Packing of Cholesterol Molecules in Human Low-Density Lipoprotein†

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ABSTRACT: High-resolution, proton-decoupled ^{13}C nuclear magnetic resonance spectra (90.55 MHz) of human low-density lipoprotein (LDL) have been employed to investigate the physical state of unesterified cholesterol molecules in this particle. Approximately half of the cholesterol molecules in LDL were replaced with [4-^{13}C]cholesterol by exchange from Celite. About two-thirds of the cholesterol molecules contribute to a resonance at δ 41.8 from the C-4 atom. This signal is assigned to cholesterol molecules located at the surface of the LDL particle in a mixed monolayer with phospholipid molecules; the spin–lattice relaxation of the C-4 nucleus of such cholesterol molecules is enhanced by the presence of Mn^{2+} ions in the aqueous phase. The remaining one-third of the cholesterol molecules are apparently neither associated with phospholipid nor exposed to the aqueous phase; these cholesterol molecules are presumed to be located in the core of the particle. Cholesterol molecules in the two microenvironments are in slow exchange on the NMR time scale but in fast exchange on a biological time scale, so that the cholesterol molecules in LDL behave physiologically as one pool. There is a loss of about 20% of the intensity of the $N(CH_3)_3$ resonance from phosphatidylcholine and sphingomyelin molecules in the LDL spectrum; this is attributed to the presence of apolipoprotein B in the surface of LDL particles, which may immobilize some of the phospholipid polar groups. Spin–lattice relaxation time measurements suggest that the fast axial motions of cholesterol molecules in the surface of LDL are the same as in high-density lipoprotein (HDL). However, comparison of the line widths of the [4-^{13}C]cholesterol resonance from molecules in the surfaces of LDL and HDL suggests that the cholesterol oscillatory motions are slower and/or more restricted in the surface of LDL. There are probably three contributions to this effect: (1) the greater acyl chain saturation of the phosphatidylcholine in LDL compared to that in HDL, (2) the higher sphingomyelin/phosphatidylcholine ratio in the former lipoprotein, and (3) the higher unesterified cholesterol/phospholipid molar ratio in the surface of LDL compared to that in HDL. The relatively slow rate of exchange of cholesterol from LDL compared to that from HDL is attributed to the tighter packing of the phospholipid/cholesterol monolayer in the surface of LDL.

LDL[1] is one of the main carriers of cholesterol in human blood and as such is important in the regulation of cholesterol metabolism in tissues. This effect arises because cells can endocytose LDL by a receptor that recognizes apo B (Goldstein & Brown, 1977) so that both unesterified and esterified cholesterol molecules in the LDL particle become part of the cell cholesterol pool. In view of this important function and its implications for the development of atherosclerosis (Steinberg, 1981), there has been a great deal of interest in the structure of LDL.

A variety of physical techniques have been utilized to probe LDL, and there is now agreement on the general structure. Human LDL is a quasi-spherical particle some 200–300 Å in diameter in which a core of cholesteryl ester (CE) molecules is encapsulated by a phospholipid monolayer in which apo B molecules are located [for reviews, see Laggner (1976), Keim (1979), and Kirchhausen et al. (1980)]. The CE molecules of LDL particles can pack in either a smectic-like liquid-crystalline or liquid state, depending upon the proportion of CE/TG molecules present in the core (Deckelbaum et al., 1977; Atkinson et al., 1977). The physical state of the unesterified cholesterol molecules in LDL is less well-defined. Indirect evidence implies that the cholesterol molecules are primarily located in the surface of the LDL particle with perhaps a relatively small fraction situated in the core (Deckelbaum et al., 1977; Keim, 1979).

In order to fully understand the participation of LDL in cholesterol transport, it is necessary to develop a complete understanding of the molecular dynamics of cholesterol molecules in LDL. These molecules can exchange or transfer with cholesterol in HDL or red blood cells (Bell, 1984) so that, in this sense, they are part of the cholesterol in any HDL-mediated reverse cholesterol transport path from peripheral tissue to the liver (Norum et al., 1983). In an earlier study from this laboratory, it was established that unesterified cholesterol transfer from LDL by diffusion through the aqueous phase is slower than transfer from HDL (Lund-Katz et al., 1982). The interfacial flux of cholesterol molecules from LDL is less than half that from HDL_3. Since the rate-limiting step in cholesterol exchange is desorption from the lipid–water interface, the variation in cholesterol flux must arise from differences in packing and/or accessibility to the aqueous phase of cholesterol molecules in the surfaces of human LDL and HDL particles. We have previously used a ^{13}C NMR ap-

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[1] Abbreviations: LDL, low-density lipoprotein(s); VLDL, very low density lipoprotein(s); HDL, high-density lipoprotein(s); apo B, apolipoprotein B; NMR, nuclear magnetic resonance; LCAT, lecithin: cholesterol acyltransferase; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PL, phospholipid; PC, phosphatidylcholine; CD, circular dichroism; chol, cholesterol; CE, cholesteryl ester; TG, triglyceride; TLC, thin-layer chromatography; $t_{1/2}$, half-time; δ, chemical shift; T_1, spin–lattice relaxation time; T_2, spin–spin relaxation time; $\upsilon_{1/2}$, line width; DSC, differential scanning calorimetry.

