# Deuterium Magnetic Resonance Study of Cholesteryl Esters in Membranes†

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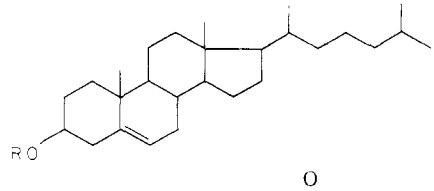
ABSTRACT: The ternary systems EYL:$H_2O$ (50:50 wt %) containing 1 and 5 mol % cholesteryl palmitate-$d_{31}$ or 1 and 5 mol % cholesteryl palmitate-*16,16,16-d*$_3$ have been studied. Cholesteryl palmitate-$d_{31}$ gave a unique deuterium magnetic resonance spectrum corresponding to a homogeneous ($\Delta\nu_Q$ = 3 and 12 kHz) and a solid phase ($\Delta\nu_Q$ = 38 and 118 kHz). From the characteristic spectra and spin–lattice relaxation times, a procedure for calculating the amount of each phase present in the ternary mixture is given resulting in a maximum value of homogeneously dissolved cholesteryl palmitate of 0.2 ± 0.1 mol % and a solid fraction above 0.2 mol %. The most probable order parameter for the $(-CD_2)_n$ portion of the homogeneous fraction of cholesteryl palmitate-$d_{31}$ was determined from the quadrupolar splittings to be $S$ = 0.1 which is less than one-half that of the order parameter found for the lecithin chains. Possible explanations for the diminution of the order parameter for cholesteryl ester in bilayers are discussed.

Recently, it has been demonstrated that low concentrations of the saturated cholesteryl ester CP[1] alter the permeability of membranes (Forrest & Cushley, 1977). Equilibrium dialysis of unilamellar EYL liposomes (vesicles) containing spin-labeled cholesteryl 16-doxylstearate and gel filtration using cholesteryl [1-$^{14}$C]palmitate (Forrest, 1978) indicated that up to 5 mol % CP can be incorporated into EYL bilayers. Janiak et al. (1974), using polarizing light microscopy and X-ray diffraction methods, found that small amounts of the unsaturated ester cholesteryl linolenate are incorporated into the lamellar structure formed by hydrated EYL. The incorporation of the linolenate ester CL was shown to vary from 2 to 4.5 wt % (2.4–5.4 mol %) depending upon the degree of hydration. Larger concentrations of CL resulted in the formation of an excess phase of solid CL readily observed by polarizing light microscopy and confirmed by X-ray diffraction.

Molecules of CP (or CL) are quite different from EYL molecules in that they do not have hydrophilic polar head groups. Therefore, it is likely that the major portion of the CP molecule is to be found in the hydrophobic, acyl chain region of the bilayer. However, since the length of the CP molecule (~40 Å when fully extended) is much greater than half the thickness of the EYL bilayer, it is likely that some unusual conformations of the CP molecule are favored. Nine possible conformations for CP intercalated into the bilayer are illustrated in Figure 1. Conformations 1, 4, and 5 may be ruled out a priori since the apparent surface area for the related ester, CL, is only 70 Å$^2$ (Janiak et al., 1974), much too small to accommodate large portions of CP or CL at the aqueous interface. Conformers 1, 4, and 5 would also place the hydrophobic cholesteryl and/or acyl moieties in an aqueous environment and are, thus, unfavorable on energetic grounds.

Grover et al. (1979) have obtained ESR data based on the alignment and angular dependence of spectra of cholesteryl 5-doxylpalmitate and cholesteryl 16-doxylstearate in EYL multilayers. The 5-doxyl ester gave spectra characteristic of slow tumbling (i.e., correlation times $\gtrsim 10^{-8}$ s (Freed, 1976)) and showed that the 2p$\pi$ orbital of the nitroxide was aligned at an angle of tilt of 47° with respect to the normal to the surface of the membrane. The 16-doxyl ester possessed essentially the same ESR characteristics as 16-doxylstearic acid incorporated into the multilayers. Additionally, the rate of L-ascorbate-induced decay of the 5-doxylpalmitate and 16-doxylstearate esters, upon addition of the reducing solution to the multilayers, indicated that both nitroxide groups were situated well below the membrane surface. The spin-labeling results must be considered merely semiquantitative since the effect of the perturbing nitroxide moiety must be taken into account; however, the results favor the horseshoe conformation, number 9, Figure 1.

Deuterium magnetic resonance ($^2$H NMR) has proven to be an excellent *nonperturbing*, sensitive microprobe of the local orientational order and mobility of lipid bilayers (Seelig, 1977; Mantsch et al., 1977). The present study was undertaken in order to obtain more detailed information on the conformational states associated with cholesteryl palmitate dissolved in the EYL bilayer. For this purpose we have prepared the two deuterated cholesteryl esters shown (see structures I and II).




