Organization of the Core Lipids of Lipoproteins from Normal and Cholesterol-Fed Rabbits. A Proton Nuclear Magnetic Resonance Study†

Paulus A. Kroon* and Joel Seidenberg

ABSTRACT: When rabbits are fed a diet supplemented with cholesterol, their plasma cholesterol levels increase markedly, and they develop atherosclerosis. Most of the plasma cholesterol exists as cholesteryl esters in very low density lipoproteins (VLDL) and intermediate-density lipoproteins (IDL). The triglyceride content of the lipoprotein cores decreases sharply during cholesterol feeding. This change is most marked for VLDL in which it decreases from 74% to 5%, while the cholesteryl ester content increases from 26% to 95%. The IDL and low-density lipoprotein (LDL) fractions from cholesterol-fed rabbits have a triglyceride content of 2% or less in their cores. The mobility of the core cholesteryl esters has been studied by proton nuclear magnetic resonance spectroscopy. Changes in the mobility were assessed by measuring the temperature dependence of the amplitude of the methylene resonances. The decrease in spectral amplitude for VLDL, IDL, and LDL from cholesterol-fed rabbits between 55 and 15 °C shows that the mobility of the core cholesteryl esters is temperature dependent and that the cholesteryl esters display thermal order–disorder transitions with midpoints of 42, 40, and 38 °C, respectively. At physiological temperatures, the core cholesteryl esters of lipoproteins from cholesterol-fed rabbits therefore exist in a partially ordered state. In contrast, the core cholesteryl esters of VLDL, IDL, and LDL from normal rabbits show no evidence for an order–disorder transition. This is consistent with their high core triglyceride content which precludes the existence of an ordered cholesteryl ester phase within the core. The core cholesteryl esters of normal rabbit lipoproteins therefore exist in a liquid state at physiological temperatures. High-density lipoproteins (HDL) from normal and cholesterol-fed rabbits fail to display an order–disorder transition. This is attributed to the constraints imposed by the small HDL core diameter, which prevents the existence of an ordered arrangement of cholesteryl esters, irrespective of the core triglyceride content.

Cholesterol feeding in rabbits produces hypercholesterolemia and atherosclerosis (Anitschkow, 1933; Shumaker, 1956). Hypercholesterolemia is accompanied by a large increase in the VLDL[1] and IDL cholesterol concentrations and a moderate increase in the LDL cholesterol concentration (Shumaker, 1956; Camejo et al., 1973; Shore et al., 1974). HDL cholesterol levels have been reported to decrease (Shore et al., 1974) or to remain constant (Pinon & Bridonx, 1977). Cholesterol feeding also has a major effect on the composition of the lipoprotein particles (Camejo et al., 1973; Shore et al., 1974; Pinon & Bridonx, 1977; Rodriguez et al., 1976). Camejo et al. (1973) have shown that the VLDL cholesteryl ester content increased from 4.3% to 40.3% while the triglyceride content decreased from 54.1% to 15.1% when rabbits were fed a diet supplemented with 1% cholesterol. Such compositional changes can have a profound effect on the structural characteristics of the lipoprotein core structure (Deckelbaum et al., 1977a).

Studies using techniques such as low angle X-ray scattering, differential scanning calorimetry (DSC), and nuclear magnetic resonance (NMR) spectroscopy (Deckelbaum et al., 1977a; Laggner et al., 1977; Tall et al., 1977a; Atkinson et al., 1978; Kroon, 1981; Kroon & Krieger, 1981) have shown that cholesteryl ester rich lipoproteins with a radius of 70 Å or greater display a broad reversible transition which is associated with a change of their core cholesteryl esters from an ordered smectic[2]-like state to a disordered liquid state. The transition temperature depends on the cholesteryl ester to triglyceride ratio and on the degree of saturation of the cholesteryl ester fatty acids (Deckelbaum et al., 1977a; Tall et al., 1978; Kirchhausen et al., 1979). For human LDL, the transition encompasses body temperature (Deckelbaum et al., 1977a). Studies of LDL from miniature swine and monkeys on atherogenic diets have shown that their LDL was considerable more ordered than that of control animals (Tall et al., 1978; Kirchhausen et al., 1979; Pownall et al., 1980). Similar observations have been made for VLDL from cholesterol fed rabbits (CR-VLDL) (Morrisett et al., 1980; Ploplis et al., 1979; Kroon et al., 1982). Below the transition temperature, the core cholesteryl esters of CR-VLDL are immobilized, while they exist in a liquid-like state above the transition (Morrisett et al., 1980; Kroon et al., 1982). LDL ($1.006 < d < 1.063$ g/mL) from cholesterol-fed rabbits behaves in much the same way (Kroon et al., 1982). Taken together these observations have led to the suggestion that there may be a causal relationship between the physical state of the cholesterol ester rich lipoproteins and their atherogenicity.

