

Laser Raman Spectroscopic Study of Specifically Deuterated Phospholipid Bilayers[†]

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ABSTRACT: Raman spectra of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholines specifically deuterated in the 2 chain at one of positions 3, 4, 6, 10, 12, and 14 have been obtained as a function of temperature. The frequencies of the CD₂ vibrational stretching modes depend on the position of the labeled CD₂ group, being maximum at position 3 of the acyl chain and then decreasing until they become constant beyond position 6. This frequency dependence is interpreted in terms of the inductive effect of the charge distribution of the acyl chain carboxyl group. In both gel and liquid-crystal phases, the Raman line widths depend on the position of the CD₂ group,

being minimum at position 6 and increasing toward both ends of the hydrocarbon chain. The width of the CD stretching bands abruptly increases at the phase transition temperature. The magnitude of the increase depends upon the position of the label, increasing almost linearly up to position 10 and then decreasing for positions 12 and 14. The spectra for the CD₂ group at position 3 and the terminal CD₃ group are almost the same in both phases. These results are interpreted in terms of the effects of hydrocarbon chain organization on the vibrational modes.

The phospholipid bilayer has been extensively used as a model membrane system with which to investigate both the structural and functional aspects of biological membranes. In particular, the structural changes that accompany the gel to liquid-crystal phase transition in lipids have been studied by using a wide variety of physical techniques, and much qualitative and some quantitative information has been obtained. In an effort to better understand the disordering mechanism, and how the disorder varies at different points within the lipid bilayer, we have begun a Raman spectroscopic investigation of specifically deuterated phospholipid bilayers. The results presented in this publication may form a background with which to compare results obtained on more complex bilayer systems, such as protein-lipid or lipid-cholesterol systems, and perhaps eventually on intact biomembranes themselves.

In recent years many investigators have applied Raman spectroscopy to study the structure of lipids in both natural and model membranes (Gaber & Peticolas, 1977; Yellin & Levin, 1977; Gaber et al., 1978a; Verma & Wallach, 1975). These studies have shown that several bands in the Raman spectrum are sensitive to the changes in conformation and lateral packing that accompany the gel to liquid-crystal phase transition. In particular, the bands corresponding to the CH stretching vibrations of the methylene groups undergo significant changes in intensity and shape upon melting of the hydrocarbon chain portion of the lipid molecule (Gaber & Peticolas, 1977; Snyder et al., 1978). However, since all the CH₂ groups of the hydrocarbon chain contribute to the observed intensity, the spectral changes reflect changes summed over the bilayer thickness. Moreover, the CH vibrations from

CH, CH₂, and CH₃ groups in the head group (or any other molecule introduced into the bilayer) further complicate interpretation of the observed spectral changes. While these latter complications may be avoided by using phospholipids deuterated on one or both chains (Gaber et al., 1978b,c; Mendelsohn et al., 1976; Mendelsohn & Maisano, 1978), averaging over the entire bilayer thickness still remains. To overcome this problem, we have obtained Raman spectra for *specifically* deuterated phospholipids. Although such deuterated phospholipids have been studied previously by using deuterium nuclear magnetic resonance spectroscopy (Seelig, 1977; Oldfield et al., 1978a), there have been no Raman studies of these compounds, due presumably in part at least to the poor signal to noise ratios obtained from a single CD₂ group compared with the spectrum of a fully deuterated lipid (the Raman intensity should be directly proportional to the number of CD₂ groups in the molecule). However, by the use of signal averaging techniques, we show that it is possible to obtain high-quality Raman spectra for single CD₂ groups in specifically deuterated phospholipid molecules. We have obtained Raman spectra over a range of temperatures for dispersions of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholines (DMPC's)¹ that have been specifically deuterated on the 2 chain at one of the positions 3, 4, 6, 10, 12, and 14. Our data indicate that the frequencies of the CD₂ stretching vibrations are influenced by the charge distribution of the carboxy group, showing a significant increase for positions very close to this group. We find also that the CD₂ vibrational spectra show large line width changes upon melting of the hydrocarbon chains of the lipid bilayer and show that it is possible to use the width of the Raman bands as an empirical parameter to monitor the phase transition. In both phases, we observed an unusual variation of the Raman line width as a function of the position of the labeled group. The width is minimum around position 6 and increases toward both the polar and the methyl-terminus ends. These results are explained in terms of the effects of chain motions and the charge distribution of the polar group on the vibrational modes. Our results are

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¹ Abbreviations used: DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; FWHH, full width at half-maximum intensity; NMR, nuclear magnetic resonance.

compared and contrasted with those obtained by using other techniques. The spectral parameters for pure DMPC that have been determined from this study should provide the basic data necessary to analyze Raman spectra of more complex bilayer systems in future studies.

