# Carbon-13 Nuclear Magnetic Resonance Studies of Cholesterol–Egg Yolk Phosphatidylcholine Vesicles†

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ABSTRACT: Proton-decoupled natural-abundance $^{13}C$ nuclear magnetic resonance spectra at 63 kG were obtained for isolated single-bilayer egg yolk phosphatidylcholine–cholesterol vesicles containing a variable phospholipid/cholesterol ratio. Numerous well-resolved single carbon resonances of phospholipid and cholesterol carbons were observed. Carbon resonances from different parts of the phospholipid show markedly different behavior as a function of cholesterol content of the vesicles. The line widths of resonances for carbon atoms in the head-group region and the *sn*-3 carbon of the phospholipid glycerol backbone are relatively independent of cholesterol content. In contrast, resonances from the *sn*-1 and *sn*-2 carbon atoms of the glycerol backbone and the envelopes containing the olefinic and aliphatic carbon resonances of the fatty acyl chains of the phospholipids broaden markedly with increasing content of cholesterol. The most prominent cholesterol ring resonance is that for C6. This is, in part, due to its location in a clear window of the spectrum, where it is unobscured by interfering phospholipid resonances. However, resonances for cholesterol ring carbons C9 and C14,17, which should also appear in clear regions of the spectrum, are not observable. It is suggested that these resonances are broadened by dipolar interactions with neighboring protons. Anisotropic rotation of the cholesterol molecule about its long axis is suggested to be the major mechanism responsible for decreased dipolar interactions for the C6 carbon, while retaining the large dipolar coupling of the C9 and C14,17 carbons with their neighboring protons. The temperature dependence of spectra of single-bilayer phosphatidylcholine vesicles containing epicholesterol ($\alpha$-cholesterol) is different from that of corresponding cholesterol-containing vesicles. Resonances from the C9 and C14,17 carbons of epicholesterol in EYPC vesicles are detectable at 35 °C whereas the corresponding resonances from $\beta$-cholesterol are not. These data suggest that in vesicles the rotation of cholesterol is more anisotropic than that of epicholesterol and that the stereochemistry of the C3 hydroxyl group of cholesterol is at least partly responsible for the highly anisotropic rotation of the steroid ring within the bilayer.

Numerous studies of phospholipid model membranes and naturally occurring membranes have shown that the mobility of membrane lipid molecules is closely associated with several physical and biological properties (Jain & Fourcans, 1974). The relationships between lipid mobility and membrane function have stimulated efforts to understand the effects of various factors which can modify membrane fluidity, including temperature, the length and degree of unsaturation of the fatty acyl chains, the structure of the polar head group, and the presence of cations, anesthetics, and cholesterol.

The most extensively studied agent capable of altering membrane fluidity is cholesterol, due in part to its wide distribution in biological systems and to the early demonstration of its direct interaction with phospholipid (Leathes, 1925). The results of numerous studies have served as the basis for several models describing the interaction of cholesterol with phospholipids (Brockerhoff, 1974; Huang, 1976, 1977a,b). While these models have provided an explanation for a large portion of the experimental data, there remain several features of the phospholipid–cholesterol interaction which require further definition. Thermodynamic, magnetic resonance, neutron and X-ray diffraction, and bilayer permeability data have indicated that the molecular structure in the "hydrogen belt" region of the bilayer is of critical importance in the phospholipid–cholesterol interaction (Brockerhoff, 1974; Jain, 1975; Demel & de Kruyff, 1976). However, very little is known about the effects of cholesterol on the detailed molecular dynamics in this region of the bilayer. In addition, although epicholesterol (the 3$\alpha$-OH stereoisomer of cholesterol) is known not to exhibit several of the properties characteristic of cholesterol, little is known about the consequences of the altered stereochemistry on the dynamics of the sterol within the bilayer. Consequently, we have undertaken a study of the dynamics of cholesterol in sonicated egg yolk phosphatidylcholine vesicles using $^{13}C$ nuclear magnetic resonance (NMR).[1] Although $^{13}C$ NMR has been widely employed to study lipid systems, its use at high magnetic fields has been limited. The increased sensitivity and resolution afforded by the 63-kG field, together with relatively large and homogeneous sample preparations, have allowed observation of additional details of the phospholipid–cholesterol interaction not seen previously.

## Experimental Procedures

### *Materials*

Phosphatidylcholine was isolated from fresh egg yolks according to the procedure of Singleton et al. (1965). Alumina (alumina oxide, Woelm Neurtal), distributed by ICN Pharmaceuticals, Ohio, was used as received. The purified phospholipid was stored as a $CHCl_3$ solution under nitrogen in the dark at −15 °C. Phospholipid concentration was determined

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[1] Abbreviations used: EYPC, egg yolk phosphatidylcholine; DISPA, plot of dispersion vs. absorption; DPPC, dipalmitoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; CH, cholesterol; epi-CH, epicholesterol; $X_{CH}$, mole fraction of cholesterol; $T_m$, gel to liquid-crystalline phase transition temperature; NMR, nuclear magnetic resonance; ESR, electron spin resonance; $T_1$, spin–lattice relaxation time; $T_2$, spin–spin relaxation time; NOE, nuclear Overhauser effect, defined as the ratio of signal intensity with proton decoupling to the intensity without decoupling; CSA, chemical shift anisotropy; LDL, low-density lipoprotein (*d* 1.019–1.063 g/mL); EDTA, ethylenediaminetetraacetic acid.

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gravimetrically at frequent intervals. The isolated product was regularly checked for purity by using thin-layer chromatography (TLC) on silica gel plates (J. T. Baker Chemical Co., Phillipsburgh, NJ) in two solvent systems, $CHCl_3$/$CH_3OH$/$H_2O$ (65:25:4) and $CHCl_3$/$CH_3OH$/glacial acetic acid/$H_2O$ (260:100:32:14). TLC spots were visualized by exposure to $I_2$. Cholesterol was purchased from Sigma Chemical Co., St. Louis, MO, and epicholesterol from Schwarz/Mann, Orangeburg, NY. Both sterols were recrystallized twice from ethanol and checked for purity by thin-layer chromatography. [1,2-$^3H_2$]Cholesterol and $^{14}C$-labeled algal phosphatidylcholine were obtained from New England Nuclear, Boston, MA, and used as received. The scintillation fluid employed throughout was Aquasol, New England Nuclear. Sepharose 4B and 2B were purchased from Pharmacia (Uppsala, Sweden). The buffer employed was 100 mM NaCl, 10 mM sodium phosphate, 1 mM EDTA, and 1 mM $NaN_3$, pH 7.2.

*Methods*

*Vesicle Preparation.* Sonicated phospholipid–cholesterol vesicles were prepared by modification of gel filtration techniques previously described (Huang & Thompson, 1974). Fractions eluting from a Sepharose 4B column (2.8 × 50 cm) containing single-walled vesicles were pooled and concentrated to 137–185 mg/mL phospholipid by pressure ultrafiltration (<10 mm) on Amicon XM100 membranes previously equilibrated with vesicles. Lipid loss by adsorption on the ultrafiltration membrane or the agarose gel was undetectable. After purification, vesicles were transferred to NMR tubes and overlayed with argon or nitrogen, and NMR analysis was completed within 9 days. The concentration and composition of vesicle preparations were determined radiochemically. The radioactivity was determined in Aquasol by a Beckman L5-230 liquid scintillation counter, with corrections for channel spillover. For several samples, the composition was also determined by chemical analysis using the method of Bartlett (1959) for the phosphorus determination, and the method of Bowman & Wolf (1962) or the Boehringer-Mannheim enzymatic kit (Boehringer-Mannheim Biochemicals, Indianapolis, IN) for cholesterol determination. Results from the radiochemical and chemical methods agreed to within 3%.

The profiles of vesicle preparations eluted from the Sepharose 4B column were similar to those reported by Huang & Thompson (1974). Single vesicle preparations showed a significant variation in cholesterol content across the gel filtration profile. Those fractions eluting on the leading side of the peak were enriched in cholesterol relative to the trailing fractions. However, the variation in composition for 80% of those fractions pooled for NMR analysis was less than 0.01 mole fraction. Gel chromatography of several purified vesicle preparations on a calibrated Sepharose 2B column (1.2 × 90 cm) showed the presence of more than 96% single-walled vesicles having average Stokes radii identical with those reported in the literature (Huang, 1969; Gent & Prestegard, 1974a,b). Rechromatography of several vesicle preparations after NMR analysis and, in one case, after storage at 4 °C for 8 weeks showed that greater than 95% of the radioactivity eluted as single-walled vesicles, demonstrating that the vesicle preparations were stable under the conditions used for NMR analysis. Thin-layer chromatography of lipid extracts of vesicles after NMR analysis showed no detectable lipid degradation products.

