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An infrared spectroscopic study of specifically deuterated fatty-acyl methyl groups in phosphatidylcholine liposomes

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The region of the infrared spectrum corresponding to C-^{2}H stretching vibrations (2050–2250 cm^{-1}) has been examined for liposomes composed of dimyristoylphosphatidylcholine deuterated specifically at the methyl ends of either one (*sn*-2) or both the fatty acyl chains. This label is intended to provide information on lipid dynamics in the contact region between monolayers. The two most prominent bands observed correspond, respectively, to antisymmetric (2212 cm^{-1}) and symmetric (2075 cm^{-1}) C-^{2}H stretching vibration. The antisymmetric band consists of two overlapping peaks, whose positions vary with the gel or liquid-crystalline state of the lipid. The separation between the peaks making up the antisymmetric band increases with temperature, and is maximum above the T_c transition temperature; this rules out the previously proposed assignment of these two peaks to different rotational modes of the methyl group relative to the adjacent methylene. The position and width of the symmetric band at 2075 cm^{-1} are also sensitive to the physical state of the lipid. The presence of cholesterol at an equimolar ratio with the phospholipid abolishes all the phase-dependent changes observed. The intrinsic polypeptide gramicidin A, at a 5:1 lipid/peptide mol ratio, is seen to enlarge the lipid thermotropic transition, with small effects above T_c. Cytochrome *c*, an extrinsic protein, at a 10:1 mole ratio, does not modify the phase-dependent behaviour of the terminal methyl groups, but consistently shifts all the observed bands to lower-frequency positions, which suggests a long-range effect of the protein along the phospholipid fatty acyl chains.

Introduction

The dynamics of phospholipids in model and cell membranes has been the object of extensive studies, in view of the important consequences for membrane biology and technology [1,2]. One important conclusion from those efforts is the realisation of the existence of an order profile or flexibility gradient, due to different segmental motion at the various points of the phospholipid acyl chains. First described by Chapman and co-workers [3–5] from proton magnetic resonance studies, the flexibility gradient was later characterised by spin label techniques [6], ^{13}C-NMR relaxation times [7] and, in a more quantitative way, by the calculation of order parameters in selectively deuterated lipids, using ^{2}H-NMR [8,9]. According to the ^{2}H-NMR order parameter, the central region of the bilayer is highly disordered with respect to the region close to the lipid/water interface [6]. Deuterium T_1 relaxation times [10] suggest as well that the central part of the bilayer is highly fluid (i.e., shows high rates of motion).

Deuterated lipids have been used to study the dynamics of membrane components not only by NMR, but also by other techniques, namely infrared [11–13] and Raman spectroscopy [12,14,15]. Mantsch and co-workers [11,12] established the thermotropic behaviour of a series of specifically deuterated dipalmitoylphosphatidylcholines, as detected by IR and Raman spectroscopy. O'Leary and Levin [14] have studied DMPC–cholesterol interactions by Raman spectroscopy, using lipids selectively deuterated in the *sn*-2 chain at the 3′, 6′ and 10′ positions. More recently, the effect of cholesterol on the order gradient in DPPC (specifically labelled at positions 4′, 6′ and 12′), has been followed by the C^{2}H$_2$ rocking absorption [16].

Abbreviations: DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DMPC-d_3, DMPC labelled with deuterium at the 14′ position of the *sn*-2 chain; DMPC-d_6, DMPC labelled with deuterium at the 14′ position of both *sn*-1 and *sn*-2 chains; NMR, nuclear magnetic resonance; FTIR, Fourier-transform infrared; T_c, main gel to liquid-crystalline transition temperature of a phospholipid.

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In this work, we have intended to explore in further detail the C-^{2}H stretching bands arising from fatty acyl C^2H_3 groups in dimyristoylphosphatidylcholine bilayers, as well as their perturbation by cholesterol or proteins. Mixtures of phosphatidylcholines with sterols or proteins have been well characterised by a variety of techniques [1,13,17–20]. Our results provide new evidence, based on infrared spectroscopy, on the dynamic properties of fatty acyl methyl ends in membranes, and on the effects that sterols, extrinsic and intrinsic proteins may have on such properties.