Experimental Section

Raman Spectroscopy. Raman spectra were obtained by using the 5145-Å line of an Ar⁺ ion laser (Spectra Physics Model 164) and a Spex Ramalog IV spectrometer (Spex Industries, Metuchen, NJ). The spectrometer slits were set at 250/300/250 μm which corresponds to a spectral resolution of approximately 5 cm⁻¹, and the power at the sample was kept at 80–100 mW. In order to obtain good signal to noise ratios for the CD₂ spectra, we averaged 100 scans, each recorded at a rate of 1 cm⁻¹/s, using a Nicolet 1180 data system (Nicolet Instrument Corp., Madison, WI). On the other hand, CH₂ spectra of comparable or better quality could be obtained by using only five scans because of the larger number of CH₂ groups present.

Spectra were recorded for both CD and CH vibrations over a range of temperatures. The capillary tube containing the sample was maintained at a constant temperature inside a cell mounted between the plates of a thermoelectric module. With this device, temperature could be regulated to better than 0.2 °C. The temperature was measured with a copper-constantan thermocouple mounted on the sample cell as close to the illuminated region as possible. The temperature readings for this external position of the thermocouple were calibrated against the temperature measured in a test sample into which a thermocouple was introduced and positioned at the exact spot from which the Raman scattering was measured.

Sample Preparation. DMPC's specifically deuterated at one of the positions 3, 4, 6, 10, 12, or 14 of the 2 chain were from the samples whose preparation and purification are described elsewhere (Oldfield et al., 1978b). Labeled DMPC's were pumped under vacuum overnight in order to remove any residual organic solvent and were then dispersed in distilled water (at a 12.5 wt % solid concentration) by gently agitating a warm mixture (approximately 60 °C) on a vortex mixer for about 5 min. Samples were syringed into melting point capillaries, concentrated by spinning in a low-speed centrifuge, and sealed for Raman spectroscopy.

Theoretical Background. The Raman active vibrations from a single CD₂ group in a polymethylene chain are essentially those of an isolated CD₂ group. An isolated molecule of the type XY₂ has three normal modes of vibration, corresponding to a bending of the angle between the two XY bonds and a symmetric and antisymmetric stretching of the two XY bonds. In this paper we focus our attention on only the stretching vibrations since the bending vibration falls in a region where there are strong Raman peaks due to other vibrations of the lipid molecule. The stretching vibrations are decoupled from the bending mode for groups like CH₂ or CD₂ because of the large frequency difference between the two modes. With this simplification, the frequencies for the symmetric (ω_s) and antisymmetric (ω_a) CD₂ stretching vibrations are given by

$$\omega_s = \frac{k^{1/2}}{2\pi c} \left[\frac{1}{M_D} + \frac{1}{M_C}(1 + \cos \alpha) \right]^{1/2} \quad (1)$$

and

$$\omega_a = \frac{k^{1/2}}{2\pi c} \left[\frac{1}{M_D} + \frac{1}{M_C}(1 - \cos \alpha) \right]^{1/2} \quad (2)$$

respectively (Wilson et al., 1955). Here k is the bond

stretching force constant, M_C and M_D are the masses of carbon and deuterium atoms, and α is the angle between the two CD bond vectors. In eq 1 and 2 we have neglected the interaction force constant between the two CD bonds. This approximation introduces an error of ~1% in the predicted frequencies. The force constant may be determined from the measured value of the CD stretching frequency in a CHD group, since $\omega(\text{CD}) = k^{1/2}(1/M_C + 1/M_D)^{1/2}/(2\pi c)$. Using the observed value of 2138 cm⁻¹ for $\omega(\text{CD})$ measured in [12-²H]stearic acid (Sunder et al., 1976) and [12-²H]distearoylphosphatidylcholine (Day, 1979), we obtain $k = 4.626$ mdyn/Å and, hence, $\omega_s = 2088$ cm⁻¹ and $\omega_a = 2187$ cm⁻¹ from eq 1 and 2.

A similar analysis for the vibration of the CD₃ groups shows that the frequencies of the symmetric mode and doubly degenerate antisymmetric modes are given by

$$\omega_s(\text{CD}_3) = \frac{k^{1/2}}{2\pi c} \left[\frac{1}{M_D} + \frac{3}{M_C} \cos^2 \phi \right]^{1/2} \quad (3)$$

and

$$\omega_a(\text{CD}_3) = \frac{k^{1/2}}{2\pi c} \left[\frac{1}{M_D} + \frac{3}{2M_C} \sin^2 \phi \right]^{1/2} \quad (4)$$

where ϕ is the angle between the CD bond vector and the threefold symmetry axis of the CD₃ group. For tetrahedral arrangement $\phi = 70^\circ 32'$; thus, $\omega_s = 2034$ cm⁻¹ and $\omega_a = 2188$ cm⁻¹.

