Biochimica et Biophysica Acta, 640 (1981) 263—273

BBA 79049

CHOLESTEROL ROTATION IN PHOSPHOLIPID VESICLES AS OBSERVED BY ^{13}C-NMR

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(Received June 24th, 1980)

Key words: Cholesterol; ^{13}C-NMR; Chloroform; Phosphatidylcholine vesicle; Axial rotation

Summary

^{13}C-NMR spectra of cholesterol 90% enriched at C-4 with ^{13}C have been obtained in $CHCl_3$ and in sonicated egg phosphatidylcholine vesicles. ^{13}C spin-lattice relaxation times, nuclear Overhauser effects and spin-spin relaxation times have been measured for the C-4 carbon of cholesterol in phosphatidylcholine bilayers as a function of cholesterol content and temperature. All the data are consistent with a correlation time for axial rotation of about 10^{-10} s. This rotation is one or two orders of magnitude faster than axial rotation of the phospholipid molecule.

Cholesterol behavior in membranes has proven a difficult subject to study due to a lack of direct measures of cholesterol properties. The effect of cholesterol on phospholipids has been well documented by many investigators from measurements of phospholipid behavior. These effects include elimination of phospholipid phase transitions [1,2], increased ordering of phospholipid hydrocarbon chains [3,4], condensation of phospholipid monolayers [5,6] and interruption of phospholipid headgroup interactions [7]. These data have been recently reviewed [8].

Few data exist, however, from direct measurements of cholesterol behavior. Considerable motional ordering of cholesterol in membranes has been observed using ^{2}H-NMR of ^{2}H-labelled cholesterol [9,10]. ^{13}C-labelled cholesterol (with ^{13}C in position C-4) has recently been shown to produce a visible and measurable resonance in the ^{13}C-NMR spectra of sonicated phosphatidylcholine vesicles [11].

The purpose of this study is to exploit the latter observation to examine cholesterol motion in phospholipid bilayers. It is concluded that cholesterol rotates more rapidly about its molecular axis than do phospholipids.

Materials and Methods

Egg phosphatidylcholine was purchased from Avanti Biochemicals. It produced a single spot on thin-layer chromatography in $CHCl_3/CH_3OH/H_2O$ (65 : 25 : 4, v/v) when developed with iodine vapor. Cholesterol enriched to 90% in ^{13}C at position 4 was obtained from Merck, Inc. It produced a single spot when analyzed on thin-layer chromatography in petroleum ether/diethyl ether/glacial acetic acid (90 : 10 : 1, v/v), with visualization by acid charring. It comigrated with normal cholesterol.

Mixed phosphatidylcholine/cholesterol vesicles were prepared as follows. The lyophilized components were weighed to obtain the stated mole ratios. They were dissolved in $CHCl_3$ to mix completely the two components, dried under a stream of N_2 and then under a vacuum. The mixture was hydrated in 100 mM NaCl, 10 mM Tris, pH 7.6 (15% ^2H_2O), and sonicated in an iced-water bath with a Branson 350 sonifier for two 5-min periods separated by a 5 min interval. To maximize the sensitivity of the experiments, this material was used directly. From 100 to 200 mg of egg phosphatidylcholine were used in each experiment, along with the appropriate content of added cholesterol.

^{13}C-NMR measurements were performed on a Bruker WP 200 Fourier transform spectrometer at 50.3 MHz. The Bruker temperature controller was used to maintain the temperatures to within ±1°C of the stated temperature. The temperature was measured as the stream of air bathing the sample entered the receiver coil. Therefore, the sample was allowed to equilibrate for a minimum of 10 min in the receiver coil before making any measurements. ^2H_2O internal lock was used. For each spectrum, 8K data points were obtained in the time domain over a 10 kHz spectral width. Exponential multiplication was used to smooth the spectra, with the resultant line broadening listed in the figures.