I, R = $CD_3(CD_2)_{14}C(=O)-$

II, R = $CD_3(CH_2)_{14}C(=O)-$

The use of $^2$H NMR to obtain meaningful results for biological systems, in which signals are relatively weak and complex, has recently been enhanced by the use of the quadrupolar echo technique (Davis et al., 1976) which is capable of providing essentially distortion-free $^2$H NMR spectra with high sensitivity. The present study, which involves amounts of CP as low as 4 mg and nearly maximum possible

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[1] Abbreviations used: CP, cholesteryl palmitate; CL, cholesteryl linolenate; EYL, egg yolk lecithin; cholesteryl 16-doxylstearate, 3$\beta$-[2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxyl]cholest-5-ene; cholesteryl 5-doxylpalmitate, 3$\beta$-[2-(3-carboxypropyl)-2-undecyl-4,4-dimethyl-3-oxazolidinyloxyl]cholest-5-ene; ESR, electron spin resonance; $^2$H NMR, deuterium magnetic resonance; rf, radiofrequency; FT, Fourier transform.

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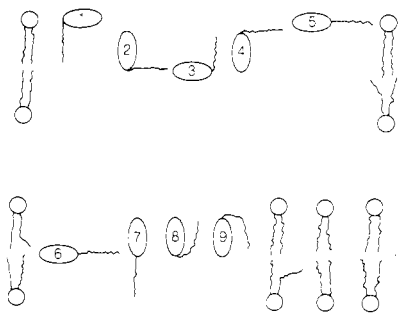


FIGURE 1: Nine possible conformations of CP in a phospholipid bilayer. The ovals around the numbers represent the cholesteryl moiety of the CP molecules; the wiggly lines represent the fatty acyl chains. The unnumbered structures on the right and on the left indicate some of the phospholipid molecules of the bilayer.

quadrupolar splittings for some of the $^2$H NMR spectra, would not have been possible otherwise.

For a superposition of randomly oriented domains of bilayers, a characteristic symmetric "powder spectrum" is obtained for a specific deuteron site (Davis et al., 1976; Seelig, 1977; Mantsch et al., 1977), which is characterized by two peaks separated by a frequency

$$\Delta\nu_Q = \frac{3}{4}\frac{e^2qQ}{h}|S| \qquad (1)$$

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

$$S = \left\langle \frac{3\cos^2\gamma - 1}{2} \right\rangle \qquad (2)$$

is the orientational order parameter for the C–D bond, $\gamma$ is the angle between the C–D bond and the axis of symmetry for reorientational motions of the C–D bond, and the brackets $\langle\rangle$ represent an average over all conformations of the molecule.

In the present study of cholesteryl palmitate in EYL bilayers, we will compare the distribution of quadrupolar splittings and order parameters obtained from the deuterium nuclei on the palmitoyl chains of CP-$d_{31}$ (I) and CP-*16,16,16*-$d_3$ (II) with the corresponding quantities observed in identical acyl chains of phospholipid molecules in model and biological membranes (Seelig, 1977; Stockton et al., 1977).

In the course of our study on the order parameters of CP dissolved in EYL, we discovered that the $^2$H NMR technique provided a convenient method of measuring the relative amounts of CP associated in a homogeneous phase with EYL and CP in the form of solid particles. We found that the critical concentration of CP above which it condenses into a separate solid CP phase is 10–20 times lower then the critical concentration indicated for CL by polarizing light microscopy measurements (Janiak et al., 1974). The arguments leading to this conclusion are presented in detail in the Results and Discussion section.

## Experimental Section

*Materials.* Cholesterol and palmitic acid were obtained from Fisher Scientific Co. Deuterium depleted water was purchased from Aldrich Chemical Co. and had a deuterium content of less than 1% of the natural abundance. EYL was extracted from fresh egg yolks using the method of Singleton et al. (1965). Palmitic-$d_{31}$ acid was prepared using a published procedure (Nguyen & Stenhagen, 1967), whereas palmitic-*16,16,16*-$d_3$ acid was purchased from Serdary Research Laboratories, London, Ontario, Canada. The cholesteryl esters

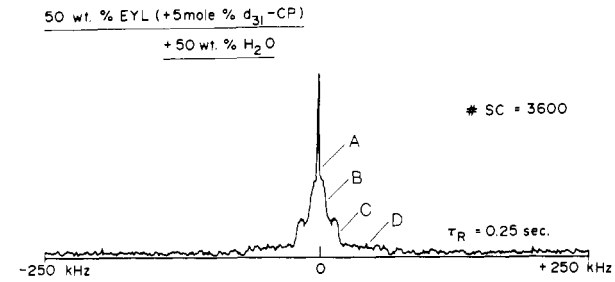


FIGURE 2: $^2$H NMR spectrum of 50 wt % EYL (plus 5 mol % cholesteryl palmitate-$d_{31}$) + 50 wt % $H_2O$. $\tau_R$ = repetition time between quadrupolar echo sequences; #SC = number of accumulations; spectral width = 500 kHz; rf pulse width = 4 $\mu$s ($\pi/2$ pulse); no. of data points = 4096; line broadening (LB) = 400 Hz. The letters A to D refer to the main features of the spectrum (see text).

