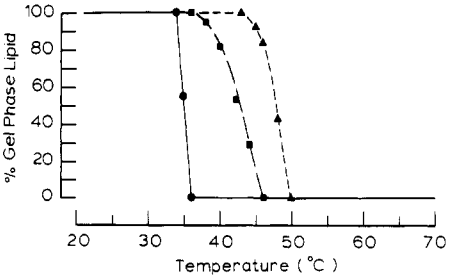


FIGURE 6: Gel-phase fraction as a function of temperature for (●) DPPC-$d_{62}$, (■) DPPC-$d_{62}$ + 20 mol % palmitic acid, and (▲) DPPC + 20 mol % PA-$d_{31}$.

for the DPPC-$d_{62}$ + 20 mol % palmitic acid sample, and the decline in the fraction of gel-phase lipid in the second half of the coexistence range appears to be more abrupt in the PA-$d_{31}$-containing sample than that observed in the DPPC-$d_{62}$ + 20 mol % protiated palmitic acid sample.

Liquid-crystal phase spectra from the samples were "de-Paked" to give the equivalent oriented spectra (Bloom et al., 1981). Figure 7 shows representative de-Paked spectra including the number of deuterons associated with resolved peaks. It is apparent that although there are systematic differences in the positions of the peaks (in particular, the peaks in the palmitic acid containing samples are distributed over a larger frequency range) the shapes of the distribution profiles are very similar for all of the samples. Order parameter profiles determined from the de-Paked spectra are discussed later.

The decay of the quadrupolar echo was measured for the samples at one temperature in both the gel and liquid-crystalline states (Figure 8). For pure DPPC-$d_{62}$, the variation with time is exponential at short times. No substantial difference in the $1/T_{2e}$ value was observed between the gel phase at 21 °C or the liquid-crystalline phase at 51 °C (Figure 8). However, for the samples containing fatty acid, the echo decay in the gel phase cannot be fitted by a single exponential (Figure 8a). Rather, at short $\tau$ values, the echo decay in these samples varies linearly with $\tau^2$ (Figure 8a, inset). This $\tau^2$ dependence was also observed for the DPPC + 20 mol % PA-$d_{31}$ sample at 26 °C (data not shown). In the liquid-crystalline phase, the echo amplitude decayed exponentially (at short times) for both of the fatty acid containing samples (Figure 8b, data not shown for DPPC + 20 mol % PA-$d_{31}$). Figure 9 illustrates the dependence of $1/T_{2e}$ on chain position in the liquid-crystalline phase. All of the samples show an increase in $1/T_{2e}$ from a minimum at the terminal methyl to a maximum within the first five segments of the chain followed by a decrease to a low value in the plateau region. Transverse relaxation rates for deuterons at all positions down the length of the chain for the pure DPPC-$d_{62}$ sample were on the average twice those observed for the DPPC-$d_{62}$ + palmitic acid mixture, and the DPPC + PA-$d_{31}$ sample had values which fell midway between the two extremes.

## Discussion

The results of this study agree with earlier work (Eliasz et al., 1976; Mabrey & Sturtevant, 1977) which has shown that the addition of palmitic acid to bilayers of DPPC eliminates the pretransition and elevates the onset temperature as well as increases the range over which the phospholipid gel to liquid-crystalline phase transition occurs. The previous results, which were obtained by differential scanning calorimetry, indicated that bilayers consisting of DPPC + 20 mol % palmitic acid begin the phase transition approximately 2 °C above that observed for pure DPPC and that the transition spans 7