Materials and Methods

Dimyristoylphosphatidylcholine labelled with deuterium at the 14′ position of chain *sn*-2 (DMPC-d_3), or of both chains (DMPC-d_6), was synthesised as described by Oldfield et al. [21]. Its purity was checked by gas-liquid chromatography, thin-layer chromatography and differential scanning calorimetry. Both lipids show a narrow gel-to-fluid transition at a $T_c \approx 22°C$. Gramicidin A was purchased from Koch-Light, and cholesterol from Fluka. The phospholipid was dissolved in chloroform and, when required, mixed with cholesterol or gramicidin A at the appropriate ratios. The solvents were evaporated and the resulting film hydrated carefully in double distilled H_2O above the T_c of the pure lipid. Multilamellar liposome formation was routinely checked by negative-staining electron microscopy. When required, cytochrome *c* in H_2O solution was used to hydrate the lipids, at a final lipid/protein mol ratio of 10 : 1. In all cases, lipid concentration in the sample was about 150 mg/ml.

Fourier transform infrared spectroscopy was carried out in a Nicolet 620 spectrometer, equipped with a DTGS detector. Samples were placed in a thermostatted cell with CaF_2 windows. A pathlength of 25 μm was used in all experiments. 300 scans (sample) and 300 scans (background) were taken for each spectrum, using a shuttle device. H_2O spectra, obtained under exactly the same conditions, were subtracted from the liposome spectra in order to eliminate the H_2O association band. Spectral data were transferred to a personal computer, and processed using standard software [22]. Fourier derivation (with a power of three and a breakpoint of 0.3) and Fourier self-deconvolution (using Lorentzian bandwidths of 10 cm^{-1} and a resolution enhancement factor of 1.75) were used to separate the component bands of the C-^{2}H antisymmetric stretching band. Band positions were determined by the centre-of-gravity method. Bandwidths were calculated at 75% of the band height.

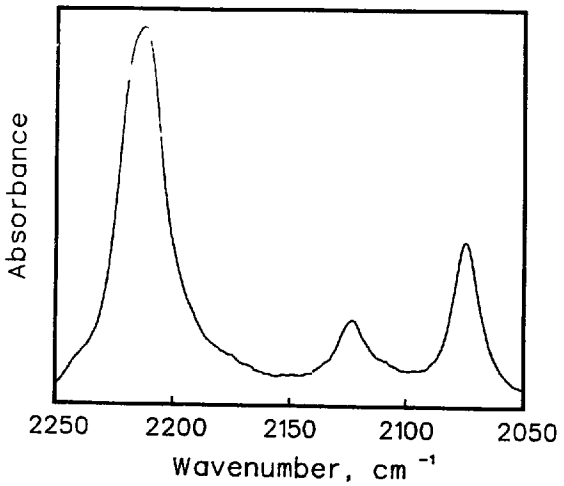

Fig. 1. The 2050–2250 cm^{-1} region of the infrared spectrum of DMPC-d_6 at 40 °C, after subtraction of the H_2O band.

Results and Discussion

Pure lipid dispersions

The C-$^2H_3$ stretching vibration of DMPC-d_3 and DMPC-d_6 gives rise to spectral absorption in the 2050–2250 cm^{-1} region. The spectrum shown in Fig. 1 corresponds to DMPC-d_6 at 40 °C; the same spectrum is obtained when DMPC-d_3 is examined. No appreciable differences are detected either between spectra recorded at above or below T_c. Consequently, our DMPC-d_6 spectrum is very similar to that published by Cameron et al. [11] for DPPC-d_3. The main spectral features are three absorption bands, that have been assigned respectively to C-^{2}H antisymmetric stretching vibration (2212 cm^{-1}) [11,12]. The two bands around 2100 cm^{-1} result from Fermi-resonance interaction between the symmetric C-^{2}H stretching fundamental and an overtone of the C-^{2}H bending vibration [12]. Our study has been focused on the two more prominent bands, namely those centred at 2212 and 2075 cm^{-1}.

The position of the antisymmetric vibration band at 2212 cm^{-1} remains virtually unchanged during the gel to liquid-crystalline phase transition (Fig. 2A). However, when band narrowing techniques are used, in particular Fourier self-deconvolution and Fourier derivation [22], the antisymmetric band is seen to consist of two peaks (Figs. 2B and C). This observation had been made, by means of spectral subtraction, for DPPC-d_3 below T_c [11], and we consistently find the two components in our derivative spectra both in the gel and the liquid-crystalline states. The position of each component may be accurately fixed from the derivative spectra determining the centre of gravity of the peaks, and is found to vary clearly with temperature. The results in Fig. 3 correspond to DMPC-d_3; the behaviour of DMPC-d_6 dispersions was similar, although the separation between the components was somewhat less pronounced. The high frequency component vibrates at ≈ 2218.5 cm^{-1} in the gel state, and is shifted upwards by 1 cm^{-1} at the T_c of the lipid (Fig. 3A), while the low-frequency component (at ≈ 2211

