The States of the Lipids in Biological Membranes As Visualized by Deuterium NMR

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I. INTRODUCTION

Biological membranes represent a tremendous challenge to spectroscopists as they contain a wide variety of constituents - proteins, phospholipids, neutral lipids, and carbohydrates. The arrangements of these components with respect to one another, and the conformations of the various individual components, are thought to play an important role in the diverse biological functions performed by the membrane (1). The current view of the overall structure is that much of the lipid is present in a bilayer structure, with the hydrophilic groups of each lipid monolayer facing outwards towards the medium or inwards towards the cellular fluid, and the hydrophobic moieties facing one another to constitute a relatively impermeable barrier to water-soluble compounds. The protein components are thought to have a variety of locations, facing outwards or inwards from the bilayer, or traversing the bilayer. The fatty acyl chains of the phospholipids are considered to exist in a liquid-crystalline state at physiological temperatures, with the possibility of some fraction present in a relatively immobilized state due to lipid-protein interaction or to the phase-forming properties of the lipids themselves.

The problem facing the spectroscopist interested in such systems is how to observe a particular component of this complex mixture without interference from all the others. Probes of various types have been used, such as fluorescent (2) or paramagnetic (3) lipids, but these suffer from the fundamental objection that they are extrinsic to the system and may measure mainly the properties of the perturbation they introduce. Isotopic substitution provides an ideal non-perturbing probe, which has become increasingly easier to observe due to technological improvements. This has been accomplished using NMR of lipids labelled specifically with ^{13}C (4) or ^{2}H (5), and by IR (6) and Raman (7) spectroscopic investigations of ^{2}H-substituted lipids. In addition, NMR studies of the naturally-occurring ^{31}P (8) and ^{14}N (9) nuclei, which occur in relatively few environments, have also provided useful insight into the properties of these environments in membranes. This article will deal with ^{2}H NMR of only biological membranes, since the many studies on model membrane systems have been adequately reviewed (10,11).

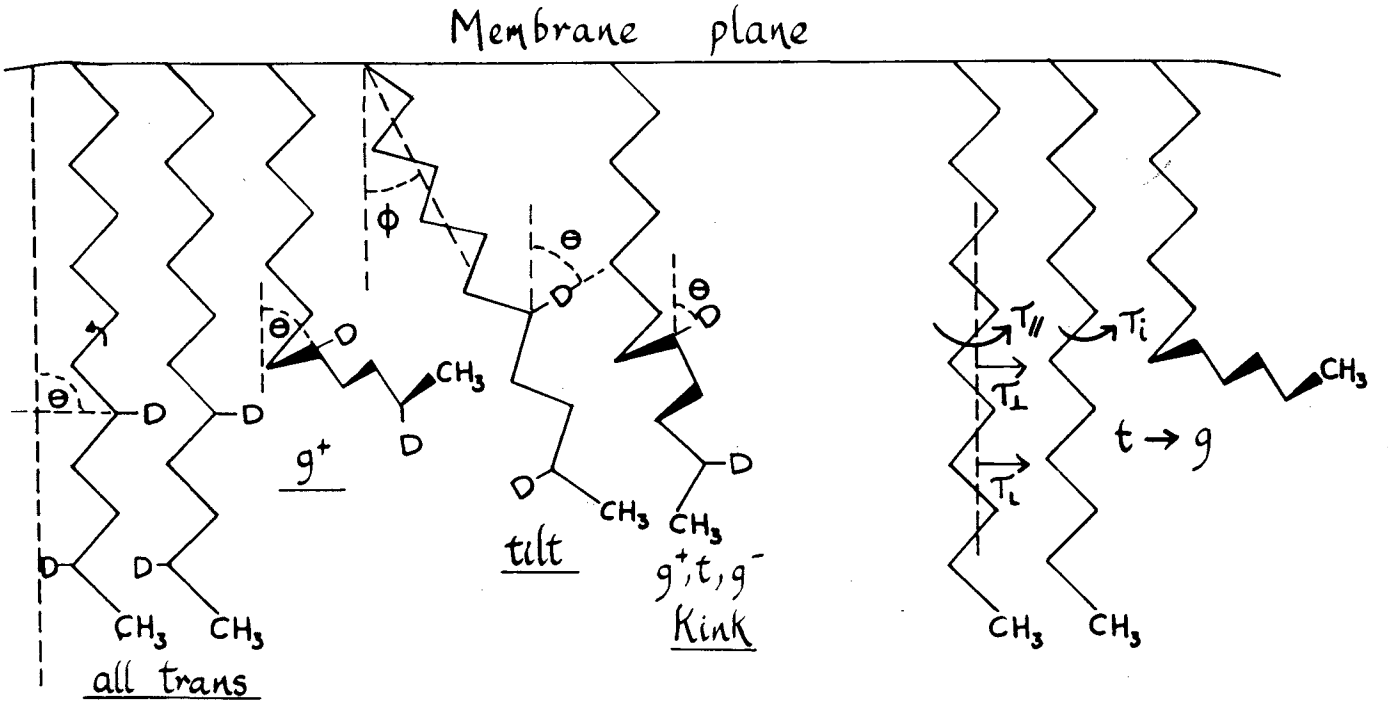

Figure 1. Schematic of the parameters used to characterize the degree of order and mobility of the fatty acyl chains of membrane lipids. The angle θ describes the instantaneous angle made by a C-D (C-^{2}H) bond with respect to the axis about which the lipids are ordered, whereas ϕ describes a possible tilting of the long molecular axes of the chains with respect to the normal. The correlation times τ are for the various possible motions of the chains: $\tau_{//}$, motion about the long molecular axis; $\tau_{\perp}$, motion normal to the long axis; τ_L, lateral motion of the entire molecule parallel to the membrane surface; τ_i, rotation about the ith C-C single bond such as is involved in *trans-gauche* conformation interconversion. Note the representation of a kink, a *gauche*$^+$-*trans*- *gauche*$^-$ conformational triad.

II. PROPERTIES OF MEMBRANE LIPIDS

It is important at the beginning of such studies to define carefully the properties one hopes to observe. Figure 1 shows the fatty acyl chains of a phospholipid as they are thought to exist in the liquid-crystalline state of a lipid bilayer. The presence of a large number of carbon-carbon single bonds allows the possibility at each such position of two *gauche* and one *trans* conformer. The relative populations of each conformer may vary greatly with position along the chain. In Figure 1 the all-*trans* state of the chains is the longest, thinnest, and most-easily packed. A single *gauche* conformer causes a large change in the length, effective width, and packing ability of the chains. This change can be partly overcome by cooperative conformational combinations, such as the *gauche*$^+$-trans-gauche$^-$ link shown in Figure 1. The ensemble-average distribution of *gauche* and *trans* conformers thus provides a measure of the molecular order of the fatty acyl chains - this can be quantitated in terms of the bond order parameter, S_{CD}, which is the ensemble and time average of the function $(3\cos^2\theta - 1)/2$, where θ is the angle between a carbon-deuterium bond and the axis of ordering.

When the appropriate transformation is made to account for the angle between the C-D bond and the long axis of the molecule, the resulting parameter, S_{mol}, has the value 1 for the *trans* conformer, and 0 for equal populations of all three conformers. This simple picture must be modified somewhat if the long molecular axis makes a net tilt with respect to the membrane normal (Figure 1). In addition, if rapid axial motion about the long molecular axis is not pre-

sent, two order parameters are needed to describe the average orientations of the chains.