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proach to evaluate the physical state of cholesterol in HDL and phosphatidylcholine (PC) small unilamellar vesicles, so that the location and motion of these molecules in the surface of HDL and phospholipid bilayers are known. This information is not available for LDL so a similar strategy has been employed to investigate the physical state of cholesterol molecules in LDL. Here we present the results of a ^{13}C NMR study of the behavior of [4-^{13}C]cholesterol molecules incorporated into human LDL particles. The NMR data demonstrate that the packing of cholesterol molecules is different in the surfaces of LDL and HDL particles and provide a basis for understanding why the interfacial flux of cholesterol molecules out of LDL is slower than that from HDL.

Experimental Procedures

Materials

Crystalline [4-^{13}C]cholesterol with 90 atom % ^{13}C enrichment was obtained from Merck Company (Montreal, Canada), and [7-$^{3}H(N)$]cholesterol (specific activity, 34.6 Ci/mmol) was purchased from New England (Boston, MA). The sterols were more than 99% pure as judged by thin-layer chromatography. Deuterium oxide was purchased from Stohler Isotopes (Waltham, MA) or Merck (Rahway, NJ) and was routinely deoxygenated and stored under nitrogen. Manganous chloride (ACS grade) obtained from Fischer Scientific Co. (Fairlawn, NJ) was dried for 18 h at 110 °C before use. Na^{125}I (carrier free) in 0.1 N sodium hydroxide was purchased from New England Nuclear (Boston, MA). Other reagents were analytical grade. All organic solvents were redistilled prior to use.

Human LDL (1.006 g/mL < d < 1.063 g/mL) was isolated from either outdated plasma from the blood bank or fresh plasma of normal donors by sequential ultracentrifugation in KBr (Havel et al., 1955). The fresh plasma was obtained by collecting blood in vacuum packs containing 2 mM EDTA. Red and white cells were immediately sedimented by centrifugation, and 50 mM *N*-ethylmaleimide was added to the plasma to inhibit the action of lecithin–cholesterol acyltransferase (LCAT). The lipoprotein fractions were dialyzed extensively against saline (0.15 M NaCl, 0.001 M EDTA, and 0.02% NaN_3, pH 7.6) prior to use and stored at 4 °C under a nitrogen atmosphere. The purities of the lipoprotein fractions were assessed by agarose gel electrophoresis (Noble, 1968) using precast agarose slides (Bio-Rad Laboratories, Rockville Center, NY); the LDL gave a single band on staining with Sudan Black. ^{125}I-Labeled LDL with a specific activity of 33 dpm/ng of apoprotein was prepared according to Billheimer and co-workers' modification (Billheimer et al., 1972) of the iodine monochloride method of McFarlane (1958).

Methods

Cholesterol Labeling of LDL Using Celite. The Celite (grade 545) used for dispersing cholesterol was obtained from Harleco (Philadelphia, PA) and pretreated as described previously (Lund-Katz & Phillips, 1984). Pooled, outdated human LDL was heated at 55 °C for 30 min to inactive LCAT and then labeled with [7-$^{3}H(N)$]cholesterol and [4-^{13}C]-cholesterol by using the Celite procedure described by Avigan (1959), Lundberg et al. (1982), and Lund-Katz et al. (1982). In a typical experiment, 0.5 g of Celite and 15 mg of [4-^{13}C]cholesterol, doped with [^{3}H]cholesterol to give a final specific activity of 24 μCi/mmol, were dispersed in chloroform in an Erlenmeyer flask; the radiotracer was added to monitor the uptake of [^{13}C]cholesterol by LDL. About 200 mg of LDL in 25 mL of saline–EDTA was added to the Celite–cholesterol mixture and incubated as described elsewhere (Lund-Katz & Phillips, 1984). Approximately 150 mg of LDL in about 18 mL of saline solution was recovered from the Celite; the total unesterified cholesterol content was 12 mg of which about half was [4-^{13}C]cholesterol.

After the Celite incubation, the lipids from an aliquot of LDL were extracted by the method of Bligh and Dyer (1959). The purities of PC, cholesterol, and CE were assessed by thin-layer chromatography on silica gel G in three solvent systems: (1) petroleum ether–diethyl ether–acetic acid (75:24:1 v/v/v) to check for oxidation products of cholesterol, (2) chloroform–methanol–water (65:25:4 v/v/v) to monitor oxidized products of polar lipids, and (3) benzene–ethyl acetate (60:40 v/v) to examine the neutral lipid distribution. Lipids were visualized by spraying developed thin-layer plates with a 50% sulfuric acid solution and charring the sprayed plates at 200 °C for 15 min. The LDL was examined by NaDodSO_4–PAGE before and after incubation with Celite.

