Biochemistry

 Volume 14, Number 18 September 9, 1975

^{13}C Nuclear Magnetic Resonance Study of the Dynamic Structure of Lecithin–Cholesterol Membranes and the Position of Stearic Acid Spin-Labels†

Patrick E. Godici‡ and Frank R. Landsberger*,§

ABSTRACT: The dynamic structure of phosphatidylcholine–cholesterol bilayers has been investigated by measuring the ^{13}C nuclear spin–lattice relaxation times and line widths of sonicated egg yolk lecithin–cholesterol (2:1) dispersions. The present studies suggest that cholesterol diminishes the long-range swinging motion of the fatty acyl chains but does not appreciably affect the rate of rapid rotational isomerizations. Data are presented which suggest that the motion of the polar head groups of the lecithin bilayer is independent of cholesterol content. Furthermore, the positioning of the C_5 stearic acid derivative spin-label is not detectably altered by the incorporation of cholesterol into phosphatidylcholine bilayers. Spin-label electron spin resonance studies of the partitioning of a small nitroxide, Tempo (2,2,6,6-tetramethylpiperidinyl-1-oxy), between the aqueous and lipid phases suggest that the lecithin–cholesterol (2:1) vesicles are largely in a fluid state at physiological temperatures.

Cholesterol is a major component of many membranes. Its exact biological function has not been fully elucidated. Model membrane studies, however, have been useful in gaining an understanding of the nature of the cholesterol–phospholipid interaction. The gel → liquid crystalline phase transition characteristic of the "melting" of the phospholipid fatty acyl chains is broadened by the addition of cholesterol. It has been reported that at a phosphatidylcholine (PC)[1]–cholesterol molar ratio of 2:1 the transition vanishes (Engelman and Rothman, 1972; Hinz and Sturtevant, 1972). Lippert and Peticolas (1971) have suggested that cholesterol decreases the interaction between adjacent hydrocarbon chains, making the phase transition a noncooperative event. In the gel phase, cholesterol fluidizes the phosphatidylcholine hydrocarbon chains (Oldfield and Chapman, 1971, 1972; Keough et al., 1973) by disrupting the tight hydrocarbon chain packing and thus inhibiting crystallization of the chains (Ladbrooke et al., 1968). In the liquid crystalline phase, however, cholesterol decreases the lipid chain flexibility (Chapman and Penkett, 1966; Long et al., 1970; Darke et al., 1971, 1972; Levine and Wilkins, 1971; Oldfield and Chapman, 1971, 1972; Lee et al., 1972; McConnell and McFarland, 1972; Mendelsohn, 1972; Rothman and Engelman, 1972; Keough et al., 1973; Saito et al., 1973; Gent and Prestegard, 1974). The decreased fluidity of the phosphatidylcholine hydrocarbon chains correlates with the condensation observed in PC monolayers upon addition of cholesterol (Shah and Schulman, 1967; Chapman et al., 1969; Tinoco and McIntosh, 1970; Ghosh et al., 1971, 1973; Demel et al., 1972a,b; Ghosh and Tinoco, 1972). The interaction is large for naturally occurring 1-saturated 2-unsaturated lecithins (Ghosh et al., 1971) and depends on the number and distribution of double bonds in the phosphatidylcholine (Demel et al., 1972b). Studies of the lipid bilayers of influenza and parainfluenza virions have similarly suggested that an increase in the cholesterol content decreases the fluidity of the bilayer in biological membranes (Landsberger et al., 1973).

Previously, using ^{13}C nuclear magnetic resonance (NMR) of stearic acid derivative spin-labeled vesicles, the long-range swinging motion of the fatty acyl chains in sonicated aqueous egg yolk phosphatidylcholine (EYL) dispersions has been discussed (Godici and Landsberger, 1974). In this paper, we report the effect of cholesterol on the ^{13}C NMR spectrum of the lipid fatty acyl chains and the polar head groups in EYL–cholesterol (2:1) dispersions. The results suggest that the presence of cholesterol does not inhib-

† From the Department of Chemistry, Indiana University, Bloomington, Indiana 47401. *Received March 10, 1975.* This work was supported by National Science Foundation Grant GB-43872 and U.S. Public Health Service Grant RR7031.

§ Present address: The Rockefeller University, New York, New York 10021.

‡ Supported by a Predoctoral Fellowship from the Indiana Heart Association. Present address: Exxon Research and Engineering Co., Linden, New Jersey 07036

[1] Abbreviations used are: PC, phosphatidylcholine; EYL, egg yolk phosphatidylcholine; Tempo, 2,2,6,6-tetramethylpiperidinyl-1-oxy; CPK, Corey-Pauling-Koltun.

