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STRUCTURAL STUDIES OF BIOLOGICAL MEMBRANES AND RELATED MODEL SYSTEMS BY RAMAN SPECTROSCOPY

SPHINGOMYELIN AND 1,2-DILAUROYL PHOSPHATIDYLETHANOLAMINE*

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SUMMARY

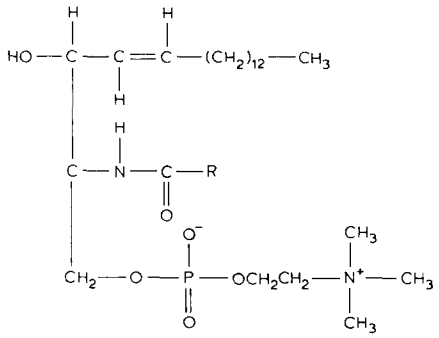
Raman spectra are reported at relatively high resolution ($1.5\ cm^{-1}$) for sphingomyelin and for 1,2-dilauroyl phosphatidylethanolamine above and below their melting temperatures. The spectra of 1,2-dilauroyl phosphatidylethanolamine below T_m show the hydrocarbon chains to be less ordered in the solid phase than the fatty acid of the same chain length, without the significant occurrence of *gauche* isomers. The spectra of sphingomyelin show significant formation of *gauche* isomers below T_m, indicating a less rigid environment for this molecule in the solid state.

INTRODUCTION

It is generally acknowledged that a detailed understanding of biological membrane structure requires the application of a variety of physicochemical techniques. Spectroscopic methods have been particularly emphasized as a probe of solution conformation, while three-dimensional structural data have been confined to X-ray diffraction measurements on selected model systems in the solid state (see for example refs 1 and 2). Raman spectroscopic studies offer a unique method that bridges the gap between solution and solid state conformation, as spectra are obtainable from both phases. In the current communication, we report Raman spectra for a naturally occurring phospholipid, sphingomyelin, at several temperatures above and below the melting point (T_m), and discuss its solid state conformation. In addition, spectra are reported for the synthetic phospholipid, 1,2-dilauroyl phosphatidylethanolamine for which the three-dimensional structure has been established [3]. The molecular structures of sphingomyelin and dilauroyl phosphatidylethanolamine are shown below.

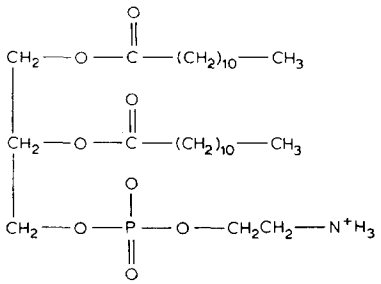
* An N.R.C.C. paper.

Sphingomyelin



R = C_{18}(O) or C_{24} (1)

Dilauroyl phosphatidylethanolamine



Lauric acid

$CH_3(CH_2)_{10}COOH$

EXPERIMENTAL

(1) *Materials*

Bovine brain sphingomyelin was obtained in a highly purified form from several sources including Serdary Research Labs, London, Ontario, K and K Labs, Plainview, N. Y., and Schwarz Mann, Orangeburg, N. Y. The molecule was stored in vacuo at −4 °C until used. Raman spectra from the three sources were essentially identical with the exception of occasional traces of fluorescent impurity from one source or another which could be reduced by exposure to the laser beam. Dilauroyl phosphatidylethanolamine was obtained from Serdary Research Labs and used without further purification. Samples for Raman spectroscopy were dried over P_2O_5 prior to usage. The sample melting point was 90 °C, lower than the literature value [2] of 104 °C for the anhydrous material. This difference is probably due to the presence of tightly bound water.

(2) *Methods*

Raman spectra were obtained either with a Jarrell-Ash model 25-102 or a Spex 1400 double monochromator equipped with Spectra Physics No. 164 and Coherent Radiation No. 52 A Argon ion lasers, respectively. The samples were contained in melting point capillaries and were illuminated at right angles to the tube axis. Scattered light was collected at right angles to both the incoming beam and

capillary axis (transverse mode). Standard photon counting detection and strip chart recording was used throughout. Power levels incident on the sample were about 400 mW of 5145 Å radiation.