*NMR Measurements.* $^{13}C$ NMR spectra at 63.4 kG were obtained as previously described (Brainard et al., 1980). Spectra of one vesicle sample ($X_{CH}$ = 0.22) were obtained with 16 384 time domain points, after modifications of the spectrometer which included a Bruker 15-mm probe, quadrature phase detection and coherent broad-band decoupling (Grutzner & Santini, 1975). Additional accumulation and data processing conditions are given in the figure legends. When it was necessary to compare integrated intensities of selected resonances, the proton decoupler was gated to eliminate the nuclear Overhauser effect. In these experiments, the irradiation for proton decoupling was switched off for a period of at least 9 times the longest $T_1$ of the resonances compared. The integrated areas were determined digitally after a base-line correction was applied, and by weighing tracings of the resonances, we found that both methods gave comparable results.

$^{13}C$ NMR spectra at 23.4 kG were recorded on a Varian Associates XL-100-15 TT-1010 spectrometer system equipped with a Nicolet 1080 computer. Time domain spectra were accumulated in 8192 addresses using a spectral width of 6329 Hz.

$T_1$ values were determined by using several pulse sequences, inversion recovery (Vold et al., 1968), progressive saturation (Freeman & Hill, 1971), and fast inversion recovery (Canet et al., 1975), with care taken to minimize the systematic errors associated with each experimental method by careful selection of instrumental parameters (Levy & Peat, 1975; Sass & Ziessow, 1977). $T_1$ values were calculated by fitting the experimental data to the exponential expression $I(\tau) = A + B \exp(-\tau/T_1)$, using at least seven $\tau$ values.

DISPA plots (Marshall, 1979; Marshall & Roe, 1978; Roe et al., 1978) of selected $^{13}C$ NMR resonances were generated directly from the digital data contained in the corresponding real (absorption) and imaginary (dispersion) portions of Fourier-transformed spectra. The accumulation conditions for the spectra used to generate the DISPA plots were selected so that neither the digital resolution (0.85 Hz) nor the pulse interval (3.19 s) compromised the diagnostic sensitivity of the DISPA plots in detecting various mechanisms for NMR spectral line broadening.

## Results

Figure 1 shows $^{13}C$ NMR spectra of (A) purified EYPC vesicles containing no cholesterol, (B) purified EYPC–CH vesicles containing approximately 0.3 mole fraction of cholesterol, and (C) cholesterol dissolved in $CHCl_3$. The assignments are based on previously reported spectra (Levine et al., 1972; Shapiro et al., 1975).

The spectral differences which occur upon incorporation of cholesterol into egg yolk phosphatidylcholine vesicles reflect both compositional and dynamic differences between the two systems. The discussion of experimental results will be structured into two parts: (i) those results which may be interpreted to suggest changes in the dynamics of phospholipid motions occurring upon interaction with cholesterol, and (ii) those spectral features which may reflect upon the dynamics of cholesterol and epicholesterol in sonicated vesicles.

*Phospholipid Carbon Resonances.* Figure 2 demonstrates the effect of cholesterol upon selected phospholipid carbon resonances. This figure shows expanded unsaturated, choline–glycerol backbone, and saturated carbon regions of $^{13}C$ NMR spectra of sonicated EYPC–CH vesicles containing approximately 0.4, 0.2, and 0.0 mole fraction of cholesterol. The envelopes containing resonances from the saturated and unsaturated carbons of the fatty acyl chains are broadened markedly in those vesicles containing cholesterol. In addition, the single carbon resonances at 63.3 and 70.9 ppm arising from the *sn*-1 and *sn*-2 carbons of the phospholipid glycerol backbone exhibit marked increases in widths upon cholesterol

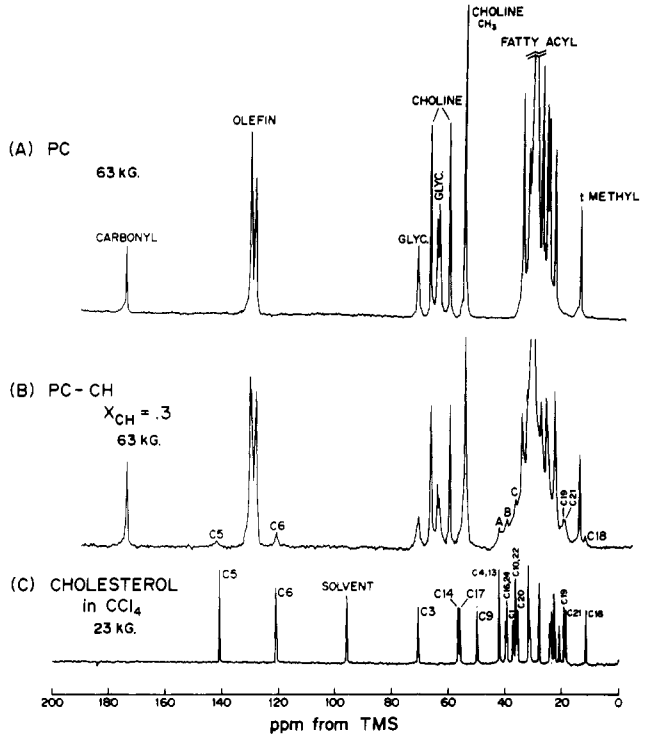


FIGURE 1: Proton-decoupled $^{13}C$ NMR spectra at 63 kG of (A) EYPC vesicles, $X_{CH}$ = 0, 185 mg/mL phospholipid, 16 384 accumulations; (B) EYPC–CH vesicles, $X_{CH}$ = 0.32, 172 mg/mL phospholipid, 32 768 accumulations; and (C) 23-kG $^{13}C$ NMR spectra of cholesterol (25 mg/mL) in $CHCl_3$. Both spectra at 63 kG were obtained at 34 °C with a pulse interval of 0.571 s and processed with 3.2-Hz digital broadening. Technical difficulties in dissipating heat from the sample require use of a relatively narrow bandwidth and low power for proton decoupling. Consequently, three spectra were accumulated for each sample under identical conditions, except for decoupler frequency. Qualitatively, the three spectra recorded were identical, but resonances outside the decoupler bandwidths showed significant broadening due to inadequate decoupling. Consequently, as an aid in the visual presentation of the data, the full (0–200-ppm) spectra presented in this figure are composites of the three spectra obtained under different decoupling conditions. Regions from 0 to 50 and from 150 to 200 ppm were taken from printouts of spectra recorded with the decoupler frequency centered at 1.52 ppm downfield from $Me_4Si$ (TMS in figure), regions between 50 and 73 ppm are from spectra with the decoupler set at 3.80 ppm from $Me_4Si$, and regions between 73 and 150 ppm are from spectra with the decoupler set at 4.95 ppm from $Me_4Si$.

addition. In contrast, line widths of those resonances arising from the choline methyl (54.5 ppm) and methylenes (59.7 and 66.3 ppm) and from the *sn*-3 glycerol backbone carbon (63.9 ppm) show no dependence upon the cholesterol content of the vesicles. The line-width dependence of selected single carbon resonances on the cholesterol content of the vesicle preparation is depicted quantitatively in Figure 3.

$T_1$ values for selected carbon resonances of egg yolk phosphatidylcholine vesicles containing approximately 0.0, 0.2, 0.3, and 0.4 mole fraction of cholesterol are summarized in Table I. In contrast to the line-width behavior of some of the carbon resonances, the $T_1$ values for all resonances show little dependence, outside of experimental error, on the cholesterol content of the vesicles.