Proton NMR spectroscopy has been used to investigate order–disorder transitions in plasma lipoproteins (Kroon, 1981; Kroon & Kreiger, 1981). Such measurements are based on the observation that cholesteryl esters in the cholesteric[2] and smectic phases do not yield high-resolution NMR spectra, while liquid cholesteryl esters do. Order–disorder transitions for neat cholesteryl esters can therefore be followed by measuring the variation of the spectral amplitude of the methylene resonance with temperature. A sudden decrease in the spectral amplitude indicates the onset of the transition (Kroon, 1981). The situation is clearly more complex in lipoproteins, in that phospholipids are present in addition to

† From the Merck Institute for Therapeutic Research, Merck Sharp & Dohme Research Laboratories, Rahway, New Jersey 07065. *Received May 3, 1982.*

[1] Abbreviations: VLDL, IDL, LDL, and HDL, very low, intermediate-, low-, and high-density lipoproteins, respectively; CR-lipoproteins, lipoproteins from cholesterol-fed rabbits; NR-lipoproteins, lipoproteins from normal chow-fed rabbits; DSC, differential scanning calorimetry; NMR, nuclear magnetic resonance; EDTA, ethylenediaminetetraacetic acid.

[2] The smectic liquid-crystalline state of cholesteryl esters is believed to consist of planar arrays stacked with a repeat distance of 36 Å. In the cholesteric liquid-crystalline state, cholesteryl ester molecules are ordered in helices about an axis at right angles to the long molecular axis.

0006-2960/82/0421-6483$01.25/0

the neutral core lipids. The lipoprotein phase transition is characterized by a curve which consists of three linear segments whose intersecting transition points correspond to calorimetrically determined phase transition temperatures (Kroon, 1981; Kroon & Krieger, 1981). During the transition, changes in the physical state of the phospholipids can be monitored simultaneously from the spectral amplitude of the phospholipid choline methyl resonance (Kroon, 1981).

In the present study we use proton NMR spectroscopy to investigate the physical state and structure of individual lipoprotein classes from normal and cholesterol-fed rabbits. Our results show that LDL from normal rabbits differ from that of other species including man, in that it does not appear to undergo a clear order–disorder transition in the temperature range studied (10–55 °C). On the other hand, with the exception of HDL, each of the lipoproteins from cholesterol-fed rabbits undergoes a clear order–disorder transition in the range of or above physiological temperatures. As a consequence, the lipoproteins from cholesterol-fed atherosclerotic rabbits exist in a partially ordered smectic-like state at physiological temperatures while those of normal chow-fed rabbits are in a disordered liquid-like state.

Materials and Methods

Cholesteryl oleate and triolein were obtained from Applied Science Laboratories (State College, PA).

Animals. Male New Zealand white rabbits were purchased from HARE (Hewitt, NJ). Experimental rabbits were fed a Purina rabbit chow diet supplemented with cholesterol (0.5% w/w) and corn oil (10% v/w). Control rabbits were fed a Purina rabbit chow. The animals were fed ad libitum. Rabbits were bled by cardiac puncture following overnight fasting. EDTA, at a final concentration of 4 mM, was used as an anticoagulant. Blood cells were removed by centrifugation at 2000*g* for 20 min.

Lipoproteins. VLDL ($d < 1.006$ g/mL), IDL ($1.006 < d < 1.019$ g/mL), LDL ($1.019 < d < 1.063$ g/mL) and HDL ($1.063 < d < 1.021$ g/mL) were isolated from plasma by sequential flotation using a Beckman Ti 60 rotor. The plasma density was adjusted by adding an appropriate volume of a 0.196 M NaCl–7.572 M NaBr solution with a density of 1.4744 g/mL. Tubes were fractionated with a Beckman tube slicer. Following isolation, lipoprotein samples were dialyzed extensively at 4 °C against 0.15 M NaCl–0.01% EDTA, pH 7.4. For NMR studies this was followed by dialysis against 0.15 M NaCl–0.01% EDTA in D_2O, pD 7.4, where pD is the pH meter reading plus 0.4.

CR-VLDL lipids were extracted by the method of Bligh & Dyer (1959). Cholesteryl esters were isolated from this mixture by silicic acid (Unisil, Williamsburg, PA) chromatography using hexane with an increasing concentration of diethyl ether (0–2%) as the eluant. The elution was monitored by thin-layer chromatography using hexane–diethyl ether–glacial acetic acid (70:30:1 v/v/v) as a solvent. The cholesteryl ester mixtures gave a single spot by thin-layer chromatography using the above solvent.

Triglycerides were saponified and assayed as glycerol with Worthington Diagnostics reagents (Freehold, NJ). The lipoprotein cholesteryl ester content was measured by gas chromatography as described previously (Kroon et al., 1982).