Spin-lattice relaxation time (T_1) measurements were performed using the inversion-recovery sequence, 180°-τ-90°. A 90° pulse equalled 10 μs. This was determined with the C-4 resonance from the ^{13}C-enriched cholesterol in C^2HCl_3, by varying the pulse width until a null, or 180° pulse was obtained. A relaxation delay between pulses of $4-6 \times T_1$ was used in each T_1 measurement. Nuclear Overhauser effects were evaluated by comparing the intensity from continuous broadband ^1H-decoupled spectra with that from spectra obtained with the ^1H decoupler gated to remove the nuclear Overhauser effects; 90° pulses were used with relaxation delays of $4-6 \times T_1$. T_1 values were determined from the slope of $\ln(A_\infty - A_\tau)$ vs. τ, where A_∞ is the intensity of the resonance after the sequence 180°-τ-90°, (where τ as well as the relaxation delay was $4-6 \times T_1$) and A_τ is the same for various smaller values of τ. Six or seven points were obtained for each T_1 determination, and T_1 was evaluated using an unweighted least-squares analysis of the slope.

Theory

This study was designed to measure the motional properties of cholesterol in membranes using ^{13}C-NMR, predominantly from the spin-lattice relaxation times of the C-4 carbon of cholesterol. Since this carbon is part of the fused-

ring nucleus of the cholesterol molecule, the C-4 carbon should reflect the motional behavior of the ring system as a whole.

Since this carbon has two hydrogens directly bonded, it can be expected that dipole-dipole interactions between the protons and the carbon nucleus will dominate the spin-lattice relaxation mechanism of the carbon. Available theory provides, in such a situation, a way of determining relevant correlation times for the motion which the molecule is experiencing. Since cholesterol is to be studied in a membrane, simple isotropic relaxation theory is inadequate. Cholesterol is oriented in the membrane perpendicular to the membrane surface (for a review, see Ref. 8). One of the motions cholesterol can experience is rotation about the long axis of the molecule, perpendicular to the surface of the membrane. This rotational axis is commonly referred to as the director. Since at 10 mol% cholesterol this system consists predominantly of small vesicles with radius 109 Å [12], an additional motion arises because the vesicle as a whole can rotate isotropically at approx. 10^{-6} s. The steroid ring system is rigid and thus segmental motion, important in the relaxation behavior of ^{13}C nuclei in phospholipids, is not important for the C-4 carbon of cholesterol. The expected motional behavior of cholesterol in a membrane can

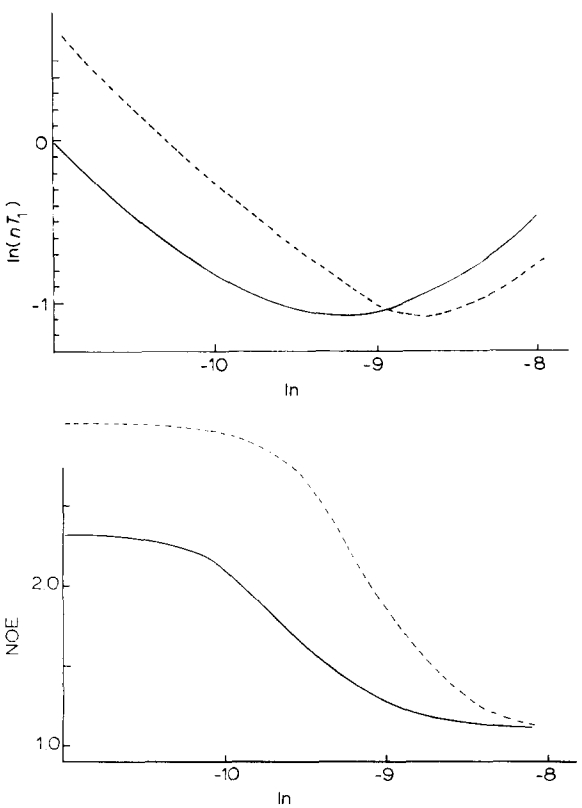

Fig. 1. (A) (———) Relationship between nT_1 and τ_G, the anisotropic rotational correlation time, using Eqn. 1 and $r = 1.08$ Å, for the field strength used in this study. Also shown (— — —) is the isotropic relationship; (B) as in A for nuclear Overhauser effect (NOE).

then be reasonably described by rapid anisotropic rotation about the director and slower isotropic rotation of the vesicle as a whole.

