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ORIENTATIONAL ORDER OF UNSATURATED LIPIDS IN THE MEMBRANES OF *ACHOLEPLASMA LAIDLAWII* AS OBSERVED BY ^{2}H-NMR

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Summary

Oleic acid specifically deuterated at fifteen different positions along the chain, including the double bond, was biosynthetically incorporated into the membrane lipids of the microorganism *Acholeplasma laidlawii* B. A detailed study of the dynamic conformation of these chains was carried out using deuterium nuclear magnetic resonance. The deuterium spectra of fourteen different samples were recorded as a function of temperature over the range 0—41°C. Spectra were obtained down to −52°C for the sample enriched with oleic acid deuterated at the C-12′ position. Above 20°C, where the lipids are in the liquid crystal phase, a single quadrupolar powder pattern was observed for each C^2H_2 segment, except for the C-2′ position which gave rise to a three-component spectrum characteristic for this position in both model and biological membranes. Simulation of this spectrum indicates that there are two conformations of the lipid molecule in the region of the C-2′ segment of the *sn*-2 chain. The orientational fluctuations of the fatty acid chain segments in the *A. laidlawii* membranes are described by the deuterium order parameters, and a striking similarity is shown to exist between the oleate chain conformation of the *A. laidlawii* membrane and a phospholipid model membrane. Remarkable similarities are also demonstrated in the *A. laidlawii* membranes enriched in palmitic and oleic fatty acids when the order parameter profiles are plotted at

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Abbreviations: DPPC, 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine; POPC, 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine; DMPC, 1,2-dimyristoyl-3-*sn*-phosphatidylcholine; DPPS, 1,2-dipalmitoyl-3-*sn*-phosphatidylserine; DSC, differential scanning calorimetry.

the same reduced temperature. Below 15°C a second component, due to gel phase lipid, starts to appear in the spectra. This broad gel phase component grows at the expense of the liquid crystal phase component as the temperature is reduced. The spectra indicate that the center of the phase transition is at about −12°C, in good agreement with DSC studies.

Introduction

A detailed knowledge of the structure and dynamics of the lipid molecules in biological membranes is essential to the understanding of membrane function and cellular activities in general. An elucidation of the conformation and dynamics of the hydrocarbon chains of membrane lipids represents an important contribution to this knowledge. Deuterium nuclear magnetic resonance (^{2}H-NMR) of labelled lipids has been the method of choice for the determination of lipid chain arrangement as it is an essentially non-perturbing probe which is very sensitive to the detailed structure of the membrane. Only recently has this technique been employed in comprehensive studies of biological membranes [1—6]. The purpose of the work reported here is to continue the characterization of the hydrocarbon chain conformation of lipids in biological membranes. In particular, the physical implications of a *cis*-unsaturated bond in the acyl chain are explored. Biological membranes have a large percentage of *cis*-unsaturated lipids and it is well known that the proportion of saturated to unsaturated lipids greatly influences the thermal behaviour of the membrane. The microorganism *Acholeplasma laidlawii* was chosen as the system to study because it readily incorporates exogenous ^{2}H-labelled fatty acids into its membrane lipids and, since it has only a single plasma membrane with no internal membrane structures, relatively homogeneous membrane preparations are possible. Furthermore, in view of previous work [2,4], a direct comparison of conformations and thermal behaviour of saturated and unsaturated chains can be carried out.

In the generally accepted fluid mosaic model [7] of the membrane the lipid molecules form a liquid crystalline bilayer fluid enough to allow the membrane proteins to function. The dynamical structure of a biological membrane is best discussed in the terms used for anisotropic fluids. The structural properties of the lipid molecules within the bilayer can therefore be described in terms of order parameters [8]. Because the molecules are flexible, a complete description would involve an order parameter tensor for each rigid unit. This second-order tensor is symmetric and traceless, and the components are defined as

$$S_{\alpha\beta} = \langle (3 \cos\theta_\alpha \cos\theta_\beta - \delta_{\alpha\beta})/2 \rangle \qquad (1)$$

The subscripts α and β refer to axes (x, y, z) fixed in the molecular subunit, with θ_α and θ_β being the angles between these axes and a direction normal to the bilayer. The $< >$ brackets denote a time or ensemble average. It should be noted that the components $S_{\alpha\beta}$ depend not only on the angular distribution of fluctuations experienced by the molecular subunit but also on the average orientation of the molecular axes.

Nuclear magnetic resonance (NMR) of selectively deuterated lipid molecules [9,10] has proven to be a perturbation-free technique for the determination of the order parameter, namely S_{C^2H}, associated with a particular C-^{2}H bond direction. The deuterium nuclear quadrupole interaction causes a splitting of the Zeeman resonance giving rise to, in an unoriented sample, a characteristic powder pattern. This spectrum has two distinct peaks which are separated in frequency by $\Delta\nu_Q$, where

$$\Delta\nu_Q = \frac{3}{4}\left(\frac{e^2qQ}{h}\right)S_{C^2H} \tag{2}$$

This equation is valid when it can be assumed that the electric field gradient at the deuteron site is axially symmetric about the C-^{2}H bond direction. Only the absolute value of the deuterium order parameter can be determined since the sign of the quadrupole splitting is generally unknown. The static deuterium quadrupole coupling constant (e^2qQ/h) is about 170 kHz for alipathic C-^{2}H bonds [11] and 175.3 kHz for olefinic C-^{2}H bonds [12]. In both cases the asymmetry parameter is very nearly zero. Knowledge of S_{C^2H}, however, does not in general determine the complete order parameter tensor $S_{\alpha\beta}$. In particular, for a rigid C^2H_2 group it is necessary to determine independently S_{C^2H} and $S_{^2H^2H}$ [13] for a complete description of the molecular subunit order.

There have now been published a number of detailed profiles of $|S_{C^2H}|$ as a function of label position in model lipid bilayers, i.e. DPPC [14,15], POPC [16,17], DMPC [18], egg-yolk lecithin [19], and DPPS [20]. In addition, a detailed order profile has been published for the membrane of the microorganism *A. laidlawii* which had been biosynthetically enriched with deuterated palmitic acid [2]. While the study reported in this paper was in progress an order profile was partially established for *Escherichia coli* membranes incorporating palmitic and oleic acid selectively deuterated at ten sites in total [6].

