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Deuterium Magnetic Resonance of Selectively Deuterated Cholesteryl Esters in Phosphatidylcholine Vesicles†

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ABSTRACT: Deuterium nuclear magnetic resonance (^{2}H NMR) experiments have been performed on selectively deuterated cholesteryl palmitate (CP) and cholesteryl stearate (CS) in egg phosphatidylcholine (PC) unilamellar vesicles. Egg PC vesicles were found to incorporate up to 5 mol % ester and to have a mean diameter of 22 nm. Addition of 20 mol % cholesterol decreased the solubility of cholesteryl ester in the bilayer to ~2–3 mol %, but the vesicle size remained unchanged. The ^{2}H NMR results reveal that the acyl chains of CP and CS are highly disordered ($S_{CD} < 0.10$) both in the presence and in the absence of cholesterol. T_1 measurements for selectively deuterated CP and CS in egg PC vesicles indicate that the high degree of disorder of the ester molecule is not associated with an increase in the rate of gauche–trans chain isomerization. Possible explanations for the low order parameters in terms of molecular motions and orientations are offered.

The onset of atherosclerosis is characterized by rapid accumulation of cholesteryl esters in aortic intimal and medial layers (Cornwell et al., 1975). It has also been reported that the permeability to lactate, iodide, and glucose is much higher in sclerotic human tissue than in normal tissue (Kirk, 1962). Since a recent study (Forrest & Cushley, 1977) has shown that incorporation of the saturated ester cholesteryl palmitate (CP)[1] increases the permeability of model membranes, data on the structural organization of saturated cholesteryl esters

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[1] Abbreviations used: CP, cholesteryl palmitate; ESR, electron spin resonance; PC, phosphatidylcholine; ^{2}H NMR, deuterium nuclear magnetic resonance; CS, cholesteryl stearate; rf, radio frequency; DPPC, dipalmitoylphosphatidylcholine.

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in bilayers are of current interest.

Incorporation of CP into egg PC multibilayers has been studied by using ESR spectroscopy (Grover & Cushley, 1979a; Grover et al., 1979), and it was found that, at concentrations above ~0.8 mol % spin-labeled CP, two distinct forms of ester were detected: (a) a homogeneous form with ester intercalated between phospholipid molecules and adopting a "horseshoe" conformation, i.e., with the ester linkage near the aqueous surface and the acyl chain and cholesteryl moiety extending deep into the hydrophobic region of the bilayer, and (b) patches of solid ester which remain associated with the membrane bilayer. Below ~0.8 mol % only the "homogeneous" form of the ester, i.e., capable of giving liquid-crystalline ESR spectra, was found (Grover & Cushley, 1979a).

When deuterated CP was studied in egg PC multilamellar liposomes by using ^{2}H NMR (Valic et al., 1979), the amount of homogeneous ester maximized at 0.2 mol % with the remainder being found in the form of solid patches. Using deuterium as a label minimizes the perturbing effects of the label with regard to both size and charge effects; hence, the higher solubility of the spin-labeled ester may be partially due to the presence of the nitroxide group.

Deuterium NMR has also proven to be an extremely useful technique for determining the local orientational order in a lipid bilayer (Seelig, 1977; Mantsch et al., 1977). The most probable order parameter S_{CD} for perdeuterated ester, CP-d_{31}, was found in our previous study to be 0.1, while S_{CD} for the terminal CD_3 segment of CP-*16,16,16*-d_3 was found to be 0.02 (Valic et al., 1979). The latter value was identical with that reported for the CD_3 segment of palmitic-d_{31} acid in egg PC multilamellar liposomes (Stockton et al., 1976), whereas the former value (S_{CD} = 0.1) was ~2.5 times lower than the corresponding value for palmitic-d_{31} acid. The low solubility of CP in egg PC multilamellar liposomes (0.2 mol %) precluded the use of esters selectively deuterated along the acyl chain for further NMR studies.

In the present communication we present results of a ^{2}H NMR study of selectively deuterated saturated cholesteryl esters, CP and CS, in egg PC unilamellar liposomes (vesicles). In vesicles the ^{2}H NMR line widths are much narrower than in multilamellar liposomes due to the much more rapid rotational reorientation and high degree of curvature of the smaller structures. This fact, combined with a higher solubility of CP and CS in vesicles, yields strong resonance signals for esters deuterated at a single position on the palmitoyl or stearoyl chain. We have further determined the effect of cholesterol on the transverse and longitudinal relaxation times of selectively deuterated cholesteryl esters in egg PC vesicles.

Experimental Procedures

Materials. Cholesterol was obtained from Fisher Scientific Co. and recrystallized from benzene before being used. Cholesteryl palmitate was obtained from Sigma Chemical Co., deuterium-depleted water was from Aldrich Chemical Co., and deuterium oxide (99.7%) was from Merck Sharp & Dohme Canada Ltd. Egg PC was isolated by using the method of Singleton et al. (1965). Hexadecanoic-d_{31} acid, hexadecanoic-*5,5,6,6*-d_4 acid, and hexadecanoic-*11,11,12,12*-d_4 acid were purchased from Merck Sharp & Dohme Canada Ltd., whereas hexadecanoic-*16,16,16*-d_3 acid was obtained from Serdary Research Laboratories, London, Ontario, Canada. Hexadecanoic acid and octadecanoic acid were purchased from Fisher Scientific Co. Syntheses of octadecanoic-*8,8*-d_2 and -*13,13*-d_2 acids were described previously (Tulloch, 1977).

α-Deuterated Fatty Acids. The hexadecanoic-*2,2*-d_2 and octadecanoic-*2,2*-d_2 acids were prepared essentially as described by Atkinson et al. (1968) except that two instead of five exchanges were used and the bath temperature was 220 °C (exchange 1) and 245 °C (exchange 2). Sample data: from 550 mg of palmitic acid was obtained 520 mg of palmitic-*2,2*-d_2 acid (93% crude yield); ^{1}H NMR, complete absence of triplet at δ 2.35; mass spectrum *m/e* (rel intensity) 258 (100, $M^{+\cdot}$), 215 (13.5, M − 43), 214 (19.1, M − 44), 187 (15.9, M − 71), 173 (10.1, M − 85), 157 (13.7, M − 101).

