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^{13}C NMR STUDIES ON [4-^{13}C]CHOLESTEROL INCORPORATED IN SONICATED PHOSPHATIDYLCHOLINE VESICLES

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Summary

1. 90.5 MHz ^{13}C NMR linewidth measurements were performed on mixed sonicated [4-^{13}C]cholesterol/phosphatidylcholine vesicles of different fatty acid composition.

2. From the Dy^{3+}-induced shift of the C_4 resonance of cholesterol it suggested that this part of the molecule is localized in the ester bond region of the bilayer.

3. The local motion of the cholesterol ring system is restricted and independent of fatty acid composition.

4. At cholesterol concentrations below 30 mol% the ring system becomes more immobilised when the fatty acids of the phosphatidylcholine molecules enter the gel state.

Introduction

Despite the numerous studies on the phospholipid-cholesterol interaction in bilayers (for review see ref. 1) only very recently it has become possible to investigate the state of molecular motion of cholesterol in the bilayer. Proton NMR studies of cholesterol in sonicated vesicles of deuterated 16 : 0/16 : 0-phosphatidylcholine have revealed different mobilities of the cholesterol nucleus and its aliphatic tail [2]. The motion of the isopropyl moiety of the tail was shown to be essentially unrestricted as compared to the ring system. A preliminary deuterium NMR study of 50 mol% [3-^{2}H]cholesterol containing bilayers of unsonicated 16 : 0/16 : 0-phosphatidylcholine liposomes demonstrated that axial rotation of both cholesterol and 16 : 0/16 : 0-phosphatidylcholine in the equimolar mixture are possible but that torsional oscillations perpendicular to this axis are severely restricted [3].

^{13}C NMR is a valuable technique in the study of the molecular motion of

membrane lipids due to the large chemical shift range and the relatively limited resonance broadening under conditions of restricted motion. This is illustrated by relaxation measurements on cholesterol containing egg phosphatidylcholine and 16 : 0/16 : 0-phosphatidylcholine bilayers which clearly showed the reduction in chain motion by cholesterol above the transition temperature [4—8]. Alternatively ^{13}C NMR linewidth measurements on liposomes prepared from a variety of synthetic and natural phosphatidylcholines demonstrated that the effect of cholesterol on the motional state of various parts of the phospholipid molecule is strongly dependent on the chain length and type and degree of unsaturation [9,10]. In this study ^{13}C NMR measurements are reported for [4-^{13}C]cholesterol incorporated in vesicles prepared from a variety of different phosphatidylcholines.

Materials and Methods

Chemicals

[4-^{13}C]Cholesterol was obtained from Merck and Sharpe Dohme (Munchen, Germany). Cholesterol was obtained from Fluka (Buchs, Switzerland). Both sterols were more than 99% pure as judged by thin layer chromatography.

1,2-Dilauroyl-*sn*-glycero-3-phosphocholine (12 : 0/12 : 0-phosphatidylcholine), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (14 : 0/14 : 0-phosphatidylcholine), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (16 : 0/16 : 0-phosphatidylcholine), 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (18 : 0/18 : 1-phosphatidylcholine) and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (18 : 1/18 : 1-phosphatidylcholine) were synthesized and purified as described before [11]. Soya phosphatidylcholine, which is a highly unsaturated phosphatidylcholine containing predominantly linoleic acid, was the kind gift of Dr. H. Eikermann from Natterman (Koln, G.F.R.). Dy_2O_3 was purchased from B.D.H. (Poole, U.K.) and was converted to its chloride by HCl.

Preparation of multilayered liposomes and sonciated vesicles

Multilayered liposomes were prepared by dispersing phosphatidylcholine with the appropriate amount of cholesterol in 1.5 ml ^2H_2O containing 25 mM Tris · HCl, p^{2}H = 7.0, and 0.2 mM EDTA by agitation on a vortex mixer. In all experiments the cholesterol concentration was 25 mM. Vesicles were prepared by ultrasonication of the liposome dispersion as described before [12].