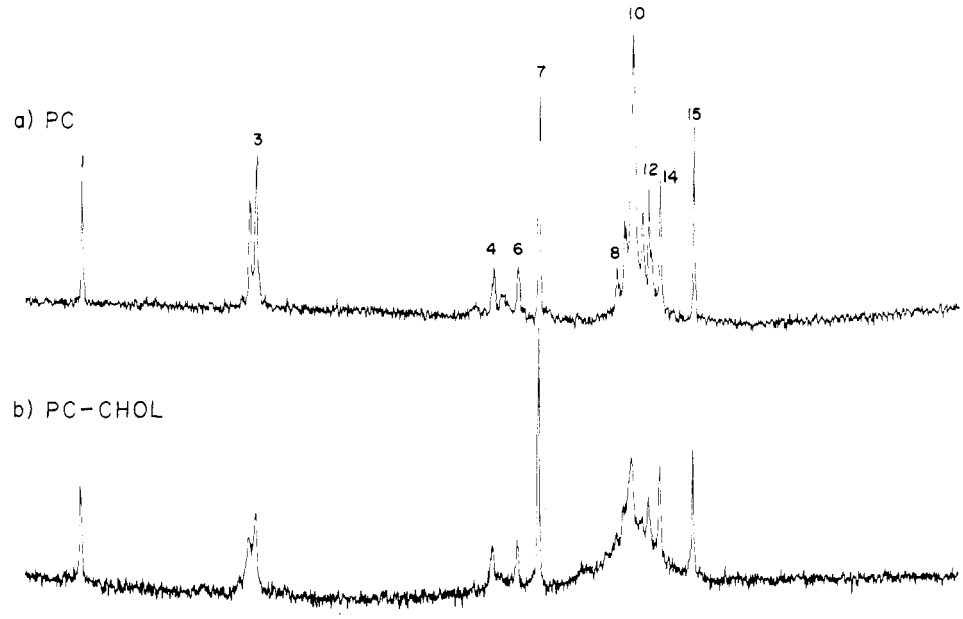
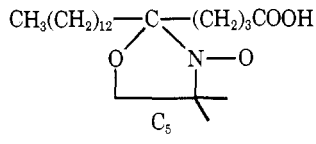


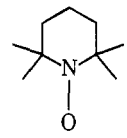
FIGURE 1: Normal Fourier transform ^{13}C NMR spectra of sonicated aqueous dispersions of (a) egg yolk phosphatidylcholine (PC) (2048 accumulations) and (b) PC-cholesterol (2:1) (4096 accumulations). The chemical shift range is 5-250 ppm upfield from CS_2.

it localized high frequency motions of the lecithin chains, such as rapid rotations about carbon-carbon bonds, but that the amplitude of the long-range swinging motion of the fatty acyl chains is diminished. The greatest reduction of the long-range swinging motion seems to occur in the central portion of the fatty acyl chains including the olefinic positions, while the perturbation of the motion of the methyl chain terminus is much smaller. The mobility of the choline moiety appears to be unaffected by the presence of cholesterol. It is also suggested that the position of the stearic acid spin-labels in lipid biolayers is not detectably altered by the addition of cholesterol.

Materials and Methods

Egg yolk phosphatidylcholine was isolated from fresh egg yolk according to the procedure of Singleton et al. (1965) with only minor modifications. The stearic acid derivative spin-label C_5 was purchased from Syva Corporation, Palo





Tempo

Alto, Calif. Tempo (2,2,6,6-tetramethylpiperidinyl-1-oxy) was synthesized as described by Briere et al. (1965). Cholesterol, purchased from the Hormel Institute, Austin, Minn. (>99% pure), and stored desiccated at −20°, was used without further purification. The cholesterol was demonstrated to be free of autoxidation products by thin-layer chromatography on silica gel G plates (J. T. Baker Chemical Company, Phillipsburg, N.J.) developed in hexane-ethyl acetate (1:1) (Smith and Foell, 1962), in acetone-hexane (1:1) (Smith et al., 1967), and in petroleum ether-ethyl ether-acetic acid (35:65:2) (Fioriti and Sims, 1967).

The stearic acid spin-labeled and unlabeled EYL-cholesterol (2:1) vesicle preparations were obtained by sonication of a dispersion of the lipids in 5 m*M* Tris-100 m*M* KCl buffer followed by centrifugation as previously described (Godici and Landsberger, 1974). The stearic acid spin-label to EYL ratio was 1:60. The final phosphatidylcholine concentration in the EYL-cholesterol (2:1) preparations was 0.13 g/ml and in the pure EYL preparations was 0.2 g/ml. To determine the lecithin-cholesterol molar ratio after the centrifugation step, the supernatant from the centrifugation step was lyophilyzed and chromatographed on silica gel G plates developed in petroleum ether-diethyl ether-acetic acid (35:65:2). The bands were visualized with a uv lamp and removed. The cholesterol was eluted with diethyl ether and the lecithin was eluted with methanol (Christie et al., 1970). Gravimetric analysis indicated the lecithin-cholesterol molar ratio to be 2.0 ± 0.3.

In the Tempo partitioning experiments, 80 μl of 5×10^{-3} *M* aqueous Tempo solution was combined with 0.5 ml of the EYL-cholesterol dispersion. The sample was transferred to a 50-μl capillary pipet used as a sample cell.

All electron spin resonance (ESR) spectra were recorded with a Varian E-4 ESR spectrometer equipped with a Varian V-6040 variable temperature controller. The temperature was measured using a copper-constantan thermocouple with a Leeds and Northrup potentiometer. The ^{13}C NMR measurements were obtained with a home-built spectrometer operating at 15.08 MHz, as previously described (Allerhand et al., 1971), at approximately 34°. The spin-lattice (T_1) relaxation times were measured using a $\pi-\tau-\pi/2$ pulse sequence (Vold et al., 1968), where τ is the delay time between the π and $\pi/2$ pulses. The pulse sequence was repeated after a delay at least four times greater than the largest T_1 value to be measured. The usual procedure was to obtain either 2048 or 4096 accumulations prior to a 4K Fourier transform.