Another important aspect of structural studies of membranes is the temperature dependence of the molecular order. All single component preparations of pure phospholipids show a sharp first-order phase transition [21] from a liquid crystalline state to a gel state as the temperature is lowered through the transition region. The transition arises from a cooperative freezing of the hydrocarbon chains located in the interior of the bilayer. The transition temperature depends upon the head-group, the length and amount of branching of the acyl chains, and the degree and type of unsaturation of the chains. The membrane lipids of *E. coli* [22—24] and *A. laidlawii* [25—27] also undergo relatively well-defined liquid crystal to gel transitions, in spite of the heterogeneity of the lipid head-groups and fatty acyl chains and the presence of protein. While the influence of molecular order on the biological functions of the membrane, in particular the enzymatic functions, has not yet been firmly established [28, 29], there is evidence that the degree of membrane fluidity definitely gives rise to a number of important effects. A consequence of the phase transition is that the rate of translational diffusion of molecules in the membrane increases by several orders of magnitude on going from the gel to liquid crystal state [30, 31]. It is well established that the phase change influences the growth rates in *A. laidlawii* [32] and in fact these cells will not grow when the lipids are all in the gel state. The maximum temperature at which a cell will grow seems to

depend, at least in part, on the fluidity of the membrane [28,32,33]. While it has been suggested that it is necessary for the two phases to be coexistent for cell growth [29], there is evidence to indicate that at least in *A. laidlawii* this is not a necessary criterion [32]. Another significant effect of temperature is that cells will change the composition of the acyl chains to adapt to the temperature of their environment [34,35]. Microorganisms grown at increasing temperature tend to incorporate more saturated, less branched, and longer chains into their lipids, with the result that the phase transition in these membranes occurs at progressively higher temperatures.

Deuterium magnetic resonance was initially limited to the liquid crystal phase due to the difficulties in observing the broad spectrum of the gel phase. Now, however, with the improvements in spectrometer design and the use of the quadrupolar echo technique [36] it is possible to follow the quadrupolar spectrum into the gel region [3,4] and to obtain quantitative information about coexistence of the two phases in the transition region.

Materials and Methods

The microorganism *Acholeplasma laidlawii* B was grown at 37°C in an initially fatty acid-free tryptose broth supplemented with specifically dideuterated oleic acids ([2′-$^2H_2$]-, [3′-$^2H_2$]-, [4′-$^2H_2$]-, [5′-$^2H_2$]-, [6′-$^2H_2$]-, [7′-$^2H_2$]-, [8′-$^2H_2$]-, [9′,10′-$^2H_2$]-, [11′-$^2H_2$]-, [12′-$^2H_2$]-, [14′-$^2H_2$]-, [16′-$^2H_2$]-, [17′-$^2H_2$]-oleic acids) and with [18′-$^2H_3$]oleic acid [37]. The cells were harvested in the late log phase, osmotically lysed in distilled water and freeze-dried for storage at −15°C. Other details of the microbial growth and membrane preparation can be found in Ref. 1. The acyl chain distribution of the various samples, as determined by gas chromatography [38], is given in Table I. While there is a small variation in the distribution of acyl chains from sample to sample, the percentages of 18 : 1 and 16 : 0 are roughly constant at 64% and 23%. The

TABLE I

FATTY ACID COMPOSITION OF THE TOTAL MEMBRANE LIPIDS OF *ACHOLEPLASMA LAIDLAWII* GROWN ON SPECIFICALLY-DEUTERATED OLEIC ACID AT 37°C

Labelled carbon atom	Fatty acids found (mol %)				
	12:0	14:0	16:0	18:0	18:1
2	1.8	5.0	23.9	5.6	61.5
3	1.2	4.7	22.0	2.6	67.4
4	—	5.6	20.1	2.9	70.1
5	—	4.5	25.6	3.6	66.4
6	0.9	3.5	20.1	4.4	62.5
7	—	7.6	25.1	3.1	64.2
8	1.5	4.0	23.4	4.2	67.0
9,10	2.9	6.6	23.5	2.1	61.3
11	3.3	4.6	20.0	2.8	63.8
12	—	4.7	26.7	5.4	63.1
14	4.5	5.6	25.7	3.1	61.1
16	2.0	5.4	22.1	3.5	61.3
17	2.1	4.6	26.0	3.5	63.9
18	2.6	6.5	23.6	3.1	59.0

samples used for the NMR experiments consisted of 100—150 mg of the freeze-dried membrane which was hydrated with deuterium-depleted water (Aldrich Chemical Co., Milwaukee, WI) in a weight ratio of about 1 : 1.2. The samples were mixed by centrifugation through a constriction, then sealed in glass ampules.

The ^{2}H-NMR spectra were obtained at a frequency of 41.3 MHz using a home-built spectrometer and a Bruker superconducting solenoid. Digitization and Fourier transformation of the nuclear signal were carried out using a Nicolet 1090 AR digital oscilloscope interfaced to a Digital PDP-8/A minicomputer. Because the samples are not oriented, the observed spectrum is a quadrupolar 'powder' pattern [9] spanning 10^3—10^5 Hz. A modified quadrupolar echo technique was used to overcome the problem of the loss of the initial part of the nuclear free induction decay due to the receiver recovery time. The basic quadrupolar echo sequence [36] consists of an initial $\pi/2$ pulse applied along the x direction in the rotating frame followed by a $\pi/2$ pulse in the y direction at a time τ later. The echo peak occurs at a time approximately τ after the second pulse. Using this sequence it was found that the magnetoacoustic ringing [39,40] induced by the radio frequency pulse interfered with the echo for the small values of τ used. The spin-spin relaxation times were relatively short so that increasing τ and waiting for the ringing to die away was not a practical solution. To overcome the problem the echo sequence was modified by changing the phase of the first $\pi/2$ pulse by 180° on successive repetitions and then alternately adding and subtracting the nuclear signals when signal averaging in the computer.

Typically the $\pi/2$ pulses were 5—6 μs in length and τ was 50—60 μs. The pulse sequence was repeated about three times a second except for the methyl group, where the rate was reduced to about once a second. The frequency of the spectrometer was set at the center of the symmetric quadrupolar powder pattern, and in most cases the spectrum was folded so that the two halves were superimposed, increasing the signal-to-noise ratio by a factor of $\sqrt{2}$.

The sample was enclosed in a copper oven in the NMR probe. The oven temperature was electronically regulated to within ±0.5°C and the temperature gradient across the sample was estimated to be less than 0.25°C.

Results and Discussion

Liquid crystal phase

The deuterium spectra of 14 different samples were recorded as a function of temperature over the range 0—41°C. For temperatures above 20°C, where all lipid was in the liquid crystal phase, a single quadrupolar powder pattern was observed, with two exceptions. The exceptions were the samples where the oleoyl chains were deuterated at the C-2′ and at the double bonded C-9′,10′ positions. Spectra from six samples at 25°C are shown in Fig. 1.