NMR Spectroscopy. Proton NMR experiments were carried out on a Varian SC-300 spectrometer operating at 300 MHz in the Fourier transform mode. Pure cholesteryl ester and triolein samples consisted of a thin film between a 5-mm, flat-bottomed NMR tube and a 4-mm, flat-bottomed concentric tube. This arrangement was necessary in order to reduce the magnitude of the signal reaching the spectrometer. The inner tube contained 100% D_2O to lock the instrument. Lipoprotein samples contained 0.1–1.0 mg of protein/mL and were studied in 5-mm tubes. Samples were equilibrated for 15 min at each temperature before a spectrum was taken. Temperatures quoted are accurate to ±1 °C. Pulse widths were typically 65°; spectra were acquired every 1.0 or 2.0 s. For the neat cholesteryl ester and triglyceride samples, 1–10 scans were obtained for each spectrum. Lipoprotein samples required between 10 and 500 scans per spectrum.

Spectra were plotted in the absolute intensity mode which allows a comparison of consecutive spectra obtained at different temperatures with identical instrument settings. Peak heights and widths were measured from a base line which extended over a 3000-Hz spectral width. Peak heights were normalized for each experiment to give a value of 100 to the tallest peak.

Differential Scanning Calorimetry. Calorimetric studies were performed on a Perkin-Elmer DSC-2 differential scanning calorimeter at full-scale settings of 0.5 mcal/s with heating and cooling rates of 5 °C/min. Cholesteryl esters were sealed in aluminum (10-μL) sample pans. Reference pans contained an identical volume of distilled water. Temperature calibration was performed by using indium and distilled water.

Table I: Triglyceride and Cholesteryl Ester Content of Rabbit Lipoprotein Core Lipids

diet	lipoprotein	core triglyceride content (%)[a]
chow	VLDL	74
	IDL	23
	LDL	17
	HDL	18
cholesterol	VLDL	5
	IDL	1
	LDL	2
	HDL	8

[a] Calculated as the percentage of the lipoprotein triglyceride plus cholesteryl ester content.

Results

Composition of Lipoprotein Cores. Rabbits maintained on a normal chow diet have an average plasma cholesterol concentration of 60 mg/dL. VLDL from such rabbits (NR-VLDL) has a triglyceride-rich core, in which triglyceride accounts for more than 74% of the neutral core lipids (Table I). Its core composition is therefore similar to that of human VLDL (Deckelbaum et al., 1977b). In contrast, the core composition of intermediate- and low-density lipoproteins from normal rabbits (NR-IDL and NR-LDL, respectively) differs substantially from that of their human counterparts. Although each of these lipoproteins has a cholesteryl ester rich core, the rabbit lipoproteins contain a much larger amount of triglyceride. As the results in Table I show, triglyceride makes up 23% and 17% of the NR-IDL and NR-LDL core lipids, respectively, while human LDL core lipids contain approximately 5% triglyceride (Deckelbaum et al., 1977a).

When rabbits are fed a chow diet supplemented with cholesterol (0.5% w/w) and corn oil (10% v/w), serum cholesterol levels rise to over 1000 mg/dL within 1 month. The largest increase is found in the VLDL fraction. The data in Table I show that cholesterol feeding results in significant changes in the core composition of the plasma lipoproteins. Unlike NR-VLDL, VLDL from cholesterol fed rabbits contains mostly cholesteryl ester and very little (5%) triglyceride. The

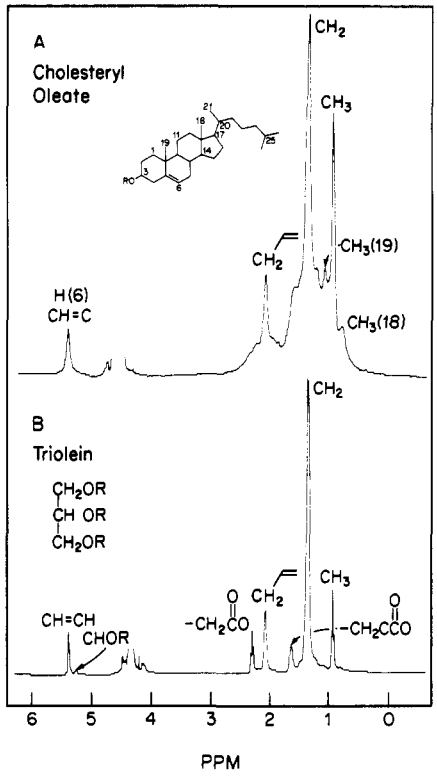

FIGURE 1: 300-MHz proton NMR spectra of (A) liquid cholesteryl oleate and (B) triolein, both at 50 °C. R = $-OC(CH_2)_7CH{=}CH(CH_2)_7CH_3$. The acyl chain olefinic (CH=C), allylic ($CH_2CH{=}$), methylene (CH_2), and terminal methyl (CH_3) resonances and cholesteryl olefinic [H(6)] and methyl [$CH_3(18)$ and $CH_3(19)$] resonances are labeled in panel A. In panel B, the acyl chain olefinic, allylic, methylene, and terminal methyl resonances are labeled as in panel A. In addition, the fatty acyl methylene $-CH_2C(=O)O$ and $-CH_2CC(=O)O$ and the glyceryl CH or resonances are labeled.

composition of IDL and LDL change in similar manner upon cholesterol feeding; CR-IDL and CR-LDL cores each contain less than 2.5% of triglyceride.