Results

The normal Fourier transform spectra of sonicated EYL and EYL-cholesterol (2:1) dispersions are given in Figure 1. The ^{13}C resonance assignments have been previously reported (Metcalfe et al., 1971; Birdsall et al., 1972; Sears, 1972; Stoffel et al., 1972; Hamilton et al., 1973; Godici and Landsberger, 1974), and are given in Table I. No ^{13}C NMR resonances corresponding to those reported for cholesterol (Reich et al., 1969) are detected in the EYL-cho-

Table I: ^{13}C NMR Parameters of Sonicated EYL and EYL–Cholesterol Dispersions.[a]

Peak[b] No.	Assignment[f]	$(T_1)_o$ (sec) ±10%[f]	(T_1)chol (sec) ±15%	$(T_1)_{C_5}$ (sec) ±10%[f]	$(T_1)_{C_5}$chol (sec) ±15%	$(1/T_{1N})_5$ (sec^{-1}) ±15%[f]	$(1/T_{1N})_5$chol (sec^{-1}) ±20%	$\Delta\nu_{1/2}^{lec}$ (sec^{-1})[d]	$\Delta\nu_{1/2}$chol (sec^{-1})[d]	$1/T_{2C}$ (sec^{-1})
1	>C=O	1.8	1.9	0.38	0.26	2.1	3.3	9 ± 1	9 ± 1	0 ± 4
2	$-CH=\underline{C}H-CH_2-CH_2-$	0.57	0.50	0.34	0.35	1.2	0.9	11 ± 2		
3	$-CH=\underline{C}H-CH_2-\underline{C}H=CH-$	0.75	0.65	0.39	0.32	1.2	1.6	11 ± 2	16 ± 2	16 ± 9
4	Choline $-N-\underline{C}H_2-$	0.30						16 ± 1	17 ± 1	3 ± 4
5	Glycerol $-\underline{C}H_2-$									
6	Choline $-O-\underline{C}H_2-$	0.41						11 ± 1	11 ± 1	0 ± 4
7	Choline $-N(\underline{C}H_3)_3$	0.62	0.57	0.37	0.33	1.1	1.3	9 ± 1	9 ± 1	0 ± 4
8	$-\underline{C}H_2-CO_2-$	0.26								
9	$-\underline{C}H_2-CH_2-CH_3$	0.64		0.48		0.52				
10[c]	Fatty acyl $(CH_2)n$	0.40	0.43	0.29	0.30	0.95	1.0	19± 2	31 ± 2	38 ± 9
11	$-CH=CH-\underline{C}H_2-CH_2$	0.53	0.52	0.35	0.30	0.97	1.4	9 ± 2		
12	$-CH=CH-\underline{C}H_2-CH=CH-$	0.66	0.49	0.40	0.30	0.98	1.3	7 ± 2	17 ± 2	31 ± 9
13[e]	$\underline{C}H_2-CH_2-CO_2-$									
14	$-\underline{C}H_2-CH_3$	1.4	1.2	0.88	0.9	0.42	0.28	7 ± 1	11 ± 1	13 ± 4
15	$-CH_3$	2.8	2.5	1.3	1.6	0.43	0.23	6 ± 1	7 ± 1	3 ± 4

[a] The spin–lattice relaxation times represent averages of between two and four separate determinations; an estimate of the percentage error is given. The line widths represent averages of up to eight separate determinations; the estimated uncertainties are given. [b] Corresponds to labeling in Figure 1. [c] Methylene resonance of composite envelope. [d] $\Delta\nu_{1/2}^{lec} = (1/\pi)(1/T_2^*)_o$ and $\Delta\nu_{1/2}$chol $= (1/\pi)\cdot (1/T_2^*)$chol are the line widths at half-height for the EYL and EYL–cholesterol dispersions, respectively. [e] Shoulder. [f] Godici and Landsberger, 1974.

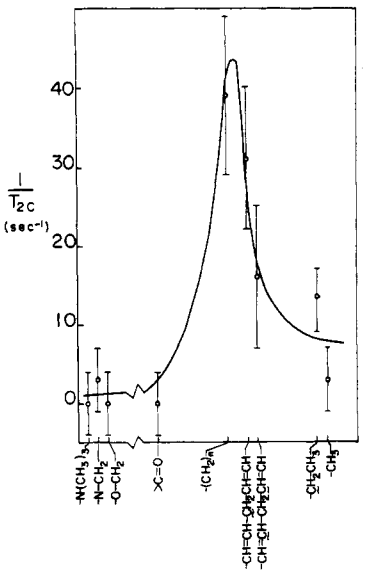


FIGURE 2: Plot of $1/T_{2C}$ vs. approximate carbon position in the extended EYL molecule.

lesterol spectrum which presumably reflects the relative immobility of the rigid steroid structure. The following comparisons can be made between the two spectra: (1) The fatty acyl methylene and olefinic resonances are markedly decreased in amplitude and broadened upon addition of cholesterol to EYL. (2) The effect of cholesterol on the resonance line width of the chain terminal methyl group is less marked with the line remaining relatively intense and not significantly broadened. (3) Within experimental resolution, there is little effect on the choline resonances and the fatty acyl carbonyl group. These qualitative observations are in agreement with the results of ^1H NMR studies (Chapman and Penkett, 1966; Darke et al., 1971, 1972; Lee et al., 1972).

