Molecular Motion and Conformation of Cholesteryl Esters in Reconstituted High Density Lipoprotein by Deuterium Magnetic Resonance*

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Reconstituted high density lipoprotein has been prepared by sonication and preparative ultracentrifugation of mixtures containing the apoprotein of high density lipoprotein, egg phosphatidylcholine, cholesteryl oleate, and acyl chain deuterated cholesteryl palmitate in aqueous buffer. The resulting structures have a size and chemical composition very similar to native high density lipoprotein. Deuterium NMR spectra and longitudinal relaxation times were obtained at approximately 25 °C. The variation of the 2H NMR line width with chain position is consistent with an average conformation such that the ester acyl chain is extended. In addition, 2H NMR line widths and longitudinal relaxation times indicate that the ester acyl chains possess significant mobility.

High density lipoproteins are of considerable current interest as there is a negative correlation between plasma HDL concentration and the risk of heart disease (Miller and Miller, 1975). The structure and physical properties of HDL have been studied by a wide variety of techniques including electron microscopy (Forte et al., 1968), x-ray diffraction (Lagnnr and Muller, 1978), ESR (Brainard et al., 1980), and NMR (Finer et al., 1975; Henderson et al., 1975; Hamilton et al., 1974; Wassall et al., 1982). HDL is proposed to be a quasi-spherical particle of 6.5-10 nm diameter (Forte et al., 1968; Scanu, 1979). The apoprotein is thought to be embedded in an outer, micellar structure formed by phospholipids and cholesterol, while the hydrophobic core of the micelle is filled with the neutral lipids, cholesteryl esters, triglycerides, and cholesterol.

The lipids have been shown to possess considerable mobility by 13C and 2H NMR (Hamilton and Cordes, 1978; Finer et al., 1975), but the details of the motion are inaccessible to those methods, due to the overlap of acyl chain resonance of cholesteryl esters, phospholipids, and triglycerides. This problem has been overcome to a certain extent by the use of 13C enriched lipids (Stoffel et al., 1974; Assmann et al., 1974), but little additional information was presented. Molecular order, as determined from ESR (Brainard et al., 1980), suffers from the serious drawback of using highly sterically perturbing probes (Taylor and Smith, 1980).

Deuterium NMR is an excellent technique for studying molecular order and dynamic structure as it monitors a non-perturbing probe. Selective deuteration, combined with the low natural abundance of deuterium, gives unambiguous assignments and, since the quadrupolar interaction is so strong, dipolar interactions with neighboring nuclei may usually be ignored. Recently, it has been shown that molecular order in lipoproteins may be monitored by 2H NMR (Wassall et al., 1982). In the present study, we report 2H NMR spectra and spin-lattice relaxation times for cholesteryl palmitate, selectively deuterated along the acyl chain, incorporated into reconstituted HDL particles. Line width analysis shows that the ester acyl chains undergo considerable anisotropic molecular motion. In addition, proposed models of cholesteryl ester conformation in HDL are discussed in light of the present results.

EXPERIMENTAL PROCEDURES

Lechitin was extracted from hen egg yolk (Singleton et al., 1965) and purified on a silica gel column (Richter et al., 1977).

Deuterium-depleted water and cholesteryl oleate were purchased from Sigma, while palmitic acid and cholesterol were obtained from Fisher. Cholesterol was recrystallized from benzene before being used. [5,6,6-2H3]Palmitic and [1,11,12-2H3]palmitic acid were purchased from Merck Sharp and Dohme, Canada Ltd.; [16,16,16-2H3]palmitic acid was obtained from Serdary Research Laboratories, London, Ontario; and [4,4-2H4]palmitic acid was a generous gift from Dr. A. P. Tulloch, Prairie Regional Laboratory, Saskatchewan, Saskatchewan.

Dry human blood (≤3 days old) was obtained from the Canadian Red Cross, Vancouver Branch. HDL was isolated in the density range of 1.125-1.210 g/ml (Havel et al., 1955), and its apoprotein and total lipids were isolated according to the methods of Scanu et al. (1969).