The spectra for the C-2′ position show three overlapping powder patterns. Seelig and Seelig [14] first observed the three pattern spectra for the C-2′ position in the model system DPPC. By selectively deuterating each acyl chain separately [15] they showed that the peaks with the largest splitting came from

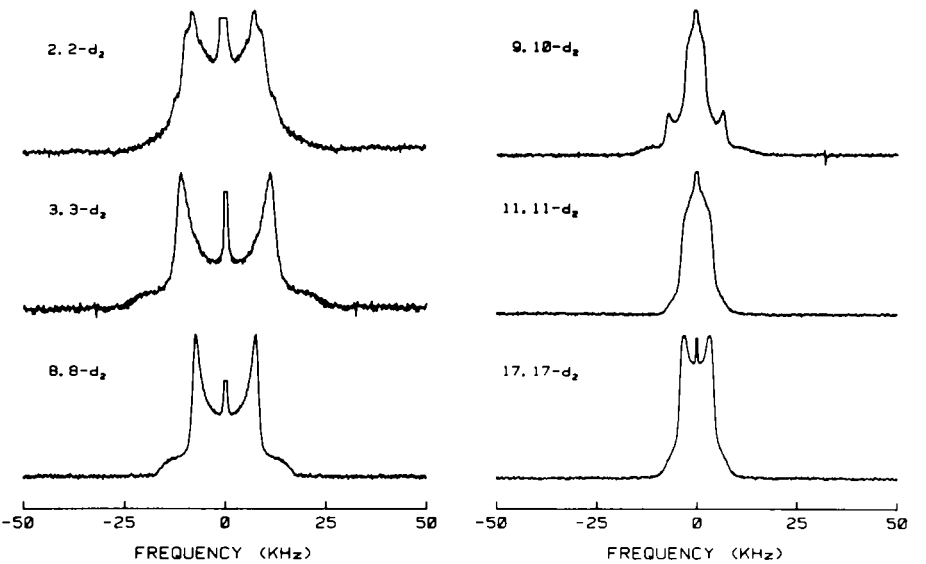

Fig. 1. ^{2}H-NMR spectra of the oleoyl chains in membranes of *A. laidlawii*, with the ^{2}H-label in the positions indicated and at a temperature of 25°C. The spectra were obtained using the quadrupolar echo technique, with a $\pi/2$ pulse duration of 5.5 μs, a pulse spacing of 40—60 μs, and a recycle time of 0.20—0.33 s. The number of scans was 32 000—128 000.

the C-2′ position on the *sn*-1 chain, while the other two splittings were due to the *sn*-2 chain. They have suggested that the different quadrupolar splittings for the two chains are due to different orientations of the start of the two chains with respect to the normal to the bilayer [15]. The *sn*-1 chain is parallel to the normal while the *sn*-2 chain starts out perpendicular to the normal and then is bent parallel to it after the C-2′ segment. This model is supported by X-ray data for crystalline phospholipid [41] and by neutron diffraction studies of DPPC bilayers [42,43]. The origin of the two-splittings for the *sn*-2 chain could be due to inequivalence of the two deuterons of the C-2′ site or to two different conformations of the *sn*-2 chain which are in slow exchange on the NMR time scale. The latter explanation seems the more likely for two reasons, the first being that with DPPC only one splitting shows a significant variation with temperature, whereas the other shows almost no temperature dependence [15]. The second reason is that simulations of our spectra for the C-2′ position indicate that the integrated intensities are not the same for the two powder patterns which give rise to the two splittings for the *sn*-2 position. One would expect equal integrated intensities if the two splittings were due solely to the inequivalence of the two C-2′ deuterons. The simulations were done by adding together three Gaussian broadened quadrupolar powder patterns with the appropriate splittings and varying the relative intensities to obtain a good fit to the experimental spectra. The lineshape function used to generate a single quadrupolar powder pattern was

$$F(\xi, \omega_Q) = F_+(\xi, \omega_Q) + F_-(\xi, \omega_Q) \quad (3)$$

where

$$F_{\pm}(\xi, \omega_Q) = \int_0^1 \frac{du}{\sigma} \exp[-\omega_Q^2(\xi \pm (3u^2 - 1)/2)^2/2\sigma^2] \quad (4)$$

$$\xi = \omega/\omega_Q \quad (5)$$

$$\omega_Q = 2\pi \Delta\nu_Q \quad (6)$$

and

$$\sigma = A + B(3u^2 - 1) \quad (7)$$

where $u = \cos\theta$, θ being the angle between the axis of motional averaging (i.e. the bilayer normal) and the external magnetic field. The functional form used for the line-broadening parameter σ was empirically chosen so as to fit the experimental spectra. It was found necessary to have an angular-dependent term in σ in order to round off the shoulders of the quadrupolar powder pattern without excessively broadening the peaks. An example of this simulation is shown in Fig. 2, along with the experimental spectrum for comparison. The ratio of the integrated intensities of the three constituent powder patterns in the simulations is 0.9 : 2.5 : 1.0, in order of decreasing quadrupolar splittings; the estimated error in these values is 15%. This is also the ratio of the number of deuterons giving rise to each quadrupolar powder pattern, due to the fact that the integrated intensity of a powder pattern is directly proportional to the number of contributing deuterons. Of the two splittings for the *sn*-2 chain, 2.5 times as many deuterons are contributing to the outer splitting

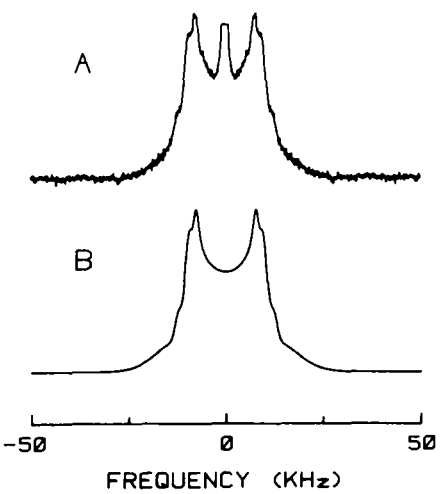

Fig. 2. Comparison between an experimental ^{2}H-NMR spectrum of [2′-$^2H_2$]oleoyl chains in *A. laidlawii* membranes and a spectral simulation. (A) Spectrum for [2′-$^2H_2$]oleoyl chains, obtained using 5.5 μs π/2 pulses, pulse spacing 50 μs, recycle time of 0.20 s, and number of scans 128 000. (B) Simulated spectrum with three overlapping Gaussian-broadened quadrupolar powder patterns. Quadrupole splittings are 25.5 kHz, 20.0 kHz, and 16.0 kHz, with the ratio of integrated intensities of the contributing patterns being 0.9 : 2.5 : 1.0.

as to the inner one. This means that the two splittings cannot be due simply to the inequivalence of the two C-2′ deuterons, but rather must involve two conformations of the lipid molecule in the region of the C-2′ segment of the *sn*-2 acyl chain. There does not appear to be a significant temperature dependence of the relative probabilities for the two conformations over the range 16—37°C. In spite of these results, however, it would be most desirable to introduce one deuteron stereospecifically into the C-2′ position of the *sn*-2 chain to confirm the idea that there are two conformations in this region of the lipid molecules.

It must be emphasized at this point that the exact ratio of integrated intensities will be dependent on the lineshape function chosen, and so without a theoretical justification for the lineshape used we must say that our values give only a qualitative indication of the relative distribution of deuterons which give rise to the three splittings. Support for the present ratio results from the fact that it indicates that 80% of the oleoyl chains are attached to the *sn*-2 position of the membrane lipids. From Table I we see that for the [2′-$^2H_2$]oleoyl-labelled sample 61.5% of the total fatty acid content is oleic acid, which means that of the total fatty acid content 49% is oleic acid attached at the *sn*-2 position of the lipids, with the rest of the oleic acid and most of the various other saturated fatty acids being attached at the *sn*-1 position. This is in agreement with the fact that in lipids of biological origin there is a strong positional preference of the saturated and unsaturated fatty acids for the *sn*-1 and *sn*-2 positions, respectively.