Since the above equations also apply to the vibrations of an isolated CH₂ or CH₃ group, we note that the main effect of deuterium substitution is to lower the stretching frequencies by a factor of approximately 1/2^{1/2}, which shifts all the vibrational frequencies into the 2000–2200-cm⁻¹ region. Since there are no other Raman fundamentals of either lipids or proteins in this region, deuterium substitution should provide a very convenient means of studying the structure of the hydrocarbon interior of complex model and biological membranes.

Results and Discussion

In this section we discuss two aspects of the Raman spectra of specifically deuterated phospholipids: first, the dependence of vibrational frequency on the position of the CD₂ label and, second, the temperature dependence of the Raman spectra. Our aim is to determine which spectral parameters are the best indicators of chain conformation and chain motion at varying distances from the membrane's polar surface in both gel and liquid-crystal phases. Once these parameters have been determined for the pure lipid bilayer, they should be useful in examining the properties of multicomponent lipid bilayer systems.

Dependence of Frequency on Position of CD₂ Group. Figure 1 shows the Raman spectra in the 2000–2250-cm⁻¹ range of dispersions of DMPC labeled at one of positions 3, 4, 6, 10, 12, or 14 of the 2 chain. These spectra are all for lipid in the gel phase. As expected on theoretical grounds, the lower frequency peak corresponds to the symmetric ("in phase") stretching of the C–D bonds, and the higher frequency peak corresponds to the antisymmetric ("out of phase") stretching of the CD bonds. The observed frequencies for 2-[6,6-²H₂]-DMPC are $\omega_s = 2096$ cm⁻¹ and $\omega_a = 2174$ cm⁻¹, which agree to an accuracy of 0.4% (approximately 8 cm⁻¹) with the values predicted on the basis of the simple model of the isolated CD₂ group discussed above. The CD₃ labeled compound shows three peaks (cf. Figure 1f) instead of the two that are predicted from the simple model discussed in the preceding section. As is well established, this is due to the strong Fermi resonance

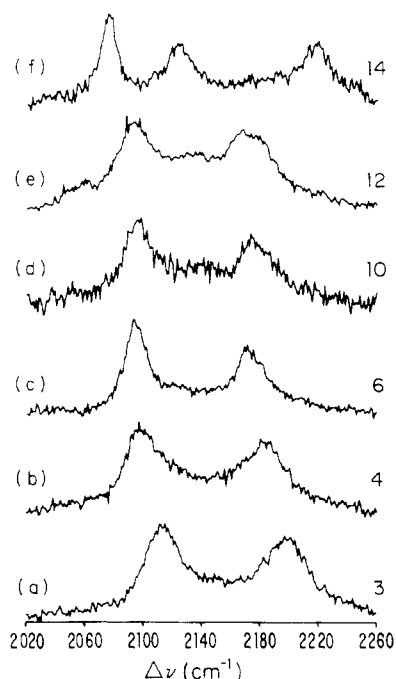


FIGURE 1: Raman spectra in the CD stretching vibration region of dispersions in excess water of DMPC labeled as CD₂ or CD₃ in the 2 chain at the positions indicated. All spectra were recorded with 80–100 mW of 5145-Å laser irradiation by using a Spex Ramalog IV instrument with spectrometer slit settings of 250/300/250 μm (=5-cm⁻¹ resolution). Sample temperature was 17.5 ± 1 °C.

interaction between the CD₃ symmetric stretching vibration and the overtone of the deformation mode (Sunder et al., 1976; Gaber et al., 1978b), giving rise to the two modes at 2074 and 2125 cm⁻¹, respectively. The peak at 2218 cm⁻¹ is the anti-symmetric stretching vibration, in agreement with the assignments for CD₃ frequencies in fully deuterated DPPC (Gaber et al., 1978b) and [18,18,18-²H₃]stearic acid (Sunder et al., 1976).