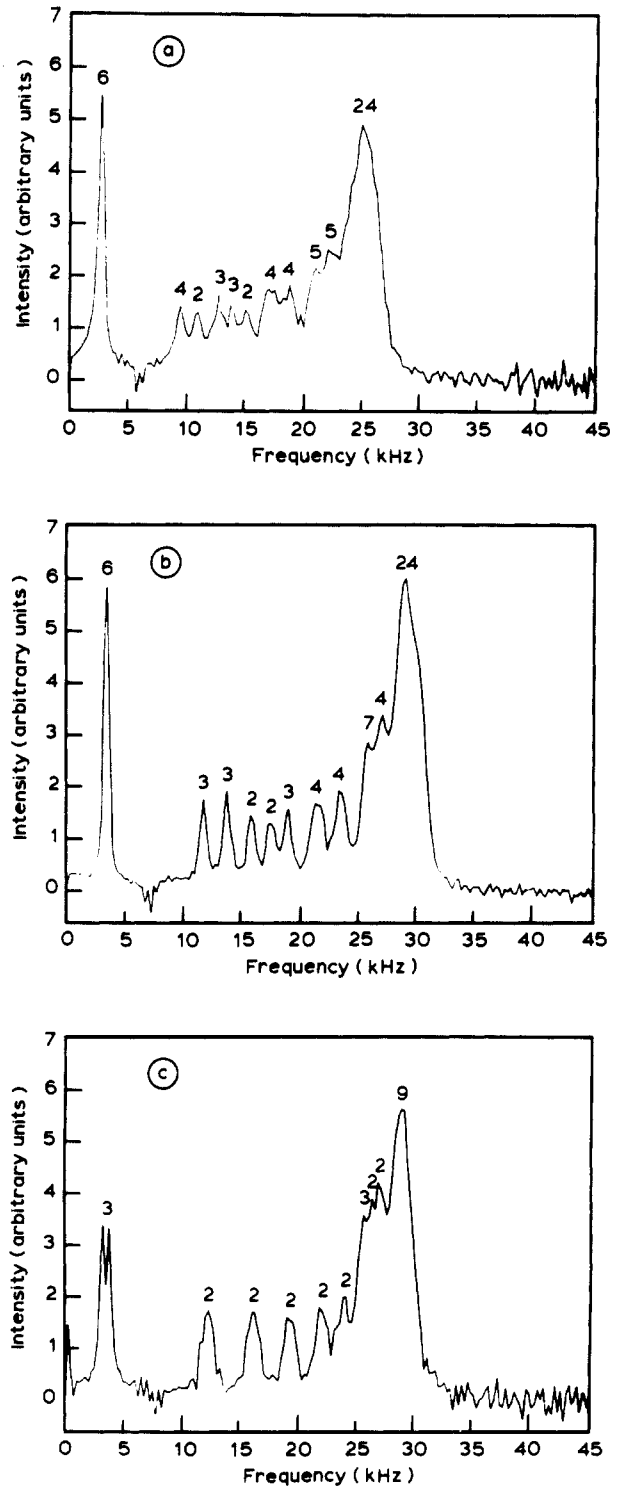


FIGURE 7: de-Paked spectra of liquid-crystalline phase (a) DPPC-$d_{62}$ at 51 °C, (b) DPPC-$d_{62}$ + 20 mol % palmitic acid at 51 °C, and (c) DPPC + 20 mol % PA-$d_{31}$ at 56 °C. Numbers over peaks indicate the number of deuterons which contribute to that peak. The numbers are proportional to integrated intensities between two minima on either side of the peak. Numbers were rounded off to the nearest integer, with the constraints that the totals equal 62 for the DPPC-$d_{62}$-containing samples and 31 for the PA-$d_{31}$-containing sample. No intensity corrections were made to take into account the differences in $T_{2e}$ for resolvable peaks (see Figure 9).

°C (Mabrey & Sturtevant, 1977) or 9 °C (Schullery et al., 1981). Thus, the results of the present study obtained by NMR, which show a 2 °C increase in the onset temperature of the phase transition and a 10 °C range for the transition observed for the DPPC-$d_{62}$ + 20 mol % palmitic acid sample, are in agreement with the calorimetric studies. It has been suggested that the effects of long hydrocarbon chains on the phase properties of phosphatidylcholines can be attributed to their ability to fill voids in the nonpolar region created by crowding of the head groups which have a larger excluded area in the plane of the bilayer than the side chains (Mabrey & Sturtevant, 1977; McIntosh, 1980). This arrangement would decrease the destabilizing effect of head-group crowding and increase the strength of the van der Waals forces in the hydrocarbon region.

