Molecular Motions and Thermotropic Phase Behavior of Cholesteryl Esters with Triolein†

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ABSTRACT: The phase behavior of cholesteryl esters with triglyceride has been characterized by differential scanning calorimetry (DSC), light microscopy, and polarizing light microscopy (PLM). Temperature-dependent molecular motions determined by ^{13}C NMR spectroscopy were correlated with thermotropic phase behavior. Two systems, cholesteryl oleate (CO) and a 3/1 w/w mixture of cholesteryl linoleate (CL) and CO, were examined in the presence of small amounts of triolein (TO). Both systems exhibited metastable cholesteric and smectic (or only smectic) phases. Increasing amounts of TO progressively lowered the liquid-crystalline phase transition temperatures and eventually abolished the cholesteric phase, but at differing amounts of TO for the two systems (between 4% and 5% with CL/CO and between 7% and 10% with CO). DSC and PLM showed a progressive broadening of the phase transitions as well as an overlapping of the temperature ranges of the cholesteric and smectic phases. At ≥4% TO, a separate isotropic liquid phase coexisted with liquid-crystalline phases. ^{13}C NMR spectroscopy was used to monitor the molecular motions of the cholesteryl ester steroid ring and acyl chain in liquid and liquid-crystalline phases. In the liquid phase, no significant changes in fatty acyl motions, as reflected in spin–lattice relaxation time (T_1) and nuclear Overhauser enhancement (NOE) values, were found on addition of TO. The line width ($\nu_{1/2}$) of the steroid ring resonances increased markedly near (1–5 °C above) the isotropic liquid → liquid-crystal phase transition temperature (T_{LC}). However, the C3/C6 $\nu_{1/2}$ ratio at 1 °C above T_{LC} was greater for mixtures exhibiting an isotropic → cholesteric transition than for mixtures exhibiting an isotropic → smectic transition. Rotational correlation times calculated for motions about the long molecular axis and the nonunique axis showed (i) that the ring motions became more anisotropic as T_{LC} was approached and (ii) that the motions were more anisotropic at T_{LC} + 1 °C for systems exhibiting a cholesteric phase than for systems exhibiting only a smectic phase. ^{13}C line widths in spectra of the cholesteryl ester liquid-crystalline phases suggested that TO perturbed the cholesteryl ester intermolecular interactions and increased the rates of cholesteryl ester molecular motions relative to neat esters.

Cholesteryl oleate (CO)[1] and cholesteryl linoleate (CL) are the most predominant cholesteryl esters in plasma lipoproteins (Skipski, 1972) and in some atherosclerotic plaques (Smith & Slater, 1973; Katz et al., 1976, 1980). The physical properties of these cholesteryl esters have been examined by a variety of techniques (Small, 1970, 1985) including ^{13}C NMR spectroscopy (Sears et al., 1976; Hamilton et al., 1977; Quinn, 1982; Ginsburg et al., 1982), which is a highly effective method for monitoring temperature-dependent molecular motions. However, in biological systems, cholesteryl esters are generally found in association with triglycerides, and even as a minor component, triglycerides can have a profound effect on the physical properties of cholesteryl esters. For example, triglyceride in low concentrations can abolish the cholesteric phase, and in higher concentrations the smectic phase, of cholesteryl esters (Ekman & Lundberg, 1976; Hamilton et al., 1977; Deckelbaum et al., 1977; Kroon, 1981; Small, 1985). Similar effects may be quite important in lipoproteins (Hamilton et al., 1979; Deckelbaum et al., 1977), in cellular systems (Adelman et al., 1984), and in atherosclerotic plaques (Hamilton et al., 1979; Waugh & Small, 1984). However, an absence of systematic and detailed studies of model systems restricts our understanding of the complex biological systems.

In this study, we have examined in detail the effects of small amounts of triolein (TO) on the phase behavior of CO and a 3/1 (w/w) CL/CO mixture.[2] TO is liquid above 4 °C (Small, 1970) and serves as a simplified model for the biological mixed-chain triacylglycerols which are liquid at, or near, body temperature. ^{13}C NMR spectroscopy is used to give a detailed, quantitative description of molecular motions of cholesteryl esters in the presence of TO. These results are correlated with data from other physical techniques, particularly polarizing light microscopy and differential scanning calorimetry.

We show that the phase behavior of these two- and three-component model systems is complex and that phase separation occurs even at low (~4 wt %) amounts of triolein. However, important features of the molecular dynamics of the triolein-containing systems can be understood from previous studies (Hamilton et al., 1977; Quinn, 1982; Ginsbug et al., 1982) of unsaturated cholesteryl esters; i.e., steroid ring motions become

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[1] Abbreviations: TO, triolein; CO, cholesteryl oleate; CL, cholesteryl linoleate; T_{LC}, isotropic liquid to liquid-crystal phase transition onset temperature; DSC, differential scanning calorimetry; PLM, polarizing light microscopy; S/N, signal to noise ratio; Me_4Si (TMS in figures), tetramethylsilane; $\nu_{1/2}$, line width; T_1, spin–lattice relaxation time; NOE, nuclear Overhauser enhancement; $T_{I\rightarrow Ch}$, isotropic liquid to cholesteric phase transition onset temperature; $T_{Ch\rightarrow Sm}$, cholesteric to smectic phase transition onset temperature; $T_{I\rightarrow Sm}$, isotropic liquid to smectic phase transition onset temperature; τ_{rz}, correlation time for cholesteryl ester reorientation about the long molecular axis of symmetry; τ_{rx}, correlation time for cholesteryl ester reorientation about the orthogonal nonunique molecular axis; C, cholesteryl ring carbons (standard steroid nomenclature).

[2] The 3/1 ratio of CL to CO approximates that found in normal plasma lipoproteins (Skipski, 1972) and some atherosclerotic lesions (Smith & Slater, 1973; Katz et al., 1976).

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highly anisotropic near the liquid–liquid-crystal phase transition temperature, and they are more anisotropic for mixtures having both a cholesteric and a smectic phase than for mixtures with only a smectic phase.

Experimental Procedures

Materials. Cholesteryl *cis*-9,10-octadecanoate (cholesteryl oleate), cholesteryl *cis*-9,10,*cis*-12,13-octadecanoate (cholesteryl linoleate), and trioleoylglycerol (triolein) were obtained from Nu Chek Prep Inc. (Elysian, MN). ^{13}C NMR spectra of the cholesteryl esters in organic solvents were consistent with those previously reported (Sears et al., 1976; Hamilton et al., 1977; Ginsburg et al., 1982). The melting temperatures and the isotropic liquid → liquid-crystal transition onset temperatures (T_{LC}) of the pure cholesteryl esters, as determined by visual inspection of samples (see below), were in good agreement with those previously determined by other techniques (Small, 1970; Ginsburg & Small, 1981).

Preparation of Triolein/Cholesteryl Ester Mixtures. Mixtures were prepared containing TO (1, 2, 3, 4, 7, or 10 wt % of the total lipid) with CO or TO (0, 2, 4, or 5%) with a 3/1 (w/w) mixture of CL/CO. Neat cholesteryl esters were weighed into 13 × 100 mm Kimax tubes. TO was added by using a calibrated microsyringe (Hamilton); the weight was calculated from the volume, assuming a density of 0.9 g/mL for pure TO at 20 °C (Singleton, 1960). The samples were melted and the tubes flushed with nitrogen and capped. The tubes were suspended in a water bath maintained 5–10 °C above the melting point of the pure ester for 2–3 h. The samples were periodically removed for agitation to mix the components.

The mixtures of cholesteryl esters with TO were characterized as a function of temperature by (a) visual inspection, (b) differential scanning calorimetry (DSC), (c) light and polarizing light microscopy (PLM), and (d) ^{13}C NMR spectroscopy.

Visual Inspection. The sample was heated in a stirred water bath to 5 °C above the melting point. The bath was cooled, and the sample was observed for the formation of cholesteric and smectic phases (Gray, 1962). Temperature was measured with a thermocouple suspended in the water bath next to the sample tube.

Polarizing Light Microscopy. PLM was carried out on a wedge-shaped sample as described previously (Small, 1970) so that both thick and thin regions of the sample could be observed. A Zeiss polarizing microscope equipped with a heating/cooling stage permitted the temperature to be raised and lowered 1–2 °C/min. Crossed polarizers were used to identify cholesteric and smectic textures; in some cases, a quarterwave plate was used to determine the sign of birefringence in order to verify phase identification. The macroscopic appearance of the thick portion of the sample was also noted under direct illumination.