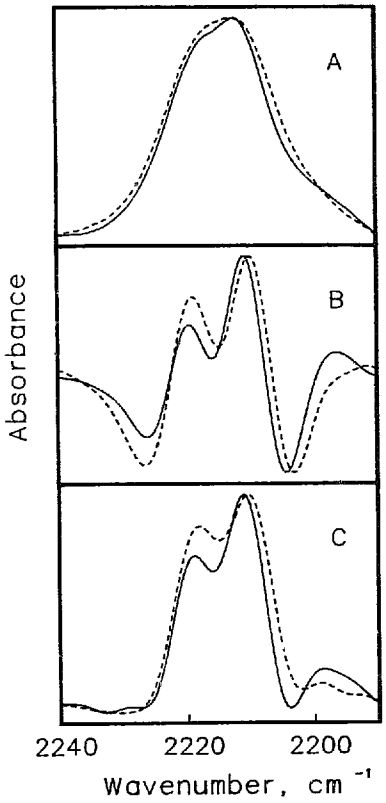

Fig. 2. The antisymmetric C-^{2}H stretching band of DMPC-d_6 at 2212 cm^{-1} and its components. (A) Original spectrum. (B) Derivative spectrum. A breakpoint of 0.3 was used. (C) Deconvoluted spectrum. Continuous line: below T_c. Broken line: above T_c.

cm^{-1}) shifts actually from a higher to a lower wavenumber (Fig. 3B). The separation between the two components of the 2212 cm^{-1} band is shown in Fig. 4;

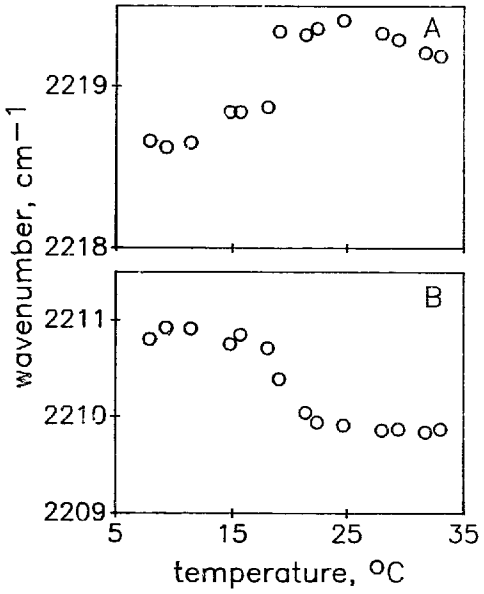

Fig. 3. The temperature dependence of the high- and low-frequency components of the antisymmetric C-^{2}H band. (A) DMPC-d_3, high frequency; (B) DMPC-d_3, low frequency.

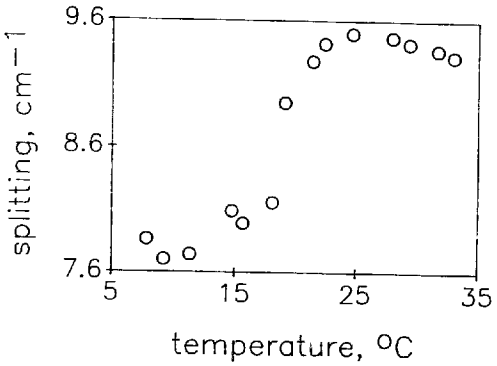

Fig. 4. The temperature dependence of the splitting between the two overlapping peaks in the antisymmetric C-^{2}H band of DMPC-d_3.

it increases with temperature, reaching a maximum just above T_c.

The band at 2075 cm^{-1} contains, as far as we can detect, just a single component. Both its position and width depend on the physical state of the phospholipid, as shown in Fig. 5 for DMPC-d_6. Both parameters increase with temperature, reaching a plateau at or near the T_c. The same results are found with DMPC-d_3 (not shown).

The major conclusion to be drawn from the above results is that, according to IR measurements, the terminal methyl groups of phospholipid fatty acyl chains reflect the main gel to fluid transition as a change in their vibrational properties. This had been shown for the whole antisymmetric C-^{2}H vibration [11], and is demonstrated here for each of the two components of that band, as well as for one of the symmetric stretching vibration peaks. Our results are in agreement with ^{2}H-NMR studies of DMPC selectively deuterated at various positions [15], that show a substantial increase in