Nuclear magnetic resonance (NMR)

A Bruker WS 360 spectrometer operating at 90.5 MHz was used for all measurements. The spectrometer was equipped with broad-band proton decoupling (8 W input power) and variable temperature unit (accuracy of the temperature in the sample ±1°C). A spectral width of 20 KHz was used using 16 K data points. 2000—10 000 transients were accumulated with an interpulse time of 1 s using 90° pulses. The contribution of the field inhomogeneity to the linewidth was a maximum of 2 Hz, which was the linewidth of a resonance of Tris in the buffer. The maximum error in the determination of the linewidth of the [4-^{13}C]cholesterol resonance in sonicated vesicles was estimated to be 5%. Chemical shifts are given relative to external 1,4-dioxane. The peak intensities

and linewidths were independent of time (for the length of the measurements) and thermal history of the sample. Furthermore the opalescent nature of the vesicle solutions did not change visibly during the experiments, indicating that no signification fusion or aggregation of the vesicles occurred.

Differential scanning calorimetry

The phase transitions in sonicated vesicles were determined with a Perkin-Elmer DSC-2 calorimeter as described before [13].

Results

In Fig. 1B the ^{13}C NMR spectrum of unsonicated liposomes containing 20 mol% [4-^{13}C]cholesterol and 16 : 0/16 : 0-phosphatidylcholine at 50°C is shown. From a comparison of the spectrum of [4-^{13}C]cholesterol in chloroform (Fig. 1A) it can be concluded that the C_4 atoms of cholesterol give rise to a very broad resonance (width about 1 KHz) centered around 24 ppm. The resonance is only partly resolved due to the resonances of the naturally-abundant ^{13}C of 16 : 0/16 : 0-phosphatidylcholine. This is in agreement with the results obtained by Opella et al. [14] who under similar conditions also failed to obtain resolved nuclear resonance signals from the steroid nucleus of cholesterol in unsonicated liposomes. To avoid very long data accumulations and the use of difference spectroscopy to take into account the naturally-abundant ^{13}C background of the lipids it was decided to use sonicated vesicles which give high resolution spectra.

In Fig. 2A the spectrum of 16 : 0/16 : 0-phosphatidylcholine/[4-^{13}C]-

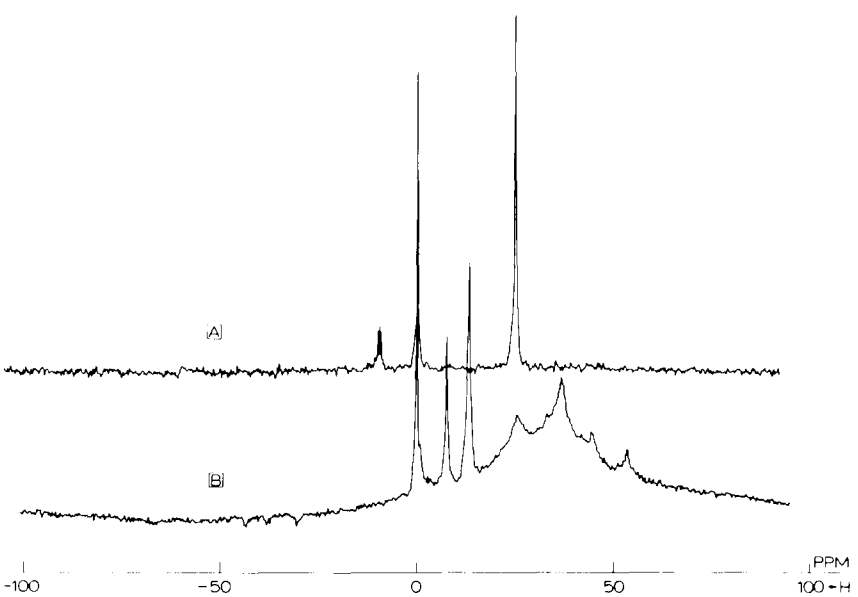


Fig. 1. 90.5 MHz ^{13}C NMR spectra at 50°C of (A) [4-^{13}C]cholesterol in C^2HCl_3 and (B) 16 : 0/16 : 0-phosphatidylcholine/[4-^{13}C]cholesterol (4 : 1) unsonicated liposomes. The resonance at 0 ppm is from external 1,4-dioxane.