Woessner [13] has described spin relaxation for a two-spin system undergoing uncorrelated isotropic and anisotropic motions. When adapted to the present situation, which resembles the treatment of Doddrell et al. [14], the angle between the C-H vectors of the C-4 position and the director is of importance. An effective angle of 60° was chosen for this analysis from measurements of a space-filling model of cholesterol. The spectral density functions of Woessner [13], when examined for the effect of an isotropic rotational correlation time of 10^{-6} s, demonstrate that the effect on T_1 is negligible, providing the anisotropic correlation time, τ_G is much shorter. Since this proves to be the case here, an expression for T_1 can be derived which contains only τ_G. Fig. 1 is a plot of this equation through the region of interest for this study. Included on the plot is a curve representing pure isotropic motion over the same time scale. These curves are plotted for the field strength of the instrument used in this study. Expressions for T_2 and nuclear Overhauser effects can also be derived from Woessner's development.

The expression for the anisotropic T_1 used here is Eqn. 1. While the angle chosen (60°) is near the 'magic' angle, only one of three terms in the expressions of Woessner cancels and the T_1 calculated is not strongly affected.

$$\frac{1}{T_1} = K\tau_G\left\{\frac{27}{2}\left(\frac{1}{1+(\omega_c-\omega_H)^2 9/4\tau_G^2}+\frac{3}{1+\omega_c^2 9/4\tau_G^2}+\frac{6}{1+(\omega_c+\omega_H)^2 9/4\tau_G^2}\right)+\right.$$
$$\left.72\left(\frac{1}{1+36(\omega_c-\omega_H)^2\tau_G^2}+\frac{3}{1+36\omega_c^2\tau_G^2}+\frac{6}{1+(\omega_c+\omega_H)^2\tau_G^2}\right)\right\} \quad (1)$$

where $K = (3/640)\hbar^2\gamma_c^2\gamma_H^2 r^{-6}$, $\hbar$ is Planck's constant, γ_c and γ_H are the magnetogyric ratios of the carbon nucleus and the proton, respectively, r is the internuclear C-H vector, and ω_c and ω_H are the resonance frequencies at the applied field strength of ^{13}C and ^1H.

The expression for the ^{13}C (^1H) nuclear Overhauser effects (NOE) is modified by the anisotropic motion:

$$\mathrm{NOE} = 1 + N^{-1}\frac{\gamma_H}{\gamma_c}\left\{6\left(\frac{27}{2}\frac{1}{1+(\omega_c+\omega_H)^2 9/4\tau_G^2}+72\frac{1}{1+36(\omega_c+\omega_H)^2\tau_G^2}\right)\right.$$
$$\left.-\left(\frac{27}{2}\frac{1}{1+(\omega_c-\omega_H)^2 9/4\tau_G^2}+72\frac{1}{1+36(\omega_c-\omega_H)^2\tau_G^2}\right)\right\} \quad (2)$$

where $N = T_1^{-1}K^{-1}\tau_G^{-1}$.

An expression for T_2 can also be derived:

$$\frac{1}{T_2} = \frac{6}{2560}\hbar^2\gamma_c^2\gamma_H^2 r^{-6}\left\{4\tau_R+\tau_G\left(\frac{27}{2}\left(\frac{1}{1+(\omega_c-\omega_H)^2 9/4\tau_G^2}+\frac{3}{1+\omega_c^2 9/4\tau_G^2}+\right.\right.\right.$$
$$\left.\left.\left.\frac{6}{1+(\omega_c+\omega_H)^2\,9/4\tau_G^2}+\frac{6}{1+\omega_H^2 9/4\tau_G^2}+12\right)\right.$$

$$+ 72\left(\frac{1}{1 + 36(\omega_c - \omega_H)^2\tau_G^2} + \frac{3}{1 + 36\omega_c^2\tau_G^2} + \frac{6}{1 + 36(\omega_c + \omega_H)^2\tau_G^2} + \frac{6}{1 + 36\omega_H^2\tau_G^2} + 12\right)\Bigg\} \quad (3)$$