*Cholesterol Carbon Resonances.* Spectra of EYPC vesicles containing cholesterol show several relatively broad resonances which can be assigned to cholesterol ring and side-chain carbons. These resonances are detectable in spectra of vesicles containing between 0.2 and 0.5 mole fraction of cholesterol. The most prominent of these arises from the C6 carbon of the ring. The dependence of the cholesterol C6 line width on the cholesterol content of the vesicle is included in Figure 3. Also

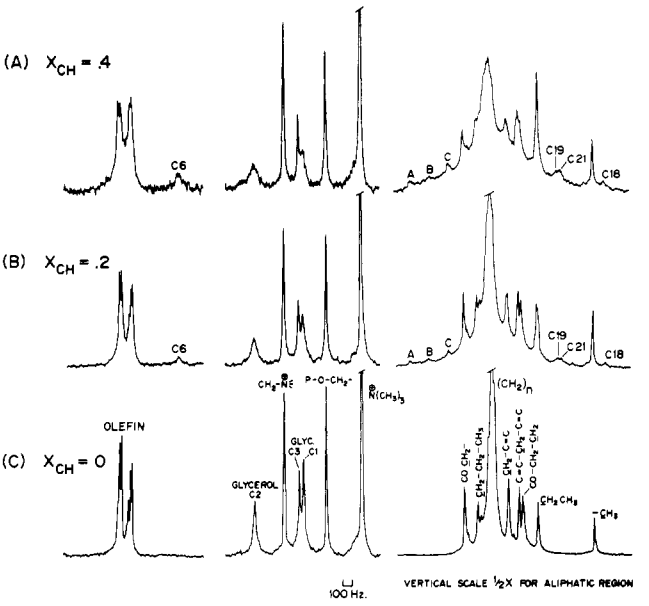


FIGURE 2: Expanded regions of proton-decoupled $^{13}C$ NMR spectra at 63 kG of (A) EYPC–CH vesicles, $X_{CH}$ = 0.41, 171 mg/mL phospholipid, 16 384 accumulations; (B) EYPC–CH vesicles, $X_{CH}$ = 0.19, 172 mg/mL phospholipid, 32 768 accumulations; (C) EYPC vesicles, $X_{CH}$ = 0, 185 mg/mL phospholipid, 16 384 accumulations. All spectra were obtained at 34 °C with a pulse interval of 0.571 s and processed with 3.2-Hz digital broadening.

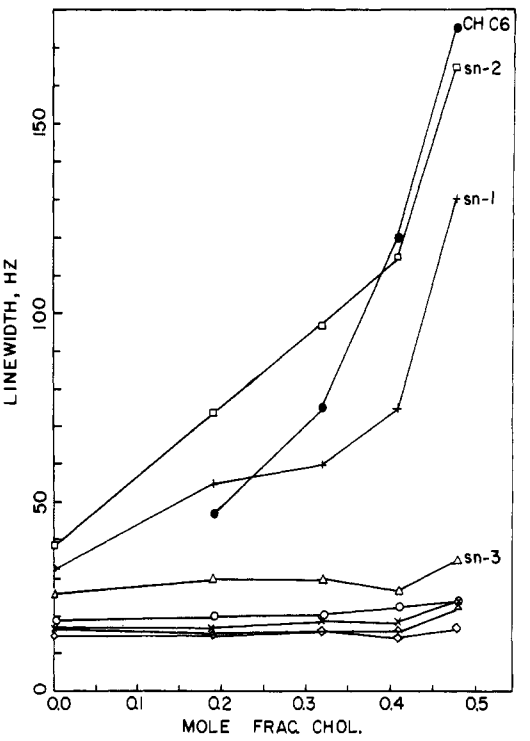


FIGURE 3: Dependence of the line width at one-half height of several phospholipid single carbon resonances and the C6 cholesterol resonance upon the cholesterol content of the vesicles. Instrumental contribution ($H_0$ inhomogeneities and drift, and 3.2-Hz exponential line broadening) is estimated to be less than 9 Hz. (●) C6 cholesterol; (□) *sn*-2 glycerol; (○) choline methylene ($CH_2$–N); (△, *sn*-3) *sn*-3 glycerol; (+) *sn*-1 glycerol; (×) choline methylene (O–$CH_2$); (◇) choline methyl; (▲) fatty acyl $CH_3$.

observable are resonances from the methyl-group carbons C18, C19, and C21, and from the C5 carbon of the cholesterol ring system. Figure 4 shows the unsaturated carbon region of a $^{13}C$ NMR spectrum obtained by using a longer pulse interval in which the cholesterol C5 resonance is more clearly resolved. Significantly, the $^{13}C$ resonance from the nonprotonated C5

Table I: $T_1$ Values for Selected Carbon Resonances of Cholesterol-EYPC Vesicles at 63 kG

| resonance assignment | $T_1$ (ms) $X_{CH}$ = 0 | $X_{CH}$ = 0.22 | $X_{CH}$ = 0.32 | $X_{CH}$ = 0.41 |
|---|---|---|---|---|
| unsaturated carbons | | | | |
| 129.9 ppm | 774 ± 44[a] | 654 ± 50 | 493 ± 61 | 548 ± 74 |
| 129.6 ppm | 600 ± 26 | 504 ± 46 | 417 ± 121 | 591 ± 65 |
| polyunsaturated carbons | | | | |
| 128.2 ppm | 932 ± 48 | 746 ± 71 | 441 ± 204 | 515 ± 83 |
| 127.9 ppm | 876 ± 19 | 649 ± 53 | 467 ± 125 | 629 ± 75 |
| cholesterol C6 | | 193 ± 18 | 141 ± 16 | 119 ± 47 |
| *sn*-2 glycerol backbone | 119 ± 64 | 200 ± 5 | 195 ± 28 | 244 ± 90 |
| $-CH_2N$ | 369 ± 16 | 320 ± 31 | 321 ± 40 | 375 ± 25 |
| *sn*-3 glycerol backbone | 131 ± 17 | 132 ± 4 | 120 ± 13 | 89 ± 21 |
| *sn*-1 glycerol backbone | 122 ± 37 | 133 ± 3 | 113 ± 8 | 99 ± 25 |
| $-OCH_2-$ | 356 ± 15 | 291 ± 25 | 307 ± 12 | 358 ± 45 |
| $-\overset{+}{N}(CH_3)_3$ | | 687 ± 65 | | |

[a] The uncertainties given are those calculated from the error matrix by the nonlinear least-squares fitting program BMDX85 (Dixon, 1969). The rather wide variation in the uncertainties is a reflection of the variation in signal to noise ratios between different resonances and samples, and of the variation in the ratios longest $\tau/T_1$ and pulse interval/$T_1$ [see, for example, Levy & Peat (1975) or Sass & Ziessow (1977)].

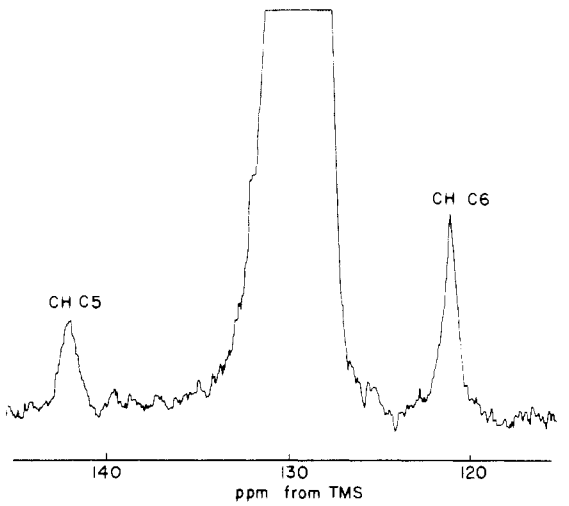


FIGURE 4: Expanded unsaturated region of a proton-decoupled $^{13}C$ NMR spectrum at 63 kG of sonicated EYPC–CH vesicles containing 0.22 mole fraction of cholesterol and 140 mg/mL phospholipid. Other conditions were the following: 8192 accumulations, quadrature detection, 2 × 8192 time domain points, pulse interval = 3.19 s, $^1H$ decoupling frequency centered at 4.76 ppm from $Me_4Si$ (TMS in figure) and modulated by 100-Hz square wave, 3.2-Hz digital broadening applied during processing.

carbon has a line width approximately equal to that for the protonated carbon, C6. In addition, three broad resonances labeled A, B, and C in Figures 1 and 2 appear as shoulders on the low-field side of the intense envelope containing the saturated carbon resonances, which may also be assigned to cholesterol carbons. The unambiguous assignment of these three broad resonances is difficult because of their breadth and position next to an intense phospholipid resonance. The spectrum of EYPC–cholesterol vesicles is also characterized by the conspicuous absence of several cholesterol carbon resonances. This is graphically illustrated in Figure 5, which compares the unsaturated and glycerol backbone carbon regions of the $^{13}C$ NMR spectra of (A) cholesterol in EYPC