Differential Scanning Calorimetry. Small samples (1–10 mg) of the melted mixtures were transferred to preweighed aluminum pans by using a preheated Pasteur pipet. The pans were weighed, sealed, and, if not used immediately, stored at −20 °C. DSC was carried out as previously described (North & Small, 1977) using a Perkin-Elmer DSC-2 and heating and cooling rates of 10 or 5 °C/min. An empty aluminum pan was used as a reference standard.

NMR Spectroscopy. Natural abundance proton-decoupled Fourier transform ^{13}C NMR spectra were obtained at 4.7 T (50.3 MHz for ^{13}C) with a Bruker WP-200 spectrometer equipped with an Aspect 2000 data system. Neat mixtures of cholesteryl esters with TO in a coaxial insert were placed in a 10-mm NMR tube containing ^2H_2O as an external lock. In experiments requiring a greater signal to noise ratio (S/N) per unit time, the sample was placed directly in a 10-mm NMR tube, and the external lock was provided by ^2H_2O in a coaxial insert.

Sample temperatures were controlled to within 1 °C with the Bruker B-VT-1000 variable-temperature unit. The NMR probe temperature was calibrated by using a nonionic viscous sample (ethylene glycol) equal in volume to the sample volume. Probe temperature was measured as previously described (Ginsburg et al., 1982). At a given setting of the variable-temperature unit, temperatures were identical (±1 °C) for both sample configurations (sample in tube or in insert).

NMR samples were melted and mixed by mechanical agitation while maintaining the sample 15–20 °C above T_{LC} for 20–30 min. The melted sample was equilibrated in the probe at the desired temperature for an additional 20 min prior to the acquisition of each spectrum. After NMR experiments conducted at temperatures near (within 3 °C) T_{LC}, the sample was ejected from the probe for visual inspection. Chemical shifts were referenced to the fatty acyl CH_3 carbon of the cholesteryl esters [14.10 ppm relative to tetramethylsilane (Me_4Si)] (Hamilton et al., 1977). Broad-band proton decoupling (1.0 W) centered 3.4 ppm downfield from the ^1H resonance of Me_4Si was used. Typically, spectra were acquired with a 90° pulse (15.5 μs) and a spectral width of 10 000 Hz using 16 384 time domain addresses and a recycle time of 0.82 s. To obtain an S/N adequate for accurate line-width ($\nu_{1/2}$) measurements of the broadest resonances, 2000–12 000 accumulations (for samples in the coaxial insert) or 250–500 accumulations (for samples in the 10-mm NMR tube) were used.

Spin–lattice relaxation time (T_1) values were measured by the fast inversion recovery method (Canet et al., 1975) and calculated by using a program supplied by Bruker Co., which utilized a nonlinear three-parameter fit (Sass & Ziessow, 1977). Nuclear Overhauser enhancement (NOE) values were measured as the ratio of peak heights in spectra obtained with continuous broad-band decoupling to those in spectra obtained with gated decoupling. In all cases, peaks in both spectra had similar line shapes and $\nu_{1/2}$ values. To assure that both spectra were obtained at the same temperature, free induction decays under both decoupling conditions were alternatively summed at separate computer addresses (Ginsburg et al., 1982).

Calculations of theoretical $\nu_{1/2}$, T_1, and NOE values were performed on a Digital Equipment Corp. LSI-11/2 computer using a modeling program by Quinn (1982).

Results

Visual Characterization. (A) Triolein/Cholesteryl Oleate. Mixtures containing ≤7% TO exhibited isotropic to cholesteric to smectic phase transitions. For example, when the 4% TO + 96% CO system was cooled (0.5–1.0 °C/min) from the colorless isotropic liquid, it exhibited a faint blue-purple color at 37 °C, indicating the formation of the cholesteric phase. As the temperature was lowered, the intensity of the color increased, and the color changed from purple to blue. About 3 °C below the temperature at which color was first apparent, the sample became increasingly turbid, and at 33 °C, the blue color was replaced by a white waxy texture characteristic of the smectic phase. If the sample was slowly heated (1–2 °C/min) and then subsequently cooled, the above changes were reversible. Cooling to 20 °C produced a smectic texture from which a solid formed within ~1 h. Both the isotropic liquid to cholesteric onset temperature ($T_{I\rightarrow Ch}$) and the cholesteric to smectic onset temperature ($T_{Ch\rightarrow Sm}$) were dependent

Table I: Thermal Transitions of Cholesteryl Esters and Cholesteryl Ester/Triolein Mixtures

system	transition[a]	method: visual inspection[b]	method: DSC[b] (°C)	method: PLM[a,d]
100% CO	I → Ch	46	44	
	Ch → Sm	43	41	
1% TO + 99% CO	I → Ch	43		
	Ch → Sm	40		
2% TO + 98% CO	I → Ch	41	41	Ch
	Ch → Sm	36	35	Sm
3% TO + 97% CO	I → Ch	38	38	
	Ch → Sm	34	34	
4% TO + 96% CO	I → Ch	37	38	Ch
	Ch → Sm	33	33	Sm[e]
7% TO + 93% CO	I → Ch	33	34	Ch
	Ch → Sm	31	30	Sm[e]
10% TO + 90% CO	I → Sm	31	30	Sm[e]
100% CL/CO[c]	I → Ch	34	34	Ch
	Ch → Sm	32	31	Sm
2% TO + 98% CL/CO[c]	I → Ch	30	29	Ch
	Ch → Sm	28	23	Sm
4% TO + 96% CL/CO[c]	I → Ch	25		Ch
	Ch → Sm	23		Sm[e]
5% TO + CL/CO[c]	I → Sm	23–25		Sm[e]

[a] I = isotropic liquid; Ch = cholesteric phase; Sm = smectic phase. [b] Onset temperatures reported to the nearest 1 °C. All transition temperatures were measured on samples cooled from the isotropic liquid. [c] CL/CO is a 3/1 (w/w) mixture of CL and CO. [d] This is the predominant phase identified by PLM. In all mixtures containing TO, there was some overlap of cholesteric and smectic phases (see text). [e] Contained some isotropic liquid (see text).

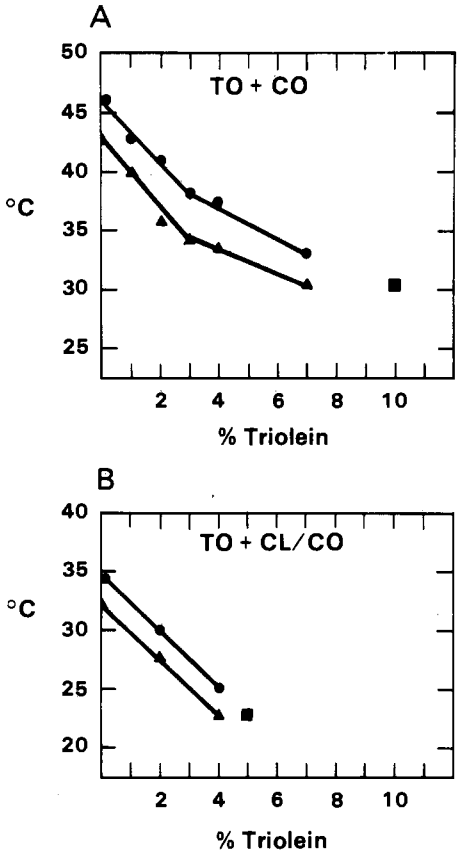

FIGURE 1: Plots of onset temperature(s), on cooling from the isotropic liquid, of liquid-crystalline phases vs. percent triolein for (A) TO/CO and (B) TO/CL/CO. Onset transition temperatures were determined visually (see text) and in selected cases were corrobroated by DSC (Table I). Symbols on the temperature axes indicate transition temperatures for the pure cholesteryl ester systems, (●) isotropic → cholesteric transition onset temperature; (▲) cholesteric → smectic transition onset temperature; (■) isotropic → smectic transition onset temperature. The straight lines represent linear least-squares fits of the experimental data. In panel A, the data for both transitions could be fitted to straight lines between 0% and 3% TO (correlation coefficients ≥0.99) and between 3% and 7% TO (correlation coefficients ≥0.99). For panel B the data for each transition could be fitted to a single straight line (correlation coefficients ≥0.99).

on the amount of TO present (Table I, Figure 1A).

The behavior of the 10% TO + 90% CO system was different. Upon cooling of the isotropic liquid, no purple or blue color was seen; instead, a white waxy smectic texture appeared at 31 °C. However, at 31 °C, a white solid formed on the walls of the tube. At 20 °C, this solid grew from the smectic texture until the entire sample became solid with small interspersed drops of oil. The smectic phase could be converted to a colorless isotropic liquid by heating above 31 °C, the isotropic liquid to smectic liquid-crystal onset temperature ($T_{I \rightarrow Sm}$), but the solid material did not melt until 47–50 °C, the melting temperature predicted for a crystalline TO/CO mixture (Small, 1970, 1985).

