Packing of Cholesterol Molecules in Human High-Density Lipoproteins†

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ABSTRACT: High-resolution, proton-decoupled ^{13}C nuclear magnetic resonance spectra (90.55 MHz) of human high-density lipoproteins (HDL) have been employed to investigate the physical state of unesterified cholesterol molecules in such particles. The cholesterol molecules in HDL_2 and HDL_3 were replaced with [4-^{13}C]cholesterol by either particle reconstitution or exchange from Celite. Two well-defined resonances from [4-^{13}C]cholesterol molecules in HDL_2 and HDL_3 were observed at chemical shifts (δ) of 41.70 and 42.20 ppm, indicating that cholesterol molecules are present in two distinct environments. The signal at δ 41.70 arises from the C-4 atom of cholesterol molecules associated with the phospholipid monolayer at the surface of the particles. The resonance at δ 42.20 is due to the 4-^{13}C atom of cholesterol molecules dissolved in the cholesterol ester/triglyceride core. Decomposition of the two [4-^{13}C]cholesterol resonances shows that approximately 40% of the signal arises from molecules in the apolar core, with the remainder due to molecules in the surface. Spin–lattice relaxation time and line-width measurements indicate that the cholesterol molecules dissolved in the core are relatively disordered and mobile. The cholesterol molecules located among phospholipid molecules in the surface of the particle undergo relatively restricted, anisotropic motions. The chemical shifts and relaxation enhancements induced by the addition of paramagnetic ions to the aqueous phase indicate that the surface cholesterol molecules in HDL_2 and HDL_3 are exposed to the water and that the 4-^{13}C atom of cholesterol is located in the region of the phospholipid acylcarboxyl groups. The NMR data indicate that the residence time for cholesterol molecules in either the surface or the core pools of HDL is $\geq$10 ms. However, more than 90% of the unesterified cholesterol molecules in HDL are in a single kinetic pool for exchange with cholesterol molecules in other lipoprotein particles or cells. It follows that cholesterol molecules in the two microenvironments undergo fast exchange on the biological time scale and can equilibrate between the surface and core of HDL in the time scale 10 ms–ca. 300 s. Neither the surface nor the core microenvironments of human HDL particles are saturated with cholesterol.

High-density lipoproteins (HDL)[1] of human plasma apparently serve as an important means of transport of cholesterol between peripheral tissues and the liver [for reviews, see Tall & Small (1980) and Scanu et al. (1983)]. It seems that this class of human lipoprotein confers protection in the sense that the risk of coronary artery disease is inversely proportional to the concentration of cholesterol in HDL (Miller, 1980). The mechanisms by which HDL achieves this effect are not understood fully although it is realized that HDL/cell interactions must play a fundamental role. An essential prerequisite to elucidation of the mechanism of transfer of cholesterol is knowledge of the structures of normal and abnormal HDL particles. An important facet of the transfer process is the transition from the particle being a donor of cholesterol rather than an acceptor of cellular cholesterol at some characteristic content of unesterified cholesterol [e.g., see Rothblat et al. (1978)]. It follows that in order to fully understand the metabolism of HDL at the molecular level it is necessary to solve the structure of HDL and to know the physical state of cholesterol molecules present in the particle.

The behavior of unesterified cholesterol molecules in lipoprotein particles has proven a difficult subject to study due to the lack of methods which can monitor the physical properties of such molecules directly. On the basis of compositional and space- and surface-filling requirements, models of HDL structure have been proposed. The generally accepted structural model of HDL is an ~90-Å diameter sphere which features a surface layer of protein and amphiphilic lipid molecules encapsulating a nonpolar core of cholesterol ester and triglyceride molecules [e.g., see Smith et al. (1978), Edelstein et al. (1979), Tall & Small (1980), and Brewer (1981)].