I and II were synthesized by reaction of the appropriate deuterated fatty acid, activated by carbonyldiimidazole, with cholesterol (Grover & Cushley, 1979).

*Preparation of Samples for NMR.* EYL was dissolved at known concentration in chloroform, stored under nitrogen at −20 °C, and dispensed volumetrically. Cholesteryl esters were weighed directly into the sample tubes, the EYL solution was added, and the solvent was removed by evaporation under a stream of nitrogen. Final traces of chloroform were removed by pumping under high vacuum for at least 2 h. Multilamellar liposomes were prepared by adding an equal weight of water (or deuterium depleted water) to the dry lipid mixture and vigorously shaking with a vortex mixer.

*Nuclear Magnetic Resonance Methods.* $^2$H NMR experiments were performed in a Nalorac superconducting magnet; rf measurements were carried out at 34.4 MHz using a Bruker SXP 4-100 console and a Spin-Tech Electronics probe head interfaced to an Intel 8080 microprocessor based computer by means of a Nicolet 1090AR Explorer digital oscilloscope, FT spectra were obtained using a Nicolet B-NC 12 computer.

In the quadrupolar echo method, two rf pulses separated by a delay time $\tau$ are applied to the deuterium spin system. The free induction decay signal immediately following the first $\pi/2$ pulse is severely distorted because of the large range of deuterium quadrupolar splittings present in the systems studied here and because the high sensitivity receiver takes at least 10 $\mu$s to recover from the effect of the strong rf pulse. As a result of the second pulse, however, the transverse nuclear magnetization responsible for the free induction decay signal is refocused at a time $2\tau$. For a $(\pi/2|_{0°}-\tau-\pi/2|_{90°})$ pulse pair sequence (Davis et al., 1976), the refocussing is complete and the proper $^2$H NMR spectrum is obtained as the Fourier transform of the echo signal starting at time $2\tau$. In a typical experiment we use values of $\tau$ between 65 and 100 $\mu$s depending on the signal-to-noise ratio of the sample. The two-pulse sequence for the quadrupolar echo is repeated at time intervals $\tau_R$. Alternate pulse sequences have their first pulse shifted in phase by 180° and the signals arising from them are added and subtracted to eliminate systematic rf interference effects.

In the spin–lattice relaxation time ($T_1^{(i)}$) measurements the 180° phase shift in alternate pulse sequences was replaced by a $\pi$ pulse which preceded the alternate two-pulse sequences by a time $\tau_1$. The difference in the echo amplitudes arising from alternate pulse sequences, $D(\tau_1)$, was studied as a function of $\tau_1$ and fitted to the relaxation equation

$$D(\tau_1) = D(0)\exp[-\tau_1/T_1] \qquad (3)$$

Care was taken to ensure that $\tau_R \geq 5T_1$ in all such experiments.

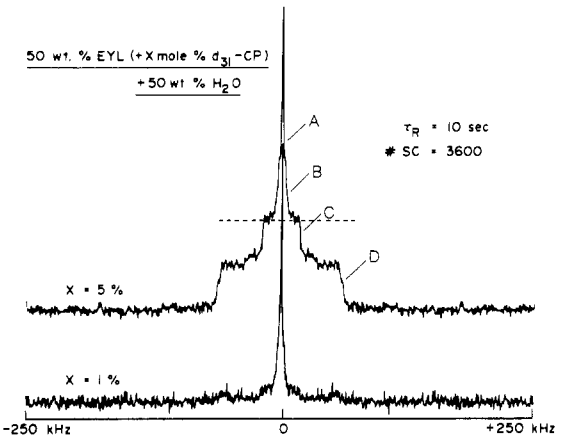


FIGURE 3: $^2$H NMR spectrum of multilamellar liposomes composed of 50 wt % EYL (plus $X$ mol % cholesteryl palmitate-$d_{31}$) + 50 wt % $H_2O$. (Top spectrum) $X$ = 5 mol % CP-$d_{31}$; (bottom spectrum) $X$ = 1 mol % CP-$d_{31}$. Parameters as described in Figure 2. The dashed line indicates the base line chosen for the separation of the A + B and C + D signals (see Appendix B).

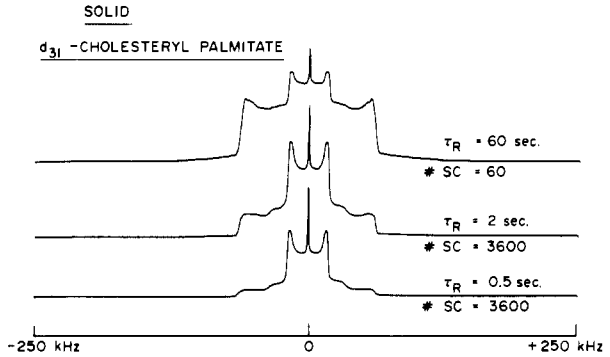


FIGURE 4: $^2$H NMR spectrum of dry, polycrystalline cholesteryl palmitate-$d_{31}$ for various values of the repetition time, $\tau_R$. Parameters as in Figure 2, except LB = 75 Hz.