(B) Triolein/Cholesteryl Linoleate/Cholesteryl Oleate. The temperature-dependent visual appearance of the CL/CO system with no TO was quite similar to that observed for CO with no TO. The addition of small amounts of TO (2% or 4%) depressed the isotropic to cholesteric transition temperature (Figure 1B) and produced temperature-dependent changes in the visual appearance of the samples similar to those observed for CO with ≤7% TO (Table I). However, 5% TO in CL/CO was enough to abolish the blue cholesteric phase, as compared with a value between 7% and 10% TO in CO (Figure 1A). CL/CO containing 5% TO formed a waxy white smectic texture directly from the isotropic liquid. As with CO containing 10% TO, solid material formed from the smectic phase at 20 °C but it took longer (about 2 h) to form. All liquid-crystalline phase changes were reversible with heating and cooling (0.5–1 °C/min) unless the solid formed.

Polarizing Light Microscopy. (A) Triolein/Cholesteryl Oleate. Systems containing TO (2, 4, 7, or 10%) and CO were examined as a function of temperature by PLM to correlate microscopic texture(s) of phases with their macroscopic appearance. All mixtures containing ≤7% TO exhibited similar textures both upon melting and upon cooling from the isotropic liquid; the transition temperatures were dependent on the composition. For example, when a mixture of 4% TO + 96% CO (Figure 2) was crystallized from the isotropic liquid on the microscope slide, the needlelike crystals melted upon heating directly to the isotropic liquid at ~50 °C. The melting temperature for this mixture and all other mixtures agreed with previous results (Small, 1970, 1985). Upon cooling (~1 °C/min) the isotropic liquid, the sample turned purple in the thick section at 37 °C. When the coverslip was pressed, this phase became birefringent with a negative sign, indicative of the cholesteric phase (Figure 2A). Decreasing the temperature to 36 °C caused batonnets (smectic phase) to nucleate and coexist with the cholesteric phase (Figure 2B). By 34 °C, the batonnets coalesced into a mosaic smectic phase, but islands of isotropic liquid remained, clearly demonstrating the separation of an isotropic phase from the smectic phase (Figure 2C). Continued cooling decreased the size of the isotropic islands, but even at 20 °C, they were observed. The changes observed by microscopy for the 7% TO mixture were quite similar, although the transition temperatures were lower. CO with 2% TO exhibited similar phase changes except that islands of isotropic liquid were not observed. This demonstrates that the maximum incorporation of TO into the smectic phase was >2% and <4%. Heating (to 38 °C) the smectic textures in samples with ≥4% TO resulted in the appearance of patchy

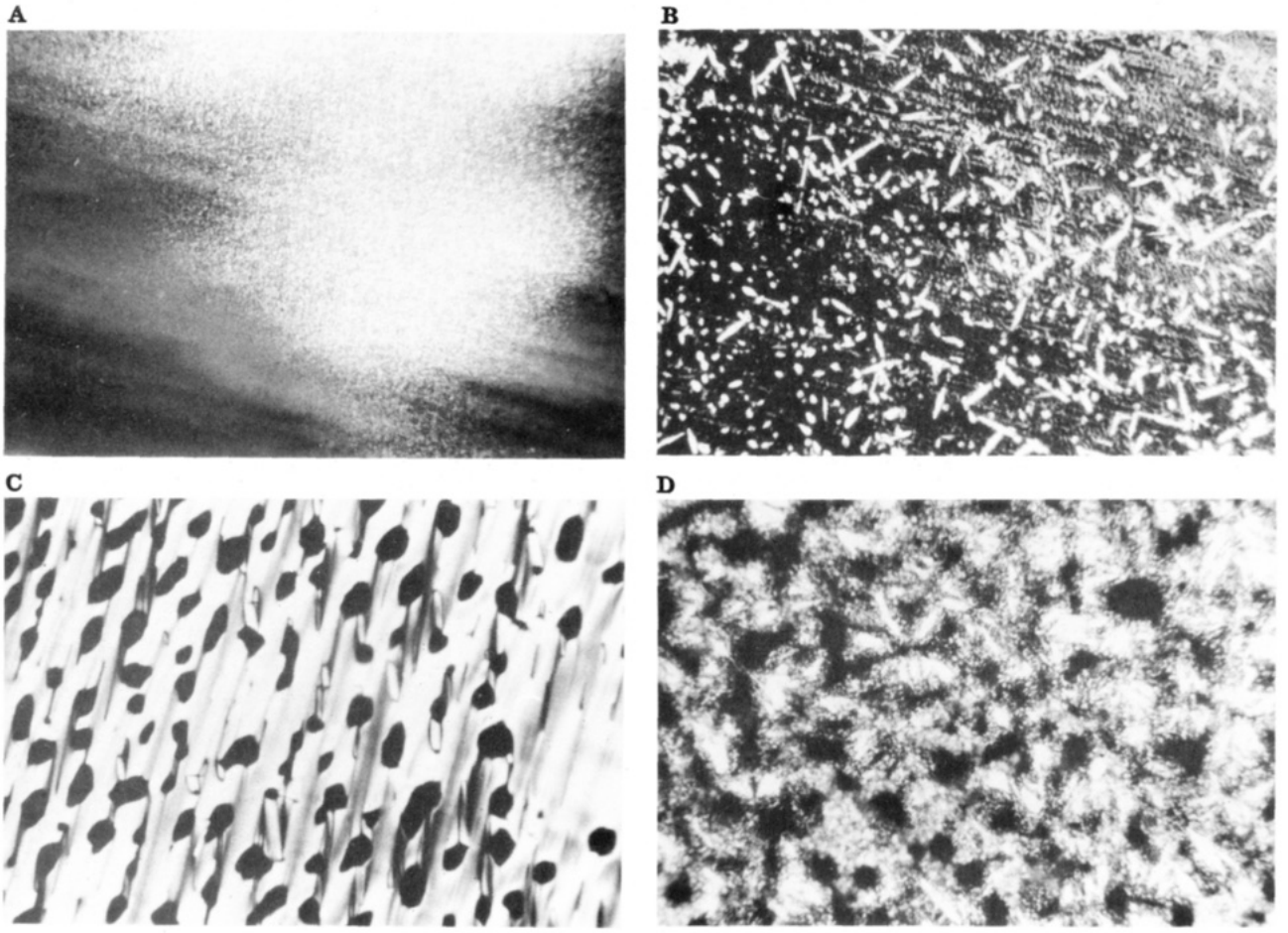

FIGURE 2: Selected polarizing light micrographs showing the liquid-crystalline and isotropic phases for 4% TO + 96% CO. All micrographs were taken with crossed polarizers at 64× magnification. (A) Formation of cholesteric texture (white area) on cooling from the isotropic liquid (black area) at ~37 °C. (B) Rodlike batonnets (smectic phase) formed on cooling to 36–34 °C but some cholesteric material remained (grainy textures). (C) By 34 °C, these batonnets coalesced to form a mosaic smectic phase with islands of isotropic liquid (black areas). (D) Heating (~34–38 °C) of the smectic textures resulted in the appearance of patchy granular cholesteric textures interspersed with an isotropic phase (dark areas).

cholesteric textures interspersed with the isotropic phase, as illustrated for a 4% TO mixture (Figure 2D); recooling resulted in the appearance of batonnets and other smectic textures within the cholesteric patches. Eventually all liquid-crystalline textures, as well as the isotropic phase, formed crystals at temperatures between 30 and 49 °C, showing the metastability of the liquid-crystalline phases.

Heating a sample of 90% CO + 10% TO, which had been crystallized from the isotropic liquid on the microscope slide, produced no changes until the sample melted at 48 °C directly from needlelike crystals to the isotropic liquid. When cooled, the sample exhibited no cholesteric phase; the directly illuminated sample on the stage did not become blue. At 31 °C, batonnets grew directly out of the isotropic liquid. The batonnets grew larger with time (temperature held constant at 31 °C) and eventually fused to produce patches of smectic mosaic and fan textures interspersed with isotropic liquid. In addition, focal conic textures with a positive sign of birefringence (characteristic of a smectic phase) were observed. When heated (1–2 °C/min), the smectic textures melted abruptly at 32 °C to an isotropic liquid and re-formed smectic textures (containing some isotropic liquid) when cooled to 31 °C.

(*B*) *Triolein/Cholesteryl Linoleate/Cholesteryl Oleate.* CL/CO mixtures containing 0, 2, 4, or 5% TO were examined by PLM. With ≤4% TO, samples exhibited temperature-dependent textures similar to those observed for CO with 4% TO, as described above. CL/CO with 5% TO gave results similar to CO with 10% TO. The temperatures at which the microscopic textures occurred depended on the amount of TO in the mixture (Table I).