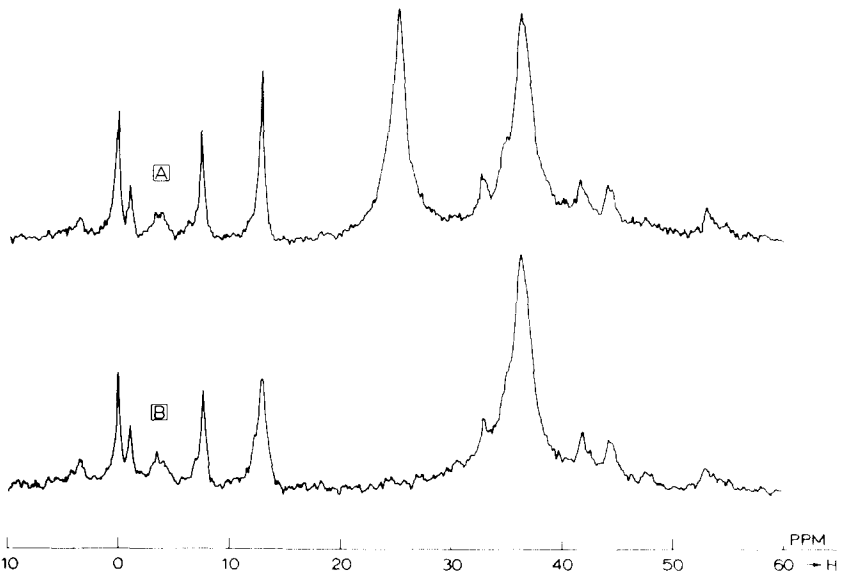
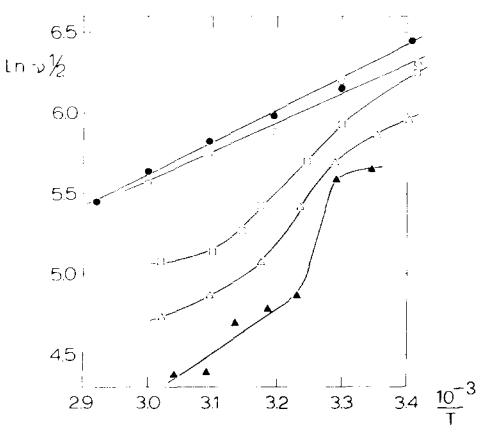


Fig. 2. 90.5 MHz ^{13}C NMR spectra at 50°C of (A) sonicated 16 : 0/16 : 0-phosphatidylcholine/[4-^{13}C]-cholesterol (4 : 1) vesicles and (B) sonicated 16 : 0/16 : 0-phosphatidylcholine/cholesterol (4 : 1) vesicles. The resonance at 0 ppm is from external 1,4-dioxane and at 8 ppm is from Tris. The other resonances came from the natural-abundance ^{13}C in 16 : 0/16 : 0-phosphatidylcholine.