Temperature was controlled by flowing cold N_2 gas over the sample tube which was contained in an unsilvered vacuum jacketed container [5]. The temperature was monitored with a thermocouple placed near the laser focus and the indicated temperatures are accurate to ± 2 °C, as determined from the Raman spectra of fatty acids with well established melting points.

RESULTS AND DISCUSSION

Several regions of the Raman spectrum of dilauroyl phosphatidylethanolamine are shown at various temperatures in Fig. 1–3 while the line positions, relative intensities, and tentative assignments are listed in Table I. Spectra of sphingomyelin in the 700–1700 and 2800–3100 cm^{-1} regions at various temperatures above and below T_m (87 °C) are shown in Figs 4 and 5. Spectra shown in Figs 1–5 have been obtained with relatively high resolution for this type of system. The spectral slit width used for dilauroyl phosphatidylethanolamine was about 1.5 cm^{-1}, while for sphingomyelin it was 1.3 cm^{-1}. The combination of relatively high resolution and low temperature allows the observation of impressive detail in the spectra.

Assignment of the features is greatly facilitated by the extensive data available on the vibrational spectra of hydrocarbons [6–10]. These have been applied to phospholipid structure in a number of cases, especially by Peticolas and co-workers [11–17]. Certain regions of the Raman spectrum have been shown to be sensitive to conformational changes in the phospholipid hydrocarbon chains. In the following section we discuss observed variation in the Raman spectra with temperature.

(1) *Dilauroyl phosphatidylethanolamine*

(i) *Below 400 cm^{-1}.* This frequency region contains the C-C longitudinal acoustical mode, which is predominantly a skeletal bending vibration of the hydrocarbon chains. The position of the mode has been studied extensively [9, 10], and its frequency has been shown to vary inversely with all-*trans* chain length of the hydrocarbon. Typical behaviour of the longitudinal acoustical mode is illustrated in Fig. 1, where we have included spectra above and below T_m for lauric acid which has the same hydrocarbon chain length as dilauroyl phosphatidylethanolamine. At −160 °C, the longitudinal acoustical mode in lauric acid appears as a sharp feature at 205 cm^{-1} [12]. The modes at 119 and 171 cm^{-1} are probably torsional or lattice modes. As the temperature is raised to 23 °C (still below T_m) the longitudinal acoustical mode broadens with little or no frequency shift, while the two other modes broaden and/or merge with the background. The presence of many *gauche* conformers above T_m results in an effective decrease in the average all-*trans* chain length, so that the longitudinal acoustical mode appears as a broad feature at 234 cm^{-1}.

Striking differences are observed in the corresponding spectral region of dilauroyl phosphatidylethanolamine, (Fig. 1) although lauric acid has the same number of carbon atoms in its hydrocarbon chain. At −165 °C two main features are seen at 224 and 138 cm^{-1}, with shoulders at 218 and 148 cm^{-1}, respectively. At 23 °C the shoulders merge with the main features.

TABLE I

RAMAN SPECTRUM FOR DILAUROYL PHOSPHATIDYLETHANOLAMINE AT −165 °C

$\Delta\nu$ (cm^{-1})	Intensity*	Tentative assignment**
138	w	
148	sh	
218	sh	Longitudinal acoustical mode
224	w	
404	w	
594	w	
716	vvw	
752	m	
761	sh	
820	m	
843	vw	
859	vvw	
871	w	
888	sh	
892	m	
911	w	
964	w	
1018	w, br	
1065	vs	ν(C–C) skeletal optical
1080	w	
1089	w	
1101	m	ν_{sym}(O–P–O)
1109	w	
1130	vs	ν(C–C), skeletal optical
1270	w	
1299	vs	CH_2 twist
1421	m	CH_2 bending
1441	vs	
1446	m, sh	
1457	w, sh	
1471	m	
1723	w	
1732	m	ν(C=O)
2732	wbr	
2848	s	$\nu_{sym}CH_2$
2872	sh	
2885	s	$\nu_{as}CH_2$
2903	w, sh	
2955	wsh	
2965	m	ν_{as}, CH_3

* w, m, s, v, sh, br stand for weak, medium, strong, shoulder and broad, respectively.

