as dimyristoyllecthin, functional inactivation occurs at the transition temperature of the pure lipid (Warren et al., 1974a). In these complexes, which exemplify the normal type of behavior of many proteins, we assume that crystallization of the extraannular lipid at the transition temperature imposes sufficient rigidity on the annular lipids to switch off activity.

Finally we wish to emphasize that we are not suggesting that the structural interaction of the annular lipid with the ATPase is independent of the properties of the lipid. This would imply a uniform temperature–activity profile for all complexes which is incompatible with previous data for the ATPase and many other membrane-bound enzymes. The specific conclusion which the anomalous behavior of the DPL–ATPase allows us to make is that the lipid phase transition is not greatly perturbed beyond the immediate lipid neighbors of the protein, and we suggest that the same conclusion will apply also to membrane proteins which show a normal dependence of function on lipid phase transitions.

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Temperature-Dependent ^{13}C Nuclear Magnetic Resonance Studies of Human Serum Low Density Lipoproteins†

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ABSTRACT: The natural abundance ^{13}C nuclear magnetic resonance (NMR) spectrum of human serum low density lipoproteins (LDL) shows significant temperature-dependent changes. These temperature-dependent spectra have been used to monitor changes in the organization of cholesterol esters within the LDL particle. Comparison with ^{13}C NMR spectra of both cholesterol linoleate and an aqueous codispersion of cholesterol linoleate and egg phosphatidylcholine suggests that at low temperatures (10 °C), the cholesterol esters in LDL are organized in a smectic-like, liquid-crystalline arrangement. At temperatures above the order–disorder transition exhibited by the cholesterol esters of LDL, the cholesterol esters appear to be partially melted but still are motionally restricted compared with liquid cholesterol esters.

Electron microscopy and small angle x-ray scattering indicate that human serum low density lioprotein (LDL[1]) is a spherical particle about 220 Å in diameter (Forte et al., 1968; Mateu et al., 1972). Recent differential scanning calorimetry and x-ray scattering studies show that the core of LDL is occupied mainly by cholesterol esters which undergo a temperature-dependent structural reorganization in the range of body temperature (Deckelbaum et al., 1975). Furthermore, the x-ray scattering shows that below this thermal transition, the

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[1] Abbreviations used: LDL, low density lipoproteins; NMR, nuclear magnetic resonance; Me_4Si, tetramethylsilane; EDTA, (ethylenedinitrilo)tetraacetic acid.

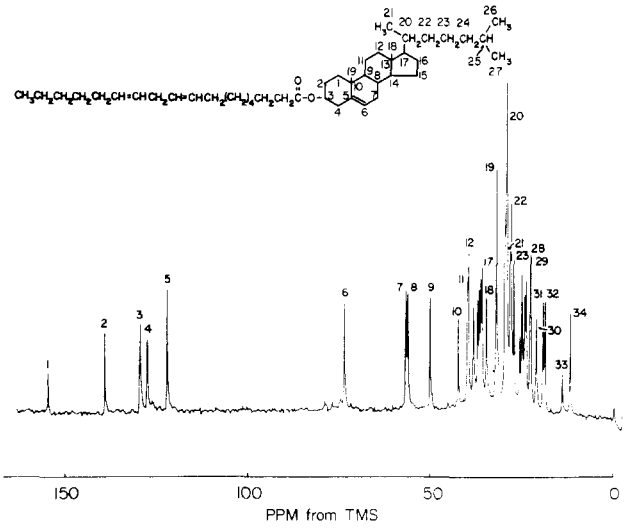

FIGURE 1: Natural abundance ^{13}C NMR spectrum of cholesteryl linoleate (0.5 M) dissolved in $CDCl_3$ at 15.03 MHz at 39 °C; 8K data points in a 2.5K spectral width and 1000 accumulations with a recycle rate of 1.7 s was used to obtain the spectrum. The peak numbers are the same as those shown in parentheses in Table I.

cholesterol esters are organized in a smectic-like phase, whereas above the transition the organization of the cholesterol esters is more liquid like.

Relaxation time measurements using natural abundance ^{13}C nuclear magnetic resonance (NMR) spectroscopy have indicated that the T_1 times of the acyl chain resonances in LDL at 36 °C (Hamilton et al., 1974) are considerably shorter than they are in phospholipid dispersions at similar temperatures (Sears, 1975). However, no attempts were made to distinguish the relative contributions made to the acyl chain relaxation times by the various lipid species of LDL or to investigate the effect of temperature on the LDL spectrum.

In order to further investigate the structural origin of the thermal behavior of LDL, we have used natural abundance ^{13}C NMR spectroscopy to study LDL and its major lipid components, cholesterol ester and phospholipid. ^{13}C NMR only gives information concerning the rotational motion of a molecule. However, it is possible to correlate the motions of the cholesterol esters with various known structural states as determined by more direct structure-probing techniques, such as x-ray diffraction. Our results indicate that the cholesterol moiety of cholesterol ester undergoes preferential immobilization in various liquid-crystalline states compared with the acyl chain. Furthermore, the physical properties of the cholesterol esters are similar in the neat state and in the aqueous codispersion with phospholipids. The ^{13}C NMR results are consistent with the cholesterol esters in LDL being in the smectic-like phase below the transition, but that above the transition the cholesterol esters are more restricted in their motion than would be expected for the isolated cholesterol esters.