The narrower width for the transition (7 °C) observed with the DPPC + 20 mol % PA-$d_{31}$ sample is probably due to the preferential partitioning of the fatty acid probe molecules into the gel phase, thus making them less sensitive to the phospholipid phase transition onset. The phase diagram for the DPPC–palmitic acid system determined by Schullery et al. (1981) indicates that all of the probe molecules would be in the gel phase at the onset of the transition and that the liquid-crystalline phase is gradually enriched with free fatty acid as the temperature is raised, to the limit of 20 mol % when the sample is completely fluid. Similarly, the shape of the quadrupolar splitting temperature profiles for the fatty acid containing samples can also be accounted for by the partitioning behavior of palmitic acid between the gel and liquid-crystalline phases of DPPC. The observed rise in value of the quadrupolar splittings in the transition region, for all positions as the temperature is raised, reflects the ordering effect of palmitic acid on the phospholipid side chains which becomes more pronounced as the liquid-crystalline phase becomes enriched with fatty acid. The maximum values occur at the termination of the phase transition for both samples, and the subsequent decreases in quadrupolar splitting values, which occur as the temperature is raised, reflect increased motional averaging of the quadrupolar interaction.

Phospholipid dispersions in the liquid-crystalline state have been shown to have an order parameter profile which is very similar to that observed for biological membranes (Stockton et al., 1977; Davis, 1979). A characteristic feature of these plots is a plateau in the variation of order parameters for the first five to eight methylenes after the carboxyl in the acyl chains (Seelig & Seelig, 1974; Davis & Jeffrey, 1977; Davis, 1979). In the spectra from perdeuterated phospholipids, this plateau manifests itself as an increase in intensity at the edges resulting from an overlap of several peaks with similar quadrupolar splittings. The addition of palmitic acid increased the frequency range of the liquid-crystalline powder pattern spectra but had little effect on their overall shapes. The latter indicates that the shapes of the order parameter profiles for fatty acid containing samples are likely to be similar to those observed for aqueous dispersions of pure phospholipid.

Order parameter profiles were estimated for the samples used in this study by assuming that order parameter values decrease monotonically down the length of the chains (except for position 2 of the *sn*-2 chain) and that the two chains are staggered by approximately three carbons with respect to the depth to which they penetrate into the membrane (Hauser et al., 1981; Büldt & de Haas, 1982) and by using $S_{CD}$ values calculated from the quadrupolar splittings and the number of deuterons associated with each peak in the de-Paked spectra. In Figure 10a, the profile obtained in this way for DPPC-$d_{62}$ at 44 °C is compared to that calculated previously by using a least-squares fit of the three parameters $S(o)$, $\mu$, and $\nu$ for the empirical function $S(x) = S(o)(1 - \mu x^{\nu})$ to $M_n/M_1^{n-1}$ from the first four moments of DPPC-$d_{62}$ (Bloom et al., 1978; Davis et al., 1980). The profile obtained in the present study shows separate curves for the two fatty acid chains in the region