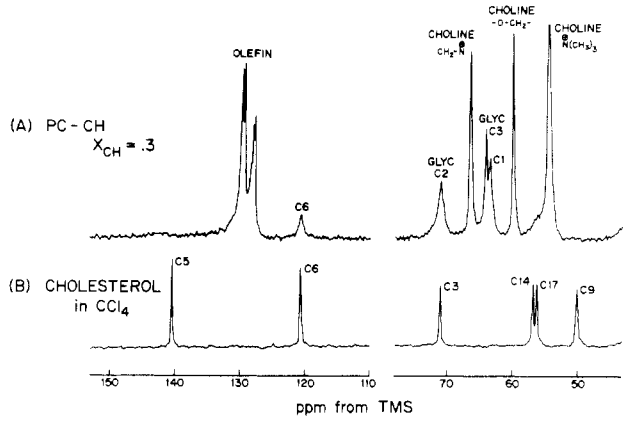


FIGURE 5: Expanded regions of proton-decoupled $^{13}C$ NMR spectra of (A) EYPC–CH vesicles at 63 kG and 34 °C, $X_{CH}$ = 0.32, 172 mg/mL phospholipid, 16 384 accumulations, 0.571-s pulse interval, 3.2-Hz line broadening; and (B) cholesterol in $CHCl_3$ at 23 kG, 25 mg/mL, 512 accumulations, 5.13-s pulse interval.

vesicles (mole fraction cholesterol 0.32) with (B) cholesterol in $CHCl_3$. On the basis of the chemical shifts observed in $CHCl_3$, resonances from cholesterol carbons C9 and C14,17 are expected to fall in regions of the $^{13}C$ spectrum sufficiently removed from intense phospholipid resonances to be detectable. The asymmetry observed at the base of the phospholipid $N(CH_3)_3$ resonance may be due to detectable intensity from the C14,17 carbon resonance. However, the asymmetry surrounding the $N(CH_3)_3$ resonance varied among different samples and spectra and was highly dependent upon the phase correction applied to the spectra. Furthermore, $^{13}C$ spectra of epicholesterol in EYPC vesicles show the C14,17 resonance clearly resolved from the phospholipid choline methyl resonance.

In order to determine if the C3 cholesterol carbon was contributing significant intensity to the resonance observed at 70.9 ppm, integrated intensities obtained by gated decoupling were compared for the resonances at 120 and 70.9 ppm from tetramethylsilane ($Me_4Si$) in vesicle preparations containing 0.32 and 0.41 mole fraction of cholesterol. The intensity ratios agreed better with the mole ratio of cholesterol to phospholipid rather than the mole fraction, suggesting that the C3 cholesterol ring carbon does not contribute significant intensity to the resonance at 70.9 ppm observed in cholesterol–EYPC vesicles. The quantitative integration of NMR resonances is technically difficult because of the uncertainties associated with selecting a base line and the limits for integration. These difficulties are particularly acute for broad resonances, where the true line shape may be super-Lorentzian. Conventional integration of broad resonances may result in underestimating the area. At $X_{CH}$ = 0.3 and 0.4, the cholesterol C6 line width is approximately equal to, or greater than, the line width of the resonance at 70.9 ppm. Consequently, introduction of systematic error from the integration should not affect the *ratio* of areas appreciably. In the worst case, it may result in underestimating the ratio. These data indicate that resonances from carbon atoms C3, C9, and C14,17 of the cholesterol ring system are not detectable under conditions for which there is appreciable intensity observed for the $^{13}C$ resonance from the cholesterol C6 carbon.

*Epicholesterol Carbon Resonances.* The temperature dependence of proton-decoupled $^{13}C$ NMR spectra of vesicles containing 0.3 mole fraction of cholesterol is compared with vesicles containing 0.3 mole fraction of epicholesterol in Figure 6. The spectra recorded at 31–32 °C are similar. At higher temperatures, however, significant differences appear. These

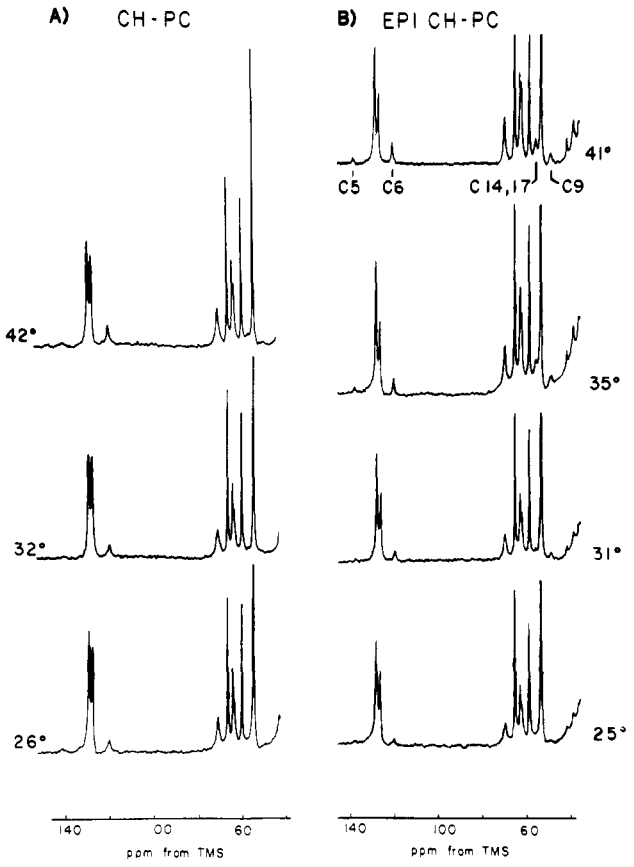


FIGURE 6: Temperature dependence of proton-decoupled $^{13}C$ NMR spectra at 63 kG of (A) EYPC–cholesterol, 0.30 mole fraction of cholesterol, 183 mg/mL phospholipid, 32 768 accumulations; and (B) EYPC–epicholesterol vesicles, 0.29 mole fraction of epicholesterol, 125 mg/mL phospholipid, 65 536 accumulations. All spectra were obtained with a 0.571-s pulse interval, the proton decoupling frequency centered at 2.1 ppm downfield from $Me_4Si$ (TMS in figure), and processed with 3.2-Hz line broadening.

differences are primarily in the detectability of cholesterol carbon resonances C9 and C14,17. At 32–35 °C, resonances from carbons C9 and C14,17 are clearly observable in vesicles containing epicholesterol, whereas spectra of cholesterol-containing vesicles at 42 °C just barely show broad resonances for these carbon atoms. In addition, at higher temperature, the resonance from the *sn*-1 carbon of the phospholipid backbone is narrower in spectra of vesicles containing epi-CH than in spectra of EYPC–CH vesicles.

## Discussion

$^{13}C$ NMR has been used extensively to investigate the dynamics of sonicated phospholipid vesicles (Keough et al., 1973; Lee et al., 1976; Gent & Prestegard, 1977; Levine et al., 1972; Godici & Landsberger, 1974, 1975; Stoffel et al., 1974; de Kruijff, 1978; Lancee-Hermkens & de Kruijff, 1977). The results of the present study are in substantial agreement with those of these previous investigations. However, the high magnetic field strength and the relatively large and homogeneous samples used in our study have overcome earlier technical difficulties in detection of broad resonances. Consequently, we have been able to study the behavior of several phospholipid and cholesterol single carbon resonances whose resolution has not been reported by previous investigators.

Practical considerations have made the spin–lattice relaxation rate, $1/T_1$, one of the parameters most frequently measured in $^{13}C$ NMR. However, $T_1$ measurements suffer from the disadvantage that in systems undergoing anisotropic motion, they are not very sensitive to the slower components of motion which may be of interest to the investigator. In contrast, the spin–spin relaxation rate, $1/T_2$, is more sensitive to these slower components of motion in anisotropic systems (Kroon et al., 1975). However, its measurement requires multiple pulse sequences with phase inversion (Carr & Purcell, 1954) in order to refocus the magnetization in the $x$–$y$ plane. An alternate way of estimating the spin–spin relaxation rate, which has been adopted by a number of investigators, is to determine a lower limit for the rate from the line width of the carbon resonance (Gent & Prestegard, 1977; Godici & Landsberger, 1974, 1975; Sears, 1975; Lancee-Hermkens & de Kruijff, 1977). In liquids, the adsorption signals usually have a Lorentzian shape, and an apparent spin–spin relaxation time may be derived from the resonance line width by eq 1.

$$T_2^* = \frac{1}{\pi \nu_{1/2}} \tag{1}$$

$T_2^*$ defined in this manner, however, contains contributions from sources which can cause line-width changes that are unrelated to the molecular motion of the nucleus being studied. Fortunately, in some systems, the contributions from these other sources may be shown to be either small or constant, so that useful conclusions about molecular dynamics may be drawn based on comparisons between line-width measurements.