Materials and Methods

Materials. Cholesteryl linoleate (Nu-Chek-Prep, Elysian, Minn.) and egg phosphatidylcholine (Lipid Products, Nutfield Ridge, England) were shown to be greater than 99% pure by thin-layer and gas-liquid chromatography methods. D_2O (99.8%) was obtained from New England Nuclear (Boston, Mass.). All other chemicals were of reagent grade. Doubly distilled water was used throughout the study.

LDL from normal human plasma was isolated by salt density ultracentrifugation between densities 1.025 and 1.050 g/ml as previously described (Deckelbaum et al., 1975). Lack of contamination by other plasma fractions was confirmed by immunodiffusion and immunoelectrophoresis analysis (Hatch and Lees, 1968). The protein content was estimated by the method of Lowry et al. (1951). The liid classes were assayed by quantitative thin-layer chromatography (Downing, 1968) after a Folch extraction of LDL. The fatty acid compositions of the isolated lipid species were determined by gas-liquid chromatography of the methyl esters of the fatty acids.

Sample Preparation. Cholesteryl linoleate (1 g) was placed in an 8-mm NMR tube which was then placed coaxially within a 10-mm NMR tube. The gap between the tubes was filled with D_2O for locking purposes. Dispersions of cholesteryl linoleate and egg phosphatidylcholine were prepared by adding D_2O (2 ml) to a colyophilized mixture of cholesteryl linoleate (1 g) and egg phosphatidylcholine (0.5 g). This solution was then centrifuged back and forth through a constricted tube at 55 °C. The equilibrated sample was then placed in a 10-mm NMR tube and layered with nitrogen.

The LDL was concentrated by methods previously described (Deckelbaum et al., 1975) and the concentrated solution was dialyzed against 0.15 M NaCl–0.1 mM Na_2EDTA in D_2O (pD 8.4). The sample was placed in a 10-mm NMR tube layered with nitrogen. Thermal disruption of LDL was achieved by heating the LDL solution in the NMR tube for 5 min at 100 °C (Deckelbaum et al., 1975). The sample was then cooled to the desired temperature and layered again with nitrogen.

Nuclear Magnetic Resonance Spectroscopy. The NMR spectrometer used was JEOL FX-60 operating at 15.03 MHz using proton decoupling. A deuterium lock was employed throughout the study. The ^1H decoupling power was 10 W; the temperature of the sample was controlled within 1 °C. In some spectra, a pulse angle of less than 90° was used to accumulate spectra. The pulse angle and repetition rates chosen were based on the T_1 times determined by Hamilton et al. (1974), and the equations of Ernst and Anderson (1966).

Polarizing Microscopy. Polarizing light microscopy was done as previously described (Loomis et al., 1974).

Results

Low density lipoproteins are complex particles containing 22–24% protein and several lipid species (Skipski, 1972). The composition of LDL and the relative distribution of its lipid components are cholesterol ester (55 mol %), phospholipid (22.8 mol %), free cholesterol (19.7 mol %), and triglyceride (2.5 mol %). The majority of the cholesterol esters are cholesteryl linoleate (58%) and cholesteryl oleate (19%). Phosphatidylcholine (70%) and sphingomyelin (28%) are the major phospholipids.

To aid the interpretation of the ^{13}C NMR data from the more complex LDL particle, the temperature dependence of the ^{13}C NMR spectra of (a) cholesteryl linoleate and (b) an aqueous codispersion of cholesteryl linoleate and egg phosphatidylcholine were studied.

^{13}C NMR of Cholesteryl Linoleate. Cholesteryl linoleate has been shown by polarizing microscopy and differential scanning calorimetry (Small, 1970), proton NMR (Small et al., 1974), and x-ray diffraction (Shipley et al., unpublished observations) to undergo several temperature dependent phase changes. At 42 °C, it melts from the crystalline state to an isotropic liquid. On subsequent cooling, it forms metastable liquid-crystalline phases: the cholesteric phase at 36.5 °C, and the smectic phase at 34 °C. Although the liquid-crystalline phases are metastable, the transitions between the liquid-

TABLE I: Chemical Shifts and Assignments of ^{13}C Resonances of Cholesteryl Linoleate.