A complementary aspect of the membrane state involves the various motions of the chains. The order parameter gives an average picture of the degree of organization, but does not provide insight into the rates of interconversion between *gauche* and *trans* conformers (τ_i), the rate of modulation of tilt angle ($\tau_\perp$), the rate of overall axial rotation of the chain ($\tau_\parallel$), or the rate of lateral diffusion of the entire phospholipid molecule (τ_L). These rates describe the mobilities of the lipid molecules. The vague concept of fluidity involves both the degree of organization and rates of movement of the lipids. These must be determined separately if the properties of the lipids are to be described unambiguously.

III. DEUTERIUM NMR OF ORDERED SYSTEMS

The relatively small quadrupole moment of deuterium makes it an ideal probe of membrane lipids. The underlying physics of these systems has been presented in two recent reviews (10,11). Briefly, rapid motion of the acyl chains results in a partial averaging of the principal components of the quadrupole splitting tensor. The resulting space- and time-averaged tensor components are direct measures of the amplitude of the allowed motion, and hence of the distribution of *gauche* and *trans* conformers (and tilt angle, if present). Figure 2 summarizes the behavior observed under such circumstances - the two principal peaks in the powder type spectrum yield the quadrupole splitting, D_q, which is directly related to the order parameter, S_{CD}. This simple picture applies in the case of rapid motional averaging; if molecular motions are slow on the ^{2}H NMR time scale, more complex line shapes can occur.

Although the quadrupole coupling constant of deuterium in a C-D bond is relatively small (*ca* 170 kHz), it can lead to spectra which are broad in the usual high resolution terms with which most of us are familiar. To observe ^{2}H NMR spectra for such systems with an adequate fidelity of lineshape, the spectrometer must have a high sensitivity, a short 90° pulse (< 10 μs), a very rapid response to signal, and the ability to digitize spectra rapidly (*ca* 500 kHz). The details of spectrometer design and methods will

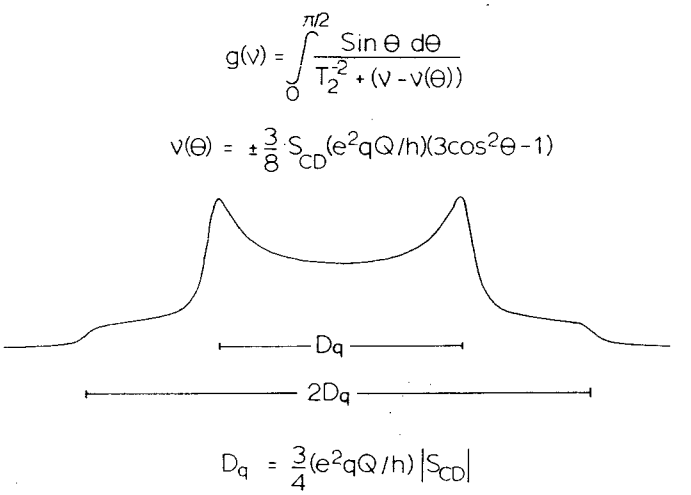

Figure 2. A theoretical ^{2}H NMR powder pattern for a C-^{2}H bond in a partially ordered system which, like a biological membrane, rotates slowly on the time scale of deuterium quadrupole splittings. The spectrum $g(\nu)$ is an envelope of the many spectra $\nu(\theta)$ for different orientations of the C-^{2}H bond with respect to the applied magnetic field of the spectrometer. The separation D_q gives a direct measure of the segmental order parameter, S_{CD}.

be covered in a forthcoming review (12). A major breakthrough was the use of the quadrupole echo technique to overcome problems of very rapid signal decay after the excitation pulse (13). Some aspects of the problem have been described recently (14).

IV. *Acholeplasma laidlawii B* - A RELATIVELY SIMPLE MEMBRANE SYSTEM

The microorganism *Acholeplasma laidlawii*, strain B, belongs to the sub-bacterial family *Mycoplasmatacae*, minute prokaryotic organisms approaching the larger viruses in size. However, unlike viruses, mycoplasmas are capable of autonomous growth and reproduction in cell-free media. They are not only the smallest self-replicating organisms, but also the simplest in ultrastructure. They have no cell walls or intracellular membranous structures, and are bounded by a single membrane, the plasma membrane (15).

The structural simplicity of *A. laidlawii* is accompanied by a relatively simple biochemistry, such that complex media are required for their growth.

Membrane lipid components or their precursors must be provided, offering a convenient pathway for regulation of fatty acid composition and introduction of isotopic labels. By depleting the medium of fatty acid, and then supplementing it with that desired, incorporation of particular fatty acids to levels of 60-80% of total fatty acid can be accomplished. Furthermore, by inclusion in the medium of the egg white protein avidin, which complexes the coenzyme biotin required for fatty acid elongation, some fatty acids can be incorporated to levels near 100% (16).

Isolated membranes of *A. laidlawii* can be prepared by the simple and mild process of osmotic lysis. They may then be freeze dried and stored without any apparent (by the NMR criteria) structural modification.

V. VARIATION OF ORDER PARAMETER WITH POSITION OF LABELLING OF THE FATTY ACYL CHAINS

We have cultured *A. laidlawii* on two different fatty acids in which the position of the deuterium label was varied from one end to the other. Palmitic acid is a saturated, sixteen-carbon chain, which is known to result in membranes of a relatively high rigidity. Oleic acid, which has a *cis* double bond at position 9 and a linear chain of eighteen carbons, is known to induce a high degree of fluidity in membranes. Calorimetric studies on *A. laidlawii* membranes enriched in these fatty acids indicate a transition from a highly-organized, relatively rigid, gel state to