Hexadecanoic-3,3-d_2 Acid. Methyl tetradecanoate (24.2 g) was reduced with lithium aluminum deuteride (3.1 g) in ether (400 mL) for 18 h, giving tetradecanol-*1,1*-d_2 which was used without purification. The mesylate was prepared by the method previously described (Tulloch, 1977) and crystallized from methanol (mp 42–44 °C). Reaction with diethyl malonate (Spener & Mangold, 1973; Tulloch, 1977) gave tetradecyl-*1,1*-d_2 malonic acid, and decarboxylation yielded hexadecanoic-*3,3*-d_2 acid (17.7 g; $bp_{0.25mmHg}$ 170 °C). The overall yield from methyl tetradecanoate was 69%. The ^{13}C NMR spectrum showed that the product was completely deuterated at C-3.

Hexadecanoic-4,4-d_2 Acid. Methyl tetradecanoate (24.2 g) was heated with sodium deuterioxide (5 g) in deuterium oxide (100 mL) in a rocking stainless steel autoclave at 200 °C for 4 days. Tetradecanoic-*2,2*-d_2 acid (22.5 g) was recovered in the usual way (Tulloch, 1977), and the mass spectrum (of the methyl ester) showed 97% dideuteration. Reduction with lithium aluminum hydride, followed by the reaction sequence described above, gave hexadecanoic-*4,4*-d_2 acid (15.7 g); the overall yield was 61%.

Deuterated cholesteryl esters were prepared from the corresponding fatty acids and cholesterol by a known method (Grover & Cushley, 1979b).

Preparation of Egg PC Vesicles. Egg PC was dissolved at a known concentration in chloroform, stored under nitrogen at −20 °C, and dispensed volumetrically. Cholesteryl esters and cholesterol were weighed directly into samples tubes, the egg PC solution was added, and the tube was shaken to ensure complete mixing. The solvent was then removed by evaporation under a steam of nitrogen and subsequent pumping under high vacuum for at least 2 h. The required amount of water or deuterium-depleted water was then added, the tube was shaken vigorously on a vortex mixer, and the resultant dispersion was sonicated for 15 min at room temperature with a Biosonik III probe-type sonicator at maximum output power. The vesicles were then centrifuged for 15 min on a small laboratory centrifuge, in order to eliminate titanium fragments and undispersed lipid. In most of the NMR experiments, the vesicles were used immediately after completion of this step. However, in those cases where the amounts of CP (or deuterated CP) and egg PC in the samples had to be determined accurately, i.e., for the experiments described under the first part of Results, the samples were subjected to an additional preparative ultracentrifugation for 3 h at 160000*g* on a Spinco Model L ultracentrifuge in order to remove any traces of residual undispersed lipid and multilamellar liposomes (Barenholz et al., 1977). The amount of phosphatidylcholine present in the suspension was quantified by phosphorus determination (Ames, 1966) while cholesteryl esters were determined as described by Rudel & Morris (1973).

Measurement of Diffusion Coefficients. Diffusion studies were carried out using a Spinco Model E analytical ultracentrifuge equipped with a Schlieren optical system and a RTIC temperature control unit, at 20 °C and 42 040 rpm, essentially as described by Newman & Huang (1975). The vesicles used for these studies were subjected to preparative

ultracentrifugation described in the previous section and were then diluted to the desired concentration with H_2O and D_2O in such a way as to bring the composition of the aqueous phase to 86 vol % H_2O–14 vol % D_2O. Under these conditions, the vesicles showed negligible sedimentation during the analytical ultracentrifugation, and the spreading of the Schlieren peak could be recorded over long periods of time.

Nuclear Magnetic Resonance. Experiments were performed at 15.4 MHz on a Varian XL 100-15 spectrometer in the Fourier transform mode and fitted with a NIC 1080 computer and 600K disc. Sufficient rf power was achieved by using a 300-W ENI power amplifier. All experiments were performed at the temperature of the probe (~28 °C). The field-frequency lock was an external ^{19}F signal. Longitudinal relaxation times (T_1) were measured either by the inversion–recovery method, (180°–τ–90°, sample and add, $\tau_R)_N$, where τ is the delay between the two pulses of the sequence, τ_R is the delay between subsequent pulse sequences ($\tau_R \geq 5T_1$ to ensure complete relaxation), and N is the number of acquisitions, or by the subtractive method, (180°–τ–90°, sample and subtract, τ_R, 90°, sample and add, $\tau_R)_N$. In all experiments a phase-alternating pulse sequence was used in order to minimize base-line aberrations.

Generally, for the NMR experiments, the vesicles were prepared by using deuterium-depleted water in order to eliminate the strong resonance peak due to the natural abundance of deuterium in water. In spite of this precaution, the HOD resonance sometimes appeared in the spectra, and we attribute this to residual water of hydration present in the egg PC and the cholesteryl esters. When accurate measurements of line widths were demanded, the HOD signal was eliminated by using difference spectroscopy.

Theory

It is well-known (Seelig, 1977) that, for a superposition of randomly oriented domains of bilayers, a characteristic, symmetric powder spectrum is observed for a specific deuteron site which is characterized by two peaks separated by a frequency

$$\Delta\nu = 3/4(e^2qQ/h)|S_{CD}| \quad (1)$$

where e^2qQ/h is the static quadrupolar coupling constant [168 kHz for a deuterium on a methyl or methylene group (Burnett & Müller, 1971; Davis & Jeffrey, 1977)] and

$$S_{CD} = \langle(3\cos^2\gamma - 1)/2\rangle \quad (2)$$

is the segmental order parameter for the C–D bond, where γ is the angle between the C–D bond and the axis of symmetry for reorientational motions of this bond, and the angular brackets represent an average over all conformations of the molecule. The order parameter measures the degree of ordering of a particular segment of the molecule, and it can be interpreted in terms of molecular motions (Schindler & Seelig, 1975; Bocian & Chan, 1978).