DSC. (*A*) *Triolein/Cholesteryl Oleate.* DSC was used to characterize mixtures of CO with 2, 3, 4, 7, or 10% TO (Table I; Figure 3). All mixtures containing ≤7% TO exhibited similar DSC results. Upon initial heating, each mixture gave a large endothermic transition at a temperature corresponding to the melting temperature determined by visual characterization and PLM and to that expected from the phase diagram (Small, 1970), as illustrated for CO with 2% TO (Figure 3A). On cooling, two broad, partially overlapping exothermic transitions were seen (Figure 3B). The onset temperature of one of these corresponded to $T_{I \rightarrow Ch}$ observed by visual inspection and PLM, while the second transition temperature corresponded to $T_{Ch \rightarrow Sm}$ (Table I). The two transitions were reversible after cooling below the lower (smectic) transition and immediately reheating (Figure 3C). However, with increasing time, a third transition whose temperature corresponded to that of the initial melting endotherm (crystal → isotropic) was seen (for example, the small shoulder at 48 °C, Figure 3C). Thus, the DSC results also showed that the liquid-crystalline states are metastable. Tracings D–F in Figure 3 compare the cooling behavior of CO samples with 2% (D), 4% (E), and 10% (F) TO. They show a decrease in T_{LC} with increasing percent TO and a broadening (Figure 3E) and eventual disapperance (Figure 3F) of the isotropic →

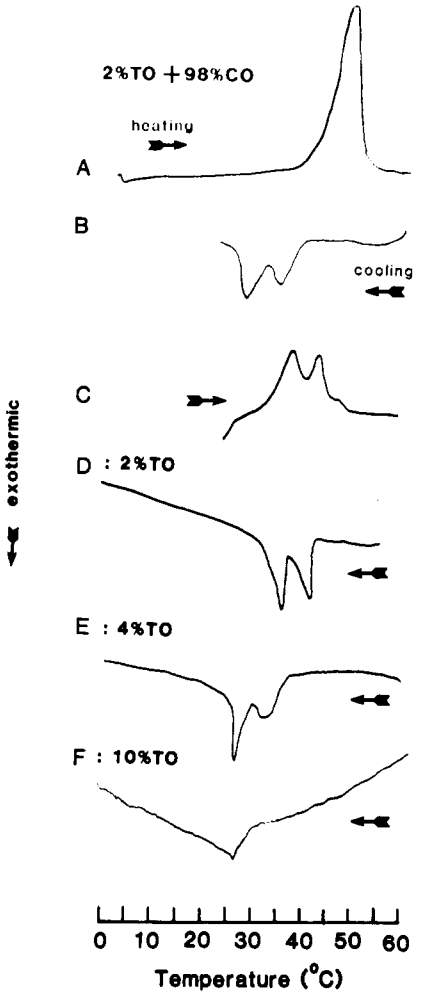

FIGURE 3: Differential scanning calorimetry (DSC) was used to characterize transitions associated with the formation of metastable liquid crystals. Transitions are identified in Table I. (A) Heating (10 °C/min) of 2% TO + 98% CO produced an endotherm for the melting of the crystal to isotropic liquid. (B) Cooling (10 °C/min) of 2% TO + 98% CO gave two broad liquid-crystalline exotherms which exhibited a slight undercooling because of the rapid cooling rate. (C) Reheating of this sample produced liquid-crystalline endotherms as well as a crystal to isotropic endotherm (small shoulder at 48 °C). A comparison of cooling (5 °C/min) curves of (D) 2% TO + 98% CO, (E) 4% TO + 96% CO, and (F) 10% TO + 90% CO shows two exotherms for liquid-crystalline transitions in curves D and E while curve F shows only a broad exotherm for the isotropic to smectic transition.

cholesteric transition. With 10% TO (Figure 3F), a single broad, asymmetric transition, which corresponded to the isotropic → smectic transition, was detected (Table I).

(*B*) *Triolein/Cholesteryl Linoleate/Cholesteryl Oleate.* DSC was also used to characterize CL/CO mixtures containing O% and 2% TO (not shown). Initial heating gave an endotherm near the melting point of the CL/CO mixture. Two transitions were observed on cooling, the higher one corresponding to $T_{I\rightarrow Ch}$ and the lower one corresponding to $T_{Ch\rightarrow Sm}$ (Table I). As with TO/CO mixtures wth ≤7% TO (see above), these transitions were reversible.

^{13}C *NMR Spectroscopy.* (*A*) *Triolein/Cholesteryl Oleate.* Mixtures of CO with 1, 2, 3, 4, or 10% TO were studied by ^{13}C NMR spectroscopy as a function of temperature. Figure 4 shows representative spectra of a cholesteric-forming mixture (4% TO + 96% CO) in the isotropic liquid at $T_{I\rightarrow Ch}$ + 10 °C (A), at $T_{I\rightarrow Ch}$ + 1 °C (B), and in liquid-crystalline phases (C–E). As the temperature was lowered near $T_{I\rightarrow Ch}$, the $\nu_{1/2}$ values of the steroid ring resonances increased differentially

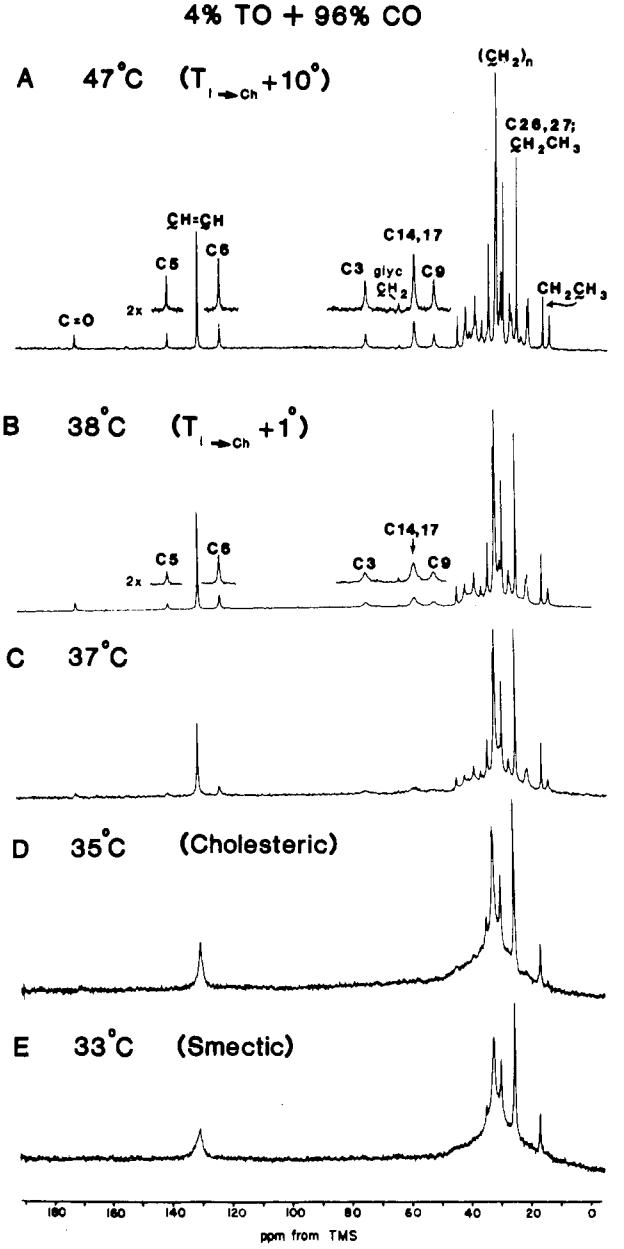

FIGURE 4: Proton-decoupled natural abundance ^{13}C Fourier transform spectra of 4% TO + 96% CO at 50.3 MHz. All spectra were recorded after 2000 accumulations, except for (B) (8000 accumulations). All spectra were recorded with a 10 000-Hz spectral width and a recycle time of 0.82 s. Assignments are shown for selected steroid ring carbons (C) by number (standard sterol nomenclature), for triolein glycerol carbons (glyc:CH_2), and for selected prominent fatty acyl chain carbons as indicated. The physical state of each sample was verified by inspection after removal from the probe immediately following acquisition of the spectrum. (A) Isotropic liquid at $T_{I\rightarrow Ch}$ + 10 °C; (B) isotropic liquid at $T_{I\rightarrow Ch}$ + 1 °C; (C) cholesteric phase and possibly some isotropic phase (see text) at the isotropic to cholesteric transition temperature; (D) cholesteric phase; (E) smectic phase. Spectra A and B were processed with 2-Hz and spectra C–E with 4-Hz line broadening.