Proton noise-decoupled $^{31}$P FT spectra were obtained at 40.5 MHz using a Varian XL-100-15 nuclear magnetic resonance spectrometer fitted with a NIC 1080 24K computer and 600K disk. The field-frequency lock was an external $^{19}$F signal.

## Results and Discussion

The spectrum of 5 mol % cholesteryl palmitate-$d_{31}$ (I) in a 50 wt % EYL–50 wt % $H_2O$ dispersion is shown in Figure 2. The spectrum has four features spread over a range of approximately ±60 kHz. These features and their spectral range are labeled in Figure 2 as: (A) 3 kHz; (B) 12 kHz; (C) 38 kHz; (D) 118 kHz. This unusual spectrum can be explained as a superposition of two complex spectra, a narrow one consisting of A + B and a broad one consisting of C + D. The latter spectrum is grossly distorted due to two types of effects as discussed in Appendix A.

The spectrum of Figure 2 was taken with a repetition time for the quadrupolar echo pulse sequence of $\tau_R$ = 0.25 s. This value of $\tau_R$ would not lead to appreciable saturation for the values of $T_1 \lesssim 0.1$ s typically observed for methylene deuterons in lipid bilayer systems (Davis et al., 1978; Brown & Seelig, private communication). However, when the spectrum was taken with a longer repetition time of $\tau_R$ = 10 s, as shown in Figure 3 (top spectrum), the broadest spectral feature D grew relative to all the other features of the spectrum. The broad features C + D are unquestionably due to solid CP. This is demonstrated in Figure 4 which depicts the $^2$H NMR spectrum of dry, polycrystalline CP-$d_{31}$, I, for several values of $\tau_R$. The broad feature has the same form as D of Figures 2 and 3 (top spectrum), while the feature with a 38-kHz splitting in Figure 4 is identified with C in Figure 2. We propose that feature D is the $^2$H NMR of the $CD_2$ groups of the palmitic acid part of CP-$d_{31}$, while C is associated with the $CD_3$ group of CP-$d_{31}$. For this interpretation to be valid, the ratio of the equilibrium signals for the two types of deuterons should be $E_0^{(D)}/E_0^{(C)} = 28/3$. The ratio of the areas under features C and D in Figure 3 (top spectrum) in the limit that $\tau_R \rightarrow \infty$ was found to be approximately 7. The difference between this ratio and the expected value of 9.33 is caused by distortion due to the finite pulse length of 4 μs used to record the spectra (see Appendix A). When a shorter pulse length of 1 μs was used to obtain the spectrum of Figure 5, the ratio was found to be $E_0^{(D)}/E_0^{(C)} = 9$, in agreement with the predictions given by the above interpretation within experimental error. In addition, the behavior of C and D with repetition time is found to be due to the different values of $T_1$ for the methyl and methylene deuterons, respectively, as illustrated in Figure 4.

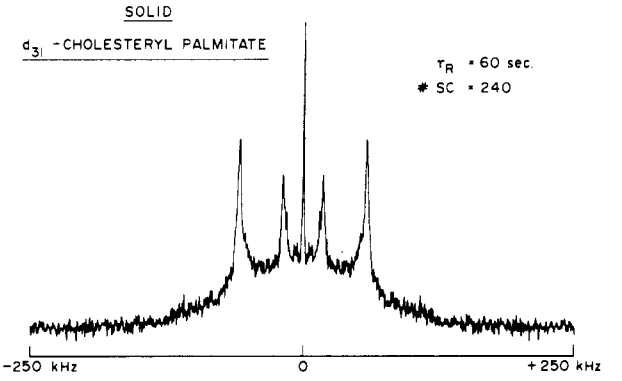


FIGURE 5: $^2$H NMR spectrum of sample used for Figure 4 except rf pulse width = 1 μs (flip angle = 22.5°); $\tau_R$ = 60 s; LB = 1 Hz.

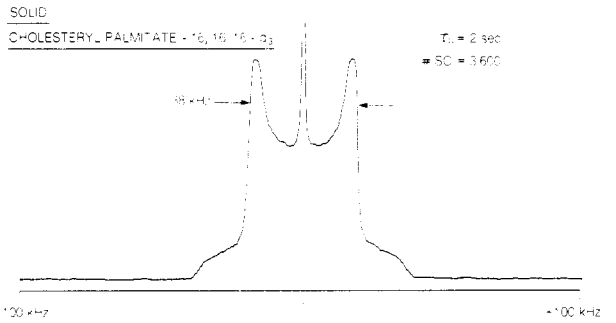


FIGURE 6: $^2$H NMR spectrum of dry, polycrystalline cholesteryl palmitate-*16,16,16-*$d_3$. Parameters as in Figure 2 except spectral width = 200 kHz and LB = 200 Hz. Only the initial portion of the central "isotropic" signal is presented.