The three splitting pattern has now been observed in a number of different model membrane systems with different head groups and both saturated and unsaturated chains [15,17,18,20]. The incorporation of elaidoyl chains, deuterated at the C-2′ position, into the membranes of *E. coli* [6] also gives rise to spectra with three quadrupolar splittings. In addition, the spectrum of the C-2′-labelled palmitoyl chains in the membrane of *A. laidlawii* [2] has an 'unusual shape' which may result from the presence of three overlapping powder patterns. The three overlapping patterns which we observe would therefore seem to be a manifestation of the same characteristic behaviour. The weight of all these studies leads to the conclusion that the inequivalence of the *sn*-1 and *sn*-2 chains is a general feature of lipid organization in all membranes and that the conformation of the beginning of each chain is about the same in all systems.

The spectra for acyl chains labelled at the C-9′,10′ double bond consist of two overlapping powder patterns of equal integrated intensity, suggesting the motional inequivalence of the C-9′ and C-10′ deuterons. Similar spectra have been observed in a model system, POPC [17] and for oleoyl chains in *E. coli* membranes [6]. If the double bond were parallel to the normal to the bilayer the C-9′ and C-10′ deuterons would be motionally equivalent and the average angle between the two C-^{2}H bonds and the normal would be the same, because the double bond is rigid. Since two splittings are observed, the double bond cannot be parallel to the normal.

The *cis* double bond has C_{2v} symmetry and there are three independent components of the order parameter tensor. The order parameters for the two C-^{2}H bonds are given by

$$S_{C^2H} = \cos^2\theta S_{zz} + \sin^2\theta S_{xx} \pm \sin\theta \cos\theta S_{xz} \quad (8)$$

where θ is the angle between the C-^{2}H bonds and the C=C bond. The z-axis is along the C=C bond and the x-axis is in the plane of the C-^{2}H bonds. The complete order parameter tensor cannot be determined by measurements of the two S_{C^2H} alone. Using an independent measurement of S_{zz} from infrared linear dichroism [17], Seelig and Waespe-Sarcevic were able to determine four possible sets of components of the order parameter tensor. The four sets arise because the signs of the two S_{C^2H} are not determined. These four sets were diagonalized by rotations about the x-axis by an angle α where $-9° < \alpha < 9°$. However, only one set of components leads to an axially symmetric order parameter tensor consistent with the uniaxial properties of the lipid membrane. The angle α is then interpreted as being the average angle between the double bond and the bilayer normal. Furthermore, the principal component of the diagonalized order tensor, which is the order parameter describing the fluctuations of the double bond segment about the bilayer normal, has a value very similar to that found in the adjacent palmitic acyl chains at the same depth in the bilayer. Seelig and Waespe-Sarcevic [17] have thus shown that a relatively small tilt angle of 7—8° between the double bond and the bilayer normal is sufficient to account for the two observed quadrupole splittings.

At the present time a complete analysis of the order parameter tensor for the double bond in the oleic-enriched *A. laidlawii* membranes is not possible because S_{zz} has not been determined. This parameter could be obtained from the dipolar splitting for two protons substituted at the double bond on an otherwise deuterated oleic acyl chain. Because of the similarity between the results for the oleoyl chains in the model system POPC and in the *A. laidlawii* membranes, it is reasonable to suggest that the origin of the two spectra at the double bond is due to the tilting of the double bond away from the direction of the bilayer normal.

The effect of varying the spacing between the two $\pi/2$ pulses of the quadrupolar echo sequence is shown in Fig. 3. The two spectra displayed are for the C-11′ position of the oleoyl chain at 25°C, and were obtained using the echo technique as described above. Spectrum A was obtained using a pulse spacing of 60 μs while the spacing used for spectrum B was 150 μs. As can be seen, the lineshapes of the two spectra differ markedly. Similar results have not previously been reported in other lipid or lipid-protein systems. The explanation of this result appears to be that there is an angular dependence across the spectrum of the transverse relaxation time T_2. Spectra have been obtained as a function of pulse spacing for several positions along the oleoyl chain, and in all cases an angular-dependent transverse relaxation was observed. In particular, the peaks for the 10′ position, which are not seen in Fig. 1, have been observed by increasing the spacing of the echo pulses, as in the case for the 11′ position. The observation of an angular-dependent T_2 lends support to the use of an angular-dependent line-broadening parameter in the simulation of the spectra for the C-2′ position.

The S_{C^2H} profile

The liquid crystal phase component dominates the spectra for all label positions even at 0°C. The quadrupolar splitting for this liquid crystal phase component was measured as a function of temperature from 0 to 41°C and as a

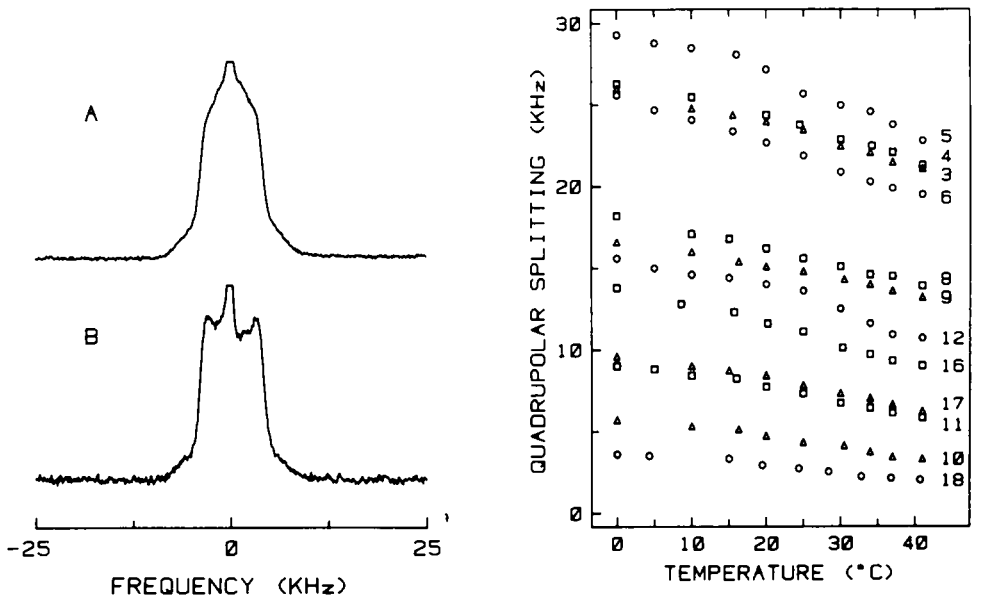

Fig. 3. Comparison between experimental ^{2}H-NMR spectra of [11'-$^2H_2$]oleoyl chains of *A. laidlawii* membranes for different spacing between the two $\pi/2$ pulses of the quadrupolar echo sequence. For both spectra the $\pi/2$ pulses were 5.5 μs in duration, the recycle time was 0.31 s, the number of scans was 64 000, and the temperature was 25°C. (A) Pulse spacing of 60 μs. (B) Pulse spacing of 150 μs.

