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BIOLOGICAL MEMBRANES ARE RICH IN LOW-FREQUENCY MOTION

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Using ^{13}C cross-polarization NMR techniques, we have found that the effect of protein on the dynamics of the hydrocarbon interior of a series of biological membranes is to depress the intensity of motion on the nanosecond timescale (i.e., T_1 becomes longer) and to enhance the intensity of motion on the timescale of tens of microseconds (i.e., $T_{1\rho}$ becomes shorter).

The role of molecular motion in the function of biological membranes has yet to be determined [1,2]. Many experimental approaches have been employed to examine the motion undergone by the phospholipids [3–5] and to a lesser extent by the proteins [6] associated with membranes. There remains, however, very little direct evidence of the dynamic interactions which occur between these membrane components. Measurements based upon the use of probe molecules suffer from the inherent uncertainty of the location, distribution, conformation and perturbation effects of these probe molecules relative to the unlabelled molecules in the membrane. Nuclear magnetic resonance spectroscopy (NMR) provides one method of avoiding many of these problems, since information may be obtained of the molecular order and motion using naturally occurring atomic species or by isotopic enrichment of chemically identical molecules.

Contrary to the results obtained using electron spin resonance or fluorescence spectroscopy, to date NMR has failed to reveal any major changes in the molecular order of the hydrocarbon interior of a membrane due to the presence of a membrane-associated protein [7–11]. By contrast, in the present work we demonstrate that a significant interaction does indeed exist between the membrane lipid and protein by observing the lipid dynamics over a range of timescales from tens of microseconds to nanoseconds.

The lower frequency modes of reorientation were measured using the NMR rotating frame spin-lattice relaxation time, ($T_{1\rho}$), which is sensitive to motion on the timescale of tens of microseconds [12]. The rapid intramolecular reorientations which occur on a timescale of nanoseconds were measured by the laboratory reference frame spin-lattice relaxation time (T_1) [13].

The NMR relaxation time in the laboratory reference frame (T_1), and in the rotating reference frame ($T_{1\rho}$), may under many circumstances be related to the spectral intensity of motion $I(\omega)$ by the approximate expressions [12–14]

$$T_1^{-1} \approx K_1 I(\omega_0)$$

and

$$T_{1\rho}^{-1} \approx K_2 I(2\omega_1)$$

where K_1 and K_2 are constants depending on the

dominant interaction contributing to relaxation and on the type and anisotropy of the motion undergone by the nuclear spins.

$I(\omega_0)$ and $I(2\omega_1)$ are the spectral intensities of this motion near the operating frequency of the spectrometer (ω_0) and near twice the frequency of precession of the nuclear spins about the applied radiofrequency field ($2\omega_1$). In the present context, these techniques probe motion on the timescale of nanoseconds and tens of microseconds, respectively.

Of the various ways in which T_1 and $T_{1\rho}$ may be measured, we have chosen to use the double-resonance, proton-enhanced technique [15,16] as it provides a number of advantages. The most important of these is that the primary result of this approach is a natural abundance carbon-13 spectrum in which the lipid methylene envelope is well resolved. No specific labels are needed and the sample does not have to be prepared in any special manner for NMR. For reasons which we will explain presently, the results of the proton enhanced carbon-13 measurement of T_1 and $T_{1\rho}$ shall be denoted T_1^H and $T_{1\rho}^H$.

The NMR measurements were made using a Bruker CXP system operating at either 90.02 MHz or 300.066 MHz for protons, and 22.63 MHz or 75.46 MHz for carbon-13. The proton 90° pulse was varied between 4 and 10 μs, corresponding to a range of H_1 values of 62.5 to 25 kHz. Typical parameters for the 90.02 MHz frequency range were a sweep width of 20 kHz, a repetition time of one per second and 500 scans per spectrum. Similar conditions were used at 300 MHz with a sweep width of 60 kHz. For the maize and spinach chloroplasts, a significant improvement in the signal-to-noise ratio of the spectrum was achieved by enriching their growth atmosphere with carbon-13 labelled CO_2. In the unlabelled material the quantity of solids in the membrane samples was typically in the range 70–200 mg. For the lipid samples as little as 10 mg of solids was used. With the carbon-13 enrichment, the chloroplast systems required an order of magnitude less sample to yield adequate spectra. An example of a carbon-13 membrane spectrum is shown in Fig. 1.

