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THE TEMPERATURE DEPENDENCE OF MOLECULAR ORDER AND THE INFLUENCE OF CHOLESTEROL IN *ACHOLEPLASMA LAIDLAWII* MEMBRANES

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Summary

^{2}H nuclear magnetic resonance (NMR) of *Acholesplasma laidlawii* membranes grown on a medium supplemented with perdeuterated palmitic acid shows that at 42°C or above, the membrane lipids are entirely in a fluid state, exhibiting the characteristic 'plateau' in the variation of deuterium quadrupolar splitting with chain position. Between 42 and 34°C there is a well-defined gel-to-fluid phase transition encompassing the growth temperature of 37°C, and at lower temperatures the membranes are in a highly ordered gel state. The ^{2}H-NMR spectra of the gel phase membranes are similar to those of multilamellar dispersions of chain perdeuterated dipalmitoyl phosphatidylcholine (Davis, J.H. (1979) Biophys. J. 27, 339) as are the temperature dependences of the spectra and their moments. The incorporation of large amounts of cholesterol into the membrane removes the gel to fluid phase transition. Between 20 and 42°C, the position dependence of the orientational order of the hydrocarbon chains of the membranes is similar to that of the fluid phase of the membranes without cholesterol, i.e., they exhibit the plateau in the deuterium quadrupolar splittings. However, the cholesterol-containing membranes have a higher average order, with the increases in order being greater for positions near the carbonyl group of the acyl chains. Below 20°C the ^{2}H spectra of the membranes containing cholesterol change dramatically in a fashion suggestive of complex motional and/or phase behaviour.

Abbreviation: DPPC, dipalmitoyl phosphatidylcholine.

Introduction

Acholeplasma laidlawii readily incorporates exogenous deuterium-labelled fatty acids into its membrane lipids. The cells can be grown in either the presence or absence of cholesterol in the growth medium. If cholesterol is present it is incorporated into the membranes. Since *A. laidlawii* has only a single plasma membrane, relatively homogeneous membrane preparations can be obtained.

It has recently been demonstrated, using ^{2}H-NMR of specifically deuterated membranes, that *A. laidlawii* membranes in the fluid phase exhibit a hydrocarbon chain flexibility gradient [1] similar to that of phospholipid multilamellar dispersions [2—4]. Further ^{2}H-NMR studies of *Escherichia coli* [5,6] and *A. laidlawii* [7] have shown that even at lower temperatures, where the membranes are in a gel state, there is a close correspondence between the ^{2}H-NMR spectra of the model [8] and the biological membranes.

The membrane lipids of *E. coli* [5,6,9—11] and *A. laidlawii* [7,12—14] undergo relatively well-defined fluid-to-gel phase transitions. Membranes which have a high cholesterol content, such as erythrocyte ghosts or *A. laidlawii* grown in the presence of a high concentration of cholesterol, do not exhibit this phase transition [15—17]. In differential scanning calorimetry studies of model systems with varying concentrations of cholesterol, the area under the endothermic peak at the phase transition decreases with increasing cholesterol content until, for example, with equimolar mixtures of dipalmitoyl phosphatidylcholine (DPPC) and cholesterol, no transition is observed [18,19]. There is evidence that cholesterol interacts preferentially with certain classes of phospholipids [19]. Electron spin resonance [20,31] and NMR studies [21,22] have indicated that the incorporation of cholesterol into model systems greatly increases the degree of order at temperatures above the phase transition.

What role the details of membrane fluidity (or order) play in the biological function of membranes (enzymatic functions in particular) is still not firmly established [23,24]. Recently, the rate of ATP hydrolysis by the membrane-bound ATPase of *A. laidlawii* has been reported to depend critically on the phase behaviour of the membrane lipids, dropping rapidly as significant amounts of gel-phase lipid form [25]. The recent improvements in the use of ^{2}H-NMR to measure the variation of membrane order with temperature [5—8] permit a more quantitative comparison of membrane order and phase behaviour with membrane function.

Obtaining the most accurate description of bilayer order by ^{2}H-NMR requires the use of specifically-deuterated samples [1—3]. However, it has been demonstrated on both model [8,21,26] and biological [5,6] membranes that completely deuterated samples provide much of the same information in a far shorter time. Even the shape of the fluid phase bilayer flexibility gradient can be obtained from the deuterium spectrum of a single perdeuterated sample [26].

The quadrupolar echo technique [27] allows one to obtain essentially distortion-free deuterium spectra even at low temperatures where the spectra are very broad [8]. The analysis of these spectra in terms of their moments [8]

has made it possible to determine the variation of chain order with temperature [5—8]. Using these techniques we compare the temperature dependence of the deuterium spectra of *A. laidlawii*, grown with and without cholesterol, with previously reported measurements on di-(perdeutero)-palmitoyl phosphatidylcholine (chain-perdeuterated DPPC) [8,26].