Confirmation of the assignment of the C signal comes from the spectrum of polycrystalline cholesteryl palmitate-*16,16,16-*$d_3$, II. The spectrum, Figure 6, taken at a repetition time $\tau_R$ = 2 s shows only the splitting of 38 kHz, i.e., feature C. From the dependence of the signal intensity of C with $\tau_R$, the $-CD_3$ relaxation time $T_1^{(C)}$ was found to be 0.24 ± 0.02 s. The $(-CD_2)_n$ relaxation time $T_1^{(D)}$ was measured directly using the pulse sequence described in the Experimental Section and found to be 11 ± 1 s.

The splitting of 38 kHz for C is consistent with the assumption that the most important molecular motion here is the rotation of the $CD_3$ group about its symmetry axis. For this motion alone, identifying $\gamma$ in eq 2 with the angle between the C–D vectors and the $CD_3$ symmetry axis, $\cos\gamma \simeq 1/3$, leading to $S = -1/3$ and the prediction that $\Delta\nu$ = 42 kHz. The reduction from 42 kHz to the observed value of 38 kHz is due to small internal librational motions of the $CD_3$ group. Of

course $\gamma \simeq 0$ for the nonrotating $CD_2$ groups, which gives $S \simeq 1$ in agreement with the splitting of the D feature when small internal librational motions are taken into account.

The appearance of a large $^2$H NMR signal identified with solid CP implies that most of the 5 mol % CP associated with the hydrated EYL bilayer exists, not as a homogeneous mixture, but in the form of an associated solid. This may also be seen from the spectrum of multilamellar liposomes of 50 wt % EYL (containing 1 mol % cholesteryl palmitate-$d_{31}$) + 50 wt % $H_2O$, with $\tau_R$ = 10 s, shown in Figure 3 (bottom spectrum). The spectrum was recorded under identical conditions to the 5 mol % CP sample (top spectrum Figure 3), i.e., identical values of $t_w$, $\tau_R$, receiver gain, no. of scans, sample mass, etc., so that one may compare the intensities of the broad components (the area under the C + D features) with the intensities of the narrow components (the area under A + B). We associate B with the $-(CD_2)_n$ groups of homogeneous CP-$d_{31}$ in EYL bilayers and the narrow central component, A, partly with homogeneous CP-$d_{31}$ in EYL—possibly the $CD_3$ group since the splitting is only 3 kHz—partly with the natural abundance of deuterium in water, and partly with an "isotropic" phase of CP-$d_{31}$. The isotropic CP-$d_{31}$, the very sharp signal, accounts for 1–2% of the total ester present and could arise from boundary ester, from CP-$d_{31}$ dissolved in small amounts of residual $CHCl_3$, etc.

From the analysis of the spectra in Figure 3 it is seen that the intensity of the $^2$H NMR signal for the homogeneous phase remained essentially constant, while the solid signal was reduced ca. fivefold on going from 5 mol % to 1 mol % incorporation. By analyzing the ratio $R$ of the intensities of the $^2$H NMR resonances for polycrystalline and homogeneous phase using arguments presented in Appendix B, it is possible to calculate the concentration of CP in the homogeneous phase. Our analysis of the spectra of Figure 3 gives the results $R$ = 14 for $f$ = 5 mol % CP-$d_{31}$ and $R$ = 2.7 for $f$ = 1 mol % CP-$d_{31}$, which yields values of $f_{diss}$ = 0.17 and 0.16 mol %, respectively, using eq B.1 and B.5. The accuracy of our measurements of $R$ is limited mainly by the uncertainty in drawing the true base line for the A + B features of the spectrum in Figure 3. We feel that a conservative estimate in the uncertainty in $f_{diss}$ is ±50%. Thus, a truly homogeneous phase of cholesteryl palmitate exists in fully hydrated EYL bilayers only up to a concentration of 0.2 ± 0.1 mol % ester and this is about 10–20 times lower than proposed for cholesteryl linolenate in EYL bilayers (Janiak et al., 1974). Above approximately 0.2 mol %, domains of crystalline or solid CP exist which we believe still remain dispersed in the EYL.

All of our studies of the intensity of the spectrum of CP homogeneously dissolved in EYL (mainly the B feature) indicate that it is independent of $\tau_R$ for $\tau_R > 0.1$ s so that $T_1^{(B)} \leq 0.1$ s, which is consistent with values of $T_1$ normally found for deuterium spins in the $CD_2$ groups of acyl chains in bilayers (J. H. Davis, unpublished results; Davis et al., 1978; Brown & Seelig, private communication). Thus, it seems that $^2$H NMR is a most sensitive technique for determining the distinct phases of the cholesteryl ester associated with EYL. Although CP and CL are structurally different, it is difficult to reconcile the 10–20-fold difference in the solubility of homogeneous phase of each in EYL on such a slight difference. The previous study by Janiak et al. (1974) using polarizing light microscopy would not be able to distinguish two types of associated CL, for instance, if the solid component were in the form of microcrystals whose size was on the order of, or less than, the wavelength of light used.