** ν(M–N), stretching vibration of M–N bond, sym and as stand for symmetric and anti-symmetric, respectively.

The behaviour of the feature at 219 cm^{-1} in dilauroyl phosphatidylethanolamine with change in temperature is similar to that at 205 cm^{-1} in lauric acid. This strongly suggests that the band has the same origin. If indeed the longitudinal acoustical mode is assigned to the band at approx. 219 cm^{-1}, the shift of about 14 cm^{-1} to higher frequencies from lauric acid indicates a shorter effective all-*trans* chain length

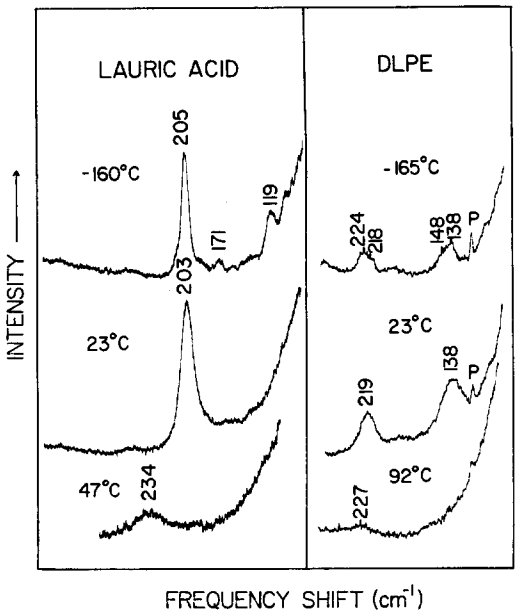


Fig. 1. Spectral region containing the longitudinal acoustical modes of lauric acid and dilauroyl phosphatidylethanolamine. Spectral slit width 1.5 cm^{-1}. The feature marked P is a non-lasing emission line of the Argon laser which was not completely eliminated by the spike filter. Excitation source, 400 mW of 5145 Å radiation.

in the phospholipid compared with the fatty acid.

Evidence that the longitudinal acoustical mode is sensitive to particular details of chain packing in phospholipid systems has been presented by Brown et al. [15] in their studies of sonicated vesicles of dipalmitoyl phosphatidylethanolamine and dipalmitoyl phosphatidylcholine. In the former, the longitudinal acoustical mode was observed at 161 cm^{-1}, at exactly the same frequency as in pure palmitic acid. In the latter, no corresponding vibration was noted and only a weak broad feature at 125 cm^{-1} was seen, although the hydrocarbon chains of both phospholipids have the same number of carbon atoms. It was suggested that differences in the structures of the pre-melting phases were responsible for differences in the behaviours of the two longitudinal acoustical modes.

In the current study, it is therefore not unreasonable to assume that the longitudinal acoustical mode is sensitive to differences in the molecular packing between lauric acid and dilauroyl phosphatidylethanolamine. Evidence that the chains in dilauroyl phosphatidylethanolamine do not pack perfectly comes from the results of the three-dimensional structure determination of the molecule [3]. The intermolecular packing in the crystal closely resembles the classical lipid bilayer structure. Rather large Debye thermal factors were noted for the methyl and methylene carbon atoms towards the centre of the bilayer [3], indicating a significant disorder in the hydrocarbon chains, even in the crystalline state. This disorder is in agreement with the observed broadening of the longitudinal acoustical mode in dilauroyl phosphatidylethanolamine at −165 °C, compared with lauric acid.