In the case of unilamellar vesicles, the quadrupolar interaction is partially averaged out due to tumbling of the vesicles and lateral diffusion of the lipid molecules, and a narrow single Lorentzian line is observed for a specific deuteron site. The width at half-height, $\Delta\delta$, for this line is dependent upon the segmental order parameter S_{CD} and the correlation times of the motions modulating the quadrupolar interactions.

Assuming that the molecular motions occurring in the system can be divided into two classes, one whose correlation time τ_c is much shorter than the inverse of the Larmor frequency ω_0 (and which we shall identify with the fast local motions of the lipid molecules) plus a second much slower class characterized by an effective correlation time τ_e ($\tau_e \gg \tau_c$) (which we identify with the vesicle reorientation), then the line width $\Delta\delta$ will be given by (Stockton et al., 1976)

$$\Delta\delta = 1/(\pi T_1) + 9\pi/20(e^2qQ/h)^2S_{CD}^2\tau_e \quad (3)$$

where T_1 is the longitudinal relaxation time of the deuterons, S_{CD} is the segmental order parameter (vide eq 2), and τ_e is the effective correlation time for vesicle reorientation. The effective correlation time, τ_e, has the form

$$1/\tau_e = 1/\tau_v + 1/\tau_d \quad (4)$$

where τ_v is the correlation time for rotational reorientation and τ_d is the correlation time for lateral diffusion of lipid molecules around the vesicle surface. The correlation time for lateral diffusion is related to D, the diffusion coefficient for the process, by

$$\tau_d = R^2/(6D) \quad (5)$$

where R is the effective distance from the center of gravity of the vesicle to the midpoint of the bilayer. The correlation time for vesicle tumbling can be estimated from

$$\tau_v = 4\pi\eta r_v^3/(3kT) \quad (6)$$

where η is the viscosity experienced by the vesicle at temperature T, k is the Boltzmann constant, and r_v is the hydrodynamic vesicle radius.

The longitudinal relaxation time, T_1, for a particular deuterated segment in vesicles is related to its correlation time, τ_c, by (Brown et al., 1979; Davis et al., 1978)

$$1/T_1 = 3/8(e^2qQ/\hbar)^2\tau_c(1 - S_{CD}^2) \quad (7)$$

which reduces to

$$1/T_1 = 3/8(e^2qQ/\hbar)^2\tau_c \quad (8)$$

for small S_{CD}, i.e., for $S_{CD} \lesssim 0.3$.

Recently, Petersen & Chan (1977) have suggested that the motions experienced by the lipid molecules in a bilayer are due to two different processes: the first corresponds to gauche–trans chain isomerizations ("kink motions"), which are assumed to be very fast ($\tau_c \approx 10^{-10}$–10^{-11} s) on the ^2H NMR time scale; the second is thought to be due to the reorientation of the entire lipid chain about the long molecular axis ("rigid stick motions") and is postulated to be several orders of magnitude slower ($\tau_c \approx 10^{-7}$–10^{-8} s). On the basis of this model, Petersen & Chan (1977) give the following expression for the ^2H NMR line width in vesicles:

$$\Delta\delta = \frac{1}{\pi T_1} + \frac{9\pi}{20}\left(\frac{e^2qQ}{h}\right)^2(S_\gamma^{CD})^2\left[S_\alpha^2\tau_e + \frac{1}{9}\left(\frac{5}{2}\sigma_\alpha^2 - 5S_\alpha^2 - 2S_\alpha + 9\mu_\alpha^2 + 7\right)\tau_\perp\right] \quad (9)$$

In eq 9 S_γ^{CD} is the order parameter associated with the gauche–trans isomerizations only.[2] S_α is the order parameter associated with the rigid stick motion, σ_α^2 is the variance associated with the molecular axis reorientation relative to the average position, τ_e is the effective vesicle correlation time (vide eq 4), μ_α is the mean of the function 2 sin $\alpha(t)$, and $\tau_\perp$ is the correlation time associated with the rigid stick motions.

[2] Equation 9 differs from eq 25 in Petersen & Chan (1977) by the term $1/(\pi T_1)$. This term can only be neglected if the spin–lattice relaxation rate is smaller than the spin–spin relaxation rate, $1/T_1 \ll 1/T_2$.

Equation 9 reduces to eq 3 in the limit where chain reorientation is not an important motion.

Results

Nature of the Egg PC–Cholesteryl Ester Vesicle. We have prepared egg PC–CP vesicles containing up to ~5 mol % CP. These vesicles appear stable, at 4 °C, for ~2 weeks. It was observed that, in order to prepare vesicles with high concentrations of ester (≳1 mol %), excess ester has to be added and, particularly with the saturating concentration (~5 mol %) (Forrest & Cushley, 1977; Forrest, 1978), a large excess of CP has to be added to the $CHCl_3$ solution containing phospholipid (see Experimental Procedures).[3] Control experiments revealed that CP alone cannot be dispersed in water in measurable amounts, even after prolonged sonication.

The concentration of CP that could be incorporated into egg PC–20 mol % cholesterol vesicles was seen to be approximately 2 times lower than for vesicles without cholesterol; i.e., the maximum solubility of CP in egg PC–20 mol % cholesterol vesicles is only ~2–3 mol %.

From sedimentation equilibrium measurements, the apparent diffusion coefficient at infinite dilution at 20 °C, D_{20}^0, of vesicles made up of egg PC, egg PC–2.5 mol % CP, and egg PC–0.6 mol % CP–20 mol % cholesterol were found to be 1.85 (±0.10) × 10^{-7}, 1.98 (±0.10) × 10^{-7}, and 1.90 (±0.10) × 10^{-7} cm^2 s^{-1}, respectively. All the Schlieren patterns were highly symmetrical, which indicates a homogeneous population of vesicles in the three cases. The measured values of the apparent diffusion coefficients for the three systems are identical within experimental error and in excellent agreement with the value of 1.87 × 10^{-7} cm^2 s^{-1} reported by Huang (1969), which indicates that the average size of the vesicles in all these preparations is the same. The mean hydrodynamic radius of the vesicles, calculated by using the Stokes–Einstein relationship, is 11.0 ± 0.7 nm.