Fig. 4. Quadrupolar splitting as a function of temperature for *A. laidlawii* membranes biosynthetically-enriched with specifically-deuterated oleic acid. The numbers along the right hand side indicate the carbon atom which was deuterated.

function of label position; the results are shown in Fig. 4. The temperature dependence is approximately linear, the quadrupolar splitting increasing with decreasing temperature, as expected. At lower temperatures there is less motion of the C-^{2}H bond due, for example to *trans-gauche* isomerization of the acyl chains, rotational and translational diffusion, and chain tilting. With the motions slowing down and/or decreasing in amplitude, they would be less effective in averaging the quadrupolar interaction and hence the quadrupolar splitting would increase. In absolute terms, the temperature dependence appears to be weakest at the methyl end of the hydrocarbon chains and strongest in the C-3' to C-6' region. The converse of this true, however, if the temperature dependence is viewed as a percentage change in the quadrupolar splittings.

Fig. 5A shows the quadrupolar splittings and order parameters for fifteen positions down the oleoyl chain at 25°C. As well, results obtained by Seelig and Waespe-Sarcevic [17] and Seelig and Seelig [16] for the model system POPC at 27°C are shown for comparison. It is remarkable that the two sets of results should be so similar, considering that the *A. laidlawii* membrane contains 80% protein by weight, has a heterogeneous fatty acid content (see Table I) and has diglucose, glucose and phosphoglycerol instead of phosphocholine as the polar head group. The dip in the $|S_{C^2H}|$ profile at the double bond of the oleate

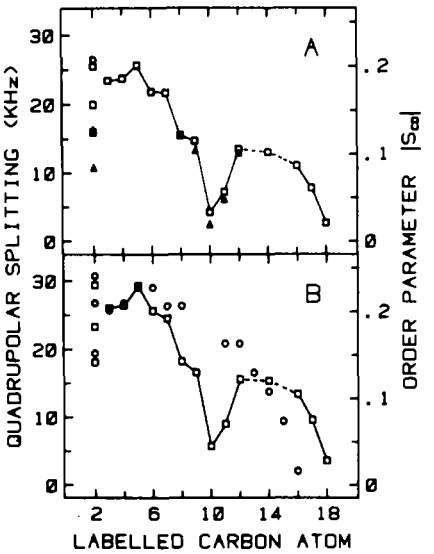

Fig. 5. Variation of the quadrupolar splitting and deuterium order parameter $|S_{C^2H}|$ with labelled carbon atom. Data for POPC taken from Seelig and Seelig [16] and Seelig and Waespe-Sarcevic [17] and data for palmitate-labelled *A. laidlawii* taken from Stockton et al. [2]. (A) □, oleate-labelled *A. laidlawii* membranes at 25°C. △, deuterium label attached to oleate chain of POPC at 27°C. ○, deuterium label to palmitate chain of POPC at 27°C. (B) □, oleate-labelled *A. laidlawii* membranes at 0°C. ○, palmitate-labelled *A. laidlawii* membranes at 42°C.

chains is due to the geometry and alignment of the double bond with respect to the bilayer normal. Apparently the double bond alignment is almost the same in the phospholipid model membrane and the *A. laidlawii* membrane. There is, however, a significant difference between the two order profiles for the 2′ position of the *sn*-2 chain. The splittings for the *A. laidlawii* membrane are approx. 5 kHz larger than corresponding splittings for the POPC model membrane. This increase in order parameters for the *A. laidlawii* membrane could be due to the presence of protein (about 80% by weight). Rice and Oldfield [44] have observed the same effect in a model system. They measured the quadrupolar splittings for samples of pure DMPC, DMPC + 15 wt.% gramicidin, and DMPC + 50 wt.% gramicidin, where the DMPC was labelled at the 2′ position in the *sn*-2 chain. The splittings for the two peptide-containing bilayers were approximately equal, but significantly higher than for the pure lipid bilayer. It is more obvious from their data that the two splittings for 2′ position are increased by approximately the same amount; one might otherwise have concluded from our data that one pair of splittings coincided and that the other corresponding pair differred by approx. 10 kHz. Their findings also make unlikely the possibility that the differences in splittings between the POPC and *A. laidlawii* membranes are due solely to the inhomogeneity of the head groups and acyl chains in the latter. It seems most likely that the proteins are causing a slight change in the conformation of the lipid molecules in the region of the 2′ segment of the *sn*-2 chain. The similarities between model and biological membranes such as *A. laidlawii* and *E. coli* [6] indicate that studies on model systems are biologically relevant and have an important role to play in the overall study of membranes.

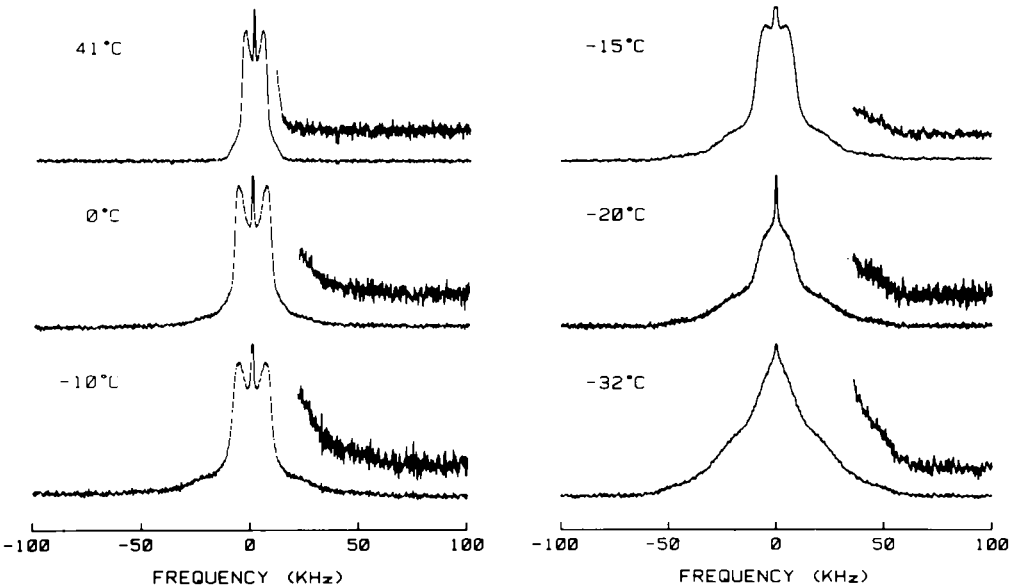

Fig. 6. ^{2}H-NMR spectra of the [12′-$^2H_2$]oleoyl chains in membranes of *A. laidlawii*, at the temperatures indicated. The $\pi/2$ pulse duration was 5.5 μs, pulse spacing 60 μs, recycle time 0.31 s, and the number of scans averaged was 64 000. Expanded segments are shown where the intensity is multipled by a factor of four.