Above T_m, the longitudinal acoustical mode appears as broad, weak feature in

dilauroyl phosphatidylethanolamine at 227 cm^{-1} (Fig. 1, 92 °C spectrum) and in lauric acid at 234 cm^{-1}. The difference in the frequencies can be explained if the average all-*trans* chain length above T_m is longer for dilauroyl phosphatidylethanolamine, indicating that fewer *gauche* isomers can form in the phospholipid. In lauric acid above T_m, relatively more are formed leading to a shorter average chain length than dilauroyl phosphatidylethanolamine and correspondingly higher longitudinal acoustical mode.

The origin of the band at 140 cm^{-1} is obscure. However, the temperature dependence of the band between −165 °C and 23 °C (Fig. 1) is similar to that of the longitudinal acoustical mode, indicating that the feature is not a lattice mode.

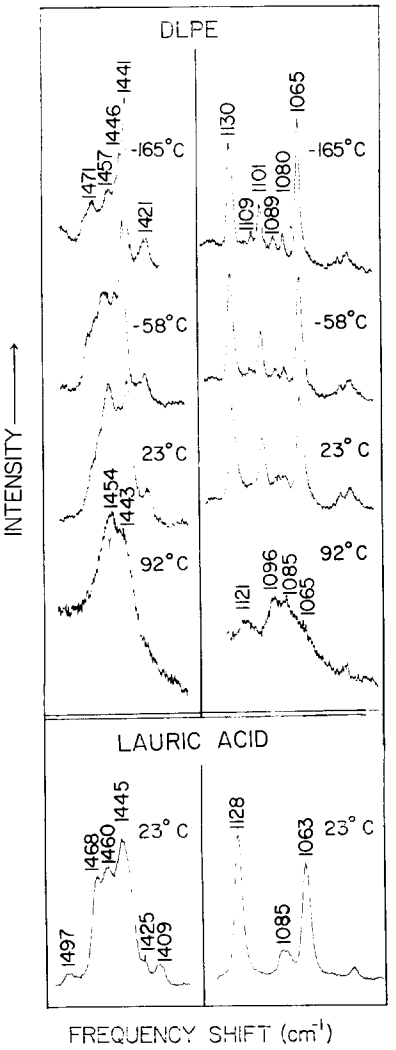


Fig. 2. The 1000–1150 and 1400–1500 cm^{-1} regions of dilauroyl phosphatidylethanolamine as a function of temperature and of lauric acid at 23 °C. Spectral conditions as in Fig. 1.

(ii) *1000–1150 cm^{-1} region.* Information concerning *gauche-trans* isomerisation in solid dilauroyl phosphatidylethanolamine is available from the 1000–1150 cm^{-1} spectral region shown in Fig. 2 (cf. refs 11–13). The region contains the skeletal optical vibrations, predominantly C-C stretching modes of the chain, along with the O-P-O symmetric stretching vibration of the phosphate group. Assignments of this spectral region are facilitated by comparison with lauric acid [12]. Bands at 1130 and 1065 cm^{-1} appear in dilauroyl phosphatidylethanolamine with relative intensities similar to the bands in lauric acid at 1128 and 1063 cm^{-1} and probably arise from similar vibrations. The band at 1101 cm^{-1} in dilauroyl phosphatidylethanolamine has no counterpart in the lauric acid spectrum and can be assigned to the O-P-O symmetric stretch, in agreement with a suggestion of Lippert and Peticolas [12]. The mode at 1085 cm^{-1} on lauric acid has a counterpart in dilauroyl phosphatidylethanolamine at 23 °C (Fig. 2) which appears to be extremely sensitive to order in the crystal. At low temperature the vibration splits into two features at 1080 and 1089 cm^{-1}. The origin of the low temperature band at 1109 cm^{-1} is unexplained.

The Raman spectrum of dilauroyl phosphatidylethanolamine above T_m is very different from its spectrum below T_m (Fig. 2). The intensity of the feature at 1065 cm^{-1} is greatly diminished upon melting, while a weak broad feature appears at 1121 cm^{-1} in place of the strong sharp band at 1130 cm^{-1}. In addition two intense features are observed at 1096 and 1085 cm^{-1} above T_m. The bands at 1130 and 1065 cm^{-1} have been assigned to the all-*trans* chain conformation, while the 1085 cm^{-1} band contains a contribution from *gauche* conformations [11].

