Breslow, E. (1964) *J. Biol. Chem. 239*, 486–496.
Conn, H. L., Jr. (1961) *J. Appl. Physiol. 16*, 1065–1070.
Emsley, J. W., Feeny, J., & Sutcliffe, L. H. (1965) *High Resolution Nuclear Magnetic Resonance Spectroscopy*, Vol. 1, Pergamon Press, Oxford.
Ewing, G. J., & Maestas, S. (1970) *J. Phys. Chem. 74*, 2341–2344.
Frauenfelder, H., Petsko, G. A., & Tsernoglou, D. (1979) *Nature (London) 280*, 558–563.
Kretsinger, R. H., Watson, H. C., & Kendrew, J. C. (1968) *J. Mol. Biol. 31*, 305–314.
Lakowicz, J. R., & Weber, G. (1973) *Biochemistry 12*, 4161–4179.
Miller, K. W., Reo, N. V., Uiterkamp, A., Stengle, D. P., Stengle, T. R., & Williamson, K. L. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4946–4949.
Parkhurst, L. (1979) *Annu. Rev. Phys. Chem. 30*, 503–546.
Samejima, T., & Yang, J. T. (1964) *J. Mol. Biol. B*, 863–871.
Schoenborn, B. P. (1965) *Nature (London) 208*, 760–762.
Schoenborn, B. P. (1969) *J. Mol. Biol. 45*, 297–303.
Schoenborn, B. P., Watson, H. C., & Kendrew, J. C. (1965) *Nature (London) 207*, 28–30.
Schulman, R. G., Peisach, J., & Wyluda, B. J. (1970) *J. Mol. Biol. 48*, 517–523.
Smith, D. W., & Williams, R. J. P. (1968) *Biochem. J. 110*, 297–301.
Sudmeier, J. L., Evelhoch, J. L., & Jonsson, B. H. (1980) *J. Magn. Reson. 40*, 377–390.

Temperature-Dependent Molecular Motions of Cholesterol Esters: A Carbon-13 Nuclear Magnetic Resonance Study†

Geoffrey S. Ginsburg, Donald M. Small, and James A. Hamilton*

ABSTRACT: Carbon-13 NMR spectroscopy at 50.3 MHz has been used to study four long-chain cholesterol esters with a double bond in the ω-9 position: cholesteryl oleate, $C_{18:1}$, ω-9; cholesteryl linoleate, $C_{18:2}$, ω-6,9; cholesteryl erucate, $C_{22:1}$, ω-9; cholesteryl nervonate, $C_{24:1}$, ω-9. The linoleate and oleate esters exhibit two metastable liquid-crystalline phases (cholesteric and smectic), whereas the longer chain esters form a stable smectic phase but no cholesteric phase [Ginsburg, G. S., & Small, D. M. (1981) *Biochim. Biophys. Acta 664*, 98–107]. Line widths ($\nu_{1/2}$), spin–lattice relaxation times (T_1), and nuclear Overhauser enhancements (NOE) were measured for all well-resolved resonances from ring and fatty acyl (FA) carbons at different temperatures in the isotropic liquid of each ester. T_1 and NOE values of FA resonances were constant between the FA-2 carbon and olefinic region of each acyl chain and increased markedly for carbons near the chain terminus. FA carbon motions are thus restricted and/or highly correlated in the region between the ring and the olefinic carbons, suggesting that strong interactions occur between cholesterol ester molecules in this region of the FA chain. These results also suggest that the FA chains are approximately extended in the isotropic liquid. Steroid ring methine C-6 and C-3 $\nu_{1/2}$'s increased differentially on cooling to the liquid → liquid crystal transition temperature (T_m) of each ester, indicative of increasingly anisotropic ring rotations. The rotational anisotropy was quantitated by using a prolate ellipsoid model for the cholesterol ester molecule for which two correlation times (corresponding to rotations about the long and short molecular axes) were calculated from the C-3 and C-6 $\nu_{1/2}$ values. The C-3/C-6 $\nu_{1/2}$ ratio was directly proportional to the anisotropy of the ring motions as measured by the ratio of the two correlation times. At any given temperature relative to T_m, the C-3 and C-6 $\nu_{1/2}$'s and the C-3/C-6 $\nu_{1/2}$ ratios were larger for cholesterol esters which have a cholesteric phase than for esters which have no cholesteric phase, showing that steroid ring motions were more restricted and more anisotropic prior to the formation of a cholesteric phase. Cholesteryl erucate and cholesteryl nervonate have longer regions of FA chain interactions which result in greater chain cooperativity, apparently preventing the preordering of steroid rings to the degree necessary for formation of a cholesteric phase. Thus, these esters form the smectic phase directly from the isotropic liquid. These results are applied to the cholesterol ester transition in plasma low-density lipoproteins.

The physical state of cholesterol esters at physiological temperatures may be an important determinant in the development of atherosclerosis (Small, 1970). In systems such as cholesterol ester rich lipoproteins and atheromatous lesions, the cholesterol esters (primarily cholesteryl linoleate and cholesteryl oleate) undergo a phase transition from a smectic to a disordered phase at or above body temperature (Deckelbaum et al., 1975; Tall et al., 1978; Katz et al., 1976). However, in isolated pure systems, these cholesterol esters not only have slightly higher transition temperatures but also exhibit an additional intermediate cholesteric phase (Small, 1970; Ginsburg & Small, 1981). Elucidation of the factors which affect the phase behavior and transition temperature of pure cholesterol esters is thus important in order to understand the phase behavior of cholesterol esters in biological systems.

Several studies have shown that the domain size (Armitage et al., 1977) and the fatty acid composition of pure cholesterol esters influence their phase behavior (Small, 1970; Ginsburg & Small, 1981). In biological systems, such as plasma low-density lipoproteins (LDL),[1] the presence of other lipids such

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[1] Abbreviations: LDL, low-density lipoprotein; NMR, nuclear magnetic resonance; T_1, spin–lattice relaxation time; $\nu_{1/2}$, line width in hertz; NOE, nuclear Overhauser enhancement; Me_4Si (TMS in the figures), tetramethylsilane; FA, fatty acyl; C, cholesteryl ring carbons (standard steroid nomenclature used); T_m, temperature of the highest liquid → liquid crystal transition.

0006-2960/82/0421-6857$01.25/0

as triglycerides, as well as the cholesterol ester fatty acid composition, affects the phase behavior of the constituent cholesterol esters (Deckelbaum et al., 1977; Tall et al., 1978). However, the specific differences in cholesterol ester molecular motions and intermolecular interactions responsible for these effects are not known.

^{13}C nuclear magnetic resonance (NMR) spectroscopy at low magnetic field (24 kG) has been used to study the molecular dynamics of neat cholesterol esters as a function of temperature and has shown that well-resolved resonances from both the steroid ring system and the fatty acyl chain are observed in spectra of the isotropic liquid phase (Hamilton et al., 1977). Line-width measurements of individual steroid ring carbon resonances as a function of temperature showed that ring motions become increasingly anisotropic as the liquid → liquid-crystalline transition is approached. A similar line-width analysis was extended to cholesterol esters in LDL and in atherosclerotic plaques to study the liquid → liquid crystal transition in these systems (Hamilton et al., 1979).

In this report we present natural abundance ^{13}C NMR studies of the two most prevalent biological cholesterol esters, cholesteryl linoleate ($C_{18:2}$) and cholesteryl oleate ($C_{18:1}$), and two naturally occurring but much less common sterol esters, cholesteryl erucate ($C_{22:1}$) and cholesteryl nervonate ($C_{24:1}$). The fatty acyl chains of the four esters range in length from 18 to 24 carbons but have the common structural feature of the double bond in the ω-9 position. Additionally, cholesteryl linoleate has a double bond in the ω-6 position. The minor structural differences in this group of esters results in markedly different phase behaviors which alter both the phase transition temperatures and the nature of the phases formed (Ginsburg & Small, 1981). Upon cooling from the isotropic liquid, the linoleate and oleate esters form metastable cholesteric and smectic liquid-crystalline phases (Small, 1970), whereas the erucate and nervonate esters form a stable smectic liquid-crystalline phase and do not form a cholesteric phase (Ginsburg & Small, 1981).

Analysis of ^{13}C NMR spin–lattice relaxation times (T_1), line widths ($\nu_{1/2}$), and nuclear Overhauser enhancement (NOE) values of single-carbon resonances allowed detailed and quantitative comparisons of the molecular motions of the steroid ring and fatty acyl carbons of each ester. Further, a quantitative treatment of these data using a theoretical model for a prolate ellipsoid undergoing anisotropic motion has been used to obtain rotational diffusion correlation times of the steroid ring in the isotropic liquid state. The results show the relative importance of ring interactions and fatty acyl chain interactions in determining the phase behavior of these esters and can be used to understand the molecular motions of cholesterol esters in biological systems which do not exhibit a cholesteric phase such as plasma LDL.

Materials and Methods

Cholesteryl *cis*-9,10-octadecenoate (cholesteryl oleate), cholesteryl *cis*-9,10,*cis*-12,13-octadecadienoate (cholesteryl linoleate), cholesteryl *cis*-13,14-docosenoate (cholesteryl erucate), and cholesteryl *cis*-15,16-tetracosenoate (cholesteryl nervonate) were obtained from Nu Chek Prep, Inc. (Elysian, MN). Thin-layer chromatography of the esters in 1:4 diethyl ether–petroleum ether showed a single spot even with overloading (100–200 μg). ^{13}C NMR spectra of these cholesterol esters were consistent with other reported spectra of cholesterol esters (Hamilton et al., 1977; Sears et al., 1976; Reich et al., 1969). A small additional resonance at 32.6 ppm was present in some samples. This peak was assigned to the trans isomer (Gunstone et al., 1977) and amounted to ≤7% of the sample as measured by integrated peak area ratios. The phase behavior and transition temperatures of the purest cholesteryl erucate sample (<2% trans isomer) were identical with that for the least pure sample (7% trans isomer).