In our case, the measured line-widths of $^{13}C$ resonances may be expected to contain contributions from the following sources:

$$\nu_{1/2} = \frac{1}{\pi T_2} + \mathrm{LW_{ins}} + \mathrm{LW_{chem}} \tag{2}$$

where $1/(\pi T_2)$ is the contribution from the spin–spin relaxation processes that are modulated by the molecular motion of the nuclei. For $^{13}C$ nuclei of lipids at 63 kG, $^{13}C$–$^1H$ dipolar interactions and possibly chemical shift anisotropy are expected to be the dominant processes leading to spin–spin relaxation. $\mathrm{LW_{ins}}$ describes the contribution to the measured line width from instrumental artifacts, such as $H_0$ inhomogeneities and drift, insufficient proton decoupling power, or digital filtering applied during data processing. $H_0$ drift was monitored for each spectrum recorded and found to be less than 2.7 Hz for all spectra reported herein. The homogeneity of the field was better than 3 Hz at the beginning and end of data accumulation as determined from the line width of the ethylene glycol resonance. The digital broadening applied during data processing (3.2 Hz) was identical for all spectra. Consequently, contributions to resonance line widths from instrumental artifacts are expected to be less than 9 Hz. In addition, these contributions are expected to be constant from run to run and for different resonances in a single spectrum. Therefore, it is unlikely that the line-width differences observed are the result of changes in the instrumental contributions to the line width.

A third source of line broadening can originate from resonances which are unresolved due to their chemical exchange or chemical shift nonequivalence. Small differences in the chemical shifts of nuclei located at the inner and outer monolayers of bilayers have been observed in $^1H$ (Sheetz & Chan, 1972; Kostelnik & Castellano, 1973), $^{19}F$ (Longmuir & Dahlquist, 1976), and $^{31}P$ (McLaughlin et al., 1975a,b) NMR spectra. In addition, formation of a phosphatidylcholine–cholesterol complex might also be expected to contribute to line broadening if the nuclei in the complex have a magnetic environment different from that of uncomplexed cholesterol or phospholipid. Recently, a method has been reported for determining whether a spectral line is a superposition of Lorentzians with different frequencies or with different line

widths (Marshall & Roe, 1978). Construction of DISPA plots for the *sn*-2 carbon resonance of the phospholipid glycerol backbone, the fatty acyl terminal methyl resonance, and the cholesterol C6 resonance from a $^{13}C$ NMR spectrum of sonicated EYPC–CH vesicles showed that these resonances were not superpositions of unresolved peaks with different chemical shifts or the result of exchange between molecular species with different chemical shifts since the DISPA points did not fall significantly above the reference semicircle. However, DISPA plots for the choline methyl and methylene resonances showed points above the reference semicircle. The choline methyl resonance is a good candidate for broadening by chemical shift nonequivalence since its magnetic environment is likely to be affected by the molecular packing differences between the inner and outer monolayers. This hypothesis is corroborated by the observation that the line width of the choline methyl resonance is field dependent. The methylene resonances are likely to be broadened by dipolar coupling to phosphorus, since the line widths of these resonances were not field dependent and $^{13}C$–$^{31}P$ coupling constants of 5–6 Hz have been observed for the phosphorylcholine group (Birdsall et al., 1972).

Fortunately, none of the resonances giving DISPA plots characteristic of chemical shift nonequivalence showed variations in their line widths with cholesterol content. This suggests that cholesterol does not change the chemical shift nonequivalence or the coupling constants of these nuclei, or the motions leading to spin–spin relaxation. For these resonances whose line widths are affected by the presence of cholesterol, the changes observed are unlikely to be the result of contributions from instrumental artifacts, chemical exchange, or chemical shift nonequivalence; consequently linewidth comparisons may be useful in investigating the molecular dynamics of lipids in bilayers.

There is consensus that the relaxation mechanism for $^{1}H$ and $^{13}C$ nuclei of phospholipids in membrane systems is dominated by magnetic dipole interactions (Keough et al., 1973; Seiter & Chan, 1973; Gent & Prestegard, 1977; Levine et al., 1972; Stoffel et al., 1974; Horwitz et al., 1972; Chan et al., 1971). This is most easily demonstrated for $^{13}C$ nuclei where a NOE 2.99 may be observed for the methylene and methyl carbons (Gent & Prestegard, 1977). However, most of these previous studies have been done at magnetic field strengths of 23 kG or less, and since the chemical shift anisotropy Hamiltonian is dependent on the square of the magnetic field strength, it is possible that at 63.4 kG, chemical shift anisotropy may contribute significantly to the relaxation mechanism (see below).

In an attempt to establish the relaxation mechanism of carbon nuclei of phospholipids in sonicated vesicles, the field dependence of several single carbon resonances was studied (Table II). This study revealed that, except for the choline methyl resonance and the cholesterol C5 and C6 carbon resonances, the single carbon resonance line widths showed little variation with magnetic field strength. This observation suggests that chemical shift anisotropy relaxation does not contribute significantly to the experimentally observed line widths of these carbon nuclei. Moreover, the values for the shielding tensors of aliphatic carbons are small ($\Delta\sigma \lesssim 20$ ppm), and theoretical calculations predict that contributions to the relaxation rates of these carbon atoms from chemical shift anisotropy are negligible relative to dipolar contributions (Norton et al., 1977). The magnitudes of the shielding tensors for olefinic carbons are higher ($\Delta\sigma \lesssim 170$ ppm), but theoretical calculations also predict that for most orientations of protonated olefinic carbons within the bilayer, the dipolar

Table II: Field Dependence of the Line Width of Selected Single Carbon Resonances in EYPC–CH Vesicles Containing 0.22 Mole Fraction of Cholesterol[a]

| | line width (Hz) | |
|---|---|---|
| | 23 kG | 63 kG |
| cholesterol C5 | 20–25 | ~60 |
| cholesterol C6 | 28–30 | ~60 |
| *sn*-2 glycerol backbone | 58–64 | 68 |
| $-CH_2-N$ | 19 | 20 |
| *sn*-3 glycerol backbone | 20–24 | 28 |
| *sn*-1 glycerol backbone | | 46 |
| $-CH_2O-$ | 15–16 | 19 |
| $-\overset{+}{N}(CH_3)_3$ | 10 | 16–17 |

[a] Line widths are given in hertz measured at one-half peak height; this includes 3.2-Hz digital broadening applied during processing.

mechanism is still expected to dominate. In addition, the $T_1$ values measured in this study for the fatty acyl unsaturated and the choline methyl carbon nuclei (Table I) are in substantial agreement with those measured at lower fields in similar systems (Lee et al., 1976; Gent & Prestegard, 1977; Godici & Landsberger, 1974, 1975), which together with the observation that phospholipid segmental motions fall inside the motional narrowing region (Petersen & Chan, 1977) suggest that chemical shift anisotropy is not an effective relaxation mechanism for these carbons.

Consequently, we believe that the small field dependence observed for the line widths (except for the choline methyl carbon resonance, where chemical shift nonequivalence may contribute, and the cholesterol C5 and C6 resonances, which will be discussed later) is a result of differences in the amount of line broadening introduced by instrumental artifacts between the two spectrometers and that the relaxation mechanism is predominantly dipolar for the methine, methylene, and olefinic carbon nuclei of phospholipids in bilayers.