Seelig and Browning [20] have demonstrated that if a reduced temperature $\theta = (T - T_c)/T_c$ is defined, where T_c is the temperature of the transition from the liquid crystal to the gel phase, then at a common θ a variety of chains attached to a variety of head groups have a very similar profile of order versus position. Further evidence of this is displayed in Fig. 5B, where the order profile at 0°C for the oleoyl-enriched *A. laidlawii* membranes is plotted along with the results for the palmitoyl-enriched membranes [2] at 42°C, roughly the same reduced temperature. The temperature of the phase transitions for the *A. laidlawii* membranes were taken from the calorimetric data of McElhaney [32]. The dip in the $|S_{C^2H}|$ profile at the double bond of the oleoyl chains is due to the geometry and alignment of the double bond with respect to the bilayer normal. As mentioned above, the fluctuations about the average orientation of this segment of the chain are expected to be about the same as that found in the corresponding position in the palmitoyl chain. This is evident in the close similarity of the order parameters for the top and bottom of the oleoyl and palmitoyl chains, as seen in Fig. 5B.

Gel phase

A study was made of the temperature dependence of the various spectra; a representative set for the C-12′ position of the oleoyl chain is shown in Fig. 6. Above 20°C the spectra are characteristic of the liquid crystal phase. There is a well-defined powder pattern with distinct peaks, and shoulders at twice the quadrupolar splitting. As the temperature is lowered below 20°C, a second broad component, due to gel phase lipid, with a width of about 60 kHz begins

to appear in the spectra while the liquid crystal spectrum decreases in relative amplitude. Below −5°C the spectra change very rapidly, particularly in the region of −13°C, which is the center of the thermal transition covering the range from −5 to −22°C [32]. The well-defined peaks of the liquid crystal component are dramatically broadened between −10°C and −15°C. The fact that there are two components in these spectra indicates that the two phases coexist in the membrane with an interconversion rate which is less than the difference in the quadrupole splittings ($<10^5$ s^{-1}).

The broadening of the peaks in the liquid crystal phase part of the spectrum appears to be due to a very marked reduction in the spin-spin relaxation time T_2 in the vicinity of the phase transition. Such behaviour has also been observed in lyotropic liquid crystals (Wong, T.C. and Jeffrey, K.R., unpublished results). One possible explanation of the reduction in T_2 is that T_2 is determined by diffusion of the molecules from one domain to the next, causing sudden changes in the strength of the quadrupolar interaction. The rate of exchange is slow enough that an average spectrum is not observed but fast enough to be an effective spin-spin relaxation mechanism [4,5]. At high temperature when the sample is completely in the liquid crystal phase, diffusion from one domain to the next causes a relatively small change in the quadrupolar splitting. In the phase transition region movement from one domain to the next may bring the molecule from the liquid crystal to the gel state, causing a relatively large change in quadrupolar splitting.

The narrow central component in the spectra due to liquid crystal phase lipid is seen to persist even down to −32°C, 10° below the temperature at which DSC studies [32] indicate the phase transition is complete. Kang et al. [46] have observed the same phenomenon in the DMPC-cytochrome oxidase system. By varying the lipid-protein ratio they found that the narrow component persists to lower temperatures in those samples with the smallest lipid to protein ratios. This seems to indicate that the narrow component is due to lipid molecules associated with the protein. The proteins in the *A. laidlawii* membrane appear to be capable of disordering the surround 'boundary' lipid to very low temperatures.

The gel phase component of the low temperature spectra is very broad and relatively featureless. As the temperature is decreased below −32°C, intensity starts to build up at ±60 kHz, which would indicate that the rotation of the acyl chains is being progressively frozen out. However, even down to −73°C (unpublished results) there is still significant intensity in the middle part of the spectra. Further study is necessary, including a detailed lineshape analysis of the spectra, to elucidate the nature of the motions of the lipid molecules at these low temperatures; this work is currently in progress.

It is necessary at this point to comment on the fidelity of our broad spectra. To obtain an undistorted spectrum directly (i.e. using no correction factors) it is necessary that the spectral density of the radio frequency pulses at the outer edges of the spectrum differ from that at the center by no more than approx. 5%. This requires that $t_p < 1/\pi\Delta\nu_{sp}$ [47], where t_p is the width of the radio frequency pulse and $\Delta\nu_{sp}$ is the total spectral width. To obtain an undistorted spectrum of width 127.5 kHz (half of the rigid lattice value for deuterium) it is necessary to use pulses of 2.5 μs duration or less. We did this to check the

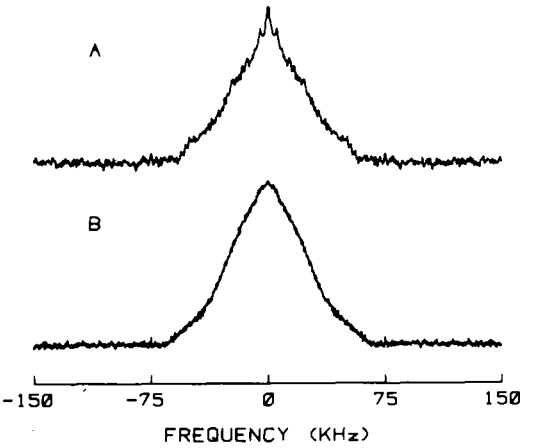

Fig. 7. Comparison of ^{2}H-NMR spectra obtained using different pulse lengths for [12'-$^2H_2$]oleoyl chains in membranes of *A. laidlawii* at −42°C. (A) Spectrum was obtained using 40° pulses of duration 2.5 μs, 50 μs pulse spacing, recycle time 0.21 s, spectral width 1 MHz, and averaging 128 000 scans. (B) Spectrum was obtained using 90° pulses of duration 5.5 μs, 50 μs pulse spacing, recycle time 0.31 s, spectral width 500 kHz, and averaging 64 000 scans.

fidelity of our spectra, and the result is shown in Fig. 7. Both spectra A and B were taken at −42°C. For spectrum A 40° pulses of duration 2.5 μs were used, with a recycle time of 0.21 s, spectral width of 1 MHz, and number of scans 128 000. For spectrum B 90° pulses of duration 5.5 μs were used, with a recycle time of 0.31 s, spectral width of 500 kHz, and number of scans 64 000. As expected, there is some power fall-off in the wings of spectrum B compared to the undistorted spectrum A, but other than that there are no gross differences. The reason that the 2.5 μs pulse is not used routinely is that for the B_1 fields that our spectrometer is capable of producing a 2.5 μs pulse width represents only a 40° pulse angle for deuterons, due to the low magnetogyric ratio of deuterons. Using 40° pulses rather than 90° pulses still produces an echo [48] but with a considerable loss in the signal-to-noise ratio, as is evident in Fig. 7.

