

# Phase Behavior and Crystalline Structures of Cholesteryl Ester Mixtures: A C-13 MASNMR Study

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**ABSTRACT** Cholesteryl esters are a transport and storage form of cholesterol in normal physiology but also a significant lipid in atherosclerotic plaques. To understand better the molecular properties of cholesteryl esters in tissues and plaques, we have studied the polymorphic and mesomorphic features of pure and mixed cholesteryl esters by solid state C-13 NMR with magic angle sample spinning (MASNMR). The temperature-dependent properties of two single components (cholesteryl linoleate (CL, C18:2) and cholesteryl linolenate (CLL, C18:3)), four binary systems (cholesteryl palmitate (CP, C16:0) with CL, CLL or cholesteryl oleate (CO, C18:1), and CO/CL), one ternary system (CO/CP/CL), and one quaternary system (CO/CP/CL/CLL) were studied. The mixing ratios were based on the composition of an atherosclerosis plaque dissected from a cholesterol-fed New Zealand white rabbit. C-13 MASNMR determined the phase transition temperatures, identified the phases present in all systems, and provided novel information about molecular structures. For example, solid CL exhibited a disordered structure with multiple molecular conformations, whereas pure CLL had a crystalline structure different from the three most commonly characterized forms (*MLII*, *MLI*, *BL*). In binary mixtures, the crystalline structure of each cholesteryl ester species was identified by its own characteristic resonances. It was found that CP always existed in its native *BL* form, but CL and CO were influenced by the composition of the mixture. CL was induced to form *MLII* crystals by the coexisting CP (55 wt %). When CO was cooled from the isotropic phase, it existed as a mixture of *MLII* and an amorphous form. The presence of CP significantly accelerated the conversion of the amorphous form to the *MLII* form. For the ternary mixture co-dried from chloroform, CL cocrystallized with CO in the *MLII* form and CP existed in *BL* form. Addition of a small amount of CLL slightly increased the heterogeneity of the solid mixture, but had little effect on the crystal structures or the phase transitions. C-13 MASNMR represents a powerful method for physical characterization of cholesteryl ester mixtures reflecting the composition of biological samples.

## INTRODUCTION

Esterification of cholesterol to fatty acids enhances cholesterol transport in human plasma and storage in cells. Cholesteryl esters (CEs) as a lipid class have general properties of a weakly polar, nonswelling lipid but specific properties dependent on the specific fatty acyl chain (Small, 1986). Thus, the phase transition temperatures and types of liquid crystalline phases and crystalline structures depend on the acyl chain length and unsaturation (Small, 1986). Because of their importance in normal human physiology and in pathophysiology (most importantly, atherosclerosis), CEs have been studied intensively by a variety of physical techniques (Small, 1986), including high resolution C-13 NMR spectroscopy (Hamilton et al., 1977, 1979; Ginsburg et al., 1982; Croll et al., 1985, 1986).

In a recent study we systematically examined eight different CEs in their pure form in different phases by a new

approach, solid state NMR with magic angle sample spinning (MASNMR). Novel properties of liquid crystalline and crystalline phases were revealed, and a protocol was established to distinguish the various phases of CE (Guo and Hamilton, 1993). High resolution C-13 spectra of the crystalline CE were obtained by MASNMR with cross-polarization transfer. The three known crystallographic types of CE (monolayer I, *MLI*; monolayer II, *MLII*; and bilayer, *BL* (Craven, 1986)) can be distinguished by unique chemical shifts. Specifically, *MLI* and *BL* forms are characterized by twin signals for certain carbons, such as C5, C6, C18, C13, C10, C19, C=O, and  $\omega\text{CH}_3$  in the *BL* form, and C=O, C5, C6, C9, and C18, and in the *MLI* form, reflecting the two crystallographically inequivalent molecules. CEs in the *MLII* form are characterized by a single peak for each carbon because all the molecules are equivalent in this crystalline structure. Details on the spectral differences and peaks assignments in these three crystalline forms have been provided previously (Guo and Hamilton, 1993).

The non-crystalline phases of CE, smectic (*Sm*) or cholesteric (*Ch\**) liquid crystalline, and isotropic (*Iso*), can be differentiated by C-13 MASNMR. Crystalline CE phases can be detected effectively only by MASNMR with cross-polarization transfer, whereas liquid crystalline CE can be detected with or without cross-polarization transfer. Proton-decoupled C-13 MASNMR spectra for the *Sm* and *Iso* phases are very similar, but these two phases are easily differentiated by off-magic angle sample spinning, in which case the signals for the *Sm* phase broaden significantly, whereas the signals for the *Iso* phase remain narrow. Unlike the crystalline, *Sm*, or *Iso*

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Abbreviations used in this article: CPMAS, MASNMR with cross-polarization transfer; CL, cholesteryl linoleate; CLL, cholesteryl linolenate; CO, cholesteryl oleate; *Ch\**, cholesteric phase; CP, cholesteryl palmitate; CS, cholesteryl stearate; Cryst, crystalline phase; *Sm*, smectic phase; SSB, spinning side band.

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phases, which all give narrow peaks with MAS, the Ch\* phases are characterized by broad signals, particularly for carbons with a large chemical shift anisotropy, such as C5, C6, and C=O. These broad linewidths are not significantly affected by the decoupling power, temperature, or spinning rate and are standard features for the recognition of the Ch\* phase (Guo and Hamilton, 1993). We attributed this line broadening to a broad chemical shift distribution caused by motional modulation of the shielding resonance. The biaxial nature of the Ch\* phase might also contribute to such line broadening.

CE pools in biological systems such as plasma low density lipoproteins, intracellular droplets, and atherosclerotic plaques consist of CE with different acyl chains. The composition determines the plasma behavior of such CE-rich mixtures and is a major determinant of the pathophysiology of atherosclerosis (Small, 1988, 1990). The primary unsaturated CEs in most biological pools are cholesteryl oleate (CO) and cholesteryl linoleate (CL). CP is the primary saturated CE. CP is also the major saturated species in atherosclerotic plaques and could solidify in plaques at 37°C because of its tendency to fractionate in CE mixtures (Dorset, 1990a). Conventional C-13 NMR spectroscopy is useful for characterization of isotropic CE phases in atherosclerotic plaques but is unable to detect solid phases and can only partially characterize liquid crystalline phases (Hamilton et al., 1979).

In the present work C-13 MASNMR is used to study CE in mixtures of increasing complexity. First, we characterize the crystalline and liquid crystalline phases of two pure CEs, CL and cholesteryl linolenate (CLL). CL is an important and abundant CE in biological systems, whereas CLL is generally a minor component. The polymorphic and mesomorphic properties of these two CEs have been reported (Small, 1970), but these esters have not previously been characterized by C-13 MASNMR, and there is no published information on their crystalline structures. We then extend the characterization of pure CEs to mixtures modeling CEs in atherosclerotic plaques in rabbit arteries, first to binary mixtures of the four principal CEs. Binary mixtures of CEs have been investigated by other techniques as models of biological CE mixtures and as models for interactions of CEs with different chain lengths in the liquid crystalline and crystalline states (Dorset, 1987, 1988, 1990a,b; Dorset and Pangborn, 1992; McCourt et al., 1994). At a higher level of complexity, we characterize a ternary and quaternary mixture of CE. A detailed analysis of the phase behavior of CE in intact lesions will be presented elsewhere (W. Guo, R. Cohen, and J.A. Hamilton, manuscript in preparation).