Nuclear Magnetic Resonance Studies. In order to provide a basis for subsequent discussions, we first present NMR spectra of each of the two major core components of lipoproteins. Figure 1 shows NMR spectra of a triglyceride (triolein) and of a cholesteryl ester (cholesteryl oleate), both at 50 °C. Assignments shown were determined from a comparison with the same samples dissolved in $CDCl_3$. The spectrum of triolein consists of sharp, well-resolved resonanced characteristic of a nonviscous liquid. Triolein remains liquid, and its resonances sharp at all temperatures studied (20–70 °C). Cholesteryl oleate is an isotropic liquid at 50 °C (Kroon, 1981; Small, 1970) with a viscosity of about 210 cP (Hamilton et al., 1977). Spectral line widths of cholesteryl oleate shown in Figure 1A are characteristic of such a viscous liquid (Kroon, 1981). Like most biological cholesteryl esters, liquid cholesteryl oleate undergoes two transitions when cooled from its melting point: a liquid → cholesteric transition at 46 °C and a cholesteric → smectic transition at 42 °C (Small, 1970). In a previous study we have shown that no high-resolution proton NMR features can be discerned for cholesteryl esters in the cholesteric or smectic phases because of a decreased mobility of smectic and cholesteric cholesteryl esters (Kroon, 1981).

Figure 2 shows proton NMR spectra (at 37 °C) of lipoproteins from normal and cholesterol-fed rabbits at 37 °C. We showed earlier that the NR-VLDL neutral core lipids consisted primarily of triglyceride (74%) with a smaller amount of cholesteryl ester (26%). The spectrum shown in Figure 2A clearly reflects this composition: the sharp and well-resolved

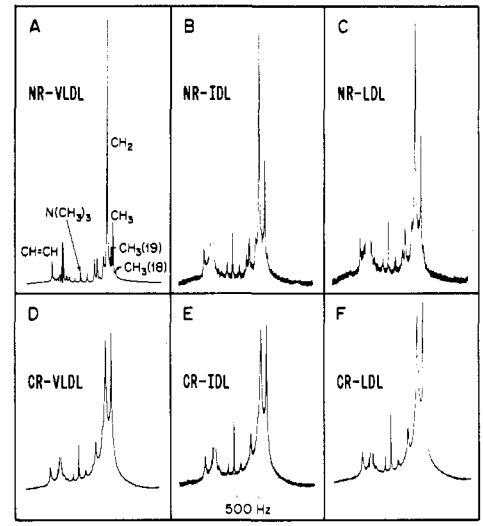

FIGURE 2: 300-MHz proton NMR spectra of lipoproteins from normal (A–C) and cholesterol-fed rabbits (D–F), all at 37 °C.

methylene and methyl resonances resemble those of liquid triolein (Figure 1B). The only evidence for the presence of cholesteryl esters are peaks from the cholesteryl $CH_3(18)$ and $CH_3(19)$ methyl groups on either side of the main methyl peak. In contrast, the spectrum of CR-VLDL is characterized by broad methylene and methyl resonances. A comparison of the CR-VLDL spectrum with the spectrum of liquid cholesteryl oleate (Figure 1A) shows that the CR-VLDL methylene and methyl resonances are substantially broader. In so far as the line widths reflect the motional state of the cholesteryl esters, these data show that the CR-VLDL cholesteryl esters, at 37 °C, are much less mobile than liquid cholesteryl oleate. This conclusion is in part based on the fact that the cholesteryl ester fatty acyl chains make up more than 60% of the total (phospholipid plus cholesteryl ester) fatty acyl chains and therefore dominate the spectrum.

The spectra of NR-IDL and NR-LDL resemble that of liquid cholesteryl oleate (Figure 1A), with the addition of a phospholipid choline methyl resonance and with a substantially larger CH_2/CH_3 peak height ratio. The sharpness of the resonances is consistent with a liquid-like state at 37 °C. The larger CH_2/CH_2 peak height ratio is due to the relatively high triglyceride content of these lipoproteins which results in a combination of the spectral features exhibited by cholesteryl oleate and triolein. In contrast, spectra of CR-IDL and CR-LDL have substantially broader resonances than the spectrum of liquid cholesteryl oleate and resemble that of CR-VLDL, consistent with lipoproteins whose core cholesteryl esters have a substantial degree of order at physiological temperatures. Spectra of NR-HDL and CR-HDL (not shown) are both well resolved at 37 °C and resemble that of liquid cholesteryl oleate, indicating that the HDL cholesteryl esters are in a disordered state at 37 °C.