Prior to NMR measurements, the [4-^{13}C]cholesterol-enriched LDL sample was concentrated to about 100 mg of LDL/mL by dialysis against Water-Lock A-220 (Grain Processing Corp., Muscatine, IA). The sample was finally dialyzed against a solution containing 0.15 M NaCl, 0.001 M EDTA, and 0.02% NaN_3, pH 7.6. 20 μL of 1,4-dioxane was added, and then D_2O as the NMR lock compound was added to increase the volume by 20% to 1.5 mL. The lipoprotein was transferred to 10-mm NMR tubes under nitrogen and used within 24 h.

Nuclear Magnetic Resonance Measurements. The apparatus and methods used to obtain ^{13}C NMR spectra and to measure T_1 values have been described elsewhere (Lund-Katz & Phillips, 1984). All proton-decoupled ^{13}C NMR spectra were recorded at 90.55 MHz by employing a Bruker WH 360 spectrometer and broad-band, phase-modulated proton decoupling. Mn^{2+} in the form of chloride was added to the aqueous phase of the LDL samples in order to study the relaxation enhancement of PC and cholesterol resonances. The mole ratio of $MnCl_2$ to that of the LDL phospholipid was 0.06.

^{125}I-Labeled LDL Interaction with Fibroblasts. Degradation of control and [^{13}C]cholesterol-enriched ^{125}I-labeled LDL was determined by using a human fibroblast GM3468 cell line (Human Genetic Mutant Cell Repository, Camden, NJ) and established procedures (Goldstein et al., 1983; Cassel et al., 1984).

Differential Scanning Calorimetry. DSC was performed by using a Perkin-Elmer DSC-2 as previously described (Collins & Phillips, 1982). Appropriate amounts of the LDL sample were loaded into 75-μL stainless steel sample pans and scanned at 5 °C/min with pans containing water as the reference.

Analytical Procedures. The concentrations of free and esterified cholesterol in LDL were determined by gas–liquid chromatography using coprostanol as an internal standard (Bates & Rothblat, 1974). Protein determination was carried out following the NaDodSO_4–Lowry method of Markwell et al. (1978), and phospholipid was monitored by phosphorus analysis (Sokoloff & Rothblat, 1974). Negative stain electron microscopy (Collins & Phillips, 1982) was used to assess the particle size of the LDL preparations.

Results

Characterization of LDL. In a previous ^{13}C NMR study of cholesterol molecules in HDL, [4-^{13}C]cholesterol was incorporated by reconstitution of the entire lipoprotein particle (Lund-Katz & Phillips, 1984). Since the insolubility of apolipoprotein B precludes reconstitution of the LDL particle with all 450 unesterified cholesterol molecules replaced by

Table I: Comparison of the Dynamics of Cholesterol Molecules in Lipoproteins and Phospholipid Vesicles at 37 °C

particle type	radius[a] (nm)	chol content[a] (mol of chol/particle)	T_1 of surface [4-^{13}C]chol resonance[b] (ms)	$\upsilon_{1/2}$ of surface [4-^{13}C]chol resonance[b] (Hz)	$t_{1/2}$ for chol desorption[c] (min)
10 mol % chol/egg PC vesicles	11	255	160	73	87
HDL_3	3.9	13	140	56	2.9
LDL	9.6	475	130	126	45

[a] Data for vesicles taken from Newman and Huang (1975) and for HDL_3 and LDL from Shen et al. (1977). [b] Data for vesicles and HDL_3 taken from Lund-Katz and Phillips (1984). $\upsilon_{1/2}$ values include 2-Hz exponential line broadening applied during processing. [c] Data for vesicles taken from McLean and Phillips (1982) and for HDL_3 and LDL from Lund-Katz et al. (1982).

[4-^{13}C]cholesterol, a method to transfer cholesterol into LDL was employed. Incubation of fresh LDL with cholesterol deposited on Celite leads to formation of cholesterol-rich LDL (Lundberg et al., 1982), which was deemed undesirable in this study given the focus on cholesterol packing in LDL; in addition, the amount of [^{13}C]cholesterol that can be incorporated would prohibit the NMR study given the low sensitivity of the technique. In order to achieve significant loading with [^{13}C]cholesterol without increasing the total unesterified cholesterol content of LDL, the strategy of first depleting LDL of cholesterol by the action of LCAT and then repleting from Celite was employed. Outdated plasma was used in which LCAT action had decreased the LDL cholesterol/PL mole ratio to about 0.6 compared to about 1 for LDL isolated from fresh, LCAT-inhibited plasma. The substrate in plasma for LCAT is HDL where the enzyme activity converts cholesterol to cholesteryl ester. The decrease in cholesterol content of HDL creates a concentration gradient so that cholesterol then redistributes from LDL to HDL. Transfer of [4-^{13}C]cholesterol from Celite repleted the LDL so that the cholesterol/PL mole ratio was restored to 1. This corresponds to the addition of ~210 [4-^{13}C]cholesterol molecules to ~260 [^{12}C]cholesterol molecules originally present in the LDL particle. The final particle content of ~470 cholesterol molecules is similar to the literature value (Table I). The composition of the LDL samples was otherwise unaltered by the Celite incubation although only ~75% of the LDL was recovered [cf. Lundberg et al. (1982)].