Results

Fig. 2A shows the 50.3 MHz ^{13}C-NMR spectrum of cholesterol 90% enriched in ^{13}C at position 4 in C^2HCl_3. Because of the high level of enrichment, only the C-4 carbon resonance is observed. Fig. 2B presents the ^{13}C-NMR spectrum of sonicated egg phosphatidylcholine vesicles containing 10 mol% ^{13}C-enriched cholesterol prepared as described previously. The C-4 carbon resonance of cholesterol in the vesicles is clearly observed. Although it is 90% enriched in ^{13}C, while all the phospholipid resonances are at a 1.1% natural abundance, the resonance does not dominate the ^{13}C-NMR spectrum due to a vastly increased linewidth. This has been observed previously [11] and is due to the motional constraints imposed on the cholesterol by the phospholipid bilayer. In fact, the broadening effects are so severe that no resonances of unenriched cholesterol are apparent in the unenriched spectrum of this same system [15,16]. These motional constraints reveal themselves in the spin-spin relaxation rate, and probably refer to inhibition of off-axis motion of cholesterol. As will be seen shortly, axial rotation as measured by the spin-lattice relaxation rate is not as strongly affected by orientation of cholesterol in a phospholipid bilayer.

The parameter which will prove to be of greatest use for motional analysis is the spin-lattice relaxation time, T_1. This value has been determined for

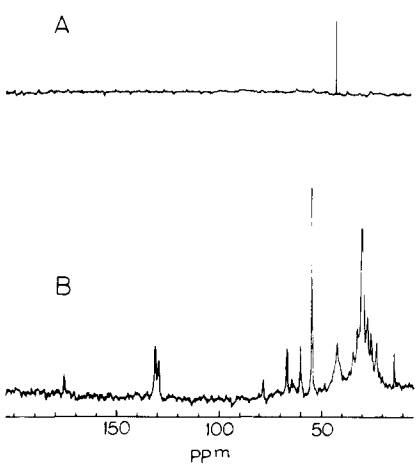

Fig. 2. ^{13}C-NMR spectra at 50 MHz of cholesterol labelled at C-4 with ^{13}C at 30°C. (A) Cholesterol in C^2HCl_3, 5 mg/ml, 1 Hz linebroadening, 32 scans, 1.7 s repetition rate. (B) Cholesterol, in sonicated egg phosphatidylcholine vesicles, at a composition of 10 mol% and a concentration of 10 mg/ml, 20 Hz linebroadening, 250 scans, 0.5 s repetition rate. Abscissa is the chemical shift in ppm from external tetramethylsilane.

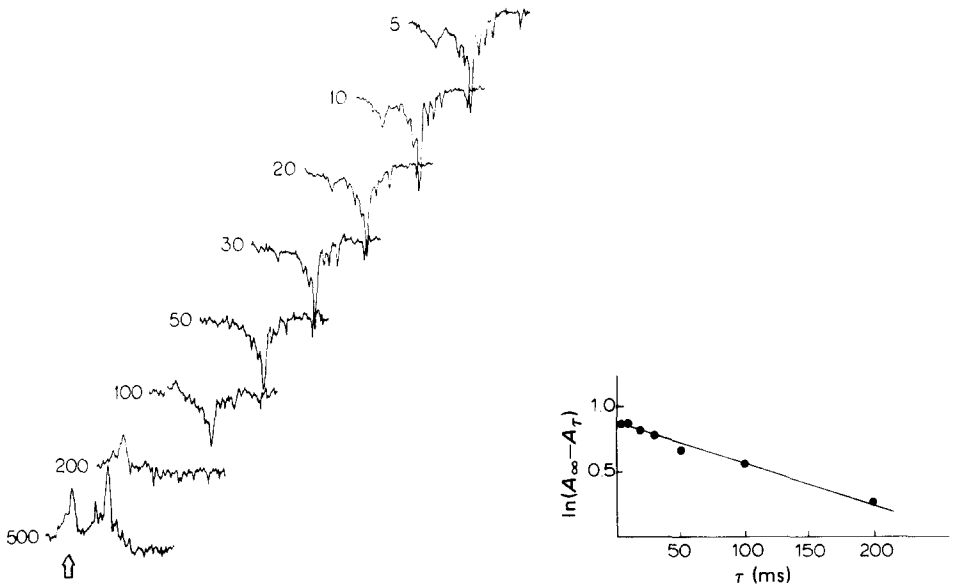

Fig. 3. ^{13}C spin-lattice relaxation of the C-4 carbon of cholesterol at 50.3 MHz, 30°C, 10 mol%, in sonicated egg phosphatidylcholine vesicles, 20 Hz line broadening, 250 scans, 0.5 s repetition rate. (A) Partially relaxed spectra, with τ values written in ms, the arrow marks the C-4 cholesterol resonance; (B) plot of $\ln(A_\infty - A_\tau)$ vs. τ for the data in A.