Preparation of Reconstituted High Density Lipoprotein—Egg phosphatidylcholine, cholesteryl oleate, and deuterated cholesteryl palmitate, in the ratio of 3:1:1 (w/w), were dissolved in CHCl3. The solvent was removed by evaporation under a stream of nitrogen and subsequent overnight pumping under high vacuum. The dried lipid was incubated at 48 °C for 10 min in NaHCO3/Na2CO3 buffer (pH 8.6) (Scanu, 1966), prepared in deuterium-depleted water. The density was adjusted to 1.063 with NaCl/KBr (Havel et al., 1955) in deuterium-depleted water. The lipid dispersion was shaken on a Vortex mixer until it appeared homogeneous.

Dry apoprotein was added to the lipid dispersion (50:50; protein:total lipid) and allowed to incubate at 48 °C for approximately 10 min or until all of the solid protein particles appeared to have dissolved. The resultant cloudy mixture was sonicated at approximately 40-48 °C for five 1-min periods, using a Biosonic III probe-type sonicator. After sonication, the recombined particles were isolated in the density range of 1.063-1.210 g/ml (Hirz and Scanu, 1970) and then concentrated to approximately 8 mg of protein/ml, prior to the NMR experiments, using Millipore immersible CX-30 membrane units.
Viscosity measurements of reconstituted HDL were performed using an Ostwald viscometer. The amount of protein present in reconstituted HDL was quantified according to the method of Lowry et al. (1951), using egg albumin as a standard. Phospholipid was quantified by phosphorus determination (Ames, 1966), while cholesterol esters were determined as described by Rudel and Morris (1973) or by NMR (see below).

Electron Microscopy—Lipoprotein particles were negatively stained with 2% ammonium molybdate, pH 8.0, placed on 200-mesh Formvar carbon-coated grids, and allowed to air dry. The specimens were examined in a Philips 300 electron microscope operating at 80 kV.

Nuclear Magnetic Resonance—Deuterium NMR experiments were carried out at 38.8 MHz, using a Nalorac 5.9 Tesla superconducting magnet and home-built spectrometer. Collection and Fourier transformation of the free induction decays were performed on a Nicolet BNC-12 computer. Longitudinal relaxation times were obtained by the inversion-recovery method (Vold et al., 1968). The sample temperature was approximately 25 °C.

$^3$P NMR spectra were acquired at 40.5 MHz on a Varian XL-100-15 spectrometer, operating in the Fourier transform mode and interfaced to a NIC 1080 computer. Magnet field stabilization was by means of an external "F field frequency lock. Free induction decays were recorded in the presence of $^1$H noise-decoupling having a 1 kHz bandwidth. In order to minimize sample heating due to the radiofrequency field (10 watts) used for decoupling either the decoupler was gated on only during acquisition of the free induction decay or, when decoupling was applied continuously, sample cooling by a gas flow system was employed. The temperature of the experiments was approximately 25 °C. Chemical shifts were measured with respect to an external $^3$P$_{o}$PO$_{4}$ (85%) sample.

A phase-alternating pulse sequence was used in all experiments in order to minimize base-line aberrations.

Quantification of Deuterated Cholesteryl Palmitate in Reconstituted HDL—The amount of deuterated cholesteryl palmitate present in reconstituted HDL was determined by recording a $^3$P NMR spectrum for reconstituted HDL containing cholesteryl$^{[16,16,16,16-^2H]}_{6}$ palmitate in which a capillary tube containing 30 μl of CHCl$_3/$CHCl$_3$ (9/1, v/v) was coaxially inserted into the sample tube. Sufficient time to ensure complete relaxation (≥5 T$_1$) was left between successive radiofrequency pulses during data acquisition. Comparison of the integrated intensity of the CHCl and cholesteryl ester signals, separated by 280 Hz, enabled quantitation of the ester.