The phase transition temperatures of the cholesterol esters were measured by polarizing light microscopy as described before (Ginsburg & Small, 1981). Cholesteryl oleate and cholesteryl linoleate melted at 51.0 and 42.0 °C, respectively, and on cooling formed a cholesteric phase at 46.0 and 36.5 °C, respectively. The smectic phases formed at 41.0 and 34.0 °C, respectively. Cholesteryl erucate and cholesteryl nervonate both exhibited stable smectic phases which formed directly on heating from the crystal with no cholesteric phase. The crystal → smectic transitions for the erucate and nervonate esters were at 45.5 and 37.0 °C, respectively, and the smectic ↔ isotropic transitions were at 50.5 and 53.0 °C, respectively. The latter transitions were fully reversible with *no* undercooling; however, the smectic phase of cholesteryl erucate and nervonate could be undercooled to room temperature without crystallization (Ginsburg & Small, 1981). Our microscopy measurements agreed with the transitions observed by calorimetry, by macroscopic inspection of the NMR sample in a controlled temperature bath, and with literature values (Small, 1970; Ginsburg & Small, 1981) within ±1.0 °C.

NMR Spectroscopy. Natural abundance proton-decoupled Fourier transform ^{13}C NMR spectra were obtained at 47 kG (50.3 MHz for ^{13}C) with a Bruker WP-200 spectrometer equipped with an Aspect 2000 data system. Neat cholesterol esters were placed in 10-mm NMR tubes with C_6D_6 in a coaxial insert as the external lock. For spectra obtained in organic solvent, $CDCl_3$ was used as both solvent and lock, and tetramethylsilane (Me_4Si) was used as an internal reference for chemical shift assignments. Broad-band proton decoupling centered at 3.4 ppm downfield from the proton resonance of Me_4Si and 1.0-W decoupler power was used in the acquisition of routine spectra.

Sample temperature was controlled (±1 °C) with the Bruker B-VT-1000 variable-temperature unit. The NMR probe temperature was calibrated for these studies by using a nonionic, viscous sample equal in volume to that used for the esters (1.5 mL of ethylene glycol). After equilibration at different probe temperatures for 15 min under the same experimental conditions as employed for the cholesterol esters, the ethylene glycol sample was ejected from the probe, a thin thermocouple was inserted, and the temperature was recorded at 15-s intervals from the time of sample ejection for 3 min. The probe temperature was determined by extrapolation to zero time.

Neat cholesterol ester samples were melted to an isotropic liquid outside the probe and then allowed to equilibrate inside the probe for 20 min prior to acquisition of data. In the smectic or cholesteric phase of a given ester, identical spectra were obtained regardless of whether the liquid-crystalline phase was formed outside or within the probe. For the esters which formed stable mesophases, identical spectra were obtained when the smectic phase was formed upon heating from the crystal and upon cooling from the isotropic liquid. At the end of each accumulation the sample was removed from the probe and the presence of a single homogeneous phase in the sample was verified by visual inspection and by the macroscopic criteria for either the smectic or cholesteric phase (Gray, 1962).

T_1 values were measured by the fast-inversion recovery method (Canet et al., 1975) and calculated by using a program supplied by the Bruker Co. which utilized a nonlinear three-

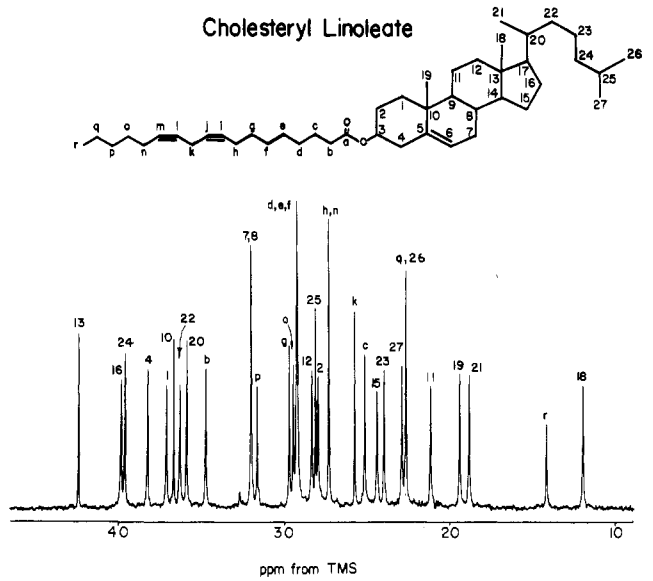

FIGURE 1: The 0–45-ppm region of the proton-decoupled natural abundance ^{13}C Fourier transform NMR spectrum of cholesteryl linoleate in deuteriochloroform (0.2 M) recorded at 50.3 MHz after 200 accumulations with the use of a 10000-Hz spectral width, 65536 time-domain addresses, and a recycle time of 3.28 s. Digital line broadening of 0.5 Hz was introduced to increase the signal-to-noise ratio. Numbered peaks corresponding to numbered carbons of the steroid moiety as denoted in the illustration above (standard steroid nomenclature used). Letters correspond to carbons on the fatty acyl moiety as indicated. The schematic drawing of the ester is not intended to suggest a preferred conformation of the molecule. Peaks from ring carbons 3, 5, 6, 9, 14, and 17 and from fatty acyl carbons a, i, j, l, and m occur downfield from 45 ppm and are not shown. The small unlabeled peak at 32.6 ppm is from the FA-8,14 carbons of the all-trans isomer.

parameter fit (Sass & Ziessow, 1977). NOE was measured as the ratio of the integrated intensities with broad-band decoupling to those with gated proton decoupling (Opella et al., 1976). To assure that both spectra were obtained at the same temperature, we used a program sequence which summed consecutively acquired broad-band decoupled scans and gated proton-decoupled scans, respectively, in different regions of computer memory. This procedure gave an equilibrated sample temperature intermediate between that obtained with full decoupling and gated decoupling.

Calculations of theoretical spin–lattice relaxation times, line widths, and nuclear Overhauser enhancement was performed on a Digital Equipment Corp. LSI-11/2 computer by using a modeling program provided by Dr. Daniel M. Quinn (personal communication).

Results

Natural abundance ^{13}C NMR spectra of the four cholesterol esters in $CDCl_3$ were highly resolved, as exemplified by the aliphatic region (0–45 ppm) from a spectrum of cholesteryl linoleate (Figure 1). Because of improved resolution compared to the previously published spectrum of cholesteryl linoleate in $CDCl_3$ at lower field (Sears et al., 1976), several new resonances were observed. Assignments for the steroid ring carbons were based on previous assignments for short-chain cholesterol esters and cholesterol in organic solvents (Reich et al., 1969). Assignments for fatty acyl chain carbons were based on assignments for fatty acids (Stoffel et al., 1972; Gunstone et al., 1977) and on rules for paraffins (Lindemann & Adams, 1971). In particular, the assignment of resonances for cholesterol ring carbons C-2, C-4, C-7, and C-8 were made for peaks at 27.8, 38.2, and 31.9 ppm (carbons C-7 and C-8 appeared as a single resonance). Peaks at 29.6 and 29.4 ppm were assigned to fatty acyl carbons FA-7 and FA-15, respectively (designated "g" and "o" in Figure 1). The two resonances at 29.2 and 29.1 ppm were assigned to the three remaining aliphatic carbons (FA-4, -5, and -6, designated "d", "e", and "f").

Natural abundance ^{13}C NMR spectra were recorded at several temperatures in the isotropic liquid state on cooling from 20 or 21 °C above the highest liquid-crystalline transition temperature (T_m) and at selected temperatures below T_m. Figures 2 and 3 show spectra of neat cholesteryl oleate and neat cholesteryl nervonate, respectively, at selected temperatures in the isotropic liquid (T_m + 10 °C, T_m + 1 °C) and in the liquid-crystal phase(s).

The isotropic liquid spectra are similar to those previously published for cholesteryl linoleate (Hamilton et al., 1977; Sears et al., 1976), but the improved resolution resulting from the higher magnetic field allowed a more accurate assessment of temperature-dependent changes in intensity, $\nu_{1/2}$, T_1, and NOE values. Compared with the spectra of dilute "monomeric" esters in chloroform (Figure 1), the spectra of the bulk-phase isotropic liquid at high temperature (T_m + 10 °C; Figures 2A and 3A) exhibited poorer spectral resolution because *all* resonances were significantly broader. However, the protonated steroid ring carbon resonances were broadened more than the protonated fatty acyl carbon resonances.

On cooling from T_m + 10 °C, most resonances broadened with a consequent loss of spectral resolution (Figures 2 and 3). The broadening was most marked for protonated steroid ring carbon resonances and occurred to a lesser degree for fatty acyl chain resonances from carbons close to the steroid ring, whereas negligible broadening was observed in fatty acyl chain resonances from carbons near the terminal methyl group. Futhermore, as T_m was approached on cooling, the steroid ring methine resonances (C-6, C-3, C-14,17, and C-9), which were well-resolved from other resonances, exhibited *differential* line broadening. Figure 4 shows a plot of $\nu_{1/2}$ as a function of temperature for the ring methine C-3 and C-6 resonances and for the fatty acyl ω-carbon resonance for all four cholesterol esters. For a given cholesterol ester, the C-14,17 and C-9 resonances had a line-width behavior nearly identical with that for the C-3 resonance (data not shown). For each ester, at any given temperature relative to the T_m, the C-3 resonance was broader than the C-6 resonance; additionally, the C-3/C-6 line-width ratio increased as a function of decreasing temperature in the isotropic liquid. In contrast, the $\nu_{1/2}$ of the ω-carbon resonance from the fatty acyl chain was unaffected by temperature changes between T_m + 20–21 °C and T_m. Similar differential line-broadening results have been reported for cholesteryl linoleate and cholesteryl oleate at lower magnetic field (Hamilton et al., 1977).