The samples used in the NMR studies had the following compositions [%(w/w) ± 5%]: protein, 21 [analyses were normalized to this value—cf. Shen et al. (1977)]; phospholipid, 20; cholesteryl ester, 32; cholesterol, 9; with the remainder being triglyceride. The CE content of 32% is somewhat lower than the value of 37% reported for native LDL (Shen et al., 1977). This presumably arises because, concomitant with the action of LCAT discussed above, there is an exchange by plasma transfer protein of CE in LDL with TG in VLDL during storage of the outdated plasma. Consistent with the higher TG/CE ratio in our samples, examination by DSC of the thermal transition of the CE in the core of the LDL particles showed that the fresh LDL had a transition in the range 25–45 °C with a peak temperature of 30–37 °C [cf. Deckelbaum et al. (1977)] whereas in various outdated LDL samples the transition temperatures were decreased by at least 20 °C. This modification of the thermal properties presumably arises because incorporation of TG decreases the temperature of the liquid crystal to liquid transition of CE [cf. Deckelbaum et al. (1977)]. The addition of cholesterol by the Celite procedure to LDL from either fresh or outdated plasma did not lead to any alteration in the melting properties of the LDL core.

Electron microscopic examination of the LDL samples before and after exposure to Celite showed that there was no significant alteration in particle size and that the radius was consistent with the value cited in Table I. A small increase in electrophoretic mobility of LDL on agarose gels was observed after the Celite incubation [cf. Lundberg et al. (1982)]. Inspection of the LDL lipids by TLC after cholesterol loading by Celite incubation showed that no detectable decomposition had occurred. Similarly, NaDodSO$_4$–PAGE indicated that the apo B was not significantly degraded. In addition, the rates of degradation of LDL before and after cholesterol loading from Celite by human fibroblasts growin in culture were identical. Thus, the GM3468 cells degraded 800 ± 100 ng of ^{125}I-labeled apo LDL/mg of cell protein in 6 h when either control or cholesterol-loaded LDL was present in the extracellular medium at a concentration of 10 μg of LDL protein/mL. The 2- and 4-h time points also showed that both types of LDL were degraded equally. This evidence indicating that the apo B in our LDL samples is not denatured is consistent with the observation that the CD spectrum of LDL is unaltered by the Celite treatment (Lundberg et al., 1982).

In summary, the above evaluation of the LDL samples used for the ^{13}C NMR study indicates that its surface structure is similar to circulating LDL in that the cholesterol/PL mole ratio is normal and that the apo B is in its biologically active conformation. The only obvious difference from native LDL is that the core CE and TG are more fluid as indicated by the altered melting properties.

^{13}C NMR Spectra of LDL. The above inferences about the structural similarities between control LDL and LDL containing [4-^{13}C]cholesterol added from Celite are supported by the ^{13}C NMR spectrum shown in Figure 1. This spectrum for [4-^{13}C]cholesterol-enriched LDL is similar to those for native LDL in the literature [cf. Hamilton et al. (1974, 1978), and Sears et al. (1976)]. The chemical shifts (δ) and line widths ($\upsilon_{1/2}$) of corresponding resonances are similar in both native and [^{13}C]cholesterol-enriched lipoprotein, with the only difference being the expected appearance of the signals from the [4-^{13}C]cholesterol. The acyl-terminal CH_3 resonance (δ 13.9) has a line width of 13 ± 2 Hz (this includes the 2-Hz exponential line broadening applied during processing), which is equal to that in HDL_3 and 10 mol % cholesterol/egg PC vesicles (Lund-Katz & Phillips, 1984). However, the value of 13 ± 2 Hz observed for the $N(CH_3)_3$ resonance in LDL is somewhat smaller than that of 20 ± 2 Hz for HDL_3 or vesicles. There are two prominent resonances at δ 41.8 and 42.2 in LDL enriched with [4-^{13}C]cholesterol (see inset in Figure 1), which coincide with the δ values observed for [4-^{13}C]cholesterol in HDL_2 and HDL_3 (Lung-Katz & Phillips, 1984). The downfield resonance is narrower than the peak at δ 41.8, which has $\upsilon_{1/2}$ = 126 ± 7 Hz. The data in Table I show that the $\upsilon_{1/2}$ of this resonance from [4-^{13}C]cholesterol associated with PL in LDL is significantly broader than the equivalent signal in spectra of HDL_3 or 10 mol % cholesterol/egg PC vesicles.

