A STUDY OF MOBILITY AND ORDER IN MODEL MEMBRANES USING ^2H NMR RELAXATION RATES AND QUADRUPOLE SPLITTINGS OF SPECIFICALLY DEUTERATED LIPIDS.

Gerald W. Stockton, Carl F. Polnaszek, L.C. Leitch[†], A.P. Tulloch[‡], and Ian C.P. Smith, Division of Biological Sciences, National Research Council of Canada, Ottawa, Ontario, Canada K1A 0R6.

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SUMMARY

^2H NMR spectra have been observed for several selectively deuterated phospholipid and fatty acid probes intercalated in the liquid crystalline phase of egg phosphatidylcholine in aqueous dispersion. For unsonicated lamellar dispersions and planar multibilayers, quadrupole splittings may be observed which lead directly to a value for the order parameter for the carbon-deuterium bond. Sonicated dispersions yield high-resolution spectra, from which spin-lattice relaxation rates and correlation times for rotational diffusion can be obtained. The presence of cholesterol in the dispersion has no effect on the quadrupole splittings and relaxation rates for ^2H in the choline methyl groups, in contrast to its profound effect on the spectra for ^2H in the hydrocarbon chains.

INTRODUCTION

Both real and model biological membranes have been frequently studied using electron spin (1) and nuclear magnetic resonance spectroscopy, the latter technique most often employing proton (2), carbon (3), and phosphorus (4) relaxation times. Deuterium NMR has been much less exploited: Saitô *et al.* (5) have shown that the line width of the ^2H resonance of ω-trideuteriolauric acid intercalated in egg phosphatidylcholine bilayers is sensitive to the condensing effect of cholesterol; Seelig *et al.* (6) have used wide-line deuterium NMR to study quadrupole splittings and to obtain molecular order parameters in model membranes; Finer (7) has used ^2H NMR of heavy water to study phosphatidylcholine solvation. We wish to present here some preliminary but exciting observations of deuterium spin-lattice and spin-spin relaxation times and order parameters for specifically deuterated phospholipid and fatty-acid probes intercalated in dispersions of egg phosphatidylcholine (PC).

MATERIALS AND PROCEDURES

Egg yolk PC, lyso PC, and phosphatidylethanolamine were

[†]Division of Chemistry.

[‡]Prairie Regional Laboratory, NRCC, Saskatoon, Sask., Canada S7N 0W9.

supplied by Lipid Products Ltd., S. Nutfield, England, and cholesterol by Steraloids, Pawling, N.Y. Selectively deuterated fatty acids were prepared by previously described methods (8, 9, 10). ω-Trideuteriooctadecanoic acid was converted to acyl chloride, which was then used to acylate egg lyso PC as described by Baer and Buchnea (11). Phosphatidylethanolamine (0.4 g) was converted to its choline analogue by reaction with trideuteriomethyl iodide (1 g) in chloroform-methanol (20 ml of 1:1) in the presence of potassium bicarbonate (2 g), stirring at *ca.* 25°C under nitrogen for 14 days. The conversion was quantitative, as demonstrated by thin layer chromatography (12). Chloroform solutions of lipid mixtures were evaporated in a stream of nitrogen and pumped under vacuum for no less than 2 hours. Dispersions were prepared by adding water and shaking in a vortex mixer. Sonication was performed under nitrogen at *ca.* 10°C with a bath-type sonicator for not less than 1 hour. Planar multibilayers were prepared by evaporating chloroform solutions of lipids on clean microscope slide cover glasses, hydrating the films in Ringer's solution, and stacking the plates with slight compression. Deuterium NMR experiments were carried out in 12 mm o.d. sample tubes with a Varian XL-100-15 spectrometer operated at 15.4 MHz in the pulse Fourier-transform mode, signal-averaging on a Varian 620 L computer. Proton noise decoupling was necessary only when a spectrum contained lines narrower than *ca.* 5 Hz. Spin-lattice relaxation times were measured by the inversion recovery (180-τ-90) method.