Since the ^{2}H NMR signals characteristic of liquid–crystalline homogeneous ester, i.e., ester intercalated between phospholipid molecules, and solid patches (Valic et al., 1979) could not be detected simultaneously with our spectrometer, a new method was devised for determining the amount of CP homogeneously dissolved in the bilayer. Deuterium NMR spectra were determined for egg PC vesicles in deuterium-depleted water containing various amounts of CP-d_{31}. The spectrum of CP-d_{31} in egg PC vesicles appeared as a relatively narrow superposition of Lorentzian lines having a width at half-height of ~26 Hz and extending up to about ±300 Hz from the center frequency. The line width, as well as the line shape, was independent of the concentration of CP-d_{31} in the lipid. The intensity of the absorption was proportional to the amount of deuterated ester in the vesicles, up to the maximum concentration of ~5 mol %. An additional experiment was then performed in which egg PC–CP-d_{31} vesicles were prepared by using a carefully weighed amount of normal water, and the signal of the deuterated ester was compared to the signal of the natural abundance of deuterium in water. Care was taken that the delay between successive pulses was at least 5 times the largest longitudinal relaxation time in the system in order to obtain correct intensities (T_1 values for deuterons in CP-d_{31} are ≤0.25 s; T_1 for HOD = 0.5 s). This experiment showed that the observed intensity of the narrow ^{2}H NMR resonance signal for CP-d_{31} was >95% of that expected.

[3] A referee has suggested that vesicles containing relatively high amounts of CP, although stable, are most probably supersaturated in CP. This is consistent with the finding that excess CP must be added to form vesicles with >1 mol % CP.

Table I: Deuterium Transverse Relaxation Times for Cholesteryl Palmitate and Cholesteryl Stearate in Vesicles

ester	line width[a] (Hz)	$T_2^* \times 10^3$ (s)
(A) Without Cholesterol		
CS-*2,2*-d_2	145 ± 15[b]	2.2[b]
CP-*2,2*-d_2	160 ± 15	2.0
CP-*3,3*-d_2	135 ± 15	2.4
CP-*4,4*-d_2	85 ± 10	3.7
CP-*5,5,6,6*-d_4	63 ± 15	5.1
CS-*8,8*-d_2	72 ± 10	4.4
CP-*11,11,12,12*-d_4	59 ± 15	5.4
CS-*13,13*-d_2	46 ± 10	6.9
CP-*16,16,16*-d_3	10 ± 2	31.8
(B) With 20 mol % Cholesterol		
CS-*2,2*-d_2	160 ± 15	2.0
CP-*2,2*-d_2	145 ± 20	2.2
CP-*3,3*-d_2	205 ± 30	1.6
CP-*4,4*-d_2	160 ± 20	2.0
CP-*5,5,6,6*-d_4	115 ± 10	2.8
CS-*8,8*-d_2	125 ± 20	2.5
CP-*11,11,12,12*-d_4	270 ± 50	1.2
CS-*13,13*-d_2	145 ± 25	2.2
CP-*16,16,16*-d_3	23 ± 3	13.8

[a] The width at half-height ($\Delta\delta$) is related to relaxation time by $T_2^* = 1/(\pi\Delta\delta)$. [b] Values recorded are averages of all runs.

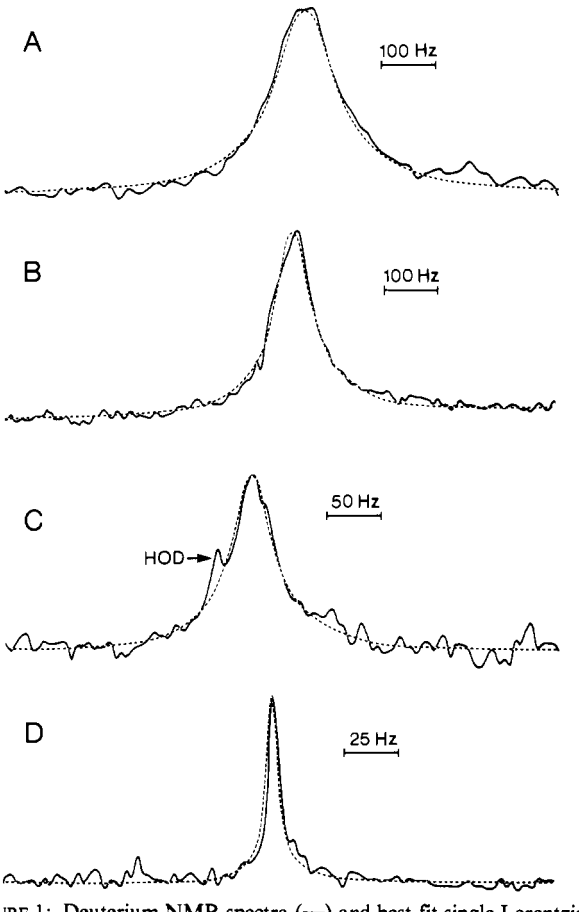

FIGURE 1: Deuterium NMR spectra (—) and best-fit single Lorentzian line shapes (---) of selectively deuterated cholesteryl palmitate in egg PC vesicles: (A) *3,3*-d_2; (B) *4,4*-d_2; (C) *11,11,12,12*-d_4; (D) *16,16,16*-d_3. In (D) the simulated spectrum is slightly displaced for clarity. Spectral parameters: pulse width = 60 μs (90° flip angle); sweep width = 2500 Hz; pulse delay = 0.4 s (A–C) and 1.3 s (D); number of data points = 2000; number of acquisitions = 3000–100000. The starting frequencies for each expansion are not the same.