Temperature dependence of the second moment

As has been pointed out by Smith et al. [4] the use of the second moment M_2 is a method of analyzing quantitatively the temperature dependence of the quadrupolar powder spectra and provides further insight into the effects of molecular motion on the spectra. Denoting the quadrupolar lineshape function by $F(\omega - \omega_0)$ where ω_0 is the center frequency of the symmetric powder pattern, the nth moment of the spectrum is given by

$$M_n = \frac{\int_0^\infty x^n F(x)\,dx}{\int_0^\infty F(x)\,dx} \tag{9}$$

where $x = \omega - \omega_0$. We have used the fact that our spectra are symmetric about ω_0 in order that we may use both the even and odd moments defined by Eqn. 9. Otherwise, if we had taken the integration limits as $\pm\infty$, the odd moments would have vanished. The moments of a spectrum decrease as the efficiency of the molecular motion in averaging the electric field gradient components and magnetic dipole-dipole interactions increases. The temperature dependence of the second moment for the C-12′ position is shown in Fig. 8. In the temperature region 41—20°C, where only the liquid crystal spectrum is present, there is a small increase in M_2 brought about primarily by the increase in the quadrupolar splitting as the temperature decreases. The steep increase in M_2 below 20°C is due to the increase in the fraction of the membrane lipids in the gel state. It must be pointed out that, due to power fall-off in the wings of the very broad low temperature spectra, the second moments will be only qualitatively correct for the lowest temperatures. However, even taking the reduced intensity in the wings into account, the value of M_2 at −52°C is still much less than the rigid lattice value of $1.28 \cdot 10^{11}$ s^{-2}. It appears that there is still considerable motion of the lipid molecules even at these very low temperatures.

Above 20°C the spectra are characterized by a single, broadened quadrupolar powder pattern, giving rise to a single order parameter S_{C^2H}. Below 20°C however, the membranes are in a mixed phase containing regions of both gel and liquid crystal lipid. There may then be a broad distribution of order parameters since there are large differences between values of S_{C^2H} found in gel and liquid crystalline regions and there may be defect structures causing a variation of S_{C^2H} in each of these regions. The description of the orientational order of such a complex system requires an order parameter distribution function $P(S)$ where, for a quasi-continuous distribution of S, $P(S)\mathrm{d}S$ is the probability of finding an orientational order parameter between S and $S + \mathrm{d}S$. Denoting the moments of the order parameter distribution function by

$$S_n = \int_0^\infty S^n P(S)\,\mathrm{d}S \tag{10}$$

one can define a parameter Δ_2 [49,50] as

$$\Delta_2 = \frac{S_2 - S_1^2}{S_1^2} = \frac{M_2}{1.35 M_1^2} - 1 \tag{11}$$

where the second equality is a straightforward derivation valid for cases where transverse relaxation due to the time-dependent quadrupolar interaction or the time-independent dipolar interactions can be neglected. This parameter gives the relative mean square width of the distribution of orientational order parameters. A plot of Δ_2 versus temperature for the C-12′ position is shown in Fig. 9. A system having only a single order parameter gives $\Delta_2 = 0$ when transverse relaxation is neglected. The non-zero Δ_2 values in the region 20—40°C could, in principle, be due to a small distribution of order parameters in the liquid crystal phase. However, spectra broadened by transverse relexation will also have non-zero Δ_2 values; spectral simulations of single quadrupolar powder patterns which include the effects of transverse relaxation give rise to Δ_2 values approximately equal to those determined experimentally in the region above

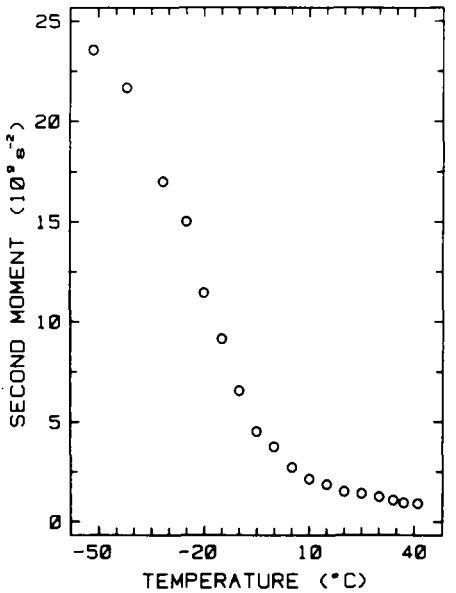

Fig. 8. Temperature dependence of the second moments of the ^{2}H-NMR spectra of *A. laidlawii* membranes biosynthetically enriched in [12'-$^2H_2$]oleic acid.

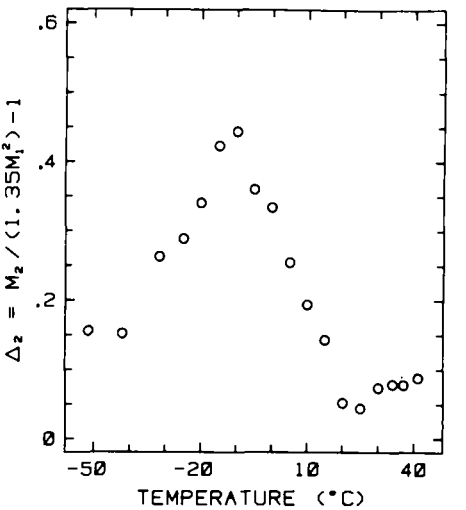

Fig. 9. Temperature dependence of the Δ_2 parameter for the ^{2}H-NMR spectra of the [12'-$^2H_2$]oleoyl chains in *A. laidlawii* membranes.

20°C. The small increase of Δ_2 as the temperature is raised above 20°C is believed to be due to the increased significance of the broadening due to transverse relaxation. The Δ_2 parameter depends not on the absolute value of the line-broadening, but rather on the ratio of line-broadening to quadrupolar splitting. If the transverse relaxation time remains relatively constant, then as the quadrupolar splitting decreases with increasing temperature the ratio of broadening to splitting will increase, and hence so will the Δ_2 parameter.

The Δ_2 parameter is very sensitive to sample inhomogeneities such as the coexistence of phases. As can be seen in Fig. 9, there is a sudden dramatic increase in Δ_2 upon entering the transition region from the high temperature side. Further decreasing the temperature, Δ_2 reaches a maximum at approx. −12°C, and falls off more slowly on the low temperature side of the peak. The peak of the Δ_2 plot coincides very closely with the center of the thermal phase transition as determined by DSC [32]. It is at this temperature that the membrane lipid order is the most inhomogeneous, as one would expect with there being large regions of both liquid crystal and gel phase lipid. The results shown in Fig. 9 indicate that the Δ_2 parameter provides a very sensitive means of mapping out the phase transition region.

Concluding remarks

Deuterium nuclear magnetic resonance has proven to be an extremely sensitive method of detecting structural and dynamical changes in membrane

systems. Thus, it provides an excellent means to determine the effects of the incorporation of a *cis*-unsaturated hydrocarbon chain into the lipids of a biological membrane. The close similarity between the order profiles for the oleoyl chains and the palmitoyl chains of the membrane lipids of *A. laidlawii*, when plotted at the same reduced temperature, must be considered as strong evidence that the conformations available to saturated and unsaturated lipids are almost idential. It seems that the major influence of the double bond is to cause an organizational perturbation in its immediate vicinity, which is sufficiently strong to regulate the relative amounts of gel and liquid crystalline phase lipid which coexist in a membrane at a given temperature; as the degree of unsaturation increases the ratio of gel to liquid crystal phase lipid decreases and the membrane becomes more 'fluid'. The viability of *A. laidlawii* at 37°C where the membrane lipids are entirely in the liquid crystalline phase indicates that, at least for this organism, it is not necessary for the growth temperature to be encompassed within the phase transition region of the membrane lipids.