cholesterol (4 : 1) vesicles is compared with a spectrum of similar vesicles prepared from unlabelled cholesterol. It may be noted that the C_4 resonance of cholesterol is well separated from the resonances of the naturally-abundant ^{13}C nuclei in the phospholipid molecule. In order to obtain an indication as to the availability of the C_4 atom to the aqueous medium the effect of paramagnetic shift reagents on the C_4 resonance was studied. Dy^{3+} was chosen because this lanthanide produces the largest shifts [15,16]. Furthermore a cholesterol concentration of 10 mol% was used which gave the best-resolved NMR spectrum. At the highest concentration of Dy^{3+} tested (25 mM; Dy^{3+}/cholesterol, 1 : 1), a small downfield shift of 0.4 ppm of the C_4 resonance in 10% [4-^{13}C]cholesterol containing 16 : 0/16 : 0-phosphatidylcholine vesicles at 50°C was observed. The resonances of the naturally abundant ^{13}C atoms were assigned using literature data [4—8,17]. The resonances from the 16 : 0/16 : 0-phosphatidylcholine molecules in the outer monolayer were shifted by 3 and 4 ppm for the -*C^H-O-CO-R and *CH_2-O-P-choline resonances, respectively, and 4 and 4.4 ppm for the -*CH_2-$N(CH_3)_3$ and -$N(*CH_3)_3$ resonances. This is in agreement with the results obtained with the less effective shift reagent Pr^{3+} which also shifts the ^{13}C resonances of the choline and glycerol groups of the outside facing phosphatidylcholine molecules [17]. It may be noted that the largest shift is observed for the ^{31}P resonance of the negatively charged phosphate group where the shift reagent binds most strongly [17]. If the cholesterol 3β-OH group is hydrogen-bonded to the phosphate group, as has been proposed [18], it may be considered likely that the adjacent C_4 atom would sense the presence of the shift reagent in a similar way as the resonances of the glycerol and choline groups, thus causing a downfield shift in the order

of 3—4 ppm. That a much smaller shift is observed suggests that the C_4 atom is further away from the phosphate group. Dy^{3+} produced a downfield shift of 1.3 and 0.5 ppm, respectively, for the -*CO-O- and -*CH_2-CO resonance of the outside-facing 16 : 0/16 : 0-phosphatidylcholine molecules. The shift of the latter resonance is very close to the value observed for the C_4 resonance of cholesterol. This would suggest that the C_4 atom of cholesterol and thus the adjacent 3β-OH group is localised in the ester bond region of the bilayer. Such a possibility has been suggested on both theoretical arguments [19,20] and experimental data [21,22,34]. However, precise localisation of the C_4 atom with respect to Dy^{3+} is not possible because the induced shift depends not only on the distance between Dy^{3+} and the C_4 atoms but is also affected by the chemical configuration around the nucleus among other factors. The linewidth of the C_4 resonance of cholesterol in mixed 16 : 0/16 : 0-phosphatidylcholine vesicles is shown in Fig. 3. The linewidth increases with increasing cholesterol concentrations. A particularly abrupt increase is observed between 30 and 40 mol% cholesterol (Fig. 4). Under no condition was more than one signal observed from cholesterol. Opella et al. [14] observed under certain conditions two signals from [4-^{13}C]cholesterol in unsonicated 16 : 0/16 : 0-phosphatidylcholine bilayers using proton enhanced ^{13}C NMR difference spectroscopy. The reason for this discrepancy is not fully understood. Possible vesicle tumbling would average the two different dominant singularities of the observed spectrum [14]. At 10 mol% cholesterol the linewidth undergoes a sharp change between 30 and 35°C. This change in linewidth becomes less with increasing cholesterol concentration (Fig. 3). That the lipid phase transition of 16 : 0/16 : 0-phosphatidylcholine in the vesicles is responsible for this change is indicated in Fig. 5A where the linewidth of the -$(CH_2)_n$ resonance of 16 : 0/16 : 0-phosphatidylcholine in the presence of 10 mol% cholesterol also undergoes a large increase below 40°C. At 50 mol% cholesterol concentration such a change does not seem to occur (Fig. 5B).



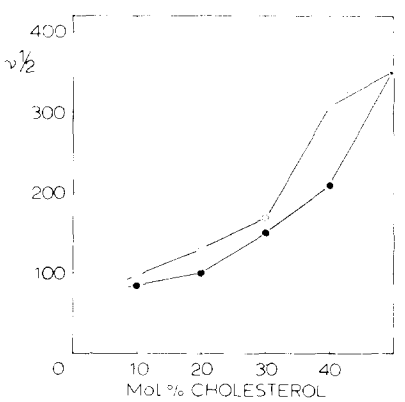


Fig. 3. Effect of [4-^{13}C]cholesterol concentration and temperature upon the 90.5 MHz ^{13}C NMR linewidth of the C_4 resonance of cholesterol in sonicated 16 : 0/16 : 0-phosphatidylcholine vesicles. ▲, 10 mol%; △, 20 mol%; □, 30 mol%; ○, 40 mol% and ●, 50 mol% [4-^{13}C]cholesterol.