## MATERIALS AND METHODS

Cholesteryl esters (>99%) were purchased from Nu-chek Co. (Elysian, MN) and used without further purification. Thin-layer chromatography (TLC) of the esters in 96:4:1 hexane-diethyl ether-acetic acid (Ginsburg et al., 1982) showed a single spot. For the mixed systems each pure component was weighed according to the desired ratio, CHCl<sub>3</sub> was added to achieve uniform mixing, and the CHCl<sub>3</sub> evaporated according to the procedure of Dorset (1990a) except when otherwise indicated. All NMR measurements were performed on a Bruker (Billerica, MA) AMX-300 (75 MHz for C-13) equipped with a BL-7 MAS probe and a high power amplifier unit. The actual probe temperature was calibrated by correlating the changes in the C-13 MASNMR spectrum with the known phase transition temperatures of pure cholesteryl esters. The melting of crystalline CE into the Iso, Sm, or Ch\* phase is accompanied by a large increase in signal-to-noise ratio in the single pulse C-13 experiment (without cross-polarization transfer); the Sm → Ch\* transition is accompanied by characteristic line broadening of the C=O, C5, and C6 peaks; the Sm → Iso transition is accompanied by the disappearance of SSB, and the Ch\* → Iso transition by unique line narrowing as described previously (Guo and Hamilton, 1993).

The mixing ratios of the CE mixtures were based on the analytical data in a rabbit plaque (Table 1). Differential scanning calorimetry (DSC) experiments were performed on a DSC-7 instrument (Perkin Elmer). The phase transition temperatures measured for each CE or CE mixture were in good agreement with the corresponding transition temperatures measured by MASNMR.

## RESULTS AND DISCUSSION

### CL

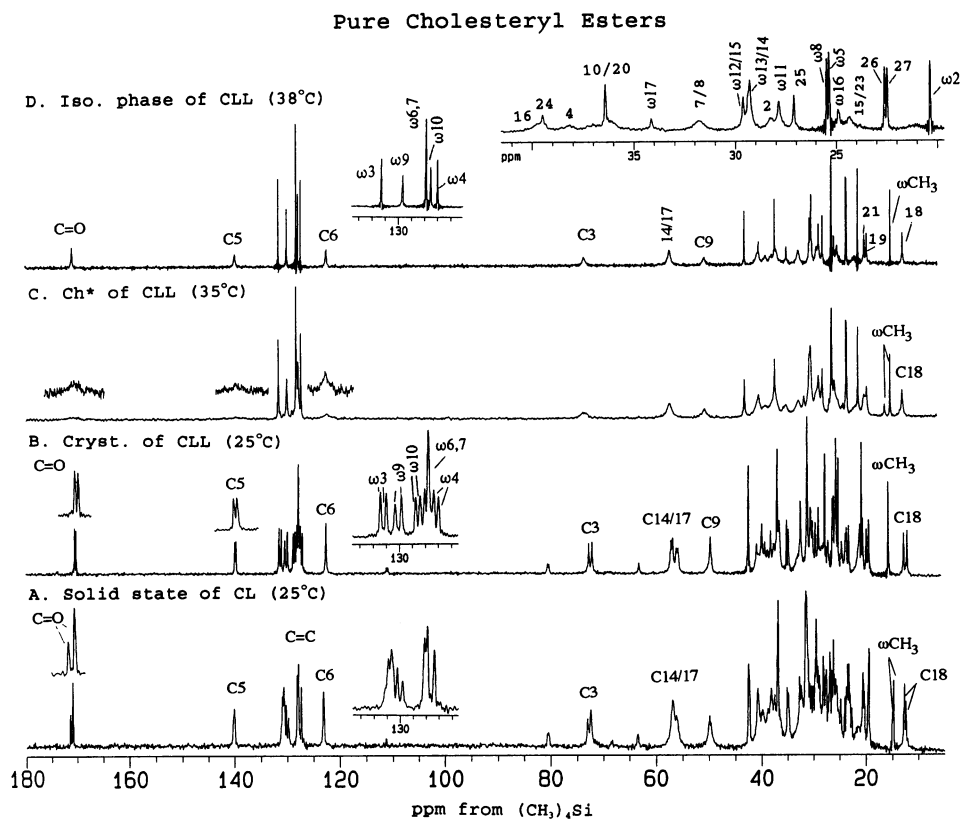
Pure CL is known to be very difficult to crystallize, and the single crystal structure has not been reported (Dorset, 1990b). The lamellar long spacing of CL in its solid state at ambient temperature (40 Å) is different from the long spacings of crystalline CE with other 18 carbon acyl chains (Small, 1986). Since MASNMR does not require single x-ray quality crystals, we attempted to obtain a polycrystalline sample by recrystallization from CHCl<sub>3</sub>. After drying the resultant waxy material, a C-13 MASNMR spectrum was obtained at 25°C (Fig. 1 A). This spectrum does not resemble that of any crystalline CE previously studied (Guo and Hamilton, 1993). Similar spectra were obtained for a solid state sample of CL received directly from the manufacturer (stored at 4°C for >1 year) or for a sample solidified from the isotropic phase, except for some irregular variations in the peak intensity (not shown). The appearance of multiple signals for the same carbon with anomalous peak intensities indicates the CL sample does not have a uniformly ordered crystalline structure. On the other hand, the chemical shifts of these multiple signals were independent of the heating history, implying that the solid CL was not a totally disordered structure. Instead, it is likely that the solid CL is composed of two (or

**TABLE 1** Composition of rabbit atherosclerotic plaque

CE	Lipid composition (wt %)				Acyl chain composition of CE (wt %)											
	TG	FFA	Chol	PL	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:1	20:2	20:3	20:4	22:1
42	3.7	5.1	24	26	1.1	14.4	4.4	4.3	58.5	11.6	1.4	1.4	1.5	0.4	0.6	0.4

CE, cholesteryl ester; TG, triacylglycerol; FFA, free fatty acid; Chol, cholesterol; PL, phospholipid.

FIGURE 1 C-13 MASNMR spectra at 75 MHz. (A) CL in the solid state at 25°C; (B) crystalline CLL at 25°C; (C) CLL in the cholesteric phase at 35°C; (D) CLL in the Iso phase at 38°C. All spectra in this figure and figures below were obtained with a sample spinning rate of 4.5 kHz, except as noted, and were processed without line broadening after 1000 scans. Spectra A and B were obtained with cross-polarization transfer; spectra C and D were obtained without cross-polarization transfer and sample spinning rate of 3 KHz. Steroid carbons are labeled numerically according to standard nomenclature after the letter C, except in the region 10–45 ppm, where letter C was omitted. Acyl chain carbons are labeled numerically relative to the terminal ( $\omega$ ) carbon.



more) types of microcrystals that have different crystallographic habits, each giving its own C-13 resonances (B. Craven, personal communication).

In the Iso, Ch\*, and Sm phases, CL gave spectra (not shown) characteristic of other pure CE in the corresponding phases (Guo and Hamilton, 1993).

## CLL

Because of the lack of single crystal data for CLL, details about its actual structure are not known. The long spacing of crystalline CLL (27.85 Å) determined by x-ray diffraction (Loomis et al., 1974) is substantially larger than that of CO (18.5 Å, *MLII*), but shorter than that of CL (40 Å, unknown structure) or CS (54 Å, *BL*). This implies that CLL may not fall into any type of structure determined for these 18 carbon chain CEs. C-13 NMR spectra of CLL have not been previously reported, and chemical shifts and assignments for each phase are listed in Table 2. The C-13 spectrum of the solid state CLL (25°C, Fig. 1 B), in contrast to that of CL (Fig. 1 A), shows distinct twin signals for many well isolated carbons, such as C=O, C5, C3, C18, C14,17 and the  $\omega(3,6,9)$  double bond carbons in the acyl chain. In our previous work characterizing the crystalline structures of eight CEs (Guo and Hamilton, 1993), twin signals were observed for CE in *MLI* and *BL* crystals. The separations between the twin peaks of CLL ( $\leq 0.8$  ppm) are generally much smaller than those in the *MLI* structure (up to 2.0 ppm), especially for the C=O, C5, and C3 peaks. The twin-peak separations of CLL are quantitatively similar to those in the *BL* structure, with some

significant differences; twin signals are observed for the C3 signal of CLL but only a singlet for CEs with a *BL* structure, whereas twin signals for C6, C13, and  $\omega\text{CH}_3$  are seen in the *BL* structure but only singlets for CLL. Thus the C-13 MASNMR result also excludes the possibility of an *MLII*, *MLI*, or *BL* structure for CLL, in agreement with the lamellar d-spacing measurements. Instead, CLL represents a new type of crystalline structure containing two crystallographically inequivalent molecules. The shielding environments in these two types of molecules are different, especially around the C=O, C5, C3, C18, C14,17, and  $\omega(3,6,9)$  carbons. For CLL the C5 gives a twin signal but the C6 gives a single peak, in contrast to CE in either *MLI* or *BL* crystalline forms, which gave twin signals for both C5 and C6 peaks (Guo and Hamilton, 1993). However, the origin of this observation is not clear because of the lack of the single crystal configuration. The separation (in ppm) between each twin peak is rather small, probably implying a monolayer structure with a slight displacement between the two neighboring molecules to accommodate the cis- $\omega(3,6,9)$  double bonds in the acyl chains. Considering that CLL has a longer d-spacing than CO, we suggest that the tilt angle between the layer normal and the molecular long axis is smaller than that of CO.