Materials and Methods

Perdeuteropalmitic acid (95% ^2H) from Larodan Lipids, Malmö, Sweden was biosynthetically incorporated into the membrane of *A. laidlawii* as described previously [28]. The growth medium of the preparation containing cholesterol was enriched with 20 mg of perdeuterated palmitic acid per liter and 15 mg of cholesterol per liter. The acyl chain composition, in mol%, was determined by gas-liquid chromatography (GLC) (as described in Refs. 3 and 28); for the sample without cholesterol, 12 : 0 = 7.0%, 14 : 0 = 26.2%, 14 : 1 = 2.0%, 16 : 0 = 64.5%, and for the sample with 39% cholesterol (the molar concentration of cholesterol was determined by GLC and is given as the percentage of total lipid in the membrane) the acyl chain composition was 12 : 0 = 5.5%, 14 : 0 = 22.2%, 14 : 1 = 3.2%, 16 : 0 = 68.8%. The NMR samples, which contained approx. 0.5 g of lyophilized *A. laidlawii* membranes, had a volume of 1 ml and contained approx. 60—70% H_2O. Measurements on *E. coli* have determined that lyophilization of the membranes has no effect on the ^2H-NMR spectra [5]. The samples used for differential scanning calorimetry had the same composition as the NMR samples and weighed approx. 10 mg.

The calorimetry was done on a Perkin-Elmer DSC-1b differential scanning calorimeter with a temperature scanning rate of 10 degrees/min. The temperature scale was calibrated by measuring the gel to fluid transition in fully hydrated dipalmitoyl phosphatidylcholine.

The ^2H-NMR spectra were taken at 34.4 MHz with a Bruker SXP 4-100 pulsed spectrometer using the quadrupolar echo technique [27] with a pulse separation of 60 μs, and signal averaging for approx. 2 h. All spectra were recorded on resonance so that the negative frequency half of the symmetric spectrum is folded over on top of the positive frequency half on Fourier transformation. A spectral width of 250 kHz was used for all spectra. No filters were used in the data acquisition and no phase corrections were applied to the transformed spectra. Two $\pi/2$ pulses of 4.5-μs length were used. When the spectrum covers a wide frequency range, the finite length of the pulse leads to some distortion of the wings of the spectrum. This distortion causes a systematic error in the calculation of the moments of the spectra. This error is negligible in the fluid phase but is expected to lead to an underestimate of the second moment, M_2, by approx. <25% at 0°C [8] (Bloom, M., Davis, J.H. and Valic, M.I. unpublished results).

The nth moment of the ^2H-NMR spectrum is obtained by evaluating

$$M_n = \int_0^{\omega_m} d\omega \omega^n g(\omega) / \int_0^{\omega_m} d\omega g(\omega) \quad (1)$$

where the Larmor frequency of 34.4 MHz is taken as $\omega = 0$, ω_m is the

frequency beyond which there is no signal and $g(\omega)$ is the spectral lineshape function of the experimental spectrum.

Results and Discussion

^{2}H-NMR spectra of the fluid phase

Fig. 1 (a, b and c) shows three deuterium spectra of the membranes of *A. laidlawii* grown on the medium supplemented with perdeuteropalmitic acid. At 42°C, Fig. 1a, the membrane lipids are in the fluid phase. This is clearly demonstrated by the expansion of the baseline region which indicates that there is no gel phase component to the spectrum. This spectrum exhibits the square shape and sharp cut-off in intensity which is characteristic of the 'plateau' in the variation of quadrupolar splitting with chain position found in a variety of model [8,29] and biological membranes [5,7,15]. The quadrupolar splitting ($\Delta\nu$) of the sharp edges of this spectrum gives the plateau C-^{2}H bond order parameter $|S_{C^2H}|_{plateau} \simeq 0.23$ ($\Delta\nu = (3e^2qQ/4h)\, S_{C^2H}$, where e^2qQ/h = 167 kHz, is the quadrupolar coupling constant). The orientional order param-

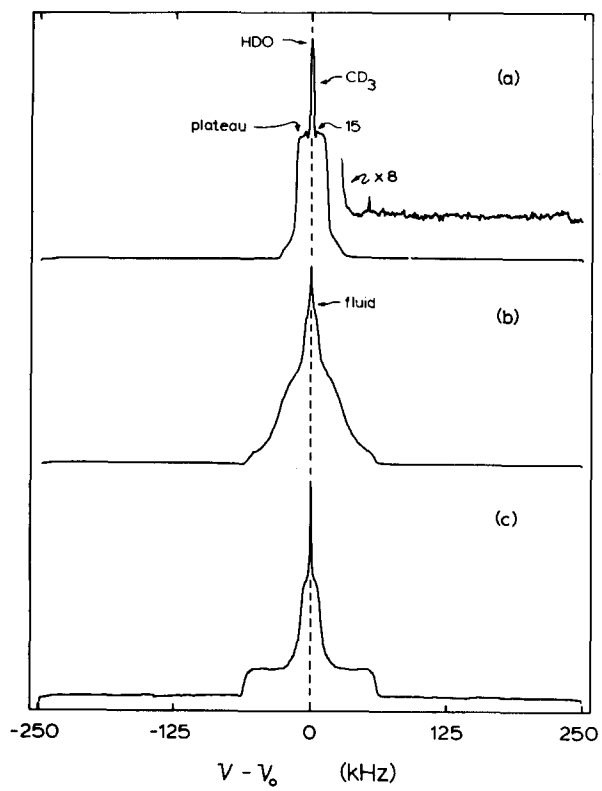


Fig. 1. ^{2}H-NMR spectra at 34.4 MHz of perdeuteropalmitate enriched *A. laidlawii* membranes; 30 000 scans at 4 scans/s. (a) T = 42°C, the vertical scale has been increased by a factor of 8 on the right hand side to show the sharp cut-off in intensity; (b) T = 22°C, and (c) T = 0°C. D, ^{2}H.