Deuterium Nuclear Magnetic Resonance Spectra. Transverse relaxation times T_2^*, where $T_2^* = 1/(\pi\Delta\delta)$ and $\Delta\delta$ is the deuterium line width at half-height, for a number of selectively deuterated palmitate (CP) and stearate (CS) esters

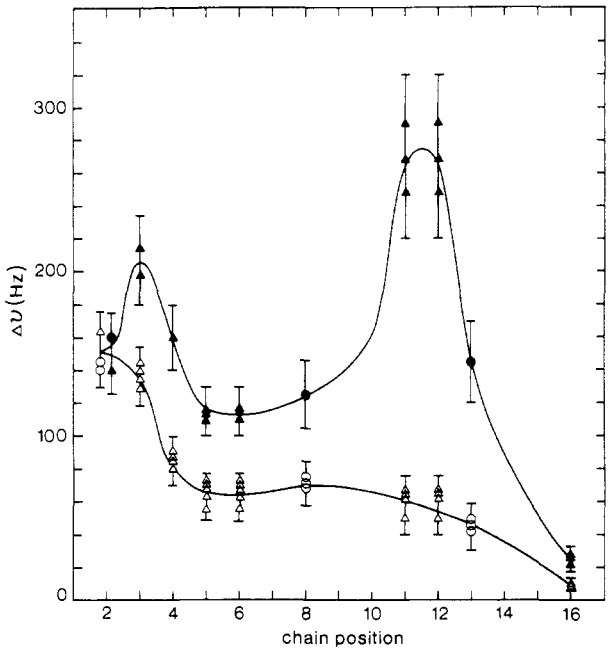

FIGURE 2: Deuterium NMR line widths of selectively deuterated cholesteryl esters in egg PC vesicles or egg PC–20 mol % cholesterol vesicles vs. chain position. (△) Cholesteryl palmitate in egg PC vesicles; (○) cholesteryl stearate in egg PC vesicles; (▲) cholesteryl palmitate in egg PC–20 mol % cholesterol vesicles; (●) cholesteryl stearate in egg PC–20 mol % cholesterol vesicles.

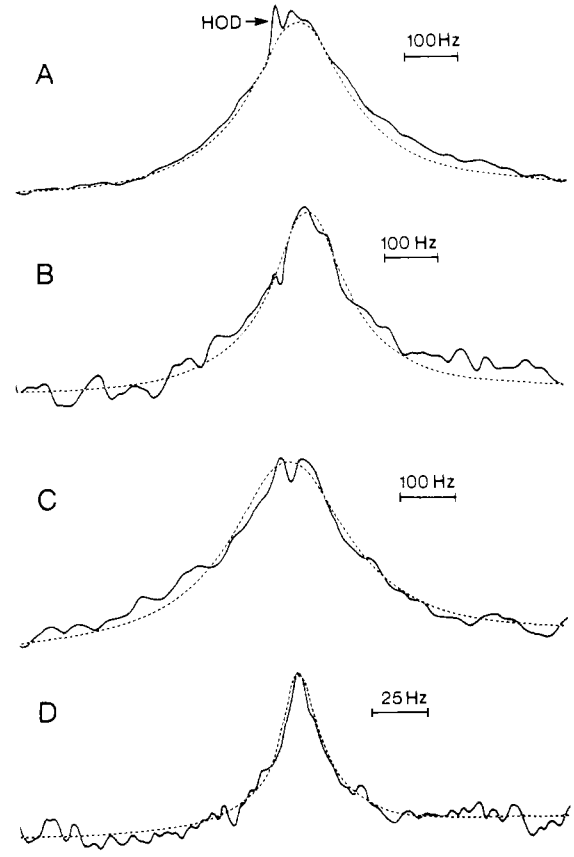

FIGURE 3: Deuterium NMR spectra (—) and best-fit Lorentzian line shapes (- - -) of selectively deuterated cholesteryl palmitate in egg PC–20 mol % cholesterol vesicles: (A) *3,3-d_2*; (B) *4,4-d_2*; (C) *11,11,12,12-d_4*; (D) *16,16,16-d_3*. Spectral parameters are as in Figure 1, except number of acquisitions = 5000–300 000.

are given in Table I, part A. The system consisted of vesicle solutions composed of 20 wt % lipid (97.5 mol % egg PC plus 2.5 mol % deuterio-CP) and 80 wt % deuterium-depleted water, at 28 °C. A selection of spectra from four deuterio-CP's chosen to represent the label at various positions along the entire acyl chain is shown in Figure 1. A single Lorentzian line was fitted to each of the spectra and appears superimposed on the experimental resonances. In these cases, and in the case of the other selectively deuterated esters which we have used, a good fit of the experimental spectrum to a single Lorentzian line is observed. Control experiments showed that the ^2H NMR line shape and, hence, $\Delta\delta$ *remained unchanged* whether the vesicles were homogeneously sized by preparative ultracentrifugation (Barenholz et al., 1977) or not. Consequently, many of the systems reported in Figure 1 and Table I are not subject to final preparative ultracentrifugation. The complete picture of the measured line widths for deuterated cholesteryl palmitate and cholesteryl stearate in egg PC vesicles is shown in Figure 2. It is readily seen that the line widths are greatest for deuterons near the ester linkage of the molecules and decrease gradually toward the terminal methyl group. A "plateau" region is observed between segments 5 and 13 in which the measured line width is practically constant. This is reminiscent of the plateau observed between positions 2 and 10 for deuterated fatty acids in egg PC bilayers (Stockton et al., 1976); however, the line widths are very different in both cases. While Stockton et al. (1976) report a line width of ~1200 Hz for deuterated fatty acids in egg PC vesicles, we find a much reduced line width of ~50–70 Hz for the deuterated cholesteryl esters. The smallest line width observed for the deuterated cholesteryl esters studied here corresponds to CP-*16,16,16-d_3*, having $\Delta\delta$ = 10 Hz. This is identical in value with $\Delta\delta$ observed for the CD_3 group of palmitic-*16,16,16-d_3* acid intercalated into egg PC liposomes (Stockton et al., 1976).

