HIGH RESOLUTION DEUTERIUM MAGNETIC RESONANCE – AN APPROACH TO THE STUDY OF MOLECULAR ORGANIZATION IN BIOLOGICAL MEMBRANES AND MODEL SYSTEMS*

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1. Introduction

Magnetic resonance techniques have been widely employed for the study of biological membranes and model systems. Both the spin probe method [1] and nuclear magnetic resonance [2] have provided useful information about the motion of molecules in membranous assemblies. A large amount of work has been done using proton and carbon magnetic resonance. Phosphorus [3, 4], fluorine [5] and broad line deuterium resonance [6–8] have been used to a lesser extent.

We report here the results obtained from the high resolution deuterium resonance (^{2}H NMR) spectra of selectively deuterated lauric acid intercalated in sonicated egg lecithin bilayers. Deuterium resonance has the advantage that selective deuteration can be easily effected and that the linewidths are principally determined by quadrupole relaxation. Intermolecular effects (dipole–dipole interaction) which complicate proton relaxation mechanisms are eliminated in ^{2}H NMR. Earlier ^{2}H NMR studies on perdeuterated dimyristoyl lecithin at 30°C [6] suggested that the linewidths were very large (4.2 KHz for the terminal C^2H_3 group). However, the linewidths for the C^2H_3 group of selectively deuterated lauric acid in sonicated egg lecithin bilayers are narrow (17 ± 1 Hz). Addition of 20 mole % cholesterol results in an increase to 65 ± 5 Hz. The effect of introducing the deuterium probe was monitored using the ESR spectra of intercalated spin probes. High concentrations of the fatty acid probe resulted in increased fluidity of the lipids in the bilayers. Correlation times for internal motion of the C^2H_3 group are in good agreement with those calculated for fatty acid spin probes in similar systems.

2. Experimental

The deuterated probe, 1,2,2,12,12,12-hexadeuteriolauric acid (C^2H_3-CH_2)$_9$-C^2H_2-COO^2H, LA), prepared according to Isabelle and Leitch [9], was a generous gift from Dr. L.C. Leitch. Egg yolk lecithin (Lipid Products, S. Nutfield, England) was used without further purification. Cholesterol (recrystallized twice from methanol) was from Steraloids, Pawling, N.Y. Chloroform solutions of LA and lecithin (and cholesterol) were evaporated under wet nitrogen and samples were left under vacuum for no less than 2 hr. Dispersions were prepared by adding water and shaking in a Vortex mixer. Sonication was performed under N_2 with a bath type sonifier at 0°C for not less than 1 hr. Planar bilayers for electron spin resonance (ESR) experiments were prepared as described previously [10].

^{2}H NMR experiments were performed at 30°C in 12 mm o.d. sample tubes with a Varian XL-100-15 spectrometer operated at 15 MHz both in continuous wave and Fourier transform modes. The ^{1}H resonance of the solvent was used for the field-frequency control. Improvements of signal-to-noise ratio were made by

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time-averaging on a Varian 620 L computer. The ESR experiments were performed on a Varian E-9 spectrometer at 22 ± 1°C. Spin probes were products of Syva Associates.

3. Results and discussions

Fig. 1A shows the ^{2}H NMR spectrum of LA in acetone. Two peaks separated by 21.6 Hz appear and have been assigned to the C^2H_3 (linewidth at half height, $\Delta\nu_{1/2}$ = 1.6 Hz) and C^2H_2 ($\Delta\nu_{1/2}$ = 2.8 Hz) groups, respectively. The linewidth of the C^2H_2 group increases considerably ($\Delta\nu_{1/2}$ = 11.5 Hz) in a water–methanol mixture (7:3 v/v, pH 8.6), whereas the linewidth of the C^2H_3 group ($\Delta\nu_{1/2}$ = 2.9 Hz) is much less changed (fig. 1B). This implies the formation of an aggregate where motion is more restricted at the polar region.

^{2}H NMR spectra were taken in the Fourier transform mode for samples of LA incorporated in egg lecithin. Only a peak due to natural abundance deuterium in water (with a chemical shift δ = 5.03 ppm from an external reference consisting of the natural abundance deuterium resonance of tetramethylsilane, TMS) is seen for unsonicated samples (fig. 2A). After sonication to translucency in a water–ice bath under a nitrogen stream, a peak ($\Delta\nu_{1/2}$ = 17 ± 1 Hz) assigned to the C^2H_3 group appears at δ = 1.13 ppm from TMS (fig. 2B). The linewidth of deuterium in water is not