eter S_{C^2H} is defined by the relation

$$S_{C^2H} = \langle \tfrac{1}{2}(3\cos\theta_{C^2H} - 1)\rangle \quad (2)$$

where θ_{C^2H} is the angle between the symmetry axis of the motion (here presumed to be the normal to the bilayer) and the C-^{2}H bond vector, and the angular brackets indicate an average over all motions which are faster than the inverse of the quadrupolar interaction strength.

While the order parameter S_{C^2H} certainly depends on the average static orientation of the C-^{2}H bond vector (see e.g., Refs. 2 and 29) in the present case the values of the order parameters are predominantly determined by the motional averaging.

This value of $|S_{C^2H}|_{plateau}$ is very similar to those found for *E. coli* [5], DPPC [8] and with specifically deuterated palmitoyl chains in *A. laidlawii* membranes [1]. The only other identifiable sharp features of this spectrum are the contribution from the methyl groups, whose splitting gives $|S_{C^2H}|_{methyl} \simeq 0.019$ and a small peak, due to the deuterons in the 15 position, whose splitting gives $|S_{C^2H}|_{15} \simeq 0.079$ in agreement with the value obtained in the specific label studies [1]. The other positions on the lipid chains have a distribution of quadrupolar splittings or C-^{2}H bonds order parameters whose values lie between the two extremes [1]. This distribution, which may be due to the heterogeneity of the membrane, as well as to the variation of the quadrupolar splitting with chain position, results in the smooth shape of the spectrum, similar to that observed for *E. coli* [5]. The overall form of the spectrum observed for pure chain-perdeuterated DPPC/water is similar to that shown in Fig. 1a, but for the model system some sharp, resolvable features are observed [8]. When dealing with biological membranes one can expect to find the type of spectrum shown in Fig. 1a, and it is for the analysis of such spectra that the moment technique was developed.

Moment analysis of the ^{2}H-NMR spectra

The moments of the fluid phase deuterium quadrupolar powder pattern spectrum, defined by Eqn. 1, are simply and directly related to the moments of the distribution of quadrupolar splittings (Refs. 8, 26 and Bloom, M., Davis, J.H. and Dahlquist, F.W., unpublished results).

$$S_n = \int_0^\infty \mathrm{d}S P(S)\, S^n \quad (3)$$

The S_n are the moments of the order parameter distribution function $P(S)$ where $P(S)\mathrm{d}S$ is the probability of finding an order parameter between S and $S + \mathrm{d}S$. The moments of the spectrum are given in terms of the S_n by

$$M_n = A_n\left(\frac{3}{4}\,\frac{e^2qQ}{\hbar}\right)^n S_n \quad (4)$$

where the constants, $A_1 = 2/3\sqrt{3}$, $A_2 = \frac{1}{5}$, $A_3 = \frac{2}{35}(1 + 2/\sqrt{3})$, and $A_4 = \frac{3}{35}$, etc. are calculated from the expression for the quadrupolar powder pattern lineshape function [30]. From the expressions for the first two moments of $P(S)$

we can define a parameter Δ_2

$$\Delta_2 = \frac{S_2 - S_1^2}{S_1^2} = \frac{M_2}{1.35M_1^2} - 1 \tag{5}$$

which is the fractional mean squared width of the distribution of quadrupolar splittings.

As has been demonstrated for DPPC [26] we can obtain the hydrocarbon chain C-^{2}H bond order parameter profile from the spectrum of a perdeuterated sample using the values of the moments of the spectrum. The empirical function ν_Q of the continuous variable x, where x replaces the discrete variable, k, (= 2,3, . . . ,16) labelling the carbons on the chains,

$$\nu_Q(x) = \nu_Q(0)\{1 - [(\nu_Q(0) - \nu_Q(1))/\nu_Q(0)]\, x^{\mu}\} \tag{6}$$

can be used to describe the overall shape of the variation of quadrupolar splitting (or equivalently, the C-^{2}H bond order parameter) with position x. The quantities $\nu_Q(0)$ (the splitting at $x = 0$), $\nu_Q(1)$ (the splitting at $x = 1$) and the exponent, μ, are the three parameters which must be determined from the moments of the spectrum. It is possible to calculate the moments from this function $\nu_Q(x)$ in terms of the three parameters by assuming a statistical weight function $p(x) = 1$ for $0 \leqslant x \leqslant 1$ and $p(x) = 0$ otherwise, so that

$$S_n = \int_0^1 [S(x)]^n \, dx \tag{7}$$

The resulting system of non-linear equations for the three parameters can then be solved. However, it is more appropriate to do a least-squares fit of the calculated moments to the experimental moments. In order to give the higher moments the same weight as the lower moments, we have chosen to minimize the function