The effect on the ^2H line width of adding 20 mol % of cholesterol to vesicles composed of 97.5 mol % egg PC–2.5 mol % deuterioester is shown in part B of Table I. Figure 3 presents four representative ^2H NMR spectra illustrating the effect of the added cholesterol along with the Lorentzian line shape function fitting the spectrum best. Direct comparison with Figure 1 quantifies the effect of adding cholesterol. Due to the much lower solubility of cholesteryl ester in egg PC–20 mol % cholesterol vesicles, the NMR signals are much weaker and, therefore, we have a greater uncertainty in the measurement of the line widths. However, we still see a substantial increase in line width at every position except C-2, indicating a shorter T_2*. Note the especially large increase in line width for CP-*11,11,12,12-d_4*. A somewhat less dramatic but still significant maximum in the line-width profile also occurs at position C-3. The plateau region observed between position 5 and 13, in the case of vesicles without cholesterol, is much reduced for vesicles containing cholesterol and only spans positions 5–8. The line width for the terminal CD_3 segment of CP-*16,16,16-d_3* is 23 ± 3 Hz, which is very similar to the value of ~20 Hz observed for the CD_3 segment in stearic-*18,18,18-d_3* acid by Stockton et al. (1974).

We have also obtained longitudinal relaxation times, T_1, for several deuterated cholesteryl esters in egg PC vesicles, and these are tabulated in Table II and graphically illustrated in Figure 4. Very short ($T_1 \leq 0.02$ s) values were measured for all deuterated segments except for the terminal CD_3, which has T_1 = 0.25 s. This behavior is very similar to that observed for deuterated fatty acids in egg PC bilayers (Stockton et al., 1976) and for micelles of perdeuterated potassium palmitate (Davis et al., 1978) and, indeed, is reminiscent of the T_1 behavior of the fatty acyl chains of the phospholipids themselves, as shown by ^{13}C NMR measurements (Godici &

Table II: Deuterium Longitudinal Relaxation Times (T_1) for Cholesteryl Palmitate and Cholesteryl Stearate in Vesicles

ester	T_1[a] (s)
(A) Without Cholesterol	
CS-*2,2*-d_2	0.01
CP-*2,2*-d_2	0.02
CP-*3,3*-d_2	0.02
CP-*4,4*-d_2	0.02
CP-*5,5,6,6*-d_4	0.01
CS-*8,8*-d_2	0.02
CP-*11,11,12,12*-d_4	0.02
CS-*13,13*-d_2	0.02
CP-*16,16,16*-d_3	0.25
(B) With 20 mol % Cholesterol	
CP-*5,5,6,6*-d_4	0.01
CP-*11,11,12,12*-d_4	0.02
CP-*16,16,16*-d_3	0.19

[a] Error limits are approximately ±50% for the short T_1 values (≤0.03 s) and approximately ±10% for the long T_1 values (>0.10 s).

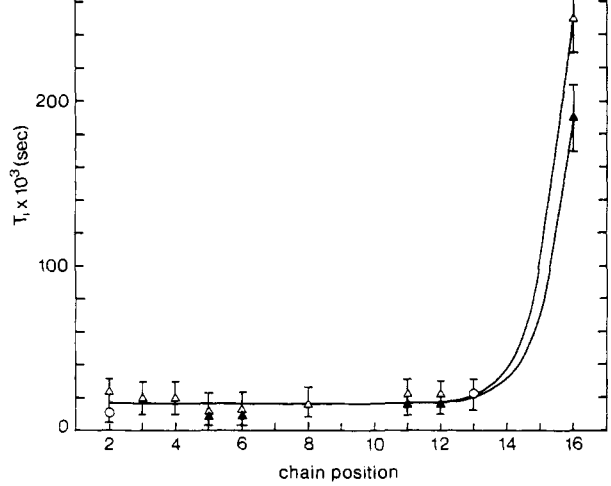

FIGURE 4: Longitudinal relaxation times (T_1) of selectively deuterated cholesteryl esters in egg PC or egg PC–20 mol % cholesterol vesicles. (△) Cholesteryl palmitate in egg PC vesicles; (○) cholesteryl stearate in egg PC vesicles; (▲) cholesteryl palmitate in egg PC–20 mol % cholesterol vesicles; (●) cholesteryl stearate in egg PC–20 mol % cholesterol vesicles.

Landsberger, 1974). The general behavior is that T_1 remains short and essentially constant with a large increase in the last two to three carbons at the end of the chain, culminating in a much longer relaxation time for the terminal methyl group.

Due to the low solubility of ester in the cholesterol-containing vesicles and the large line widths, which make necessary a large number of scans in order to obtain a sufficient signal to noise ratio, we have measured the longitudinal relaxation times, T_1, only for cholesteryl palmitate containing more than two deuterons on the fatty acyl chain in mixed CP–cholesterol vesicles. These values are also included in Table II and Figure 4. Only at the terminal CD_3 group do we see a significant change in the longitudinal relaxation time (0.19 vs. 0.25 s) upon cholesterol incorporation. For CP-*5,5,6,6*-d_4 and CP-*11,11,12,12*-d_4, the measured T_1 values are somewhat shorter than for the same ester in vesicles containing no cholesterol, but we cannot attribute significance to these changes due to the considerable experimental uncertainties. For the case of selectively deuterated CP in egg PC vesicles, $1/T_2$ is only 2–10 times greater than $1/T_1$, and, hence, the contribution of $1/T_1$ to the deuterium NMR line width is significant and must be included in any calculations.

Discussion

The sonicated aqueous dispersions of egg PC–cholesteryl ester yield stable, unilamellar vesicles with a narrow size distribution about an average diameter of 22 nm. The presence of 20 mol % cholesterol in egg PC–cholesteryl ester vesicles does not change the vesicle average diameter. This result agrees with the work of Newman & Huang (1975), who saw an increase in the size of egg PC vesicles of less than 10% when adding up to 25 mol % cholesterol.

Our previous work on multilayers (Grover & Cushley, 1979a) and liposomes (Valic et al., 1979) showed that saturated cholesteryl esters exist in two forms in these systems. By use of cholesteryl palmitate perdeuterated along the acyl chain (CP-d_{31}), it was found that only 0.2 mol % ester was homogeneously dissolved in the bilayer formed in egg PC liposomes, i.e., intercalated between PC molecules, while amounts > 0.2 mol % CP were proposed to exist in microcrystalline patches (Valic et al., 1979). In the vesicle system utilized for the present study, we find a much higher solubility of up to ~5 mol % CP. All of the ester incorporated into the vesicles is homogeneously dissolved, and we find no evidence for the existence of microcrystalline patches such as the ones postulated for CP in multilamellar liposomes.