RESULTS AND DISCUSSION

Phospholipid Probes. Figure 1a shows the ^2H NMR spectrum of N,N,N-trimethyl-d_3-phosphatidylcholine probe in unsonicated aqueous dispersion. The spectrum corresponds to a random distribution of oriented molecules, as might be expected for a slowly rotating lamellar multibilayer structure. The lineshape is an envelope of many relatively narrow lines with different quadrupole splittings due to a distribution of bilayer orientations with respect to the applied magnetic field. The splitting $\Delta\nu(\theta)$ for a single bilayer orientation is given by

$$\Delta\nu(\theta) = 3/4(e^2qQ/h)(3\cos^2\theta - 1)S_{CD} \qquad (1)$$

where S_{CD} is the order parameter for the C-D bond, e^2qQ/h is the quadrupole coupling constant, and θ is the angle between the normal to the bilayer and the applied magnetic field. The lineshape function $g(\nu)$ is simply the integral over all possible orientations with respect to the field.

$$g(\nu) = \int_0^{\pi/2} \frac{\sin\theta\, d\theta}{T_2^{*-2} + (\nu-\nu(\theta))^2} \qquad (2)$$

where T_2^* is the effective spin-spin relaxation time and $\nu(\theta) = \pm{}^1/_2\Delta\upsilon(\theta)$. The separation D_q between the maxima is $^3/_4(e^2qQ/h)|S_{CD}|$.

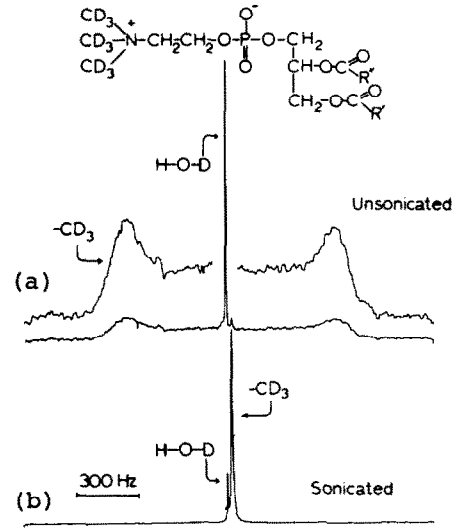

Figure 1. ^2H NMR spectra of N,N,N-trimethyl-d_3-PC probe, 32 mgs/ml, in aqueous dispersion at 31°C. (a) unsonicated, 11000 transients; (b) sonicated, 500 transients.

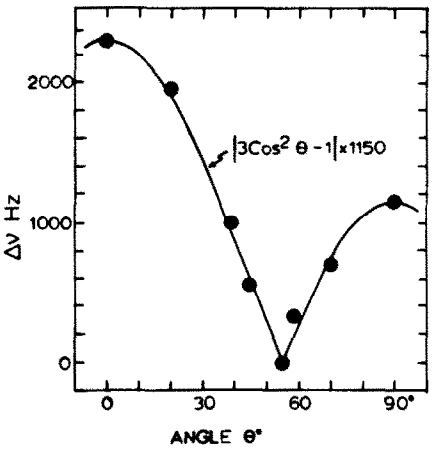

Figure 2. Angular-dependence of the quadrupole splitting $\Delta\nu$ for the N,N,N-trimethyl-d_3-PC probe in planar multibilayers at 31°C.

Values of $|S_{CD}| = 0.008$ and $T_2^* = 0.003$ sec. were obtained by fitting the observed lineshape to Eq. (2). The quadrupole coupling constant was assumed to be 170 KHz. The small value for S_{CD} signifies relatively weak ordering of the choline methyl groups.

The profound effect of sonication on the dispersion is shown in Figure 1b. The quadrupole splittings vanish and all the intensity becomes centred about a single frequency with a linewidth of only 8 Hz, corresponding to $T_2^* = 0.040$ sec. The measured spin-lattice relaxation time T_1 is 0.046 sec. It is important to note that a narrow line does not necessarily imply the absence of order. The mean diameter of unsonicated lamellae is several thousand Ångstroms, whereas the mean diameter of single bilayer vesicles is *ca.* 250 Å. The Stokes-Einstein equation may be used to predict the rotational correlation time τ_c for spherical body tumbling: τ_c = *ca.* 10^{-6} sec. for vesicles, and >*ca.* 10^{-2} for lamellae. Thus, for the small vesicles any quadrupole splitting will be averaged to zero by the overall rotation, which accounts for the narrow resonance observed after sonication.