$$\phi(\nu_Q(0), \nu_Q(1), \mu) = \sum_{n=1}^{4} W_n^2 \left[\frac{M_n^{\exp}}{(M_1^{\exp})^{n-1}} - \frac{m_n(\nu_Q(0), \nu_Q(1), \mu)}{[m_1(\nu_Q(0), \nu_Q(1), \mu)]^{n-1}} \right]^2 \tag{8}$$

where $M_n^{\exp}$ is the experimental nth moment of the spectrum, and

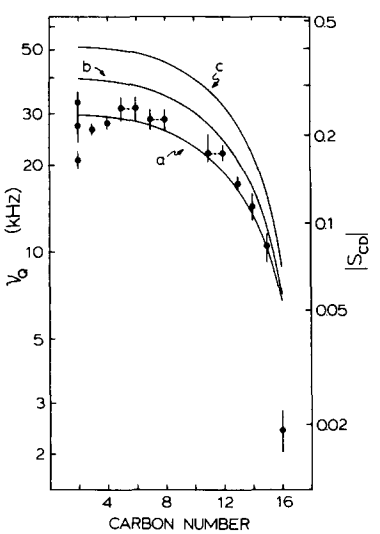
$$m_n(\nu_Q(0), \nu_Q(1), \mu) = A_n\{\nu_Q(0)\}^n \sum_{k=0}^{n} \binom{n}{k} \left(\frac{1}{\mu k + 1} \right) \left[\frac{\nu_Q(1) - \nu_Q(0)}{\nu_Q(0)} \right]^n \tag{9}$$

The weights, W_n, are given by $W_n = (A_n/A_1^{n-1})$, and are used to ensure that each of the first four moments have equal significance in the fitting procedure.

The parameters of the fit, $\nu_Q(0)$, $\nu_Q(1)$, and μ have a simple physical interpretation. $\nu_Q(0)$ and $\nu_Q(1)$ are the quadrupolar splittings of the two endpoints of the chain. The exponent, μ, describes the variation of $\nu_Q(x)$ between the two endpoints. If μ is large, there is a strong curvature in the profile, such as is found when there is a 'plateau'. If $\mu = 1$, then $\nu_Q(x)$ varies linearly with x and there is no plateau. Since $\nu_Q(x)$ is a continuous function of chain position x, the endpoints do not necessarily coincide with carbon-deuteron positions 2 and 16. The identification of the points $x = 0$ and 1 with positions of the hydrocarbon chains is somewhat arbitrary. We have chosen to make the corre-

spondence between x and k (the carbon number) through the relation $x = (k - k_0)/15$. Then, $x = 0$ corresponds to $k = k_0$ which does not necessarily coincide with the position of a carbon atom, i.e., k_0 may or may not be an integer. Since the position of the first methylene group corresponds to $k = 2$, we require that $k_0 \leqslant 2$. There is no other logical restriction on the value of k_0 and, since it does not enter into our analysis of the moments, we have chosen the value $k_0 = 2$ which gives the best overall fit when comparing our empirical $S(x)$ with actual measurement of $S(k)$.

Fig. 2 compares the order parameter profile obtained by the fit to the moments of the spectrum in Fig. 1a, with the profile obtained from the ^{2}H-NMR spectra of specifically deuterated membranes of *A. laidlawii* at 42°C [1]. The discrepancy between the solid surve, labelled a in this figure, and the points obtained from the specific label studies may be the result of a lower phase transition temperature for the *A. laidlawii* membranes containing large amounts of perdeuterated palmitic acid. In spite of this slight discrepancy, it is clear that the moment analysis of the spectrum of the perdeuterated membrane gives the same overall picture of the membrane order parameter profile as is obtained from the spectra of the specifically deuterated membranes.



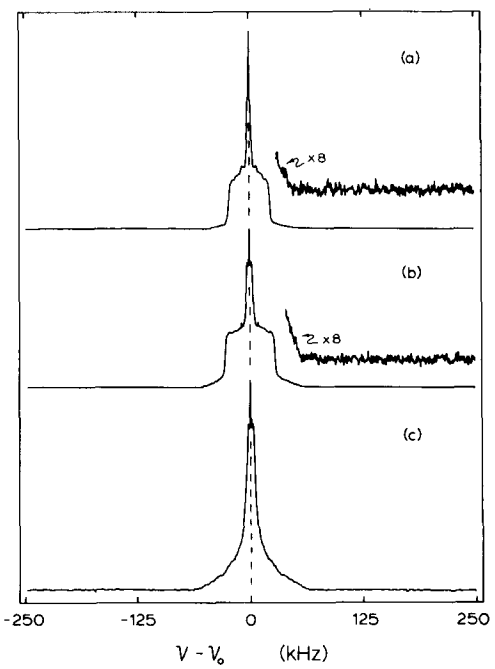


Fig. 2. The order parameter profile of *A. laidlawii* membranes. The experimental points giving the splitting of the specific deuterium labels at 42°C are from Stockton et al. [1]. The curve labelled a is obtained from the moment analysis of the spectrum of perdeuteropalmitate enriched *A. laidlawii* membranes at 42°C. Curves b and c are obtained from the moment analysis of spectra of perdeuteropalmitate and cholesterol-enriched *A. laidlawii* membranes at 42 and 22°C. D, ^{2}H.