The spectra in Figure 3 show that the degree of ordering of the core cholesteryl esters of CR-VLDL is temperature dependent. The spectrum at 49 °C resembles that of liquid cholesteryl oleate (Figure 1) and shows that the core cholesteryl esters exist in a liquid state at this temperature. The increased line widths observed at 20 °C show that the core cholesteryl esters are much less mobile at this temperature.

In order to determine whether an order–disorder transition of the CR-VLDL cholesteryl esters was responsible for the observed temperature dependence, CR-VLDL cholesteryl esters were extracted, purified, and subsequently studied by calorimetry and by NMR spectroscopy. Figure 4 shows a

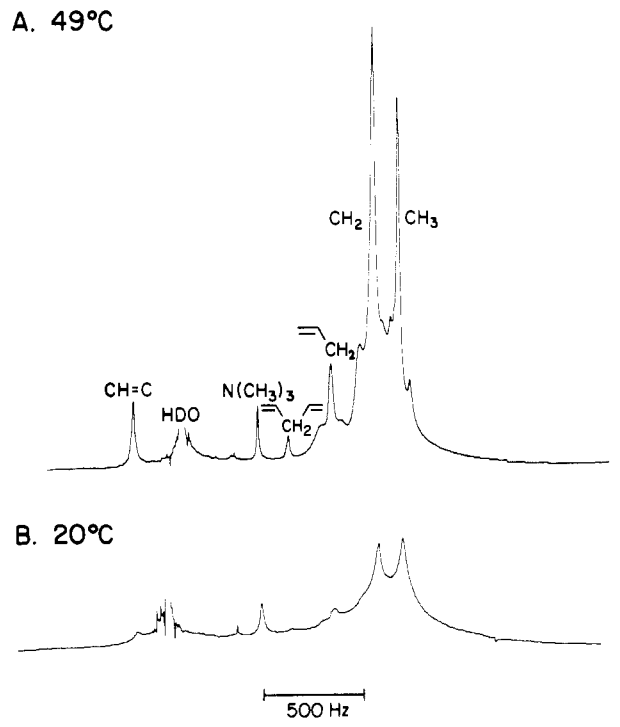

FIGURE 3: 300-MHz proton NMR spectra of CR-VLDL at 49 °C (A) and at 20 °C (B). The olefinic (CH=C), water (HDO), choline methyl [$N(CH_3)_3$], doubly allylic (=$CHCH_2CH$=), allylic (=CH-CH_2), methylene (CH_2), and terminal methyl (CH_3) resonances are labeled. Spectra in panels A and B were taken by using identical instrument settings, in the absolute intensity mode.

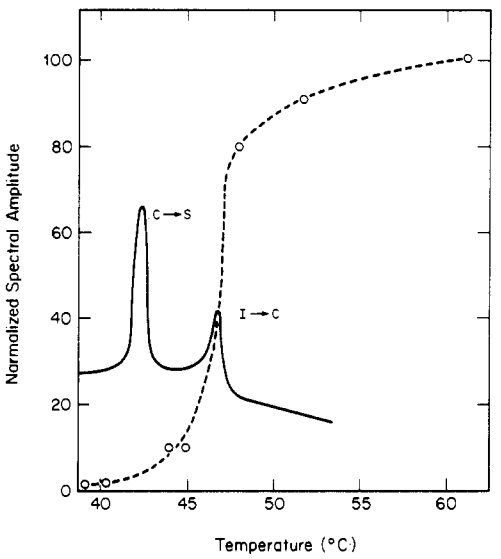

FIGURE 4: Temperature dependence of the NMR spectral amplitude for the methylene protons of cholesteryl esters extracted from CR-VLDL (---) and differential scanning calorimetry heating curve for the same cholesteryl ester sample (—). The NMR spectra were collected by equilibrating the sample at the highest temperature indicated and then lowering the temperature in steps for the measurements. The spectral amplitudes were measured and placed on a relative scale as described under Materials and Methods. The differential scanning calorimetry curve was obtained at a heating-cooling rate of 5 °C/min, with a full-scale sensitivity of 0.2 mcal/s.

DSC scan of the CR-VLDL cholesteryl esters. Consistent with previous studies of biological cholesteryl esters, two transitions are observed: the first corresponds to a liquid → cholesteric transition with a peak temperature of 47 °C and the second to a cholesteric → smectic transition with a peak temperature at 42 °C. Superimposed on the same figure is a plot of the