The nuclear Overhauser effect is suppressed in the spectrum of LDL (Figure 1) so that the signal intensities are a true

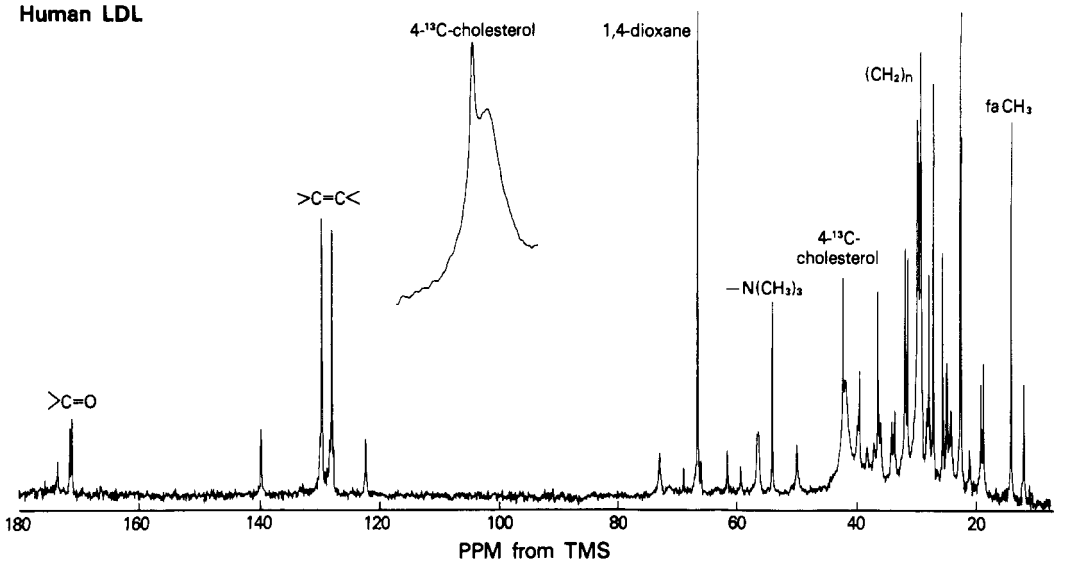

FIGURE 1: Proton-decoupled ^{13}C NMR spectrum (90.55 MHz) at 37 °C of human LDL containing [4-^{13}C]cholesterol, with 3100 accumulations. Gated decoupling and a recycling time of 2.9 s were employed so that the nuclear Overhauser effect was suppressed for the cholesterol resonances. The spectrum was processed with 2.0-Hz exponential filtering. The expanded region has the horizontal axis expanded by a factor of about 20.

reflection of the number of ^{13}C atoms contributing to each resonance (Wehrli & Wirthlin, 1978). Deconvolution of the two [4-^{13}C]cholesterol resonances (Figure 1) by procedures described elsewhere (Lund-Katz & Phillips, 1984) shows that 65 ± 10% of the signal is associated with the broad line at δ 41.8. However, the sum of the intensities of the δ 41.8 and 42.2 resonances is about 15% greater than the value expected on the basis of the mass of [4-^{13}C]cholesterol (i.e., the sum of 90% ^{13}C-enriched and natural abundance cholesterol molecules) present in the LDL. As pointed out to us by Dr. James Hamilton, the reason for this is that the C-13 atom of CE that is present at natural abundance resonates close to δ 42.2 (Hamilton et al., 1974). Under identical spectral conditions, comparison of the intensities [normalized with respect to the intensities of the $N(CH_3)_3$ resonance] of the δ 42.2 resonance in spectra of native LDL and LDL containing [4-^{13}C]cholesterol indicates that ~25% of the intensity of the composite peak at δ 42.2 in the spectrum of the [4-^{13}C]-cholesterol-containing LDL arises from the C-13 CE nucleus. This contribution underlies the greater than expected total intensity of the δ 41.8 and 42.2 resonances; these data suggest that all the [4-^{13}C]cholesterol molecules in LDL are sufficiently mobile to contribute to the high-resolution spectrum. The composite δ 42.2 resonance originating from the 4-^{13}C nucleus of cholesterol and the C-13 nucleus of CE in Figure 1 has a T_1 of 250 ms compared to the value of 1.9 s for the natural abundance C-13 CE resonance in LDL to which [4-^{13}C]cholesterol has not been added. The spin–spin relaxation of the natural abundance C-13 CE resonance in LDL is also different because $\nu_{1/2}$ = 12 ± 2 Hz as compared to a value of 26 ± 3 Hz for the δ 42.2 resonance in Figure 1. Since the physical state of the CE in LDL samples such as that of Figure 1 is more fluid than in native LDL, we cannot be sure of how our information on the distribution and behavior of any cholesterol molecules in the core of LDL relates to the in vivo situation. However, the spectral characteristics of the resonances in Figure 1 originating from lipids in the surface of LDL can be used to deduce information about the surface structure of LDL.

The fact that there is a resonance from [4-^{13}C]cholesterol in LDL at δ 41.8 is strong evidence that the cholesterol molecules in the surface of LDL are associated with PL. Integration of the $N(CH_3)_3$ peak in Figure 1 indicates that 82 ± 5 (±SD, n = 4) of the phospholipid polar groups contribute to the spectrum. This is different from the situation with HDL_2 and HDL_3, where all $N(CH_3)_3$ carbon atoms are sufficiently mobile to contribute to the high-resolution NMR spectrum (Lund-Katz & Phillips, 1984) but is consistent with earlier ^{31}P and ^1H NMR studies of human and porcine LDL where 20% and 33%, respectively, of the PL polar groups do not contribute to the spectra (Yeagle et al., 1977; Finer et al., 1975). The T_1 value for the $N(CH_3)_3$ resonance in Figure 1 is 540 ms, which is similar to the T_1 at lower field for LDL (Hamilton et al., 1974) and is the same as the value observed from $N(CH_3)_3$ groups in HDL_3 and 10 mol % cholesterol/egg PC vesicles (Lund-Katz & Phillips, 1984). The T_1 of the δ 41.8 resonance from [4-^{13}C]cholesterol molecules associated with PL is the same for LDL, HDL_3, and vesicles (Table I). Also, the apparent T_1 value of 540 ms for the envelope of $-(CH_2)_n-$ resonances in LDL is the same as in the other types of particles.