(Figure 4A,B). The carbonyl resonance also broadened as $T_{I\rightarrow Ch}$ was approached; in contrast, most resonances from the acyl chain remained relatively sharp (Figure 4A,B). Similar results were obtained with all other mixtures.

Spectra C–E in Figure 4 were obtained at temperatures corresponding to $T_{I\rightarrow Ch}$ (C), the cholesteric phase (D), and the smectic phase (E) of CO with 4% TO. The cholesteric phase spectra contained fairly narrow resonances from the olefinic and certain other acyl chain carbons. Residual in-

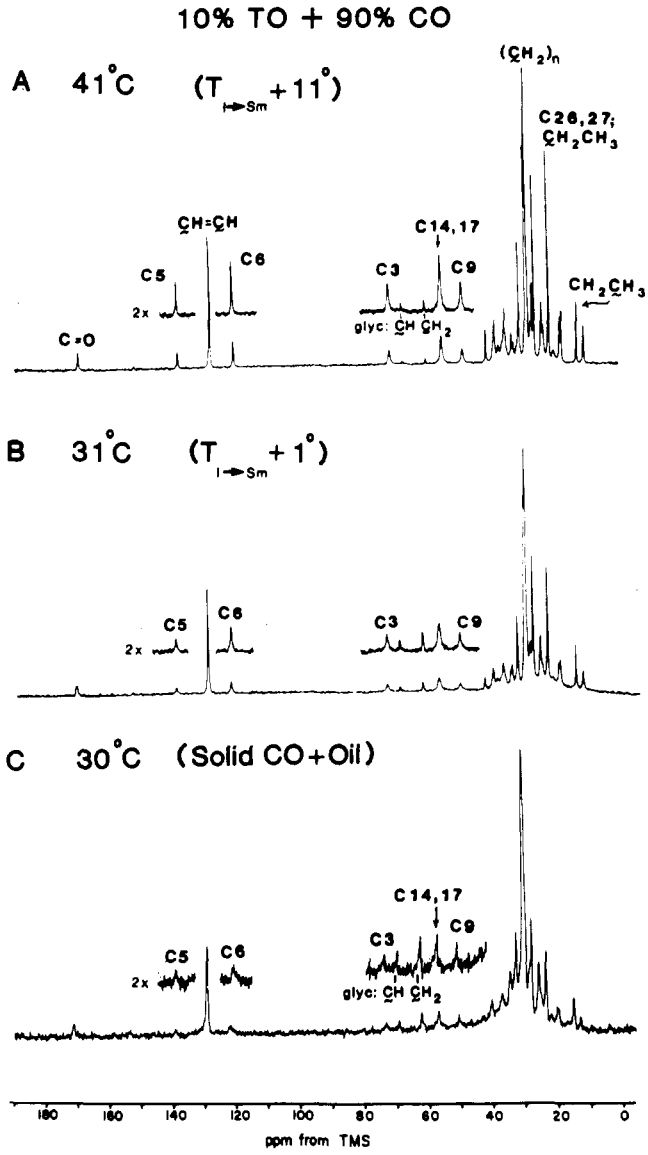

FIGURE 5: Proton-decoupled natural abundance ^{13}C Fourier transform NMR spectra of 10% TO + 90% CO in (A) the isotropic liquid at $T_{I\rightarrow Sm}$ + 11 °C (2000 accumulations), (B) the isotropic liquid at $T_{I\rightarrow Sm}$ + 1 °C (2000 accumulations), and (C) following solidification and phase separation as described in the text (10 000 accumulations). Spectra are labeled as in Figure 4. Note that the vertical expansion of spectrum C is much greater than the expansion for spectra A and B.

tensity from the steroid ring carbons, not seen with the pure ester systems (Hamilton et al., 1977; Ginsburg et al., 1982), was observed in the spectrum (Figure 4C) at 37 °C ($T_{I\rightarrow Ch}$). By visual inspection, this sample appeared to be cholesteric phase. On lowering the temperature 1–2 °C in the cholesteric phase, the steroid resonances broadened and became unobservable; all other resonances broadened only slightly (Figure 4D). In spectra of the smectic phase, the resonances observed for the cholesteric phase broadened (Figure 4E) but not to the extent observed for pure unsaturated cholesteryl esters (Ginsburg et al., 1982). In fact, in the present case, the cholesteric → smectic transition was difficult to distinguish by NMR (compare spectra D and E of Figure 4).

Figure 5 shows spectra of CO with 10% TO, a mixture which exhibited a smectic but no cholesteric phase upon cooling. With decreasing temperature in the isotropic liquid, the steroid ring resonances broadened differentially (Figure 5A,B). However, the $\nu_{1/2}$ changes of the C3 and C6 resonances near (1–2 °C) T_{LC} (see below) were different from the changes

Table II: Line Widths ($\nu_{1/2}$) of C3 and C6 Steroid Ring Carbons of Triolein/Cholesteryl Ester Systems

system	temp (°C)	$\nu_{1/2}$(C3)[a] (Hz)	$\nu_{1/2}$(C6)[a] (Hz)	$\nu_{1/2}$(C3)/ $\nu_{1/2}$(C6)[b]
100% CO[c]	$T_{I\rightarrow Ch}$ + 1	60	18	3.4
	$T_{I\rightarrow Ch}$ + 10	16	8	2.0
1% TO + 99% CO	$T_{I\rightarrow Ch}$ + 1	58	18	3.2
	$T_{I\rightarrow Ch}$ + 8	20	9	2.2
2% TO + 98% CO	$T_{I\rightarrow Ch}$ + 1	112	30	3.7
	$T_{I\rightarrow Ch}$ + 10	24	10	2.4
3% TO + 97% CO	$T_{I\rightarrow Ch}$ + 1	110	23	4.8
	$T_{I\rightarrow Ch}$ + 10	23	11	2.1
4% TO + 96% CO	$T_{I\rightarrow Ch}$ + 1	86	25	3.4
	$T_{I\rightarrow Ch}$ + 12	24	11	2.2
10% TO + 90% CO	$T_{I\rightarrow Sm}$ + 1	44	23	1.9
	$T_{I\rightarrow Sm}$ + 11	28	12	2.3
100% CL/CO[d]	$T_{I\rightarrow Ch}$ + 1	79	25	3.2
	$T_{I\rightarrow Ch}$ + 11	29	11	2.6
2% TO + 98% CL/CO	$T_{I\rightarrow Ch}$ + 1	175	37	4.7
	$T_{I\rightarrow Ch}$ + 12	42	15	2.8
5% TO + 95% CL/CO	$T_{I\rightarrow Sm}$ + 1	78	34	2.3
	$T_{I\rightarrow Sm}$ + 12	39	20	2.0

[a] Line widths to the nearest hertz. [b] Estimated error of 20% arising from an uncertainty of 10% in the line widths. [c] From Ginsburg et al. (1982). [d] CL/CO is a 3/1 (w/w) mixture of CL and CO.

Table III: Temperature Dependence of T_1 and NOE for Selected Steroid Ring Carbons of 4% TO + 96% CO

carbon no.[a]	NT_1 (s)[b] $T_{I\rightarrow Ch}$ + 1 °C	NT_1 (s)[b] $T_{I\rightarrow Ch}$ + 10°C	NT_1 (s)[b] $T_{I\rightarrow Ch}$ + 20 °C	NOE[c] $T_{I\rightarrow Ch}$ + 1 °C	NOE[c] $T_{I\rightarrow Ch}$ + 10 °C	NOE[c] $T_{I\rightarrow Ch}$ + 20 °C
3	0.14	0.14	0.15	1.5	1.5	1.6
6	0.18	0.13	0.12	1.4	1.3	1.3
8	0.13	0.15	0.16	1.6	1.4	1.6
14,17[d]	0.13	0.15	0.16	1.4	1.5	1.6

[a] Standard steroid ring numbering. [b] NT_1 value ± 0.03 s. [c] NOE ± 0.2. [d] Resonances not resolved.

for the mixtures with ≤4% TO. Below the isotropic → smectic transition at 31 °C, the sample with 10% TO solidified rapidly (within 10–20 min), making it impossible to obtain a spectrum of the smectic phase. The ^{13}C NMR spectrum of the sample at 30 °C, which contained a small amount of oil, is shown in Figure 5C. The low S/N reflects the fact that most of the material solidified and did not contribute to the spectrum. The intensities from the TO glycerol backbone resonances relative to CO resonances were greatly increased compared to spectra of the liquid phase above T_{LC} (Figure 5A,B), suggesting that the spectrum originated from a liquid containing CO but rich in TO.