Assignment[b]	Chemical Shift (ppm)[a]			
	Cholesteryl Linoleate in $CDCl_3$	Isotropic	Cholesteric	Smectic
$>C{=}O$	173.4 (1)	171.2 (1)	171.2 (1)	
C-5	139.8 (2)	139.9 (2)	139.9 (2)	
$-CH{=}CHCH_2$	130.0 (3)	129.9 (3)	129.9 (3)	
$-CH{=}CHCH_2CH{=}CH-$	128.0 (4)	128.0 (4)	128.0 (4)	129.1 (1)
C-6	122.6 (5)	122.2 (5)	122.2 (5)	
C-3	73.7 (6)	72.9 (6)	72.9 (6)	
C-14	56.8 (7)	56.7 (7)	56.7 (7)	
C-17	56.3 (8)			
C-9	50.2 (9)	50.2 (8)		
C-13	42.4 (10)	42.4 (9)	42.4 (8)	
C-16	39.8 (11)			
C-24	39.6 (12)			
Undetermined	38.2 (13)			
C-1	37.1 (14)			
C-10	36.6 (15)	36.6 (10)	36.6 (9)	
C-22	36.3 (16)			
C-20	35.8 (17)			
$-\overset{O}{\overset{\|}{C}}CH_2$	34.7 (18)			
$CH_3CH_2CH_2-$	31.9 (19)	31.8 (11)	31.8 (10)	
$-(CH_2)_n-$	29.1 (20)	29.7 (12)	29.7 (11)	29.7 (2)
C-12	28.3 (21)			
C-25	28.0 (22)			
$-CH{=}CHCH_2-$	27.2 (23)	27.3 (13)	27.3 (12)	27.3 (3)
$-CH{=}CHCH_2CH{=}CH-$	25.7 (24)	25.7 (14)	25.7 (13)	
$CH_2CH_2\overset{O}{\overset{\|}{C}}-$	25.1 (25)			
C-15	24.3 (26)			
C-23	23.9 (27)			
C-27, $-CH_2CH_3$	22.7 (28)	22.7 (15)	22.7 (14)	22.7 (4)
C-26	22.6 (29)			
C-11	21.1 (30)			
C-19	19.3 (31)	18.9 (16)	18.9 (15)	
C-21	18.8 (32)			
$-CH_3$	14.1 (33)	14.1 (17)	14.1 (16)	14.1 (5)
C-18	11.9 (34)	11.9 (18)	11.9 (17)	

[a] Chemical shifts are in parts per million downfield from Me_4Si. The terminal methyl group of the fatty acid acyl chains at 14.1 ppm downfield from this was used as an internal reference, except in cholesteryl linoleate dissolved in $CDCl_3$ in which Me_4Si was added to the sample. The numbers in the parentheses after the chemical shifts are the peak designations in the spectra (see Figures 1 and 2). The chemical shifts are accurate to ±0.2 ppm. [b] Unless otherwise noted, the assignments refer to the fatty acyl chain. The italic carbon indicates the specifically assigned carbon. Cholesterol carbons are designated by a C preceding the position in the cholesterol moiety (see Figure 1).

crystalline and isotropic phases are reversible (Small, 1970).

Cholesteryl linoleate gives a high resolution ^{13}C NMR spectrum when dissolved in $CDCl_3$ (Figure 1). The chemical shift assignments shown in Table I were determined using previously published ^{13}C NMR data on cholesterol and fatty acids (Johnson and Jankowski, 1972). The natural abundance ^{13}C NMR spectra of cholesteryl linoleate at temperatures corresponding to the liquid-crystalline and liquid states are shown in Figure 2. Figure 2A shows the high resolution spectrum of cholesteryl linoleate in the isotropic state at 44 °C. The chemical shift assignments are shown in Table I. The most prominent resonances of the cholesterol ring system carbon atoms are as follows: C-5 (peak 2), C-6 (peak 5), C-3 (peak 6), C-14 and -17 (peak 7), C-9 (peak 8), and C-18 (peak 18). These resonances serve as useful probes of the physical state of the cholesterol ring carbons of the ester because they are distinct from any overlapping contributions of the acyl chain carbons in the chemical shift region 20–40 ppm from Me_4Si. Compared with cholesteryl linoleate dissolved in $CDCl_3$, the carbonyl carbon resonance (peak 1) of cholesteryl linoleate in the isotropic liquid is chemically shifted by more than 2 ppm (see Table I).

At 34 °C, cholesteryl linoleate is in the cholesteric phase. Compared with the isotropic liquid, the resonances from the cholesterol ring system carbon atoms are broadened (Figure 2B). This is shown for the C-3 (peak 6), C-14 and C-17 (peak 7), C-9 (no high resolution resonance in the cholesteric phase), and C-18 (peak 17) resonances. The C-5 (peak 2) and C-6 (peak 5) resonances from the double bond in the cholesterol ring system have not broadened as much as those from other carbons in the ring system.

At 30 °C, cholesteryl linoleate is in the smectic phase. In this liquid-crystalline state, a marked change is seen in the ^{13}C NMR spectrum (see Figure 2C). There are no longer any high resolution resonances which can be assigned to the cholesterol ring system carbons. In addition, the carbonyl carbon has become so immobilized that it also fails to show a high resolution signal and there is now marked broadening of the resonances from the acyl chain carbons (peaks, 2, 3, and 4 in Figure 2C). Finally, the previously distinct resonances of the double bonds in the acyl chain (peaks 3 and 4 in Figures 2A and 2B) have broadened and merged into a single resonance (peak 1, Figure 2C).