It is of interest that, for the four biologically important 18 carbon chain CEs, the ease of crystallization from the isotropic liquid follows the trend of  $\text{C18:0} \approx \text{C18:3} \gg \text{C18:1} \gg \text{C18:2}$ . The significant differences in the C-13 MASNMR spectra of solid state CE with 18 carbon acyl chains (C18:2 (Fig. 1 A); C18:3 (Fig. 1 B); C18:0 and C18:1 (Guo and

**TABLE 2** The chemical shift values of cholesteryl linolenate in various physical states

# C	Cryst.	Sm	Ch*	Iso
C=O	171.01, 170.76	170.90	170.80	170.90
C5	140.31, 140.05	139.63	139.58	139.58
$\omega$ 3	131.86, 131.41	131.30	131.33	131.32
$\omega$ 9	130.73, 130.26	129.68	129.70	129.70
$\omega$ 6/7	128.17, 128.17	127.90	127.92	127.92
$\omega$ 6/7	128.46, 128.17	127.90	127.92	127.92
$\omega$ 10	129.16, 128.81	127.52	127.56	127.56
$\omega$ 4	127.76, 127.40	127.00	127.03	127.03
C6	122.86	122.13	122.06	122.07
C3	72.87, 72.21	72.83	73.08	72.92
C14/17	57.19, 56.90	56.51	56.57	56.48
	56.19, 55.86			
C9	49.69	49.77	49.84	49.86
C13	42.45	42.22	42.22	42.20
C16	40.91	39.88	39.52	39.76
C24	39.90	39.52	39.52	39.47
C4	38.25	38.21	38.21	38.15
C1	37.64	37.00		37.02
C10/22	36.99	36.45	36.43	36.40
C20	36.56	36.45	36.43	36.07
$\omega$ 17CH <sub>2</sub>	34.80, 35.18	34.23	34.28	34.13
C7/8	32.45	31.82	31.85	31.75
$\omega$ 12	30.50	29.72	29.68	29.62
$\omega$ 15	30.14	29.61	29.43	29.36
$\omega$ 13/14	29.00, 29.57	29.61	29.43	29.28
C2		28.30	28.38	28.24
$\omega$ 11	27.77	27.94	27.92	27.86
$\omega$ 5	25.65	25.45	25.35	25.34
$\omega$ 8	25.65	25.32	25.47	25.46
C25		27.18	27.18	27.10
$\omega$ 16	25.22	25.00	24.84	24.89
C15/23	24.57			24.32
C26/27		22.55	22.63, 22.52	22.48, 22.60
C11	21.10	21.07	21.09	21.07
$\omega$ 2CH <sub>2</sub>		20.35	20.36	20.35
C19	19.82	19.29	19.24	19.14
C21	19.31	18.72	18.70	18.66
$\omega$ CH <sub>3</sub>	15.65	14.00	14.14	14.11
C18	12.64, 11.97	11.84	11.75	11.75

Hamilton, 1993)) and in their d-spacing (C18:2 (40.2 Å); C18:3 (27.5 Å); C18:1 (18.5 Å); C18:0 (54.5 Å) (Small, 1986)) show that the position and number of unsaturated carbons play an important role in determining their crystal structure. Previously it was suggested that, with the exception of C16:1 ( $\omega$ -7) and C24:1 ( $\omega$ -9), all of the cis-unsaturated cholesteryl esters studied are isostructural with cholesteryl oleate, i.e., *MLII* (Ginsburg et al., 1984). Our results show that CLL is not isostructural with CO, and that CL did not crystallize into a form that is isostructural with CO, either.

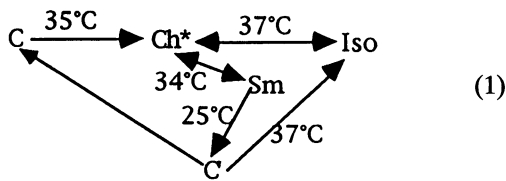
Upon heating, the crystalline CLL melted into the Ch\* phase at 35°C, and gave a spectrum typical of CE in the Ch\* phase (Fig. 1 C). (At this temperature, there is still a small amount crystalline phase in equilibrium with the Ch\* phase, as shown by  $\omega$ CH<sub>3</sub> peaks). Notably, the C5 and C6 resonances are broader compared to other phases. The origin of such line broadening has been discussed in detail (Guo and Hamilton, 1993) and is also briefly described above. The CLL thus observed represents a "stable" liquid crystalline phase, in contrast to the previously report that CLL only forms metastable liquid crystalline phases (Small, 1970). The Ch\* phase melted to the isotropic phase at 37°C (Fig. 1 D,

38°C). Complete C-13 assignments of CLL in liquid crystalline phase and the isotropic phase are summarized in Table 2. Because of the high unsaturation, more of the acyl chain carbons give rise to distinct resonances than other CEs previously studied (Hamilton et al., 1977; Ginsburg et al., 1982, Croll et al., 1986).

On cooling, CLL exhibited an Iso-Ch\* transition at 37°C and a Ch\*-Sm transition at 34°C. The MASNMR spectrum of the Sm phase closely resembled that of the Iso phase except for the presence of SSBs (not shown). In both the Iso and liquid crystalline phases (Ch\* and Sm), the inequivalent chemical environments were lost and single peaks were observed for each carbon, as for other CEs. The spectra of the Ch\*, Sm, and Iso phases of CLL have the same features of other typical CEs in the corresponding mesophases (Guo and Hamilton, 1993). The melting of the crystalline phase caused large changes in the chemical shifts of the  $\omega$ (3,6,9) carbons (Table 2). All these resonances moved upfield, in the order of  $\omega$ (9,10) >  $\omega$ (6,7) >  $\omega$ (3,4); i.e., the absolute change decreased toward the end of the acyl chain, implying the poly-unsaturated acyl chain is highly ordered in the crystalline structure.

In the Sm phase, the long spacing of CLL (35 Å) is close to that of other 18 carbon unsaturated CEs (CO (35.5 Å) and CL (35 Å); Small, 1986), implying that they have the same SmC layered structures. (The long spacing of the saturated 18-carbon chain CE(CS) is 37.5 Å because it exists in SmA structure with the layer normal along the long molecular axis (Ginsburg et al., 1984)). The Sm structure was metastable for >1 h when the sample was cooled to 25°C, but complete recrystallization occurred within 24 h as evidenced by the loss of signals from the Sm phase. The C-13 MASNMR spectrum of this crystalline phase (not shown) was identical to that of crystalline CLL before the heating cycle (Fig. 1 B). When the crystalline phase thus formed was reheated again (without long-term storage at low temperature), it melted to the Iso phase at 37°C without going through the Ch\* or Sm phases, but the phase behavior on cooling was the same as that described above. Such an observation indicates that the crystalline phase newly formed from the Sm phase at 25°C is different from that after long-term storage at low temperature. The conversion between these two forms is probably a slight transitional displacement instead of a reconstruction process, i.e., one with little change in the nearest shielding environment, because both forms have the same C-13 spectral features. According to the literature, there is another crystalline form of CLL (C<sub>2</sub>), which is in equilibrium with Sm phase at 6°C, and forms Ch\* at 29.7°C before melting into Iso phase at 30°C (Small, 1986). In the current work, the low temperature phase transition was not explored; instead, the Sm phase was incubated at 25°C, and the crystalline phase formed in this way might not be the same as the C<sub>2</sub> reported before. The transition temperatures and phase behavior of CLL observed in this work are summarized

in the following diagram:



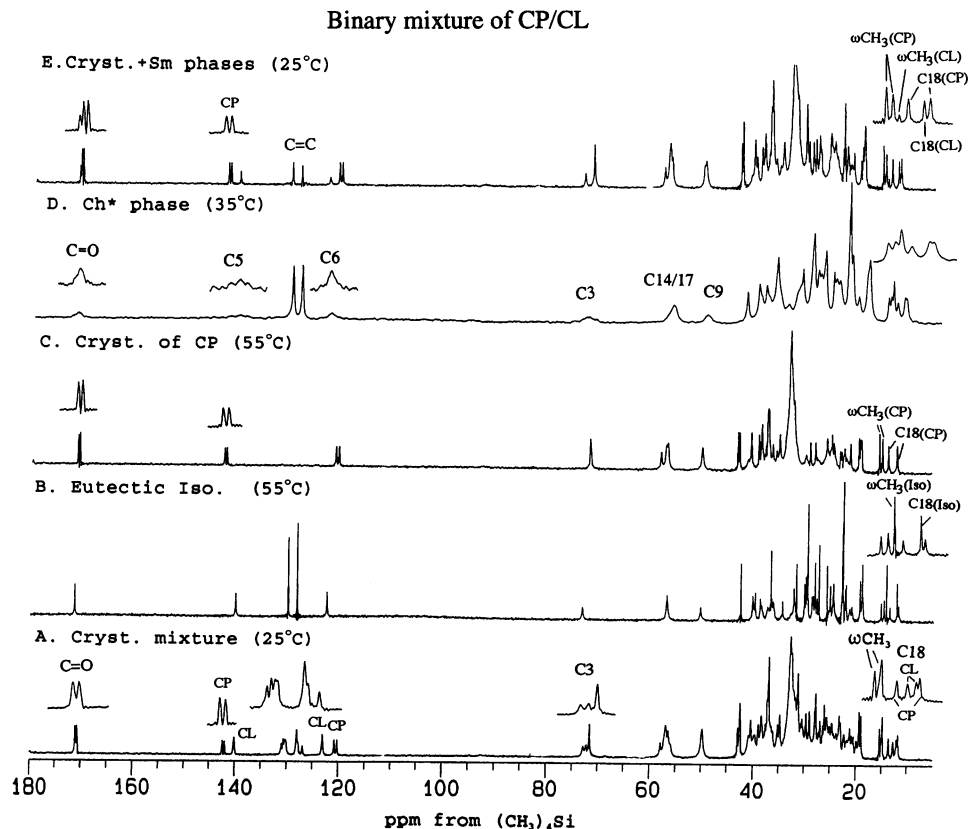
The small differences compared with the reported phase behavior (Small, 1986) are probably due to the different heating history.

### CP/CL (55:45 wt %)

The CP/CL system with varying mixing ratios has been investigated by x-ray diffraction (Dorset, 1990a). In this work the mixing ratio was chosen to reflect the CP:CL ratio found in the atherosclerotic plaque in a rabbit artery (Table 1). For this binary system in the solid state, the long spacings were 52.6, 26.3, and 18.0 Å, and it has been characterized as a completely fractionated system (Dorset, 1990a). The lamellar long spacing of 52.6 Å corresponds to CP crystals in the *BL* form (Small, 1986), and 26.3 Å is half of the long spacing of the same index. The third long spacing (18.0 Å) is close to the long spacing of CO in the *MLII* form (18.5 Å, Small, 1986), suggesting a mixture of *BL* and *MLII* crystalline phases.

The C-13 spectrum of the crystalline CP/CL sample at 25°C (Fig. 2 A) yielded two sets of signals, one attributable to the CP in the *BL* form (characterized by twin peaks for the C=O, C5, C6, C13, C18, and  $\omega$ CH<sub>3</sub> carbons), and the other with chemical shifts values close to those of CO in the *MLII* form (e.g., C5, C6, C18). Several unique resonances for CP and CL are shown in Fig. 2 A. Importantly, the irregular multiple peaks observed for pure CL (Fig. 1 A) were absent. There are two groups of resonances for the acyl chain unsaturated carbons separated by ~2 ppm. In the downfield group, there are four resolvable peaks of approximately equal intensities, whereas in the upfield group, there are two peaks of approximately equal intensities and one about twice as intense. Since there are only four olefinic carbons in the acyl chain of CL, and true *MLII* crystals yield only one signal for each carbon, it is likely that CP promotes the crystallization of CL into the *MLII* form in this mixture, but the crystalline structure of CL is slightly different from the *MLII* structure formed by pure CO. Since the difference is mostly limited to the acyl chain region, it is probably caused by the polyunsaturation in the CL acyl chain. Therefore, from both x-ray and MASNMR considerations, the CL component of this mixture exists in a slightly modified *MLII* form. Close inspection of Fig. 2 A also shows three peaks for the C3 carbon; the most intense peak at 71.0 ppm corresponds to CP in the *BL* form, and the peak at 72.8 ppm is close to the chemical shift of CO in the *MLII* form (Guo and Hamilton, 1993). The peak between these two (72.0 ppm) is at an anomalous chemical shift. (To eliminate the possibility that these signals

FIGURE 2 C-13 MASNMR spectra of a binary mixture of CP and CL. (A) Crystalline mixture formed from CHCl<sub>3</sub> at 25°C; (B) Iso phase above the eutectic point at 55°C; (C) crystalline CP coexisting with the eutectic Iso phase at 55°C; (D) Ch\* phase formed from the Iso phase at 35°C; (E) cryst. and Sm phases coexisting at 25°C. Spectra A, C, and E were obtained with cross-polarization transfer and spectra B and D without cross-polarization transfer. Selected resonances specific for CP and CL are indicated.



are from SSB, a different spinning rate was used (3 KHz) and no difference in this region was seen (not shown)). Since the conformation of C3 is closely related to the conformation of the acyl chain, it is possible that these two peaks (72.0 and 72.8 ppm) originate from the inequivalent chain arrangement in the CL crystals. When the mixture was heated to the eutectic point (40°C), part of the sample melted into the Iso phase, and all the signals that did not correspond to the *BL* form disappeared from the CPMAS spectrum. Heating to 70°C melted the solid phase completely, and the spectrum of the Iso phase was typical (Guo and Hamilton, 1993).

The phase behavior of CP/CL was monitored by C-13 MASNMR with and without cross-polarization transfer, which can identify the type of phase present in pure CE (above and Guo and Hamilton, 1993). In the binary mixture, it was possible to monitor the phase behavior of each CE. On cooling from the Iso phase, CP recrystallized into the *BL* form directly without forming liquid crystalline phases. At 55°C, spectra obtained without cross-polarization showed an Iso phase (Fig. 2 B) and with cross-polarization, a *BL* crystalline phase (Fig. 2 C). In Fig. 2 B, the  $\omega\text{CH}_3$  and C18 peaks from the crystalline CP are also observed because these two groups are mobile enough to be detected without cross-polarization transfer.