Fig. 4. Effect of [4-^{13}C]cholesterol concentration in 16 : 0/16 : 0-phosphatidylcholine vesicles on the C_4 resonance linewidth at 50°C. ○———○, measured linewidths; ●———●, calculated linewidths (see text).

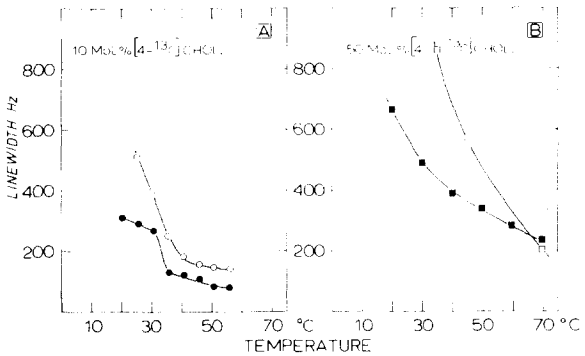


Fig. 5. 90.5 MHz ^{13}C NMR linewidths of the C_4 resonance of cholesterol (●,■) and the $-(CH_2)_n$ resonance of 16 : 0/16 : 0-phosphatidylcholine (○,□) in sonicated vesicles containing (A) 10 mol% [4-^{13}C]cholesterol and (B) 50 mol% [4-^{13}C]cholesterol.

By measuring the intensity of the C_4 resonance with respect to the naturally-abundant chain methyl resonance of 16 : 0/16 : 0-phosphatidylcholine it could be concluded that at 50°C 70—90% of the C_4 carbon atoms were observed in the spectrum of the 10—50 mol% [4-^{13}C]cholesterol containing vesicles. There was no clear correlation between the intensity of the C_4 resonance and the percentage of cholesterol in the bilayer. The 10—30% of the C_4 nuclei not observed in the narrow resonance at 24 ppm are most probably present in some larger vesicles which will give rise to much broader lines which are most probably lost in the baseline.

Since the phase transition in unsonicated 16 : 0/16 : 0-phosphatidylcholine bilayers is sharp and occurs at 41°C, the phase transition of mixed cholesterol/16 : 0/16 : 0-phosphatidylcholine sonicated vesicles was investigated in more detail by differential scanning calorimetry. In the absence of cholesterol the phase transition of 16 : 0/16 : 0-phosphatidylcholine in sonicated vesicles is broad and ranges from 30—42°C (Fig. 6A) in agreement with several other studies [13,23—26]. The larger width of the transition arises most likely from the small size of the vesicle which limits the size of the cooperative unit [26].

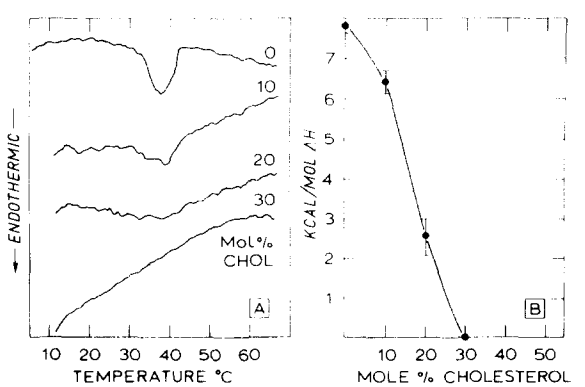


Fig. 6. A. Differential scanning calorimetric scans of sonicated 16 : 0/16 : 0-phosphatidylcholine vesicles containing increasing amounts of cholesterol. B. Effect of cholesterol incorporation on the heat of transition in sonicated 16 : 0/16 : 0-phosphatidylcholine vesicles.