In order to probe the proximity of cholesterol molecules in LDL to the aqueous interface, Mn^{2+} was added to the aqueous phase to see how it enhanced the spin–lattice relaxation of the [4-^{13}C]cholesterol resonances and PC and sphingomyelin $N(CH_3)_3$ resonances [cf. Lund-Katz & Phillips (1984)]. The relaxation enhancements observed when Mn^{2+} was added to LDL at a Mn^{2+}/PL molar ratio of 0.06 were similar to those reported previously from this laboratory for HDL_3 and cholesterol/PL vesicles (equivalent levels of Mg^{2+} were used to monitor changes in relaxation time due to alterations in molecular motion induced by ion binding). The acyl chain $-(CH_2)_n-$ T_1 value for LDL is unaffected by the addition of Mn^{2+} while the surface [4-^{13}C]cholesterol and $N(CH_3)$ T_1 values were reduced from 130 to 80 ms and from 590 to 160 ms, respectively. Application of the analysis presented before (Lund-Katz & Phillips, 1984) to these T_1 data enables the distance ratio $r_{Mn\text{-}chol}/r_{Mn\text{-}N(CH_3)_3}$ to be computed. This distance ratio in LDL has a value of unity, which is the same as that observed for HDL_3, HDL_2, and cholesterol/egg PC vesicles. The exposure of the cholesterol molecules to the aqueous phase surrounding the particles enables them to desorb into the aqueous phase [cf. Lund-Katz et al. (1982)]. The half-times for this process in 10 mol % cholesterol/egg PC vesicles, HDL_3,

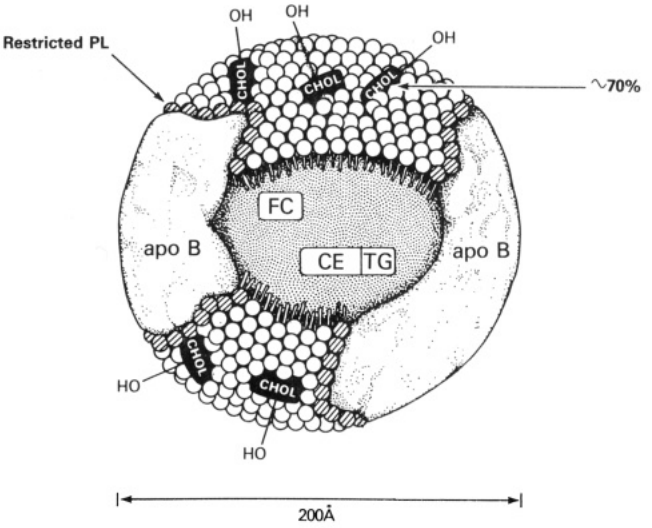

FIGURE 2: Oil-drop model of human plasma low-density lipoprotein. The emphasis is on the surface structure, which comprises apolipoprotein B and a mixed phospholipid/cholesterol monolayer exposed to the aqueous phase. About two-thirds of the total unesterified (free) cholesterol (FC) in the particle is in this location. About 20% of the phospholipid (PL) polar groups (shown with crosshatching) are immobilized by interaction with apo B; the restricted PL may be in a boundary layer or may be trapped by apo B. The physical state of the cholesteryl esters (CE) and triglycerides (TG) in the core of the particle have been discussed in detail elsewhere (Deckelbaum et al., 1975; Atkinson et al., 1977; Laggner et al., 1984).

and LDL are summarized in Table I.

Discussion

The NMR data described above have to be interpreted in terms of the current information on the structure of human LDL. A schematic representation of an LDL particle that summarizes some of this information is depicted in Figure 2. In common with the other serum lipoproteins, LDL can be viewed in terms of an "oil-drop" model where a core of cholesteryl ester and triglyceride molecules is encapsulated by a monolayer comprising phospholipid and apolipoprotein B. The molecular organization of the core below the thermal transition involves CE molecules that are thought to be extended and oriented radially (Deckelbaum et al., 1977; Atkinson et al., 1977; Laggner et al., 1984) and at 37 °C appear to be in a smectic liquid-crystal-like state by X-ray criteria. However, NMR studies show that they are less motionally restricted than in the neat smectic phase (Hamilton et al., 1977; Kroon, 1981). All phospholipids with mobile polar groups are at the surface of the LDL particle (Finer et al., 1975; Yeagle et al., 1978), and the apo B moiety is apparently located, on average, 5–10 Å further out from these polar groups toward the aqueous environment (Laggner et al., 1981). The tertiary structure of the apo B is unknown, as are the details of the protein–lipid interactions. However, as shown in Figure 2, it has been suggested that some 20% of the PL polar groups are immobilized by interaction with apo B (Finer et al., 1975; Yeagle et al., 1977), and this is supported by the present ^{13}C NMR data. This restriction in polar group phosphate or choline motion is attributed to a protein–lipid interaction rather than a cholesterol–lipid interaction because trypsin treatment of LDL removes the effect (Yeagle et al., 1977) and cholesterol located in PC bilayers does not immobilize the $N(CH_3)_3$ groups (Darke et al., 1972). Since all the PL polar groups in HDL_2 or HDL_3 particles are sufficiently mobile to contribute to the high-resolution ^{13}C NMR spectrum (Lund-Katz & Phillips, 1984), it can be inferred that the interaction of apo B with PL is different from that of either apo A-I or apo A-II with PL.