Previous investigators have observed that the spin–lattice relaxation rates of carbon nuclei change very little when cholesterol is added (Godici & Landsberger, 1975; Stoffel et al., 1974). Similar results have been obtained in our studies at 63 kG. In addition, we have observed that the $T_1$ values for the *sn*-1, -2, and -3 carbons of the phospholipid glycerol backbone are also greatly changed by cholesterol. Godici & Landsberger (1975) have argued that the local fast motions of the fatty acyl chains, such as $\beta$-coupled gauche isomerizations, are not inhibited by the presence of cholesterol, based on the demonstration that spin–lattice relaxation times in anisotropic systems are sensitive mainly to the more rapid components of motion (Seiter & Chan, 1973; Kroon et al., 1975). The $T_1$ values for the glycerol backbone region of the phospholipid molecule suggest that, first, the motions responsible for spin–lattice relaxation are more restricted for the glycerol backbone carbon nuclei than for carbon nuclei in the fatty acyl chains or in the head-group region, in agreement with the idea of motional gradients extending in both directions from the glycerol backbone region (Lee et al., 1976; Godici & Landsberger, 1974). This gradient appears to be maintained in the presence of cholesterol. Second, the invariance of the spin–lattice relaxation rates with cholesterol content suggests that the motions of the glycerol backbone carbon atoms which result in spin–lattice relaxation are not changed very much by cholesterol. This second conclusion is compromised somewhat by the large experimental uncertainties associated with the $T_1$ measurement; there are trends in the data which because of the large uncertainties may not presently be rigorously considered significant. Decreases in spin–lattice

relaxation times for the glycerol backbone carbons, the olefinic resonances (particularly at 128.2 and 127.9 ppm), and for cholesterol C6 with increasing mole fraction of cholesterol suggest that the motions responsible for spin–lattice relaxation of the unsaturated carbons and for the cholesterol ring system may be restricted by cholesterol.

Previous investigators have established that the line widths of $^1H$, $^2H$, and $^{13}C$ NMR resonances of fatty acyl methylene groups of phospholipids in the liquid-crystalline phase are markedly broadened by cholesterol (Keough et al., 1973; Stockton et al., 1974, 1976; Godici & Landsberger, 1975; Lancee-Hermkens & de Kruijff, 1977; Gent & Prestegard, 1974a,b; Darke et al., 1972). In contrast, the line widths of resonances of nuclei in the polar head-group region are not detectably altered by cholesterol. Again, these results have been substantially confirmed by our studies at 63 kG. However, the additional sensitivity at 63 kG has revealed a notable difference in the behavior of the glycerol backbone carbon resonances with increasing cholesterol content. The *sn*-3 carbon resonance shows behavior characteristic of the head-group region of the phospholipid, where the line widths are not detectably changed by cholesterol. In contrast, the *sn*-2 and *sn*-1 carbon resonances broaden markedly with increasing cholesterol content (Figure 3). The line-width data strongly suggest that those motions of the *sn*-1 and -2 carbons of the phospholipid backbone affecting the carbon spin–spin relaxation rates are strongly affected by the presence of cholesterol in the bilayer. In addition, cholesterol also appears to change the motions of the olefin and methylene carbon of the fatty acyl chains, based on the behavior of the envelopes containing their resonances. However, definitive conclusions based on the line shapes of envelopes with contributions from multiple carbon resonances are difficult to reach due to the difficulties associated with separating the multiple contributions to line shape.

The motions which determine the spin–spin relaxation rates in the regions surrounding the terminal methyl groups of the fatty acyl chains, the *sn*-3 carbon of the glycerol backbone, and the choline methylene and methyl groups are not detectably altered by the addition of cholesterol to the bilayer.

The theory of nuclear spin relaxation in ordered or semiordered systems is not fully understood in terms of the rates and/or modes of the various types of motions involved. Consequently, spin–lattice relaxation times and line widths can presently provide only a qualitative, or at best semiquantitative, picture of the motions in bilayer systems. Seiter & Chan (1973) and Gent & Prestegard (1977) have demonstrated that for anisotropic motions such as those occurring in a phospholipid bilayer, the spin–lattice and spin–spin relaxation rates are sensitive to different types of motions. The spin–lattice relaxation times are determined primarily by motions with frequencies equal to or in excess of the Larmor frequency. In a bilayer, these motions are expected to be rather restricted in amplitude. In contrast, the spin–spin relaxation rates, and thus their contribution to the line width, are determined by slower, larger amplitude motions within the bilayer. Increases in spin–lattice relaxation times for carbon nuclei in bilayers may be interpreted in a qualitative way to suggest that the faster components of motion are either increasing in rate or becoming less restricted in their angular fluctuations. Likewise, increases in the spin–spin relaxation rate (or decreases in the line width) suggest the same changes for the slower components of motion.

The data from this study suggest that cholesterol introduced into sonicated EYPC vesicles restricts the slower motions of the C–H internuclear vectors of the *sn*-1 and *sn*-2 carbon atoms of the phospholipid glycerol backbone but that it does not detectably affect the slower motions of the *sn*-3 glycerol backbone carbon, the choline methyl or methylene carbons, or the terminal methyl carbons of the fatty acyl chains. Moreover, cholesterol does not detectably affect the fast motions responsible for spin–lattice relaxation for all carbon nuclei in the phospholipid molecule, at least within the limits of the experimental uncertainties in the $T_1$ values.

$^2H$ NMR relaxation measurements have demonstrated that cholesterol markedly broadens the deuterium resonances from specifically deuterated fatty acids intercalated in sonicated EYPC vesicles (Stockton et al., 1974, 1976). This result suggests that the behavior observed in this study for the envelopes containing the $^{13}C$ resonances of the unsaturated and aliphatic carbons of the fatty acyl chains is a result of motional restrictions in the fatty acyl chains caused by the presence of cholesterol in the bilayer.

Although it is clear that cholesterol restricts motion(s) in certain parts of the phospholipid molecule, the molecular basis of this restriction is difficult to define. For a carbon nucleus undergoing dipolar relaxation, relaxation rates are sensitive to motions of its $^{13}C$–$^1H$ internuclear vector(s). The internuclear vectors of phospholipid or cholesterol carbon nuclei contained in sonicated vesicles may undergo motions arising from a variety of sources. First, the vector may reorient as a result of internal motions within the phospholipid molecule, or within the aliphatic side chain or methyl groups of the cholesterol molecule. A variety of internal motions are expected to occur, ranging from rigid-body motions of the fatty acyl chain, to kink diffusion, to simple methyl group rotation. Second, the vector may undergo motions as a result of the entire cholesterol or phospholipid molecule tilting, rotating, or diffusing within the bilayer. Third, the internuclear vector may undergo motion due to the overall tumbling in solution of the sonicated vesicle. The dependence of the line widths upon the mole fraction of cholesterol could arise from restrictions in local motions of the phospholipid molecules, and/or alterations in the conformation of phospholipid molecules, and/or from the effect of increasing vesicle radius. Vesicles containing larger mole fractions of cholesterol have an increased average radius (Gent & Prestegard, 1974a,b; Newman & Huang, 1975); consequently, their rotational correlation times are expected to be longer, and the changes observed in the $^{13}C$ line widths may be partly associated with the longer correlation time for vesicle rotation. The sensitivity of the natural line width of protonated carbons to the rate of vesicle tumbling is highly dependent upon the degree to which the internal motion averages the dipolar interactions. For groups undergoing rapid, unrestricted motion within the bilayer (such as the choline methyl groups), the line widths of the carbon nuclei are not sensitive to the rate of vesicle tumbling. For groups undergoing more restricted internal motion(s), the line widths may be expected to show marked dependence on the vesicle tumbling rate. Since the motions of the lipids within the bilayer are not yet sufficiently well characterized to write an exact correlation function describing the motion of the pertinent $^{13}C$–$^1H$ internuclear vector, the contribution from the effect of increasing vesicle size is difficult to estimate. However, there are two arguments that suggest that the line-width changes observed at least partly reflect restrictions in local motions.

Several investigators have suggested that $^{13}C$ and $^{31}P$ line widths from lipids in sonicated vesicles may be expected to have two contributions, one depending on the local anisotropic

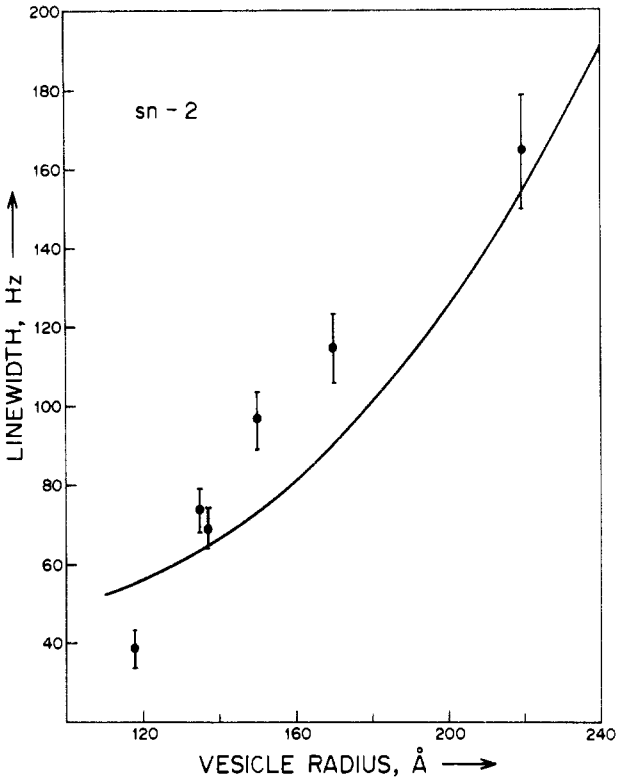


FIGURE 7: Variation in the line width of the *sn*-2 glycerol backbone $^{13}C$ resonance with vesicle radius. (●) Experimental data; (—) best nonlinear least-squares fit to the equation $LW = Ar^3 + B$ (see text).

