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MOLECULAR ORDER IN *ACHOLEPLASMA LAIDLAWII* MEMBRANES AS DETERMINED BY DEUTERIUM MAGNETIC RESONANCE OF BIOSYNTHETICALLY-INCORPORATED SPECIFICALLY-LABELLED LIPIDS

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Summary

The first application of deuterium magnetic resonance of specifically labelled lipids to the study of a natural biological membrane is described. Palmitic acid labelled at the terminal methyl group with deuterium was incorporated biosynthetically into the lipids of the plasma membrane of *Acholeplasma laidlawii*. The deuterium nuclear magnetic resonance spectra contain quadrupole splittings which yield directly order parameters for this region of the membrane. Below the growth temperature (37 °C) the spectra are indicative of lipid in both gel and liquid crystalline states. Above this temperature they demonstrate the existence of an entirely liquid crystalline membrane whose order parameter decreases rapidly with increasing temperature. Comparison with egg phosphatidylcholine over the same temperature range shows a more rapid change in order with temperature for the *A. laidlawii* membranes.

Several recent articles have demonstrated the value of deuterium magnetic resonance spectroscopy (^{2}H NMR) in studies of molecular motion and orientational-order of specifically deuterated lipids in model-membrane bilayers [1—5]. Unlike the nitroxide-containing electron spin resonance (ESR) probes, the deuterium probe does not significantly perturb its environment [4]. Isotopic enrichment with deuterium provides a level of selectivity normally unavailable with natural abundance ^{1}H or ^{13}C NMR at much lower cost than enrichment with ^{13}C. Furthermore, the electric quadrupole moment

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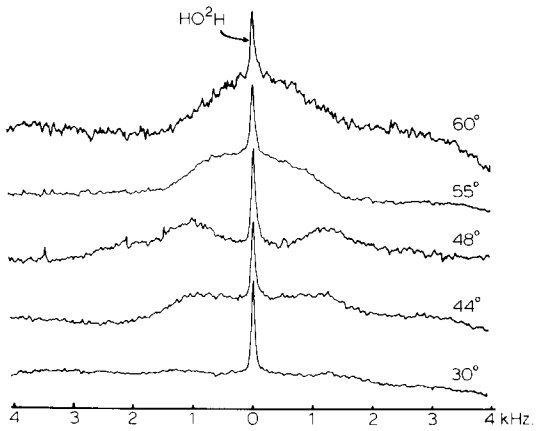
of deuterium allows direct measurement of the molecular order parameter [3,4]. Only one previous study of a natural biological membrane using ^2H NMR has appeared in the literature; Oldfield et al. [6] incorporated perdeuterated lauric and palmitic acid into the membrane lipids of *Acholeplasma laidlawii* by supplementing the growth medium with the labelled fatty acid. The ^2H NMR spectra of the post-lysis cytoplasmic membranes recorded at the growth temperature contained a broad unstructured envelope of numerous overlapping resonances due to the fully deuterated hydrocarbon chains. However, the need for incorporation of specifically deuterated lipids, to facilitate measurement of the dynamic quadrupole splittings, was made clear.

We have grown *A. laidlawii* strain B cells (originally obtained from Dr. R.N. McElhaney) at 37°C in an initially fatty acid-free tryptose broth [7] supplemented with $16d_3$-palmitate (25 $\mu g \cdot ml^{-1}$, Serdary Research Laboratories, London, Ontario). The cells were harvested in late-log phase, washed with β-buffer (0.5 M NaCl in 0.025 M Tris·HCl, pH 8.5), osmotically lysed in distilled water, washed three times in distilled water, and freeze-dried. Several batches were combined and a portion was analyzed for acyl chain composition by a process involving solvent extraction, saponification, methylation, and gas chromatography. The acyl chain distribution in *A. laidlawii* grown in supplemented media is 9.2% 12:0, 11.8% 14:0, and 79.0% 16:0, and in unsupplemented media 5.7% 12:0, 32.1% 14:0, 53.6% 16:0, 6.3% 18:0, and 2.3% 18:1. From several previous studies [6,7] it is known that the exogenous fatty acid is preferentially incorporated and the analysis indicates that 79% of the lipid acyl chains was derived from the labelled fatty acid. The acyl chains are shown to exist in the membrane in the form of carotenoid esters, glucosyl diglycerides, phosphatidyl glycerol and its amino acyl ester, and phosphatidyl glucose [8]. The relative amounts of these lipids vary with growth conditions but the acyl chain distribution is known to be similar for each lipid [8].