There is also the possibility that the splitting D_q observed for unsonicated liposomes is partially averaged by slow but sig-

nificant overall rotation. Two experiments were performed to elucidate this point. In the first, boiled starch solution was added to the lamellar dispersion in order to greatly increase the viscosity and to decrease the rate of rotation. No change in the spectrum was observed. In the second experiment, planar multibilayers were prepared on microscope slide cover plates as described above. For a planar bilayer, a pair of relatively narrow lines with a separation $\Delta\nu$ given by Eq. (1) is expected. Figure 2 shows the observed angular dependence of the separation $\Delta\nu$ and also the curve predicted by Eq. (1). The excellent fit of the data to Eq. 1 confirms that the ordering axis is perpendicular to the surface of the bilayer. The order parameter evaluated from these data is identical, within experimental error, with that found for the liposomes. Thus the rotation of the unsonicated liposomes is so slow that it has no effect on a quadrupole splitting of *ca.* 1 KHz. This observation is of great value in planning further experiments involving unsonicated liposomes.

The inclusion of 20 mole % cholesterol has no effect on the quadrupole splitting or on the relaxation rates for unsonicated and sonicated liposomes. This demonstrates that cholesterol has little or no effect on the mobility and ordering of the choline methyl groups, in contrast to the powerful condensing effect on the hydrocarbon chains (5, 13).

Figure 3 shows ^2H NMR spectra for several systems containing a synthetic phosphatidylcholine with the natural distribution of fatty acids at position 1 and ω-trideuteriostearate at position 2. For this probe in chloroform solution, the line width of the ω-CD_3 resonance is *ca.* 2 Hz. Intercalated in egg PC lamellae, the resonance is too broad to be observed with the available sensitivity and sweep widths, but sonication reduces the line width to 8 Hz, corresponding to $T_2^* = 0.04$ sec. The measured spin-lattice relaxation time T_1 is 0.32 sec. It should be noted that $T_1 >> T_2^*$, which may indicate incomplete averaging of the quadrupole splittings or sensitivity of T_2 to slow motions. The presence of cholesterol causes a considerable increase in line-width (*eg.* for 15 mole % cholesterol, $W_{1/2}$ = 14 Hz), presumably due to the well-known condensing effect of cholesterol (13).

Fatty Acid Probes. Table I lists values of T_1 and T_2^* for several fatty acid probes intercalated in egg phosphatidylcholine dispersions. As with the phospholipid probes, narrow lines were

Table 1. ^2H NMR Spin-Lattice and Spin-Spin Relaxation Times and Rotational Correlation Times (all in seconds) for Deuterated Fatty Acid Probes Intercalated in Sonicated Egg Phosphatidylcholine Dispersions.[a]

CHAIN LENGTH	POSITION OF ^2H	TYPE OF GROUP	31°C T_1	31°C T_2^{*c}	31°C τ_c	55°C T_1	55°C T_2^{*c}	55°C τ_c
12[b]	2	CD_2		$<10^{-3}$				
12[b]	12	CD_3		0.020				
18	12	CHD		$<10^{-3}$			$<10^{-3}$	
18	16	CD_2		$<10^{-3}$			$<10^{-3}$	
18	17	CD_2	0.060	0.005	4.0×10^{-11}	0.23	0.013	1.0×10^{-11}
18	18	CD_3	0.32	0.040	7.4×10^{-12}	0.58	0.080	4.1×10^{-12}

[a]Concentrations: 0.02 mmoles of probe, 0.2 mmoles of egg PC, 1 ml of water.

[b]Taken from reference (5).

[c]It is estimated that signals of width greater than *ca.* 300 Hz cannot be observed with the low concentration of probe used because of limited signal-to-noise ratio. For this reason, when a signal was expected but not observed, T_2^* is listed as $<10^{-3}$ sec.

observed only after sonication. T_1 is consistently about an order of magnitude greater than T_2^*, and both relaxation times increase with temperature. It is noteworthy that T_1 and T_2^* for the ω-trideuteriostearic acid probe are identical with those of the ω-trideuteriostearoylphosphatidylcholine probe at the same concentration and temperature. It is commonly observed in NMR studies of oriented molecules that $T_1 > T_2^*$, T_2^* is angular-dependent, and T_1 is angular-independent (14). It is probable that T_1 depends largely upon motion and that T_2^* depends upon both motion and order (15). We are presently developing a theory to separate these effects. "Effective" rotational correlation times τ_c may be computed from the observed values ot T_1 using standard formulae (16). The correlation time, which decreases with increasing rate of motion, is used to characterize mobility at different positions in the molecule. The observed values of T_1 (hence $1/\tau_c$) and T_2^* increase rapidly towards the methyl end of the fatty acid chain. A similar trend was observed for ^{13}C relaxation times of sonicated egg PC (17). The values of τ_c derived from the deuterium probes are listed in Table I and they are in good agreement with those estimated from the ^{13}C data.