The spectrum of a sample of 1 mol % cholesteryl palmitate-$d_{31}$ in liposomes containing 50 wt % EYL–50 wt % $H_2O$ (deuterium depleted in order to eliminate contribution of HDO to the central peak) using a rapid repetition time ($\tau_R$ = 0.1 s) so as to suppress the solid CP $^2$H NMR signal is recorded in Figure 7. The most striking feature of this spectrum of cholesteryl palmitate-$d_{31}$ in EYL is that, except for the central portion where the $CD_3$ peaks are not resolved, it is very similar in form to that obtained for perdeuterated acyl chains in phospholipid model membranes such as DPL (Davis et al., 1976; Davis, private communication). It should be noted that, although the pioneering work in establishing the characteristic "plateau" signature of $S$ vs. hydrocarbon chain position for phospholipid bilayer systems was established using specifically labeled acyl chains, the plateau, a consequence of the large constant quadrupolar splittings for C(2)–C(10), is manifested in a most striking way in the spectra of perdeuterated chains (Davis et al., 1976; Davis & Jeffrey, 1977; Stockton et al., 1977) in that the $^2$H NMR spectra are characterized by a sharp outer edge. The separation of the sharp edges of the homogeneous CP signal (feature B) is 12 kHz and, consequently, the most probable order parameter ($S$ = 0.1) is less than that found for palmitic-$d_{31}$ acid in EYL by a factor of greater than two (Stockton et al., 1976).

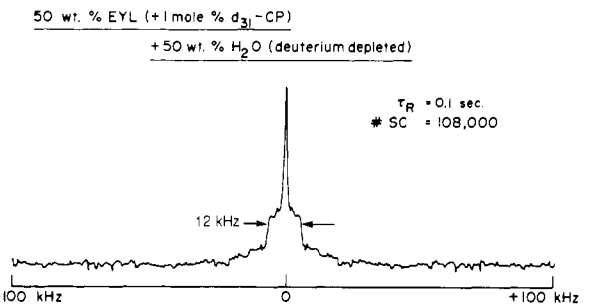


FIGURE 7: $^2$H NMR spectrum of liposomes composed of 50 wt % EYL (plus 1 mol % cholesteryl palmitate-$d_{31}$) + 50 wt % deuterium depleted $H_2O$; rapid repetition rate, i.e., $\tau_R$ = 0.1 s; spectral width = 200 kHz; LB = 100 Hz.

One possible cause of the approximate twofold reduction in the quadrupolar splitting would be the existence of a hexagonal phase for the membrane (Seelig, 1977). In contrast to bilayers, the hexagonal phase consists of long cylindrical rods of phospholipid molecules and the approximate twofold reduction in splitting occurs because of rapid rotation about the cylinder long axis. The hexagonal phase has been shown to greatly enhance incorporation of CL in EYL (Loomis et al., 1974). A hexagonal phase yields a proton-noise-decoupled $^{31}$P NMR spectrum characterized by a chemical shift anisotropy with reversed asymmetry and one-half the magnitude of the lamellar bilayer phase spectrum (Cullis & De Kruyff, 1978). No sign of hexagonal phase was detected in the proton-noise-decoupled $^{31}$P NMR spectrum of a 50 wt % EYL (containing 5 mol % CP) + 50 wt % $H_2O$ dispersion. Therefore we must attribute the reduced quadrupolar splittings of CP-$d_{31}$ homogeneously distributed in EYL membranes to the combined effects of motional averaging associated with rapid local changes in isomerization of the palmitate chains (e.g., the type of motion often associated with "kinks" (Schindler & Seelig, 1975)) and the geometry of preferred conformations of CP of the type illustrated in Figure 1.

The spectrum of 5 mol % cholesteryl palmitate-*16,16,16-$d_3$*, II, incorporated into EYL liposomes measured using a repetition time of $\tau_R$ = 0.12 s is presented in Figure 8. Because of the short $T_1$ for the $-CD_3$ group (vide supra) the 38-kHz splitting is still present. A small splitting of 3 kHz ($S$ = 0.02) is found for the homogeneous portion of II. The order pa-

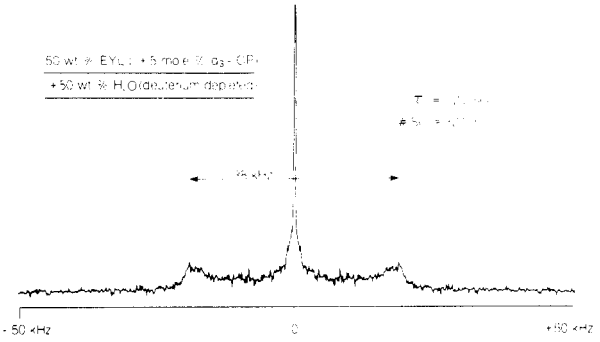

FIGURE 8: $^2H$ NMR spectrum of liposomes of 50 wt % EYL (plus 5 mol % cholesteryl palmitate-*16,16,16-d*$_3$) + 50 wt % deuterium depleted $H_2O$. Parameters as in Figure 2 except spectral width = 100 kHz and LB = 50 Hz. The number of data points was 512, zero-filled to 4096 before FT.

rameter of 0.02 is the same as that found by Stockton et al. (1976) for palmitic-*16,16,16-d*$_3$ acid in EYL bilayers.