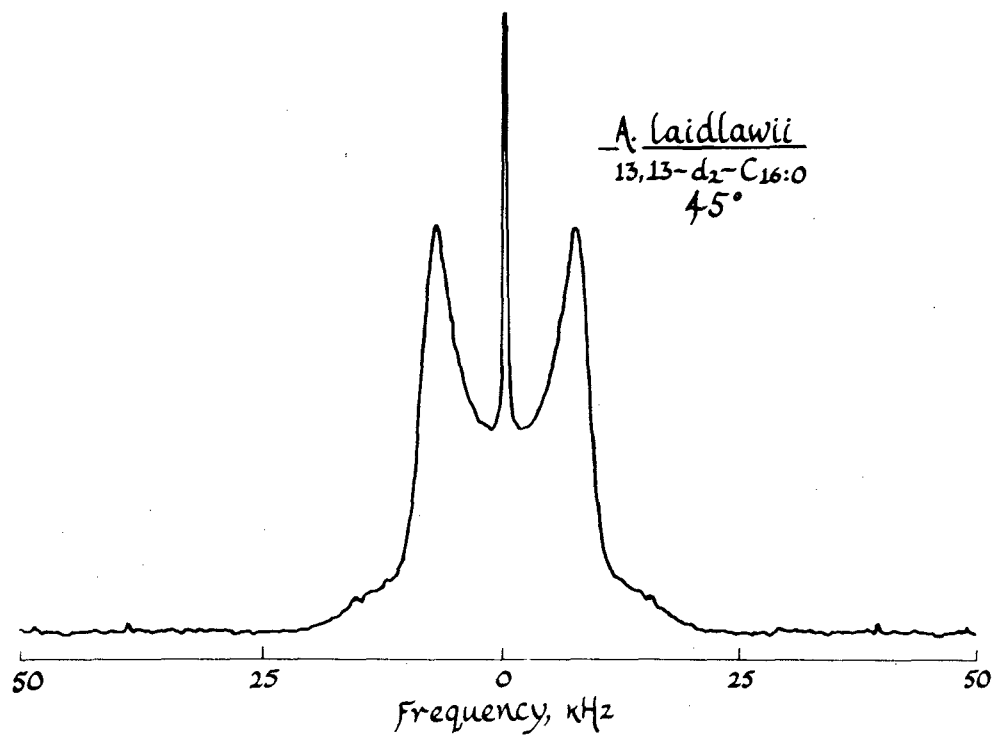

Figure 3. ^2H NMR spectrum (34.4 MHz) of membranes of *Acholeplasma laidlawii* enriched to 75% in 13,13-$^2H_2$-palmitic acid. The organism was grown at 37°C and membranes prepared according to methods described in (5). Experimental conditions: 45°C; 110,000 accumulations; pulse angle 90° (9 μs); quadrupole echo sequence 90°_x-τ-90°_y-echo, τ = 60 μs; recycle time, 0.25 s; Δ_2 = 0.029. The narrow line in the centre of the pattern is due to deuterium at natural abundance in water. The spectrum was taken in single phase, on resonance, and reflected about the centre frequency.

a less-ordered, relatively fluid, liquid-crystalline state over the temperature range 20 to 50°C (palmitic acid) and −22 to −4°C (oleic acid) (17).

Figure 3 shows the ^2H NMR spectrum of *A. laidlawii* membranes enriched in 13,13-d_2-palmitic acid, at a temperature above the midpoint of the gel-liquid crystal phase transition. The spectrum shows no sign of components due to gel state lipid, and a moment analysis (*vide infra*) indicates that the membrane lipid is highly homogeneous. Similar spectra for the other positions yield the data shown in Figure 4. These data constitute the first detailed picture of the variation of molecular order with position in a biological membrane (18). The plateau-like behavior for the first ten positions, and the steep drop in order

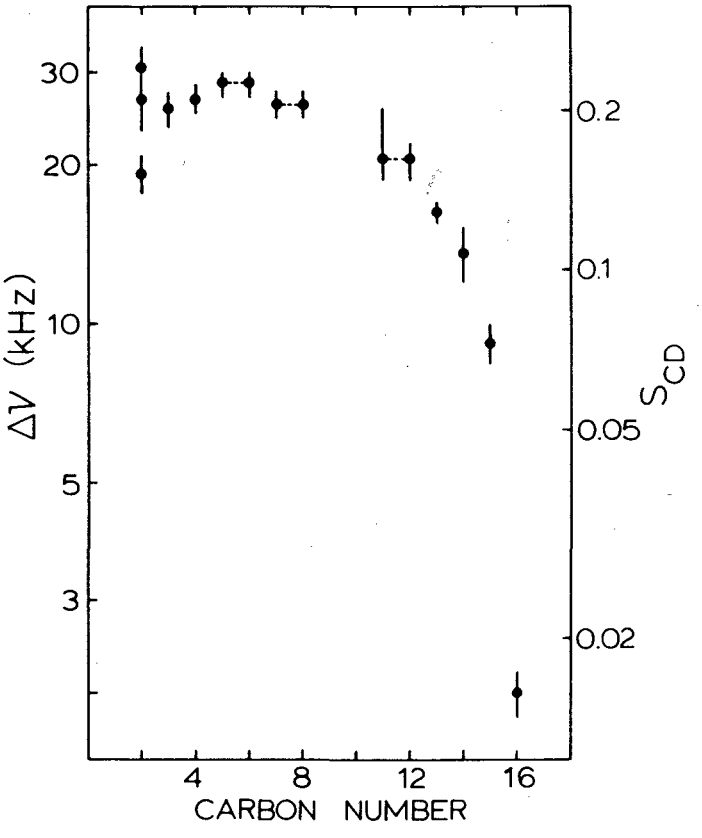

Figure 4. Variation of the quadrupole splitting ($\Delta\nu$), or the segmental order parameter (S_{CD}), on position of labelling in the palmitic acid-enriched membranes of *A. laidlawii*. The molecular order parameter, S_{mol}, is calculated from S_{CD} by multiplication by 2 for the methylene groups, or by 6 for the methyl group (18).

with successive positions, is remarkably similar to that seen in model membranes of dipalmitoyl phosphatidylcholine (19) and egg yolk phosphatidylcholine (20). A general decrease of ordering on proceeding deeper into the bilayer is expected in view of the covalent attachment of the fatty acyl carbonyl group to the hydrophilic head group region of the bilayer and the lack of any covalent restriction at the methyl terminus. The constancy for the first 8-10 positions is not easy to rationalize, but can be thought of as due to the proximity of two covalently attached fatty acyl chains and the balance between electrostatic forces at the surface of the membrane and hydrophobic (Van der Waals) forces at the centre. Thus, the outer edges of the bilayer are highly organized ($S_{mol} \sim 0.4$), whereas the centre is very disorganized. The observation of three separate quadrupole splittings for carbons at position 2 of the fatty acyl chains may due to the inequivalence of the *sn*-1 and *sn*-2 chains (21), with a further inequivalence of the two deuterons on the *sn*-2 chain, as clearly demonstrated for dipalmitoyl phosphatidylcholine by Seelig and coworkers (22). Another possible source of the multiple splittings is the heterogeneity of head group composition in the lipids of *A. laidlawii* B (23,24). We are presently isolating substantial quantities of these labelled lipids to investigate this possibility in multilamellar dispersions of the pure lipids.

Turning now to the same measurement for the membranes enriched in oleic acid (25), shown in Figure 5, we see both similarities and differences on comparison with the data of Figure 4. The presence of the *cis* double bond at position 9 results in a severe drop in order parameter, as expected. However, further from this position, the profile is more similar to that of the palmitate-enriched membranes. The profile in Figure 5 is essentially identical to that seen for the oleoyl chains in the model membrane 1-palmitoyl-2-oleoyl phosphatidylcholine (26). There is also a great similarity to the several points that have been determined for *Escherichia coli* membranes enriched in ^2H-labelled oleic acid (27).

The foregoing leads to the conclusion that, for a given type of fatty acyl chain measured at the same reduced temperature ($(T - T_c)/T_c$, where T_c is the midpoint of the gel-liquid crystal phase transition), the variation of molecular ordering with position of labelling is very similar for all systems studied, whether model or biological membrane (28).