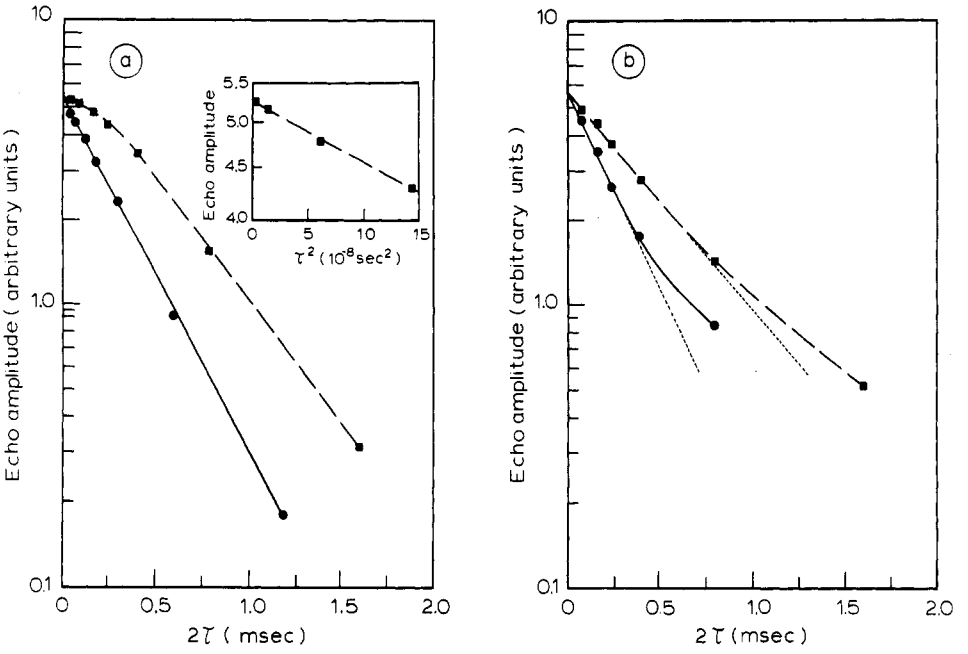


FIGURE 8: Quadrupolar echo decay plotted as a function of twice the spacing between pulses at 21 (a) and 51 °C (b) for DPPC-$d_{62}$ (●) and DPPC-$d_{62}$ + 20 mol % protiated palmitic acid (■). The inset at 21 °C shows the echo amplitude decay vs. $\tau^2$ for DPPC-$d_{62}$ + 20 mol % palmitic acid.

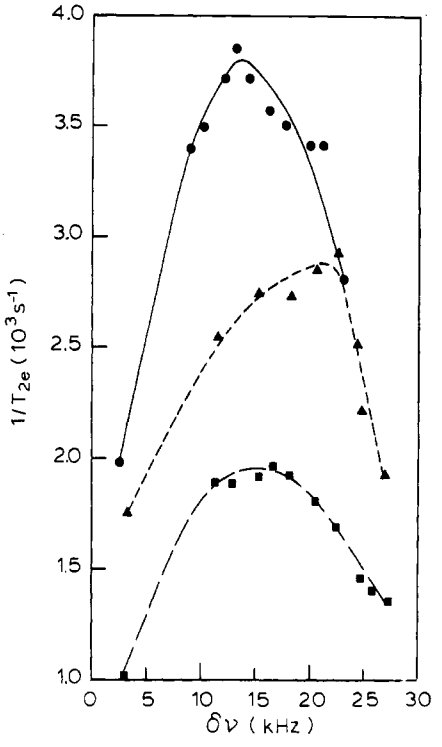


FIGURE 9: Plots of the quadrupolar echo decay rates for resolvable peaks in liquid-crystalline phase spectra of (●) DPPC-$d_{62}$ at 51 °C, (■) DPPC-$d_{62}$ + 20 mol % palmitic acid at 51 °C, and (▲) DPPC + 20 mol % PA-$d_{31}$ at 56 °C.

beyond the plateau. This reflects the fact that the two chains are out of step and is a consequence of the way in which the fatty acids are esterified to the glycerol portion of the phospholipid. The glycerol carbons together with the carboxyl group of the fatty acid esterified to the *sn*-1 position are oriented approximately perpendicular to the bilayer plane, whereas the initial part of the fatty acid esterified to the *sn*-2 position extends parallel to the membrane plane and bends at the second carbon to become parallel to the other fatty acid (Hauser e t al., 1981). This also accounts for the three order parameter values at position 2, two arising from the fatty acid esterified to the *sn*-2 position. The profile obtained previously by Bloom et al. (1978) can be considered to be an average for the two chains. Figure 10a shows that the latter falls midway between the two curves determined for the side chains in the present study. This illustrates the close agreement between these two methods for estimating order parameter profiles from perdeuterated systems.

Figure 10b shows order parameter profiles estimated in the manner described above for the fatty acid containing samples compared at temperatures which are 15 °C above the transition temperature of the pure phospholipids. The curve for perdeuterated palmitic acid (20 mol % in DPPC) falls midway between that observed for the two chains of DPPC-$d_{62}$ + 20 mol % protiated palmitic acid. Since the number of assumptions required to estimate the profile for the DPPC + 20 mol % PA-$d_{31}$ sample are much fewer than those required for the perdeuterated phospholipid profile, the close agreement between the curves for the two samples suggests that this simple accounting procedure does give physically meaningful results. Furthermore, this specific comparison indicates that free fatty acid probes monitor accurately the molecular order of phospholipid chains in the mixed bilayer.