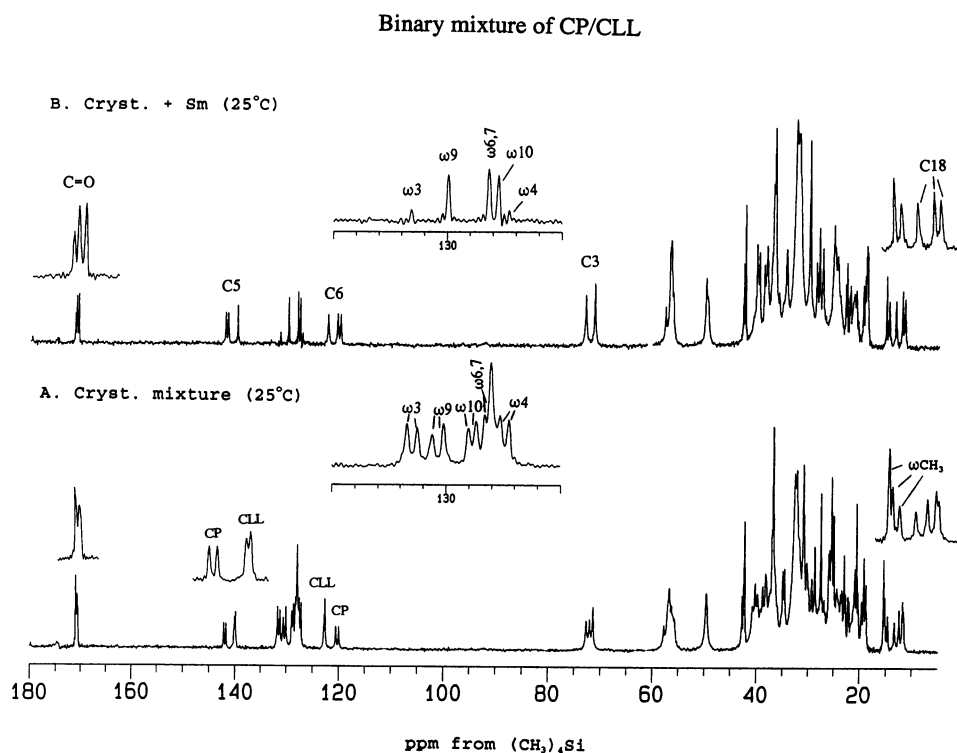
On cooling to 35°C, the CL-rich liquid phase formed the *Ch\** phase, as evidenced by broad signals for several steroid carbons such as the C5 and C6 (Fig. 2 D). Further cooling led to the *Ch\**-Sm transition, and the CPMAS spectrum revealed the coexistence of crystalline CP (*BL*) and liquid crystalline CL (Sm) phases (Fig. 2 E). Several spectral features show the two phases clearly, e.g., the C=O, C5, C6, and C18,  $\omega\text{CH}_3$  regions (Fig. 2 E, insets). Incubation of this mix-

ture at 25°C for 24 h completed the conversion of the Sm phase into *MLII* crystals, and the spectrum (not shown) was the same as that before the heating process. Thus, the mixture of CP/CL (55:45) is most stable in the fractionated solid state, with CP in the *BL* and CL in the *MLII* crystal form, regardless of whether the crystalline phase was formed from mixing the two components in organic solvent or from the Iso melt. Also, since pure CL does not form *MLII* crystals under usual conditions, the coexistence of CP in the CL bulk phase must promote this structure. This also suggests that CL crystallizes through the heterogeneous nucleation mechanism: the crystalline particles of CP dispersed in the CL-rich mesophases might help to promote nucleation for the CL crystallization process. To find out whether a small amount of CP solubilized in liquid crystalline CL would be sufficient to promote nucleation, a CP/CL mixture with 4 wt % CP was investigated. No crystallization was detected, and a spectrum obtained was similar to Fig. 1 A. This further implies that the existence of larger domains of crystalline CP is essential for the crystallization of CL.

### CP/CLL (50:50 wt %)

CLL is a minor constituent of biological CE mixtures and amounted to 1.4% of the CE in our plaque sample. Since this level is too low to detect by NMR, the proportion of CLL in our sample was augmented. The C-13 spectrum at 25°C of the sample co-crystallized from  $\text{CHCl}_3$  (Fig. 3 A) reveals the features of the two individual crystalline phases of CP and CLL, implying fractionated crystalline mixtures: CP (*BL*) and CLL. The two esters are clearly distinguished in the C5 and C6 regions of the spectrum, where CLL has twin peaks

FIGURE 3 C-13 MASNMR spectra of a binary mixture of CP and CLL. (A) Crystalline mixture formed from  $\text{CHCl}_3$  at 25°C; (B) crystalline CP and smectic CLL coexisting at 25°C after cooling from the Iso phase. Both spectra were obtained with cross-polarization transfer.



for C5 upfield of the twin peaks of CP and a single peak for C6 downfield of the twin peaks of CP (see Fig. 1 B, and Guo and Hamilton, 1993). When the sample was heated to its eutectic temperature (36°C), signals for CLL disappeared from the CPMAS spectrum, signifying complete melting of CLL; some CP melted into the Iso phase and some remained in the *BL* crystalline form (spectra not shown). The residual CP finally melted completely at 65°C, as evidenced by the disappearance of signals for crystalline CP.

When the CP/CLL mixture was cooled, the Ch\* and Sm phases formed at 60 and 55°C, respectively. Both mesophases had one set of C-13 resonance signals (spectra not shown) characteristic of typical pure CE in the corresponding phases (Guo and Hamilton, 1993). The Sm → Cryst transition was not detected because of undercooling of the metastable Sm phase; as the mixture was further cooled to 25°C, CP crystallized into *BL* first and coexisted with CLL in the Sm phase (Fig. 3 B). The C5/C6 region, e.g., shows the twin peaks for crystalline CP, as in Fig. 3 A, but single peaks for CLL. The twin peaks for unsaturated carbons in crystalline CLL (Fig. 3 A, *inset*) are also collapsed to single peaks in the liquid crystalline phase (Fig. 3 B, *inset*), as expected (Guo and Hamilton, 1993). For CLL in the Sm phase, the resonances for the  $\omega(3,6,9)$  double bond carbons responded to the cross-polarization transfer in an order of  $\omega(9,10) > \omega(6,7) \gg \omega(3,4)$ , showing an increasing mobility towards to end of the acyl chain. After incubation at 25°C for 24 h, the sample showed no signals for the Sm phase, and the spectrum (not shown) was the same as that before heating (Fig. 3 A), showing two fractionated crystalline phases. Clearly, CP and CLL are not compatible in the crystalline state because of the

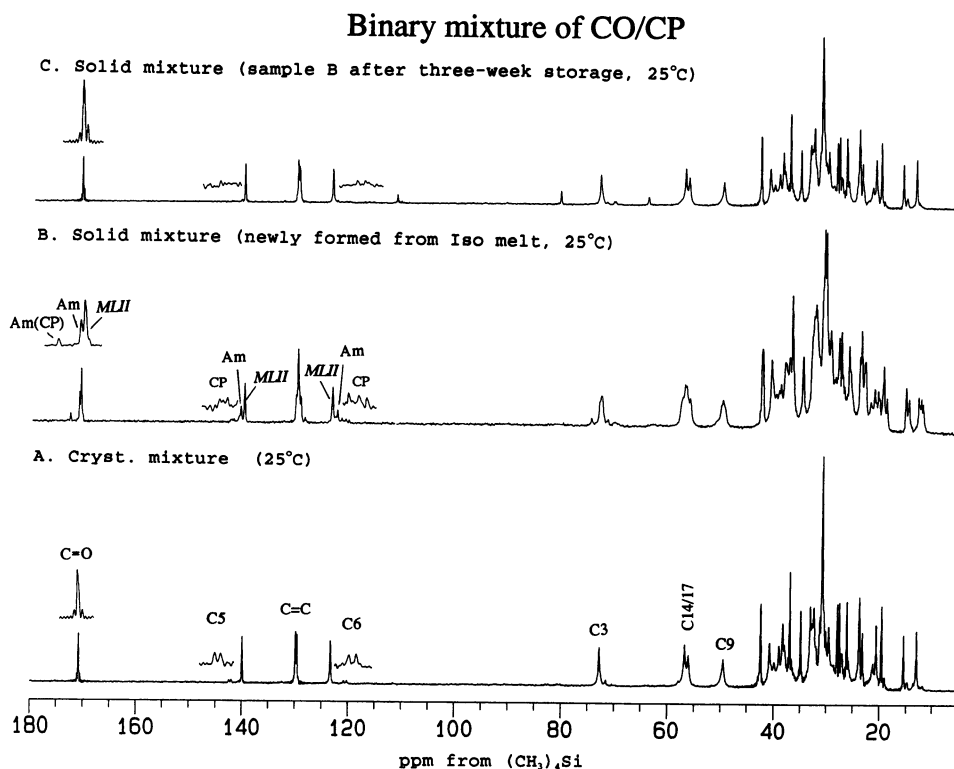
difference in their acyl chain conformations and their different crystal structures, as predicted (Dorset, 1988).