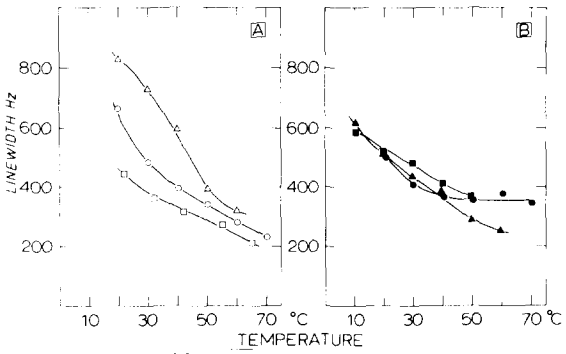


Fig. 7. 90.5 MHz ^{13}C NMR linewidths of the C_4 resonance of sonicated [4-^{13}C]cholesterol/phosphatidylcholine (1 : 1) vesicles. △———△, 14 : 0/14 : 0-phosphatidylcholine; ○———○, 16 : 0/16 : 0-phosphatidylcholine; □———□, 12 : 0/12 : 0-phosphatidylcholine; ■———■, soya phosphatidylcholine; ▲———▲, 18 : 0/18 : 1-phosphatidylcholine; ●———●, 18 : 1/18 : 1-phosphatidylcholine.

Cholesterol incorporation gradually decreases the heat of transitions such that above 30 mol% no phase transition can be detected any more (Fig. 6B). A similar decrease in transition heat of 16 : 0/16 : 0-phosphatidylcholine by cholesterol has been observed for multilayered liposomes [27,28].

Fig. 7 compares the linewidths of the C_4 resonances of 50 mol% [4-^{13}C]-cholesterol incorporation in different saturated and unsaturated phosphatidylcholines. The order of the C_4 linewidth in the saturated species is 12 : 0/12 : 0-phosphatidylcholine < 16 : 0/16 : 0-phosphatidylcholine < 14 : 0/14 : 0-phosphatidylcholine. In the temperature range 10—40°C the linewidth is very similar for the phospholipids containing one, two and four double bonds. The temperature dependence of the linewidth in these vesicles follows the general behaviour as for the other phosphatidylcholines with the exception of 18 : 1/18 : 1-phosphatidylcholine, where the linewidth from 40—70°C is relatively independent of temperature.

Discussion

Methylene carbons in solids have a chemical shift anisotropy of about 33 ppm [14]. The linewidth of the C_4 resonance of cholesterol in unsonicated 16 : 0/16 : 0-phosphatidylcholine liposomes is still approx. 10 ppm (Fig. 1) demonstrating that only part of the chemical shift anisotropy is averaged, most probably due to fast rotation around the long axes of the molecule, but that more isotropic motions around axes perpendicular to the long axis are severely hindered. The observed line narrowing upon sonication of the liposomes can be explained by two mechanisms: 1, the local motion of cholesterol in the bilayer of the sonicated vesicle is much higher than in the liposome; 2, because of the small size of the vesicle part of the ^{13}C-^{1}H dipolar interaction is averaged due to a fast lateral diffusion of cholesterol or by vesicle tumbling.

The similar effects of cholesterol on the phase transition of 16 : 0/16 : 0-phosphatidylcholine in both bilayer types suggest that the cholesterol/16 : 0/16 : 0-phosphatidylcholine interaction is not much affected by the sonication. The second possibility therefore seems most plausible.

The linewidth of the C_4 resonance of cholesterol in vesicles may be assumed to have two contributions, one tumbling independent which depends on the local motion of the ring system in the bilayer and one part which is dependent upon the lateral diffusion and vesicle tumbling. By analogy with the results of McLaughlin et al. [29] and Cullis [30] for ^{31}P NMR it may be expected that the linewidth varies as $1/T_2 = M_2\tau_c + C$, where M_2 is the residual second moment obtained after the averaging due to the restricted anisotropic motion of cholesterol in the bilayer and C is the portion of the linewidth which is independent of τ_c, which is the correlation time for rotational diffusion which arises both from vesicle tumbling and from lateral diffusion.