In this study, attention has been focused on the location and physical state of unesterified cholesterol molecules in HDL particles by using high-field ^{13}C NMR spectroscopy to monitor ^{13}C-enriched cholesterol molecules present in reconstituted human HDL_2 and HDL_3. Two well-defined resonances from [4-^{13}C]cholesterol molecules in HDL_2 and HDL_3 are observed in the high-resolution spectra, indicating that cholesterol molecules are present in two distinct environments. Analysis of the two [4-^{13}C]cholesterol resonances shows that about 40% of the signal is associated with a narrow resonance at 42.2 ppm which arises from cholesterol molecules in the neutral lipid core of the HDL particle. The remaining 60% of the cholesterol molecules are associated with phospholipid molecules in the surface of the particle and have a chemical shift of 41.7 ppm. The [4-^{13}C]cholesterol molecules in the two locations are undergoing fast exchange on the biological time scale, indicating that >90% of the free cholesterol molecules in HDL are in a single kinetic pool for exchange with cholesterol molecules in other lipoprotein particles or cells.

Experimental Procedures

Materials

Crystalline [4-^{13}C]cholesterol with 90 atom % ^{13}C enrichment was obtained from Merck Co. (Montreal, Canada), and

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; HDL, high-density lipoprotein(s); LCAT, lecithin:cholesterol acyltransferase; LDL, low-density lipoprotein(s); $NaDodSO_4$, sodium dodecyl sulfate; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; T_1, spin–lattice relaxation time; T_2, spin–spin relaxation time; La^{3+}, lanthanide; δ, chemical shift; $\Delta\nu_{1/2}$, line width; chol, cholesterol.

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[7-^{3}H(N)]cholesterol (specific activity 34.6 Ci/mmol) was purchased from New England Nuclear (Boston, MA). The sterols were more than 99% pure as judged by thin-layer chromatography. Chromatographically pure egg PC and triolein were obtained from Calbiochem (La Jolla, CA) and Sigma Chemical Co. (St. Louis, MO), respectively, and were used without further purification. D_2O and $CDCl_3$ were purchased from Stohler Isotopes (Waltham, MA) or Merck (Rahway, NJ); D_2O was routinely deoxygenated and stored under nitrogen. Magnesium chloride ($MgCl_2$; Mallinckrodt, Paris, KY), manganous chloride ($MnCl_2$; A.C.S. grade) obtained from Fisher Scientific Co. (Fairlawn, NJ), and ytterbium chloride ($YbCl_3$, Pfaltz and Bauer, Stamford, CT) were dried for 18 h at 110 °C before use. Praseodymium chloride ($PrCl_3$; Pfaltz and Bauer) was used as supplied. The purities of the rare earth salts were checked spectroscopically (Stewart & Kato, 1958; Banks & Klingman, 1956) and found to be >99%; $YbCl_3$ and $PrCl_3$ were used as 0.1 M solutions in D_2O adjusted to a nominal pH of 7.6 with sodium hydroxide. Other reagents were analytical grade. All organic solvents were redistilled prior to use.

Human HDL_2 ($1.065 < d < 1.125$ g/mL) and HDL_3 ($1.125 < d < 1.21$ g/mL) were isolated from human serum by sequential ultracentrifugation in KBr by the method of Havel et al. (1955) as modified by Marsh (1976). 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 N_2 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 HDL_3 gave a single band on staining with Sudan Black, and the HDL_2 showed ≤5% contamination with LDL.

Methods

Vesicle Preparation. [4-^{13}C]Cholesterol (10 mol %) was mixed with 100–120 mg of egg phosphatidylcholine (PC) in chloroform and dried under N_2 to a thin film on the walls of a test tube. Traces of remaining solvent were removed by drying for 2 h at 40 °C under vacuum. The dry lipid mixtures were taken up in 1.2 mL of 0.15 M NaCl, 0.001 M EDTA, and 0.02% NaN_3, pH 7.6, and immersed in an ice bath (4 °C). The lipid was dispersed by sonication under N_2 using a Branson Model 350 sonifier at setting 5 with a tapered microtip. The dispersion was sonicated for 1 h using a 50% duty cycle. Multilamellar particles, undispersed lipid, and titanium were sedimented by centrifugation for 2 h at 40000 rpm ($139700g_{max}$) in a Beckman Type 65 rotor. The clear supernatant was removed, 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%. The vesicles were transferred to 10-mm NMR tubes under N_2, and NMR analysis was completed within 24 h.