### CO/CP (80:20, wt %)

For this mixed system, a sample prepared by cocrystallization in CHCl<sub>3</sub> yielded a C-13 CPMAS spectrum that shows the coexistence of *BL* and *MLII* structures (Fig. 4 A). The spectra of *MLII* crystals have a single resonance for each carbon at a characteristic chemical shift (Guo and Hamilton, 1993), and CO in the *MLII* form is readily identified. Although CP is a minor component in this mixture, the characteristic twin peaks for C=O, C5, and C6 are well resolved and easily identified (Fig. 4 A, *insets*). The twin peaks from the C=O of CP also flank the intense C=O peak of CO. This mixture melted to the Iso phase at 50°C (spectrum not shown). The current mixing ratio is close to the eutectic ratio (16 wt % CP, Dorset, 1990a), and a melting point of 50°C agrees with the eutectic temperature for the CO/CP binary system (Dorset, 1990a). According to the prediction of the phase diagram for CO/CP (Small, 1986, Snow and Phillips, 1990), a small amount (4%) of the total CP should remain in the solid state, but this minor fraction was not detected in the NMR spectrum. To make certain no crystalline phase remained in the mixture, the sample was heated to 60°C, 7°C above the melting point predicted by the phase diagram.

Upon cooling this homogeneous Iso phase, the Ch\* phase appeared at 52°C, and the Sm phase at 46°C, as detected by NMR. The spectra of the Iso, Ch\*, and Sm phases were typical of those of pure CEs in the corre-

FIGURE 4 C-13 CPMAS spectra of a binary mixture of CO and CP at 25°C. (A) Crystalline mixture formed from CHCl<sub>3</sub>; (B) solid state formed by cooling the isotropic melt to 25°C for 24 h; (C) sample B after 3 weeks storage. All spectra were obtained with cross-polarization transfer. In spectrum B, selected peaks specific for CP, and specific for Am and *MLII* solid phases, are indicated.



sponding phases (not shown). The  $Sm \rightarrow$  cryst transition was not detected because of undercooling of the  $Sm$  phase. After incubation at  $25^\circ C$  for 24 h, the mixture solidified completely. The C-13 spectrum (Fig. 4 B) reflected a mixture of  $MLII$  crystals and an amorphous phase, as in the case of pure CO solidified from its melted state (Guo and Hamilton, 1993). Typically, the amorphous phase yields peaks for C5 and C=O slightly downfield, and C6 slightly upfield, of the peaks in the  $MLII$  form seen in Fig. 4 B. Weak signals for C5 and C6 of CP in the  $BL$  form are still visible, but they are no longer well defined twin peaks as in the crystalline mixture (Fig. 4 A, *inset*) because of the mixing effect. Also, there is a small peak at 172.5 ppm that does not correspond to any peak in the solid state of pure CE. This peak is neither an SSB nor an impurity from sample decomposition, as confirmed by TLC analysis of the sample following NMR studies. After storage of this sample at  $4^\circ C$  for 3 weeks, the amorphous solid was completely converted to the  $MLII$  structure, and features of a  $BL$  form characteristic of pure CP, such as the twin peaks of C5 and C6, were barely detected (Fig. 4 C). The anomalous peak downfield from the major C=O peaks also disappeared, suggesting it represented CP molecules in the amorphous (Am) phase. Therefore, it is likely that more CP molecules are solubilized in the CO-rich  $MLII$  structure when the mixture is solidified from the Iso melt as compared with the sample prepared in  $CHCl_3$  (Fig. 4 A), possibly because the viscosity of the mixture in the supercooled  $Sm$  phase is too high to allow CP molecules to segregate into their native structure.

The presence of CP in an amorphous phase would also explain the reduced intensities of peaks for CP in the  $BL$  crystal (e.g., C5 and C6 twin peaks). Previous work has shown that pure CP (saturated CE) forms a smectic ( $Sm$ )A phase, and pure CO (unsaturated CE) forms an  $SmC$  phase (Ginsburg et al., 1984). The lamellar structures in these  $SmA$  and  $SmC$  phases are similar, but the long molecular axis in  $SmA$  aligns with the layer normal, whereas that in  $SmC$  forms an angle related to the layer normal (Ginsburg et al., 1984). It might be feasible for a small number of CP molecules to be incorporated into the  $SmC$  structure at elevated temperature and then become trapped in the Am phase. The C=O resonance of such CP molecules might give a higher chemical shift value compared to that for a regular bilayer structure. After the Am-Cryst rearrangement, these molecules are rearranged into the  $MLII$  layers, and the shielding on the C=O group returns to its normal value.

Reheating this sample gave a Cryst- $Ch^*$  transition at  $46^\circ C$  and a  $Ch^*$ -Iso transition at  $52^\circ C$  with the phase behavior on cooling as described above. This observation shows that after the first heating cycle, the sample is no longer a eutectic mixture; instead, it behaves more like a solid solution, i.e., a single uniform phase with reproducible phase transition temperatures. The observation of a stable  $Ch^*$  phase before the appearance of the Iso phase

agrees with the phase diagram in the literature showing a stable  $Ch^*$  in a CO-rich region (70–90 wt %, Small, 1986; Snow and Phillips, 1990).

### CO/CL (83:17, wt %)

Extensive work has been done on this biologically important CE binary system, yet previous studies are not in agreement. It has been reported that CO/CL forms a solid solution when  $CL > 40$  wt %, and a solid solution ( $CL = 40$  wt %) plus crystalline  $MLII$  CO when  $CL < 40$  wt % (Small, 1986). On the other hand, the CO/CL binary system has been reported to give fractionated eutectics without the formation of a solid solution (Dorset, 1990). The C-13 MASNMR spectrum of this binary mixture at  $25^\circ C$  consists of multiple sets of peaks, one of which correlates with an  $MLII$  structure; the others do not correlate with any known crystalline CE structure, implying a more disordered amorphous phase. The olefinic and carbonyl region (Fig. 5 A) is sufficient for the analysis of the phases present. It is known that when CO and CL are co-dissolved in  $CHCl_3$ , CO readily crystallizes into the  $MLII$  form, but CL is very difficult to crystallize, and yields an Am

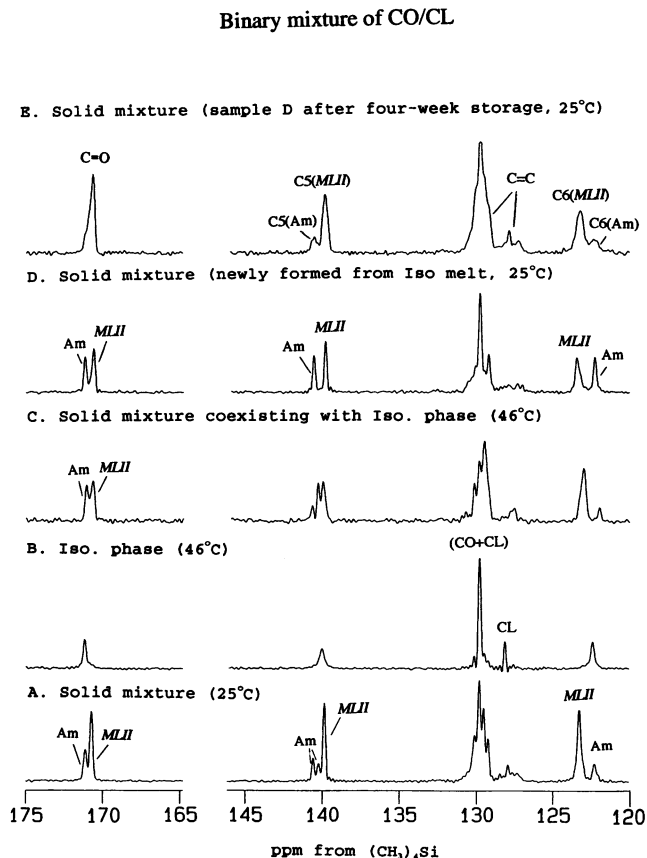


FIGURE 5 C-13 MASNMR spectra of a binary mixture of CO and CL. (A) Solid mixture formed from  $CHCl_3$  at  $25^\circ C$ ; (B) Iso phase at  $46^\circ C$ ; (C) solid mixture coexisting with the isotropic phase at  $46^\circ C$ ; (D) solid state formed by cooling the Iso phase to  $25^\circ C$  for 24 h; (E) sample B after 3 weeks storage. Spectrum B was obtained without, and all other spectra with, cross-polarization transfer. Selected resonances are indicated for CL and CO components and for Am and  $MLII$  solid phases.