Examination of the CD₂ Raman spectra, for different hydrocarbon chain positions of the CD₂ label, reveals a sharp decrease in frequency as the labeled site moves away from the polar carboxy group region, with the frequencies becoming essentially constant for $n > 6$. The magnitude of this type of effect depends on the nature of the polar group substituent (Table I; Gotoh & Takanaka, 1961), implying that the increase in frequency is caused by the electric charge distribution of the polar group affecting the polarizability of the C–D bond in such a way as to increase the force constant; i.e., the effect is essentially that of a “solvent shift”. Since both the induced charge and electric field due to the polar group decrease as the distance from the polar group increases, one would expect both these effects to lead to a decrease in the bond stretching force constant and thus a decrease in the vibrational frequencies. Although a rigorous calculation of the contribution of inductive charge and electric field effects is probably not feasible for such complex species as the phospholipids, simple electrostatic considerations would suggest that the induced charge decreases exponentially whereas the electric field decreases only according to some power law. The inductive effect has previously been used to explain the dependence of terminal methyl group frequencies in monosubstituted hydrocarbons upon the chain length (Gotoh & Takanaka, 1961). The same predictions are applicable to the frequencies of the individual deuteriomethylene groups, and the fractional shift in frequency is given by

$$\delta\omega = (\omega - \omega^0)/\omega^0 = A \exp[-B(n-1)] \quad (5)$$

Table I: Raman Vibrational Frequencies of Specifically ²H-Labeled 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholines and Myristic Acids

	n , position labeled ^a	ω symmetric (cm ⁻¹) ^b	ω antisymmetric (cm ⁻¹) ^b
DMPC ^c	3	2114	2196
	4	2101	2183
	6	2096	2174
	10	2093	2174
	12	2094	2173
myristic ^d acid	3	2119	2206
	4	2103	2186
	6	2092	2174

^a Both DMPC and myristic acid are labeled as CD₂ at the position indicated. ^b Obtained from spectral simulations; see the text and Figure 4 for details; error is ±2 cm⁻¹. ^c 1-Myristoyl-2-[*n*-²H₂]myristoyl-*sn*-glycero-3-phosphocholine labeled at position n ; samples were hand dispersions in excess water at 17.5 °C. ^d Tetradecan-1-oic acid, labeled as CD₂ at one of the positions indicated; sample temperature was 11.8 °C. Melting point of myristic acid is 58 °C.

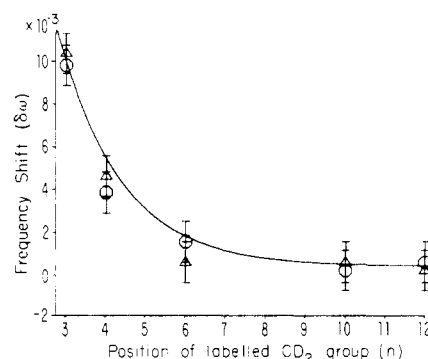


FIGURE 2: Dependence of CD₂ vibrational frequencies on n , the position of the labeled group in 2-[*n,n*-²H₂]DMPC's. (○) CD₂ symmetric mode; (Δ) CD₂ antisymmetric mode. The data are plotted as the fractional shift in frequency $\delta\omega = (\omega - \omega^0)/\omega^0$ where $\omega_s^0 = 2093.5$ cm⁻¹ and $\omega_a^0 = 2173.5$ cm⁻¹. The solid curve is calculated by using eq 5: $\delta\omega = A \exp[-B(n-1)]$ with $A = 0.0360$ and $B = 0.64$.

where ω is the frequency of one of the CD₂ modes, ω^0 is the corresponding frequency in the absence of any perturbation, n is the chain position of the labeled CD₂ group, A is a constant characteristic of the polar substituent, and $e^{-B} = \sigma$ is the ratio of the induced charges of two adjacent carbon atoms in the alkyl chain. The values of ω^0 chosen are the limiting values shown in Table I ($\omega_s^0 = 2093.5$ cm⁻¹; $\omega_a^0 = 2173.5$ cm⁻¹).

As shown in Figure 2, the observed frequency shifts for both CD₂ stretch modes for DMPC agree very well with the predictions of eq 5. A similar variation is also observed for the CD₂ vibrational stretching frequencies in myristic acid labeled at different positions (Table I). The frequency shifts are only slightly different in the two cases, implying that, as expected, the major contribution to the inductive effect comes from the carboxyl group.