Reconstitution of HDL. Lipid-free proteins, apo-HDL_2, and apo-HDL_3, obtained by extraction with 3:2 (v/v) ethanol:diethyl ether at 0 °C (Scanu & Edelstein, 1971), were stored dry at −20 °C. The ethanol–diethyl ether extracts, containing the lipids from HDL_2 and HDL_3, were cleared of any precipitate by filtration and brought to dryness by rotary evaporation under reduced pressure, and the lipids were stored in 2:1 (v/v) chloroform:methanol at −20 °C under N_2. The polar and nonpolar lipids were isolated from the organic phase and the latter separated into the individual lipid classes by thin-layer chromatography. Samples and appropriate standards were applied to precoated silica gel G plates (2000 μm) (Analtech, Newark, DE), and a 100:5 (v/v) benzene:ethyl acetate mixture was employed as the solvent system. After double development of the plate, the lipids were visualized with iodine vapor, outlined, and extracted. All procedures were carried out under a N_2 atmosphere. The cholesterol was removed, quantitated by gas–liquid chromatography, and then replaced by [4-^{13}C]cholesterol. The reassemblies of HDL_2 and HDL_3 were achieved by using the procedure of Hirz & Scanu (1970); the reconstituted particles were isolated by ultracentrifugal flotation in the density range 1.065–1.125 and 1.125–1.21 g/mL for HDL_2 and HDL_3, respectively. The yields of HDL were in the range 65–85%. HDL_3 particles containing [4-^{13}C]cholesterol, but no cholesterol ester or triglyceride, were reconstituted in a similar fashion.

Prior to NMR measurements, native and reconstituted HDL samples were concentrated to 100 mg/mL by dialysis against Permasorb 30, kindly provided by Edward Richman (National Starch and Chemicals, Bridgewater, NJ), or against Sephadex G-200. The samples were 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%. The lipoproteins were transferred to 10-mm NMR tubes under N_2 and used within 24 h.

Cholesterol Labeling of HDL Using Celite. The Celite (grade 545) used for dispersing cholesterol was a product of Harleco (Philadelphia, PA). Prior to use, the Celite was thoroughly washed with dilute HCl, deionized water, and methanol and then dried under vacuum. Human HDL_3 was heated at 55 °C for 30 min to inactivate lecithin:cholesterol acyltransferase and then labeled with [7-^{3}H(N)]cholesterol and [4-^{13}C]cholesterol to a [^{3}H]cholesterol specific activity of 14.4 μCi/mmol and a level of about 4 mg of [4-^{13}C]-cholesterol by a modification of the procedures of Avigan (1959), Lundberg et al. (1982), and Lund-Katz et al. (1982). Celite (1.4 g) and [4-^{13}C]cholesterol (15 mg) doped with [^{3}H]cholesterol at a specific activity of 22.5 μCi/mmol were dispersed in chloroform in an Erlenmeyer flask; the radiotracer was added to monitor the uptake of [^{13}C]cholesterol by HDL. The solvent was evaporated under N_2 and reduced pressure, and lipoprotein solution was added to give a final ratio of 1 mg of protein/10 mg of Celite. The mixture was capped under N_2 and incubated at 37 °C for 72 h in a shaking water bath. Celite was pelleted from the solution by centrifugation (18000 rpm, 30 min). The supernatant containing HDL was removed and filtered through a 0.45-μm Millipore filter (Millipore Corp., Bedford, MA) with a filter guard.