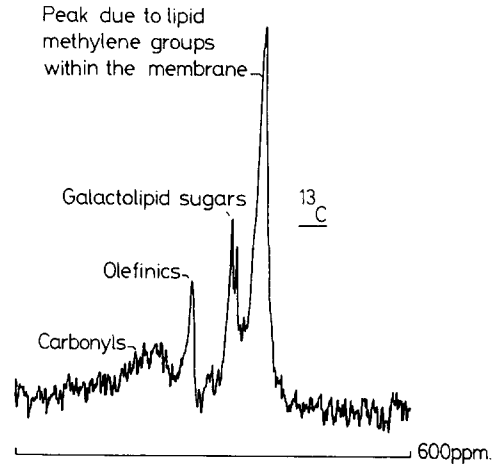

Fig. 1. Proton enhanced, cross-polarization, carbon-13 NMR spectrum of maize chloroplast membranes obtained using a sweep width of 60 kHz, a Hartman Hahn 90° pulse duration of 8 μs in 8 min at one acquisition per s (500 scans). The temperature was 12°C and the line broadening of this particular spectrum 75 Hz. The plot width for the spectrum shown above is 600 ppm.

Biological membranes used in this study included bovine brain myelin [17], spinach [18] and maize mesophyll chloroplasts [19], the purple membrane of *Halobacterium halobium* [20], rat liver mitochondrial membranes free of matrix proteins [21], red blood cell membranes [22] and the inner membranes of spores of *Bacillus megaterium* [23] and were prepared as discussed in the appropriate reference. For this study the membranes were concentrated by centrifugation at 180 000 × g for 1–12 h to give preparations which contained by weight between 20- and 3-fold excess of water to total solids.

Functional tests of chloroplasts [24], *Halobacterium* membranes [25,26] and rat liver mitochondria [21] indicated a negligible loss of biochemical activity as a result of being examined by NMR. Measurements were performed during no more than a 1 h period for a single estimate of $T_{1\rho}^H$ or T_1^H, which ensured the integrity of biochemical function but at the expense of signal quality. Any trends seen in the relaxation time were then extrapolated back to zero time. No systematic changes were detected in the relaxation times over the first 8 h. However, measurements spaced by days showed a progressive trend to longer values of $T_{1\rho}^H$ and shorter values of T_1^H. A possible origin for this effect is the pooling of lipid within the sample resulting in lipid spectra less

influenced by the membrane protein. Lipid extracts and in some instances fractionation of these extracts into their component lipids were also studied.

Myelin total lipid was extracted by the method of Hara and Radin [27]. The membranes of *Halobacterium* and the spore inner membrane were extracted for their total lipid by the Bligh and Dyer technique [28]. The polar lipid fraction of a lipid extract of rat liver mitochondria was used to provide data on the phospholipids of the mitochondrial membrane lipids [21]. The chloroplast lipids, including chlorophyll were extracted by the technique of Fishwick and Wright [29]. A polar lipid extract of maize leaves was also produced. These extracts were dispersed in at least an equal weight of water and thoroughly homogenised prior to being loaded into the spectrometer.

A summary of the results we have obtained on a variety of natural membranes is shown in Table I.

The data are presented as a ratio relative to $T_{1\rho}^{H}$ or T_1^H of fluid phase dimyristoylphosphatidylcholine (DMPC) for which $T_{1\rho}^{H} = 85$ ms and $T_1^H = 210$ ms at 32°C. These ratios provide an estimate of the relative spectral density near frequencies of reorientation of the hydrocarbon chains within these membranes relative to those of the hydrocarbon chains in a fluid phase dispersion of DMPC. In Table II we compare the result from intact membranes and their lipid extract.

A clear result of this work is that the timescale of events within intact natural membranes is depressed quite dramatically to lower frequencies when compared to either a pure synthetic lipid or to a lipid extract of the membrane. This is reflected in a general large increase in the ratio of the $T_{1\rho}$ and a less marked decrease in the ratio of the T_1. The only minor deviation from this behaviour is seen in the inner membranes of *B. megaterium* spores in which the T_1 increased from 135 ms to 244 ms upon extracting the lipid. The lower frequency events on the timescale of tens of microseconds were still very much more intense in the natural membrane than in the lipid extract.