Table II: Comparison of Cholesterol Molecular Packing in the Surfaces of HDL_3 and LDL at 37 °C

property		HDL_3	LDL
lipoprotein composition	mol of surface chol/mol of PL	0.15	0.55
	unsat PC/sat PC[a]	1.3	0.6
	sphingomyelin/PC[a]	0.12	0.38
molecular motions of surface cholesterol molecules	[4-^{13}C]chol apparent spin–spin relaxation time (ms)[b]	5.8	2.5
	chol interfacial flux to aqueous phase [mol/(10 nm^2·h)][c]	10	4

[a] Data taken from Skipski (1972). [b] Computed from $\upsilon_{1/2}$ data in Table I. T_2(app) is a measure of the degree and rate of slower oscillations of cholesterol molecules (Lund-Katz & Phillips, 1984). [c] Data taken from Lund-Katz et al. (1982).

A variety of spectroscopic techniques have been utilized to investigate the location and motion of cholesterol molecules in LDL, but definitive information is not available [for a review, see Keim (1979)]. More recently, CD experiments using a derivative of ergosterol as a model of cholesterol in HDL and LDL have suggested that the sterol occupies distinctly different environments in the two lipoproteins (Yeagle et al., 1982), and ^{31}P NMR data have been interpreted as suggesting that most of the cholesterol is not associated with mobile surface PL (Yeagle et al., 1978). In contrast to the above findings, ^{13}C NMR studies from this laboratory using [4-^{13}C]cholesterol in HDL (Lund-Katz & Phillips, 1984) and LDL (Figure 1) show that in both particles most of the cholesterol molecules are located in the surface PL monolayer exposed to the aqueous phase. These conclusions are based on the observations that (1) δ 41.8 is characteristic of [4-^{13}C]cholesterol associated with PL and (2) only the [4-^{13}C]-cholesterol resonating at δ 41.8 is affected by Mn^{2+} ion added to the aqueous phase. Those cholesterol molecules that are neither associated with PL in the LDL surface nor directly exposed to the aqueous phase are presumed to be in the core. Consistent with this interpretation, in the ^{13}C NMR spectrum of native human LDL the resonance at δ 120.7 from the C-6 atom of unesterified cholesterol molecules (Hamilton et al., 1979) accounts for only about 33% of the cholesterol in LDL and is attributed to mobile, core-solubilized cholesterol (J. A. Hamilton, personal communication). As with HDL (Lund-Katz & Phillips, 1984), cholesterol molecules in the two microenvironments in LDL are in slow exchange on the NMR time scale but in fast exchange on a biological time scale so that the cholesterol molecules in LDL (see Figure 2) behave physiologically as one pool.

The NMR data summarized in Figure 1 and Table I clearly demonstrate that all the unesterified cholesterol in LDL is mobile and that ~65% of it is associated with PL with the 4-^{13}C atom near the PL carbonyl groups as discussed for HDL and egg PC vesicles (Lund-Katz & Phillips, 1984). The fast axial motions of the cholesterol molecules in LDL, HDL, and vesicles are similar because the T_1 values observed for the 4-^{13}C nucleus (Table I) are essentially the same. It can be inferred that this axial motion is insensitive to the molecular structure of the PL molecules forming the matrix in which the cholesterol molecules are embedded because the composition of the phospholipids in the three particles is quite different (Table II). The spin–spin relaxation time (T_2, Table II) of the [4-^{13}C]cholesterol nucleus, as measured from the line width of the resonance, becomes longer with increases in the rate

and degree of the slower cholesterol oscillations in the PL matrix (Brainard & Cordes, 1981). If particles tumbling were the major motion affecting the dipolar relaxation rate of the [4-^{13}C]cholesterol nucleus, the relatively broad $\upsilon_{1/2}$ of this resonance in the various particles would be proportional to the cube of the particle radius (Brainard & Cordes, 1981). In addition, $\upsilon_{1/2}$ values in LDL and egg PC/cholesterol vesicles that have the same radius (Table I) would be equal. Neither of these conditions is valid for the data in Tables I and II so oscillatory and diffusional motions of cholesterol molecules in the PL matrix also contribute to the spin–spin relaxation of the 4-^{13}C nucleus in small unilamellar vesicles [cf. Brainard & Cordes (1981)] and lipoproteins (Hamilton & Morrisett, 1986). It follows that these cholesterol molecular motions are relatively restricted and/or slower in the surface of LDL compared to those in HDL_3. Moieties in lipoprotein particles or PL vesicles such as the $N(CH_3)_3$ and acyl-terminal CH_3 groups that undergo relatively unrestricted segmental motions give narrow NMR resonances whose line widths are not dependent on the particle size [cf. Brainard & Cordes (1981)]. The reason for the narrower $N(CH_3)_3$ resonance in the LDL spectrum (Figure 1) compared to that HDL_3 (Lund-Katz & Phillips, 1984) is not clear but may be due to the increased sphingomyelin in LDL.