The line-width results in Figure 4 fall into two general groups. The C-3 and C-6 line widths and the C-3/C-6 line-width ratio at any given temperature relative to the T_m of each ester were smaller for the longer chain esters (nervonate and erucate) than for the esters of shorter chain length (oleate and linoleate). For example, cholesteryl nervonate and cholesteryl erucate had C-3/C-6 $\nu_{1/2}$ ratios of ~1.55 at T_m + 20 °C and 2.80 and 2.90, respectively, at T_m + 1 °C. Cholesteryl oleate and cholesteryl linoleate had a C-3/C-6 $\nu_{1/2}$ ratio of ~1.75 at T_m + 20 °C and 3.4 and 3.0, respectively, at T_m + 1 °C. Furthermore, the $\nu_{1/2}$ of C-3 resonance for cholesteryl oleate and cholesteryl linoleate at 1 °C above the isotropic → cholesteric transition (64 ± 4 Hz) was more than 2 times greater than for cholesteryl erucate and cholesteryl nervonate at 1 °C above the isotropic → smectic transition (23 ± 1 Hz). A

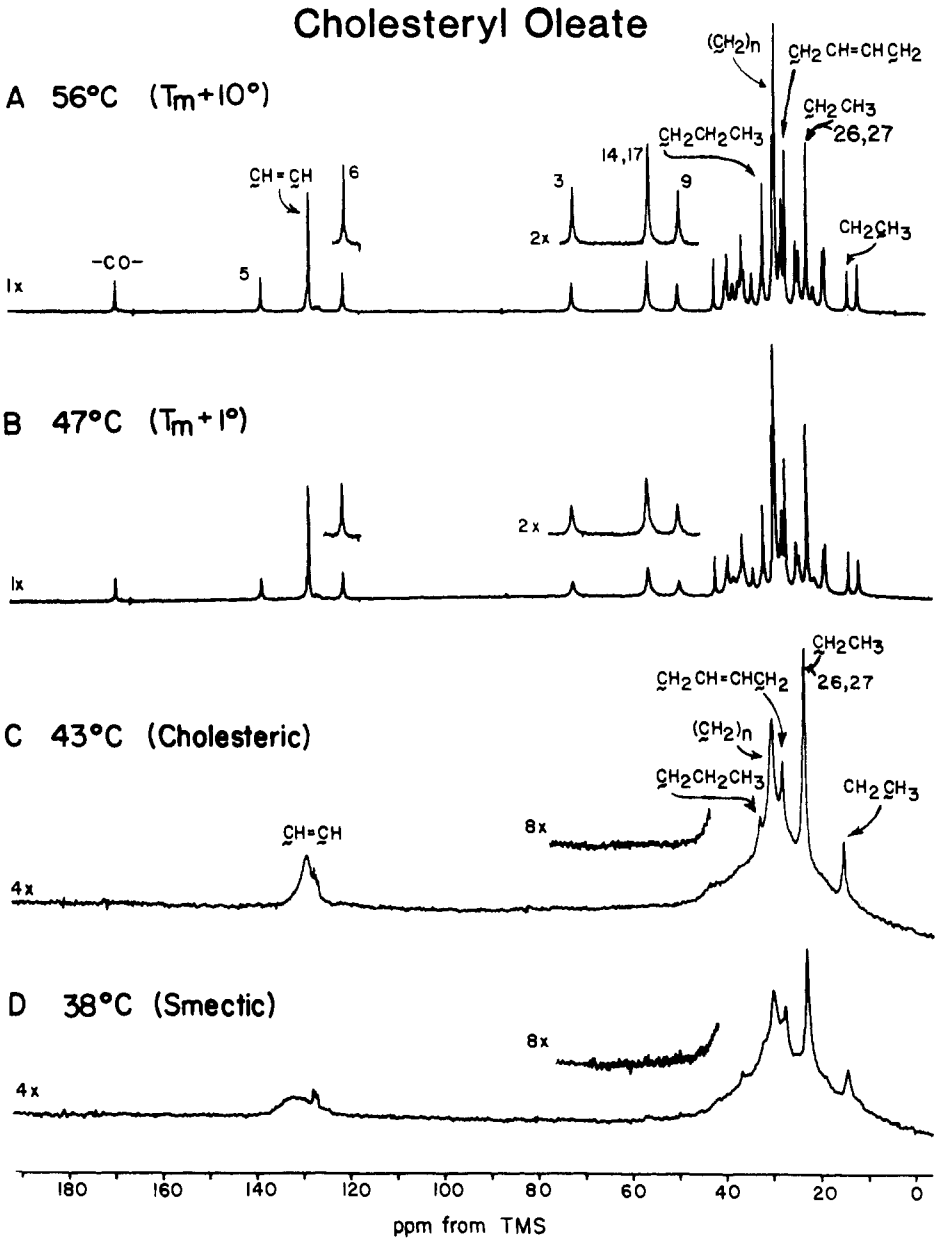

FIGURE 2: Proton-decoupled natural abundance ^{13}C Fourier transform NMR spectra in the isotropic liquid phase (A and B), the cholesteric phase (C), and the smectic phase (D) of (neat) cholesteryl oleate. Spectra were recorded at 50.3 MHz after 250 accumulations with the use of a 10000-Hz spectra width, 32768 time-domain addresses, and a recycle time of 1.62 s. Spectra A and B were recorded at 10 deg (T_m + 10 °C) and at 1 deg (T_m + 1 °C) relative to the isotropic liquid ↔ cholesteric phase transition temperature (T_m), and 1-Hz digital line broadening was applied to increase the signal-to-noise ratio. Spectra in the liquid-crystalline phases (C and D) were processed with 3-Hz digital line broadening. All spectra were normalized to spectrum A, and thus a 4-fold vertical expansion was used in printing spectra C and D. All insets were printed with a 2-fold vertical expansion of the main spectrum. The inset in spectrum D is from the same data set as the main spectrum except after 1500 accumulations. The assignments are indicated as follows: numbered peaks correspond to ring carbons as in Figure 1; fatty acyl carbons are indicated by chemical formula with the symbol ~ beneath the specific carbon(s) represented by the peak. The triplet at ~128.0 ppm is from the C_6D_6 lock.

similar result was obtained for the $\nu_{1/2}$ of the C-6 resonance.

For all four esters, cooling the sample *below* the T_m resulted in an abrupt broadening of the steroid ring resonances (generally beyond the limits of detection; see Figures 2C,D and 3C,D) and a differential broadening of the fatty acyl resonances. Resonances for the first two carbons of the fatty acyl chain (the carbonyl and the adjacent methylene group) were not detected, whereas resonances for the terminal methyl and adjacent methylene carbon were relatively narrow; the olefinic resonances were broad but detectable. The spectra of cholesteryl oleate in the cholesteric and smectic phases (Figure 2) were qualitatively similar; the same resonances were observed in each spectrum and a uniform broadening (~2-fold) of all resonances occurred below the cholesteric → smectic transition. Spectra of cholesteryl linoleate were obtained in the cholesteric phase (34 °C) and in the smectic phase (31 °C) (not shown). Contrary to an earlier report (Sears et al., 1976), no discernible resonances from steroid ring carbons were observed. The high-resolution features of the liquid-crystal spectra were entirely due to fatty acyl resonances from the double bond region and the terminal methyl region and were nearly identical with those obtained at lower field by Hamilton et al. (1977).

Spectra obtained for the smectic phase of cholesteryl nervonate at 45 and at 25 °C (undercooled) shown in Figure 3 were nearly identical; thus, there was no change in $\nu_{1/2}$ of observed resonances over a large temperature range once the stable smectic phase had formed. These spectra were similar