When considered in the context of the relatively small changes seen in deuterium NMR order parameters for lipid dispersions in the presence of protein [7] it is probable that the effects we observe in $T_{1\rho}^{H}$ and T_1^H are more a consequence of the rate at which certain events occur rather than a major change in the amplitude of motion undergone by the lipid chains.

Implicit in the approach we have adopted, are two assumptions: (a) that the methylene resonance peak is dominated by the membrane lipid, and (b) that the relaxation pathway for the methylene carbons is dominated by the relaxation times of the protons adjacent to the methylene carbons and for this reason we have denoted the relaxation times $T_{1\rho}^{H}$ and T_1^H, respectively.

In justifying these two assumptions, we make

TABLE I

INTENSITY OF MOTION UNDERGONE BY THE HYDROCARBON CHAINS IN A VARIETY OF NATURAL MEMBRANES RELATIVE TO FLUID PHASE DMPC AT 32°C FOR RATES OF REORIENTATION ON THE TEN MICROSECOND AND NANOSECOND TIMESCALE

A larger number indicates a greater relative intensity of motion in comparison with DMPC. The relative intensities of motion per interval of frequency on the microsecond and nanosecond timescales are approximated here by $T_{1\rho}^{H}$ (DMPC fluid phase)/$T_{1\rho}^{H}$ (membrane) and T_1^H (DMPC fluid phase)/T_1^H (membrane). For fluid-phase DMPC, $T_{1\rho}^{H} = 85$ ms and $T_1^H = 210$ ms. Apart from the gel-phase DMPC, which was recorded at 1°C, for all other samples the membrane lipids were predominantly in the fluid phase.

	Relative intensity of motion	
	Ten microsecond timescale	Nanosecond timescale
Gel phase DMPC (1°C)	28.9	0.9
Purple membrane of *Halobium* (10°C)	27.4	0.4
Myelin (10°C)	27.7	1.0
Maize chloroplasts (12°C)	16.7	0.5
B. Megaterium (32°C) inner spore membranes	15.2	1.6
Spinach chloroplasts (12°C)	13.7	1.1
Rat liver mitochondria (10°C)	12.3	0.7
Red blood cells (32°C)	7.1	1.0
Fluid-phase DMPC (32°C)	1.0	1.0

TABLE II

INTENSITY OF MOTION UNDERGONE BY THE HYDROCARBON CHAINS IN A VARIETY OF NATURAL MEMBRANES RELATIVE TO THAT OF THE HYDROCARBON CHAINS IN THEIR LIPID EXTRACTS DISPERSED IN WATER FOR RATES OF REORIENTATION ON THE TEN MICROSECOND AND NANOSECOND TIMESCALE

A large number indicates a greater relative intensity of motion in the membrane than in rehydrated dispersions of the endogeneous lipid. The intensity of motion per interval of frequency on the microsecond and nanosecond timescales are approximated here by $T_{1\rho}^{H}$ (lipid extract)/$T_{1\rho}^{H}$(membrane) and T_1^H (lipid extract)/T_1^H (membrane). The ratios shown in the above table are the relaxation times in ms. All samples were run at temperatures high enough to ensure that their membrane lipids were predominantly in the fluid phase.

	Relative intensity of motion	
	Ten microsecond timescale	Nanosecond timescale
Purple membrane (10°C)	15 / 3.1 ≈ 5	52/570 ≈ 0.1
Myelin (10°C)	24.1/ 3 ≈ 8	222/822 ≈ 0.3
Maize chloroplasts (12°C)	35 / 5.1 ≈ 7	173/405 ≈ 0.4
Rat liver mitochondria (10°C)	75 / 6.9 ≈ 11	207/303 ≈ 0.7
B. megaterium spore (32°C) inner membranes	18 / 5.6 ≈ 3	244/135 ≈ 1.8
Red blood cells (32°C)	65 /12 ≈ 5	223/213 ≈ 1.05

the following points. With the exception of the purple membrane, for all of the membranes more than 98% of the methylene groups are associated with the membrane lipid. In the case of the purple membrane we calculate this number to be greater than 70%, hence some contribution from the resonances on the membrane protein might be included in the peak we are assigning to the membrane lipid, despite the absence of any obvious multicomponent relaxation behaviour. A further indication that we are observing the properties of the membrane lipid is seen in those situations where the membranes contain a significant percentage of olefinic carbons. These resonances also possess short values of $T_{1\rho}^{H}$ similar to the methylene groups and cannot, of course, arise from the membrane protein.