Fig. 1 illustrates ^2H NMR spectra recorded at several temperatures of approx. 300 mg of *A. laidlawii* membranes (containing 60 mg of $16d_3$-palmitate) suspended in β-buffer to a total volume of 1 ml. The spectra, obtained on a Varian XL-100 Fourier transform spectrometer at 15.4 MHz using 12 mm sample tubes, resulted from the acquisition of up to $2.5 \cdot 10^5$ transients. Each spectrum contains a narrow central line due to natural abundance deuterium in water, and a broad signal symmetric about the water line attributed to the C^2H_3 resonance of partially-oriented lipid in the liquid crystalline phase of the membrane bilayers*. The appearance and intensity of the deuteriomethyl signal vary markedly with temperature. Below approx. 50°, the spectra exhibit the expected powder pattern with a characteristic double maximum and residual quadrupole splitting D_q. This lineshape is normal for a random distribution of bilayer orientations present in the inhomogeneous suspension of membrane fragments. When the temperature exceeds 50°, the residual quadrupole splitting approaches the line width

*The much broader signal from the gel phase cannot be observed on the XL-100 spectrometer, even in model systems with improved signal-to-noise ratio, because the free-induction-decay goes to zero in a time shorter than the recovery time of the probe and receiver.



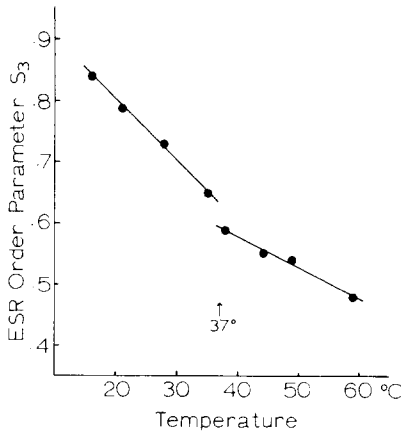


Fig. 1. 15.4 MHz ^2H NMR spectra of *Acholeplasma laidlawii*-B membranes containing incorporated $16d_3$-palmitate, at several temperatures.

Fig. 2. A plot of ESR order parameter S_3 vs. temperature for palmitate-supplemented *Acholeplasma laidlawii*-B membranes containing the intercalated nitroxide spin probe 5-doxyl stearic acid.

and the double maximum is no longer discernible.

The area under the deuteriomethyl resonance is constant, within the experimental uncertainty of approx. 20%, throughout the temperature range 44—60°C, and is roughly five times greater than at 30°C. This is indicative of the broad gel-to-liquid crystal transition previously observed for palmitate-supplemented *A. laidlawii* membranes using differential thermal analysis [9]. We have performed a study of this phase transition using standard techniques [10] and the spin probe 5-doxyl stearic acid. The variation of the ESR order parameter S_3 with temperature (Fig. 2) contains an obvious break characteristic of a gel-to-liquid crystal transition [11] in the vicinity of the growth temperature of 37°C.

If we assume a two state (gel and liquid crystal) system in the vicinity of the broad phase transition, the ^2H NMR spectra provide a facile estimate of the relative proportions of lipid in the gel and liquid crystalline states. The ^2H at neutral abundance in water provides a crude but useful intensity reference through which the absolute intensity of the lipid resonance can be estimated. The relative areas of the HO^2H and C^2H_3 signals indicate that for temperatures above 44°C most or all of the lipid is in the liquid crystalline state, while at 30°C only 20% of the lipid is present in this state. The ratio of gel-to-liquid crystal in *A. laidlawii* membranes is known to bear a direct relation to the ability of the organism to grow [12].

The main purpose of this study is to demonstrate the measurement of the molecular (chain segment) order parameter S_{mol} for lipid bilayers in a natural membrane by the ^2H NMR method. The powder patterns in Fig. 1 conform to the lineshape function:

$$g(\nu) = \int_{\theta=0}^{\pi/2} \sin\theta \, [T_2^{*-2} + \{\nu \pm 3/8 \, (e^2 qQ/h)(3\cos^2\theta - 1) S_{C^2H}\}_2]^{-1} \, d\theta \qquad (1)$$