Figure 6 shows plots of line widths of the C3 and C6 resonances from spectra of CO with 2%, 4%, and 10% TO in the isotropic liquid. The mixtures which exhibited a cholesteric phase (1–4% TO) showed a large increase in the C3 and C6 $\nu_{1/2}$ values and in their ratio near T_{LC} (insets, Figure 6A,B; Table II). In contrast, for the system with an isotropic → smectic phase transition (10% TO + 90% CO), the C3/C6 $\nu_{1/2}$ ratio was smaller and exhibited an anomalous slight decrease near T_{LC} (Figure 6C, Table II).

The mixture of CO with 4% TO was chosen to investigate the effects of TO on T_1 and NOE values of ^{13}C resonances from liquid CO. Table III shows T_1 and NOE values for selected steroid ring resonances. These values are the same (±10%) as those reported for corresponding resonances from pure CO (Ginsburg et al., 1982). T_1 and NOE values for steroid ring carbons showed little variation with temperature (Table III). The acyl chain T_1 values were similar to those obtained for the acyl chain of the pure cholesteryl esters

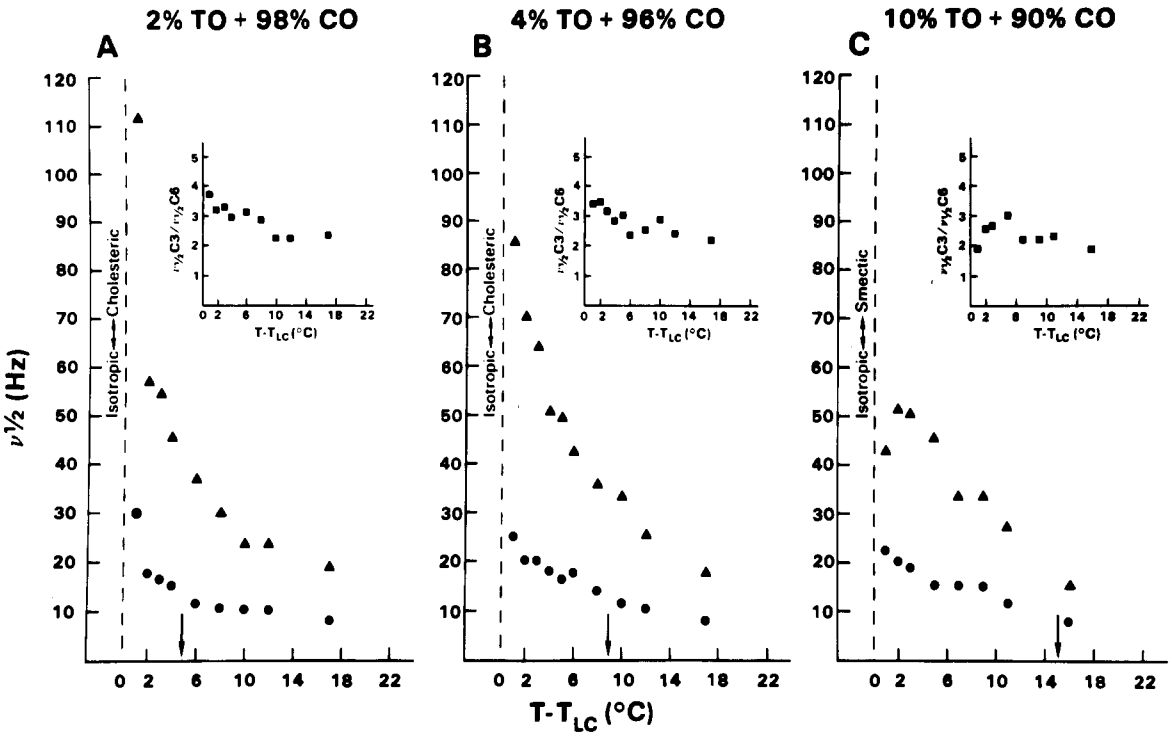

FIGURE 6: Plots of line widths ($\nu_{1/2}$) for the C3 (▲) and C6 (●) steroid ring resonances for mixtures of TO + CO vs. $T - T_{LC}$, the temperature relative to the isotropic liquid to liquid-crystal phase transition temperature (T_{LC}). The phase transition is represented as a dashed vertical line, and the type of transition is noted in each plot. The vertical arrow above each temperature axis indicates the isotropic liquid to cholesteric phase transition temperature for the pure ester. The insets are plots of $\nu_{1/2}(C3)/\nu_{1/2}(C6)$ vs. $T - T_{LC}$ for (A) 2% TO + 98% CO, (B) 4% TO + 96% CO, and (C) 10% TO + 90% CO.

Table IV: Steroid Ring Dynamics of Triolein/Cholesteryl Ester Systems: Experimental C3 and C6 Line Widths ($\nu_{1/2}$), T_1, NOE, and Calculated Rotational Correlation Times at T_{LC} + 1 °C

system	C3 $\nu_{1/2}$ (Hz)	T_1[a] (s)	NOE[b]	C6 $\nu_{1/2}$ (Hz)	T_1[a] (s)	NOE[b]	τ_{rx} (s)	τ_{rz} (s)	motional anisotropy,[c] τ_{rx}/τ_{rz}
100% CO[d]	60	0.12		18	0.15		2.9×10^{-7}	2.8×10^{-9}	103
1% TO + 99% CO	58			18			3.0×10^{-7}	2.9×10^{-9}	103
2% TO + 98% CO	112			30			6.4×10^{-7}	4.5×10^{-9}	142
3% TO + 97% CO	110			23			6.2×10^{-7}	4.3×10^{-9}	144
4% TO + 96% CO	86	0.14	1.5	25	0.18	1.4	4.4×10^{-7}	4.2×10^{-9}	105
10% TO + 90% CO	44			23			2.1×10^{-7}	4.5×10^{-9}	47
100% CL/CO[e]	79	0.13	1.6	25	0.13	1.3	3.7×10^{-7}	3.0×10^{-9}	123
2% TO + 98% CL/CO[e]	175	0.20	1.6	37	0.13	1.3	9.7×10^{-7}	4.5×10^{-9}	215
5% TO + 95% CL/CO[e]	78			34			3.6×10^{-7}	6.0×10^{-9}	60

[a] Measured $T_1 \pm 0.03$ s. [b] Measured NOE ± 0.2. [c] Estimated error ± 20% arising from uncertainties in line-width values. [d] Ginsburg et al. (1982) [e] CL/CO is a 3/1 (w/w) mixture of CL and CO.

(Ginsburg et al., 1982), showing a roughly constant T_1 between the fatty acyl carbon adjacent to the carbonyl and carbons in the olefinic region. However, T_1 for the penultimate CH_2 and terminal CH_3 resonances was greater (10–30%) in the presence of TO. T_1 values of the acyl chain resonances showed a small increase with increasing temperature between $T_{I \rightarrow Ch}$ + 1 °C and $T_{I \rightarrow Ch}$ + 20 °C; the largest increase (30–40%) was seen for carbons at the end of the acyl chain.

NOE values (plots not shown) also exhibited a plateau in the region of the unsaturated bond(s) and increased at the chain terminus. The values of the NOE were the same as those of the pure esters (Ginsburg et al., 1982). Also, little variation of the NOE as a function of temperature was observed.

(B) Triolein/Cholesteryl Linoleate/Cholesteryl Oleate. Temperature-dependent spectra were obtained for CL/CO with 0, 2, or 5% TO. The C3 and C6 resonances broadened differentially with decreasing temperature near T_{Lc} (Figure 7, Table II) but to a larger degree for the cholesteric-forming mixtures (0% or 2% TO) (Figure 7A, B) than for the mixture (5% TO) (Figure 7C) which did not form a cholesteric phase. Thus, this ternary system appeared to be qualitatively similar to the TO/cholesteryl oleate system. Spectral features of the isotropic, cholesteric, and smectic phases were also similar to the features for the binary system. T_1 values were obtained for CL/CO with 2% TO in the isotropic liquid phase and were similar to those for the mixture of CO with 4% TO (above).