Of the relaxation pathways available to the carbon-13 nuclei, it has been found in very similar systems, such as polyethylene [30], that relaxation is dominated by the spin-lattice relaxation of the strongly coupled adjoining methylene protons. Measurements of the apparent rotating frame spin-lattice relaxation time ($T_{1\rho}^{C}$) of the carbon nuclei, gave very similar values to those obtained for $T_{1\rho}^{H}$. This suggests that the cross-coupling effects between the carbon-13 and proton populations are very fast relative to the relaxation rates of either $T_{1\rho}^{H}$ or $T_{1\rho}^{C}$. Some indications of the actual value for $T_{1\rho}^{C}$ may be obtained from the measured values of the carbon spin-lattice relaxation time (T_1^C), which are not influenced by these cross-relaxation effects. Using the technique of Torchia [31], we have found that T_1^C is approximately an order of magnitude longer than T_1^H in a number of the systems studied here. A major factor contributing to this longer value of T_1^C is the smaller carbon-proton second moment relative to the proton-proton second moment which influences the T_1^H. Since the same decrease in second moment is expected to influence $T_{1\rho}^{C}$, provided the spectral density at the carbon frequency is not too different from that at the proton frequency, we would expect to see a much longer value of the intrinsic $T_{1\rho}^{C}$.

All relaxation functions were, to the precision of the data, adequately fitted by a single exponential decay *. The possibility of a superposition of a number of decays is in the present context not very significant because of the qualitative magnitude of the effects we are reporting.

Many types of motion appear to be possible candidates for the major effect seen here. The timescale for the lower frequency motion suggests that the dominant mode of reorientation contributing to the relaxation is a collective movement of

* A further consideration is the effect of spin diffusion [32] which will average the actual values of $T_{1\rho}^{H}$ and T_1^H for the various sites along the hydrocarbon chain to give a single weighted value. As we have discussed elsewhere [14], it is unlikely that the effects of spin diffusion will be sufficiently rapid to be influenced by groups beyond the chain in which a particular carbon resonance is located.

large segments of the lipid acyl chains. One such physical picture of this type of motion may be likened to the effect of 'wind on wheat' in which large numbers of stems (lipid chains) are tilted in a common direction at a particular instant. Support for this type of motion occurring has recently been provided by the molecular dynamics simulations of Van de Ploeg and Berendsen [33]. Other possible collective motions include a variety of 'transverse wave effects'. Like the wave motions which emanate from a disturbance in a pool of water, these effects involve displacements perpendicular to the surface of the membrane which radiate outwards into the surrounding lipid away from the protein surface. Again, many authors [34,35] have considered this type of motion although almost exclusively from the point of view of molecular order rather than the timescale of the interactions which occur between the lipid and protein. Because of the present lack of both experimental data and theoretical simulations, it is unclear how far from the membrane protein such effects are still significant. It appears unlikely, however, that the range is greater than of order of ten molecular distances away from the protein. Based on molecular field calculations [36] of the influence of proteins on the formation of *trans, gauche, trans* kinks along the length of the chain, these intra-chain effects appear to relax back to equilibrium by as few as two lipid molecules away from the protein.

In summary we wish to emphasise the following results:

(a) The lipid chains in natural membranes undergo motion which is far more intense on the timescale of tens of microseconds and less intense on the timescale of nanoseconds than do the chains of fluid phase DMPC.

(b) Comparing the motion undergone by the lipid chains within an intact functioning membrane with dispersions prepared from their lipid extract we find a substantial transfer to lower frequencies of all motions. This result in a depletion of the nanosecond timescale motion and an increase in the intensity of motion on the ten microsecond timescale, respectively.

(c) By comparison with the relative insensitivity of the molecular order reported by deuterium NMR data when proteins are added to lipids, the present results suggest that the major effect we are observing is a shift of the timescale of the molecular motion to lower frequencies rather than a substantial change in the amplitude of an existing motion.

Although the details of the low frequency motion remain to be explored, it is intriguing to find that the major effect on the lipid dynamics due to the addition of protein occurs on the apparently biologically relevant timescale of tens of microseconds. These low-frequency modes of reorientation therefore need to be considered as a possible source of lipid-mediated protein-protein interaction.

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