Fig. 3. ^{2}H-NMR spectra at 34.4 MHz of perdeuteropalmitate and cholesterol enriched *A. laidlawii* membranes; 30 000 scans at 4 scans/s. (a) $T = 42$°C; (b) $T = 22$°C, the vertical scale in a and b is increased by a factor of 8, and (c) $T = 0$°C.

^{2}H-NMR spectra of the gel phase

Below 42°C the membranes undergo a transition from the fluid phase to the gel phase. The spectrum in Fig. 1b was obtained at 22°C, where most of the membrane is in the gel state. The small feature at the center of the spectrum, labelled fluid in the figure, indicates that there is still a very small amount of fluid phase lipid at this temperature. The shape of the spectrum in Fig. 1b is nearly identical to that of the spectrum observed at the same temperature in chain perdeuterated DPPC [8]. This shape seems to be characteristic of the membrane gel phase [5,8] just as the shouldered 'plateau' shape is characteristic of the fluid phase.

At 0°C, Fig. 1c, the spectrum is similar to that observed at the same temperature in DPPC [8]. The strong square shoulders have the maximum quadrupolar splitting of 126 kHz corresponding to the complete absence of motion. When the temperature is decreased from 22°C the intensity at ±63 kHz increases steadily, indicating that an increasing fraction of the chains have ceased the reorientation about their long axes [7,8]. However, the intensity of the component at the center of the spectrum indicates that, even at 0°C, an appreciable fraction of the chains are still undergoing this reorientation.

The effect of cholesterol

Fig. 3a, b and c, are spectra taken for the membranes of *A. laidlawii* cells grown on the medium supplemented with cholesterol as well as perdeuteropalmitic acid. As has been observed with fatty acid [21] and electron spin label probes in model systems [31] and in the palmitate-enriched membranes of *A. laidlawii* [32], the presence of cholesterol increases the degree of membrane order at higher temperatures [1]. Fig. 3a, the spectrum at 42°C, shows that in the presence of cholesterol there is still a well-defined plateau. The quadrupolar splitting of the plateau edge gives $|S_{C^2H}|_{plateau} \simeq 0.36$, that of the methyl group, $|S_{C^2H}|_{methyl} \simeq 0.035$, and that of the 15 position $|S_{C^2H}|_{15} \simeq 0.135$.

At 22°C, Fig. 3b, the spectrum retains the overall shape it had at 42°C, becoming somewhat broader. The plateau edge here gives $|S_{C^2H}|_{plateau} \simeq 0.44$, while the methyl group has $|S_{C^2H}|_{methyl} \simeq 0.044$. The fluid to gel phase transition has apparently been eliminated and the membrane maintains a well-defined order parameter plateau throughout this temperature region.

These spectra can be analyzed in terms of the moments in the same manner as the fluid phase spectrum of Fig. 1a. At 42 and 22°C we obtain the order parameter profiles (of the spectra in Fig. 3a and b) shown in Fig. 2, curves b and c. The primary difference between these two curves is the larger value of $\nu_Q(0)$ at the lower temperature. While both curves exhibit the 'plateau', there is an observable difference in the values of μ.

The parameters of the fit vary systematically with temperature, between 20 and 42°C. As shown in Fig. 4a, $\nu_Q(0)$ varies between approx. 51.5 kHz at 20°C and approx. 40 kHz at 42°C. This strong variation in the quadrupolar splitting in the plateau region of the chain is a reflection of the decreased motional averaging at lower temperatures. The parameter $\nu_Q(14/15)$, Fig. 4, identified with the quadrupolar splitting of the 16-position (methyl group), is, within the error, independent of temperature throughout this temperature range.

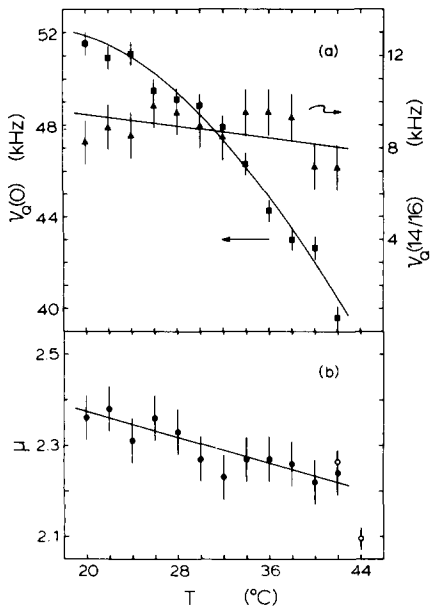


Fig. 4. The temperature dependence of the parameters of the quadrupolar splitting profiles obtained from the moment analysis of the spectra of *A. laidlawii* membranes enriched in perdeuteropalmitate plus cholesterol. (a) The quadrupolar splittings of the two ends of the chain, $\nu_Q(0)$, left-hand scale, (■) and $\nu_Q(14/16)$, right-hand scale (▲). (b) The exponent μ. Open circles at 42 and 44°C are for membranes enriched only in perdeuteropalmitate.