Nuclear Magnetic Resonance Measurements. ^{13}C NMR spectra at 90.55 MHz were obtained on a Bruker WH 360 spectrometer. Broad-band, phase-modulated proton decoupling was used (7–8 W). Sweep widths of 220 ppm were observed by using 90° pulses of 12–14-μs duration; 16384 data-point, free induction decay, signals were zero-filled to 32768 points before their Fourier transforms were calculated. Sample temperatures were controlled with the Bruker thermostat, and spectra were obtained at 37 ± 1.5 °C; sample temperatures were checked with a small thermocouple (Omega Trendicator) immediately after the sample was removed from the spectrometer. Chemical shifts are given in parts per million downfield from $(CH_3)_4Si$ with internal, aqueous 1,4-dioxane (66.55 ppm) as reference and are accurate to ±0.05 ppm. Line widths ($\Delta\nu_{1/2}$) were measured as the peak width at half-height from expanded printouts of the resonances, with an estimated error of ±10%. All spectra were processed with 2.0-Hz exponential filtering.

Spin–lattice relaxation times (T_1) were measured by the standard inversion recovery sequence (Vold et al., 1968). A $(180°-\tau-90°-PD)_n$ pulse sequence was employed where τ is the variable delay time between 180° and 90° pulses, PD is the pulse delay (2.5 s), and n is the number of times the pulse sequence was repeated. Ten to twelve τ values were obtained for each determination, and the T_1 values were derived from linear plots of ln (signal intensity) vs. τ by using a linear regression analysis; the peak heights were measured from plotted spectra. T_1 values were also calculated by using the DISNMR T_1 routine; both methods gave similar results. The T_1 values have a nonsystematic error of ±10%. When it was necessary to compare integrated intensities of selected resonances, the proton decoupler was gated to eliminate the nuclear Overhauser effect. In these experiments, the irradiation for proton decoupling was switched off for a period at least 5 times the longest T_1 of the resonances compared. The integrated areas from expanded printouts of the required resonances were determined either digitally by a DISNMR peak-picking routine, graphically by planimetry, or gravimetrically by weighing of tracings of peaks resolved by assuming Lorentzian line shapes: all methods gave comparable results.

Pr^{3+}, Yb^{3+}, Mg^{2+}, and Mn^{2+} in the form of their chlorides were added to the aqueous phase of the NMR samples in order to study ion-induced changes in the chemical shift or spin–lattice relaxation of PC and cholesterol resonances. The mole ratio of cations to that of the phospholipid was ≤4.0 for $PrCl_3$ ≤0.7 for $YbCl_3$, and 0.06 for $MnCl_2$ and $MgCl_2$. When ions were added to PC vesicles, the solution was sonicated briefly to allow cations to enter the aqueous phase encapsulated in the vesicles.

Analytical Procedures. The concentrations of free and esterified cholesterol in all of the lipoproteins 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 content was monitored by phosphorus analysis (Sokoloff & Rothblat, 1974).

Negative-stain electron microscopy (Collins & Phillips, 1982) was used to assess the particle sizes of the various HDL preparations.

Results

^{13}C NMR Spectra of HDL. As indicated by Hirz & Scanu (1970), who originated the method, and confirmed in this laboratory (Lund-Katz & Phillips, 1981), human HDL_2 and HDL_3 can be disassembled and reconstituted into particles which are indistinguishable from the original. Thus, apart from retention of the original composition, particle size, and shape, the reconstituted HDL_2 and HDL_3 particles behave the same as the native lipoproteins in a biological test; both species cause the same efflux of cholesterol from tissue culture cells (Lund-Katz & Phillips, 1981). This information, coupled with the fact that the NMR characteristics of cholesterol are identical whether it is incorporated into HDL by reconstitution of the whole particle or transferred from Celite into intact HDL, suggests that the ^{13}C-enriched cholesterol molecules present in the reconstituted HDL_2 and HDL_3 are in essentially the same physical state as the [^{12}C]cholesterol molecules in native HDL.