In order to determine via $^2H$ NMR whether any one of the conformations in Figure 1 is favored, it will be necessary to perform a series of experiments with $^2H$ nuclei in specific positions on the palmitate chain. Some preliminary experiments have been performed already; however, for a complete study we require a further improvement in the signal/noise of our already sensitive spectrometer. This is best illustrated by the following example: for a sample of 500 mg of 50 wt % EYL (+5 mol % CP-$d_{31}$) + 50 wt % $H_2O$ there are only 11 mg of CP-$d_{31}$ of which only 0.4 mg is in the homogeneous phase. With 0.4 mg of material, we obtain, after 3 h of averaging, the spectrum shown in Figure 7 with a signal/noise of $\sim$10:1. In CP-$d_{31}$ there are 31 $^2H$ nuclei while in CP selectivity deuterated on one chain position there would be only two $^2H$ nuclei, reducing the signal by a factor of greater than 15. Thus, in order to obtain a spectrum of the quality of Figure 7, we estimate the time required to obtain the spectrum could be weeks ($\sim$30 000 000 accumulations).

It is not entirely clear whether the notion of favored conformations such as those of Figure 1 has any real meaning. A more basic theoretical approach would be to examine the statistical distribution of conformations for the palmitate chain taking into account (1) a preferred orientation for the C(1)–C(2) bond direction imposed by the bonding of the palmitate chain to the rigid steroid moiety and (2) the molecular interactions between the palmitate chain and the orientationally ordered hydrocarbon chains of the phospholipid molecules of the bilayer. Such an approach has provided insight into differences in the order parameters associated with C–C bonds near the heads of the two acyl chains in DPL (Schindler & Seelig, 1975) where the interactions with neighboring phospholipid molecules were taken into account using a mean field approximation.

In this picture, the horseshoe conformation, number 9 in Figure 1, would result from a C(1)–C(2) bond orientation in a direction tilted upward from the bilayer plane, while the tail would be oriented, in a statistical sense, toward the interior of the bilayer, the average local orientation along the chain defining an arc as indicated in Figure 1. It may be that the variation of order parameters along the chain would reflect in some obvious way the geometry of this arc; e.g., one would anticipate a minimum in the plot of order parameter vs. position part way along the arc. In fact, our finding that the most probable value of the quadrupolar splitting in CP-$d_{31}$ is about one-half that obtained for palmitic acid probes in EYL (Stockton et al., 1976) is consistent with crude geometrical calculations using a short helical segment near the head of the palmitate chain to simulate the arc. We postpone a more detailed discussion of the variation of the orientational order parameter with chain position until results from cholesteryl esters deuterated at specific chain positions become available.

## Conclusion

Although a number of studies have shown that cholesteryl esters are dissolved in, or associated with, EYL in amounts up to 5 mol % ester in the ternary system ester–EYL–$H_2O$ (Janiak et al., 1974; Forrest & Cushley, 1977; Forrest, 1978), we have shown by $^2H$ NMR spectroscopy that the "dissolved" cholesteryl ester, CP, in EYL bilayers consists of two forms—a homogeneous fraction of 0.2 ± 0.1 mol %, where the ester intercalates between EYL molecules, and a solid fraction of crystalline patches for concentrations higher than 0.2 mol %. Janiak et al. (1974) claimed that 3.6 mol % CL formed a homogeneous phase with EYL in 40 wt % EYL + 60 wt % $H_2O$. Since the concentration of the homogeneous phase of CP is an order of magnitude lower in 50 wt % EYL + 50 wt % $H_2O$, it is difficult to account for the vast solubility difference between CP and CL simply on the basis of the structural differences between the two esters. If the solid phase of cholesteryl ester were to consist of microcrystalline patches of dimension less than the wavelength used for polarizing light microscopy, they would not be visualized by that technique.

The most probable order parameter calculated for the deuterated ester CP-$d_{31}$, I, is $S$ = 0.1, while, for CP-*16,16,16-d*$_3$, II, $S$ = 0.02. The latter value is identical with that found for palmitic-*16,16,16-d*$_3$ acid in EYL bilayers. The fact that the most probable order parameter for I is more than two times smaller than the corresponding value for the acyl chains of palmitic-$d_{31}$ acid implies the conformations for the palmitoyl chains are quite different. A definitive answer to this question must await the study of CP selectively deuterated along the acyl chain.

The presence of crystalline patches inside the membrane may be of importance in our understanding of the structural changes associated with the initial stages of atherosclerosis since the fatty streaks or lipid crystals found in the intimal layer are composed of from 60 to 95% cholesteryl ester (Lang & Insull, 1970) and may have their origins as microcrystalline "seeds" in the intimal cell membranes.