Correlation Times. The anisotropic motions of liquid cholesteryl esters can be described (Quinn, 1982) by a spectral density function for an ellipsoid undergoing anisotropic rotational motions (Woessner, 1962). In this model, two correlation times are used: one (τ_{rz}) describes reorientation about the long axis of the ester, while the other (τ_{rx}) describes reorientation about the orthogonal nonunique axis. Using $\nu_{1/2}$ of the steroid ring C3 and C6 resonances, we calculated τ_{rx} and τ_{rz} for all systems at T_{LC} + 1 °C. Table IV summarizes these results and shows that τ_{rz} varied between 3×10^{-9} and 6×10^{-9} s, while τ_{rx} was roughly 2 orders of magnitude longer (2×10^{-7} s $\leq \tau_{rx} \leq 1 \times 10^{-6}$ s). Mixtures which exhibited cholesteric phases had larger values of motional anisotropy ($\tau_{rx}/\tau_{rz} \geq 100$) than mixtures which formed only a smectic

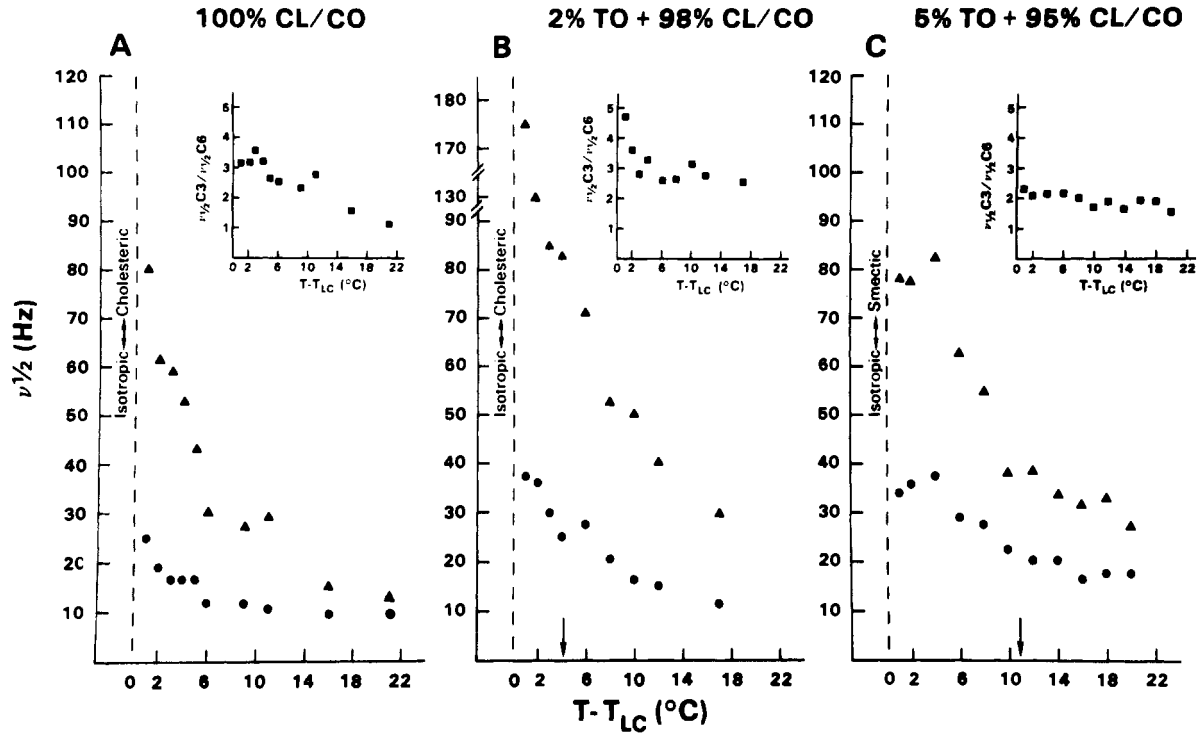

FIGURE 7: Plots of line widths ($\nu_{1/2}$) and line-width ratio (insets) for the C3 and C6 steroid ring resonances vs. $T - T_{LC}$, the temperature relative to T_{LC}, for (A) CL/CO, (B) 2% TO + 98% CL/CO, and (C) 5% TO + 95% CL/CO. The conventions for the plots and insets are as described for Figure 6. The vertical arrows above the temperature axes indicate the isotropic liquid to cholesteric phase transition temperature for CL/CO.

phase ($\tau_{rx}/\tau_{rz} \leq 60$). Using the correlation times τ_{rx} and τ_{rz} presented in Table IV, T_1 and NOE values were calculated (Quinn, 1982). The calculated values (data not shown) agreed closely with the experimental values (Table IV).

Discussion

In this study, we have made a comprehensive physical characterization of two cholesteryl ester/triglyceride systems which are simplified models for cholesteryl ester rich phases in plasma lipoproteins, fatty streaks, and atherosclerotic plaques: (i) cholesteryl oleate with triolein and (ii) a 3/1 mixture of cholesteryl linoleate and cholesteryl oleate with triolein. Qualitatively, triolein had similar effects on both systems: increasing amounts progressively lowered the temperature of the isotropic → cholesteric and cholesteric → smectic phase transitions. At low concentrations of TO (≤4%), each percent TO lowered the transition temperature by 2 ± 1 °C (Table I, Figure 1). However, the amount of TO needed to abolish the cholesteric phase differed significantly in the two systems: at 5% TO in CL/CO, an isotropic → smectic transition was seen, and no cholesteric phase was detected, as reported previously (Hamilton et al., 1977). In contrast, the cholesteric phase persisted in CO with 7% TO and was abolished at a level of 10% TO.[3] In this system, addition of TO between 4% and 10% produced a relatively small temperature depression, and the relationship between temperature depression and TO concentration was not a simple linear function (Figure 1). Using the mixture of cholesteryl esters isolated from human plasma low-density lipoprotein, Deckelbaum et al. (1977) found that >3% triglyceride (mixed triglycerides isolated from low-density lipoprotein) abolished the cholesteric phase; the average temperature decrease was <1 °C per percent triglyceride added to the cholesteryl esters. In addition, a linear relationship between temperature depression and triglyceride concentration (between <1% and 14% triglyceride) was found.

Taken together, the results of model systems predict that the precise composition of a biological sample with respect to cholesteryl ester and triglyceride will influence the phase behavior and that modest compositional changes may alter the phase behavior of the cholesteryl esters. That cholesteryl ester composition and triglyceride content can affect the phase behavior of cholesteryl esters in lipid cellular inclusions has been demonstrated by Adelman et al. (1984). Changes in physical state greatly altered the efflux of cholesteryl esters from cells (Adelman, 1984). In physical studies of macrophage lipid droplets in Tangier disease, the average melting temperature of droplets was dependent on the cholesteryl ester fatty acid composition and the content of triglyceride and cholesterol (Katz et al., 1977). In rabbit lipid-rich tissues, chemical heterogeneity has been detected within the same tissue by PLM; the regional differences in melting behavior most likely result from cholesteryl ester compositional differences and/or variations in triglyceride content (Waugh & Small, 1984).

Our microscopy results provide detailed information about the identity of different phases and the nature of the phase transitions. There are important similarities and differences between these systems and pure cholesteryl esters. First, the cholesteryl esters with triolein exhibited crystal → isotropic liquid and metastable isotropic → liquid-crystal transitions, as do neat cholesteryl esters (Small, 1970). Second, cholesteric

[3] These results differ from those of Lundburg (1976) and Kroon (1981), who reported abolition of the cholesteric phase of CO at lower levels (2–5%) of TO. However, in the first case, samples were prepared from organic solvents which even in trace amounts could affect liquid-crystal formation. In the latter case, the single broad DSC transition assigned to the isotropic liquid → smectic transition occurred at the temperature (~40 °C) at which we observed an isotropic liquid → cholesteric transition and could include both transitions. Our finding that 5% TO abolished the cholesteric phase in the CL/CO mixture but that a higher concentration (between 7% and 10%) of TO was required to abolish the cholesteric phase of pure CO may indicate that the second cholesteryl ester affects the stability of the cholesteric phase and reduces the amount of the third component (TO) needed to alter the phase behavior of CL/CO.

and smectic phases had microscopic textures similar to those of neat cholesteryl esters (Small, 1970). Third, unlike neat cholesteryl esters, isotropic liquid was observed in some mixtures well below the liquid → liquid-crystal transition. The relative amount of this isotropic liquid was small and decreased with decreasing temperature, but in the case of ≥4% TO, some liquid was seen even at the lowest temperature investigated in the smectic phase. It probably also coexisted with the crystalline phase, as predicted from the equilibrium phase diagram for the crystalline and liquid TO/CO system (Small, 1970). With increasing amounts of TO, more of this isotropic phase was seen interspersed with the smectic liquid-crystalline phase. Fourth, the phase transitions were not as discrete as for neat cholesteryl esters. In addition to the persistence of some isotropic liquid in temperature ranges in which the smectic phase was the predominant phase, there was some overlap of cholesteric and smectic phases. Thus, the phase transitions occurred over a larger temperature range, and the boundaries between different phases were somewhat obscured.