the C-4 carbon of cholesterol in egg phosphatidylcholine vesicles at three different mole ratios and two different temperatures. Fig. 3A demonstrates that while prodigous amounts of enriched material are required, an adequate signal-to-noise ratio can be obtained to provide reasonable T_1 data. Fig. 3B shows that good quality plots of $\ln(A_\infty - A_\tau)$ vs. τ can be obtained using ^{13}C-resonance peak heights. Thus, the T_1 data obtained can be used with some confidence for motional analysis. Fig. 4 shows the temperature and composition dependence of the C-4 ^{13}C T_1.

For a full motional analysis using T_1, nuclear Overhauser effect values are required. These data are presented in Fig. 5, as a function of temperature. The increase in nuclear Overhauser effects and T_1 with increasing temperature

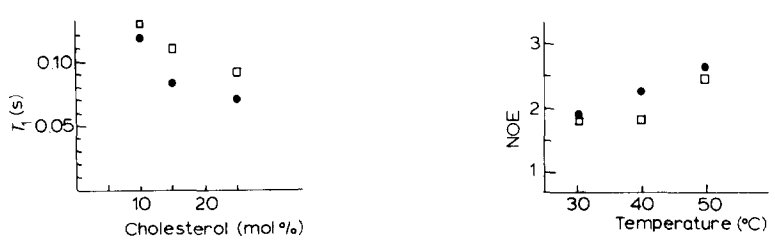

Fig. 4. ^{13}C spin-lattice relaxation of the C-4 carbon of cholesterol at 50.3 MHz, in sonicated egg phosphatidylcholine vesicles, as a function of mol% cholesterol at 30°C (●) and 50°C (□).

Fig. 5. ^{13}C (^1H) nuclear Overhauser effects (NOE) for the C-4 carbon of cholesterol in sonicated egg phosphatidylcholine vesicles as a function of temperature, at 10 mol% cholesterol (●) and 25 mol% cholesterol (□).

suggests that the nuclear Overhauser effects may be motionally limited, and that the low nuclear Overhauser effect values may not reflect contributions to T_1 from other relaxation mechanisms.

This suggestion proved to be internally consistent, because when the correlation times derived from the T_1 data were used to calculate the expected nuclear Overhauser effects (see below), reasonable agreement was obtained with the observed nuclear Overhauser effect data. In the motional analysis to follow, the dominant spin-lattice relaxation mechanism considered is a dipolar interaction with the directly bonded protons. Even if other mechanisms are contributing to ^{13}C relaxation of the C-4 carbon, the nuclear Overhauser effect data indicate that the rotational correlation times derived will not be significantly affected. (The nuclear Overhauser effect value observed for the C-4 carbon of cholesterol in C^2HCl_3 is 2.0 ± 0.2, in agreement with a previously published value of 1.9 ± 0.2 [17].)

For comparison, some of the phospholipid ^{13}C T_1 values are presented in Table I for vesicles containing 10 mol% cholesterol at 30°C. nT_1 data are calculated, where n represents the number of directly bonded protons. Also listed is the C-4 carbon T_1 for cholesterol in C^2HCl_3. This is somewhat longer than a value previously reported, but the concentration used here, 20 mM is 50-times less concentrated than that used in the previous study [17].

The ^{13}C resonance linewidth of the C-4 carbon in cholesterol as a function of cholesterol composition behaves similarly to those previously reported [11], showing little dependence on cholesterol composition unless cholesterol contents greater than 25 mol% are examined. In that case, the linewidths are too broad to allow accurate T_1 measurements. The linewidths varied from 80 ± 10 Hz at 10% cholesterol to 110 ± 10 Hz at 25% cholesterol at 30°C in this study.