The ^{13}C NMR spectra shown in parts A and B of Figure 1 for reconstituted HDL_2 and HDL_3, respectively, are similar to those for native HDL_3 in the literature [cf. Hamilton & Cordes (1978), Stoffel (1976), and Brainard et al. (1980)] and also support the idea of the physical state of the lipids in all particles being the same. The chemical shifts (δ) and line

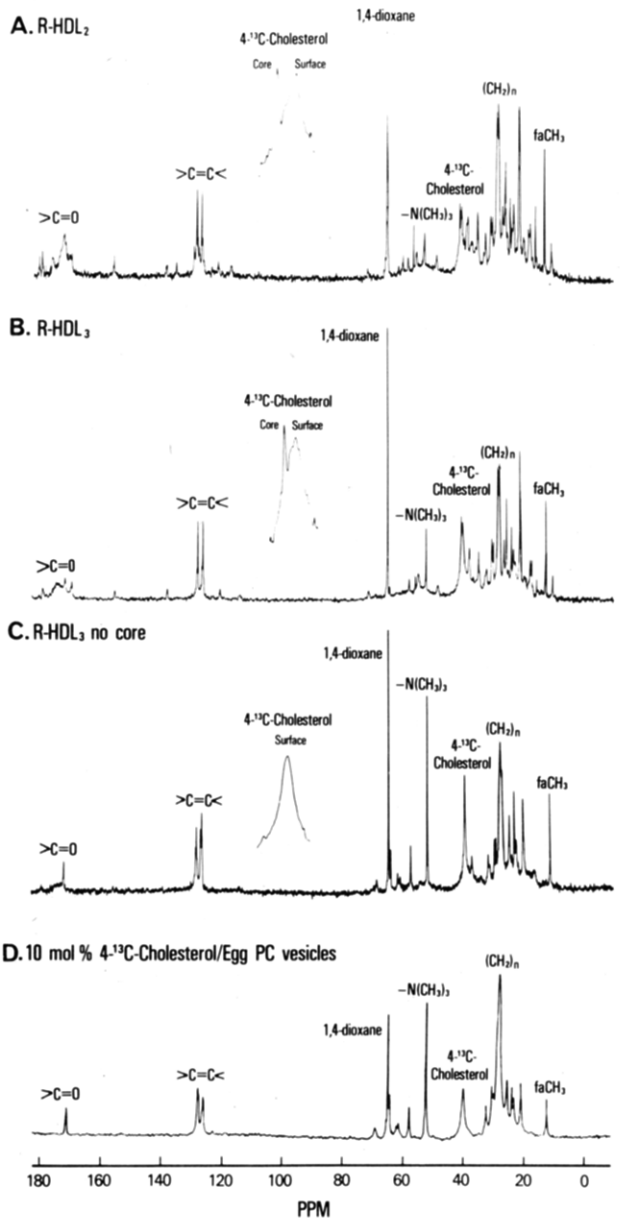

FIGURE 1: Proton-decoupled ^{13}C NMR spectra (90.55 MHz) at 37 °C: (A) human HDL_2 reconstituted with [4-^{13}C]cholesterol, 10 500 accumulations; (B) human HDL_3 reconstituted with [4-^{13}C]cholesterol, 10 000 accumulations; (C) human HDL_3 reconstituted with [4-^{13}C]cholesterol but no cholesterol ester or triglyceride, 5000 accumulations. Spectra A–C were obtained with gated decoupling and a recycling time of 2.9 s so that the nuclear Overhauser effect was suppressed. The expanded regions in spectra A–C have the horizontal axis expanded by a factor of 20. (D) 10 mol % [4-^{13}C]cholesterol/egg PC vesicles, 2000 accumulations. Spectrum D was obtained by using broad-band decoupling and a recycling time of 1.2 s. All spectra were processed with 2.0-Hz exponential filtering.

widths ($\Delta\nu_{1/2}$) are similar in both the native and the reconstituted lipoproteins, with the only difference being the expected appearance of the signals from the [4-^{13}C]cholesterol substituted in the reconstituted particles. Line widths for selected carbon resonances for HDL_2, HDL_3, and 10 mol % [4-^{13}C]cholesterol/egg PC vesicles are compared in Table I. For most resonances of HDL_2 and HDL_3, somewhat greater line widths are observed in HDL_3 [cf. Brainard et al. (1980)]. The $\Delta\nu_{1/2}$ values observed for the resonances from the egg PC/cholesterol vesicles (Figure 1D) are generally larger, especially the line widths of the [4-^{13}C]cholesterol and some of the phospholipid hydrocarbon chain resonances [cf. Lancée-Hermkens & DeKruijff (1977), DeKruijff (1978), Brainard