^{13}C NMR of Aqueous Dispersons of Cholesteryl Linoleate and Egg Phosphatidylcholine. Aqueous dispersions of cho-

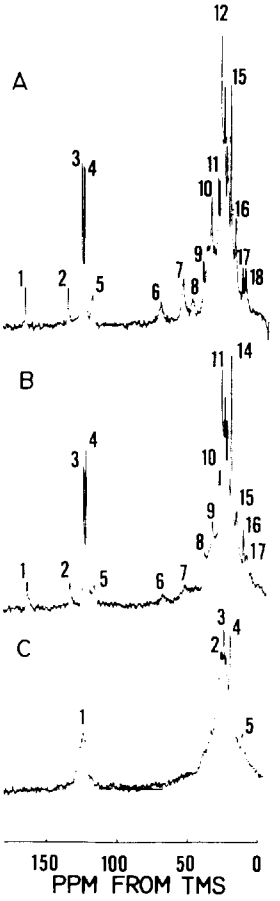

FIGURE 2: Natural abundance ^{13}C NMR spectra of neat cholesteryl linoleate. The spectra were recorded at 15.03 MHz with 8K data points in a 5K spectral width using 1000 accumulations and at a recycle rate of 0.9 s. Peak numbers are the same as those shown in parentheses in Table I. (A) T 44 °C; (B) T 34 °C; and (C) T 30 °C.

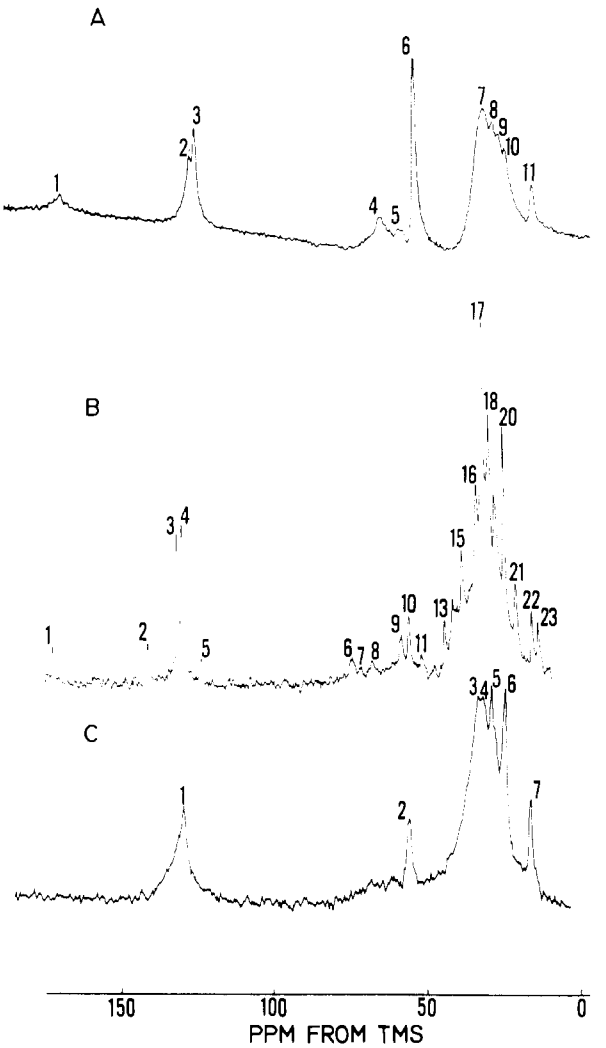

FIGURE 3: Natural abundance ^{13}C NMR spectra of aqueous dispersions of egg phosphatidylcholine and egg phosphatidylcholine plus cholesteryl linoleate. The spectra of egg phosphatidylcholine (spectra A) was taken from Sears (1975). The peak numbers of this spectrum are also assigned in Sears (1975). The remaining spectra were recorded at 15.03 MHz with 70° pulse angle, 8K data points in a 5K spectral width using 1000 accumulations with a recycle rate of 1.1 s. The peak numbers of spectra B and C are the same as those shown in parentheses in Table II. (A) Egg phosphatidylcholine T 42 °C; (B) egg phosphatidylcholine and cholesteryl linoleate, T 50 °C; and (C) egg phosphatidylcholine and cholesteryl linoleate, T 10 °C.

lesteryl linoleate and egg phosphatidylcholine (weight ratio 2:1, a similar ratio to that found in LDL) consist of two phases (Janiak et al., 1974). One is phosphatidylcholine bilayer saturated with a small amount of cholesteryl linoleate and the other is cholesteryl linoleate oil droplets probably stabilized by an outer monolayer of phosphatidylcholine. Polarizing light microscopy of these dispersions shows that, in the droplets, the cholesteryl linoleate is organized in a smectic phase at 10 °C and in an isotropic phase at 50 °C. The ^{13}C NMR spectra of this dispersion at 10 and 50 °C are shown in Figures 3B and 3C.