where θ is the angle between the normal to the bilayer and the applied magnetic field, ν is the frequency in Hz, and e^2qQ/h is the quadrupole coupling constant (approx. 170 kHz for most C-^{2}H bonds). S_{C^2H} is the order parameter for the carbon-deuterium bond, and is related to the molecular order parameter by a simple transformation of coordinates; $S_{mol} = -3S_{C^2H}$ for a deuteriomethyl group [4]. The line width W is characterized by a time constant T_2^* (where $W = 1/\pi T_2^*$). For cases where the quadrupole splitting $D_q = 3/4(e^2qQ/h)S_{C^2H}$ is greater than approx. 10 times the line width, the frequency separation between the lineshape maxima is identical with D_q. When this condition is not fulfilled, the frequency separation is less than D_q, and the quadrupole splitting must be estimated by fitting the observed lineshape to the theoretical function given by Eqn 1. This was found to be necessary for all of the spectra shown in Fig. 1. Of the two adjustable parameters, D_q and T_2^*, the latter was chosen to be constant for all spectra. Our experience with model membranes has shown that T_2^* is determined largely by inhomogeneities in the sample and ordering and is not strongly temperature dependent [3]. On the other hand, the spin lattice relaxation time T_1, which is determined by molecular diffusion, is usually several orders of magnitude longer than T_2^* and strongly temperature dependent [3].

In Fig. 3 a comparison is made between the values of S_{mol} for 16d_3-palmitate in *A. laidlawii* membranes and intercalated in an aqueous dispersion of egg lecithin. At 30°C the values of S_{mol} for the natural and model membranes are very similar, but at higher temperatures the curves diverge. The value of S_{mol} is related to conformational preferences of the flexible hydrocarbon chains. The effective length of a hydrocarbon chain in the bilayer is determined by the distribution of *gauche* and *trans* rotamers among the chain segments, and is given by the sum over all segments of the products of order parameter and length of each segment [4]. The coefficient of thermal expansion α of the chain length is the summation of the coefficients for each chain segment: $\alpha = \sum_i \alpha_i = \sum_i \Delta(S_{mol})_i/2.25\Delta T$ [4]. For the terminal segment of the palmitate chain in egg lecithin and *A. laidlawii* membranes, $\alpha_{16} = -2.6 \cdot 10^{-4}$ and $-8.9 \cdot 10^{-4}$ °K^{-1}, respectively, in the range 40—60°C. Although it can be misleading to make general inferences from measurements made at

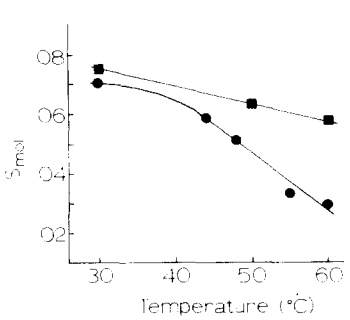


Fig. 3. A plot of deuterium NMR chain-segment order parameter S_{mol} vs. temperature for *Acholeplasma laidlawii*-B membranes containing incorporated 16d_3-palmitate (●), and for aqueous egg lecithin dispersion containing intercalated (8 mol %) 16d_3-palmitate (■). The experimental uncertainty in S_{mol} is ± 0.005.

a single chain position, these observations imply a much stronger dependence of the thickness of the bilayer on temperature in *A. laidlawii* membranes than in the lamellar liquid crystalline phase of egg lecithin.

It would be of considerable interest to compare the present ^2H data with results obtained from the corresponding spin label in order to ascertain the extent of the spin label perturbations, as has been done recently for dipalmitoylecithin [4]. However, spin labels have not yet been introduced at the terminal position of a fatty acid. The closest comparison is with the 16-doxyl stearic acid; it gives an ESR spectrum of three narrow lines, implying rapid, pseudoisotropic motion. Only complex spectral simulation could yield an estimate of the order parameter in this case [13]. In general, ESR can measure the order parameter conveniently only when $S_{mol} > 0.3$ [14], whereas ^2H NMR is most sensitive for small order parameters, involves no complicated simulations, and is always accurate.

The present study demonstrates the viability of ^2H NMR for the study of biological membranes. The sensitivity problem is overcome by achieving a high level of biosynthetic incorporation and the use of Fourier transform NMR techniques. We are presently extending the study using palmitic acid labelled sequentially up the chain from the terminal methyl group.

Deuterium NMR cannot replace nitroxide spin-labelling as a sensitive, rapid, and general method to determine order and mobility in biological membranes. However, it offers an accurate alternative for the study of major, lipid-rich membranes, and an evaluation of the inaccuracy of spin label data due to nitroxide-induced perturbations.

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