Due to the nature of the function being minimized by the fitting procedure (Eqn. 8), the parts of the spectrum with larger quadrupolar splittings are more heavily weighted than those with smaller splittings. Thus, the fit is much less sensitive to the value of $\nu_Q(1)$ than it is to the value of $\nu_Q(0)$, and the uncertainty in $\nu_Q(1)$ is correspondingly larger. The value of $\nu_Q(1)$ is also strongly dependent on the value of the exponent, μ, especially when μ is large. Using this fitting procedure, the values of $\nu_Q(0)$ and μ are determined more accurately than $\nu_Q(1)$.

The values obtained for μ are plotted vs. temperature in Fig. 4b. The exponent μ is significantly larger at lower temperatures, indicating that the plateau region of the chain is longer at lower temperatures. A similar behaviour is observed in the moment analysis of the fluid phases spectra of chain perdeuterated DPPC (Davis, J.H., unpublished result).

These results demonstrate how this moment analysis can be used to study the influence of variables such as temperature and cholesterol content on the membrane order parameter profile. The error bars on the values of the parameters, shown in Fig. 4a and b indicate the range of values over which the moments calculated from $\nu_Q(x)$ agree, within error, with the experimental moments. Table I compares, at selected temperatures, the values of the moments calculated from $\nu_Q(x)$ with the experimental moments.

The incorporation of large amounts of cholesterol into the membrane of *A. laidlawii* causes an increase in the mean acyl chain order at temperatures above the growth temperatures of 37°C. The increase in order is much larger

TABLE I

COMPARISON OF EXPERIMENTAL MOMENTS * WITH THOSE CALCULATED FROM $\nu_Q(x) = \nu_Q(0)\{1-[(\nu_Q(0)-\nu_Q(1))/\nu_Q(0)]x^{\mu}\}$

Sample **	Temperature (°C)	M_1^{exp} $\times 10^{-4}$ (s^{-1})	$m_1(\nu(x))$ $\times 10^{-4}$ (s^{-1})	M_2^{exp} $\times 10^{-9}$ (s^{-2})	$m_2(\nu(x))$ $\times 10^{-9}$ (s^{-2})	M_3^{exp} $\times 10^{-14}$ (s^{-3})	$m_3(\nu(x))$ $\times 10^{-14}$ (s^{-3})	M_4^{exp} $\times 10^{-19}$ (s^{-4})	$m_4(\nu(x))$ $\times 10^{-19}$ (s^{-4})
Sample 1	44	4.69	4.70	3.42	3.41	3.12	3.13	3.35	3.36
Sample 1	42	5.15	5.18	4.10	4.14	4.06	4.11	4.70	4.79
Sample 2	42	6.67	6.76	7.10	7.15	9.32	9.59	14.3	14.9
Sample 2	32	8.11	8.15	10.4	10.4	16.7	16.8	31.3	31.7
Sample 2	22	8.68	8.76	12.0	12.0	20.4	20.8	40.6	41.9

* Higher moments, M_4 to M_8 agree within experimental error in all cases, e.g., the values of M_8^{exp} and $m_8(\nu_Q(x))$ typically agree to better than 10%.

** Sample 1 is *A. laidlawaii* membranes enriched in palmitate-d_{31} while sample 2 is enriched in palmitate-d_{31} and cholesterol.

for positions near the carboxyl region of the acyl chains than for those near the terminal methyl groups, in agreement with earlier studies on egg phosphatidylcholine/cholesterol [21]. The order parameter plateau is well-defined between 20 and 42°C. The primary change over this temperature range is an increase in the plateau order parameter at lower temperatures. At 20°C, $|S_{C^2H}|_{plateau} \simeq 0.46$, a value which is very close to the value of 0.5 expected when the only motion remaining is a rapid rotation (or reorientation) about the long axis of the chain.

Below 20°C, the shape of the spectra changes dramatically, and the spectra can no longer be analyzed in terms of the moments of $\nu_Q(x)$. The changes in the spectrum occurring between 0 and 20°C do not seem to be characteristic of a system undergoing a gel-to-fluid phase transition. The spectrum at 0°C, shown in Fig. 3c, bears little resemblance to any of the gel phase spectra of the sample which contains no cholesterol (Fig. 1b and c). The weak shoulders at ±25 kHz and the large intensity out to ±63 kHz suggest that this spectrum may contain two components, one that has the shape of the spectrum in Fig. 3b (accounting for the weak shoulders), and another broader component.