Certain of the PLM observations were corroborated by results from other methods. For example, the DSC transitions were broader than those for neat cholesteryl esters (Small, 1970), consistent with the lack of sharp (i.e., highly cooperative) microscopic transitions. Visual inspection of the CO sample with 10% TO showed liquid droplets interspersed with the smectic phase and with the solid phase that formed from the smectic phase. The PLM results showed that there were regions of isotropic liquid in the smectic phase. The ^{13}C NMR spectrum of the solidified mixture at 31 °C (Figure 5C) revealed a liquid phase rich in triglyceride. Another instance in which ^{13}C NMR identified minor components in mixed-phase systems was in the spectrum of CO with 4% TO at 37 °C (Figure 4C). This spectrum, obtained just below the isotropic → cholesteric transition, showed fairly prominent resonances from steroid ring carbons, suggesting that the sample, which visually appeared to be cholesteric, contained a significant amount of isotropic liquid. This finding is consistent with the blurring of phase boundaries observed by microscopy.[4]

Our results show that a liquid triolein-rich phase separated from the smectic and, perhaps, from the cholesteric cholesteryl esters. In both systems (CO and CL/CO), the phase separation was first detectable (by PLM) at a concentration of 4% TO. In the case of CO, which required a higher concentration of TO to abolish the cholesteric phase than CL/CO, the addition of 4% and 7% TO lowered T_{LC} to a lesser extent than predicted from the temperature-lowering effect of 1, 2, and 3% TO (Figure 1A). This finding suggests that, for CO with 4–7% TO at temperatures below T_{LC}, TO partitions between the liquid and liquid-crystalline phases.

Steroid ring methine carbons of neat liquid cholesteryl esters exhibit increasingly anisotropic motions and consequently increased ^{13}C line widths as the isotropic → liquid-crystal transition is approached (Hamilton et al., 1977; Quinn, 1982; Ginsburg et al., 1982). The present results show that ^{13}C NMR spectroscopy detected the preordering of steroid rings near the cholesteryl ester phase transition in the presence of triglyceride. Thus, steroid ring resonances were observed below T_{LC} of neat CO (46 °C) or the CL/CO mixture (34 °C), and the large $\nu_{1/2}$ increases occurred near the isotropic → liquid-crystal transition of the particular mixture (Figures 6 and 7). Below T_{LC}, steroid resonances were not observed because of extensive line broadening, except in cases where small amounts of liquid might have persisted below the transition (see above). Thus, in liquid-crystalline phases, the motions of the ring system were highly restricted and/or anisotropic, while those of some fatty acyl carbons (the distal portion of the chain) were sufficiently rapid that relatively narrow peaks were observed, as for neat cholesteryl esters (Hamilton et al., 1977; Ginsburg et al., 1982). However, in the spectrum of CO with 4% TO in the smectic phase (Figure 4E), the fatty acyl peaks were narrower than those for CO (Ginsburg et al., 1982), showing that TO perturbed the molecular motions of the liquid-crystalline CO.

Close examination of the C3 and C6 line-width plots (Figures 6 and 7), the C3/C6 line-width ratios (insets, Figures 6 and 7; Table II), and the correlation time ratios (Table IV) shows that steroid ring motions were more anisotropic prior to formation of a cholesteric phase than prior to formation of a smectic phase. Thus, when the cholesteric phase was abolished and an isotropic → smetic transition was observed, these systems (i.e., 10% TO + 90% CO and 5% TO + 95% CL/CO) had decreased $\nu_{1/2}$ values, $\nu_{1/2}$ ratios, and anisotropy ratios (τ_{rx}/τ_{rz}) at $T_{I\rightarrow Sm}$ + 1 °C relative to the systems at $T_{I\rightarrow Ch}$ + 1 °C with lower amounts of TO. For example, for 4% TO + 96% CO, $\nu_{1/2}(C3)/\nu_{1/2}(C6)$ = 3.4 (C3 = 86 Hz, C6 = 25 Hz) and τ_{rx}/τ_{rz} = 105 (Tables II and IV). With abolition of the cholesteric transition (10% TO), the $\nu_{1/2}(C3)/\nu_{1/2}(C6)$ decreased to 1.9 and τ_{rx}/τ_{rz} to 47.[5] Results for the CL/CO system were basically similar (Tables II and IV). In contrast, the line-width ratio at 8–12 °C above T_{LC} was unaffected by the TO concentration (Table II). These results are similar to those of a recent study which compared cholesteryl esters with a single double bond at the same relative position (ω-9) and showed that cholesteryl esters exhibiting an isotropic → smectic transition (cholesteryl nervonate, $C_{24:1}$, and cholesteryl erucate, $C_{22:1}$) had a lower anisotropy ratio at T_{LC} + 1 °C than cholesteryl oleate ($C_{18:1}$) which exhibits an isotropic → cholesteric transition (Ginsburg et al., 1982).

The present results amplify the limited NMR data on mixtures of cholesteryl esters and triglycerides exhibiting liquid-crystalline phases (Hamilton et al., 1977). They show that general principles drawn from studies of long-chain unsaturated cholesteryl esters without triglyceride (Hamilton et al., 1977; Quinn, 1982; Ginsburg et al., 1982) should be applicable to biological systems containing triglycerides. In fact, temperature-dependent differential line broadening for cholesteryl ester ^{13}C resonances has been described for low-density lipoproteins (Hamilton et al., 1979), for cholesteryl ester rich very low density lipoproteins (Kroon et al., 1982; Morrisett et al., 1980, 1984), and for human atherosclerotic plaques (Hamilton et al., 1979). However, ^{13}C NMR spectra of these biological samples revealed (i) a single liquid-liquid crystal transition, (ii) a temperature depression of the differential line broadening relative to cholesteryl esters with no triglyceride, and (iii) relatively narrow fatty acyl resonances for the liquid-crystalline phase. The present study shows that all these discrepancies between biological samples and cholesteryl ester model systems can be explained, at least in part, by the exclusion of triglycerides from previous model systems. Furthermore, the decreased C3/C6 line-width ratio near the cholesteryl ester transition in lipoproteins and plaques (Hamilton et al., 1977, 1979) may result from the abolition of the

[4] Alternatively, the presence of steroid resonances may indicate that in the cholesteric phase the ring system has increased rates of molecular motions compared to the pure ester near the phase transition.

[5] Some systems showed an increase, relative to pure esters, in the line-width ratio at low TO concentrations (before abolition of the cholesteric phase). Thus, lowering T_{LC} at these levels of TO may promote stronger ring–ring interactions and increased rotational anisotropy.

cholesteric phase by triglyceride.

From the NMR data, it appears that the major perturbations of the molecular motions of CO and CL caused by TO are in the steroid ring motions. On an absolute temperature scale, the anisotropic ring motions are altered by each addition of TO. Depressing T_{LC} should not, per se, prevent formation of a cholesteric phase from the isotropic phase, since long-chain (ω-9) cholesteryl esters which exhibit a cholesteric phase have lower transition temperatures than the ω-9 analogues which exhibit only a smectic phase (Ginsburg et al., 1982). In fact, for the cholesteryl ester/TO mixtures, those systems which had an isotropic → cholesteric phase transition exhibited motions which were as anisotropic (or more so) at the depressed T_{LC} than their antecedent cholesteryl esters, suggesting that strong ring–ring interactions were still present. At higher levels of TO, the abrupt decrease in anisotropic steroid ring motions at T_{LC} + 1 °C when the cholesteric phase was abolished indicates a marked disrupting effect of TO on cholesterol ester interactions. Previous comparisons of transition enthalpies and entropies have shown that strong and specific ring–ring interactions are required for formation of a cholesteric phase (Barrall et al., 1969). Thus, low concentrations of TO disrupt steroid ring interactions, but the depression of T_{LC} may permit interactions crucial for cholesteric phase formation to be reestablished. Higher concentrations of TO disrupt ring–ring interactions to the extent that the cholesteric phase cannot form, even at the depressed T_{LC}.

Finally, our results suggest a molecular mechanism for the effects of triglyceride on cholesteryl ester phase behavior. The NMR data showing anisotropic motions of liquid cholesteryl esters near T_{LC} could reflect the presence of increasing amounts of highly anisotropic and organized domains in rapid equilibrium with the bulk liquid phase. Frenkel (1946) has shown that on approaching the temperature of a first-order phase transition (such as an isotropic liquid → liquid-crystal transition), there is an increasing concentration of domains or clusters of the second phase in the first phase. The effect of small additions of TO could be to prevent domain formation at the T_{LC} of the neat cholesteryl esters. Decreasing the temperature (and thereby the kinetic energy of the system) would permit formation of domains requisite for liquid-crystal formation. Since the cholesteric phase is an extended structure with helical periodicites of several thousand angstroms (Gray, 1962), it might be predicted that this phase could be abolished with relatively low amounts of TO, because the associations between small organized domains that serve as building blocks for the extended structure would be prevented. The microscopy results showing mixed types of patchy liquid-crystalline phases in the presence of TO may also exemplify the existence of clusters or submicroscopic domains.

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Registry No. Cholesteryl oleate, 303-43-5; cholesteryl linoleate, 604-33-1; triolein, 122-32-7.

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