The above conclusion can be drawn from the fact that more than 95% of the expected signal intensity of CP-d_{31} lies within ±300 Hz of the center frequency. If crystalline patches were present, the methylene segments of the CP molecules in these patches (S_{CD} = 1) would give rise to a deuterium line width of ~27 kHz, while the methyl segment (S_{CD} = 0.33) would give a line width of ~2 kHz (for both numbers, a correlation time for vesicle tumbling of 6.8×10^{-7} s was assumed). Since no broad lines are found in any of our ^{2}H NMR spectra of selectively deuterated CP or CS in egg PC vesicles, and since the intensity of the narrow lines reported in part A of Table I quantitatively account for all of the deuterium present in each sample, we conclude that only the homogeneous form of ester is present in vesicles.

The above result is not totally surprising since it is known that the physical behavior of unilamellar vesicles is somewhat different from that of multilamellar liposomes. Thus, the phase transition in vesicles is largely broadened and has a decreased enthalpy change as compared to that of liposomes (Van Dijck et al., 1978). It has also been proposed that the lipid packing in a vesicle is asymmetric between the two halves of the bilayer, with the average areas for the phospholipid head group and the fatty acyl chains in a vesicle being quite different for the inner and outer monolayers and differing from the values encountered in liposomes (Huang & Mason, 1978). It is therefore entirely possible that properties such as the solubility and the packing of cholesteryl esters in unilamellar vesicles differ quite substantially from those in liposomes. Evidence for a different packing of ester in vesicles as compared to liposomes will be presented below.

When 20 mol % cholesterol is present in the egg PC bilayer, the maximum solubility of cholesteryl ester is decreased from ~5 to ~2–3 mol %. This value is still considerably larger than the solubility of CP-d_{31} in liposomes of only ~0.2 mol % (Valic et al., 1979). Cholesterol is known to increase the orientational order of the phospholipid hydrocarbon chains *above* their phase transition and to fluidize these chains *below* their phase transition (Ladbrooke et al., 1968; Stockton & Smith, 1976). Since CP and the related ester, cholesteryl myristate, are known to disrupt the phospholipid bilayer

(Forrest & Cushley, 1977; Janiak et al., 1979), the tight packing of the hydrocarbon chains of the phospholipid induced by cholesterol may not allow for an effective incorporation of the ester and, hence, will result in a decreased solubility.

Deuterium Magnetic Resonance of Ester in Vesicles. For esters selectively deuterated along the acyl chain, the line widths and resulting transverse relaxation times, T_2^*, are given in part A of Table I for the various chain positions. Like previous ^2H NMR work on the acyl chain of deuterated stearic or palmitic acid intercalated into phosphatidylcholine vesicles, a decrease in deuterium line width (increase in transverse relaxation time) is found on going from the carboxy end to the terminal methyl end of the chain. However, in contrast to the results with the fatty acids, where the line widths decrease 155-fold from 1240 Hz for stearic-*3,3-*d_2 acid to 8 Hz for stearic-*18,18,18-*d_3 acid or 10.8 Hz for palmitic-*16,16,16-*d_3 acid (Stockton et al., 1976), the cholesteryl palmitate shows only a 16-fold decrease in line width from 160 ± 15 Hz for cholesteryl palmitate-*2,2-*d_2 to 10 ± 2 Hz for cholesteryl palmitate-*16,16,16-*d_3. Since CP–egg PC vesicles are identical in size with egg PC vesicles (see Results) and since we believe that the coefficient of lateral diffusion of the CP molecules is essentially the same as that for the egg PC molecules (H. Gorrissen, S. R. Wassall, and R. J. Cushley, unpublished experiments), the much smaller line widths for selectively deuterated CP or CS in these systems compared to those of the free fatty acids cannot be attributed to a changed correlation time for vesicle reorientation.

We can also discard the possibility of an enhanced rate of gauche–trans chain isomerizations for the ester as a reason for the observed narrow lines of deuterated CP and CS. These motions are known to be very fast (correlation time $\tau_c \approx 10^{-10}$–10^{-11} s) (Brown et al., 1979) and therefore affect directly the longitudinal relaxation times, T_1, for the affected chain segments (Abragam, 1961). A higher rate for these gauche–trans chain isomerizations in the fatty acyl moiety of cholesteryl esters would result in a noticeable increase of the T_1 values. This behavior is clearly not observed, since our results for T_1 of deuterated CP and CS agree well with the published data for deuterated fatty acids (Stockton et al., 1976).

In order to be able to calculate the segmental order parameters S_{CD} for the cholesteryl esters, a knowledge of the value for the effective vesicle correlation time τ_e is necessary. By use of our experimentally determined vesicle radius r_v of 11.0 nm, the viscosity η of pure water, a coefficient for lateral diffusion D of 7×10^{-8} cm^2 s^{-1} (MacKay et al., 1978), and an average distance from the center of the vesicle to the ester molecules R = 90 Å (calculated by subtracting half the estimated bilayer thickness of 40 Å from the vesicle radius), we obtain from eq 4–6 $\tau_e = 6.8 \times 10^{-7}$ s at 30 °C. This result is in good agreement with the value of $\tau_e = 5.8 \times 10^{-7}$ s obtained by Stockton et al. (1976) from a comparison of the deuterium line widths of deuterated fatty acids in vesicles with the corresponding quadrupolar splittings in liposomes. Since the segmental order parameter S_{CD} depends on the square root of the vesicle correlation time (see eq 3), the exact value of τ_e is not critical for the calculation of the S_{CD} values.

By use of $\tau_e = 6.8 \times 10^{-7}$ s, order parameters may then be obtained for the various chain positions of CP and CS in egg PC vesicles by using eq 3. Values of S_{CD} for one ester, CP, are listed in Table III. All positions of the palmitoyl chain, with the exception of position 16, show low order parameters which are much smaller than that of the corresponding chain position of free palmitic acid in egg PC bilayer vesicles or liposomes (Stockton et al., 1976) or the equivalent position on the palmitoyl chains of selectively deuterated dipalmitoylphosphatidylcholine in liposomes (Seelig & Seelig, 1974). It has to be pointed out that a priori we cannot expect a fatty acid and a cholesteryl ester to behave similarly in a phospholipid bilayer. Presumably, the hydrophilic carboxyl group of such an acid is more rigidly bound to the water layer than the ester group, in which the fatty acyl moiety is bound to the bulky alcohol, cholesterol. This could give rise to a much different motional behavior of the chain.