It should be emphasized that incorporation of the fatty acid perturbs the membrane. This is illustrated by Figure 10c which compares the order parameter profiles obtained for DPPC-$d_{62}$ in the presence or absence of 20 mol % palmitic acid. The addition of free fatty acid increased the order parameter at each position, but this effect was greatest for those positions in and closest to the plateau region. Thus, it can be concluded that deuterated fatty acids accurately monitor the molecular order of the phospholipid membranes into which they have been incorporated but that they are reporting on a system perturbed by their presence.

The variation of $\Delta_2$ with temperature shown in Figure 5 deserves some comment. Maxima in such plots are usually indicative of the coexistence of spectra having very different values for $M_1$ and $M_2$. Indeed, the spectra of the two samples containing palmitic acid are superpositions of gel and liquid-crystalline spectra over the entire range of the thermal anomalies for $\Delta_2$. However, the pure DPPC-$d_{62}$ sample only

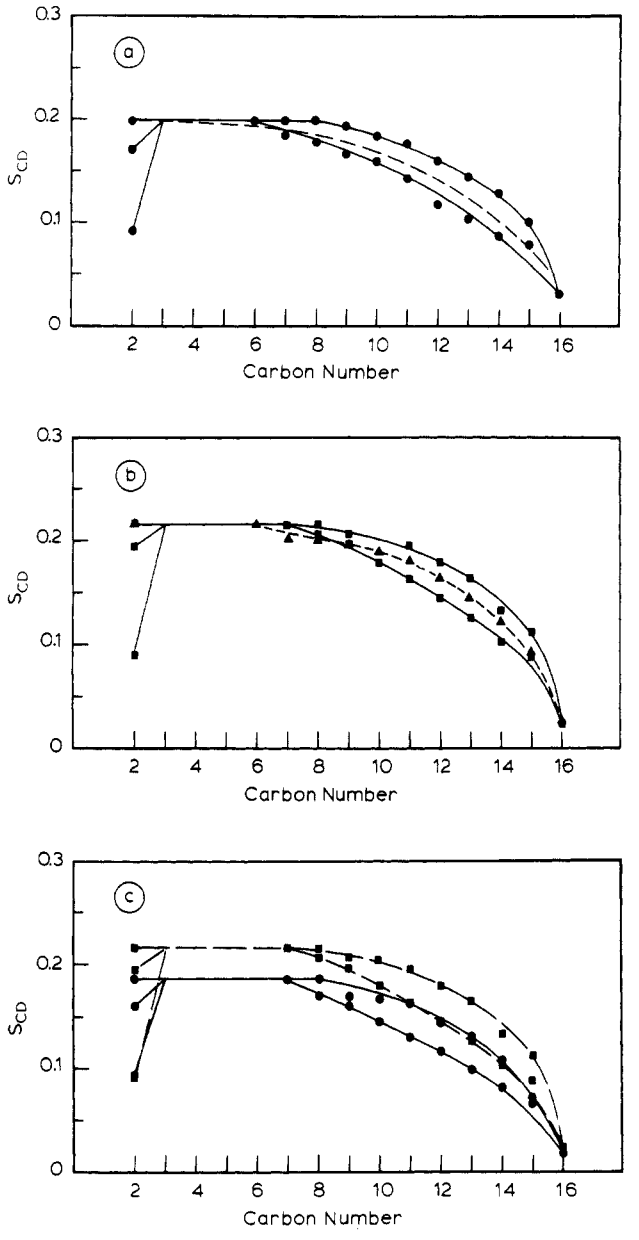


FIGURE 10: Order parameter profiles for (a) DPPC-$d_{62}$ at 45 °C from this study (●) and that obtained by the method of moments (---) (Bloom et al., 1978), for (b) DPPC-$d_{62}$ + 20 mol % palmitic acid at 51 °C (■) and DPPC + 20 mol % PA-$d_{31}$ at 56 °C (▲), and for (c) DPPC-$d_{62}$ at 51 °C (●) and DPPC-$d_{62}$ + 20 mol % palmitic acid at 51 °C (■).