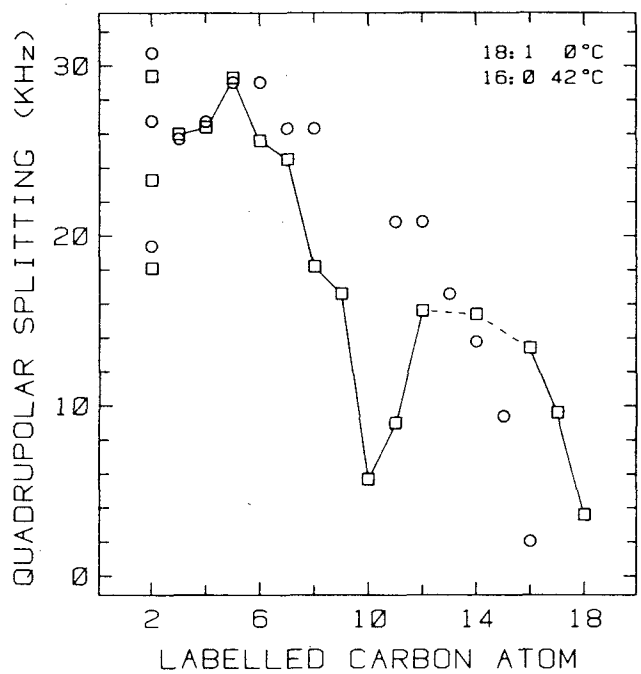

Figure 5. Comparison of the dependence of deuterium quadrupole splitting on position of labelling in *A. laidlawii* membranes enriched in oleic acid—18:1, 0°C, squares (25)—or palmitic acid—16:0, 42°C, circles (18).

VI. THE SPECTRA OF GEL STATE LIPID

Until the advent of the quadrupole echo techniques (13) the spectra of gel state lipid were inaccessible. Cooling the membranes of *A. laidlawii* below their gel-liquid crystal phase transition temperature resulted in a disappearance of the spectrum (5). With the aid of M. Bloom and J.H. Davis, we were able to visualize the spectra of gel state lipid in membranes of *A. laidlawii* enriched in palmitic acid (18). Figure 6 shows the spectra of these membranes, containing 13,13-d_2-palmitic acid, as a function of temperature (29).

As 45°C, on the upper edge of the phase transition detected calorimetrically, we see the characteristic spectrum of a liquid-crystalline lipid. In the middle of the phase transition, at 37°C, two distinct spectral components are apparent, one very similar to that seen at 45°C and another of width 60 kHz due to gel state lipid. By 25°C this latter spectrum dominates. The first conclusion to be drawn from the above data is that the two states of the membrane lipid are in slow exchange with one another on the ^{2}H NMR time scale ($< 4 \times 10^4$ s^{-1}). This means that at the growth temperature, 37°C, the membrane lipid is highly heterogeneous. Large areas of discontinuity between the phases must exist; these areas could serve as channels for the passive permeability of the membranes to small molecules. Large increases in permeability have been detected in the region of the lipid phase transition in a variety of model membranes (30,31). The second conclusion refers to the nature of the gel state lipid; a quadrupole splitting of 60 kHz is that expected for a segment of an all-*trans* chain undergoing rapid rotation about the long molecular axis. Further decrease in temperature from 25 to 3°C results in a broadening of the gel state pattern to *ca* 120 kHz. This implies that over this temperature range the rapid axial motions of the chains decrease in rate so as to be imperceptible on the ^{2}H NMR time scale ($< 6 \times 10^4$ s^{-1}). Thus, it appears that just below the phase transition the chains are highly ordered (essentially all-*trans*), but rotating rapidly about the axis of molecular ordering ($\tau_{||}$ in Figure 1 is less than 10^{-5}s), whereas at lower temperatures they are highly ordered and immobile. These conclusions have been verified by recent Fourier transform infrared spectroscopic studies on this system (6).

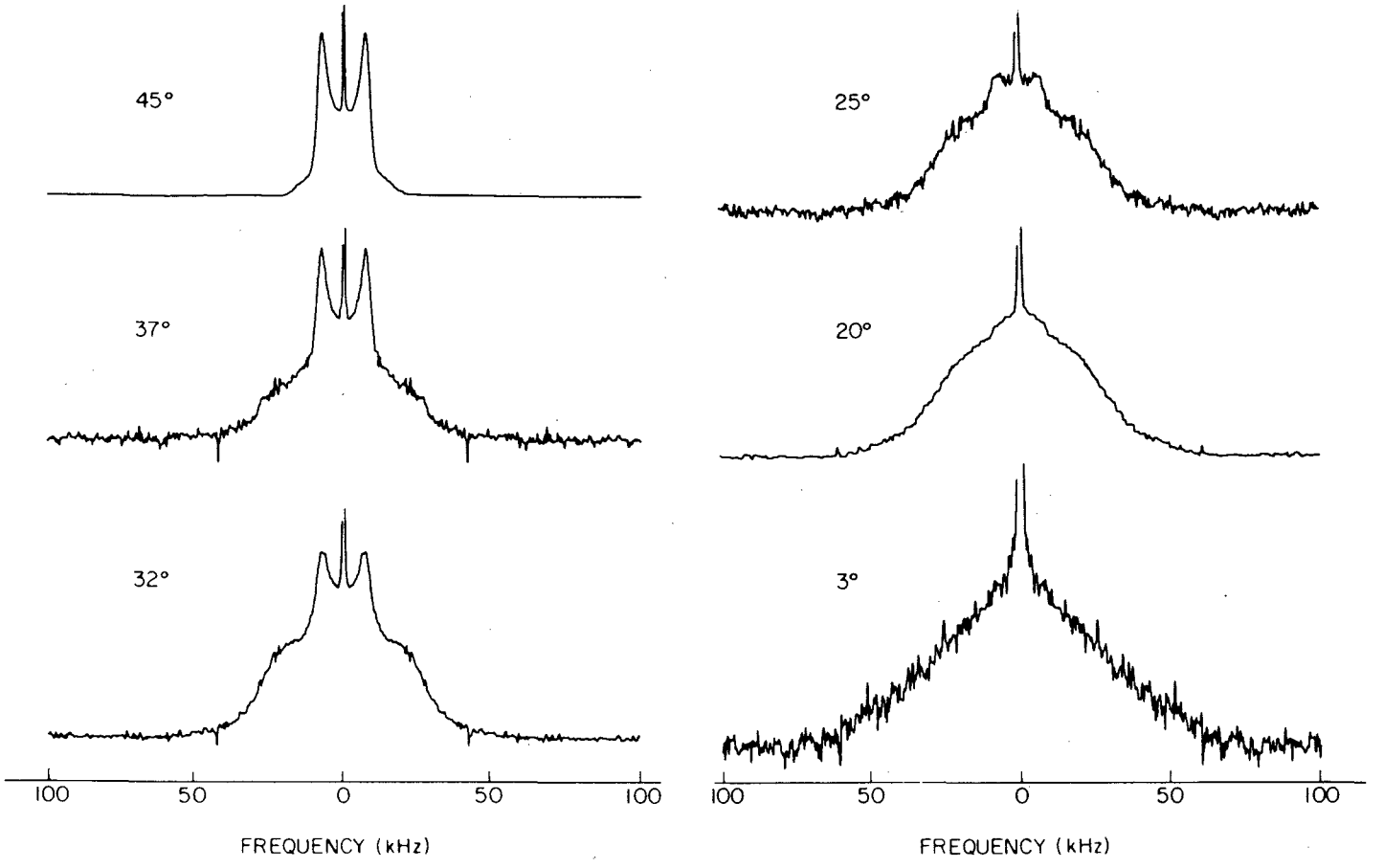

Figure 6. ^2H NMR spectra (34.4 MHz) of the 13,13-$^2H_2$-palmitoyl chains in the membranes of *A. laidlawii* at various temperatures. The spectral acquisition conditions were as in Figure 3, but increasing numbers of scans were required with decreasing temperature. A better definition of the lineshapes at low temperature, but with reduced signal-to-noise ratio, could be obtained by using 30° pulses (3 μs) in the quadrupole echo sequence (29).