Temperature dependence of the moments of spectra

The moments of the ^{2}H-NMR spectra are sensitive to the degree of acyl chain order. Since the increase in entropy of the phospholipid gel-to-liquid-crystalline phase transition is primarily due to the melting of the hydrocarbon chains [33], the moments of the spectra provide a useful picture of this phase transition [8]. The first moment, M_1, is proportional to the mean C-^{2}H bond order parameter, $\langle S_{C^2H} \rangle$, while M_2 is proportional to $\langle S^2_{C^2H} \rangle$. Fig. 5a shows the variation of the second moment, M_2, with temperature for the spectra of

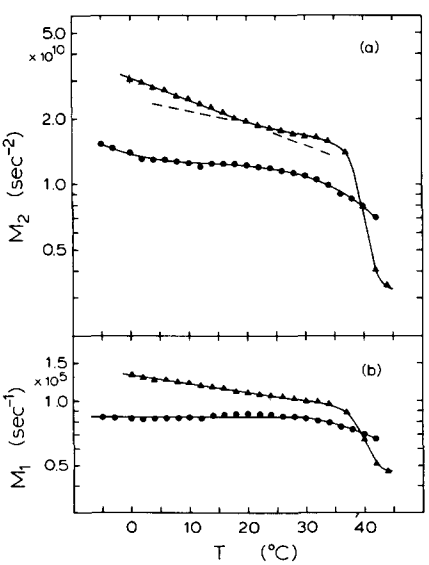


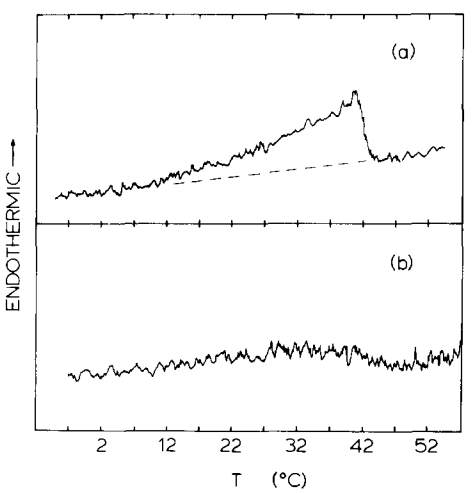
Fig. 5. The temperature dependence of the first two moments of the spectra of *A. laidlawii* membranes enriched in perdeuteropalmitate (▲) and perdeuteropalmitate plus cholesterol (●). (a) M_2 vs. T; (b) M_1 vs. T.

membranes with and without cholesterol, while Fig. 5b shows the variation of M_1. For the sample without cholesterol there is a sharp change in both M_1 and M_2 between 34 and 42°C indicating that there is a well-defined transition in the orientational order of the hydrocarbon chains. Below 37°C there is a gradual increase in M_1 and M_2 due to a decrease in the motional averaging of the quadrupolar interaction (i.e., an increase in hydrocarbon chain order) at lower temperatures. Also, there is a significant change in the slope of the variation of M_2 with temperature at approx. 20°C.

Differential scanning calorimetry of part of the cholesterol-free sample used for the ^{2}H-NMR studies also shows a clearly defined phase transition occurring near 42°C, Fig. 6a. This curve, and similar results reported on *A. laidlawii* [13,14] and *E. coli* [10], is highly asymmetric. The origin of this asymmetry is not clear.

The parameter Δ_2, defined by Eqn. 5, is shown as a function of temperature for the two samples in Fig. 7. For the sample without cholesterol, the variation of Δ_2 with temperature is reminiscent of the variation of Δ_2 for multilamellar dispersions of chain perdeuterated DPPC [8]. There is a strong maximum at the phase transition (since between 37 and 42°C the sample is in a mixed phase region) followed by a gradual decrease at lower temperatures. It is not clear whether the asymmetry in the variation of Δ_2 is related to the asymmetry in the phase transition as observed by differential scanning calorimetry. There is a local minimum in Δ_2 between 20 and 30°C, below which Δ_2 increases again and appears to level off at 0°C. The behaviour between 0 and 30°C is similar to that observed in DPPC [8].

The interpretation of these results for the *A. laidlawii* membranes without



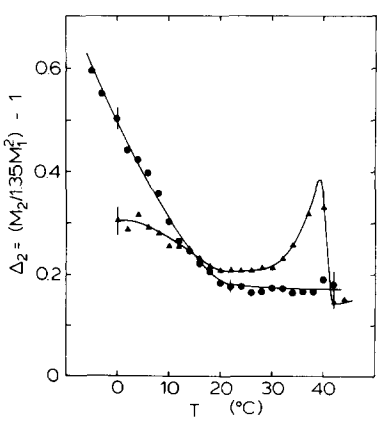


Fig. 6. Differential scanning calorimetry curves taken at a scan rate of 10°C/min. (a) *A. laidlawii* membranes enriched in perdeuteropalmitate; (b) *A. laidlawii* membranes enriched in perdeuteropalmitate plus cholesterol.

Fig. 7. The temperature dependence of the mean squared width of the distribution of quadrupolar splittings. *A. laidlawii* membranes enriched in perdeuteropalmitate membranes, ▲; membranes enriched with both perdeuteropalmitate and cholesterol (●).

cholesterol [7] is the same as that given for the model system [8]. From 20 to 30°C, the sample is in a relatively well-defined gel phase where most of the molecules are undergoing a rapid reorientation about their long axes. The fact that the spectrum in this region is not a simple two-component (one for methyl groups, one for methylenes) powder pattern spectrum (expected for identical molecules in the all-*trans* conformation) suggests that there may be some variation in order along the chain, or from one phospholipid to another giving rise to a distribution of quadrupolar splittings [8].