Temperature Dependence of Raman Spectra. We have measured the Raman spectra for each of the labeled DMPC dispersions as a function of temperature. At each temperature we recorded spectra in the 2000–2250-cm⁻¹ range (corresponding to the CD₂ modes) as well as the 2800–3000-cm⁻¹ range (corresponding to the CH₂ vibrations). Typical results are shown in Figure 3 for 2-[6,6-²H₂]DMPC. Melting of the hydrocarbon chains causes the peak intensity of the CH₂ asymmetric stretching mode at 2884 cm⁻¹ to decrease sharply (Gaber & Peticolas, 1977), and this decrease in peak height with respect to the 2850-cm⁻¹ band can be used as an internal monitor for the onset of the phase transition. Although the

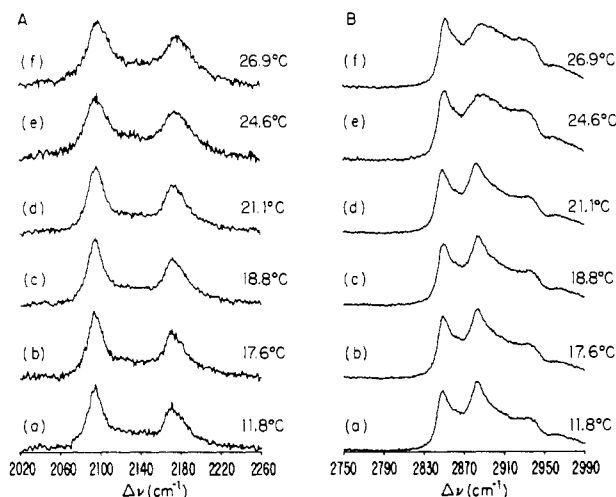


FIGURE 3: Raman spectra in the CD_2 and CH_2 stretching vibration region of 2-[6,6- $^2\text{H}_2$]DMPC, at the temperatures indicated, on either side of the phase transition temperature (23 °C). Experimental conditions were the same as for the spectra shown in Figure 1. In order to obtain approximately the same signal to noise ratios in the two regions, 100 scans were averaged in the 2000–2250- cm^{-1} range (CD_2 spectra), whereas only 5 scans were averaged in the 2800–3000- cm^{-1} range (CH_2 spectra). All spectra are plotted after background subtraction (a linear ramp).

CD_2 vibrations do not show the same differential change in peak intensity, they do show an abrupt increase in line width upon melting. The intensity variation in the CH_2 spectra has been attributed to the effects of Fermi resonance between the CH_2 symmetric mode at 2850 cm^{-1} and the first overtone of the deformation mode (Snyder et al., 1978). Similar effects are not, however, observed either in our data for isolated CD_2 groups or in the Raman spectra of fully deuterated phospholipids (Gaber et al., 1978b), implying that the pattern of Fermi resonance overlaps is different in hydrocarbons and deuteriocarbons (Snyder et al., 1978). In addition to the increase in width, melting also causes an increase of $\sim 5 \text{ cm}^{-1}$ in the frequencies of the CD_2 stretching vibrations. This result is analogous to that observed for the CH_2 stretching vibrations (Gaber & Petcolas, 1977) as well as for the C–C modes (Spiker & Levin, 1976).

Broadening of Raman lines of hydrocarbon chains upon melting has been reported by several authors (Mizushima & Shimanouchi, 1949; Mendelsohn et al., 1976; Gaber & Petcolas, 1977). On a molecular level there are at least three possible effects which may contribute to this broadening. They are as follows. The first is *rotamer broadening* (Mizushima & Shimanouchi, 1949). Due to the increased occurrence of *gauche* rotations about individual C–C bonds, there are many conformations of the hydrocarbon chain in the liquid-crystalline (or melted) state as compared with one (all-trans) or a few (*gauche* rotations near the CH_3 end) in the crystalline and gel states. This causes a spread of vibrational frequencies and hence broadens the Raman bands. The second is *reorientation effects*. The coupling of rotational and vibrational motions gives rise to a finite width in Raman and infrared spectra (Gordon, 1965; Sykora, 1972). Reorientational motion will be greatly increased in the liquid-crystal phase and this will contribute to line broadening. For small molecules, with well-defined symmetry properties, the isotropic part of the polarized Raman spectrum can be Fourier transformed to determine the rotational correlation functions. Such a detailed analysis is, however, not feasible in our case, since the CD_2 groups have been incorporated into lipid molecules and therefore have only local C_{2v} symmetry. Reorientational