RESULTS

We have prepared model HDL particles by sonication and subsequent ultracentrifugation of mixtures containing HDL$_a$ apoprotein, cholesteryl oleate, deuterated cholesteryl palmitate, and egg phosphatidylcholine in aqueous buffer, similar to that described by Hirz and Scanu (1970). Electron microscopy of these structures, using the negative-staining technique (Fig. 1), shows that they have an overall spherical structure and are quite homogeneous in size. The diameter of these structures and their chemical composition is shown in Table I.

$^3$P NMR spectra of native HDL$_a$ and of reconstituted HDL, prepared with egg phosphatidylcholine/cholesteryl oleate/cholesteryl palmitate or total HDL$_a$ lipids, respectively, are shown in Fig. 2, a–c. The $^3$P NMR spectrum of native HDL (Fig. 2a) is essentially identical to those published previously (Glonek et al., 1974; Henderson et al., 1975). The more intense upfield resonance at $-0.6$ (+0.1) ppm, which has a width at half-height $\Delta T_{1/2} = 6$ Hz, is due to phosphatidylcholine, while the less intense downfield resonance at $+0.1$ (+0.1) ppm is due to sphingomyelin. The $^3$P NMR spectrum of reconstituted HDL, containing egg phosphatidylcholine/cholesteryl oleate/cholesteryl palmitate, only consists of a single resonance at $-0.6$ (+0.1) ppm which is due to phosphatidylcholine and which has $\Delta T_{1/2} = 5$ (+1) Hz (Fig. 2b). To confirm the suitability of the reconstitution method, we have also prepared reconstituted HDL using total HDL$_a$ lipids which yields a virtually indistinguishable spectrum (Fig. 2c) to that of native HDL$_a$.

Fig. 3, a–e, depicts $^2$H NMR spectra of reconstituted HDL$_a$ containing cholesteryl palmitate selectively deuterated along the acyl chain. A single Lorentzian line gives a satisfactory fit to each of these spectra which, in addition to the absorption due to deuterated cholesteryl palmitate, sometimes contains the narrow, downfield-shifted line from residual deuterium in water. Control experiments revealed that the line intensity and line width of the $^2$H NMR spectra of deuterated cholesteryl palmitate in reconstituted HDL are unchanged over the time span of the NMR experiments (up to approximately 48 h), indicating that no significant change in structure of the sample occurs over the length of the experiments.

Table II lists $^2$H NMR line widths of selectively deuterated cholesteryl palmitate in reconstituted HDL. The value of $\Delta T_{1/2}$ is approximately constant for deuterons on C2–C6 of the acyl chain, then decreases at C11 and C12 before reaching a minimum at C16.

Longitudinal relaxation times, $T_1$, measured for selectively deuterated cholesteryl palmitate in reconstituted HDL, are also presented in Table II. Due to the low amplitude of the broad methylene signals for the ester, necessitating long periods of data collection, the $T_1$ values are subject to a relatively
large uncertainty of ±30%. On the other hand, for the narrow cholesteryl[16,16,16-2H3]palmitate resonance, the uncertainty of the T1 value is only ±10.

The viscosity of all the reconstituted HDL samples containing selectively deuterated cholesteryl palmitate was measured at 1.13 cP, except that containing cholesteryl [2,2-2H2]palmitate which, having an approximately 1.5 × higher lipoprotein concentration, had a viscosity of 1.2 cP.

**DISCUSSION**

The results from Fig. 1 and Table I show that the reconstituted HDL particles, prepared in the present study, have physical properties very similar to those of native HDL. The diameter of reconstituted HDL, as shown by electron microscopy (7.8 ± 1.2 nm), is within the range of 6.5-10 nm reported for native HDL (Scanu, 1979). In addition, 31P NMR linewidths for cholesteryl palmitate in reconstituted HDL can be obtained by calculating theoretical 'H NMR linewidths for an ester acyl chain undergoing given motions. The simplest case is that of a static all-trans chain embedded in a lipoprotein particle, such that particle tumbling is the only motion responsible for line-narrowing. The 'H NMR linewidth \( \Delta \nu_{1/2} \) is then given by (Abragam, 1961, p. 424):