The numerical line widths for the ^{13}C resonance of EYL and EYL–cholesterol (2:1) are given in Table I. For Lorentzian line shapes, the line width at half-height $\Delta\nu_{1/2}$ is given by (Farrar and Becker, 1971)

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

The quantity $1/T_2^*$ is a function of both the nuclear spin–spin relaxation time T_2 and the instrumental broadening T_2'

$$\frac{1}{T_2^*} = \frac{1}{T_2} + \frac{1}{T_2'} \tag{2}$$

The magnitude of the increase of the EYL resonance line widths resulting from the incorporation of cholesterol may be defined by

$$\frac{1}{T_{2C}} \equiv \left(\frac{1}{T_2^*}\right)^{chol} - \left(\frac{1}{T_2^*}\right)_o \tag{3}$$

where the "o" and "chol" refer to the EYL and EYL–cholesterol samples, respectively. Since T_2' is relatively constant, the parameter $1/T_{2C}$ is assumed to be essentially independent of the instrumental broadening. Although several of the resonances have contributions from chemically inequivalent carbons, it is unlikely that a change in the chemical shift inequivalence could account for the observed

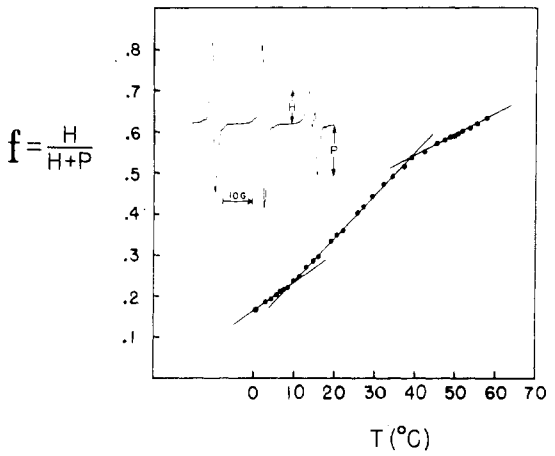


FIGURE 3: Plot of the Tempo spectral parameter f vs. temperature. The inset, an ESR spectrum of Tempo in a sonicated EYL-cholesterol dispersion, defines the spectral parameter f.

broadening upon the incorporation of cholesterol. For example, the carbonyl resonance, arising from inequivalent carbons, does not broaden upon inclusion of cholesterol in the lipid bilayer.

A plot of $1/T_{2C}$ vs. approximate position in the phospholipid is given in Figure 2. It should be noted that EYL consists of a heterogeneous distribution of fatty acyl chains. Since the length of the esterified fatty acyl chains as well as the position and degree of unsaturation vary in EYL, there are uncertainties in the precise position along the abscissa assigned to a given $1/T_{2C}$ value and in the ordering of the points corresponding to the carbon nuclei in the olefinic region of the chain. However, approximately 90% of the esterified fatty acids at the glycerol 1 position are palmitic (16:0) and stearic (18:0), and at the 2 position are oleic (18:1) and linoleic (18:2) (Tattrie et al., 1968) (detailed discussion in Godici and Landsberger, 1974). It can be seen that the largest changes occur in the main fatty acyl methylene envelope and in the olefinic resonances. The effect decreases rapidly near the methyl chain terminus. The polar head group region and the carbonyl group of the EYL fatty acyl chain are not significantly affected by cholesterol.

From the resonance amplitudes of partially relaxed Fourier transform spectra, the spin-lattice relaxation times (T_1) were calculated as previously described (Godici and Landsberger, 1974). The results are given in Table I. Within experimental error, the spin-lattice relaxation times of EYL are not affected by the inclusion of cholesterol in EYL vesicles.

It may be assumed that the inverse of the spin-lattice relaxation time of a particular nucleus in a C_5 spin-labeled lipid dispersion is the sum of two parts, one due to the relaxation mechanism present in the absence of the spin-label and the other due to the presence of the unpaired electron of the nitroxide moiety. Thus, the contribution of the unpaired electron to the spin-lattice relaxation time of a particular nucleus in a C_5 spin-labeled EYL-cholesterol dispersion may be calculated from

$$\left(\frac{1}{T_{1N}}\right)_5^{chol} = \left(\frac{1}{T_1}\right)_5^{chol} - \left(\frac{1}{T_1}\right)^{chol} \quad (4)$$

where $(1/T_1)^{chol}$ and $(1/T_1)_5^{chol}$ are the reciprocals of the spin-lattice relaxation times of the EYL-cholesterol dispersions in the absence and presence of the spin-label, respectively. The subscript "5" corresponds to the C_5 nitroxide spin-label. Similarly, the contribution of the C_5 spin-label to the spin-lattice relaxation time of labeled EYL dispersions without cholesterol may be calculated from

$$\left(\frac{1}{T_{1N}}\right)_5 = \left(\frac{1}{T_1}\right)_5 - \left(\frac{1}{T_1}\right)_0 \quad (5)$$

where $(1/T_1)_0$ and $(1/T_1)_5$ are the inverses of the spin-lattice relaxation times of the EYL dispersions in the absence and presence of the spin-label, respectively (Godici and Landsberger, 1974). The calculated values of $(1/T_{1N})_5$ and $(1/T_{1N})_5^{chol}$ are given in Table I. The perturbation of the C_5 spin-label on the spin-lattice relaxation times of the EYL ^{13}C nuclei follows a similar trend in EYL vesicles both with and without cholesterol inclusion. The observed differences arising from the addition of cholesterol to the lipid bilayer are (1) the effect of the C_5 nitroxide label on the EYL carbonyl group is increased and (2) the effect on the chain terminal methylene and methyl groups is decreased.