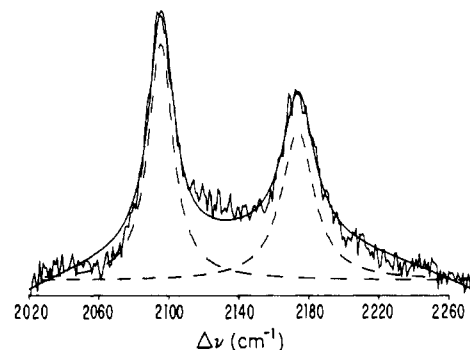


FIGURE 4: Experimental and simulated spectra of 2-[6,6- $^2\text{H}_2$]DMPC at a temperature of 21.7 °C. In the simulated spectrum the total intensity (eq 6) is shown by the solid curve and the two-component Lorentzian peaks are shown by dashed curves. The spectral parameters for the symmetric and antisymmetric peaks are $h_s = 0.380$, $\omega_s = 2096 \text{ cm}^{-1}$, $\Gamma_s = 17.1 \text{ cm}^{-1}$, $h_a = 0.320$, $\omega_a = 2175 \text{ cm}^{-1}$, and $\Gamma_a = 22.5 \text{ cm}^{-1}$. Note that the least-squares fitting is done after background subtraction.

motions also contribute to nuclear magnetic resonance (NMR) relaxation (Metcalf et al., 1971; Levine et al., 1972), but it is important to recognize that the NMR and Raman experiments deal with very different time scales (microseconds in NMR; picoseconds in Raman) and hence are not necessarily measuring the same types of motions. The third is *lattice disorder*. The breakdown of the crystalline lattice upon melting may also contribute to the broadening of Raman bands.

In order to test the usefulness of the line width as a parameter for monitoring the gel to liquid-crystal phase transition dynamics at various levels in the bilayer, we determined the widths of both the symmetric and antisymmetric modes by fitting the CD_2 spectra to a sum of two Lorentzians, given by

$$f(\omega) = \frac{1}{2\pi} \left[\frac{h_s \Gamma_s}{(\omega - \omega_s)^2 + (\Gamma_s/2)^2} + \frac{h_a \Gamma_a}{(\omega - \omega_a)^2 + (\Gamma_a/2)^2} \right] \quad (6)$$

Here $f(\omega)$ is the spectral intensity in the simulated spectrum, h_s is proportional to the integrated intensity, ω_s is the measured peak frequency, Γ_s is the full width at half-height (FWHH) of the CD_2 symmetric stretching band, and h_a , ω_a , and Γ_a are the corresponding parameters characterizing the asymmetric mode. The parameters h_s , Γ_s , h_a , and Γ_a were determined by a least-squares fitting procedure (Day, 1979). Typical results for a 2-[6,6- $^2\text{H}_2$]DMPC dispersion along with a simulated spectrum are shown in Figure 4. An excellent fit is observed with $h_s = 0.380$, $\omega_s = 2096 \text{ cm}^{-1}$, $\Gamma_s = 17.1 \text{ cm}^{-1}$, $h_a = 0.320$, $\omega_a = 2175 \text{ cm}^{-1}$, and $\Gamma_a = 22.5 \text{ cm}^{-1}$.

The variation of the widths, Γ_s and Γ_a , with temperature for each of the labeled DMPC dispersions is shown in Figure 5. We observe that there is an abrupt increase in the width at the melting temperature for positions 4–12 in DMPC, suggesting that at each of these positions there is an increased disorder due to the onset of *gauche* rotations and greater motional freedom in the liquid-crystalline phase consistent with previous ^2H NMR studies (Seelig, 1977). The behavior of the CD_2 label at position 3 is, however, anomalous, since it appears that position 3 undergoes no discrete increase in motional freedom upon melting, the line width Γ_s remaining essentially constant and the line width Γ_a increasing slightly. Of the two line widths (Γ_s and Γ_a) for 2-[3,3- $^2\text{H}_2$]DMPC, the former is the more accurately measured parameter. The measurement of Γ_a has a large error due to the presence of an unresolved spectral feature presumably due to an overtone

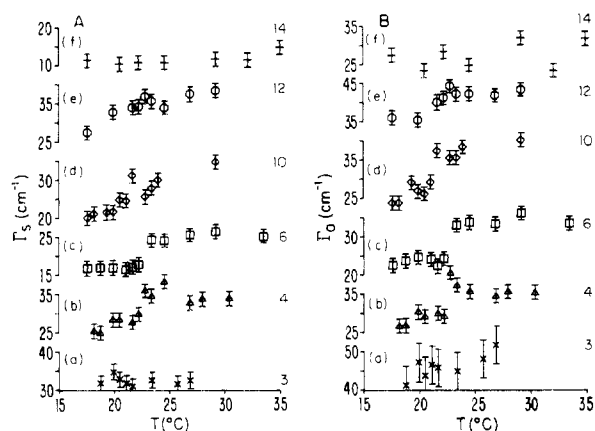


FIGURE 5: Variation of the CD₂ spectral widths (A) Γ_s (symmetric mode) and (B) Γ_a (antisymmetric mode) with temperature for the DMPC's labeled at the positions indicated. The width of the CD₂ antisymmetric mode, Γ_a , for 2-[3,3-²H₂]DMPC has a greater error than the other widths due to the presence of a small unresolved feature adjacent to this peak.