We have just completed similar studies on *A. laidlawii* membranes enriched in ^2H-substituted oleic acid (25). The same coexistence of gel and liquid-crystalline lipid was observed, albeit at lower temperatures, and the liquid-crystalline phase was found to persist to temperatures 10°C lower than the cessation of the phase transition as seen by calorimetry.

Our earlier observation, that 50% of the lipid in palmitate-enriched membranes exists in the gel state at the growth temperature, leads to the possibility that such an inhomogeneous membrane is essential for optimal growth. Comparison with the data for the oleate-enriched membranes shows that this hypothesis is invalid. In Figure 7 we compare the spectra of oleate- and palmitate-enriched membranes, labelled at similar positions, at temperatures near those for optimal growth, but corresponding to very different reduced temperatures (0.192 for 18:1, 0.0 for 16:0). Moment analysis (*vide infra*) reveals that the 18:1-enriched membranes are highly-homogeneous, with no trace of gel state or protein-immobilized lipid, whereas the 16:0-enriched membranes are very heterogeneous, and contain roughly 50% gel phase lipid. Although the growth yields for the two types of membranes are roughly equal at 37°C, the 18:1-enriched membranes appear to grow somewhat more readily than their 16:0 counterparts. Thus, gel state lipid is not essential for optimal growth of *A. laidlawii*.

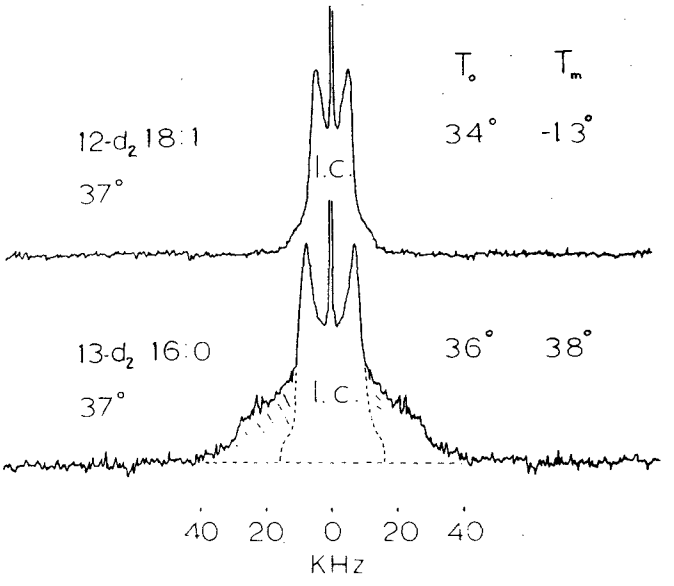

Figure 7. Comparison of the ^2H NMR spectra (34.4 MHz) taken at 37°C of *A. laidlawii* membranes grown at 37°C and enriched in oleic acid (12,12-$^2H_2$-18:1, 64 mol % of total lipid) with those of membranes enriched in palmitic acid (13,13-$^2H_2$-16:0, 75 mol %). The spectral acquisition conditions were as in Figure 3.

VII. THE USE OF SPECTRAL MOMENTS IN ANALYSIS

As the quality of the ^2H spectra becomes better, it is clear that a level of analysis using more than the quadrupole splitting or the width of the pattern is necessary to extract the greatest possible amount of information from the spectra. For many years, spectral moments have been used in the study of solids by NMR (32). The *n* th moment of a spectrum is defined by:

$$M_n = \int_0^\infty x^n F(x)dx \Big/ \int_0^\infty F(x)dx$$

where $F(x)$ is the spectral lineshape, $x = \omega - \omega_0$, and ω_0 is the central frequency. We perform our integrations from the centre of the spectra in order to conserve the odd moments, which otherwise would be identically zero due to the symmetry of the spectra. As the moments give extra weight to the wings of the spectra, they are particularly sensitive to the rates of motions which partially average the components of the quadrupole splitting tensor. The second moment is often used as a monitor of molecular motion. Figure 8 shows the temperature dependence of the second moment for the spectra of Figure 6. The onset of the phase transition is seen in the large increase in M_2 from 45° to 25°C. If all motion had ceased at 25°C, there would be no further change in M_2 with decreasing temperature; on the contrary, it increases even more steeply over the lower range, indicating the decrease in rate of axial motion referred to earlier. However, even at the lowest temperature in Figure 8, molecular motion is still occurring at a significant rate on the ^2H NMR time scale, as M_2 is far below the rigid-lattice limit of 1.28×10^{11} s^{-2}. Part of this deviation from the rigid-

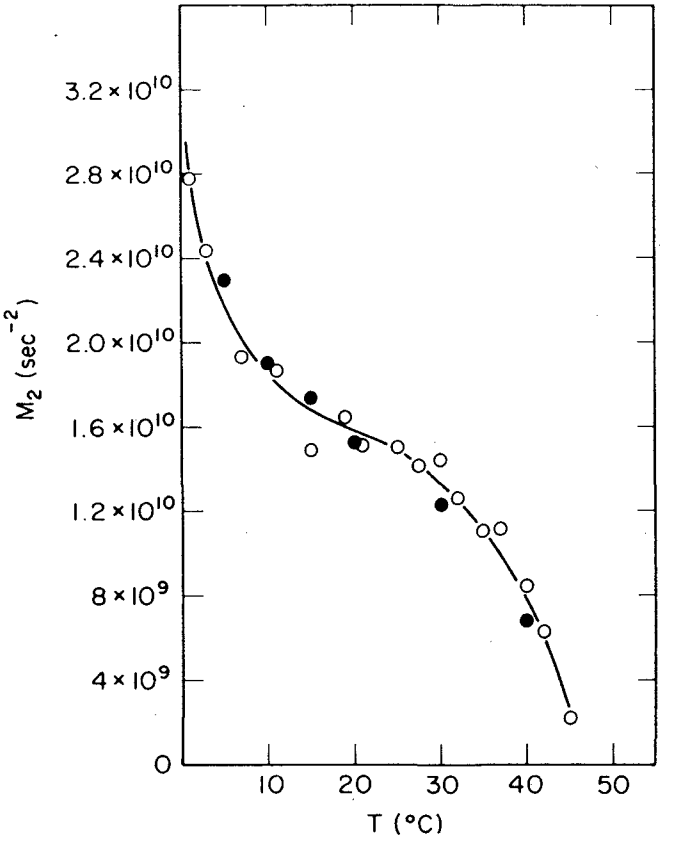

Figure 8. Variation with temperature of the second moment (M_2) of the ^2H NMR spectra (34.4 Mz) of *A. laidlawii* shown in Figure 6. The filled and open circles are for increasing and decreasing temperature, respectively (29).

lattice limit may also be due to the difficulty of obtaining the full lineshape when its extremities approach separations of 200 kHz.