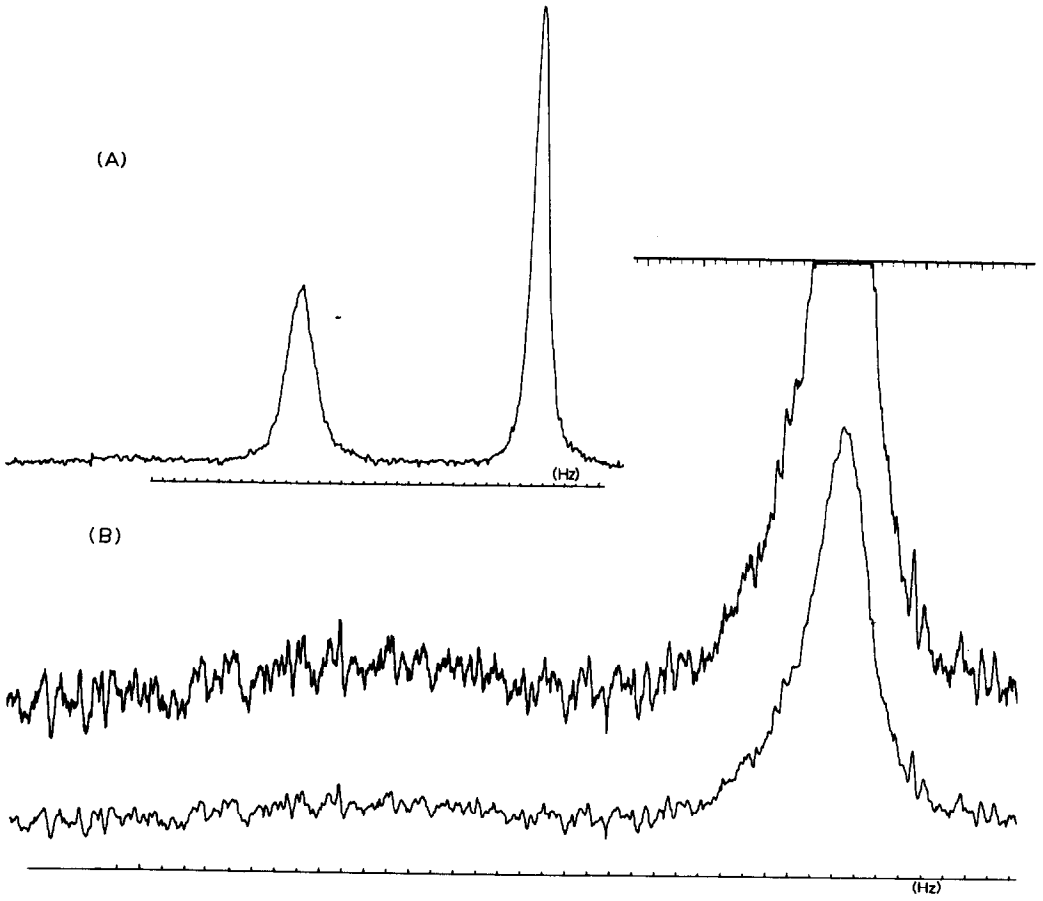

Fig. 1. A) ^{2}H NMR spectrum (1 Hz/division) of a 485 mM solution LA in acetone (27 scans). B) ^{2}H NMR spectrum (1 Hz/division) of LA (145 mM) in a water–methanol mixture (7:3, v/v, pH 8.6) (46 scans).

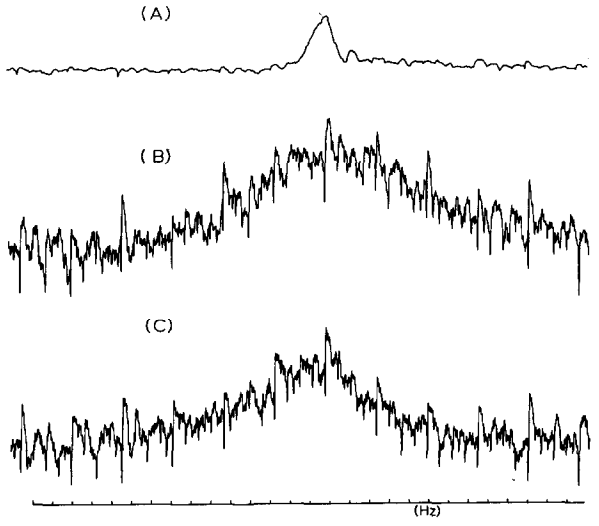

Fig. 2. Fourier transformed spectra of the C^2H_3 group of LA (20 mM) in lecithin bilayers (195 mM) in H_2O. A) Unsonicated sample (19 755 transients). The only visible peak is due to natural abundance ^2H in water. B) Sonicated sample (35 468 transients). The C^2H_3 peak is 1.13 ppm downfield from TMS. C) Sonicated sample in the presence of 10 mole % cholesterol (20 172 transients). D) Sonicated sample in the presence of 20 mole % cholesterol (62 289 transients).

affected by this treatment. Previous ^1H [11, 11a] and ^{13}C [12] NMR results have also shown that sonication is necessary in order to observe high resolution spectra.