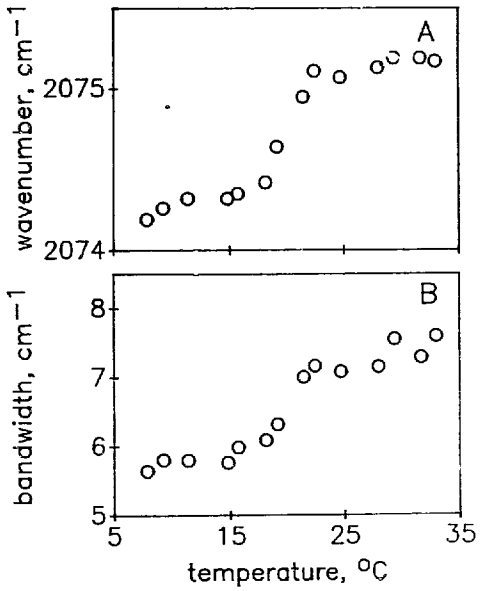

Fig. 5. The temperature dependence of the main symmetric C-^{2}H stretching band position (A), and bandwidth (B), of DMPC-d_6.

deuterium quadrupole splitting upon cooling the bilayers below their T_c. Both the deuterium quadrupole splitting and the position of stretching vibration bands have been related to the acyl chain static order [8,24]. It should be noted, however, that the shift in methyl C-^{2}H stretching that accompanies the phase transition is considerably smaller in magnitude than the corresponding methylene shifts (about 4-fold); this is consistent with previous studies according to which the terminal CH_3 group is quite disordered, even in the gel phase [15,25,26].

In their study of methyl C-^{2}H vibrations, Cameron et al. [11] interpret the two components of the antisymmetric band as a splitting of this stretching mode, related to the rate of rotation of the methyl group relative to the adjacent methylene group. They base this assignment on studies of pure hydrocarbons [27–29] in which an increase in the rate of rotation would result in the collapse of the band contour into a single peak. Actually, a splitting would only be seen for hydrocarbons in the crystalline state, which is not the case in our system. Our results for temperatures above, as well as below, the T_c (Fig. 5) indicate that the peaks do not merge, but rather the separation increases with temperature. This observation rules out the assignment proposed by Cameron et al. [11] for the antisymmetric band components, whose origin cannot be explained at present.

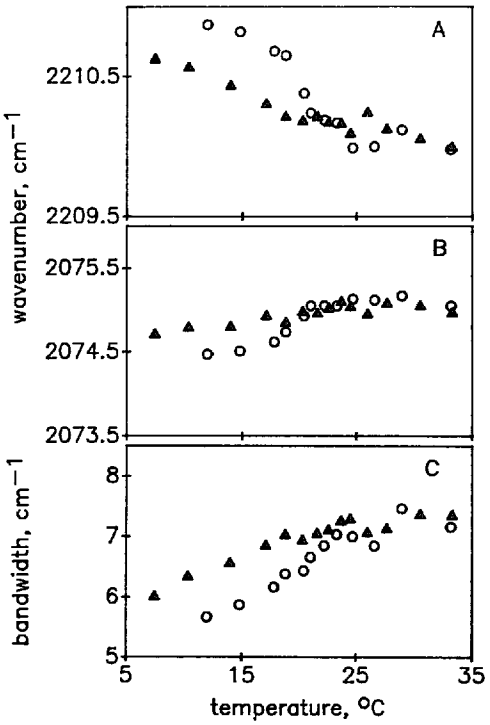

Fig. 7. The effect of gramicidin A (5:1 lipid/peptide mol ratio) on various phase-sensitive parameters of the infrared spectrum of DMPC-d_6. (A) Position of the high-frequency component of the antisymmetric C-^{2}H band. (B) Position of the main symmetric C-^{2}H stretching band. (C) Width of the main symmetric C-^{2}H stretching band. Circles: pure phospholipid. Triangles: phospholipid/peptide mixture.

Mixtures with cholesterol or protein

Sterols and proteins are, together with phospholipids, important components of cell membranes. In order to examine their effects on methyl C-^{2}H vibrations, several mixtures of DMPC-d_6 with cholesterol or proteins were studied along the lines described above. In the presence of the sterol, at equimolar proportions with DMPC-d_6, no change is seen in band positions (Fig. 6) or bandwidths (data not shown) at or near the T_c. Since cholesterol, at equimolar proportions with saturated phosphatidylcholines, is known to suppress the gel to fluid phase transition of the latter [1], this confirms that the changes seen at T_c with the pure lipid (Figs. 3–5) are indeed due to the phospholipid phase transition.