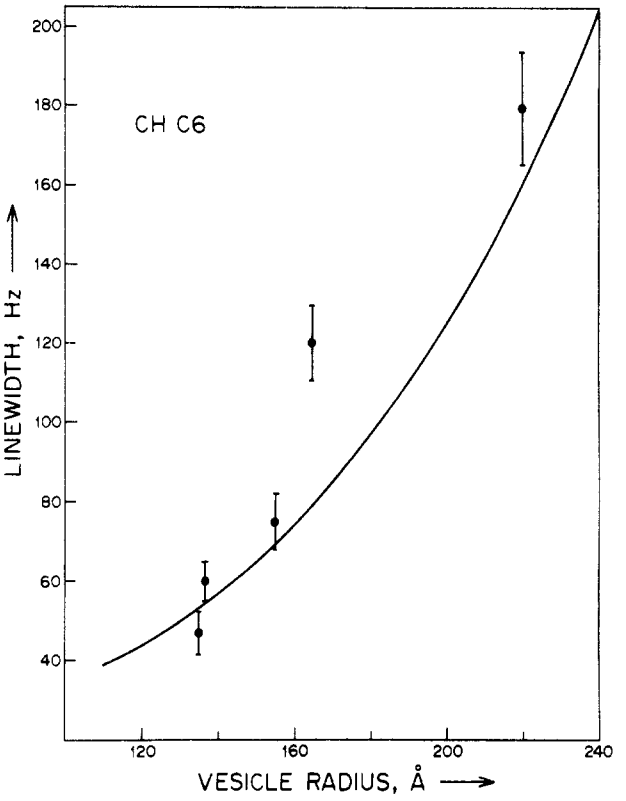


FIGURE 8: Variation in the line width of the cholesterol C6 $^{13}C$ resonance with vesicle radius. (●) Experimental data; (—) best nonlinear least-squares fit to the equation $LW = Ar^3 + B$.

motions of lipids in the bilayer and the other depending on the isotropic motion of the lipids due to vesicle tumbling and lateral diffusion of the lipids in the curved bilayers. Following the derivations of McLaughlin et al. (1975b), Cullis (1976), and de Kruijff (1978), it may be shown that the line widths may be expected to vary as

$$LW \propto \frac{1}{T_2} = \frac{M_2 r^2}{6[kT/(8\pi\eta r) + D_t]} + C \qquad (3)$$

where $M_2$ is the residual second moment, $C$ is that portion of the spin–spin relaxation time which is independent of vesicle tumbling, $k$ is the Boltzmann constant, $T$ is the absolute temperature, $\eta$ is the viscosity of the suspending medium, and $D_t$ is the lateral diffusion rate. If the effect of lateral diffusion is neglected,[2] the line width should vary as

$$LW \propto \frac{1}{T_2} = \frac{M_2 8\pi\eta}{6kT} r^3 + C \qquad (4)$$

Consequently, the line-width data should show a cubic dependence on the vesicle radius.

Best nonlinear least-squares fits of the observed line widths to a cubic equation of the form $LW = Ar^3 + B$ for $A, B > 0$ were performed by using the radii for various EYPC–CH vesicles of different composition reported by Gent & Prestegard (1974a,b). Figures 7 and 8 compare the calculated best least-squares fit with the experimentally observed line widths for the *sn*-2 glycerol backbone carbon resonance and for the cholesterol C6 carbon resonance. The experimentally observed line widths deviate significantly from the predicted cubic dependence on the vesicle radius, suggesting that vesicle size alone is not responsible for the observed line-width dependence. However, McLaughlin et al. (1975b) assume in their derivation that vesicle tumbling is much slower than the local anisotropic motions. While this assumption is clearly of value for the fast motion ($\tau_C \cong 10^{-11}$ s) which determines spin–lattice relaxation in sonicated vesicles, estimates of correlation times for the slower motions ($\tau_C \cong 10^{-7}$ s) (Gent & Prestegard, 1977; Petersen & Chan, 1977) are only an order of magnitude faster than the correlation times predicted from Stokes law for vesicle rotation [$(1–8) \times 10^{-6}$ s]. Consequently, it is not clear that separation of vesicle tumbling effects from effects of slow anisotropic motion is valid in this case. Additionally, there are significant uncertainties associated with determining the vesicle radius, lateral diffusion rates, and line widths. Hence, this treatment must be regarded as approximate. While it suggests that contributions from both increasing vesicle size and more restricted local motions are responsible for the observed line-width behavior, further experiments and more refined calculations are necessary to more firmly establish the contributions to line broadening from the two different sources.

The conclusion that cholesterol does restrict the local motions of lipids in sonicated vesicles is also corroborated by results from $^1H$, $^2H$, and $^{13}C$ NMR studies on unsonicated liposomes (Seiter & Chan, 1973; Lancee-Hermkens & de Kruijff, 1977; Oldfield et al., 1978; Stockton et al., 1976; Stockton & Smith, 1976), where the particle tumbling rates are too slow to affect the quadrupolar splittings or the NMR relaxation rates. These results have indicated that the presence of cholesterol markedly restricts the local motions and order of the phospholipid molecules in these model systems.

We turn now to discussion of the dynamics of cholesterol in sonicated phospholipid vesicles. Recently, several investigators have reported the observation of resonances from cholesterol within model membrane systems. Opella et al.

[2] This assumption is justified since lateral diffusion rates (Edidin, 1974) are <10% of rotational diffusion rates, $kT/(8\pi\eta r)$, for EYPC–CH vesicle tumbling.

(1976) have observed the $^{13}C$ resonances from cholesterol molecules enriched with $^{13}C$ at carbons 4 and 26 within unsonicated DPPC liposomes, using cross-polarization techniques. de Kruijff (1978) has observed the $^{13}C$ resonance from cholesterol enriched at C4 within sonicated phospholipid vesicles. In addition, Oldfield et al. (1978) and Gally et al. (1976) have observed the deuterium resonance from cholesterol-$3\alpha$-$d_1$ in unsonicated DMPC and DPPC liposomes. Kroon et al. (1975) observed the proton spectrum of cholesterol by incorporating it into bilayer vesicles composed of DPPC molecules in which the fatty acyl chains were 99% deuterated. These investigations illustrate the intense interest in the dynamics of the cholesterol molecule within bilayer systems, as well as the advantages associated with using cholesterol as a spectroscopic probe for the structure and dynamics of model membrane systems. To our knowledge, the present study is the first to report the detection of $^{13}C$ resonances at natural abundance from carbon nuclei of cholesterol within bilayer systems. Previous investigators have suggested that $^{13}C$ resonances from cholesterol carbons in sonicated vesicles were severely broadened by dipolar interactions and were undetectable under their experimental conditions (Godici & Landsberger, 1975; Yeagle et al., 1977).

The dependence of the cholesterol C6 line width upon the mole fraction of cholesterol (Figure 3) suggests that motions of the cholesterol molecule which determine the carbon spin–spin relaxation rate are restricted by increasing amounts of cholesterol within the bilayer. Again, the molecular basis for this restriction is difficult to define. The line-width increase could result from the decrease in the rate of those motions responsible for spin–spin relaxation, from decrease in the angular excursions of the sterol ring system within the bilayer, from the effect of increasing vesicle size, or from any combination of these effects. We believe that the increase in the C6 line width at least partly reflects restrictions in the local motions of the sterol ring system within the bilayer, on the basis of considerations similar to those discussed for phospholipid carbon resonances.

The observation that the cholesterol C6 $^{13}C$ resonance is detectable under conditions where the resonances from carbon atoms C3, C9, and C14,17 are not detectable may be interpreted in terms of the dynamics of the cholesterol ring system within the bilayer.