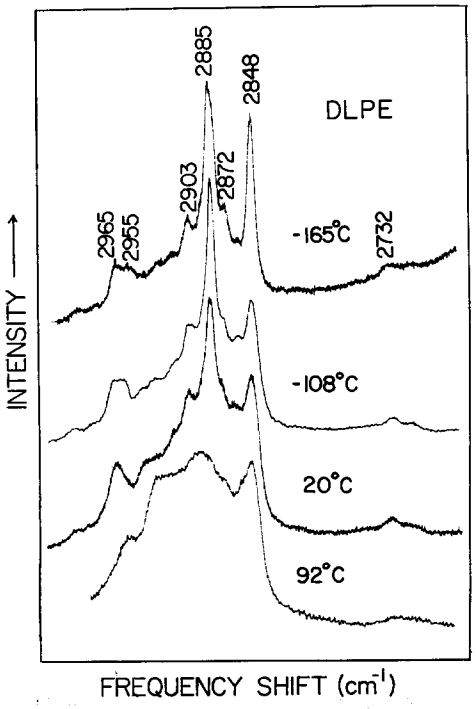


Fig. 3. Temperature dependence of the C-H stretching region (2800–3100 cm^{-1}) of dilauroyl phosphatidylethanolamine. Spectral conditions as in Fig. 1.

As the relative intensities of the bands at 1130, 1080 and 1065 cm^{-1} are approximately constant below T_m, few, if any, *gauche* isomers are formed below the melting point.

(iii) *1400–1500 and 2800–2100 cm^{-1}*. The spectral region around 1450 cm^{-1} (Fig. 2) consists primarily of CH_2 bending modes [7, 8]. Significant variation of intensity is observed in this spectral region well below T_m, as T_m is approached. The bands in this region broaden much more rapidly with increased temperature than those due to the skeletal (C-C) stretching modes. As this cannot be the result of an increased number of *gauche* conformations, (as shown from the analysis of the 1100 cm^{-1} region above), the variation must arise from increased thermal motion of the H atoms and disorder in their positions as the temperature is raised. This suggestion is further strengthened by an examination of the C-H stretching region, 2800–3100 cm^{-1} shown in Fig. 3.