The relatively restricted and/or slower cholesterol molecular motions in the surface of LDL compared to those in HDL may be a reflection of differences in the PL and apoprotein compositions as well as the cholesterol/PL ratio in the two lipoproteins. The PL in LDL is more saturated than that in HDL_3 with LDL being relatively enriched in saturated PC and sphingomyelin (Table II). Although the ratio of unsaturated to saturated fatty acids is the same for the sphingomyelin in LDL and HDL_3, the sphingomyelin contains much longer chains than the PC (Skipski, 1972). The net effect is an increased van der Waals cohesion between the relatively saturated PL hydrocarbon chains in the surface of LDL relative to HDL_3, which gives rise to a more closely packed or condensed PL/cholesterol monolayer [cf. Phillips (1972)]. By analogy to the situation with PL vesicles of varying size (Suurkuusk et al., 1976), the larger radius of curvature of the LDL surface compared to HDL_3 probably also enhances the intermolecular cohesion in the surface of the former particle. Whether or not there is a preferential interaction of cholesterol with sphingomyelin rather than PC molecules in LDL cannot be determined from the present experiments [cf. Barenholz & Thompson (1980)]. The presence of apo B in the surface of the LDL particle may also lead to restriction of the motions of cholesterol molecules. Since apo B immobilizes about 20% of the PL polar groups in the surface of LDL (cf. Figure 2) and probably also modifies the packing of their hydrocarbon chains, it may be that apo B effectively prevents these PL molecules from interacting with cholesterol molecules. Consequently, the cholesterol is constrained to the remaining PL where it is solubilized at a relatively high cholesterol/PL mole ratio. This enhancement of the cholesterol/PL ratio, which is already greater for LDL than HDL_3 even when simply averaged across all surface lipids (Table II), probably also leads to some restriction of the oscillatory motions of the cholesterol molecules. However, NMR studies of cholesterol in PC small unilamellar vesicles suggest that increasing the cholesterol/PC mole ratio does not restrict cholesterol molecular motion in a major fashion (De Kruijff, 1978; Brainard & Cordes, 1981).

In common with the cholesterol molecules in HDL and small unilamellar vesicles, unesterified cholesterol molecules in LDL can transfer or exchange with cholesterol in other lipoproteins and red blood cells [for a review, see Bell (1984)]. The mechanism of this process involves desorption of cholesterol molecules from the donor lipid–water interface and diffusion through the aqueous phase until they collide with an acceptor particle (McLean & Phillips, 1981; Lund-Katz et al., 1982). The $t_{1/2}$ values for HDL_3 and LDL listed in Table I cannot be compared due to the different number of cholesterol molecules in the various particles. In order to correct for this difference in pool size as well as the variations in particle size, interfacial fluxes of cholesterol molecules are utilized to compared the properties of the LDL and HDL_3 surfaces directly. It is apparent from Table II that the rate of desorption of cholesterol molecules per unit area of lipoprotein surface is lower for LDL than for HDL_3. The free energy of activation for formation of the transition state in the desorption of a cholesterol molecule from a PL–water interface is affected by the interactions of cholesterol with the host PL matrix (McLean & Phillips, 1984) so that the rate of cholesterol desorption is strongly dependent on nearest-neighbor interactions (McLean & Phillips, 1982). Obviously, these interactions are stronger in a more closely packed matrix so that cholesterol desorption from a saturated PC matrix is slower than that from an unsaturated one (McLean & Phillips, 1982). The $\upsilon_{1/2}$ and T_2 values of Tables I and II indicate that the cholesterol molecules undergo more restricted oscillatory motions in LDL, implying that the cholesterol/PL lateral packing density is greater in the surface of LDL than HDL_3. Consequently, the lower interfacial flux of cholesterol out of LDL is to be expected. The factors enumerated above which can alter lateral packing density in PL/cholesterol monolayers or bilayers will influence the rate of cholesterol desorption. Thus, the slower flux of cholesterol from LDL compared to that from HDL_3 can be attributed in part to the greater saturation of the phospholipids in LDL (Table II). Additional factors that probably also contribute to the slower cholesterol exchange from LDL are the greater sphingomyelin content of LDL (Table II) and the higher radius of curvature of the LDL surface (Table I). These inferences are based on recent reports that the presence of sphingomyelin reduces the rate of cholesterol exchange from PC vesicles and that the rate of exchange becomes slower as the radius of curvature of vesicles increases (Fugler et al., 1985; McLean & Phillips, 1984).

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