form (Dorset, 1990b). The lamellar long spacing of CO/CL (83:17) is  $\sim 19$  Å, (Small, 1986; Dorset, 1990b), close to CO in the *MLII* form (18.5 Å, Small, 1986). The Am phase in the CO/CL binary system may not give detectable diffractions in the x-ray diffraction, as previously observed for pure CO (Guo and Hamilton, 1993). Since CO crystallized to the *MLII* form easily in the mixture, the coexisting Am phase should be CL-rich, and might be heterogeneous, as judged from the multiple signals for individual carbons (e.g., C5, C6).

When the solid mixture was heated, the isotropic phase started to appear at 44°C, and the solid-Iso conversion was complete at 49°C. Within this temperature range, the Iso phase and the solid phase coexisted. At 46°C an Iso phase (Fig. 5 B) was detected without cross-polarization transfer, and a solid phase (Fig. 5 C) with cross-polarization transfer. Almost all the peaks observed before heating (Fig. 5 A) are still seen above the melting point, but with varied peak intensities (Fig. 5 C). The chemical shifts of the C=O, C5, and C6 resonances for CEs with different acyl chains are not distinguishable. However, comparison of the intensity of the signal at 129.8 ppm ( $\omega_{6,10}$  of CL, and  $\omega_{9,10}$  of CO) with that at 128.2 ppm ( $\omega_{7,9}$  of CL) in Fig. 5, B and C shows that a certain amount of CO exists in the Iso phase together with CL (since the downfield peak is more than twice as intense as the upfield peak, Fig. 5 B), and a certain amount of CL coexists in the solid state with CO (since signals characteristic of only CL are detected at 128 ppm). The binary system is therefore not a simple eutectic mixture; instead, the broad melting range agrees with the prediction (Dorset, 1990b) that the two components tend to segregate partially when co-crystallized from  $\text{CHCl}_3$ , so that an inhomogeneous solid mixture (CO-rich *MLII* crystals, CL-rich amorphous and CO/CL solid solution) is obtained. Cooling of the isotropic phase gave the Ch\* phase at 42°C and the Sm phase at 36°C. Spectra of the Iso, Ch\*, and Sm phases were typical of other CE and are not shown.

After cooling the melted sample from 50 to 25°C and incubating at 25°C for 3 h, a solid state with coexisting *MLII* and Am phases was detected (Fig. 5 D). This sample showed stronger signals for the Am phase compared with the sample mixed in  $\text{CHCl}_3$ , probably with an increased CO component because CO tends to form an Am phase from the melt (Guo and Hamilton, 1993). After storage at 25°C for 4 weeks, the CPMAS spectrum (Fig. 5 E) revealed significant molecular rearrangements, as indicated by the chemical shift changes and broadening of C5 and C6 C=O peaks. Reheating of this solid state mixture gave a lower melting temperature and narrower melting range (44–46°C), implying a more homogeneous mixing in the solid phases.

The above observations clearly show that when CO and CL were mixed in  $\text{CHCl}_3$  and dried, there was considerable inhomogeneity or fractionation in the mixture, as suggested by Dorset (1990b). When the sample was mixed in the isotropic phase and then cooled to solidify, it became more like a mixture of a CO-rich *MLII* crystalline phase and a CO/CL solid solution (Am form), as reported before (Small, 1986). The CO/CL solid solution (Am form) tends to convert to the

*MLII* form, but the process is very slow. The line broadening of the *MLII* signal after the sample storage also indicates that the crystalline phase has significant structure disorder caused by the incorporation of CL. These two phases have very close melting temperatures, as indicated by the narrowing of the melting temperature range.

### CO/CP/CL (69:17:14, wt %)

The C-13 spectrum at 25°C of this mixture prepared by recrystallization from  $\text{CHCl}_3$  contains the features of the *MLII* crystalline structure as its major component, as illustrated by the carbonyl and olefinic regions of the spectrum (Fig. 6 A). The most intense peaks occur at the same chemical shifts as pure CO in its *MLII* form (e.g., C=O, C5, C6, and acyl C=C peaks; Guo and Hamilton, 1993). The spectrum also reveals a small proportion of *BL* structure, indicated by the appearance of the twin peaks for C5 and C6 (Fig. 6 A), as well as C18 and  $\omega\text{CH}_3$  (not shown). A different solid state

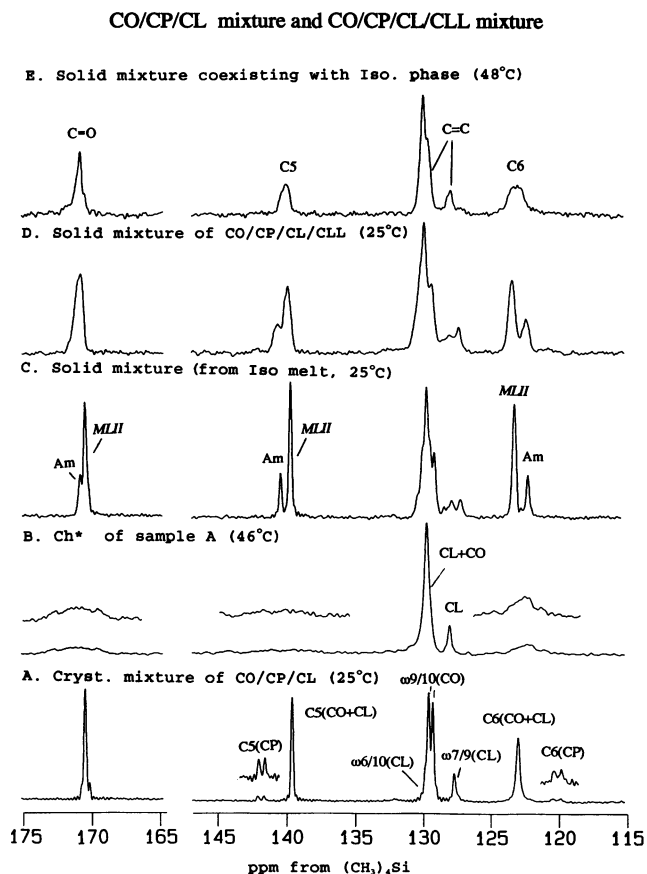


FIGURE 6 C-13 MASNMR spectra of a ternary and a quaternary mixture. (A) Crystalline mixture of CO, CP, and CL formed from  $\text{CHCl}_3$  at 25°C; (B) the Ch\* phase of sample A at 46°C; (C) solid mixture of sample A at 25°C after cooling the Iso phase to 25°C; (D) mixture of CO, CP, CL, and CLL at 25°C formed by cooling the isotropic mixture and storing for 4 weeks; (E) solid mixture of sample D coexisting with the Iso phase above the initial melting temperature at 48°C. Spectrum B was obtained without, and all other spectra with, cross-polarization transfer. Selected resonances are indicated for CO, CP, and CL components, and for Am and *MLII* solid phases.

for CL in this mixture compared with the CO/CL mixture is evident from comparison of Figs. 5 A and 6 A. A single narrow peak for the  $\omega_{7,9}$  carbons from CL is observed, implying that CL is solubilized in an *MLII* form, since it cannot be in the *BL* form, as discussed above. (In the C-13 spectrum, of CP/CO/CL mixed system prepared from  $\text{CHCl}_3$  but with a 20:39:41 wt % mixing ratio, strong multiple peaks characteristic of uncrystallized CL were observed, implying that there is a certain solubilization limit for CL in the *MLII* form dominated by CO.) Whereas CL in the CP/CL mixture crystallized into a modified *MLII* form in which the CL acyl chain exhibited two slightly different conformations, in the current mixture only one conformation of CL is detected. Thus, in the presence of CP, a small amount of CL is crystallized into an *MLII* form together with the major component CO and is dilute enough to exist as monomers surrounded by the CO molecules without causing chain packing complexity.