Table II: Raman Line Widths at Half-Maximum of Specifically ²H-Labelled 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholines

<i>n</i> ^a	Γ_s (cm ⁻¹) ^b		$\Delta\Gamma_s$ (cm ⁻¹) ^c	Γ_a (cm ⁻¹) ^b		$\Delta\Gamma_a$ (cm ⁻¹)
	17.6 °C	29.2 °C		17.6 °C	29.2 °C	
3	29	33	4	39	51	12
4	28	34	6	31	35	4
6	17	26	9	22	36	14
10	20	34	14	24	37	13
12	27	38	11	36	43	7
14 (² H ₃)	11	11	0	27	32	5

^a Position of labeled CD₂ group in DMPC. ^b Obtained from spectral simulations; see text and Figure 4 for details; error is ± 2 cm⁻¹. The 17.6 °C values are for the gel phase, and the 29.2 °C values are for the liquid-crystal phase. ^c $\Delta\Gamma_s = \Gamma_s(\text{liquid-crystal phase}) - \Gamma_s(\text{gel phase})$ is the change in width Γ_s upon melting. $\Delta\Gamma_a$ is similarly defined for the antisymmetric peak.

or combination band. The terminal CH₃ group is quite disordered even in the gel phase, consistent with previous studies (Yellin & Levin, 1977; Oldfield et al., 1978a). By contrast, the ratio of the peak heights of the CH₂ antisymmetric and symmetric stretching modes [$R = I(2884)/I(2850)$] shows a sharp inflection point in an R vs. temperature curve, irrespective of the location of the CD₂ label. In contrast with the behavior of the parameter R , the CD₂ width parameters therefore display a marked dependence on the chain position of the CD₂ label (Table II), and in this sense they are sensitive to local changes and may in principle be used to probe the phase transition at specific levels in the bilayer.

This suggestion of increasing "disorder" toward the terminal end is in general agreement with the results of spin-label (Hubbell & McConnell, 1971; Smith, 1972) and nuclear magnetic resonance studies (Seelig, 1977). However, this line of reasoning might imply that the "disorder" and hence the line widths should continue to decrease toward the polar end or should at least form a "plateau" (Seelig, 1977) in contradiction to the increase in width we observe for positions 3 and 4 relative to position 6. Since these positions are close to the polar group, we feel that the increase in width at these positions is essentially an "inhomogeneous" broadening related to the effects of the carboxy group rather than to the chain motions. As discussed earlier, the exact frequency of vibration for positions 3 and 4 is strongly influenced by the inductive effect of the carboxy group. Thus, slightly different orientations of the carboxy group or differences in conformations from C1

to C4 might easily lead to a spread of vibrational frequencies. This large effect due to the polar group could be essentially unaffected by the melting phase transition. As seen in Table II, the magnitude of the increase in width upon melting, $\Delta\Gamma_s$ and $\Delta\Gamma_a$, increases as the distance of the CD₂ group from the polar group increases, which is consistent with the observation that melting increases chain motions and that chain motions increase toward the terminal end. The decrease of $\Delta\Gamma_s$ and $\Delta\Gamma_a$ for positions 12 and 14 is presumably related to the fact that these positions are quite disordered even in the gel phase; thus changes in Γ_s and Γ_a on chain melting are small.

We have compared the line widths for DMPC dispersions with those of pure myristic acid labeled at the corresponding positions. We observe, as expected, that the gel phase of DMPC is less "ordered" than solid polycrystalline myristic acid and the liquid-crystal phase of DMPC is more "ordered" than liquid myristic acid, the magnitude of $\Delta\Gamma$ for the solid-liquid phase transition being 22 cm⁻¹ for [6,6-²H₂]myristic acid and 10 cm⁻¹ for the gel-liquid-crystal phase transition in 2-[6,6-²H₂]DMPC. It is interesting to note that the widths Γ_s and Γ_a in [3-²H₂]myristic acid are also greater than in [6-²H₂]myristic acid (for example, $\Gamma_s = 25$ cm⁻¹ for [3-²H₂]myristic acid and 11 cm⁻¹ for [6-²H₂]myristic acid, at 10 °C). This would imply that the carboxyl group has a similar effect of broadening the Raman bands in both the fatty acid and phospholipid systems investigated. We have, however, not attempted a detailed analysis of the Raman data for the fatty acids because the solid fatty acid spectra show complicated features due to the crystal field splitting of vibrational modes (Sunder et al., 1976). It is also interesting to compare our data for the widths of the specifically labeled CD₂ lipids with those of fully deuterated lipids (Mendelsohn et al., 1976; Mendelson & Maisano, 1978). The line width of the CD₂ symmetric mode at 2103 cm⁻¹ in [²H₃₄]DMPC increases from 40.5 to 45 cm⁻¹ at the melting transition (Mendelsohn & Maisano, 1978), which represents some weighted average over all the CD₂ groups. In this case $\Delta\Gamma_s = 4.5$ cm⁻¹. From Table II we see, however, that (at least in a specifically labeled DMPC) the range of $\Delta\Gamma_s$ values actually ranges from ~ 1 to ~ 14 cm⁻¹.