exhibits such a superposition over a temperature interval of approximately 1 °C in the immediate vicinity of the $\Delta_2$ maximum. The increase in $\Delta_2$ starting below the temperature of the maximum is probably due to the properties of the $P_\beta$ phase (Janiak et al., 1976). Wittebort et al. (1982) found that the $^{13}C$ NMR spectrum associated with the carbonyl group of DPPC consists of two superimposed spectra whose relative intensities changed with temperature. They proposed that these two spectra were associated with phospholipid molecules having different orientations with respect to the bilayer normal in the rippled $P_\beta$ phase. The gradual increase in $\Delta_2$ observed in this study for DPPC-$d_{62}$, beginning some 10 °C below the main transition, can also be explained in the same way since the $^2H$ NMR spectra arising from the terminal methyls, in this temperature range, are superpositions of two gel-phase powder patterns (this study, data not shown; Westerman et al., 1982). The asymmetry in the $\Delta_2$ vs. temperature plot of the DPPC + 20 mol % palmitic-$d_{31}$ acid sample has a different origin than that of the pure DPPC-$d_{62}$ sample. The sharp decrease of $\Delta_2$ at the high-temperature end of the thermal anomaly is due to the greater solubility of the palmitic acid in the gel rather than the liquid-crystalline phase of the phospholipid. Thus, the fraction of palmitic acid dissolved in the gel phase remains high throughout most of the mixed-phase region but changes rapidly just before completion of the transition to the liquid-crystalline state (Figure 6). Finally, the smaller value of $\Delta_2$ in the liquid-crystalline phase for the samples containing palmitic acid (Figure 5) may be due to the smaller Lorentzian broadening in these samples, compared to the pure phospholipid, as is indicated by the larger $T_{2e}$ values of Figure 9 and the narrower widths of the de-Paked spectra in Figure 7.

The decay of the quadrupolar echo (Figures 7 and 9), which is denoted by the time constant $T_{2e}$, is sensitive to slow fluctuations of the spin-dependent interactions which cause the nuclear spins to lose phase memory as the spacing, $\tau$, between the two pulses is increased. Previous studies with deuterated phospholipid systems (Davis, 1979, 1983) have indicated that the dominant $T_{2e}$ relaxation process was associated with modulation of the relatively large quadrupolar interactions by slow motions of the chains. The argument given for this assignment was that the relaxation curves have been observed to vary exponentially with $2\tau$ for individual $^2H$ NMR peaks. The results of the present study are in agreement with these observations for pure DPPC-$d_{62}$ but also show that addition of fatty acid significantly decreases average and individual $1/T_{2e}$ values.

The decreased relaxation rate can tentatively be interpreted to indicate that addition of free fatty acid to phospholipid bilayers inhibits slow motions of the phospholipid acyl chains. In the gel state, the inhibition of the low-frequency motions by the fatty acid seems to be sufficient to allow the relaxation due to the relatively weak dipolar interactions to manifest itself. This interpretation is based on the linearity of the decay of the echo with respect to $\tau^2$ at short times in the presence of free fatty acid (Figure 8a). The dipolar interactions responsible for this decay involve intramolecular $^2H$–$^2H$ interactions on the DPPC-$d_{62}$ and the protons on the fatty acid molecules (Boden & Levine, 1978). It should be emphasized that little can be learned about the details of molecular motion from this mechanism since the modulation of the local magnetic fields is associated with mutual flip-flops of neighboring spins due to their static (i.e., time averaged) dipolar interactions.

Some final remarks can be made about the contribution of slow motions of the phospholipid chains to relaxation (evidence for the existence of these slow motions comes from a number of studies; Petersen & Chan, 1977; Jeffrey et al., 1979; Brown, 1982). The maximum in the $1/T_{2e}$ dependence on chain position, which occurs approximately three quarters of the way down the length of the chain, has previously been observed for DPPC-$d_{62}$ but has not been explained. The results of the present study indicate that intercalated fatty acids do not alter the relaxation rate of resolvable peaks relative to each other; rather, they decrease $1/T_{2e}$ values at all positions to the same extent. Thus, the slow motions which have been proposed may involve collective motions of all carbons in the chain.

**Registry No.** DPPC, 2644-64-6; palmitic acid, 57-10-3.

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