Further insight into the information contained in the ^2H NMR powder spectra may be gained from a moment analysis proposed by Bloom and coworkers (33). They showed that the moments of the order parameter distribution, S_n, can be simply related to those of the ^2H NMR spectrum, M_n, and that a parameter Δ_2 can be defined

$$\Delta_2 = (S_2 - S_1^2)/S_1^2 = (M_2/1.35M_1^2) - 1$$

where

$$S_n = \int_0^\infty S^n P(S) dS,$$

and $P(S)$ is the probability of a given order parameter. Thus, the Δ_2 parameter is a direct measure of the mean square deviation of the order parameter, or the degree of homogeneity of the acyl chain packing. Figure 9 shows the temperature dependence of Δ_2 for 12,12-d_2-oleic acid incorporated into the membrane lipids of *A. laidlawii*. At temperatures far above that of the phase transition, this parameter has a value very near zero, indicating a narrow distribution for S and a high degree of homogeneity for the lipids. At 10°C, where the spectra show no traces of gel state lipid, Δ_2 starts to increase due to slowing down of molecular motions and to increasing spread in S. Over the range of the phase transition, Δ_2 has a high value as a consequence of the phase heterogeneity and (most likely) a wide distribution of S values. At very low temperatures, Δ_2 returns to a low value, suggesting a relatively high degree of homogeneity in the rigid, strongly ordered lipids.

Another very useful application of spectral moments is to analyze the data for perdeuterated fatty acyl chains in biological membranes. In model systems, a considerable number of individual quadrupole splittings can be measured directly (34, 35), whereas the greater linewidths found in biological membranes obscure much of this detail (18,36). By fitting the first four moments of the spectrum in terms of a polynomial, the dependence of the order parameter on position of labelling of the palmitate chains in *A. laidlawii* membranes could be determined (36). This procedure served to facilitate a study of the influence of cholesterol on the lipids of *A. laidlawii* (*vide infra*).

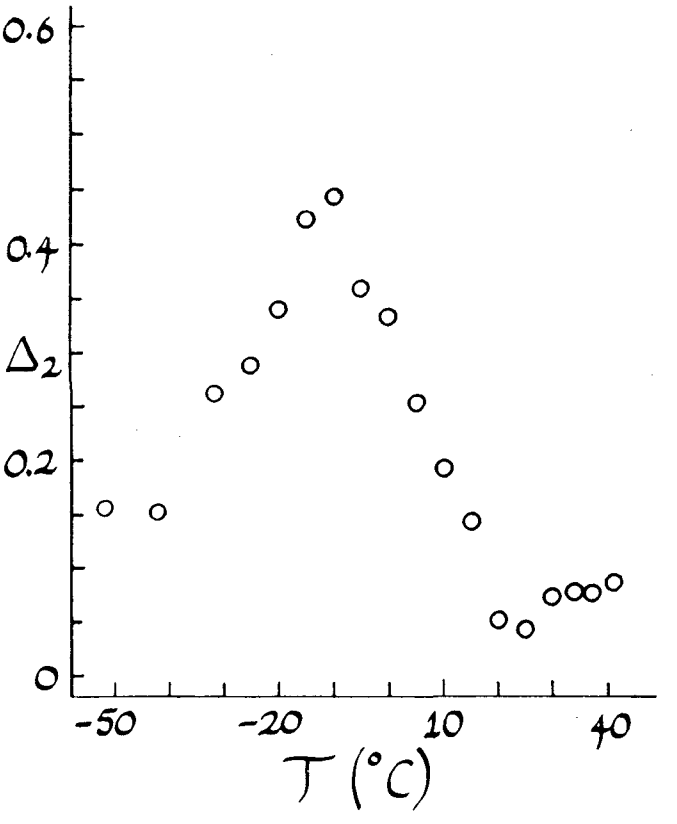

Figure 9. Temperature dependence of the parameter Δ_2, the mean square deviation in the apparent order parameter, for membranes of *A. laidlawii* enriched in 12,12-$^2H_2$-oleic acid (25).

VIII. THE INFLUENCE OF CHOLESTEROL

In model membranes, cholesterol is known to increase the degree of order in liquid-crystalline lipids, and to decrease order in gel state lipids. Using perdeuterated palmitic acid and the method of moments described earlier for spectral analysis, we have succeeded in determining the details of the effect of cholesterol on a biological membrane (36).

Figure 10 shows the ^2H NMR spectra of *A. laidlawii* membranes containing perdeuteropalmitic acid in the presence and absence of cholesterol. Due to unfavorable linewidths, only the quadrupole splittings of the terminal methyl and penultimate methylene groups can be distinguished, although the width of the pattern yields an estimate of the largest order parameter in the system, presumably due to the first 8-10 segments of the palmitoyl chains. As mentioned

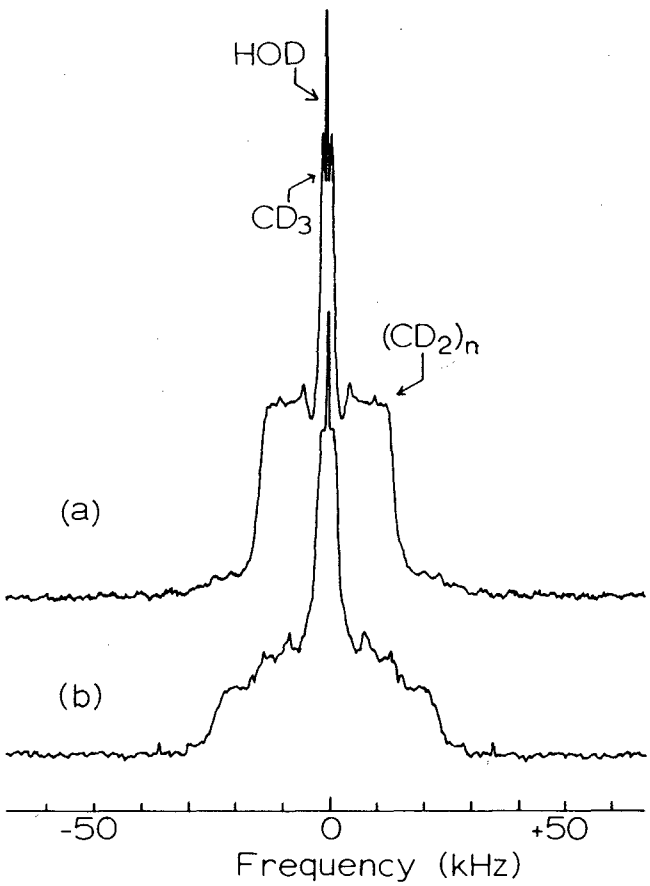

Figure 10. ^{2}H NMR spectra (13.8 MHz) of membranes of *A. laidlawii*, 42°C, containing: (a) perdeuteropalmitic acid (69 mol %); (b) perdeuteropalmitic acid plus 33 mol % cholesterol (18).