There are several possible explanations for the observation that resonances from carbon atoms C3, C9, and C14,17 from cholesterol in EYPC vesicles are not detectable in our $^{13}C$ NMR spectra. First, the chemical shifts of the C3, C9, and C14,17 resonances in sonicated vesicles may be sufficiently different from their values in organic solvents so that the resonances lie in regions of the spectrum where they are obscured by phospholipid resonances. We consider this unlikely for a number of reasons: (1) The existence of an interaction with sufficient strength to shift the carbon resonances 3–4 ppm from their values in organic solvents is unprecedented. It is likely that the magnetic environment within the hydrocarbon interior of the bilayer is very similar to that found in organic solvents. (2) The chemical shifts of the C3, C9, and C14,17 carbon resonances from cholesterol in triolein and complexed with EYPC in triolein are not significantly different from their values in organic solvents (N. O. Oppenheimer and E. H. Cordes, unpublished experiments). (3) None of the chemical shifts of cholesterol carbon resonances which *are detectable* in EYPC vesicles are shifted significantly from their values in organic solvents. It is unlikely that some carbon resonances would be shifted while other resonances would not. (4) Lastly, resonances from carbons C9 and C14,17 of epicholesterol in sonicated EYPC vesicles are detectable, and their chemical shifts are not significantly different from the values in chloroform. We consider it unlikely that the interactions of epicholesterol in the hydrophobic part of the bilayer are very different from those of cholesterol and hence expect the chemical shift of carbon atoms 9, 14, and 17 to be similar for both stereoisomers.

A second explanation for the failure to detect resonances from carbons C3, C9, and C14,17 is that the spin–lattice relaxation time may be so long that appreciable relaxation does not occur between $^{13}C$ excitation pulses, resulting in saturation of these resonances. This explanation also seems unlikely, since the resonances could not be detected when the pulse interval was increased up to 3.2 s.

A third possibility is that the spin–spin relaxation time for these carbons may be so short that the resulting resonances are broadened to such an extent that they are undetectable. We regard this as the most likely explanation. The motion of cholesterol carbons within the bilayer is expected to be restricted due to its rigid ring structure. Consequently, dipolar interactions of carbon nuclei with neighboring protons are predicted to be strong and the resulting resonances broad. A number of investigators have previously suggested that resonances from ring carbons of cholesterol contained in bilayers are broadened so that they are undetectable in high-resolution spectra (Keough et al., 1973; Williams & Cordes, 1976; Godici & Landsberger, 1975; Yeagle et al., 1977; Williams et al., 1973). de Kruijff (1978) has recently used cholesterol enriched in $^{13}C$ at C4 to facilitate the detection of the $^{13}C$ signal in sonicated vesicles and found that the resulting $^{13}C$ resonance was indeed very broad, 100–350 Hz.

Brainard & Szabo (1981) have described calculations comparing dipolar contributions to the line width of cholesterol methine carbon resonances for several representative orientations of the $^{13}C$–$^{1}H$ internuclear vector with respect to the long axis of the sterol ring system. These calculations indicate that significant differences in the line widths of methine carbon resonances may result from the anisotropic rotation of cholesterol within the bilayer and the differing orientations of the internuclear vectors. Specifically, they propose that rotation of the cholesterol molecule about its long axis is more effective in reducing the dipolar spin–spin relaxation rate for C6 than for C3 and C9, since the C6 internuclear vector is oriented very close to the "magic" angle from the long axis, whereas the C3 and C9 internuclear vectors are oriented approximately 90° from the long axis of the sterol ring system. Consequently, the C6 resonance from cholesterol is detected while the C3, C9, and C14,17 resonances are too broad to be detected under our experimental conditions. In addition, Brainard and Szabo propose that the equality of the C5 and C6 line widths observed experimentally could be accounted for by dominant contributions from chemical shift anisotropy to the line widths of both C5 and C6. The observed field dependence of the cholesterol C5 and C6 line widths supports the hypothesis that spin–spin relaxation of both cholesterol C5 and C6 has significant contributions from chemical shift anisotropy. It is important to emphasize that rotation of the cholesterol molecule about its long axis reduces the dipolar contributions to the C6 line width with respect to the dipolar contributions to the line width of other methine carbons, and with respect to the chemical shift anisotropy contribution to the C6 line width.

$^{13}C$ NMR spectra of cholesterol within sonicated EYPC vesicles show resonances for both angular methyl groups of the sterol (i.e., C18,19). This observation suggests that these

groups undergo considerable rotational motion about their 3-fold symmetry axis, since if these groups were significantly restricted in their rotation their $^{13}C$ resonances would be expected to be as broad as those for most of the protonated carbons of the rigid ring system and would not be detectable under our experimental conditions. Additionally, the $^{13}C$ NMR spectra contain evidence for significant internal motion of the aliphatic tail of cholesterol. Although the only resonance in our spectra that may be unambiguously assigned to a carbon nucleus in the aliphatic tail region is the cholesterol C21 methyl resonance at 18.9 ppm, the resonances at approximately 39.7 and 36.7 ppm may be expected to contain significant contributions from cholesterol C20, C22, and C24 of the aliphatic side chain. Akiyama et al. (1980) have recently demonstrated increased mobility of the terminal methyl groups of the cholesterol aliphatic tail using cholesterol-*26,26,26,27,27,27-d*$_6$ ($S_{mol}$ = 0.015 at 33 mol % cholesterol) in EYPC multilayers, compared to the sterol ring system of cholesterol-3-*d* in DMPC multilayers ($S_{mol} \cong 0.67$ at 33 mol % cholesterol) (Oldfield et al., 1978).

$^{13}C$ NMR spectra of epicholesterol–EYPC sonicated vesicles are remarkably similar to spectra of cholesterol–EYPC vesicles at temperatures below 32 °C (Figure 7). The spectra of epicholesterol within EYPC vesicles show a broad sterol C6 resonance of approximately the same line width as the cholesterol C6 resonance at similar temperatures. In addition, spectra of epicholesterol-containing vesicles also show the characteristic differential line broadening for the *sn*-1 and *sn*-3 carbons of the phospholipid glycerol backbone. These results suggest that below 32 °C, the dynamics of epicholesterol within the bilayer and its effect upon phospholipid dynamics are similar to those of cholesterol, at least within the sensitivity of $^{13}C$ NMR. These results are surprising in light of the spin-label experiments of Butler et al. (1970), where sterols having 3-hydroxyl groups with $\alpha$ stereochemistry showed no effect on the order parameter of 3-doxylcholestane in multilayer films. The reasons for the difference between the $^{13}C$ NMR and spin-label results are not clear, but may well be related to structural differences between vesicles and multilayers, and/or the sensitivities and the time scales of the measurements.

At higher temperatures, however, significant differences appear between $^{13}C$ NMR spectra of EYPC vesicles containing cholesterol and epicholesterol (Figure 7). The observation of $^{13}C$ resonances from C9 and C14,17 of epicholesterol suggests that the motion of epicholesterol within EYPC vesicles is significantly less restricted than the motion of cholesterol at similar temperatures. In addition, the differential line broadening observed for the phospholipid *sn*-1 and *sn*-2 carbons of the phospholipid glycerol backbone is decreased in epicholesterol–EYPC vesicles relative to cholesterol–EYPC vesicles.

Numerous studies have established the importance of the 3$\beta$-hydroxyl group for many of the characteristic effects of sterols on phospholipid bilayers and monolayers (Demel & Kruyff, 1976). However, the molecular basis for this importance is still controversial. The most popular view suggests that the 3$\beta$-hydroxyl group forms a linear hydrogen bond with the carbonyl oxygen(s) of the phospholipid ester bonds (Brockerhoff, 1974; Huang, 1976, 1977a,b). Alternatively, de Kruyff has suggested that the stereochemistry at C3 may be crucial in determining the hydration of the 3-hydroxyl group at the bilayer surface and that this hydration is of considerable importance in the sterol–phospholipid interaction (de Kruyff et al., 1973). Our results confirm that epicholesterol and cholesterol differ significantly in their interactions with phospholipids, particularly with respect to the anisotropy and/or rate of sterol motion within the bilayer. Yeagle et al. (1977) have demonstrated that lanosterol, a sterol which lacks a planar $\alpha$ face, also has considerably more mobility than cholesterol within phospholipid vesicles. The observation that sterols lacking those structural features required for producing several of the established effects of cholesterol on the functional properties of phospholipid bilayers also exhibit different dynamics within phospholipid bilayers than cholesterol strengthens the hypothesis that the dynamics of the constituent lipids are closely related to membrane function.

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