Shimshick and McConnell (1973b) have recently suggested that in cholesterol containing dipalmitoyllecithin and dimyristoyllecithin membranes there can coexist both a fluid and a solid phase at a temperature above the phase transition temperature of the pure phospholipid. In order to determine whether lateral phase separation of the lipids (Shimshick and McConnell, 1973a,b; Shimshick et al., 1973) might also occur in the EYL-cholesterol (2:1) vesicles, the partitioning of the nitroxide spin-label Tempo between the aqueous and lipid phases was recorded as a function of temperature. The Tempo spectral parameter (Shimshick and McConnell, 1973a), f, is given by

$$f = H/(H + P) \quad (6)$$

where H and P are measured from the experimental electron spin resonance (ESR) spectra (cf. inset Figure 3) and are to a first approximation proportional to the partitioning of Tempo in the lipid and aqueous phases, respectively. A plot of the Tempo spectral parameter vs. temperature is given in Figure 3. It contains points obtained by both heating and cooling. The abrupt changes in the slope of the f vs. T plot at 9° and 39° (indicated by the intersection of the solid lines in Figure 3) correspond to the onset and completion of a temperature dependent structural change for the EYL-cholesterol dispersion (Shimshick and McConnell, 1973b). (The same discontinuities are observed in an f vs. $1/T$ plot.)

Discussion

Construction of an approximate phase diagram of the EYL-cholesterol system (Shimshick and McConnell, 1973b) suggests that the present NMR data correspond largely to the fluid phase of the EYL-cholesterol (2:1) vesicles. The discontinuities observed in the plot of the Tempo spectral parameter f vs. temperature, at 9° and 39° (cf. Figure 3), correspond to points on the boundaries between a relatively solid (S) system, a system characterized by the coexistence of fluid and solid regions (F and S), and a fluid system (F) (Shimshick and McConnell, 1973a,b; Shimshick et al., 1973). Thus, at 34°, the temperature at which the NMR measurements were made, the lipid membrane appears to be composed of approximately 85% F phase and 15% S phase. The mole fractions of cholesterol at 34° may be estimated to be $X_{chol} \simeq 0.5$ in the S phase and $X_{chol} \simeq 0.3$ in the F phase (see footnote 2). Consequently, the distri-

bution of the total EYL between the F and S phases is roughly 90 and 10%, respectively at 34°. The ^{13}C NMR spectrum of EYL–cholesterol (1:1 molar ratio) at 27° (all lipids presumably in the S-phase) indicates that the fatty acyl chain resonances are dramatically diminished in intensity and broadened (barely detectable above the base line) whereas the trimethylammonium resonance remains relatively intense. Based on these observations, it appears that the solid phase of the EYL–cholesterol (2:1) system contributes less than 3% to the observed ^{13}C NMR spectral intensity and that the spectrum essentially reflects the state of the fluid component. Although arguments based on integrated intensities of ^{13}C NMR spectra are difficult, comparison of the approximate relative integrated intensities of the phospholipid resonances from vesicles of pure EYL and of EYL–cholesterol (2:1) indicates approximate agreement with the Tempo experiments and does not appear to support the model proposed by Darke et al. (1972) and Phillips and Finer (1974) in which 1:1 EYL–cholesterol complexes coexist with uncomplexed EYL in cholesterol-containing lecithin membranes. Similarly, Shimshick and McConnell (1973b) using spin-label methods and Lee et al. (1972, 1974) using ^{1}H NMR have found no evidence for 1:1 lecithin–cholesterol complexes coexisting with free lecithin in lecithin–cholesterol membranes.

Cholesterol has a dramatic effect on the ^{13}C NMR spectrum of EYL (cf. Figure 1). The nature of the perturbation of the motion of the EYL fatty acyl chains and polar head group by cholesterol can be ascertained by inspecting the effect of cholesterol on the EYL T_1 values and line widths. The ^{13}C nuclear spin–lattice relaxation times of the EYL fatty acyl chain and choline carbons are essentially unaffected by the incorporation of cholesterol into the lipid bilayer (cf. Table I). Cholesterol does cause a significant broadening of the fatty acyl methylene and olefinic resonances with only a slight broadening of resonances from carbons near the end of the chain. The choline resonances are not detectably broadened by cholesterol (cf. Figure 2 and Table I). The trend of the $1/T_{2C}$ plot is in general agreement with the plot of rigidity vs. chain position obtained from NMR line-width measurements (Darke et al., 1972; Finer, 1972) and spin-label ESR results (McConnell and McFarland, 1972) which suggest that the motion is most restricted for the region extending from approximately the glycerol backbone into the central portion of the lecithin hydrocarbon chains.

In both sonicated and unsonicated PC dispersions, the ^{1}H spin–spin relaxation time is less than the spin–lattice relaxation time (Horwitz et al., 1972; Feigenson and Chan, 1974). These results have been interpreted in terms of the anisotropic motion of the PC fatty acyl chains (Chan et al., 1972; Horwitz et al., 1972, 1973; Seiter and Chan, 1973; Feigenson and Chan, 1974). Two correlation times have been considered (Chan et al., 1972; Horwitz et al., 1972, 1973; Seiter and Chan, 1973; Feigenson and Chan, 1974). One corresponds to the motion of the fatty acyl chain axis ($\tau_\perp$) and the other corresponds to the reorientation of a group about the chain axis ($\tau_\parallel$) (Chan et al., 1972; Horwitz et al., 1972, 1973; Seiter and Chan, 1973; Feigenson and Chan, 1974) with $\tau_\parallel < \tau_\perp$.