This influence of sonication may be due to one of two factors: i) the rate of tumbling of large multilamellar structures is much slower than the 250 Å external diameter single-walled spherical vesicles obtained after sonication; ii) a different arrangement at a molecular level within the bilayers might occur in the two structures – the multibilayers would allow for regions resembling planar structures (in that case, the available area per molecule is the same for the head group and for the hydrocarbon tails), whereas in the single-bilayer vesicles the packing depends on the relative proportion of the molecules in the inner and outer monolayers (whatever this ratio is, the area per head group in both the inner and outer monolayers will be different from the area for the terminal methyl groups per molecule).

The calculations by Horwitz et al. [13] and by Levine et al. [14] and the experimental work of Sheetz and Chan [11a] indicate that it is not a change in macroscopic viscosity (case i) and hence in the diffusion rate of phospholipid aggregates that causes changes in linewidth. Therefore, it is more likely that microscopic alterations occur as a consequence of phospholipid reorganization, as suggested in [11a] and also by ESR experiments [15, 16].

In order to monitor the organization of the lipid bilayers after incorporation of LA, parallel ESR experiments were performed. Planar multibilayers containing a steroid spin probe and a fatty acid spin probe (the *N*-oxyl-4′,4′-dimethyloxazolidine derivatives of 5α-cholestane-3-one, CSL, and 5-keto stearic acid, 5-SASL) were prepared.

ESR spectra were taken with the magnetic field perpendicular to the plane of the bilayers. As the content of LA was increased the spectra of CSL were indicative of a larger distribution of probe orientations, which was mainly revealed by a decrease in the intensity of the low field line and the appearance of a new spectral line at lower field. Computer simulations have indicated that such spectral behaviour occurs when the probes' long molecular axes are distributed over a wide range of orientations [17].

In dispersions of egg lecithin and egg lecithin plus LA containing 5-SASL it was observed that LA produced an increase in the amplitude of motion of the fatty acid chains in the bilayer; the magnitude of the increase depended on the LA content. Such effects were measured by a decrease in the difference between the separation of the outer and inner extrema of the ESR spectra (determined as described in [18]) of the spin probe. Therefore, the NMR experiments were done at the lowest possible LA concentration yielding workable ^2H NMR spectra.

The incorporation of LA into the phospholipid bilayers was further confirmed by comparison of the ESR spectra with those of probes in samples of pure LA. When LA is deposited on a flat surface no anisotropy (difference between spectra run with the magnetic field parallel and perpendicular to the surface on which the lipid is deposited) is observed in the spin probe ESR spectra. Dispersions of LA also did not yield spectra indicative of anisotropic motion. Also,

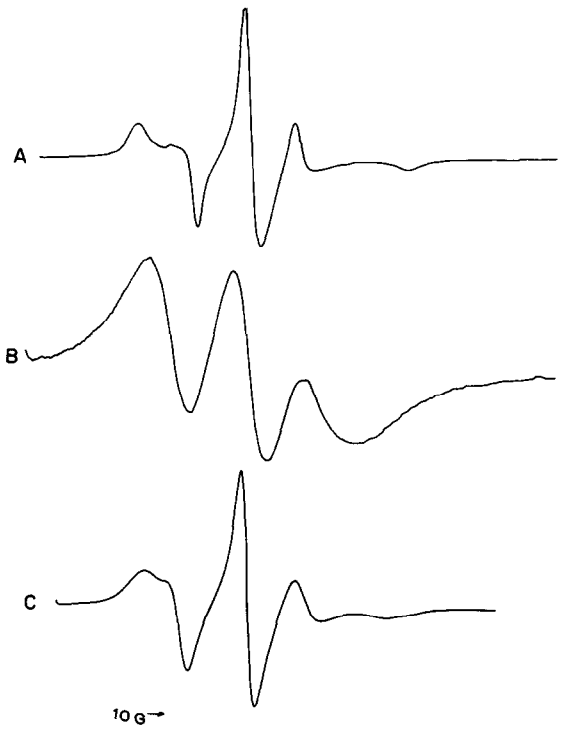

Fig. 3. A) ESR spectrum of probe 5-SASL in 10 mM lecithin dispersions. B) ESR spectrum of probe 5-SASL in 20 mM LA dispersions. C) ESR spectrum of probe 5-SASL in 10 mM lecithin – 0.87 mM LA dispersions. (Although the absolute "concentrations" of lipids were not the same in the NMR and ESR experiments, the relative amount of LA to lecithin was constant at 8 mole %.)