The chemical shifts and the assignments of the various resonances are listed in Table II. The interpretation of these spectra is more complex since some resonances (peaks 3 and 4 in Figure 3B) arise from the acyl chains of both the cholesterol ester and the phospholipid. However, a number of resonances are present which can be assigned uniquely to either the cholesterol ester (e.g., the cholesterol ring system resonances such as C-5 (peak 2), C-6 (peak 5), C-3 (peak 6), C-14 and -17 (peak 9), C-9 (peak 11), and C-18 (peak 23)) or the phospholipid (e.g., the polar head group resonances (peaks 7, 8, and 10)), particularly the N-methyl carbons of the choline moiety (peak 10) in Figure 3B.

To illustrate the effect of cholesteryl linoleate on phosphatidylcholine dispersions, the ^{13}C NMR spectrum of an unsonicated egg phosphatidylcholine dispersion (Sears, 1975) is shown in Figure 3A. In comparison, the ^{13}C NMR spectrum from the dispersion of cholesteryl linoleate and egg phosphatidylcholine (Figure 3B) shows a number of differences. These differences include the presence of a sharp carbonyl resonance (peak 1), relatively sharp acyl chain resonances from 20 to 40 ppm from Me_4Si, and an increased amplitude of the double-bond resonances (peaks 3 and 4) compared with the choline resonance (peak 10). These differences in the spectra from the two dispersions show that the cholesteryl linoleate dominates the resonances arising from the fatty acid moieties of the cholesteryl linoleate–egg phosphatidylcholine dispersion.

There are also differences in the resonances from the polar head group of phosphatidylcholine in the presence and absence of cholesteryl linoleate. In particular, the methylene carbon of the polar head group (peak 5 in Figure 3A) is no longer present, whereas the glycerol resonance (peak 7 in Figure 3B) is now present. These observations suggest that the presence of cholesteryl linoleate alters the conformation of the polar head group of egg phosphatidylcholine.

The ^{13}C NMR spectrum of a molecule gives information

TABLE II: Chemical Shifts and Assignments of ^{13}C Resonances of Dispersions and LDL.

Assignment[c]	Chemical Shift (ppm)[a] PC-CL Dispersion[b] T 50 °C	PC-CL Dispersion[b] T 10 °C	LDL[b] T 50 °C	LDL[b] T 46 °C	LDL[b] T 10 °C	Disrupted LDL[b] T 46 °C
>*C*=O	170.9 (1)		171.0 (1)	170.9 (1)		170.9 (1)
C-5	140.0 (2)		140.0 (2)	139.7 (2)		139.8 (2)
*C*H=CHCH$_2$	129.9 (3)	130.6 (1)	129.8 (3)	129.7 (3)	129.7 (1)	129.7 (3)
*C*H=CHCH$_2$CH=*C*H–	128.2 (4)	129.1 (1)	128.1 (4)	128.0 (4)	127.9 (2)	128.0 (4)
C-6	122.3 (5)		122.5 (5)	122.2 (5)		122.1 (5)
C-3	72.9 (6)					72.5 (6)
Glycerol CH	69.3 (7)					
Choline CH$_2$—N	66.3 (8)					66.1 (7)
Choline CH$_2$—O—P						
C-14, 17	56.7 (9)		56.7 (6)	56.6 (6)		56.8 (8)
Choline $\overset{+}{N}$(CH$_3$)$_3$	54.2 (10)	54.2 (2)	54.4 (7)	54.2 (7)	54.3 (3)	54.2 (9)
C-9	50.4 (11)		50.6 (8)			50.0 (10)
C-13	42.7 (12)		42.5 (9)	42.4 (8)		42.4 (11)
C-24	39.9 (13)		39.4 (10)	39.6 (9)		39.7 (12)
C-10	36.7 (14)		36.7 (11)	36.5 (10)		36.5 (13)
–*C*H$_2$C(=O)–				33.9 (11)		34.2 (14)
CH$_3$CH$_2$*C*H$_2$–	31.9 (15)	31.7 (3)	31.7 (12)	31.8 (12)		31.8 (15)
–(*C*H$_2$)$_n$–	29.6 (16)	29.5 (4)	29.5 (13)	29.5 (13)	29.7 (4)	29.6 (16)
C-25			28.1 (14)			28.3 (17)
–CH=CH*C*H$_2$–	27.5 (17)	27.5 (5)	27.3 (15)	27.4 (14)		27.4 (18)
–CH=CH*C*H$_2$CH=CH–	26.0 (18)		25.7 (16)	25.9 (15)		25.8 (19)
–*C*H$_2$CH$_2$C(=O)–						25.0 (20)
C-26,27 CH$_3$*C*H$_2$–	22.8 (19)	22.9 (6)	22.7 (17)	22.8 (16)	22.7 (5)	22.9 (21)
C-19, -21	19.3 (20)		19.0 (18)	19.2 (17)		19.2 (22)
*C*H$_3$–	14.1 (21)	14.1 (7)	14.1 (19)	14.1 (18)	14.1 (6)	14.1 (23)
C-18	12.0 (22)		11.9 (20)	11.9 (19)		12.0 (24)