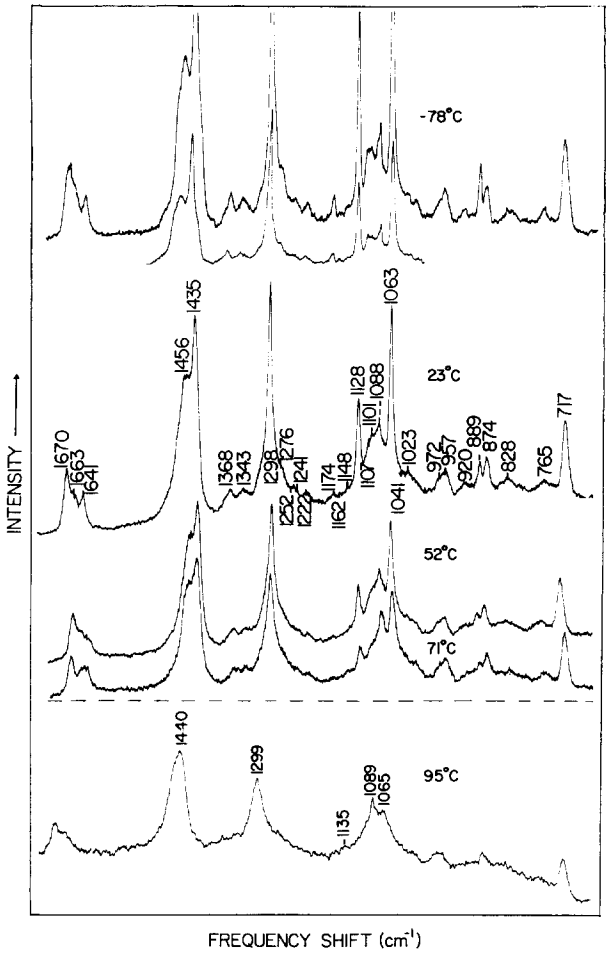


Fig. 4. Raman spectra in the 700–1700 cm^{-1} region for sphingomyelin as a function of temperature. Spectral slit width approx. 1.3 cm^{-1}. Power level approx. 450 mW of 5145 Å radiation. The horizontal scale for the spectrum at 95 °C is slightly different than the others. The frequencies are shown for the spectra obtained at 23 and 95 °C.

Once again significant changes occur below T_m. The minimum near 2860 cm^{-1} is filled in and an intensity increase at 2903 cm^{-1} occurs as T_m is approached. Above T_m, further changes occur in both the C-H bending and stretching regions, primarily consisting of mode broadening due to increased *gauche* isomer formation. The sensitivity of the C-H stretching region to the melting of hydrocarbon chains in lipids has been shown previously [15, 18].

(2) *Sphingomyelin*

The spectra of solid sphingomyelin in the 700–1700 and 2800–3100 cm^{-1} regions are shown in Figs 4 and 5. The measured melting point range of the molecule was 85–87 °C, and is in accord with that reported by Shipley et al. [19] for dry sphingomyelin. As is clear from Figs 4 and 5, dramatic changes in the spectra are observed well below T_m. One difficulty in interpretation, which is expected to be a general one in the analysis of membrane spectra, is the presence of a distribution of hydrocarbon chains. (For example in sphingomyelin the R group is usually either C_{18} with no double bonds or C_{24} with one; however, other chain lengths can and do occur.) For a given vibrational mode each of the chains of a different length may have a slightly different position, and the effect is to broaden a particular vibrational region, rendering it relatively insensitive to changes in structure, order and molecular motion.

The spectral region containing the skeletal optical vibrations near 1100 cm^{-1}

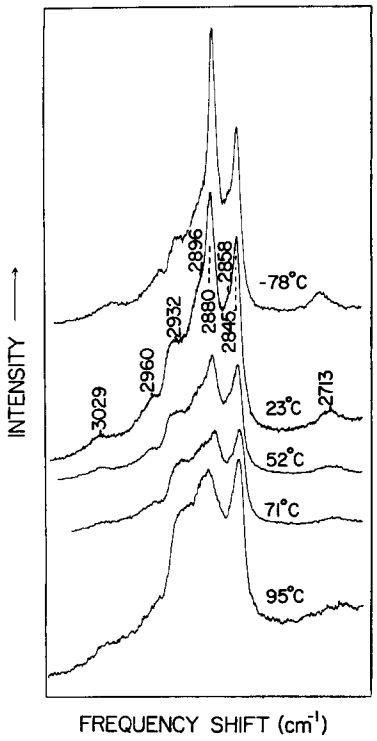


Fig. 5. Raman spectra the C–H stretching region 2800–3100 cm^{-} for sphingomyelin at various temperatures. Conditions as in Fig. 4.

shows the mode at 1088 cm^{-1} to progressively gain in relative intensity compared with those at 1128 and 1063 cm^{-1} below T_m, and finally becomes the most intense band in this spectral region above T_m in the liquid crystal phase. These changes indicate formation of *gauche* isomers in the solid state below T_m as T_m is approached. In addition, we have obtained preliminary spectra of sphingomyelin in aqueous dispersion and observe the presence of a significant fraction of gauche isomers at room temperature. The effect of conformational changes below T_m and their possible relevance to membrane structure are currently being probed. In the liquid crystal phase, above T_m, the spectrum is similar to that of dilauroyl phosphatidylethanolamine with only broad features appearing.