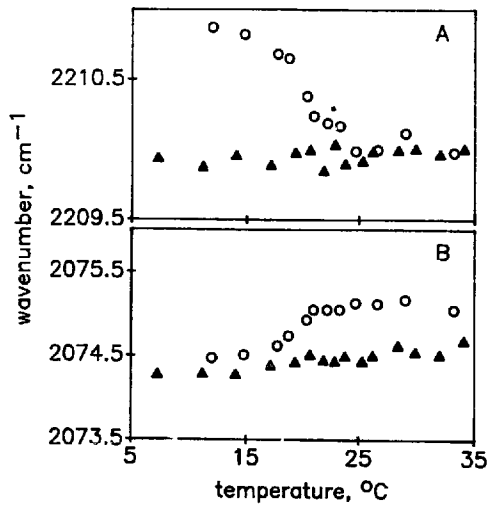

Fig. 6. The effect of cholesterol, at a 1:1 phospholipid/sterol mol ratio, on the infrared spectrum of DMPC-d_6. (A) Position of the high-frequency component of the antisymmetric C-^{2}H stretching band. (B) Position of the main symmetric C-^{2}H stretching band. Circles: pure phospholipid. Triangles: phospholipid/cholesterol mixture.

Gramicidin A is a hydrophobic peptide that can be readily incorporated into phospholipid bilayers, and is often used as a model integral protein. The interaction of gramicidin A with phosphatidylcholines has been studied in detail [19,20]; according to these studies, the peptide widens the gel-to-fluid transition of the lipid, but has little effect on the phospholipid order above T_c. Raman spectroscopy [19] and ^{2}H-NMR of DMPC-d_3/gramicidin A mixtures [20] indicate a small increase in molecular order in the fluid state, at least for peptide concentrations of up to 30 wt%, corresponding to a 5:1 lipid/peptide mole ratio. According to our observations (Fig. 7) gramicidin A, at a 5:1 lipid/peptide mol ratio, has the effect of widening the lipid phase transition; above T_c, the lipid disorder at the methyl end of fatty

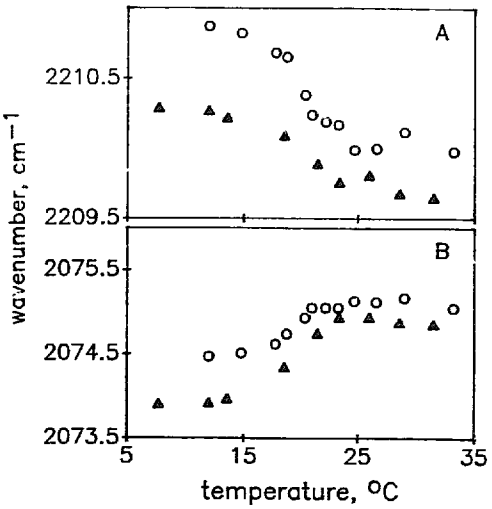

Fig. 8. The effect of cytochrome *c* (10:1 lipid/peptide mol ratio) on various phase-sensitive parameters of the infrared spectrum of DMPC-d_6. (A) Position of the high-frequency component of the antisymmetric C-^{2}H band. (B) Position of the main symmetric C-^{2}H stretching band. Circles: pure phospholipid. Triangles: phospholipid/cytochrome *c* mixture.

acyl chains appears to be similar with or without the peptide. The width of the band at 2075 cm^{-1} is increased below T_c in the presence of gramicidin A (Fig. 7C). The C-H stretching bandwidth has been described as sensitive to the degree of librational and torsional motion of the lipid acyl chains [13,29]. Thus gramicidin A appears to increase the motional freedom of the terminal portion of lipid acyl chains. In a study with perdeuterated DMPC, gramicidin A was also responsible for an increase in bandwidth of the symmetric C-^{2}H stretching vibration [13].

Cytochrome *c* is an example of a peripheral or extrinsic protein, although it is known to interact with the hydrophobic moieties of phospholipids [30]. According to our observations, cytochrome *c* does not modify the amplitude or width of the lipid phase transition, as seen through changes in the position of methylene C-H bands (not shown); this is to be expected from a peripheral protein [31]. However, it appears to shift slightly towards lower wavenumbers the C-^{2}H stretching vibration bands at all temperatures under study (Fig. 8). It is significant that, although cytochrome *c* in liposomes appears to be located in the lipid/water interface, in contact at the most with the part of the acyl chains closer to the glycerol backbone [30], still its presence is detected by the terminal portion of the same chains. In contrast to gramicidin A, cytochrome *c* does not modify the width of the symmetric stretching band at 2075 cm^{-1} (data not shown).

In conclusion, our results demonstrate: (a) that various vibrational parameters corresponding to fatty acyl methyl end groups in phospholipid bilayers are sensitive to the physical state of the lipids, and (b) that those parameters reflect in different, specific ways, the perturbations produced by sterols, intrinsic and peripheral proteins when incorporated to the bilayers.

Acknowledgments

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