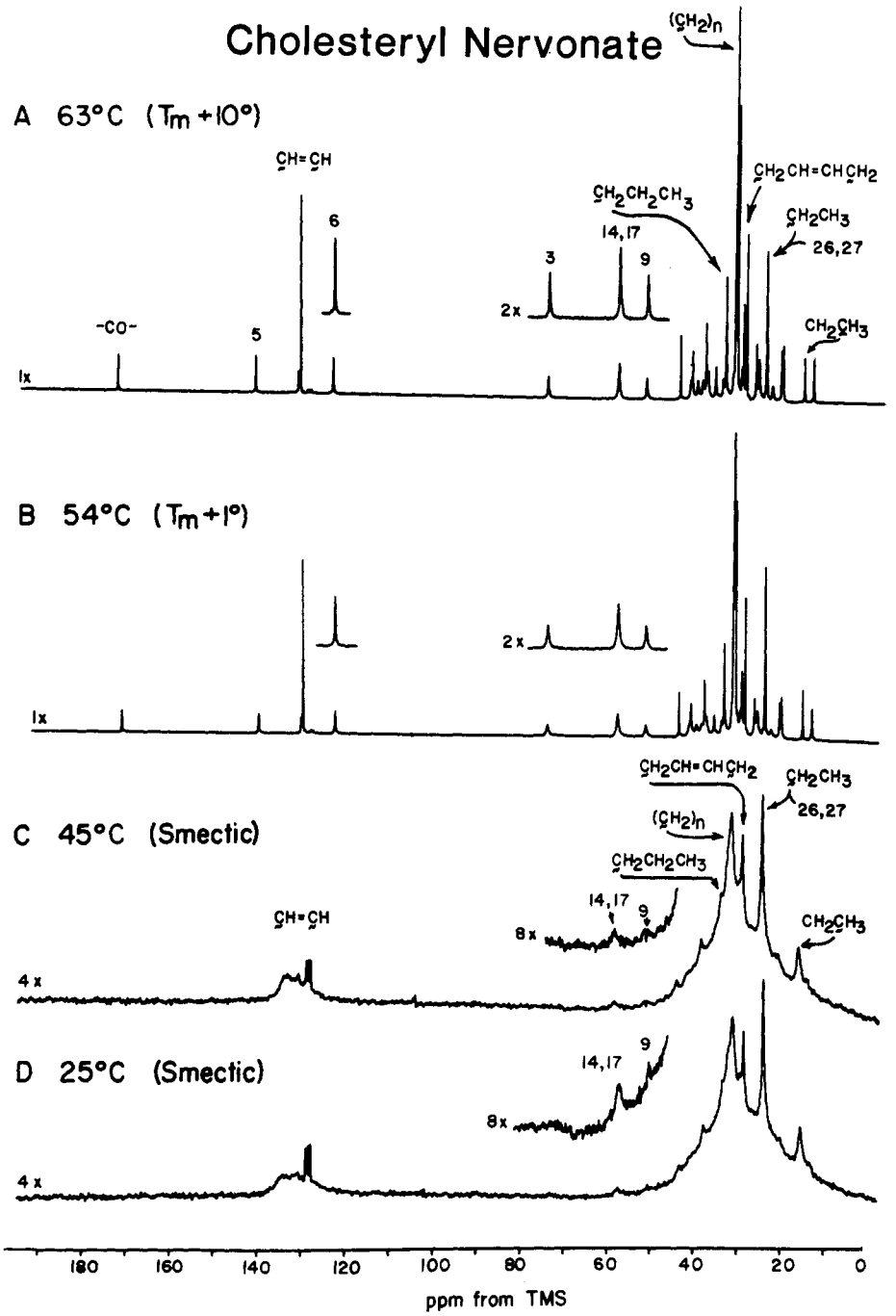

FIGURE 3: Proton-decoupled natural abundance ^{13}C Fourier transform NMR spectra in the isotropic liquid phase (A and B) and in the smectic phase (C and D) of (neat) cholesteryl nervonate. Spectra A and B were recorded at 10 deg (T_m + 10 °C) and at 1 deg (T_m + 1 °C) relative to the isotropic ↔ smectic phase transition temperature (T_m). Spectra C and D were recorded in the smectic phase. Accumulation, processing, and printing conditions were the same for the corresponding spectra in Figure 2. Spectra are labeled as in Figure 2. The triplet at ~128.0 ppm is from the C_6D_6 lock.

to the liquid-crystal spectra obtained for cholesteryl oleate (Figure 2), except for the presence of very weak resonances for the steroid ring methine C-14,17 and C-9 (see the insets to Figure 3C,D). Spectra for cholesteryl erucate in the smectic phase at 45 and 25 °C were nearly identical with those for the cholesteryl nervonate spectra in the smectic phase.

Spin–Lattice Relaxation Times. T_1 values were determined for all resolved resonances in spectra of cholesteryl linoleate and cholesteryl erucate in $CDCl_3$ at a single temperature (30 °C) and in spectra of all four neat esters at two temperatures in the isotropic liquid state (T_m + 1 °C and T_m + 10 °C). Data for selected carbons of the steroid ring and isooctyl side chain of cholesteryl linoleate are presented in Table I. These data are representative of all four esters studied since T_1 values of corresponding resonances for any of the esters were not significantly different under each of the three conditions studied ($CDCl_3$ solution, isotropic liquid at T_m + 1 °C, isotropic liquid at T_m + 10 °C). For the neat esters, the T_1's of all resonances of the cholesteryl ring moiety were temperature insensitive (±10%), except for the isooctyl side chain and angular methyl carbons (Table I). The NT_1 values (where N is the number from directly attached protons) of all ring carbon resonances were identical, as illustrated for the methine ring carbons in Table I. In $CDCl_3$, the ring carbon T_1's were 3–4 times longer than for the neat esters while differences observed for the angular methyl groups and for the isooctyl

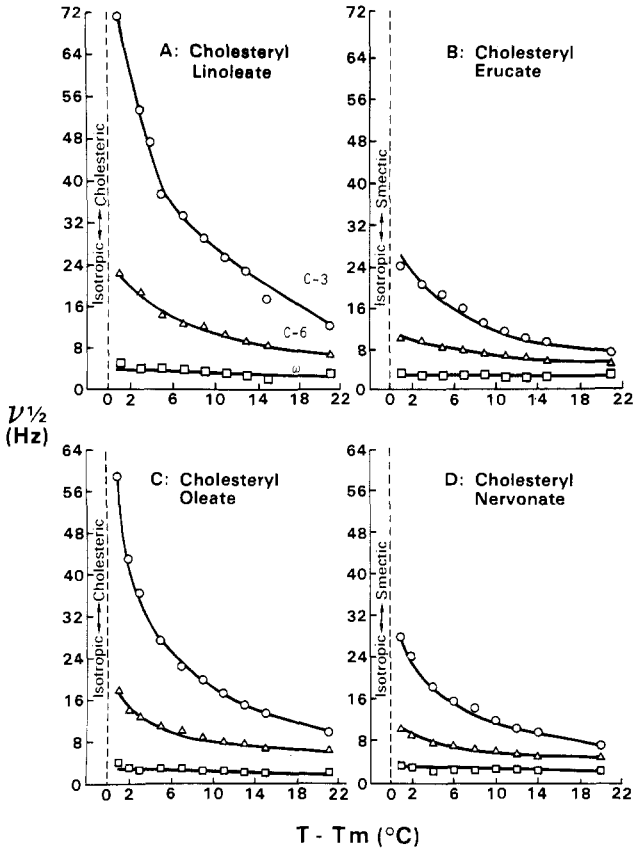

FIGURE 4: Plots of line widths of resonances for cholesterol ester ring carbons C-3 (○) and C-6 (△) and fatty acyl terminal methyl (□) resonances vs. temperature relative to the isotropic liquid ↔ liquid-crystal transition temperature (T_m) for (A) cholesteryl linoleate, (B) cholesteryl erucate, (C) cholesteryl oleate, and (D) cholesteryl nervonate. Data was acquired under the same conditions as in Figures 2 and 3 (A and B). In all cases the digital line broadening introduced to increase the signal-to-noise ratio was subtracted from the measured line width to give the values plotted. The temperature at which a liquid-crystal phase forms from the isotropic liquid (T_m) is indicated by a vertical dashed line. The type of liquid crystal below this temperature is indicated to the left of this line (printed vertically) for each system.

Table I: Spin-Lattice Relaxation Times and Nuclear Overhauser Enhancements of Ring Methine Carbon Resonances and Angular Methyl Carbon Resonances of Cholesteryl Linoleate in Deuteriochloroform and in the Isotropic Liquid Phase

cholesterol carbon no.[a]	NT_1 (s)			NOE		
	$CDCl_3$	T_m + 10 °C	T_m + 1 °C	$CDCl_3$	T_m + 10 °C	T_m + 1 °C
methine						
3	0.48[b]	0.14	0.12	2.50[c]	1.30	1.40
6	0.55	0.16	0.12	2.60	1.35	1.25
9	0.51	0.17	0.15	2.30	1.30	1.45
14	0.48	0.16[d]	0.16[d]	2.30	1.35[d]	1.30[d]
17	0.50	0.16[d]	0.16[d]	2.30	1.35[d]	1.30[d]
methyl						
18	3.36	1.95	1.74	2.84	1.95	1.85
19	2.94	1.68	1.62	2.69	1.90	1.90
21	2.19	1.44	1.02	2.74	2.00	2.05

[a] Standard steroid nomenclature; see Figure 1. [b] NT_1 values ± 0.02. [c] NOE ± 0.05. [d] The C-14 and C-17 resonances are not resolved in the isotropic liquid spectra.

side chain carbons were smaller.