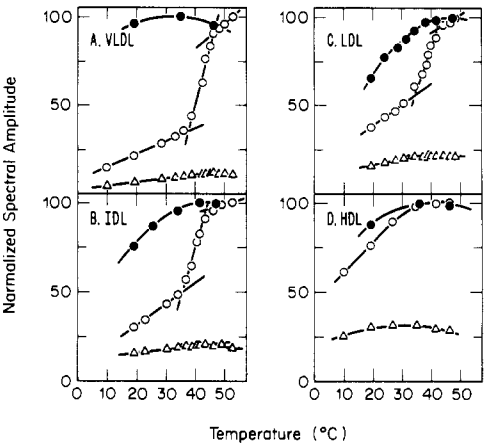

FIGURE 5: Temperature dependence of NMR spectral amplitudes for the methylene protons of lipoproteins from normal rabbits (●) and from cholesterol-fed rabbits (○) and for the choline methyl resonances of lipoproteins from cholesterol-fed rabbits (△). The NMR spectra were collected by equilibrating the sample at the highest temperature indicated and then lowering the temperature in steps for measurements. Subsequently, several spectra were taken with increasing temperatures to ensure that the changes observed were reversible. The spectra amplitudes were measured and placed on a relative scale as described under Materials and Methods. Transition temperatures are defined by the temperatures at which the central linear portion of the spectral amplitude curve intersects the high- and low-temperature linear portions.

spectral amplitude of the methylene resonance as a function of temperature. The sharp drop in spectral amplitude at 47 °C is indicative of a significant reduction of the mobility of the cholesteryl esters in the cholesteric phase (Kroon, 1981). These data support the notion that a transition of the CR-VLDL core cholesteryl esters are responsible for the temperature dependence of the CR-VLDL spectra.

In order to study the temperature-dependent spectral changes in more detail, NMR spectra of each of the lipoprotein fractions were obtained at temperatures ranging between 55 and 10 °C. The spectral amplitudes of the methylene peaks were measured, and the normalized spectral amplitudes were plotted as a function of temperature. The data for normal rabbit lipoproteins are shown as closed circles in Figure 5. The methylene spectral amplitude for NR-VLDL changes very little with temperature. These data and the spectrum in Figure 1A show that the NR-VLDL core triglycerides remain in a liquid-like state over the temperature range studied. Both NR-IDL and NR-LDL contain considerable amounts of cholesteryl ester, yet the variation of the methylene spectral amplitude in Figure 5B,C shows no evidence for a clear order–disorder transition over the temperature range studied. Instead the spectral amplitude decreases slowly with decreasing temperature. A similar pattern is observed with reconstituted LDL whose core cholesteryl esters have been replaced with cholesteryl oleate–triolein mixtures and which contain enough triolein to remove both the liquid → cholesteric and cholesteric → smectic transitions.[3] The choline methyl spectral amplitudes for the NR lipoproteins show little variation over the temperature range studied (data not shown).

The data for lipoproteins from cholesterol-fed rabbits are shown in open circles in Figure 5. In contrast to the spectral amplitude curves for normal rabbit lipoproteins, the spectral amplitude curves for VLDL, IDL, and LDL from cholesterol-fed rabbits are composed of three, approximately linear segments. This triphasic variation of the methylene spectral

[3] P. A. Kroon, unpublished results.

Table II: Phase Transition Temperatures for Lipoproteins from Cholesterol-Fed Rabbits

	thermal transition temperatures (°C)		
	range		
lipoprotein	low	high	midpoint
CR-VLDL	38	46	42
CR-IDL	36	44	40
CR-LDL	35	41	38

amplitude with temperature is characteristic of an order–disorder transition of the lipoprotein core cholesteryl esters (Kroon, 1981; Kroon & Krieger, 1981). The transition temperatures defined by these curves are summarized in Table II. Each of the transitions occur over a range of 6–8 °C with midpoints of 42 °C for CR-VLDL, 40 °C for CR-IDL and 38 °C for CR-LDL. At 37 °C each of these lipoproteins is therefore at the low temperature end of the core transition.

The decrease in spectral amplitude during the transition is accompanied by the disappearance of the cholesteryl $CH_3(18)$ and $CH_3(19)$ methyl resonances and by a broadening of each of the fatty acyl resonances. During the transition there is little change in the choline methyl spectral amplitude and therefore in its line width. This is consistent with the view that the lipoprotein phospholipids are not involved in the order–disorder transitions (Kroon, 1981). Nevertheless, a decrease in the spectral amplitude of the choline methyl peaks is observed below the lowest transition temperatures. We have made a similar observation for human LDL and have attributed this effect to a transition of the core order–disorder transition to the surface phospholipids (Kroon, 1981).

The spectral amplitude curve for CR-HDL is similar to that for NR-HDL. There is no evidence for an order–disorder transition for either of these lipoproteins. The HDL core lipids therefore retain a liquid-like mobility over the temperature range studied.