Table I: Selected ^{13}C Resonances of Phospholipid and Cholesterol in Human HDL_2, HDL_3, and Vesicles at 37 °C

resonance	10 mol % [4-^{13}C]chol/egg PC vesicles		HDL_2		HDL_3	
	δ	$\Delta\nu_{1/2}$ [a]	δ	$\Delta\nu_{1/2}$	δ	$\Delta\nu_{1/2}$
phospholipid carbonyl	173.40	19.7 ± 2	173.60	19.7 ± 2	173.40	21.9 ± 2
monoolefin	129.50	15.6 ± 2	129.50	13.7 ± 2	129.50	14.8 ± 2
polyolefin	127.75	15.7 ± 2	127.80	12.2 ± 1	127.80	13.6 ± 1
choline CH_2N	66.00	21.4 ± 3	65.40	19.6 ± 3	65.40	21.9 ± 2
choline CH_2O	59.40	21.4 ± 3	59.50	19.6 ± 2	59.50	21.9 ± 2
choline $(CH_3)_3N$	54.00	19.7 ± 1	54.00	17.6 ± 1	54.00	19.9 ± 2
cholesterol C-4, core			42.20	21.5 ± 2	42.20	27.6 ± 2
cholesterol C-4, surface	41.70	73.0 ± 6	41.70	54.8 ± 4	41.70	56.0 ± 4
fatty acyl $(CH_2)_n$ [b]	29.30	79.4 ± 8	29.30	56.8 ± 6	29.40	63.6 ± 7
$CH_3CH_2CH_2$	22.55	24.0 ± 3	22.50	19.6 ± 3	22.60	24.4 ± 3
fatty acyl terminal CH_3	13.70	13.3 ± 1	13.95	12.7 ± 1	13.95	13.7 ± 2

[a] $\Delta\nu_{1/2}$ in hertz (line width of 1,4-dioxane internal standard was the same in all spectra). Reported values include 2.0-Hz exponential line broadening applied during processing. [b] $\Delta\nu_{1/2}$ values are increased by chemical shift anisotropy.

& Cordes (1981), and Yeagle (1981)]. The peak expansions of the [4-^{13}C]cholesterol resonances in the reconstituted lipoproteins (Figure 1A,B) show clearly that the downfield signal has a narrower $\Delta\nu_{1/2}$ (Table I). When cholesterol is dissolved in bulk organic phases such as $CDCl_3$, triolein, or 4:1 (v/v) *n*-hexadecane:$CDCl_3$ at 37 °C, $\Delta\nu_{1/2}$ values of about 10–13 Hz are observed for the 4-^{13}C resonance.

Chemical Shifts of [4-^{13}C]Cholesterol in HDL and Other Solvent Systems. The cholesterol molecules in HDL give rise to two resonances at δ 41.7 and 42.2 (Table I), which are essentially the same as the δ values for the 4-^{13}C resonance of cholesterol dissolved in egg PC bilayers at pH 8 (41.7 ppm) and deuterated chloroform (42.1 ppm), respectively. Two resonances with identical δ values were also observed from [4-^{13}C]cholesterol molecules incorporated into HDL by transfer from Celite rather than by reconstitution of the whole particle. The chemical shift for [4-^{13}C]cholesterol solubilized in triolein (42.7 ppm) is slightly higher than that reported for cholesterol molecules in the core of HDL. This difference may be due to the presence of cholesterol ester in the core of the lipoprotein. The chemical shift for [4-^{13}C]cholesterol in triolein is 0.5 ppm higher than that reported previously from this laboratory (Lund-Katz & Phillips, 1981) due to a difference in the chemical shift reference: in the present study, internal aqueous 1,4-dioxane (δ 66.55) is used rather than internal hydrocarbon chain CH_3. Addition of water in a mole ratio of 20:1 H_2O:cholesterol to a [4-^{13}C]cholesterol/triolein sample (~3 mg/mL) does not affect the chemical shift of the cholesterol. This implies that the difference in δ of [4-^{13}C]-cholesterol molecules in the surface and core of HDL is not due to the lack of hydration of cholesterol molecules in the core.