The T_1 data for the fatty acyl resonances are presented in Figure 5 in the form of semilogarithmic plots of NT_1 vs. carbon number in the aliphatic chain (carbonyl carbon = 1). The general shape of the plot was similar for each ester in the

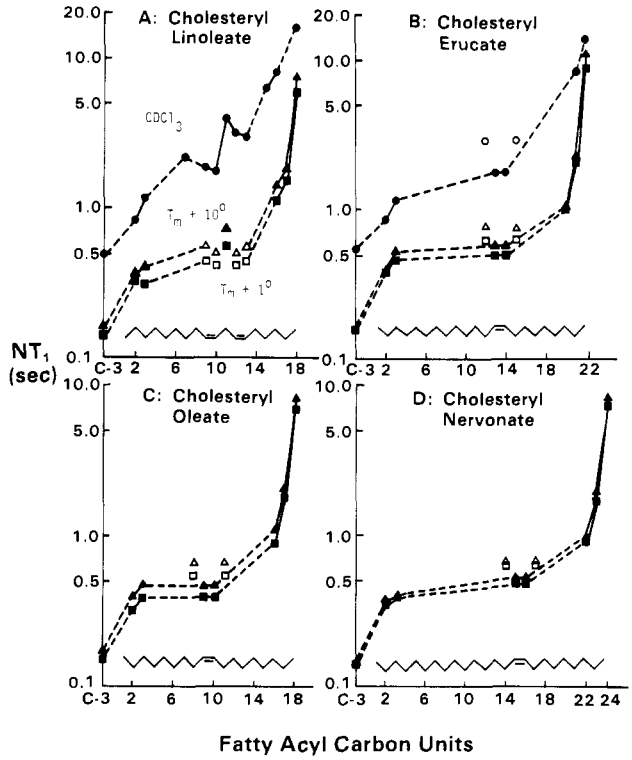

FIGURE 5: Semilogarithmic plots of NT_1 (where N is the number of directly bonded protons and T_1 is the measured spin–lattice relaxation time) vs. carbons along the fatty acyl chain in deuteriochloroform (0.2 M) (○, ●), at T_m + 10 °C (▲, △), and at T_m + 1 °C (□, ■) for cholesteryl linoleate (A), cholesteryl erucate (B), cholesteryl oleate (C), and cholesteryl nervonate (D). The abscissa represents the fatty acyl chain of each system; C-3 is the point of attachment to the cholesterol ring carbon 3 and the acyl chain carbons are numbered consecutively from the carbonyl. Closed symbols are values measured for a well resolved peak assigned to a single carbon or to two adjacent carbons. Open symbols are values measured from a peak assigned to two nonadjacent carbons. A solid line connects values measured for adjacent carbons. A dashed line connects values for nonadjacent carbons. It is important to note that although values are not plotted for unresolved methylene carbon resonances, T_1 values were obtained for partially resolved resonances for these carbons and none exceed the value of the dashed line between carbon 3 and the double bond. Note also that the FA ω-1 resonance includes the steroid C-26,27 methyl carbons.

isotropic liquid state: the NT_1 was low at the point of attachment to the sterol ring (the C-3 carbon), was approximately constant between the FA-2 carbon and the olefinic region, and was much longer for the three terminal carbon resonances (ω-2, ω-1, ω). NT_1 values for partially resolved resonances from carbons between FA-3 and the olefinic carbons equalled or were slightly lower than the plateau value indicated by the dashed lines. For a given ester the T_1 values of fatty acyl resonances increased only slightly on going from T_m + 1 °C to T_m + 10 °C.

The T_1 data for cholesteryl linoleate and cholesteryl erucate in $CDCl_3$ solutions are plotted in Figure 5 for comparison with the data for neat cholesterol esters in the isotropic liquid phase. The NT_1 values of the FA-2 and FA-3 resonances were much longer in dilute "monomeric" solutions compared to the values in the isotropic liquids while those for the terminal methyl were only slightly longer. The addition, the plateau was not as well-defined, and the NT_1 plots in $CDCl_3$ were more linear than NT_1 plots in the isotropic liquid.

Nuclear Overhauser Enhancement. The NOE was measured for all resolved resonances in spectra of cholesteryl linoleate in $CDCl_3$ at 30 °C, and in spectra of each of the four esters at T_m + 1 °C and T_m + 10 °C. NOE values for selected

Table II: Calculated and Experimental Spin-Lattice Relaxation Times and Nuclear Overhauser Enhancements for the Steroid Ring C-3 and C-6 of Four Cholesterol Esters

cholesterol ester		C-3 $\nu_{1/2}$ (Hz)		C-3 T_1 (s)		C-3 NOE		C-6 $\nu_{1/2}$ (Hz)		C-6 T_1 (s)		C-6 NOE	
		exptl[c]	calcd	exptl	calcd	exptl	calcd	exptl	calcd	exptl	calcd	exptl	calcd
CL[a]	T_m + 1 °C[b]	68.0	73.0	0.14	0.15	1.4	1.2	22.7	21.1	0.13	0.18	1.3	1.2
	T_m + 10 °C	23.0	23.5	0.12	0.12	1.3	1.3	10.0	10.7	0.16	0.12	1.3	1.3
CO	T_m + 1 °C	60.0	59.5	0.12	0.14	1.3	1.2	17.5	17.6	0.15	0.16	1.3	1.2
	T_m + 10 °C	15.8	15.6	0.16	0.12	1.5	1.5	7.8	8.4	0.12	0.10	1.3	1.3
CEr	T_m + 1 °C	24.0	22.7	0.15	0.12	1.6	1.5	8.4	9.2	0.12	0.11	1.3	1.3
	T_m + 10 °C	13.0	12.9	0.17	0.12	1.6	1.6	6.3	6.9	0.13	0.10	1.4	1.4
CN	T_m + 1 °C	22.0	22.5	0.13	0.11	1.6	1.5	8.0	8.8	0.11	0.11	1.3	1.3
	T_m + 10 °C	11.9	10.7	0.14	0.11	1.5	1.7	5.9	5.9	0.11	0.09	1.5	1.5

[a] CL = cholesteryl linoleate; CO = cholesteryl oleate; CEr = cholesteryl erucate; CN = cholesteryl nervonate. [b] T_m + 1 °C = 1 °C above the liquid → liquid-crystalline phase transition; T_m + 10 °C = 10 °C above the liquid → liquid-crystalline transition. [c] exptl = experimental; calcd = calculated (see Materials and Methods for a description of the calculations).

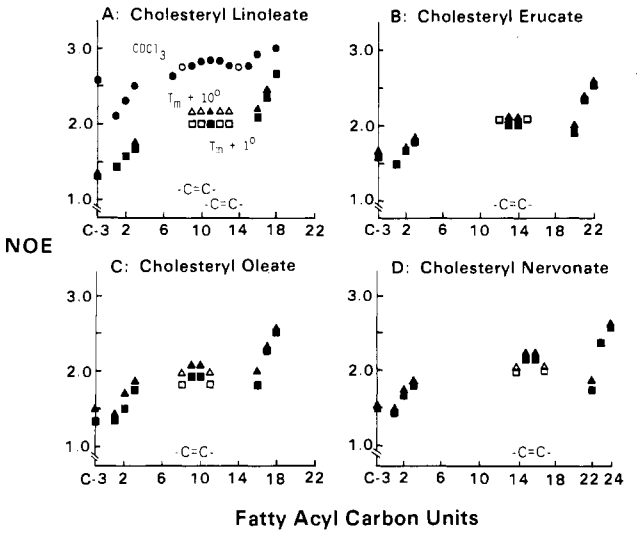

FIGURE 6: Plots of NOE (nuclear Overhauser enhancement) vs. carbons along the fatty acyl chain in deuteriochloroform (○, ●) and in the isotropic liquid at T_m + 10 °C (△, ▲) and at T_m + 1 °C (□, ■) for (A) cholesteryl linoleate, (B) cholesteryl erucate, (C) cholesteryl oleate, and (D) cholesteryl nervonate. The abscissa represents the fatty acyl chain of each system; C-3 is the cholesterol ring carbon 3, and the acyl chain carbons are numbered consecutively from the carbonyl (1). Open and closed symbols are as in Figure 5.

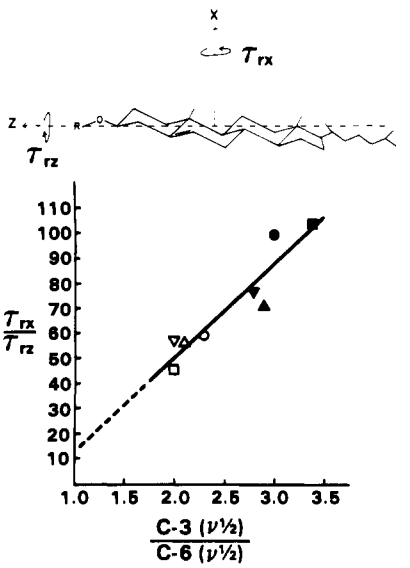

FIGURE 7: (Top) A schematic of the cholesterol ester molecule which is assumed to be a prolate ellipsoid having a long molecular axis (z) and a short molecular axis (x). The z axis, as defined by Quinn (1982) passes through the C-3 and C-13 carbon nuclei, and the x axis is nonunique and may assume any orientation perpendicular to the z axis. τ_{Rz} and τ_{Rx} are the correlation times which describe motions about these axes as shown. (Bottom) Plot of the ratio of calculated rotational diffusion correlation times τ_{Rx} and τ_{Rz} vs. the line-width ratio of the C-3 and C-6 cholesterol ring carbon resonances at T_m + 10 °C (closed symbols) and T_m + 1 °C (open symbols) for four cholesterol esters (values from Table III). (□, ■) Cholesteryl oleate; (○, ●) cholesteryl linoleate; (△, ▲) cholesteryl erucate; (▽, ▼) cholesteryl nervonate. The relationship is linear with a correlation coefficient of 0.93. The dashed line indicates extrapolation of the relationship to a line-width ratio of 1.0 (see the text).

carbons of the steroid ring and isooctyl side chain of cholesteryl linoleate are given in Table I. In $CDCl_3$ solution, the NOE values of the methyl group (C-18, C-19, C-21) approached the theoretical maximum of 2.988; the NOE values of the ring methine carbons (2.30–2.60) were significantly smaller. In the isotropic liquid, the NOE values of the steroid ring carbons were approximately half of the values in $CDCl_3$ solution and were identical for all carbons (1.30 ± 0.05). Like the T_1 values for these carbons, the NOE values were temperature insensitive between T_m + 10 °C and T_m + 1 °C. The NOE's of the C-18, C-19, and C-21 methyl groups were also smaller in the isotropic liquid than in $CDCl_3$, but the relative decrease was not as great (Table I) as for the ring carbons.