Discussion

In the present study we have used proton NMR spectroscopy to monitor the mobility of the surface and core lipids of lipoproteins from normal and cholesterol-fed rabbits. The data show that the triglyceride-rich VLDL as well as the cholesterol ester rich IDL, LDL, and HDL particles from normal rabbits have a relatively fluid core at physiological temperatures (39 °C for the rabbit). The data show no evidence for the existence of thermal order–disorder transitions over the temperature range studied. In this respect, rabbit LDL differs from LDL from other species which have been studied, which all display a clearly identifiable thermal order–disorder transition in the range of physiological temperatures (Deckelbaum et al., 1977a; Kirchhausen et al., 1979; Tall et al., 1977a; Tall, 1980). Previous studies have shown that under certain conditions lipoprotein core cholesteryl esters do not display an order–disorder transition. These conditions occur when the size of the lipoprotein core is too small to accommodate an organized layer of extended cholesteryl ester molecules (Deckelbaum et al., 1977a; Laggner et al., 1977; Tall, 1980) or when the core triglyceride content is too high to permit the existence of an ordered liquid-crystalline phase. While the cholesteryl ester fatty acyl composition could in principle by responsible for the absence of an order–disorder transition, this situation has only been observed for reconstituted LDL whose core cholesteryl esters were replaced with cholesteryl arachidonate (Kroon & Krieger, 1981). Unlike most biological cholesteryl esters, cholesteryl arachidonate does not form any liquid-crystalline phases.

Since the diameter of NR-LDL is indistinguishable from that of human LDL as determined by chromatography on Sepharose CL-2B,[3] its core can accommodate two organized layers of extended cholesteryl ester molecules (Deckelbaum et al., 1977a; Laggner et al., 1977). The size of the NR-LDL core therefore does not limit the ability of the core cholesteryl esters to undergo an order–disorder transition. The most likely reason for our inability to detect a transition is the high triglyceride content of normal rabbit LDL. While human LDL has a cholesterol ester core which contains about 5% by weight of triglyceride, normal rabbit LDL contains more than 3 times this amount of triglyceride (17%). This is important since triglycerides have a profound effect on the thermal transitions of neat cholesteryl esters. At low concentrations (about 3% w/w) they remove the liquid → cholesteric transition of cholesteryl esters, broaden the remaining liquid → smectic transition, and decrease the transition temperature (Deckelbaum et al., 1977a). As the amount of triglyceride is increased, a point is reached at which the liquid → smectic transition also disappears (Kroon, 1981; Deckelbaum et al., 1977b; Small, 1970; Hamilton et al., 1977). Thus cholesteryl oleate which contains 22% triolein remains liquid until it crystallizes (Kroon, 1981; Hamilton et al., 1977). We surmise that the amount of triglyceride in LDL from normal rabbits is large enough to abolish the liquid → smectic phase transition. The same arguments apply to IDL from normal rabbits. These particles contain a triglyceride content of 23% which is even higher than that of NR-LDL.

The core lipids of VLDL and HDL from normal rabbits also remain disordered over the temperature range studied. Similar findings have been made for human VLDL and HDL (Tall et al., 1977b). Although 26% of the VLDL core lipids are cholesteryl esters, their inability to display an order–disorder transition indicates that they are dissolved in the triglyceride-rich VLDL core. As such they are not capable of exhibiting a thermal order–disorder transition (Deckelbaum et al., 1977b). The absence of a transition for human HDL has been attributed to its size, which is too small to permit the formation of an ordered cholesteryl ester phase (Deckelbaum et al., 1977a; Tall et al., 1977b). Since the radius of rabbit HDL is similar to that of human HDL (90 Å),[3] the core cholesteryl esters are subjected to similar constraints. However, rabbit HDL also contains a substantial percentage of triglyceride which by itself may be enough to preclude the existence of a transition.

When rabbits are fed a diet supplemented with cholesterol, the lipoprotein cholesteryl ester/triglyceride ratio increases substantially. Our data show that these compositional changes have a profound effect on the organization of the lipoprotein core lipids. With the exception of CR-HDL, the core cholesteryl esters of lipoproteins from cholesterol-fed rabbits display thermal order–disorder transitions in the range of physiological temperatures. The core cholesteryl esters of these lipoproteins therefore have a considerable degree of order at physiological temperatures. The presence of order–disorder transitions in CR-VLDL and CR-IDL has also been demonstrated by ^{13}C NMR (Kroon et al., 1982) and by calorimetry (Morrisett et al., 1980; Tall, 1980). We attribute the existence of the order–disorder transitions for CR-lipoproteins to their very low triglyceride content. The absence of a transition for CR-HDL can be attributed to constraints imposed by the small size of the core (see above).

The nature of the ordered and disordered states in lipoproteins have received considerable attention. Our results show that the core cholesteryl esters of rabbit lipoproteins, like those

of human LDL (Kroon, 1981; Kroon & Krieger, 1981), exist in a liquid state above the transition temperature. This contrasts with previous suggestions that the lipoprotein cholesteryl esters exist in a radial nematic-like state above the phase transition temperature (Deckelbaum et al., 1977a; Tall et al., 1977a, 1978; Atkinson et al., 1978). The radial nematic state was viewed as arising from an interaction of the cholesteryl esters with the outer phospholipid acyl chains. The fact that the choline methyl peak height vs. temperature curve shows a break just below the transition temperature range suggests that such an interaction does indeed exist, except that the cholesteryl esters have an ordering effect on the phospholipids below the order–disorder transition temperature.