Conclusion

Raman spectroscopy of specifically deuterated DMPC's has been used to study the gel to liquid-crystal phase transition in phospholipid bilayers. It was found that the frequency of the CD₂ stretching modes depends on the position of the CD₂ group and is strongly influenced by the charge distribution of the polar carboxy group. The line width of the CD₂ mode was found to be a sensitive indicator of the local changes in hydrocarbon chain organization as a consequence of the phase transition. A quantitative measure of the line widths was obtained by fitting the spectra to a sum of Lorentzians.

The line width was observed to display an unusual dependence on position in both the liquid-crystalline and gel phase. The increase in width beyond position 6 was rationalized in terms of progressively greater motional freedom of the chains toward the CH₃ terminal end, in agreement with the results of NMR and spin-label studies. The unusual behavior of the line width was that it also increased from position 6 to position 3. This effect is probably related to the dependence of the CD₂ stretching mode vibrational frequencies for these positions on the inductive effect of the polar carboxy group, slightly different orientations of the carboxy group or conformations from C1 to C4 in different molecules giving rise to a spread of vibrational frequencies. Similar differential line widths were seen in the model system, myristic acid.

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Cholesterol-Phosphatidylcholine Interactions in Multilamellar Vesicles[†]

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ABSTRACT: We have investigated the phase behavior of dipalmitoylphosphatidylcholine-cholesterol bilayers using both the fluorescence of bilayer-associated 1,6-diphenyl-1,3,5-hexatriene (DPH) and freeze-fracture electron microscopy to elucidate specimen structure. Arrhenius analysis of the fluorescence-derived "microviscosity" parameter reveals temperature-induced structural changes in these membranes. In addition, isotherms of DPH fluorescence anisotropy and total intensity are used to detect alterations in membrane structure with varying cholesterol content. Freeze-fracture electron microscopic studies, utilizing rapid "jet-freezing" techniques, show strikingly different fracture-face morphologies for different combinations of sample cholesterol content and temperature. A phase diagram is proposed that offers a unifying interpretation of the fluorescence and freeze-fracture results. In this interpretation, inflections in temperature-scanning and

isothermal fluorescence measurements reveal phase lines in the dipalmitoylphosphatidylcholine-cholesterol membranes. Two-phase regions of the proposed phase diagram correspond to samples showing two coexisting fracture-face morphologies, while single-phase regions produce membranes having only one clearly identifiable structure. The proposed phase diagram provides an explanation for several conflicting literature proposals of stoichiometries for phosphatidylcholine-cholesterol complexes in membranes. These stoichiometric complexes correspond to the boundaries of two-phase areas in the gel region of the phase diagram. To better approximate the effect of cholesterol on natural membranes, the structure of egg phosphatidylcholine-cholesterol multilamellar vesicles was also investigated by using DPH fluorescence. The results for this complex natural phospholipid system are interpreted by comparison with the synthetic phospholipid results.

Cholesterol is a major lipid component of many mammalian cell membranes. In humans, it has long been implicated in the etiology of atherosclerosis. For these reasons, research aimed at defining the interaction of cholesterol with phos-

pholipids has been active for two decades [for a recent review, see Demel & DeKruiff (1976)]. Two lipid systems have most often been studied: egg phosphatidylcholine-cholesterol and dipalmitoylphosphatidylcholine (DPPC)¹-cholesterol mixtures. Usually, these lipid mixtures have been incorporated into either small, unilamellar vesicles or large, multilamellar vesicles. A variety of physical methods have been used to monitor

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¹ Abbreviations used: DPH, 1,6-diphenyl-1,3,5-hexatriene; DPPC, 1,2-dipalmitoyl-3-sn-phosphatidylcholine; Tempo, 2,2,6,6-tetramethylpiperidiny-1-oxy.