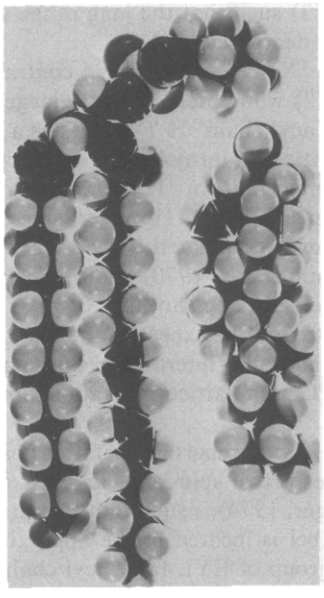


FIGURE 4: CPK models of lecithin (with palmitic and linoleic acids as the esterified fatty acids) and cholesterol. The relative vertical position is only approximate.

Although the absolute magnitudes of the ^{13}C spin–spin relaxation times have not been measured, it would appear from crude estimates that $T_2 < T_1$. At present, it is difficult to interpret quantitatively resonance line widths. However, differences in the ^{13}C NMR line widths may be assumed to reflect changes in the slower components of the motion (e.g., the motion of the local chain axis), whereas the ^{13}C T_1 values reflect the relatively faster molecular reorientations (e.g., rotations about carbon–carbon bonds) (Farrar and Becker, 1971).

In Figure 4, CPK models of an EYL and a cholesterol molecule are shown positioned next to each other as they might be in a lipid vesicle bilayer. On the basis of this figure, it is possible to suggest an explanation of the $1/T_{2C}$ and T_1 data in terms of the effect cholesterol has on the motion of the EYL head group and fatty acyl chains. The motion of the trimethylammonium group of the choline moiety is not sterically hindered by cholesterol and thus no change is detected in the line width and in the T_1 value upon addition of cholesterol. However, cholesterol severely inhibits the long range swinging motion of the carbons in the central region of the fatty acyl chains, due to the presence of its bulky rigid steroid nucleus. In comparison, the carbons near the methyl chain terminus will be less hindered. The fact that the lecithin T_1 values remain essentially unchanged in the presence of cholesterol suggests that the frequency of rotations about the fatty acyl carbon–carbon bonds is not affected by cholesterol. Thus, the line width and T_1 data taken together indicate that although in the presence of cholesterol the rate of rotation about carbon–carbon bonds in the fatty acyl chains is unaltered, those rotations are probably to some extent coupled (i.e., compensatory) (Hor-

[2] These mole fractions can be obtained from an approximate phase diagram constructed as follows. The solidus curve for a cholesterol mole fraction (X_{chol}) greater than $\frac{1}{3}$ may be naively estimated by a linear extrapolation from 9° ($X_{chol} = \frac{1}{3}$) to 148.5° (melting point of pure cholesterol) (Shimshick and McConnell, 1973a,b; Shimshick et al., 1973). Similarly, the fluidus curve may be approximated by a straight line connecting the three points ($X_{chol} = 1$, $T = 148.5°$), ($X_{chol} = \frac{1}{3}$, $T = 39°$), and ($X_{chol} = 0$, $T \simeq -5°$). [A preliminary EYL–cholesterol phase diagram using the Tempo procedure (Shimshick and McConnell, 1973a,b; Shimshick et al., 1973) agrees with the results obtained from the approximate diagram discussed here (H. C. Hasenkampf III, personal communication).]

witz et al., 1973) such that the long range swinging motion of the chains is decreased.

The present ^{13}C T_1 results are in contrast with those of Lee et al. (1972) who observed a very large perturbation of the EYL fatty acyl chain ^1H T_1 values as a result of cholesterol incorporation. Intermolecular interactions of protons appear to be important for ^1H relaxation in sonicated lipid vesicles (Lee et al., 1973). However, since the ^{13}C relaxation mechanism is dominated by the C–H dipolar interaction (Kuhlmann et al., 1970) intermolecular interactions may be relatively unimportant for ^{13}C relaxation in lipid bilayers. Thus, it might be expected, if cholesterol alters phospholipid intermolecular interaction, that a change in the ^1H but not the ^{13}C spin-lattice relaxation time might be observed.

Previously, it was reported that the position of the C_5 stearic acid derivative spin-label in EYL bilayers (Godici and Landsberger, 1974) is such that the nitroxide moiety of the C_5 spin-label is located in the approximate vicinity of the carbonyl group of EYL fatty acyl chain. The $(1/T_{1N})_5$ data (cf. Table I) show that for the EYL–cholesterol as well as for the EYL dispersions, the greatest perturbation of the EYL ^{13}C nuclear spin-lattice relaxation times by the C_5 label occurs at the fatty acyl carbonyl position. The effect of the C_5 label on the trimethylammonium T_1 value is the same in both EYL and EYL–cholesterol dispersions. Furthermore, with and without cholesterol inclusion in the lipid bilayer, the general trends of $(1/T_{1N})_5$ are similar. These results suggest that the positioning of the nitroxide moiety of the C_5 spin-label is not detectably altered by the addition of cholesterol to EYL vesicles.

Since at present no adequate theory has been developed for discussing ^{13}C relaxation processes in lipid bilayers, it is not possible to quantitatively interpret the $1/T_{1N}$ data. For the isotropic case, $1/T_{1N}$ depends on $1/r^6$ where r is the distance between the unpaired electron and a given ^{13}C nucleus, and is a function of the correlation time for the dipolar electron–nuclear interaction (Solomon, 1955; Bernheim et al., 1959). Furthermore, several important variables possibly need to be considered in comparing the $(1/T_{1N})_5$ values of the lecithin and lecithin–cholesterol dispersions: (1) differences in the proximity between the nitroxide and a given ^{13}C nucleus, (2) alteration in the amplitudes of the motion of both the lecithin and the spin-label fatty acyl chains, (3) differences in the rate of lateral diffusion of the lipids (Lee et al., 1973; Devaux and McConnell, 1972), and (4) variations in the local spin-label concentration due to phase separation of the lipids. Although ESR experiments indicate that the stearic acid derivative spin-labels are soluble to some extent in the solid phase, the degree of partitioning of the labels between the two phases has not been determined. Thus, at the present time it is not possible to interpret the $1/T_{1N}$ data in any detail.