Our NMR studies do not distinguish between different ordered states of the core cholesteryl esters. However, X-ray diffraction studies of cholesteryl ester rich lipoproteins have shown that the ordered state is best described in terms of a radial smectic arrangement (Deckelbaum et al., 1977a; Laggner et al., 1977; Atkinson et al., 1977; Tall et al., 1978). We have presented a model which views the radial smectic phase of human LDL cholesteryl esters as being substantially more disordered than the planar smectic phase of neat cholesteryl esters (Kroon, 1981). The disorder of the radial smectic phase was attributed to the geometrical constraints imposed upon the packing of the core cholesteryl esters by the high curvature of the lipoprotein core and by the presence of a fluid phospholipid monolayer surrounding the core. The core cholesteryl esters of lipoproteins from cholesterol-fed rabbits are influenced by similar constraints: CR-VLDL has an average diameter of 350 Å, CR-IDL of 270 Å, and CR-LDL of 200 Å as determined by electron microscopy[3] and the cores of each of these lipoproteins are surrouned by a fluid phospholipid monolayer. These observations suggest that the core cholesteryl esters of each of these lipoproteins are arranged in a disordered smectic state below the phase transition temperature.

The existence of smectic-like cholesteryl esters in VLDL, IDL, and LDL, at physiological temperatures, is clearly a discriminating feature between rabbits on normal and cholesterol-rich diets. It has been suggested that the physical state of lipoproteins may be causally related to their atherogenicity. In this regard, the existence of smectic core cholesteryl esters may limit the ability of lysosomal enzymes to degrade cholesteryl esters following internalization by arterial cells (Peters & DeDuve, 1974). In addition, the smectic state may also limit the ability of plasma transfer proteins to transfer core lipids between lipoproteins and thus interfere with their normal metabolic processing.

Acknowledgments

We thank Joan Kiliyanski for her secretarial proficiency in preparing this manuscript.

References

Anitschkow, N. (1933) *Atherosclerosis*, Macmillan, New York.

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

Atkinson, D., Tall, A. R., Small, D. M., & Mahley, R. W. (1978) *Biochemistry 17*, 3930–3933.

Bligh, E. G., & Dyer, W. J. (1959) *Can. J. Biochem. Physiol. 37*, 911–917.

Camejo, G., Bosch, V., Arreaza, C., & de Mendez, H. (1973) *J. Lipid Res. 14*, 61–68.

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

Deckelbaum, R. J., Tall, A. R., & Small, D. M. (1977b) *J. Lipid Res. 18*, 164–168.

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

Kirchhausen, T., Untracht, S. H., Fless, G. M., & Scanu, A. M. (1979) *Atherosclerosis (Shannon, Irel.) 33*, 59–70.

Kroon, P. A. (1981) *J. Biol. Chem. 256*, 5332–5339.

Kroon, P. A., & Krieger, M. (1981) *J. Biol. Chem. 256*, 5340–5344.

Kroon, P. A., Quinn, D. M., & Cordes, E. H. (1982) *Biochemistry 21*, 2745–2753.

Laggner, P., Degovics, G., Muller, K. W., Glatter, O., Kratky, O., Kostner, G., & Holasek, A. (1977) *Hoppe-Seyler's Z. Physiol. Chem. 358*, 771–778.

Morrisett, J. D., Stockton, R. K., & Knapp, R. D. (1980) *Atherosclerosis (Berlin) 5*, 189–196.

Peters, J. J., & DeDuve, C. (1974) *Exp. Mol. Pathol. 20*, 228–256.

Pinon, J.-C., & Bridonx, A. M. (1977) *Artery (Fulton, Mich.) 3*, 59–71.

Ploplis, V. A., Thomas, J. K., & Castellino, F. J. (1979) *Chem. Phys. Lipids 23*, 49–62.

Pownall, H. J., Jackson, R. L., Roth, R. I., Gotto, A. M., Patsch, J. R., & Kummerow, F. A. (1980) *J. Lipid Res. 21*, 1108–1115.

Rodriguez, J. L., Ghiselli, G. C., Torreggiani, D., & Sirtori, C. R. (1976) *Atherosclerosis (Shannon, Irel.) 23*, 73–83.

Shore, V. G., Shore, B., & Hart, R. G. (1974) *Biochemistry 13*, 1579–1584.

Shumaker, V. N. (1956) *Am. J. Physiol. 184*, 35–42.

Small, D. M. (1970) *Surface Chemistry of Biological Systems*, pp 55–83, Plenum Press, New York.

Tall, A. R. (1980) *Ann. N.Y. Acad. Sci. 348*, 335–351.

Tall, A. R., Atkinson, D., Small, D. M., & Mahley, R. W. (1977a) *J. Biol. Chem. 252*, 7288–7293.

Tall, A. R., Deckelbaum, R. J., Small, D. M., & Shipley, G. G. (1977b) *Biochim. Biophys. Acta 487*, 145–153.

Tall, A. R., Small, D. M., Atkinson, D., & Rudel, L. L. (1978) *J. Clin. Invest. 62*, 1354–1363.