Figure 4 shows a plot of line width $W_{1/2}$ *vs.* cholesterol concentration for ω-trideuteriostearic acid intercalated in sonicated cholesterol - egg PC dispersions. For both of these probes, the

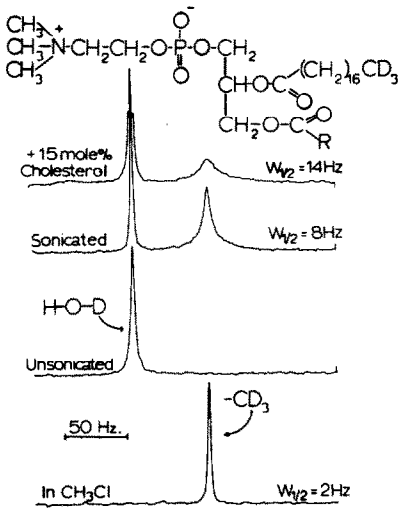

Figure 3. ^2H NMR spectra of ω-trideuteriostearoyl-PC probe at 31°C, 15 mgs/ml in chloroform, and 10 mole % in aqueous egg PC dispersions (0.2 mmole total phospholipid in 1 ml of water); *ca.* 20000 transients.

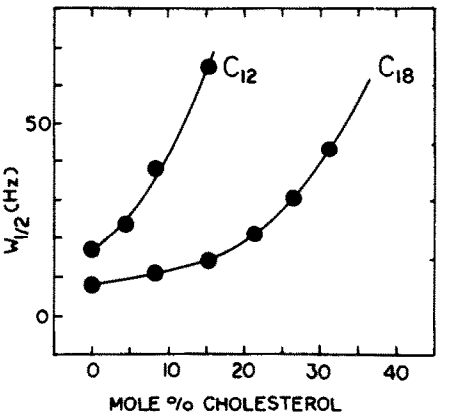

Figure 4. Dependence of the ^2H NMR linewidth $W_{1/2}$ on cholesterol concentration for ω-trideuterio lauric and stearic acid probes (.02 mmole) intercalated in sonicated egg PC dispersions (0.2 mmole in 1 ml of water) at 31°C.

line width increases with cholesterol concentration but more rapidly for the shorter chain acid. Spin-lattice relaxation times were measured for sonicated liposomes containing the ω-trideuteriostearic acid probe, and T_1 was found to be unchanged by the addition of 10 and 20 mole % of cholesterol. This implies that cholesterol has no influence on the mobility of the terminal segment of the hydrocarbon chain. The strong effect of cholesterol on the line width reinforces our contention that T_2^* is controlled by molecular order as well as mobility.

Although a ^2H NMR signal was never observed for low concentrations of probe in unsonicated liposomes, a lineshape similar to that shown in Figure 1a, with D_q = 2300 Hz, was observed at high probe and total lipid concentrations (0.18 mmole ω-trideuteriostearic acid and 0.637 mmole egg PC in 0.75 ml H_2O). The peak separation D_q increased to 2950 Hz and 3800 Hz in the presence of 14 and 32 mole % of cholesterol respectively, indicating a strong ordering effect by cholesterol. In view of the high lipid concentrations used, these values may not be directly comparable with other observations presented here, but the variation of D_q is

qualitatively in agreement with that of the line widths for sonicated liposomes containing cholesterol.

The above observations demonstrate that deuterated fatty acids and phospholipids are excellent probes for monitoring rotational diffusion and molecular ordering in model membranes. However, because of the low concentrations which must be used, the fatty acid probe suffers from inadequate sensitivity whenever broad lines are encountered. The more difficult technique of labeling phospholipids is preferred since it offers a ten-fold or more improvement in sensitivity. In addition to the continuing study of model membranes using ^2H NMR and the development of associated theory, we are presently incorporating deuterated fatty acids into the phospholipids of the micro-organism *Acholeplasma laidlawii* in an attempt to study the membranes of living cells.

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