In Figure 6, NOE results for fatty acyl resonances are presented in the form of plots of NOE vs. carbon number in the fatty acyl chain for each ester. The basic shape of these plots for the isotropic liquid resembles that for the corresponding NT_1 plots: there is an increase in NOE at the beginning of the chain, followed by a plateau between the FA-3 and the ω-2 carbon, and then an abrupt increase at terminus of the chain (last two carbons). For all four cholesterol esters, NOE's were slightly temperature dependent with a small decrease in some NOE values at the lower temperatures. The NOE values of fatty acyl carbon resonances measured for cholesteryl linoleate in $CDCl_3$ were close to the maximum value of 2.988 for all carbons with the exception of the first three carbons, effectively flattening the NOE vs. carbon number plot.

Correlation Times. A quantitative model for anisotropic molecular motions (Woessner, 1962) has been applied to the steroid ring system of cholesterol esters (Quinn, 1982). In this model the cholesterol ester is considered as an axially symmetric prolate ellipsoid with a symmetry axis corresponding to the long molecular axis ("z" direction). The motions of the cholesterol ester molecule can thus be described by two correlation times (Figure 7, top), one which describes the motion about the long axis (τ_{Rz}) and the other which describes motions about the orthogonal nonunique axes (τ_{Rx}) which are considered to be equal in length (Quinn, 1982). On the basis of

our line-width data for the C-6 and C-3 methine resonances in spectra of each ester at $T_m + 1$ °C and $T_m + 10$ °C (Table II), we have calculated correlation times τ_{Rx} and τ_{Rz} (Table III). These correlation times were then used to calculate theoretical T_1 and NOE values and are compared in Table II with the corresponding experimentally measured values.

Discussion

The high spectral resolution obtained in this study for liquid cholesterol esters provided numerous well-resolved single carbon resonances from both steroid ring carbons and fatty acyl carbons, which were used to probe the dynamics of these two components of the cholesterol ester molecule. The T_1 and line-width data presented show that the motions of the ring are qualitatively different from those of the fatty acyl chain in all cholesterol esters at all temperatures above T_m. Specifically, along the acyl chain the NT_1 values were longer and the $\nu_{1/2}$ were smaller, compared with protonated steroid ring resonances. This difference may be expected because the fused ring system prohibits internal motions of ring carbons.

A detailed analysis of fatty acyl carbon motions in the isotropic liquid using line-width data is not possible because all the resonances are narrow and the width of these resonances contains a relatively large contribution from experimental factors such as the field inhomogeneity and the digital resolution. However, the T_1 values for these carbons vary over a large range (~0.2–3.0 s) and can be used to assess differences in molecular motions along the fatty acyl chain.

Fatty Acyl Carbon Motions. The motions of a fatty acyl carbon can be approximated by assuming a single "effective" correlation time (Doddrell & Allerhand, 1971). If motions are rapid and isotropic, then the increasing NT_1's reflect decreasing "effective" correlation times (increasing rapid rotations). The relatively long correlation times of the FA-2 and FA-3 result from the close proximity of these carbons to the anchor point on the bulky ring system, and the relatively short correlation time of the terminal methyl results from the additional degrees of rotational freedom and the flexibility of the end of the long chain.

T_1 gradients along hydrocarbon chains have been documented in numerous ^{13}C NMR studies. For *n*-alkanes, NT_1 values increase away from the center of mass of the chain (Levine et al., 1974), while for phospholipids in micelles (Burns & Roberts, 1980) and in vesicles (Levine et al., 1972a,b; Gent & Prestegard, 1977) NT_1 values of fatty acyl carbons increase away from the anchor point at the ester linkage. For the neat cholesterol esters, the most striking feature of the NT_1 vs. carbon number profiles is the constancy of NT_1 in the region of the chain between the C-2 and the olefinic carbons. This result indicates that the effective correlation times are similar and that the motions are restricted in this portion of the hydrocarbon chain relative to those of the terminal methyl end of the chain. A plateau in T_1 profiles appears to be a feature of fatty acyl T_1 values of phospholipids in vesicles (Godici & Landsberger, 1974) and in micelles (Burns & Roberts, 1980) but is absent for monomeric solutions of short-chain phospholipids (Burns & Roberts, 1980). The plateau is much less pronounced for cholesterol esters in $CDCl_3$ solution, suggesting that *inter*molecular interactions are an important determinant of the acyl chain motions in the neat esters. Thus, although liquid cholesterol esters do not have a defined orientation, as do phospholipids in vesicles, strong hydrocarbon chain associations may take place, perhaps within microdomains in the liquid phase.

A more detailed description of motions of fatty acyl carbons from NMR data depends on successful modeling of the complex motions which can occur in large molecules. One model which may be appropriate for cholesterol ester fatty acyl chain motions is that of London and Avitabile which is based on the ratio of gauche to trans states about a given bond and the lifetimes of each of these configurations (London & Avitabile, 1977). The model emphasizes *correlated* internal motions rather than independent rotations about a given bond. Correlated rotations preserve the linearity of the chain while isolated gauche transformations introduce a large bend in the chain. Our T_1 and NOE results resemble the general form of the calculated NT_1 and NOE vs. carbon number plots for correlated gauche/trans isomerization and are significantly different from the results for completely independent free internal rotations (London & Avitabile, 1977).[2] Thus, our results suggest specifically that, as a result of intermolecular interactions, fatty acyl chains of the four liquid esters are in an approximately extended configuration, at least up to the double bond region. An extended configuration of the fatty acyl chain has been previously suggested for cholesterol esters in the liquid state near T_m from X-ray studies (Wendorf & Price, 1973) and recently by neutron scattering studies of deuterated cholesteryl myristate (Burks & Engelman, 1981).

A comparison of the fatty acyl carbon T_1 results among the four esters shows that the number of carbons with restricted and/or correlated motions (plateau region) increases as the ring to double bond distance (and overall chain length) increases. These differences in molecular motion may be an important determinant of the phase behavior and thermodynamic behavior of these esters, since the two esters which form stable smectic phases, erucate and nervonate, have significantly longer regions of hydrocarbon chain interactions than the two esters which form cholesteric phases, linoleate and oleate. The ratio of the ring to double bond distance for cholesteryl nervonate to cholesteryl oleate is ~1.7 (15 carbons/9 carbons), which corresponds to ratio of the enthalpy of the smectic → isotropic transition of cholesteryl nervonate [1.7 cal/g (G. S. Ginsburg and D. M. Small, unpublished results)] to the *summed* enthalpies of the isotropic → cholesteric → smectic transitions of cholesteryl oleate [1.0 cal/g (Davis et al., 1970)]. Further, since the enthalpies (and entropies) of solid → mesophase transitions for the $C_{18:1}$ (ω-9), the $C_{18:2}$ (ω-9,6), and the $C_{18:3}$ (ω-9,6,3) esters are similar to the values for the transition enthalpies of the C_9 ester (cholesteryl nonanoate), Barrall & Johnson (1974) have suggested that the hydrocarbon chain region between the ω-9 double bond and the terminal methyl does not participate in the order of the phase. This interpretation is consistent with our T_1 results showing that carbons beyond the double bond(s) have relatively rapid and/or unrestricted motions, which may exclude these carbons from strong intermolecular interactions.

Steroid Ring Motions. In contrast to fatty acyl carbon resonances, protonated carbon resonances of the fused steroid ring system exhibited a large range of line-width values which were temperature sensitive. On the other hand, NT_1 values were very similar for different ring resonances and were relatively temperature insensitive (Table I). In the isotropic liquid phase near T_m, methine carbon resonances showed a nonlinear differential broadening with decreasing temperature; the increasing C-3/C-6 line-width ratio reflects steroid ring motions which are increasingly anisotropic (Hamilton et al., 1977). Analysis of the line-width data in terms of temperatures relative to T_m (Figure 4) showed that the esters with a stable

[2] On the basis of this model, the low NOE's (Figure 6) for resonances near the beginning of the chain can be ascribed to relatively slow motions of carbons near the ring rather than to different relaxation mechanisms.

Table III: Comparison of Measured Steroid Ring C-3 and C-6 Line Widths at 50.3 MHz and Calculated Rotational Correlation Times in the Isotropic Liquid Phase of Four Cholesterol Esters

cholesterol ester		line width (Hz)			rotational correlation times (s)		
		C-3	C-6	C-3/C-6	τ_{Rx}	τ_{Rz}	τ_{Rx}/τ_{Rz}
CL[a]	T_m + 1 °C[b]	68.0[c]	22.7	3.0	3.6×10^{-7}	3.6×10^{-9}	100
	T_m + 10 °C	23.0	10.0	2.3	1.0×10^{-7}	1.7×10^{-9}	59
CO	T_m + 1 °C	60.0	17.5	3.4	2.9×10^{-7}	2.8×10^{-9}	104
	T_m + 10 °C	15.8	7.8	2.0	6.0×10^{-8}	1.3×10^{-9}	46
CEr	T_m + 1 °C	24.0	8.4	2.9	1.0×10^{-7}	1.4×10^{-9}	71
	T_m + 10 °C	13.0	6.3	2.1	5.0×10^{-8}	9.0×10^{-10}	56
CN	T_m + 1 °C	22.0	8.0	2.8	1.0×10^{-7}	1.3×10^{-9}	77
	T_m + 10 °C	11.9	5.9	2.0	4.0×10^{-8}	7.0×10^{-10}	57

[a] CL = cholesteryl linoleate; CO = cholesteryl oleate; CEr = cholesteryl erucate; CN = cholesteryl nervonate. [b] T_m + 1 °C = 1 °C above the liquid → liquid-crystalline phase transition; T_m + 10 °C = 10 °C above the liquid → liquid-crystalline phase transition. [c] Line width ± 10%.

smectic phase and no cholesteric phase (nervonate and erucate) had smaller line widths for the C-3 and C-6 resonances and smaller line-width ratios (C-3/C-6) at any temperature relative T_m when compared with the esters exhibiting a cholesteric phase (oleate and linoleate). Thus, at any relative temperature above T_m, the steroid ring motions of the nervonate and erucate esters were more rapid and less anisotropic than those of cholesteryl oleate and linoleate (for the rotational correlation times, see Table II), indicating that pretransitional steroid ring ordering is not as great.