\[
\nu_{1/2} = M_2 \tau \nu_c
\]

where \( M_2 \) is the rigid lattice second moment and \( \nu_c \) is the effective correlation time for particle tumbling. The second moment \( M_2 \) is related to the static quadrupolar splitting \( \Delta \nu_q \) of palmitate. In this case, the delay between subsequent pulse sequences was 0.1 s. Line broadening = 20 Hz (a and c), 30 Hz (b), 10 Hz (d), 1.5 Hz (e).
Assuming a spherical particle, $\tau_1$ in Equation 1 can be estimated by the Stokes-Einstein formula

$$\tau_1 = \frac{4\pi \eta r^2}{9kT}$$

(3)

where $\eta$ is the viscosity, $r$ is the particle radius, $k$ is the Boltzmann constant, and $T$ is the absolute temperature. For reconstituted HDL, with $\eta \approx 1.13$ cP, $r \approx 40$ Å and $T = 298$ Constraint, we obtain $\tau_1 \approx 7.5 \times 10^{-3}$ s; hence, from Equations 1 and 2, a line width $\Delta \nu_{1/2} \approx 3000$ Hz is estimated for a rigid $\text{C}^2\text{H}_2$ segment. For a deuterium attached to a terminal methyl group, it is also necessary to include the effect of fast rotation about the C–C bond joining it with the adjacent CH$_2$ group, which is necessary to account for the experimental line widths (Table II) for deuterated cholesteryl palmitate in reconstituted HDL.

Clearly, the experimental line widths, which encompass deuterons on C2–C6, are more than these predicted values. Therefore, we conclude that cholesteryl ester acyl chain has significant motion inside the lipoprotein particles.

The two most popular models for the conformation of cholesteryl esters in HDL particles (Fig. 4) are the “horseshoe” (Structure I) (Edelstein et al., 1979) and the extended form (Structure II) (Laggner and Muller, 1978). Treating the motions undergone by the ester acyl chain in the lipoprotein as symmetric about the labeled axis (Fig. 4), the quadrupolar splitting, $\Delta \nu_q$ in Equation 2 is modified by a factor $(3\cos^2\beta - 1)/2$, where $\beta$ is the angle between the symmetry axis for the molecular motion and the C–$^2$H bond, while the angular brackets represent a time average over all conformational states of the molecule (Oldfield et al., 1971). Hence, if cholesteryl palmitate adopted a horseshoe conformation in reconstituted HDL, a dramatic variation of the $^2$H NMR line width versus chain position in the highly curved region of the acyl chain would be expected as a consequence of the changes in the average value of $\beta$. In phospholipid liposomes containing deuterated cholesteryl ester, a local minimum of quadrupolar splitting is indeed observed at C4–C5 of the ester chain (Gorrissen et al., 1981). A variation of this form is clearly not the case for the line width of deuterated cholesteryl palmitate in reconstituted HDL. On the other hand, if the ester chain were extended, the variation of the line width versus chain position might be expected to resemble the profile of quadrupolar splittings for the sn-1 chain in phospholipid bilayers, which is known to adopt an extended conformation (Seelig and Seelig, 1977). This is, in fact, observed for deuterated cholesteryl palmitate in reconstituted HDL (Table II). In the present case, we observe a region of relatively constant line widths, which encompasses deuterons on C2–C6, while further down the chain the line widths decrease until reaching a minimum at C16.