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## Appendix A: Distortion Effects in $^2H$ NMR Spectra

In the $^2H$ NMR experiments reported in this paper, we are concerned with two types of distortions of the spectra. The first arises when the time between repetitions of successive pulse trains is comparable with or smaller than the nuclear spin–lattice relaxation time $T_1$, i.e., $\tau_R \lesssim T_1$. The amplitude of the quadrupolar echo signal, which we denote by $E(\tau_R)$, then depends on $\tau_R$ because the spin system does not have time to attain its equilibrium magnetization between successive pulse trains. For a $(\pi/2|_{0^\circ}-\tau-\pi/2|_{90^\circ})$ pulse sequence, $E(\tau_R)$ is given by

$$E(\tau_R) = E_0(1 - e^{-\tau_R/T_1}) \qquad \text{(A.1)}$$

where $E_0$ is the amplitude of the signal from a spin system in thermodynamic equilibrium with its surroundings. In dealing with a signal arising from several nonequivalent deuterons, the observed signal is a superposition of terms such as eq A.1, the $i$th species deuteron having an equilibrium signal $E_0^{(i)} \propto N_i$, where $N_i$ is the number of deuterons of type $i$. The relaxation

time $T_1^{(i)}$ associated with the $i$th species depends on its spatial location and on its dynamical motions (Davis et al., 1978).

The second type of distortion arises when the duration $t_w$ of the rf pulse is not sufficiently short to satisfy the inequality $2\pi\Delta\nu_Q t_w << 1$ and some magnetization is lost during the pulse. Some of the $^2$H NMR spectra reported in this paper are close to the maximum possible quadrupolar splitting for methylene deuterons of $\Delta\nu_Q \simeq 126$ kHz. Since we are unable to produce a $\pi/2$ rotation of the deuterium spins in less time than $t_w \simeq 4$ $\mu$s for the relatively large sample volumes ($\geq$1 mL) required for this study,[2] serious spectral distortions were observed for the large splittings. Such a limitation is fairly typical for a good, high power pulse spectrometer such as the one used for these studies and distortions of this type can be accounted for by theoretical correction factors (M. Bloom, J. H. Davis, & M. I. Valic, unpublished results). For the present paper, it will be sufficient to note that the distortion can be reduced by any desired degree providing one is prepared to sacrifice signal/noise, by reducing the value of $t_w$ well below the value required for $\pi/2$ rotation (cf. Figure 5).

A third possible type of distortion, due to different values of the transverse relaxation time, $T_2$, for nonequivalent deuterons, was found to be unimportant for the values of $\tau$ used in the experiments.

## Appendix B: Procedure for Estimating Amount of CP Homogeneously Dissolved in EYL

The concentration of CP dissolved in EYL, $f_{diss}$, is related to the total concentration of CP in the sample, $f$, by

$$\frac{f}{f_{diss}} = 1 + \frac{N_C + N_D}{N_A + N_B} = 1 + R_0 \qquad \text{(B.1)}$$

where $N_i$ = number of CP molecules contributing to feature $i$.

Let the areas of the $^2$H NMR signals, determined using a repetition time $\tau_R$, be $E_0^{(i)}(\tau_R)$ for $i$ = A, B, C, and D, and let the spin–lattice relaxation times be $T_1^{(i)}$. If $\tau_R >> T_1^{(i)}$ for $i$ = A, B, and C, and if the finite pulse width distorts feature D but not A, B, and C, so that

$$\frac{E_0^{(D)}}{E_0^{(C)}} = \frac{N_D d}{N_C} \qquad \text{(B.2)}$$

while

$$\frac{E_0^{(i)}}{E_0^{(j)}} = \frac{N_i}{N_j}; \text{ for } i, j \neq D \qquad \text{(B.3)}$$

where $d$ is the distortion factor for D, then, the measured ratio $R$ of the areas of the signals associated with solid CP to those associated with homogeneous CP is given by

$$R = \frac{E_0^{(C)} + E_0^{(D)}(1 - e^{-\tau_R/T_1^{(D)}})}{E_0^{(A)} + E_0^{(B)}} \qquad \text{(B.4)}$$

Making use of eq B.1 and B.3, we find that

$$R = \left[\frac{N_C + N_D d(1 - e^{-\tau_R/T_1^{(D)}})}{N_C + N_D}\right] R_0 \qquad \text{(B.5)}$$

Since $\tau_R$ and $N_C/N_D$ are known, $T_1^{(D)} = 11$ s has been measured, the distortion factor has been determined to be $d = 7/(28/3) = 0.75$ for $t_w = 4$ $\mu$s, then the ratio $f/f_{diss}$ is found from the measured ratio of the signal from solid CP to homogeneous CP, $R$, using eq B.1 and B.5.

[2] With the existing probe head, shorter $\pi/2$ pulses could not be achieved because the large rf voltages ($\sim$50 kV peak to peak) across the high Q tank circuit for the probe head cause electrical breakdown (arcing). For $t_w = 4$ $\mu$s, the quantity $2\pi\Delta\nu_Q t_w \sim 3$.

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