Acknowledgments

The authors gratefully acknowledge Wendy F. Boss for the synthesis of Tempo and Holly Willett for her expert assistance in the preparation of the manuscript.

References

Allerhand, A., Doddrell, D., and Komoroski, R. (1971), *J. Chem. Phys. 55,* 189.

Bernheim, R. A., Brown, T. H., Gutowsky, H. S., and Woessner, D. E. (1959), *J. Chem. Phys. 30,* 950.

Birdsall, N. J. M., Feeney, J., Lee, A. G., Levine, Y. K., and Metcalfe, J. C. (1972), *J. Chem. Soc., Perkin Trans. 2,* 1441.

Brière, R., Lemaire, H., and Rassat, A. (1965), *Bull. Soc. Chim. Fr., Ser. 5e,* 3273.

Chan, S. I., Seiter, C. H. A., and Feigenson, G. W. (1972), *Biochem. Biophys. Res. Commun. 46,* 1488.

Chapman, D., Owens, N. F., Phillips, M. C., and Walker, D. A. (1969), *Biochim. Biophys. Acta 183,* 458.

Chapman, D., and Penkett, S. A. (1966), *Nature (London) 211,* 1304.

Christie, W. W., Noble, R. C., and Moore, J. H. (1970), *Analyst 95,* 940.

Darke, A., Finer, E. G., Flook, A. G., and Phillips, M. C. (1971), *FEBS Lett. 18,* 326.

Darke, A., Finer, E. G., Flook, A. G., and Phillips, M. C. (1972), *J. Mol. Biol. 63,* 265.

Demel, R. A., Bruckdorfer, K. R., and van Deenen, L. L. M. (1972a), *Biochim. Biophys. Acta 255,* 311.

Demel, R. A., Geurts Van Kessel, W. S. M., and van Deenen, L. L. M., (1972b), *Biochim. Biophys. Acta 266,* 26.

Devaux, P., and McConnell, H. M. (1972), *J. Am. Chem. Soc. 94,* 4475.

Engelman, D. M., and Rothman, J. E. (1972), *J. Biol. Chem. 247,* 3694.

Farrar, T. C., and Becker, E. D. (1971), Pulse and Fourier Transform NMR, New York, N.Y., Academic Press.

Feigenson, G. W., and Chan, S. I. (1974), *J. Am. Chem. Soc. 96,* 1312.

Finer, E. G. (1972), *Chem. Phys. Lipids 8,* 327.

Fioriti, J. A., and Sims, R. J. (1967), *J. Am. Oil Chem. Soc. 44,* 221.

Gent, M. P. N., and Prestegard, J. H. (1974), *Biochemistry 13,* 4027.

Ghosh, D., Lyman, R. L., and Tinoco, J. (1971), *Chem. Phys. Lipids 7,* 173.

Ghosh, D., and Tinoco, J. (1972), *Biochim. Biophys. Acta 266,* 41.

Ghosh, D., Williams, M. A., and Tinoco, J. (1973), *Biochim. Biophys. Acta 291,* 351.

Godici, P. E., and Landsberger, F. R. (1974), *Biochemistry 13,* 362.

Hamilton, J. A., Talkowski, C., Williams, E., Avila, E. A., Allerhand, A., Cordes, E. H., and Camejo, G. (1973), *Science 180,* 193.

Hinz, H., and Sturtevant, J. M. (1972), *J. Biol. Chem. 247,* 3697.

Horwitz, A. F., Horsley, W. J., and Klein, M. P. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 590.

Horwitz, A. F., Klein, M. P., Michaelson, D. M., and Kohler, S. J. (1973), *Ann. N.Y. Acad. Sci. 222,* 468.

Keough, K. M., Oldfield, E., Chapman, D., and Beynon, P. (1973), *Chem. Phys. Lipids 10,* 37.

Kuhlmann, K. F., Grant, D. M., and Harris, R. K. (1970), *J. Chem. Phys. 52,* 3439.

Ladbrooke, B. D., Williams, R. M., and Chapman, D. (1968), *Biochim. Biophys. Acta 150,* 333.

Landsberger, F. R., Compans, R. W., Choppin, P. W., and Lenard, J. (1973), *Biochemistry 12,* 4498.

Lee, A. G., Birdsall, N. J. M., Levine, Y. K., and Metcalfe, J. C. (1972), *Biochim. Biophys. Acta 255,* 43.

Lee, A. G., Birdsall, N. J. M., and Metcalfe, J. C. (1973), *Biochemistry 12,* 1650.

Lee, A. G., Birdsall, N. J. M., and Metcalfe, J. C. (1974), *Methods Membr. Biol. 2,* 1.