[a] Chemical shifts are in parts per million downfield from Me_4Si. The terminal methyl group of the fatty acid acyl chains at 14.1 ppm downfield from Me_4Si was used as an internal reference. The numbers in the parentheses after the chemical shifts are the peak designation in the spectra (Figures 3–5). The chemical shifts are accurate to ±0.3 ppm. [b] The different samples are as follows: LDL, native low density lipoprotein; disrupted LDL, thermally disrupted low density lipoprotein; PC–CL dispersion, aqueous dispersion of egg phosphatidylcholine and cholesteryl linoleate. [c] Unless otherwise noted, the assignments refer to the fatty acyl chains. The italic carbon atom indicates the specifically assigned carbon. Cholesterol carbons are designated by a C- preceding the position in the cholesterol moiety (see Figure 1).

only about the rotational motions of the molecule. However, as we have shown above, the different physical organizations of cholesteryl linoleate impart different degrees of motional restrictions on selected cholesterol ring resonances. Therefore we can relate the spectral appearance of these resonances to different degrees of motional restrictions which in turn can be related to the physical organization of the cholesteryl linoleate in the aqueous dispersion.

As shown in Figure 3B, at 50 °C, the sharp resonances obtained from the cholesterol ring system carbons listed above are characteristic of cholesteryl linoleate in the isotropic state (Figure 2A). If the organization of the cholesterol esters corresponded to the cholesteric state, these resonances would have been broadened (see Figure 2B).

Upon lowering the temperature to 10 °C, broadening of most of the resonances occurs (Figure 3C). No high resolution resonances that can be attributed to the cholesterol ring system are observed and the acyl chain double bond (peaks 3 and 4 in Figure 3B) and unresolved acyl chain carbon resonances in the region 20–40 ppm are broadened. These spectral characteristics are consistent with the cholesteryl linoleate being in the smectic phase (cf. Figures 2C and 3C).

^{13}C NMR Studies of LDL. Other methods have shown that LDL undergoes a reversible thermal transition between 20 and 40 °C which is associated with changes in the organization of the cholesterol esters in the LDL particle (Deckelbaum et al., 1975). Below the thermal transition, it has been shown by x-ray scattering that the cholesterol esters are in the smectic-like phase whereas above the transition, the cholesterol esters are less organized (Deckelbaum et al., 1975).

The natural abundance ^{13}C NMR spectra of LDL above (*T* 50 °C) and below (*T* 10 °C) this transition are shown in Figure 4. Table II contains the chemical shifts and assignments of the various lipid resonances. These assignments are based on the spectra of the model systems (see above) and previously published ^{13}C NMR data on phosphatidylcholine vesicles and LDL (Sears, 1975; Hamilton et al., 1974). Although the lipid components of LDL dominate the ^{13}C NMR spectrum of LDL at 50 °C, the assignment of each resonance to a unique lipid species is difficult. For example, resonances assigned to the acyl chain carbons may arise from cholesterol ester, phospholipids, or triglycerides. However, since triglycerides are a minor component in LDL, they would be expected to contribute only slightly to the total acyl chain resonance intensity. Similarly, resonances assigned to the carbon atoms in the cholesterol ring system may arise from either free or esterified cholesterol. However, since about 75% of the cholesterol is esterified in LDL, the cholesterol ring resonances originate primarily from the cholesterol esters. The *N*-methyl carbon of the choline moiety comes only from the choline containing phospholipids, phosphatidylcholine and sphingomyelin. In comparison with LDL at 50 °C, the spectrum of LDL at 10 °C (Figure 4B)

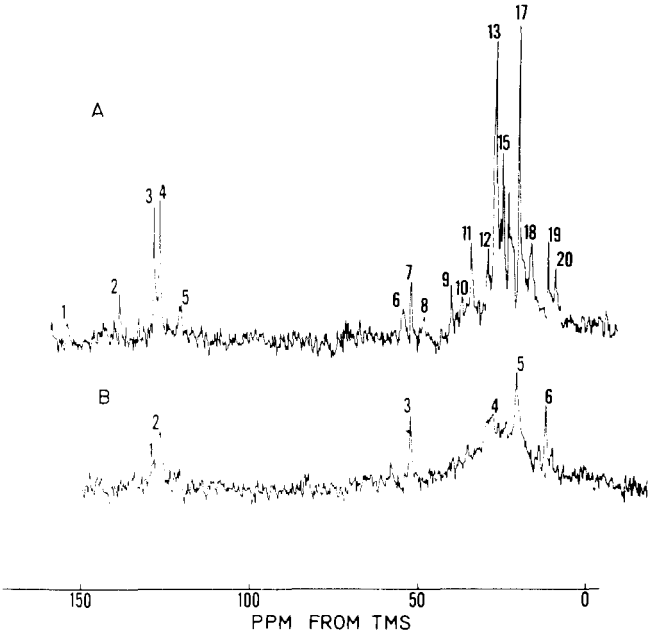

FIGURE 4: Natural abundance ^{13}C NMR spectra of low density lipoprotein (51 mg/ml). The spectra were recorded at 15.02 MHz with 8K data points in a 5K spectral width using a pulse angle of 70°. Each spectrum is the result of 12 893 accumulations with a recycle time of 1.1 s. The peak numbers are the same as those shown in parentheses in Table II. (A) T 50 °C; (B) T 10 °C.