Using the data presented in Fig. 4 and the theoretical curve in Fig. 1, it is possible to calculate a rotational correlation time for cholesterol spinning about its long axis in the membrane. Table II lists these anisotropic rotational correlation times for all the compositions and temperatures studied. Using these values for τ_G, expected nuclear Overhauser effect values were calculated. Satisfactory agreement between measured and calculated nuclear Overhauser effect values was obtained. For example, at 30°C, 10 mol% cholesterol produced a

TABLE I

^{13}C T_1 OF PHOSPHATIDYLCHOLINE AND CHOLESTEROL CARBONS IN SONICATED VESICLES WITH 10% CHOLESTEROL AT 30°C

T_1 values ± 10%.

Carbon atom	T_1	nT_1
$-N^+(CH_3)_3$	0.55	1.7
$-HC{=}CH-$	0.56	0.56
$-CH_2-$	0.58	1.2
$-CH_2OP$ (choline)	0.44	0.88
$-N-CH_2-$	0.52	1.0
Cholesterol C-4 (in vesicle)	0.14	0.28
Cholesterol C-4 (C^2HCl_3)	0.67	1.3

TABLE II

CHOLESTEROL ROTATIONAL CORRELATION TIMES

Cholesterol content	τ_G (s) ($\times 10^{-10}$)
30°C	
10%	0.5
15%	0.9
25%	1.0
50°C	
10%	0.5
15%	0.6
25%	0.7
Cholesterol in C^2HCl_3 *	0.3

* An effective τ_R is calculated using equations for isotropic rotation, and assuming that the nuclear Overhauser effect represents the percent ^1H dipolar interactions.

nuclear Overhauser effect value of 2.0 ± 0.2, while the calculated value was 2.3. At 30°C and 25 mol% cholesterol, the measured nuclear Overhauser effect value was 1.8 ± 0.2 while that calculated was 2.1.

It should be noted that two solutions are obtained for τ_G, the value listed in Table II and another set of values much longer. However, the longer τ_G values predict a negligible nuclear Overhauser effect, whereas a substantial nuclear Overhauser effect is observed. Therefore, the τ_G values listed in Table II are the relevant ones.

Discussion

As can be seen in Table I, there is a considerable difference between the ^{13}C nT_1 (where n is used to normalize the data for the number of directly bonded hydrogens) of the C-4 carbon of cholesterol and the ^{13}C nT_1 values for the comparable region on the phospholipid hydrocarbon chains. The C-3 hydroxyl of the cholesterol appears to be located in the membrane near the carbonyl groups of the phospholipid ester linkage to the hydrocarbon chains [8]. Thus, the methylenes at positions 2, 3 and 4 on the phospholipid acyl chains are in the same region in the bilayer as the C-4 carbon of cholesterol. The nT_1 values for this region of the phospholipid are in the range of 0.5–0.8 s [18] while the C-4 carbon nT_1 value reported here is 0.28 ± 0.03 for 10 mol% cholesterol in a phospholipid bilayer. Clearly, the C-4 carbon of cholesterol is in a more restricted environment than the phospholipid acyl methylenes in the same region of the phospholipid membrane.

Several factors contribute to this result. As can be seen in the ^{13}C T_1 values [18,19], there is a motional gradient along the phospholipid hydrocarbon chains, which parallels the gradient of order parameters for the same hydrocarbon chains [20]. The terminal methyl of the hydrocarbon chains experiences rapid, nearly isotropic motion. A similar result has been noted for the rate of motion of the terminus of the tail of cholesterol [21]. Both the rate and freedom of motion rapidly diminish when moving up the chain towards the polar headgroup. Thus, the section of the hydrocarbon chain of the phos-

pholipid next to C-4 of cholesterol experiences the most ordered and probably the slowest motion (though it should be noted in Fig. 1 that a decrease in T_1 can result from adoption of anisotropic motion with no change in motional rate). The steroid ring of cholesterol also experiences a relatively restricted motional environment [9], mimicking in that regard the phospholipid hydrocarbon chain. Thus, part of the reason for a short T_1 for the C-4 carbon of cholesterol is the restriction on motional freedom.

Another reason is the source of relaxation. In the hydrocarbon chains, the current model for the rapid motion necessary to contribute to T_1 is kink diffusion through the chains, caused by coupled rotations about carbon-carbon bonds. This motion is unavailable to a carbon in the rigid steroid-ring system. The C-4 carbon of cholesterol must rely on whole molecule rotation to modulate the dipolar interaction with the protons, which is not as efficient a relaxation mechanism in a membrane as is kink diffusion. Thus, the nT_1 of the C-4 carbon of cholesterol is shorter than that for carbons in the phospholipid hydrocarbon chain.