These results suggest that ring ordering is an important feature of liquid-crystalline phases of cholesterol esters but that a higher degree of ring ordering is important for the formation of a cholesteric phase. In fact, ^{13}C NMR studies of cholesteryl linoleate–cholesteryl oleate mixtures with 4 wt % triolein (J. A. Hamilton and E. H. Cordes, unpublished results), a mixture with no cholesteric phase, yielded line-width results which were similar to the results for erucate and nervonate esters and not to those for linoleate and oleate esters. Furthermore, calorimetry studies on *di*cholesterol esters have shown that these lipids undergo a cholesteric → isotropic liquid phase transition with at least *twice* the expected entropy, indicating that the steroid ring interactions are important in ordering the cholesteric phase (Barrall et al., 1969). In addition, it has been suggested that strong ring–ring interactions in crystalline cholesteryl oleate are responsible for a crystal melting temperature that is higher than the temperature range over which the liquid-crystalline phases are stable (Craven & Guerina, 1979).

The degree of motional anisotropy of the steroid ring in the isotropic liquid is illustrated by the rotational diffusion correlation times (τ_{Rz} and τ_{Rx}) calculated from our line-width data. As shown in Table III, τ_{Rz} is on the order of nanoseconds (10^{-9} s) and τ_{Rx} is ~50–100 greater (motion is slower) for all the esters. As a function of decreasing temperature from T_m + 10 °C to T_m + 1 °C in the isotropic liquid, τ_{Rz} increases ~2-fold and τ_{Rx} increases ~4-fold. The differences in rotational correlation times about the orthogonal axes are indicative of anisotropy in the rotational motions of the steroid ring unit. T_1 and NOE values calculated from the correlation times derived from the experimental line widths were in close agreement with experimentally measured values (Table II). The theoretical T_1 values verified the experimental finding that T_1 values for the C-6 and C-3 resonances are similar and are not strongly temperature dependent in the ranges investigated. Thus, T_1 is *not* sensitive to the increasingly anisotropic motions of the steroid ring in any of the four esters. Similarly, NOE is also insensitive to temperature-dependent changes in anisotropic ring motions. Since NOE is a complicated function of correlation times in cases where a single correlation time is not sufficient to describe molecular motions [see, for example, Doddrell et al. (1972) and Levy et al. (1978)], close agreement of theoretical and experimental NOE values strengthens confidence in the model for anisotropic motion of cholesterol esters.[3]

A plot of the C-3/C-6 line-width ratio vs. the correlation time ratio τ_{Rx}/τ_{Rz} using the data for all four esters at T_m + 1 °C and T_m + 10 °C is shown in Figure 7. There is a good linear correlation (r = 0.93) between these two ratios. This result shows the general applicability of the prolate ellipse model for the motions of long-chain cholesterol esters and provides a quantitative basis for relating anisotropic motions of the steroid ring to the C-3/C-6 line-width ratio, as previously proposed (Hamilton et al., 1977) and as employed above. Note that the extrapolation of the plot in Figure 7 to a C-3/C-6 line-width ratio of 1.0 gives a τ_{Rx}/τ_{Rz} ratio of 13, indicating that the line-width ratio will be sensitive to anisotropic motions only when there is at least a 10-fold difference in the correlation times. This experimental extrapolation agrees with calculations which predict a C-3/C-6 $\nu_{1/2}$ ratio of ~1.0 when τ_{Rx}/τ_{Rz} = 10 (Quinn, 1982).

The close correlation between the τ_{Rx}/τ_{Rz} ratio and the C-3/C-6 line-width ratio does not exclude the possibility that the C-3/C-6 line-width ratio may reflect differences in motions about the two shorter axes (Hamilton et al., 1977). Since the short dimensions of the steroid ring are not equivalent (5 Å × 7 Å), and the two faces of the ring are clearly not identical, it is possible that the τ_{Rx} as employed above is the time average of two nonidentical correlation times about the two unique shorter molecular axes of the cholesterol ester molecule. Ring–ring interactions of a highly specific nature may occur by transient clustering of ester molecules and consequently change the rotational diffusion correlation times about the shorter axes.

In this study three cholesterol esters were structurally homologous: cholesteryl oleate (18:1), cholesteryl erucate (22:1), and cholesteryl nervonate (24:1). All have a single double bond in the ω-9 position. Figure 8 shows the C-3 and C-6 line-width plots from Figure 4 for these cholesterol esters superimposed on the same temperature axes {the real temperature and temperature relative to the cholesteric → smectic transition of cholesteryl oleate [$T_{smectic(CO)}$]}. At temperatures greater than 52.5 °C, the curves for all esters are very similar, showing that

[3] A particularly critical assumption was that dipolar interactions are the predominant mechanism of relaxation (Quinn, 1982). In a comparison of our data for cholesteryl oleate and cholesteryl linoleate with that obtained at 24 kG (Hamilton et al., 1977), line widths of the C-6 and C-3 resonances were nearly identical at corresponding temperatures at both fields. This finding strongly suggests that dipolar relaxation predominates at the higher field.

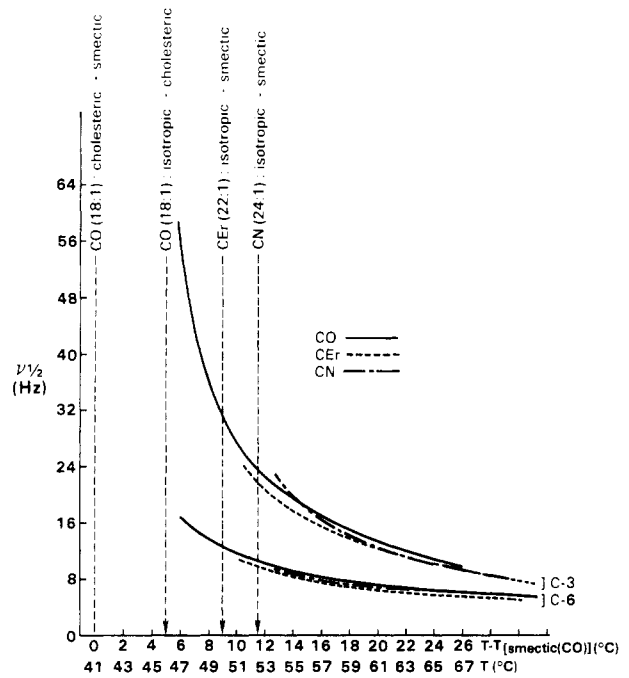

FIGURE 8: Plot of the line width of the C-3 and C-6 ring resonances for cholesteryl oleate (—); cholesteryl erucate (- - -), and cholesteryl nervonate (– – –) vs. absolute temperature (°C) and temperature *relative* to the cholesteric → smectic transition (46 °C) of cholesteryl oleate [$T_{smectic(CO)}$]. The liquid-crystalline transitions are indicated by vertical dashed lines. CO(18:1) = cholesteryl oleate; CEr(22:1) = cholesteryl erucate; CN(24:1) = cholesteryl nervonate. Values in parentheses refer to the chain length:number of double bonds. The line widths of corresponding ring resonances of all the ω-9 cholesterol esters are similar at the same temperature. Thus, the smectic phase is initiated by chain–chain interactions; the longer the chain, the higher the smectic transition.

the steroid ring motions are quantitatively similar. Although the line-width trend (and thus ring motion behavior) is similar on cooling, the long acyl chains of cholesteryl erucate and cholesteryl nervonate induce the formation of the smectic phase at high temperatures [9 and 11.5 °C, respectively, above $T_{smectic(CO)}$], precluding the ring–ring interactions which result in anisotropic ring motions and preordering necessary for cholesteric phase formation seen in the shorter chained oleate ester. Previous studies have shown that homologous ω-9 monounsaturated cholesteryl esters with a chain length greater than *20* carbons do not have a cholesteric phase; the same series of esters have smectic phases which melt at higher temperatures with increasing chain length (Ginsburg & Small, 1981). The present study shows that a high degree of rotational anisotropy (preordering) of the steroid ring in the isotropic liquid is required to form the cholesteric phase and that it is the acyl chain (length) which governs the physical state of the system.

LDL Cholesterol Esters. Cholesterol esters in human plasma LDL exhibit a disordered → ordered transition near physiological temperature. ^{13}C NMR studies have shown that the disordered state is similar to the isotropic liquid phase of neat cholesterol esters (Hamilton et al., 1977, 1979), and X-ray scattering studies have shown that the ordered phase resembles the smectic phase of neat cholesterol esters (Deckelbaum et al., 1975, 1977; Atkinson et al., 1977). Near the phase transition temperature in LDL, the C-3/C-6 line-width ratio is 1.7–1.8 (Hamilton et al., 1979), which is close to the values near T_m for esters exhibiting a stable smectic phase. The motions of cholesterol esters in LDL near the T_m are not as anisotropic as the motions of the principle constituent esters (cholesteryl oleate and linoleate) near their respective T_m's or near the T_m of a mixture of the two esters (Hamilton et al., 1977). The results of this study would predict an isotropic → stable smectic phase transition rather than an isotropic → cholesteric phase transition for the cholesterol ester core in native LDL based on the low observed C-3/C-6 line-width ratio. The presence of small amounts of triglyceride in the cholesterol ester rich core of LDL as well as the fatty acyl chains of the adjacent phospholipid surface monolayer may destabilize steroid ring interactions in favor of acyl chain interactions and thereby cause an isotropic liquid → smectic transition with no intermediate cholesteric phase.