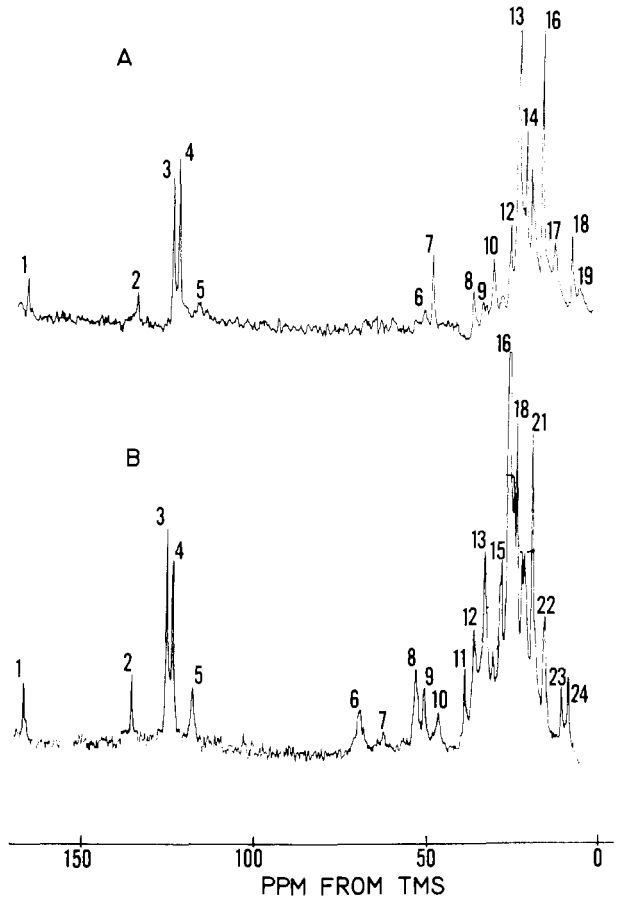

FIGURE 5: Natural abundance ^{13}C NMR spectra of native and denatured low density lipoprotein with 8K data points in 5K spectral width using a pulse angle of 70°. To obtain spectrum A, 20 000 scans were used and 65 000 scans were used to obtain spectrum B. The recycle time for both spectra was 1.1 s. The peak numbers are the same as those shown in parentheses in Table II. (A) Native low density lipoprotein; (B) disrupted low density lipoprotein, both at T 46 °C.

shows significant broadening or absence of many of the resonances seen at 50 °C. Notably, the carbonyl resonance and various cholesterol ring resonances (e.g., C-14 and -17, C-9, C-13, C-10, C-19 and -21, and C-18) are now absent at 10 °C. In addition, there is a significant broadening in the double-bond resonances and in the rest of the acyl chain resonances. Resonances associated with the cholesterol ring system are similar to those of cholesteryl linoleate in the smectic phase (Figure 2B) and also to those of the codispersion of cholesteryl linoleate and egg phosphatidylcholine at 10 °C (see Figure 3C).

Irreversible thermal disruption of the LDL particle is achieved by heating the LDL solution for 5 min at 100 °C (Deckelbaum et al., 1975). This disruption is accompanied by protein denaturation and the extrusion of the cholesterol esters of LDL into the surrounding medium in the form of oil droplets (Deckelbaum et al., 1976).

As shown in Figure 5, the ^{13}C NMR spectrum of thermally disrupted LDL cooled to 46 °C exhibits a number of differences compared with that of native LDL at 46 °C. For instance, when the amplitudes of the specific resonances are compared with the amplitude of the N-methylcholine resonance (peak 7 in Figure 5A and peak 9 in Figure 5B) which serves as an internal standard of the phospholipid contribution, the differences become obvious. Using the choline resonance as a standard, these include the appearance (e.g., C-3 (peak 6) and C-9 (peak 10)) or enhancement (e.g., C-5 (peak 2), C-6 (peak 5), C-14 and -17 (peak 8), C-18 (peak 24)) of resonances in Figure 5B that can be assigned to the cholesterol ring system in the spectrum of disrupted LDL. Furthermore, the cholesterol ring resonances (C-5 (peak 5), C-3 (peak 6), C-14 and -17 (peak 8), C-9 (peak 10), and C-18 (peak 24)) of the thermally disrupted LDL show a close similarity to those of cholesteryl linoleate in the isotropic state (see Figure 2A).

In order to ascertain the physical state of cholesterol esters in LDL, we can compare selected cholesterol ring resonances of LDL at 46 and 50 °C, and thermally disrupted LDL at 46 °C. At 46 °C in native LDL, there are no high resolution resonances from the C-3 or C-9 carbons. Other cholesterol ring resonances, C-5, C-6, C-14 and -17, and C-18 show some spectral similarities to the same resonances of cholesteryl linoleate in the cholesteric phase (Figure 2B). On the other hand, the same resonances in thermally disrupted LDL at 46 °C (Figure 5B) have a spectral appearance similar to those resonances of cholesteryl linoleate in the isotropic phase (Figure 2A). For LDL at 50 °C (Figure 4A) these same resonances appear to be intermediate between those of native and thermally disrupted LDL, both at 46 °C.