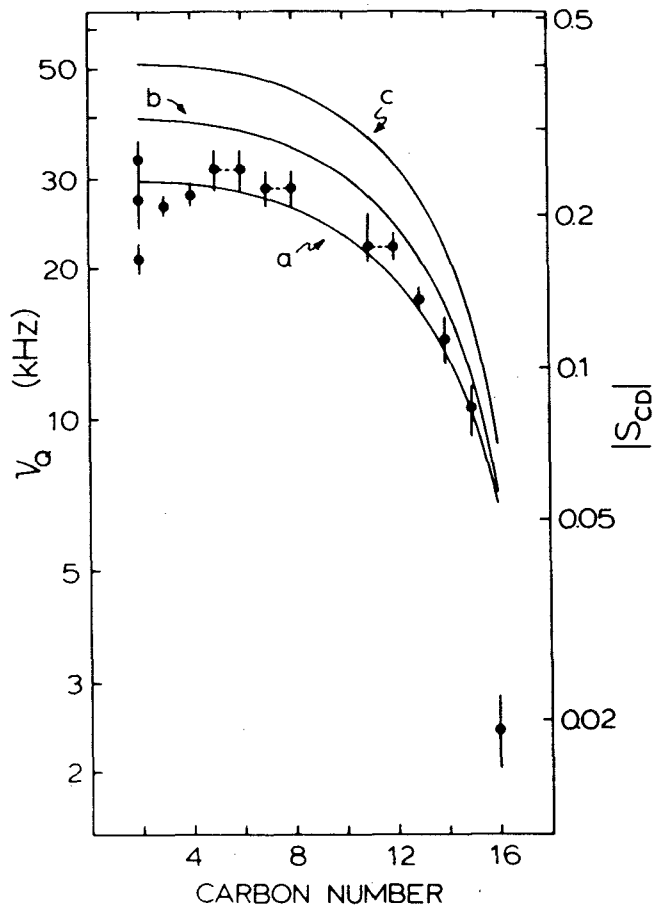

Figure 11. Dependence on position of labelling of the quadrupole splitting (ν_Q) and segmental order parameter (S_{CD}) for membranes of *A. laidlawii* enriched in palmitic acid: **(a)** smooth curve, obtained from moment analysis of the spectra of membranes containing perdeuteropalmitic acid (69 mol %), 42°C; solid circles, obtained from membranes containing specifically-deuterated palmitic acid, 42°C (18); **(b)**, obtained from moment analysis of membranes containing perdeuteropalmitic acid (69 mol % of fatty acyl chains) and cholesterol (40 mol % of total lipid), 42°C; **(c)** obtained from moment analysis of the spectra of the sample in **(d)**, but at 22°C (36).

earlier, by analysis of the first four moments of the ^{2}H NMR spectra in terms of a polynomial dependence of S on position, we were able to delineate this dependence (36). Figure 11 compares the dependence obtained from the moment analysis with that derived from direct measurement of the quadrupole splittings of specifically deuterated palmitoyl chains in the cholesterol-free membranes. The high quality of the agreement in this case gave us the confidence to apply the method to the membranes containing cholesterol. Curve b in Figure 11 is for membranes containing 40 mole % of lipid as cholesterol at 42°C; curve c is for the same sample at 22°C. Comparing curves a and b, the largest increases in molecular ordering of the acyl chains, due to cholesterol incorporation, occur for the first 10-12 carbon atoms of the chain, as has been observed earlier with egg phosphatidylcholine (34) and dimyristoyl phosphatidylcholine (37). The degree of order for the cholesterol-enriched membrane at 22°C (profile c) is even higher, but the main features of the profile are conserved. The temperature dependence of the spectra, and of the Δ_2 parameter, Figure 12, demonstrate that incorporation of cholesterol has

eliminated the liquid crystal-gel phase transition. At 20°C, Δ_2 is only slightly higher than it is for the cholesterol-free membranes at 42°C. However, further decrease in temperature results in a large increase in Δ_2, probably due to the separation of phases of different cholesterol content.

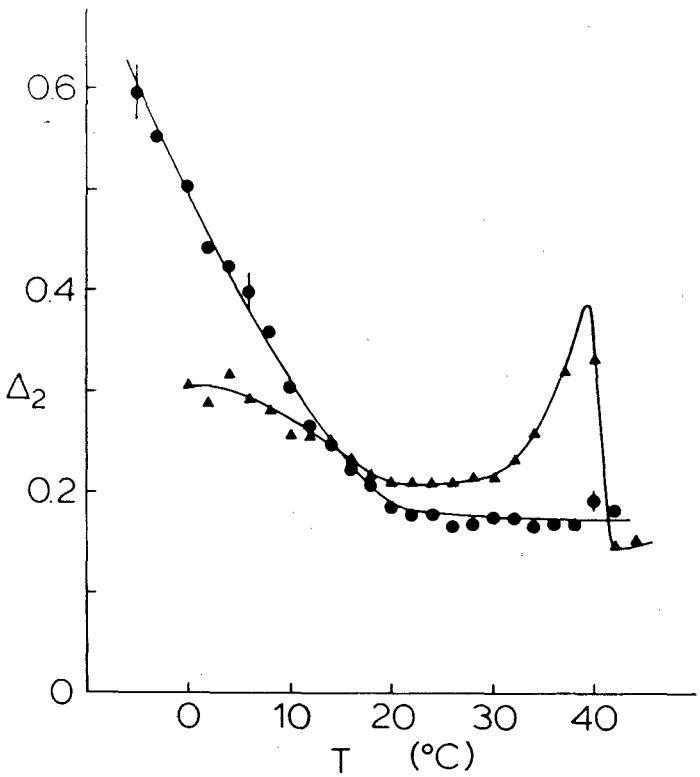

Figure 12. Temperature dependence of the moment parameter Δ_2 for the ^2H NMR spectra (34.4 MHz) of membranes of *A. laidlawii* enriched in perdeuteropalmitic acid (69 mol %, triangles) and perdeuteropalmitic acid plus cholesterol (40 mol %, circles) (36).

IX. MEMBRANES HIGHLY-ENRICHED BY THE USE OF AVIDIN

All the membrane preparations described so far have been enriched in a particular fatty acid by 60-80%. Recently, by the addition of the protein avidin to the medium according to to Silvius and McElhaney (16), we have been able to achieve incorporation of tetradecanoic acid (myristic acid, C14:0) and pentadecanoic acid (C15:0) to levels greater than 90% (38). These preparations are useful for exploring the influence of the lipid head groups, since the fatty acyl chains have an almost uniform composition.

Differential scanning calorimetric studies on the membranes enriched to 94% in myristic acid (39) revealed a gel-liquid crystal phase transition over the range 30-46°C, centered at 41°C, with a weaker pretransition centered at 25°C. This is, to our knowledge, the first evidence for a pretransition in a biological membrane, which may be a consequence of the high degree of enrichment in a particular fatty acid. The thermal data may be compared to those for aqueous dispersions of dimyristoyl phosphatidylcholine which manifest a main transition and pretransition at 23.7° and 13.5°C, respectively (40). The elevation in transition temperature for the biological membranes is no doubt due in part to the different lipid head groups, but could reflect the influence of the many proteins (60-80%, by weight of the membrane). The latter seems less likely in view of the apparent disordering influence of membrane proteins on their neighboring lipids (*vide infra*, 41-43).