Upon heating, this sample melted at 46°C into a  $\text{Ch}^*$  phase with characteristic broad C=O, C5, and C6 resonances (Fig. 6 B) before it further melted into the Iso phase. Upon cooling from the Iso phase, the  $\text{Ch}^*$  phase formed at 48°C, followed by the Sm phase (44°C). The C-13 spectra of the Iso or mesophases formed during the cooling process were the same as those observed for pure CE. (Guo and Hamilton, 1993). It is interesting that although none of the three pure components has a stable  $\text{Ch}^*$  phase, the mixed system does (Small, 1986).

The spectrum (Fig. 6 C) of the mixture solidified from the Sm phase shows a mixture of *MLII* crystals and an Am phase (Guo and Hamilton, 1993). Since CO is the major component in this mixture, it is possible that some CO recrystallized into the *MLII* form, and the rest into the Am form. The minor components of CP and CL are possibly incorporated in the Am form (amorphous solid solution). Extension of the incubation time at 4°C caused only slow and slight conversion of Am into an *MLII* form.

### CO/CP/CL/CLL

A ternary system with the above proportions of CP, CL, and CO with an additional 5 wt % of CLL was also studied to see if a small amount CLL would significantly affect the phase behavior of the major components. The four CE components were melted to a completely isotropic state (60°C) before NMR analysis and then incubated at 25°C for a week. The C-13 CPMAS spectrum of this system at 25°C, exemplified by the C=O and olefinic regions (Fig. 6 D), resembles that of the CO/CP/CL system (Fig. 6 C), except for the line broadening in the four component mixture. The  $\text{Ch}^*$  phase appeared at 45°C and coexisted with the solid phase until the mixture melted completely into the Iso phase at 50°C. The coexistence of  $\text{Ch}^*$  and solid state in a 4°C temperature region was also observed in the differential scanning calorimetry heating curve. The solid phase in this temperature region gave broad peaks (48°C, Fig. 6 E) characteristic of an inhomogeneous mixture, i.e., not a simple eutectic mixture. When cooled, the  $\text{Ch}^*$  phase formed at 47°C, and the Sm phase at 43°C, each about 1°C lower than the temperatures of the CO/CP/CL mixture without CLL. The C-13 spectra of the  $\text{Ch}^*$  and the Sm

phases were similar to those of typical CE in the corresponding phases and are not shown. The solid state formed from the Sm phase gave a spectrum the same as that before the heating, and the extension of the incubation time to 4 weeks did not cause observable change in the spectral features.

The addition of a small amount of CLL to the CE mixture did not show significant effects on the fluid phases (Iso,  $\text{Ch}^*$  and Sm); i.e., the temperature range and stability of the mesophases were approximately the same as that without CLL. In the solid state, CLL caused increased inhomogeneity, as shown by the extended temperature range in which the solid state coexisted with the  $\text{Ch}^*$  phase. This agrees with the observation that CLL molecules have the strongest tendency to form crystalline structures among the unsaturated 18 carbon CE. This strong tendency to crystallize might also explain the fact that CLL is only a very minor component, if present at all, in normal biological tissues.

### BIOLOGICAL SIGNIFICANCE

The reversal of atherosclerosis is likely to depend on hydrolysis of CEs by CE hydrolase to cholesterol, which might be removed by high density lipoprotein (Phillips et al., 1987). In hepatoma cells greater hydrolysis of isotropic, compared with liquid crystalline, CE was observed (Glick and Adelman, 1983; Adelman et al., 1984), whereas in lipid droplets isolated from cultured hepatoma cells the rate of hydrolysis was not dependent on the physical state of the CE (Lundberg et al., 1990). Whether these apparently conflicting results arose from differences in the CE hydrolase enzymes used or in the lipid droplet composition was not clear (Lundberg et al., 1990). From studies of non-human primates, there is strong evidence that cholesterol feeding affects the composition and physical state of CE in atherosclerotic plaques, and that regression of atherosclerosis is correlated with a decrease in the isotropic to liquid crystalline transition temperature of plaque CE from above to below body temperature (Small et al., 1984). However, the proposal that the physical state of CE is important in the persistence of early lesions in human was challenged by x-ray diffraction studies (Burks et al., 1989).

A shortcoming of most physical methods is that very limited information is provided about the complex chemistry and structural organization of lipid deposits in situ. A direct and simultaneous determination of the chemical components that undergo physical changes is essential to achieve a hypothesis relating physical state to biological activity. DSC and microscopic techniques give detailed information about phase transitions but do not give chemical or structural information. While x-ray diffraction methods provide some structural data, it is not always easy to determine which specific CEs give rise to diffractions in mixtures. Standard high resolution C-13 NMR provides a detailed chemical and structural description of isotropic CE but limited information about liquid crystalline phases and none about the solid state (Hamilton et al., 1977). C-13 MASNMR provides the most complete description of the phase behavior of CE mixtures to date. With C-13 MASNMR, the chemical composition and phase transitions of CE in mixtures can be monitored simultaneously. The phases and crystal structures of in-

dividual CE in mixtures can be identified. C-13 MASNMR represents a powerful new tool for characterizing complex CE-rich biological pools.

## SUMMARY

In this work we have demonstrated the feasibility of extending C-13 MASNMR from pure CE to binary, ternary, and quaternary mixtures of CE. By using C-13 NMR, we found that both CL and CLL do not crystallize into the structures commonly encountered in pure CEs. Pure CL existed in a solid state without a uniform crystalline structure, which may explain why pure CL does not form true crystals, whereas CLL crystallized into a regular structure that has not been reported before. When CP is mixed with CL, CLL, or CO, the binary systems form eutectic mixtures. Direct identification of crystal structures in mixtures can be made by reference to the chemical shifts of certain diagnostic resonances for characterized structures of CE (Guo and Hamilton, 1993; this work). CP, CLL, and CO preferred the structures they exhibit in the corresponding pure systems, and CL was induced to form an *MLII* structure. In mixed systems, CP did not mix with CL in the mesophases; instead, it crystallized into the *BL* form before the formation of CL-rich mesophases. Interestingly, in the binary mixture of CO and CL, CO was shown to crystallize in its characteristic *MLII* form, but did not induce CL to form *MLII* crystals. For the CP/CO/CL system crystallized from CHCl<sub>3</sub>, CP and CO were found in their native crystalline form, whereas CL was solubilized in the *MLII* form. A stable Ch\* phase was detected in this mixed system. Addition of a small amount (5%) of CLL to the CP/CO/CL mixed system showed no detectable effect on liquid crystalline phases, but the CLL "stabilized" the solid state mixture and resulted in a large temperature range where the solid state mixture coexisted with the Ch\* phase.

C-13 MASNMR spectra revealed interesting differences in crystalline forms and in the kinetics of crystallization, depending on the sample protocol. For example, the binary mixture of CO/CP recrystallized from organic solvent showed *BL* and *MLII* structures. When this mixture was solidified from the isotropic melt, a mixture of *MLII* crystals and an amorphous solid were detected. After long-term storage the amorphous solid converted to *MLII* crystals, and *BL* crystals were only barely detected, suggesting that part of the minor component of CP became solidified in the *MLII* crystals of the major component CO.

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