Discussion

Because of the molecular complexity of LDL, we have investigated the temperature dependence of the ^{13}C NMR spectra of cholesterol ester and aqueous codispersions of phospholipids and cholesterol esters and used these observations to interpret the more complicated spectra of LDL.

Cholesteryl linoleate is the predominant cholesterol ester in LDL. The natural abundance ^{13}C NMR spectrum of cholesteryl linoleate in the isotropic liquid (melted) state is similar to its solution spectrum in $CDCl_3$. However, two important differences are observed. First, a broadening of the resonances, particularly those from the cholesterol ring system, occurs in the liquid state. Second, the chemical shift of the carbonyl carbon of cholesteryl linoleate in the isotropic liquid state is

shifted upfield by more than 2 ppm compared with its solution spectrum in $CDCl_3$ (see Table I). The two different chemical shifts may arise from either a change in the conformation of the ester linkage, or differences due to the polarity of the solvent, $CDCl_3$. It is most likely that the chemical shift is due to the change in the solvent polarity. For example, dioleylphosphatidylcholine labeled with ^{13}C in the carbonyl group shows an upfield shift of 1 ppm in $CDCl_3$ as compared with CD_3OH (Assman et al., 1974).

The rotational motions of cholesteryl linoleate are manifest in its ^{13}C NMR spectrum. The temperature dependence of the ^{13}C NMR spectra of cholesteryl linoleate can be in turn related to the known thermotropic organization of cholesteryl linoleate in various structural organizations.

Resonances that can be assigned to the cholesterol ring system are relatively sharp in the isotropic phase, broadened in the cholesteric phase, and absent in the smectic phase. In addition, the acyl chains of cholesteryl linoleate become broadened only in the smectic phase. It appears in these various physical states that the cholesterol ring system is undergoing preferential immobilization compared with the acyl chains. Similarly, a comparison of the cholesterol ring resonances shows that, in the codispersion of cholesteryl linoleate and egg phosphatidylcholine, the cholesterol ester is in the isotropic liquid state at 50 °C and in the smectic state at 10 °C. This is verified by polarizing microscopy.

LDL is known to undergo a reversible thermal transition between 20 and 40 °C which is due to a phase transition of the cholesterol esters (Deckelbaum et al., 1975). X-ray scattering of LDL shows that the cholesterol esters of LDL are organized in a smectic-like phase below this transition. Above the transition, x-ray scattering is unable to distinguish clearly between the cholesteric and isotropic phases. However, the ^{13}C NMR spectra of cholesteric and isotropic phases of cholesterol esters are distinctive. Using the spectra of both cholesteryl linoleate and aqueous dispersions of egg phosphatidylcholine and cholesteryl linoleate, a further interpretation of the temperature dependence of the cholesterol ester organization in LDL is possible.

In LDL the presence of free cholesterol could also give resonances indistinguishable from those of the cholesterol ring system of the cholesterol esters. However, free cholesterol is a minor component compared with cholesterol esters in LDL. In addition, ^{13}C NMR studies of aqueous dispersions of egg phosphatidylcholine and free cholesterol even at 1:1 molar ratios fail to show any resonances attributable to the cholesterol ring system (Keough et al., 1973). Therefore, if we assume that all of the free choleterol is complexed with the phospholipid, it would not be expected to give a high resolution spectrum from its cholesterol ring system. Thus, the spectral appearance of the cholesterol ring resonances should mainly reflect the physical organization of the cholesterol esters in LDL.

The ^{13}C NMR spectrum of LDL at 10 °C is similar to that of cholesteryl linoleate in the smectic state and the egg phosphatidylcholine and cholesterol linoleate codispersion at 10 °C. The absence of cholesterol ring resonances indicates that the cholesterol esters in LDL are in a highly ordered state similar to the smectic phase of cholesteryl linoleate. Above the thermal transition, for instance at 46 °C, the cholesterol ring resonances of LDL resemble those of cholesteryl linoleate in the cholesteric phase and these resonances are further enhanced at 50 °C.

The ^{13}C NMR spectrum of the thermally disrupted LDL at 46 °C compared with native LDL at 46 and 50 °C suggests that, at temperatures above the thermal transition, the cholesterol esters in native LDL are in a state more ordered than the isotropic liquid.

Therefore, the ^{13}C NMR data are consistent with the interpretation that the thermal transition in LDL reflects a change in the structural organization of its cholesterol esters from an ordered to a less ordered state. However, it would appear that the structural integrity of the LDL particle maintains the cholesterol esters in a motionally restricted state even at temperatures above the thermal transition. Whether this is mediated by the protein itself (via protein–lipid interactions) or by the boundary constraints imposed by the small size of the LDL particle is being investigated.

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