Spectra of the C-H stretching region (Fig. 5) also indicate that a series of progressive changes occurs below T_m. These changes arise from both the *trans-gauche* isomerisation and from the increased intermolecular motion [5, 20, 21] and disorder [22] at higher temperatures.

Several other regions in the sphingomyelin spectrum appear sensitive to structural change, although the origin of the molecular vibrations involved is unclear. For example, as the temperature increases, the relative intensity of the modes at 889 and 874 cm^{-1} alters drastically, so that in the melt the 889 cm^{-1} line vanishes, while at −78 °C, it is 1.7 times more intense than the 874 cm^{-1} line. The 889 cm^{-1} band may therefore be indicative of the all-*trans* chain conformation in sphingomyelin.

The halfwidths of the CH_2 twisting vibrations at 1298 cm^{-1} show significant variation with temperature below T_m for both sphingomyelin and dilauroyl phosphatidylethanolamine and are plotted in Fig. 6. The bandwidths are very similar in the liquid (melt) phase, thereby indicating that the origin of the vibrational modes and the intermolecular interactions [22] are similar in both systems. The behaviour of the halfwidths at temperatures below T_m differ for the two systems. The halfwidth for sphingomyelin increases from 6.5 cm^{-1} at −78 °C to 18 cm^{-1} at 71 °C. The increase

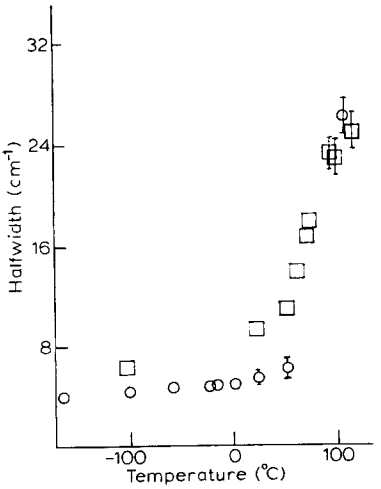


Fig. 6. Linewidth of the CH_2 twisting mode of sphingomyelin (□) and dilauroyl phosphatidylethanolamine (○) as a function of temperature. The linewidth measurement refers to the full width at half maximum.

for the same mode in dilauroyl phosphatidylethanolamine is from 4 cm^{-1} at −165 °C to 6.1 cm^{-1} at 52 °C. The linewidth for sphingomyelin, is always much greater than for dilauroyl phosphatidylethanolamine (Fig. 6) even at low temperatures where the contribution of the molecular motion is probably minimal. This is the result of significantly greater disorder in sphingomyelin, due in part to the distribution of hydrocarbon chains. At higher temperatures (still below T_m), the greater broadening of the band in sphingomyelin is the result of increased molecular motion and/or *gauche* isomer formation.

Our results for sphingomyelin in the solid state are in accord with ESR spectra for dry sphingomyelin reported by Long et al. [23]. At 37 °C, their observed values for hyderfine splittings of a cholestane spin label suggest significant fluidity for the molecule. Our observation of considerable *gauche* isomer formation well below T_m indicates that the microenvironment of sphingomyelin is non-rigid, even though sphingomyelin is a macroscopic solid until 87 °C.

CONCLUSIONS

(1) Significant gauche isomer formation is sphingomyelin occurs below T_m, and increases with temperature as T_m is approached. The molecule is therefore in a non-rigid environment in the solid state.

(2) Dilauroyl phosphatidylethanolamine exists in a relatively more ordered structure than sphingomyelin and shows little or no *gauche* isomer formation below T_m. The spectral region near 1450 cm^{-1} shows changes as T_m is approached which are the result of increasing thermal motion of the hydrogen atoms.

(3) The longitudinal acoustical mode appears sensitive to the details of the molecular structure in dilauroyl phosphatidylethanolamine, and may reflect greater disorder in the solid phospholipid compared with lauric acid.

As can be seen from the current work, different regions of the Raman spectra are sensitive to various aspects of molecular structure and motion in phospholipid systems. We hope to utilise this technique in situations where neither magnetic resonance methods nor X-ray results give completely satisfactory information.

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