Below 20°C, the spectra indicate that as the temperature decreases an increasing fraction of the molecules cease the reorientation about their long axes. At sufficiently low temperatures it is anticipated that all of the molecules become rigid and that the spectrum would exhibit the maximum quadrupolar splitting (126 kHz) [8]. A similar conclusion has been reached from ^{2}H-NMR data for *A. laidlawii* membranes enriched in [13,13-$^2H_2$]palmitic acid [7].

The membranes of *A. laidlawii* grown in the presence of cholesterol behave very differently. The plots of M_1 and M_2 (Fig. 5a and b) do not show any indication of a clearly defined phase transition. The values of M_2 at 42 and −5°C differ by only about a factor of two (compared to the factor of ten difference between the values of M_2 at 44 and 0°C for the sample without cholesterol). This weak temperature dependence of the moments does indicate that there is a gradual increase in the average acyl chain order as the temperature is lowered. However, at 0°C, the value of M_2 for the cholesterol-containing samples is almost the same as that of M_2 at 37°C (the growth temperature) for the cholesterol-free sample.

For the membranes containing cholesterol, the parameter Δ_2 (Fig. 7) is constant from 20 to 42°C, illustrating in a very striking manner that the phase transition observed in the sample without cholesterol has been suppressed. Below 20°C, there is a strong gradual increase in Δ_2 which shows no indication of leveling off even at −5°C. The differential scanning calorimetry curve in Fig. 6b, for the sample containing cholesterol, does not show any clearly defined phase transition near the growth temperature or below 20°C.

At 20°C a large fraction of the methylene groups on the hydrocarbon chains (those contributing to the plateau) has a C-^{2}H bond order parameter of approx. 0.5. This value is consistent with a rapid reorientation about the long molecular axes of molecules predominantly in the all-*trans* conformation. At lower temperatures, in order to increase the chain order substantially, the rate of this reorientation must decrease for some molecules (as happens with the cholesterol-free *A. laidlawii* membranes and with DPPC). The ^{2}H-NMR spectra are entirely consistent with this behaviour although it is more difficult to identify the contribution of the non-rotating lipids to the spectrum since the plateau at 20°C is so broad.

Below 20°C, it is possible that the phospholipid molecules are undergoing transitions between rotating and non-rotating states. This would lead to a spectrum spread out from zero frequency to the observed cut-off and hence to a large Δ_2. The value of Δ_2 would depend not only on the relative populations of the rotating and non-rotating states, but also on the correlation time for the transition between the two states. Alternatively, if a phase separation occurs,

forming regions rich and poor in cholesterol, one might expect to see the behaviour in Fig. 7, since the cholesterol-poor regions would tend to form a gel-like phase while the spectrum of those rich in cholesterol might retain the overall shape of the spectrum at 20°C. This 'mixed phase' would lead to a large distribution of quadrupolar splittings, e.g., to a large Δ_2. The value of Δ_2 would depend on the relative populations of the two components of the spectrum. Studies of specifically deuterated samples, including the relaxation behaviour, may enable us to determine whether either of these mechanisms actually occurs.

Concluding remarks

It has been previously demonstrated by ^{2}H-NMR [1,4] that, in the fluid phase, phospholipid multilamellar dispersions are useful models of the biological membrane on a microscopic scale. The strong similarities in the ^{2}H-NMR spectra of chain perdeuterated multilamellar dispersions and *A. laidlawii* membranes, and their variation with temperature (from 0 to 44°C) suggest that throughout this temperature range the model systems provide a useful description of the behaviour expected for biological membranes. The spectra of the gel phases of DPPC and *A. laidlawii* have similar shapes, and at lower temperatures both indicate that the fraction of lipids undergoing a rapid reorientation about the long molecular axis decreases gradually.

The order-parameter profile of the *A. laidlawii* membranes containing cholesterol (at temperatures between 20 and 42°C) is similar to that of the cholesterol-free *A. laidlawii* membranes above the gel to fluid phase transition. The primary difference is that the mean order parameter is much larger for the membrane containing cholesterol, in agreement with model membrane studies [21]. The spectra show unambiguously that between 20 and 42°C the membranes containing cholesterol are not composed of a mixed phase. The parameter Δ_2 of the fluid phase spectra of the membranes without cholesterol is only slightly smaller than the value of Δ_2 of the spectra of membranes with cholesterol between 20 and 42°C. This similarity in the Δ_2 values demonstrates clearly that the membrane containing cholesterol is nearly as homogeneous as the fluid phase membrane without cholesterol, and that both of these are more homogeneous than the gel-phase of the membrane without cholesterol. Below 20°C, the large increase in the value of Δ_2 for the spectra of the cholesterol-containing membranes may be an indication of the occurence of either a phase separation or a dynamic equilibrium between rotating and non-rotating states. Further experiments, especially relaxation studies (for example, the dependence of the shape of the spectrum on the spacing between the two *r-f* pulses used to form the quadrupolar echo) should resolve this uncertainty.

The moment analysis of the ^{2}H-NMR spectra of completely deuterated lipid acyl chains, whether in model or biological membranes, allows one to determine the changes in the chain C-^{2}H bond order parameter profile due to the variation of parameters such as temperature or cholesterol concentration. Correlating these variations with membrane functions should determine to what extent the physical state of the membrane affects it biological functions.

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