Levine, Y. K., and Wilkins, M. H. F. (1971), *Nature (Lon-*

don) *New Biol. 230,* 69.
Lippert, J. L., and Peticolas, W. L. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 1572.
Long, R. A., Hruska, F., Gesser, H. D., Hsia, J. C., and Williams, R. (1970), *Biochem. Biophys. Res. Commun. 41,* 321.
McConnell, H. M., and McFarland, B. G. (1972), *Ann. N.Y. Acad. Sci. 195,* 207.
Mendelsohn, R. (1972), *Biochim. Biophys. Acta 290,* 15.
Metcalfe, J. C., Birdsall, N. J. M., Feeney, J., Lee, A. G., Levine, Y. K., and Partington, P. (1971), *Nature* (*London*) *233,* 199.
Oldfield, E., and Chapman, D. (1971), *Biochem. Biophys. Res. Commun. 43,* 610.
Oldfield, E., and Chapman, D. (1972), *FEBS Lett. 23,* 285.
Phillips, M. C., and Finer, E. G. (1974), *Biochim. Biophys. Acta 356,* 199.
Reich, H. J., Jautelat, M., Messe, M. T., Weigert, F. J., and Roberts, J. D. (1969), *J. Am. Chem. Soc. 91,* 7445.
Rothman, J. E., and Engelman, D. M. (1972), *Nature* (*London*) *New Biol. 237,* 42.
Saito, H., Schreier-Mucillo, S., and Smith, I. C. P. (1973), *FEBS Lett. 33,* 281.
Sears, B. (1972), Ph.D. Thesis, Indiana University.
Seiter, C. H. A., and Chan, S. I. (1973), *J. Am. Chem. Soc. 95,* 7541.
Shah, D. O., and Schulman, J. H. (1967), *J. Lipid Res. 8,* 215.
Shimshick, E. J., Kleemann, W., Hubbell, W. L., and McConnell, H. M. (1973), *J. Supramol. Struct. 1,* 285.
Shimshick, E. J., and McConnell, H. M. (1973a), *Biochemistry 12,* 2351.
Shimshick, E. J., and McConnell, H. M. (1973b), *Biochem. Biophys. Res. Commun. 53,* 446.
Singleton, W. S., Gray, M. S., Brown, L. N., and White, J. L. (1965), *J. Am. Oil. Chem. Soc. 42,* 53.
Smith, L. L., and Foell, T. (1962), *J. Chromatogr. 9,* 339.
Smith, L. L., Matthews, W. S., Price, J. C., Bachmann, R. C., and Reynolds, B. (1967), *J. Chromatogr. 27,* 187.
Solomon, I. (1955), *Phys. Rev. 99,* 559.
Stoffel, W., Zierenberg, O., and Turggal, B. D. (1972), *Hoppe-Seyler's Z. Physiol. Chem. 354,* 1962.
Tattrie, N. H., Bennett, J. R., and Cyr, R. (1968), *Can. J. Biochem. 46,* 819.
Tinoco, J., and McIntosh, D. J. (1970), *Chem. Phys. Lipids 4,* 72.
Vold, R. L., Waugh, J. S., Klein, M. P., and Phelps, D. E. (1968), *J. Chem. Phys. 48,* 3831.

Properties of Several Sterically Modified Retinal Analogs and Their Photosensitive Pigments†

T. Ebrey,* R. Govindjee, B. Honig, E. Pollock, W. Chan, R. Crouch, A. Yudd, and K. Nakanishi

ABSTRACT: Retinal analogs which have extra (14-methylretinal and methylretinone) or displaced (13-desmethyl-14-methylretinal) methyl groups were synthesized and their isomers isolated by high-pressure liquid chromatography. The 11-cis isomer of 14-methylretinal has its λ_{max} 30 nm to shorter wavelengths than 11-*cis*-retinal; we suggest that this is due to the fact that the 14-methyl analog is unable to assume the same twisted s-cis conformation available to 11-*cis*-retinal. Thus, the seemingly anomalous long-wavelength position of the absorption band of 11-*cis*-retinal is due to its being primarily in a twisted ($\phi_{12-13} \simeq 40°$) s-cis conformation. Three isomers of 14-methylretinal, 11-cis, 9-cis, and 9,13-di-cis, reacted with cattle opsin to form photosensitive pigments. The absorption spectra, circular dichroism spectra, extinction coefficients, and photosensitivities of the 11-cis pigments of retinal (rhodopsin) and 14-methylretinal were similar. This suggests that the conformation of these two chromophores, when attached to opsin, is similar and different from that of crystalline 11-*cis*-retinal. The 9-cis isomer of 13-desmethyl-14-methylretinal also combines with opsin. No reaction of opsin with any isomer of methylretinone (15-methylretinal) was detected.

The 11-cis isomer of retinal (or its 3,4-dehydro derivative) is the chromophore of all known visual pigments (Wald, 1968). A great deal is understood about the spectroscopic properties of retinals and it now appears that many of the important differences between their spectra and those of visual pigments can be explained in terms of the protonated Schiff-base linkage that is formed between the retinal and a lysine of the apoprotein opsin. However, the detailed spectroscopic and photochemical properties of visual pigments are not well understood. In addition, a number of important problems pertaining to retinal itself are unresolved (see Honig and Ebrey, 1974; Ebrey and Honig, 1975; Honig et al., 1975). In this paper we continue our studies of the spectroscopic and photochemical properties of sterically modified retinal analogs and the photosensitive pigments that result from their combination with opsin (Chan et al., 1974).

In the absence of steric hindrance, π-electron systems may be assumed to be planar. All isomers of retinal have a

† From the Department of Physiology and Biophysics, University of Illinois, Urbana, Illinois 61801 (T.E. and R.G.), the Department of Physical Chemistry, Hebrew University, Jerusalem, Israel (B.H. and E.P.), and the Department of Chemistry, Columbia University, New York, New York 10027 (W.C., R.C., A.Y., and K.N.). *Received March 7, 1975.* Supported by Public Health Service Grants EYO-1323 and EYO-1253.