Figure 13 shows the ^2H NMR spectra of *A. laidlawii* membranes highly enriched in α,α -d_2-myristic acid. At 45°C the pattern is typical of liquid-crystalline lipid, with two clear quadrupole splittings and a suggestion of a third (see the earlier discussion of this phenomenon in Section V). By 35°C, a significant amount of gel state spectrum is present, with intensity extending over a range of 120 kHz. The temperature response of the spectra is basically the same as that described earlier for the palmitate-enriched membranes, and over a similar temperature range despite the difference in length of the fatty acyl chains. Thus, it appears that the organism has altered some other component of its membranes, possibly the properties of the various head group classes, to compensate for the change in chain length. We are presently investigating this aspect chemically.

X. PROTEIN-LIPID INTERACTION

It has long been thought that lipid in proximity to membrane protein could have properties different from that in a lipid-rich environment. Proposals for an annulus of boundary lipid have been made from spin-label ESR (44-46) data. On the other hand, ^2H NMR studies on the same system, cytochrome ox-

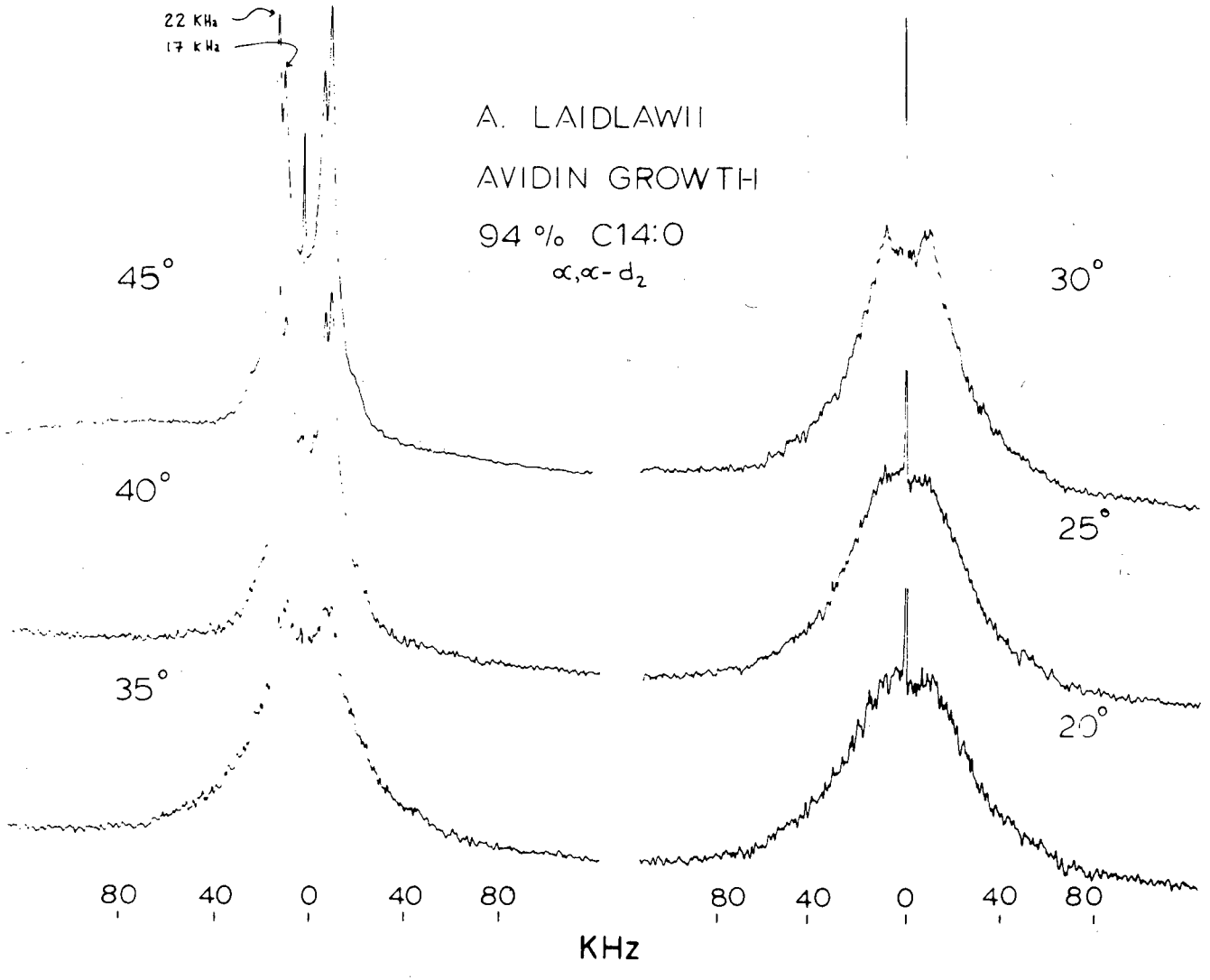

Figure 13. ^2H NMR spectra (45.1 MHz) of the membranes of *A. laidlawii* highly enriched in 2,2-$^2H_2$-myristic acid (94 mol %) at various temperatures. The spectra were obtained using the quadrupole echo technique, with quadrature detection, 90° pulses (10 μs), and a recycle time of 0.4 s. (38).

idase (42,43), and *E. coli* (41), show no evidence for a separate lipid phase. It may well be that the different time scales of the ESR and ^{2}H NMR experiments are responsible for this apparent contradiction; an exchange process that is slow in the time scale of ESR hyperfine splittings (*ca.*50 MHz) can still be fast on the scale of ^{2}H NMR quadrupole splittings (*ca* 50. kHz).

We have investigated this problem using the membranes of *A. laidlawii* (38). Initial insight is gained by consideration of the Δ_2 parameter. For the liquid-crystalline state of these membranes, regardless of which fatty acid is incorporated, low values are found for Δ_2. This indicates a very narrow distribution of order parameters for the acyl chains in this membrane of relatively low protein content, and therefore a negligible fraction of the lipid can exist in a different state of order or mobility for a time significant on the ^{2}H NMR time scale. If lipid near protein has different characteristics from that in the bulk of the bilayer, rapid exchange between these environments must average the linewidths and quadrupole splittings.

As a second test, we have compared the quadrupole splittings for specifically deuterated myristic acid in *A. laidlawii* membranes with those for multilamellar dispersions of pure lipid isolated from the same membranes (38). At temperatures above that of the lipid phase transition, the quadrupole splittings are smaller in the intact membranes than they are in the isolated lipids, indicating a greater disorder of the lipids in the presence of the membrane proteins. The tentative conclusion, which is in accord with those of other ^{2}H NMR studies, is that proteins do disorder lipids in their vicinity, but that these lipids are in rapid exchange with those which are unaffected by protein to yield a single reduced value for the net quadrupole splitting. We have obtained similar preliminary results on membranes of an unsaturated fatty acid auxotroph of *E. coli* (47).

XI. PROGNOSIS

Deuterium NMR has developed into an excellent technique for the study of molecular ordering and dynamics in biological membranes. The ability to obtain reliable spectra of very large widths, and the use of moment analysis, have made possible the study of highly ordered and immobilized lipid. Increased spectrometer sensitivity permits spectra to be obtained from packed cells in minutes, allowing the observation of time-dependent processes on a time scale that is biologically relevant. Synthetic routes to most of the deuterated fatty acids, and to some components of the head groups, are now available, and increasing use of biosynthetic pathways and auxotrophs of microorganisms should permit the labelling of particular regions in systems more complex than *Acholeplasma laidlawii*. There is little doubt that answers to a significant number of biological questions will be obtained via ^{2}H NMR of membranes.

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