This mode of relaxation, however, provides an excellent means of evaluating the rotation rate of cholesterol about its long axis in the membrane, since the only fast motion is axial rotation. As described in Theory, vesicle rotation does not contribute significantly to T_1 and an analysis can be made of τ_G, the rotational correlation time about the long molecular axis of cholesterol. The values under several conditions appear in Table II. A similar value ($3 \cdot 10^{-10}$ s) is obtained for the rotational correlation time about an axis perpendicular to the surface for a steroid spin label in a sodium decanoate/decanol bilayer [22], and in a liquid-crystalline phospholipid bilayer [23,24]. Also similar is the rotational rate calculated for the anaesthetic, tetracaine, in egg phosphatidylcholine vesicles [25]. This agreement lends considerable support to the analysis presented above.

The rotational correlation time varies by about a factor of two from 10 to 25 mol% cholesterol content. While lateral diffusion of membrane components decreases with increasing cholesterol content [26] lateral diffusion does not significantly affect the T_1 analysis, and thus probably is not the source of the variation in τ_G. Recently, it was suggested that self-association of cholesterol may occur, beginning near 20 mol% cholesterol [7]. The slowing of rotation at 25 mol% cholesterol could be explained by the cholesterol dimer formation invoked in that model. However, these data are not sufficiently resolved at this time to analyze this suggestion further.

It is of considerable interest to compare the rate of cholesterol rotation in a membrane with that of phospholipid rotation in a membrane. While a direct measure of the latter is presently lacking, some estimates can be made. ^{13}C relaxation times of the phospholipids are not helpful for this purpose, since they are dominated by carbon-carbon bond rotations, occurring at a considerably faster rate than phospholipid axial rotation.

Recent spin-label studies suggest a rotational correlation time of 10^{-9} s based on a phospholipid spin-labelled at position 5 of the hydrocarbon chain [23]. Another estimate of phospholipid rotational rate can be obtained from a ^{31}P-NMR study of phospholipids in sonicated vesicles. An effective anisotropic rotational correlation time of 10^{-9} s was obtained for the phospholipid head-

group [28]. This puts a lower limit on the rotational rate of the phospholipid molecule as a whole, because if whole molecule rotation were faster than headgroup motion the former would dominate the effective correlation time. Thus, τ_G for the phospholipid must be at least 10^{-9} s. In another analysis, Peterson and Chan [29] deduced a correlation time for reorientation of the phospholipid hydrocarbon chains of 10^{-8} s in sonicated phospholipid vesicles. One of the two contributions to this reorientation was whole molecule rotation.

From this discussion it appears that phospholipid axial rotation is one or two orders of magnitude slower than rotation of the cholesterol molecule. This conclusion argues against a strong complex between phosphatidylcholine and cholesterol at least on the NMR time scale, since the two should rotate at the same rate if complexed. However, the phospholipid most likely orients cholesterol in the membrane.

Using Eqn. 3 and the value for τ_G previously determined, it is possible to calculate an expected linewidth as a further check of the above motional model. The value obtained for the vesicles containing 10 mol% cholesterol, assuming an isotropic rotational correlation time for the vesicle of 10^{-6} s, is 70 Hz. The linewidth measured was 80 ± 10 Hz for 10 mol% cholesterol in egg phosphatidylcholine vesicles. Thus, the motional model advanced in this work is consistent with the ^{13}C linewidth measured. In contrast to the T_1 analysis, calculations for T_2 show that in this case the spin-spin relaxation is modulated predominantly by isotropic vesicle rotation. The anisotropic rotation contributes less than 10% of the linewidth. From Eqn. 3, one can see that $1/T_2$ is approximately proportional to τ_R. Thus, the observation previously that the linewidth increases with increasing cholesterol content, particularly above 30 mol% cholesterol [11], probably reflects the increase in vesicle size and thus a decrease in rotational rate with increasing cholesterol content [12].

Acknowledgements

The helpful comments of Dr. K. Ferguson are appreciated as is the use of the NMR made possible by Dr. J. Alderfer. This research was supported by a grant from the American Heart Association (79677).

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