As well as being nearly independent of the degree of saturation, the range of conformations available to the lipids does not appear to be grossly altered by the presence of large amounts of protein, which is evident in the similarity between the order profiles for *A. laidlawii* and pure phospholipid model membranes. In particular, there is no evidence for a significant population of immobile lipid around the membrane proteins of *A. laidlawii*. However, there do appear to be some small conformational differences, especially at the start of the *sn*-2 chain, which are believed to be due to the presence of the protein. Further studies of biological and model membranes are needed to elucidate the exact degree and nature of these differences.

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References

1 Stockton, G.W., Johnson, K.G., Butler, K.W., Polnaszek, C.F., Cyr, R. and Smith, I.C.P. (1975) Biochim. Biophys. Acta 401, 535—539
2 Stockton, G.W., Johnson, K.G., Butler, K.W., Tulloch, A.P., Boulanger, Y., Smith, I.C.P., Davis, J.H. and Bloom, M. (1977) Nature 269, 267—268
3 Davis, J.H., Nichol, C.P., Weeks, G. and Bloom, M. (1979) Biochemistry 18, 2103—2112
4 Smith, I.C.P., Butler, K.W., Tulloch, A.P., Davis, J.H. and Bloom, M. (1979) FEBS Lett. 100, 57—61
5 Kang, S.Y., Gutowsky, H.S. and Oldfield, E. (1979) Biochemistry 18, 3268—3272
6 Gally, H.U., Pluschke, G., Overath, P. and Seelig, J. (1979) Biochemistry 18, 5605—5615
7 Singer, S.J. and Nicholson, G.L. (1972) Science 175, 720—731
8 Saupe, A. (1964) Z. Naturforsch. 19a, 161—171
9 Seelig, J. (1977) Q. Rev. Biophys. 10, 353—418
10 Mantsch, H.H., Saitô, H. and Smith, I.C.P. (1977) Prog. N.M.R. Spectrosc. 11, 211—271
11 Burnett, L.J. and Muller, B.H. (1971) J. Chem. Phys. 55, 5829—5831
12 Kowalewski, J., Lindblom, T., Vestin, R. and Drakenberg, T. (1976) Mol. Phys. 31, 1669—1676
13 Higgs, T.P. and Mackay, A.L. (1977) Chem. Phys. Lipids 20, 105—114
14 Seelig, A. and Seelig, J. (1974) Biochemistry 13, 4839—4845

15 Seelig, A. and Seelig, J. (1975) Biochim. Biophys. Acta 406, 1—5
16 Seelig, A. and Seelig, J. (1977) Biochemistry 16, 45—50
17 Seelig, J. and Waespe-Sarcevic, N. (1978) Biochemistry 17, 3310—3315
18 Oldfield, E., Meadows, M., Rice, D. and Jacobs, R. (1978) Biochemistry 17, 2727—2740
19 Stockton, G.W., Polnaszek, C.F., Tulloch, A.P., Hasan, F. and Smith, I.C.P. (1976) Biochemistry 15, 954—966
20 Seelig, J. and Browning, J.L. (1978) FEBS Lett. 92, 41—44
21 Chapman, D. (1975) Q. Rev. Biophys. 8, 185—235
22 Overath, P., Brenner, M., Gulik-Krzywicki, T., Schechter, E. and Letellier, L. (1975) Biochim. Biophys. Acta 389, 358—369
23 Baldassare, J.J., Rhinehard, K.B. and Silbert, D.F. (1976) Biochemistry 15, 2986—2994
24 Linden, C.D., Blasie, J.K. and Fox, C.F. (1977) Biochemistry 16, 1621—1625
25 Engelman, D. (1971) J. Mol. Biol. 58, 153—165
26 McElhaney, R.N., de Gier, J. and van der Neut-Kok, E.C.M. (1973) Biochim. Biophys. Acta 298, 500—512
27 De Kruyff, B., van Dijck, P.W.M., Goldbach, R.W., Demel, R.A. and van Deenen, L.L.M. (1973) Biochim. Biophys. Acta 330, 269—282
28 Cronan, J.E. and Gelmann, E.P. (1975) Bacteriol. Rev. 39, 232—256
29 Esser, A.F. and Souza, K.A. (1976) in Extreme Environments (Heinrich, M.R., ed.), pp. 283—294, Academic Press, New York
30 Poo, M.-M. and Cone, R.A. (1974) Nature 247, 438—441
31 Kuo, A. and Wade, C.G. (1979) Biochemistry 18, 2300—2308
32 McElhaney, R.N. (1974) J. Mol. Biol. 84, 145—147
33 Huang, L., Lorch, S.K., Smith, G.S. and Haug, A. (1974) FEBS Lett. 43, 1—5
34 McElhaney, R.N. (1976) in Extreme Environments (Heinrich, M.R., ed.), pp. 255—281, Academic Press, New York
35 Souza, K.A., Kostiw, L.L. and Tyson, B.J. (1974) Arch. Microbiol. 97, 89—102
36 Davis, J.H., Jeffrey, K.R., Bloom, M., Valic, M.I. and Higgs, T.P. (1976) Chem. Phys. Lett. 42, 390—394
37 Tulloch, A.P. (1979) Chem. Phys. Lipids 24, 391—406
38 Butler, K.W., Johnson, K.G. and Smith, I.C.P. (1978) Arch. Biochem. Biophys. 191, 289—297
39 Speight, P.A., Jeffrey, K.R. and Courtney, J.A. (1974) J. Phys. E. 7, 801—802
40 Buess, M.L. and Petersen, G.L. (1978) Rev. Sci. Instrum. 49, 1151—1155
41 Hitchcock, P.B., Mason, R., Thomas, K.M. and Shipley, G.G. (1974) Proc. Nat. Acad. Sci. U.S.A. 71, 3036—3040
42 Büldt, G., Gally, H.U., Seelig, A., Seelig, J. and Zaccai, G. (1978) Nature 271, 182—184
43 Zaccai, G., Büldt, G., Seelig, A. and Seelig, J. (1979) J. Mol. Biol. 134, 693—706
44 Rice, D. and Oldfield, E. (1979) Biochemistry 18, 3272—3279
45 Woessner, D.E. (1977) Mol. Phys. 34, 899—920
46 Kang, S.Y., Gutowsky, H.S., Hsung, J.C., Jacobs, R., King, T.E., Rice, D. and Oldfield, E. (1979) Biochemistry 18, 3257—3267
47 Boden, N., Hanlon, S.M., Levine, Y.K. and Mortimer, M. (1978) Mol. Phys. 36, 519—540
48 Solomon, I. (1958) Phys. Rev. 110, 61—65
49 Davis, J.H. (1979) Biophys. J. 27, 339—358
50 Davis, J.H., Bloom, M., Butler, K.W. and Smith, I.C.P. (1980) Biochim. Biophys. Acta 597, 477—491