The correlation time for rotational diffusion is given by [30]:

$$\frac{1}{\tau_c} = \frac{6}{a^2}(D_t + D_{diff}) \quad (1)$$

where a is the outer radius of the vesicle, D_t is the rotational diffusion arising from vesicle tumbling and D_{diff} the lateral diffusion rate. For 50 mol% cholesterol containing 16 : 0/16 : 0-phosphatidylcholine vesicles D_t can be calculated from the solution viscosity and the outer radius of the vesicles (218 Å [12]) to be $14.8 \cdot 10^{-8}$ cm^2/s. D_{diff} of cholesterol is not known but for the structurally related androstane spin label a value of $1 \cdot 10^{-8}$ cm^2/s was reported [31] whereas D_{diff} of egg phosphatidylcholine in 50 mol% cholesterol containing vesicles was found to be $1.4 \cdot 10^{-8}$ cm^2/s [30]. This strongly suggests that lateral diffusion of cholesterol around the vesicle surface will not produce a significant line narrowing.

The C_4 resonance linewidth was strongly dependent upon the cholesterol concentration (Fig. 4). This can arise either from a decrease in local motion of the cholesterol nucleus with increasing cholesterol concentrations or is due to increase in size of the vesicles [12,32,33]. Using the sizes of the different 16 : 0/16 : 0-phosphatidylcholine/cholesterol vesicles [12] the relative linewidth at the various cholesterol concentrations can be calculated with respect to the linewidth observed in the presence of 50 mol% cholesterol assuming that M_2 and C are constant. The calculated linewidths agree quite well with the observed linewidths (Fig. 4), suggesting that the restricted motion of the ring system is not very sensitive to the cholesterol concentration and that the observed changes in linewidth are due predominantly to changes in vesicle size. The sharp increase in linewidth above 30 mol% cholesterol, for instance, coincides with the largest increase in vesicle size above this concentration [12]. This is further supported by the finding that the activation energy of T_2 in 40 and 50 mol% [4-^{13}C]cholesterol containing 16 : 0/16 : 0-phosphatidylcholine vesicles over the temperature range 20—60°C is 4.8 ± 0.3 and 4.0 ± 0.3 kcal/mol, respectively (Fig. 3) which is very similar to the value of 4.5 kcal/mol calculated from the Stokes-Einstein relation of vesicle tumbling over the same temperature range.

At low cholesterol/16 : 0/16 : 0-phosphatidylcholine molar ratios the linewidth of the C_4 resonance of cholesterol increases when the fatty acid chains which are not interacting with cholesterol become crystalline. This is certainly due to a decreased local motion of the ring system of cholesterol in the bilayer, since the size of the vesicle will decrease below the phase transition because of

the smaller area per molecule in the gel state, and thus would be expected to give a narrower resonance. The restricted motion must be the result of the increased lateral ordering of the bilayer due to the more crystalline state of the bilayer, even though the cholesterol molecule must be surrounded by 16 : 0/16 : 0-phosphatidylcholine molecules which cannot undergo a phase transition. Either the increased lateral ordering can be sensed by cholesterol from these molecules or a fast exchange exists between the two kinds of 16 : 0/16 : 0-phosphatidylcholine molecule.

The linewidth of the C_4 resonance of cholesterol in 50 mol% containing saturated phosphatidylcholine vesicles followed the order 14 : 0/14 : 0-phosphatidylcholine > 16 : 0/16 : 0-phosphatidylcholine > 12 : 0/12 : 0-phosphatidylcholine, which is the same order of the sizes of these vesicles which was reported as 243, 218 and 189 Å (outer radius) respectively [12]. When the linewidths are corrected for this difference in vesicle size, as shown above, the linewidths of the C_4 resonance of cholesterol become identical, within experimental error, for the different vesicles (data not shown). The unsaturated phosphatidylcholine/cholesterol (1 : 1) vesicles have similar sizes [12] and similar C_4 resonance linewidths. This suggests that the state of motion of cholesterol in vesicle bilayers is independent upon chain length and unsaturation, despite the large differences in effect of cholesterol on the molecular motion of these phosphatidylcholines [10].

Acknowledgements

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