the spectral features characteristic of LA alone were not present in the ESR spectra of egg lecithin–LA, both in planar bilayers and dispersions. These observations allow us to rule out the possibility of formation of two separate phases, i.e. phospholipid and LA. Fig. 3 demonstrates these points for the aqueous lipid dispersions.

It has already been demonstrated by a variety of techniques that cholesterol decreases the fluidity of the hydrocarbon chains of phospholipids when the system is above its gel to liquid crystal transition temperature [10, 15, 19, 22]. Addition of cholesterol up to 20 mole % (with respect to lecithin) caused line broadening of the C^2H_3 signal which was proportional to the amount of sterol: $\Delta\nu_{1/2}$ for 5 mole % was 24 ± 1 Hz, for 10 mole % it was 38 ± 2 Hz (fig. 2C, and for 20 mole % it was 65 ± 5 Hz (fig. 2D).

The motion of LA in lecithin bilayers is anisotropic and is a resultant of several components. The diffusional motion of the lipid vesicle as a whole has already been ruled out as a principal source of the observed linewidth [11a, 13, 14]. Spin labelling experiments have demonstrated rapid lateral diffusion of molecules in phospholipid bilayers [20]. The linewidth of the deuterium resonance will be mainly determined by intra-molecular relaxation mechanisms; the types of motion that need be considered are the rotation of the molecule as a whole about its long axis, the random motion of the molecule within a cone whose axis is parallel to the bilayer and whose angle is determined by the fluidity of the environment [15, 21, 22], intra-molecular chain isomerization [18], and rotation of the C^2H_3 group.

In a first approximation we have applied the formulation of Zeidler [23] (which is an extension of the work by Woessner [24] on dipole–dipole interaction) to the C^2H_3 groups in these systems. Such a formulation does not take into account the anisotropy of molecular motion – it characterizes the C^2H_3 motion by two correlation times – τ_s and τ_r, that of the molecule as a whole (or of a terminal segment) in the absence of internal rotation and that of the intra-molecular jumping process between three equilibrium positions involved in rotation of the C^2H_3 group about the C–C bond, respectively. The quadrupole relaxation effect is thus written as follows:

$$T_q^{-1} = 3/8\,(e^2Qq/\hbar)^2\,(0.11\,\tau_s + 0.89\,(1/\tau_s + 1/\tau_r)^{-1}) \quad (1)$$

where e^2Qq/h is the quadrupole coupling constant.

If we assume $\tau_r \ll \tau_s$, eg. (1) is reduced to:

$$T_q^{-1} = 3/8\,(e^2Qq/\hbar)^2\,0.11\,\tau_s \quad (2)$$

The quadrupole coupling constants of sp^3-hybridized C–^{2}H bonds have been obtained: 173 KHz in cyclohexane–$^2H_{12}$ [25], and 176 KHz in $C^2H_3 \equiv CH$ [26], respectively. Thus, we can assume a value of 170 KHz for our preliminary calculations. The correlation time τ_s calculated this way for LA in lecithin bilayers with $\Delta\nu_{1/2}$ = 17 Hz is 1.2×10^{-9} sec. Upon addition of 20 mole % cholesterol ($\Delta\nu_{1/2}$ = 65 Hz) the value of τ_s is 4.4×10^{-9} sec. The above results have been obtained from a mathematical treatment that is valid for isotropic motion and it may well be that the calculated

values of τ_s refer to a terminal segment of the molecule rather than to the molecular long axis as a whole. Nevertheless, these times are in good agreement with those estimated by spin probe studies for rotational diffusion in phospholipid bilayers [1, 18, 22, 27]. The extent of increase of rotational correlation time by addition of cholesterol to egg lecithin is also in good agreement with spin probe studies [22, 27] and once more confirms that addition of the steroid decreases the fluidity of the hydrocarbon core of lipid bilayers when the system is above its gel → liquid crystal transition temperature.

Studies with deuterated fatty acids such as $C^2H_3(CH_2)_nC^2H_2(CH_2)_mCOOH$ as probes will make feasible the analysis of chain mobility at any desired position. This is desirable for comparison with the spin probe and NMR studies on lipid systems.

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