Acknowledgments

We thank Dr. Dan M. Quinn for providing the computer modeling program for the cholesterol ester rotational correlation times. We also acknowledge Dr. David Atkinson for his critical reading of the manuscript and Anne Gibbons for preparation of the manuscript.

References

Armitage, D., Deckelbaum, R. J., Shipley, G. G., & Small, D. M. (1977) *Mol. Cryst. Liq. Cryst. 42*, 203–214.

Atkinson, D., Deckelbaum, R. J., Small, D. M., & Shipley, G. G. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1042–1046.

Barrall, E. M., & Johnson, J. F. (1974) in *Liquid Crystals and Plastic Crystals* (Gray, G. W., & Winsor, P. A., Eds.) Vol. 2, pp 254–306, Wiley, New York.

Barrall, E. M., Johnson, J. F., & Porter, R. S. (1969) *Mol. Cryst. Liq. Cryst. 8*, 27–44.

Burks, C., & Engelman, D. M. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 6863–6867.

Burns, R. A., Jr., & Roberts, M. F. (1980) *Biochemistry 19*, 3100–3106.

Canet, D., Levy, G. C., & Peat, I. R. (1975) *J. Magn. Reson. 18*, 199–204.

Craven, B. M., & Guerina, N. G. (1979) *Chem. Phys. Lipids 29*, 91–98.

Davis, G. J., Porter, R. S., Steiner, J. W., & Small, D. M. (1970) *Mol. Cryst. Liq. Cryst. 10*, 331–336.

Deckelbaum, R. J., Shipley, G. G., Small, D. M., Lees, R. S., & George, P. K. (1975) *Science (Washington, D.C.) 190*, 392–394.

Deckelbaum, R. J., Shipley, G. G., Small, D. M. (1977) *J. Biol. Chem. 252*, 744–754.

Doddrell, D., & Allerhand, A. (1971) *J. Am. Chem. Soc. 93*, 1558–1559.

Doddrell, D., Glusko, V., & Allerhand, A. (1972) *J. Chem. Phys. 56*, 3683–3689.

Gent, M. P. N., & Prestegard, J. H. (1977) *J. Magn. Reson. 25*, 243–262.

Ginsburg, G. S., & Small, D. M. (1981) *Biochim. Biophys. Acta 664*, 98–107.

Godici, P. E., & Landsberger, F. R. (1974) *Biochemistry 13*, 362–368.

Gray, G. W. (1962) *Molecular Structure and Properties of Liquid Crystals*, pp 42–54, Academic Press, New York.

Gunstone, F. D., Pollard, M. R., Scrimgeour, C. M., & Vedanayagam, H. S. (1977) *Chem. Phys. Lipids 18*, 115–129.

Hamilton, J. A., Oppenheimer, N., & Cordes, E. H. (1977) *J. Biol. Chem. 252*, 8071–8080.

Hamilton, J. A., Cordes, E. H., & Glueck, C. J. (1979) *J. Biol. Chem. 254*, 5435–5441.

Hamilton, J. A., Ginsburg, G. S., & Small, D. M. (1982) *Annu. Meet. Biophys. Soc. No. 2* (Part 2), M-PM-B1/98a.

Katz, S. S., Shipley, G. G., & Small, D. M. (1976) *J. Clin. Invest. 58*, 200–211.

Levine, Y. K., Partington, P., Roberts, G. C. K., Birdsall, N. J. M., Lee, A. G., & Metcalfe, J. C. (1972a) *FEBS Lett. 23*, 203–207.
Levine, Y. K., Birdsall, N. J. M., Lee, A. G., & Metcalfe, J. C. (1972b) *Biochemistry 11*, 1416–1421.
Levine, Y. K., Birdsall, N. J. M., Lee, A. G., Metcalfe, J. C., Partington, P., & Roberts, G. C. K. (1974) *J. Chem. Phys. 60*, 2890–2899.
Levy, G. C., Axelson, D. E., Schwartz, R., & Hochmann, J. (1978) *J. Am. Chem. Soc. 100*, 410–424.
Lindemann, L. P., & Adams, J. Q. (1971) *Anal. Chem. 43*, 1245–1252.
London, R. E., & Avitabile, J. (1977) *J. Am. Chem. Soc. 99*, 7765–7776.
Opella, S. J., Nelson, D. J., & Jardetzky, O. (1976) *J. Chem. Phys. 64*, 2533–2535.
Quinn, D. M. (1982) *Biochemistry 21*, 3548–3555.
Reich, H. J., Jautelat, M., Messe, M. T., Weigert, F. J., & Roberts, J. D. (1969) *J. Am. Chem. Soc. 91*, 7445–7454.
Sass, M., & Ziessow, D. (1977) *J. Magn. Reson. 25*, 263–276.
Sears, B., Deckelbaum, R. J., Janiak, M. J., Shipley, G. G., & Small, D. M. (1976) *Biochemistry 15*, 4151–4157.
Small, D. M. (1970) in *Surface Chemistry of Biological Systems* (Blank, M., Ed.) pp 55–82, Plenum Press, New York.
Stoffel, W., Zierenberg, O., & Tunggal, B. D. (1972) *Hoppe-Seyler's Z. Physiol. Chem. 354*, 1962–1969.
Tall, A. R., Small, D. M., Atkinson, D., & Rudel, L. L. (1978) *J. Clin. Invest. 62*, 1354–1363.
Wendorf, J. H., & Price, F. P. (1973) *Mol. Cryst. Liq. Cryst. 24*, 129–144.
Woessner, D. E. (1962) *J. Chem. Phys. 37*, 647–654.

Reaction of Human Lecithin:Cholesterol Acyltransferase with Micellar Substrates Is Independent of the Phase State of the Lipid†

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ABSTRACT: Micellar complexes with different phosphatidylcholine (PC) compositions were prepared by the dialysis of PC–cholesterol dispersions with cholate in the presence of human apolipoprotein A-I (apo A-I). The complexes isolated by gel filtration had molecular weights around 200 000, two apo A-I molecules per particle, PC to apo A-I molar ratios from 91 to 123, and cholesterol to apo A-I molar ratios from 6 to 11. The phase-transition behavior of these complexes was examined by fluorescence polarization of diphenylhexatriene: the complexes containing dimyristoyl-PC had a transition temperature (T_m) of 32 °C, the complexes with dipalmitoyl-PC had a T_m of 45 °C, and those prepared with palmitoyloleoyl-PC were mostly present in the liquid-crystalline state in the temperature range investigated (55–7 °C). The initial velocities of the enzymatic reaction with purified human lecithin:cholesterol acyltransferase decreased in the order palmitoyloleoyl-PC > dipalmitoyl-PC > dimyristoyl-PC, at saturating micellar substrate levels. Arrhenius plots of the reaction rates from 15 to 41 °C were linear, and the activation energies ranged from 20 to 30 kcal/mol. These results indicate a marked dependence of the enzymatic reaction rates on the nature of the acyl donor, a dependence which is not related to the phase state of the bulk lipid in the micellar complexes.

Lecithin:cholesterol acyltransferase (LCAT,[1] EC 2.3.1.43) is the enzyme responsible for the esterification of cholesterol and for the transformations of high-density lipoproteins (HDL) is plasma (Glomset, 1972). So far, the substrate specificity of LCAT has been investigated by using native lipoproteins or synthetic vesicles of phosphatidylcholines (PC) and cholesterol with added apolipoproteins. The human enzyme shows a marked preference for the 2-acyl position and for long-chain unsaturated fatty acids of natural and synthetic PCs in the transesterification reaction (Glomset, 1972; Sgoutas, 1972). When saturated PCs are used as acyl donors in the LCAT reaction, their order of reactivity depends on the type of substrate particle used (vesicle or HDL) and on the physical state of the lipid (Soutar et al., 1974; Yokoyama et al., 1977).

Although vesicle substrates have the advantage over native lipoproteins of being chemically defined, they present several problems: instability in the presence of apolipoproteins (Jonas et al., 1977, 1980), different apolipoprotein binding affinities and stoichiometries on stable vesicles (Yokoyama et al., 1980), a limited capacity for cholesterol ester storage (Chajek et al., 1980; Janiak et al., 1979), and major morphological differences from the natural LCAT substrates. In fact, previous observations with vesicles containing dilauroyl-PC and dimyristoyl-PC (DMPC) while the temperature was changed (Soutar et al., 1974) may have included the effects of the spontaneous transformation of vesicles into discoidal complexes of lipid and apolipoprotein. Even with PC vesicles which do not form spontaneously micellar complexes with apolipoprotein A-I (apo A-I), the extent and strength of apo A-I binding to the vesicle, and hence, LCAT activation, may be affected by the nature of the PC. Reports that optimal LCAT activity is observed at PC to cholesterol molar ratios of 4/1 in vesicle

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[1] Abbreviations: LCAT, lecithin:cholesterol acyltransferase; HDL, high-density lipoprotein(s); PC, phosphatidylcholine; apo A-I, apolipoprotein A-I, the major protein component of HDL; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; POPC, palmitoyloleoylphosphatidylcholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; T_m, gel to liquid-crystal phase transition temperature; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

0006-2960/82/0421-6867$01.25/0