Table III: Absolute Values of the Segmental Order Parameters for Deuterated Cholesteryl Palmitate in Egg PC Vesicles[a]

ester chain position	$\lvert S_{CD}\rvert$ 0 mol % cholesterol	$\lvert S_{CD}\rvert$ 20 mol % cholesterol
2	0.07	0.07
3	0.06	0.08
4	0.05	0.07
5 (6)	0.04	0.06
11 (12)	0.04	0.10
16	0.02	0.03

[a] Calculated by using a value for the effective vesicle rotational correlation time of $\tau_e = 6.8 \times 10^{-7}$ s.

We may compare the order parameters listed in Table III with the values obtained for CP-d_{31} and CP-*16,16,16-*d_3 in egg PC multilamellar liposomes (Valic et al., 1979). The value S_{CD} = 0.02 for the terminal methyl segment of CP-*16,16,16-*d_3 is identical in vesicles and liposomes. The average of the absolute values of the order parameters for the CD_2 segments in vesicles, as calculated from eq 3, is $\overline{|S_{CD}|}$ = 0.04, while in liposomes a value of 0.1 is encountered. We interpret these data, together with the enhanced solubility of cholesteryl ester in vesicles vs. liposomes, as evidence for a different packing of the ester in both systems.

The order parameters presented in Table III were calculated by means of the formula derived by Stockton et al. (1976). A more generalized treatment of the line-width equation adds a slower reorientation, due to movement of the lipid chain as a whole, to the gauche–trans isomerizations discussed above and is expressed in eq 9 (Petersen & Chan, 1977). The order parameter is, thus, represented as the product of two order parameters for the kink and rigid stick motions

$$S_{CD} = S_\gamma^{CD} S_\alpha \qquad (10)$$

where S_{CD}, S_γ^{CD}, and S_α are as defined above (cf. eq 9).

The similarity between eq 9 and eq 3 is readily apparent, the only difference being that, in eq 9, an additional term is present on the right-hand side. It is important to note that, since neither of the terms on the right-hand side of eq 9 can be negative, the value of the order parameter S_{CD} calculated by using the Petersen–Chan equation will always be equal to or less than the value obtained by using eq 3. The choice of a particular motional model for the system does not alter the fundamental conclusion that can be obtained from our ^2H NMR spectra, namely, that the order parameters for the methylene segments of deuterated CP and CS in egg PC vesicles are *much reduced* when compared to those of the corresponding fatty acids and to CP-d_{31} in egg PC multilamellar liposomes. The only difference between the approaches of Petersen and Chan and Stockton et al. is, then, the way by which this reduced order can be accomplished. On the basis of the model of Stockton et al., we can explain a much reduced order parameter either by an increased kink probability of the chain segments (this possibility was already ruled out on the basis of the observed longitudinal relaxation times, see above)

or by invoking a permanently tilted orientation of the acyl chain segments of the cholesteryl ester with respect to the bilayer normal. Such a permanent tilt would reduce the S_{CD} values by a factor $(3 \cos^2 \theta - 1)/2$, where θ is the angle of inclination of the chain axis with respect to the bilayer normal. According to Petersen and Chan, on the other hand, in addition to the above mechanisms, a reduced order parameter can also be achieved by the rigid rod motions of the acyl chains, which would reduce the S_{CD} values by a factor S_α (eq 10), depending on the type and degree of the rigid rod motion. On the basis of our NMR results we cannot differentiate between these possibilities.

When 20 mol % cholesterol is added to the egg PC–cholesteryl ester vesicles, a general increase in ^{2}H NMR line width is apparent. Since we have proved that the egg PC–CP vesicles are identical in size, whether they contain 20 mol % cholesterol or not, this change cannot be attributed to an increased correlation time for vesicle tumbling. The results are consistent with the expected condensing effect of cholesterol (Ladbrooke et al., 1968; Stockton & Smith, 1976) and prove that the acyl chains of the ester molecules are affected by the incorporation of cholesterol and exhibit a general increase in order. The ^{2}H NMR line widths at the 3 and the 11,12 positions of the acyl chain of CP show very large increases. We attribute these changes to selective interactions of the ester with cholesterol. Additional work will be required to clarify the origin of the selective line broadening at the 3 and the 11,12 positions of CP.

Conclusion

The present work utilized ^{2}H NMR spectroscopy of selectively deuterated cholesteryl stearate and palmitate in egg phosphatidylcholine unilamellar vesicles. The formation of vesicles increased the solubility of esters in the bilayer to the point that deuterium signals for a single CD_2 group in an ester are obtained, thus allowing the determination of order parameters for positions along the entire acyl chain. Diffusion experiments indicated that incorporation of up to 5 mol % CP does not increase the mean hydrodynamic radius of egg PC vesicles (r_v = 11 nm).

Deuterium NMR spectra yield narrow resonance lines of 10–160 Hz, measured at half-height, for selectively deuterated CP and CS in egg PC vesicles. Longitudinal relaxation times were found to be short ($T_1 < 0.02$ s for CD_2 and $T_1 = 0.25$ s for CD_3). An effective order parameter, S_{CD}, was calculated for each chain position of the ester in the bilayer (Stockton et al., 1976). These order parameters are very low, ranging from 0.07 for CD_2 at the 2 position to 0.02 for CD_3, with a plateau region between positions 3 and 13 with $S_{CD} \approx 0.04$.

The effect of adding cholesterol to the egg PC–CP vesicle system decreases the ester solubility in the bilayer approximately twofold. As expected, the order parameters increase upon addition of cholesterol with a very large (250%) increase in S_{CD} for CP-*11,11,12,12-d*$_4$ which we interpret as a selective interaction of that portion of the ester chain with cholesterol.

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