Hence, our observations are consistent with an extended conformation (Structure II) for cholesteryl palmitate in HDL, such as proposed previously by Laggner and Muller (1978). These authors also proposed that the cholesteryl ester molecules were radially distributed within the HDL particles. Assuming such an ordered distribution of cholesteryl palmitate in the lipoprotein, the $^2$H NMR line width of Equation 1 can be expressed by (Stockton et al., 1976)

$$\Delta \nu_{1/2} \approx \frac{3\pi}{20} \left(\frac{e^2 q Q}{\hbar}\right)^{1/2} S^{1/2}(\tau)$$

(4)

where $S = (3\cos^2\beta - 1)/2$ is the C–$^2$H order parameter, which measures the angular excursions of the C–$^2$H bond under consideration with respect to the radial direction. The absolute values of the order parameter, $|S|$, calculated from the observed $^2$H NMR line widths, are shown in Fig. 5. For comparative purposes, we have also included the values for the sn-1 chain of 1-palmitoyl-2-oleoyl phosphatidylcholine (Seelig and Seelig, 1977). While the shape of both order parameter profiles is quite similar, the values of $|S|$ for cholesteryl palmitate in reconstituted HDL are higher than those of the equivalent positions of 1-palmitoyl-2-oleoyl phosphatidylcholine (Seelig and Seelig, 1977). The shape of both order parameter profiles is quite similar, the values of $|S|$ for cholesteryl palmitate in reconstituted HDL are higher than those of the equivalent positions of 1-palmitoyl-2-oleoyl phosphatidylcholine. This indicates that the angular excursions undergone by the ester chains in lipoprotein are more restricted than those of the acyl chains in phospholipid bilayers. The highest order parameter ($S = 0.38$) for cholesteryl palmitate is observed on C2 of the acyl chain, which probably reflects the motional restriction imposed by the rigid cholesteryl moiety. However, the fact that all of the order parameters are less than the value of 0.5 expected for a chain undergoing only rapid rotation about its long axis suggests that significant angular fluctuations do occur. These fluctuations become of larger amplitude approaching the terminal methyl group, as evidenced by the progressive decrease of $|S|$ along the chain.

The longitudinal relaxation times, $T_1$, for the acyl chain of deuterated cholesteryl palmitate in reconstituted HDL are relatively constant at $\approx 15$ ms for all the $\text{C}^2\text{H}_2$ segments.

2 A referee has suggested that the small size of the HDL particle might prevent the cholesteryl ester from packing as it does in the bulk phase and lead to the chain mobility.
studied, while $T_1$ for the terminal C'H$_3$ group is much larger at approximately 150 ms. This implies that the fast molecular motions responsible for longitudinal relaxation have approximately the same rate for the C'H$_3$ segments but are significantly slower than those of the C'H$_3$ group. Such a behavior has been encountered previously for deuterated cholesteryl palmitate in phospholipid vesicles (Gorrissen et al., 1980; Cushley et al., 1980) and for deuterated phospholipids (Brown et al., 1979).

A quantitative interpretation of the longitudinal relaxation times of cholesteryl palmitate in reconstituted HDL is, unfortunately, difficult due to the small size of these particles. For deuterated cholesteryl palmitate in phospholipid vesicles, particle tumbling ($\tau_L \approx 10^{-8}$ s) is too slow to appreciably influence $T_1$, and fast segmental motions ($\omega_0 \tau_s \ll 1$) appear to dominate longitudinal relaxation (Cushley et al., 1980). This may not be the case for the much smaller lipoprotein particles ($\tau_s \approx 7.5 \times 10^{-8}$ s). In fact, a preliminary experiment at 61.4 MHz performed in this laboratory has indicated that $T_1$ is somewhat larger at the higher frequency, suggesting that not all the motions responsible for longitudinal relaxation in this system are in the short correlation time limit ($\omega_0 \tau_s < 1$). A detailed study of $T_1$ as a function of temperature and frequency would be required to clarify this point.

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**Fig. 5.** Plot of order parameter *versus* acyl chain position.

Plot of the absolute value for the C−H order parameter (|S|) *versus* chain position for selectively deuterated cholesteryl palmitate in the reconstituted HDL at approximately 25 °C ( ), and for the sn-1 chain of 1-palmitoyl-2-oleoyl phosphatidylcholine at approximately 27 °C (Seelig and Seelig, 1977) (∇).
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