In order to understand what determines the upfield chemical shift of [4-^{13}C]cholesterol dissolved in phospholipids relative to other organic solvents, vesicles under various conditions were compared. The role of any hydrogen bonding of the cholesterol hydroxyl group to the carbonyl groups of the PC was examined by using [4-^{13}C]cholesterol solubilized in dihexadecyl- and dipalmitoyl-PC vesicles (pH 8). The absence of carbonyl groups in dihexadecyl-PC led to a 0.1 ppm downfield shift to 41.8 ppm, suggesting that magnetic shielding from the nearby PC carbonyl groups contributes to the upfield shift of the [4-^{13}C]cholesterol resonance in PC systems. A 0.2 ppm upfield shift of the cholesterol C-4 resonance to 41.5 ppm was observed when the phosphate group was protonated by adding HCl to decrease the pH of egg PC vesicles to below 1. This indicates that the magnetic environment of the C-4 atom of cholesterol molecules mixed with PC molecules is affected by the charged phosphate group. However, the proximity of the negatively charged phosphate group does not account for the upfield chemical shift and increased magnetic shielding of the cholesterol C-4 atom mixed with PC rather than with a solvent such as triolein. At this time, it is not possible to attribute the magnitude of the magnetic shielding of the C-4 nucleus in cholesterol molecules located at the PC/water interface to particular electrons.

Table II: Effect of Mn^{2+} on T_1 of Selected Resonances from Human HDL_2, HDL_3, and [4-^{13}C]Cholesterol/PC Vesicles

resonances	spin-lattice relaxation time (ms)					
	HDL_2		HDL_3		10 mol % [4-^{13}C]chol/egg PC vesicles	
	$-Mn^{2+}$	$+Mn^{2+}$ [a]	$-Mn^{2+}$	$+Mn^{2+}$	$-Mn^{2+}$	$+Mn^{2+}$
[4-^{13}C]chol, surface	150	70	140	70	160	80
[4-^{13}C]chol, core	460	450	450	450		
$N(CH_3)_3$	560	120	550	100	540	130
$-(CH_2)_n-$	570	550	570	540	550	540

[a] Mn^{2+}/phospholipid (mol/mol) = 0.06.

The nuclear Overhauser effect is suppressed in the spectra of reconstituted HDL (Figure 1A–C) so that the signal intensities are a true reflection of the number of ^{13}C atoms contributing to each resonance (Wehrli & Wirthlin, 1978). Integration of the total [4-^{13}C]cholesterol and $N(CH_3)_3$ peaks indicates that 100% of the cholesterol and PC molecules, respectively, contribute to the spectrum. Decomposition of the two [4-^{13}C]cholesterol resonances (Figure 1A,B) by using the various procedures described under Methods shows that 40 ± 4% of the signal is associated with the narrow line at δ 42.2 in both HDL_2 and HDL_3 [this includes a small contribution from the C-13 atom of cholesteryl esters (Hamilton & Cordes, 1978)]. Reconstitution of an HDL_3 particle which does not contain cholesterol ester or triglyceride eliminates the [4-^{13}C]cholesterol resonance at 42.2 ppm, leaving a single resonance at 41.7 ppm (Figure 1C) arising from 100% of the cholesterol molecules in the system.

Spin–Lattice Relaxation Time Measurements. The ^{13}C spin–lattice relaxation times (T_1) for certain carbon atoms of phospholipid and cholesterol molecules in HDL_3, HDL_2, and small unilamellar vesicles are listed in Table II. The resonances selected here are those for which the resolution and signal to noise ratio under our experimental conditions are sufficient for an accurate determination of T_1. The T_1 values

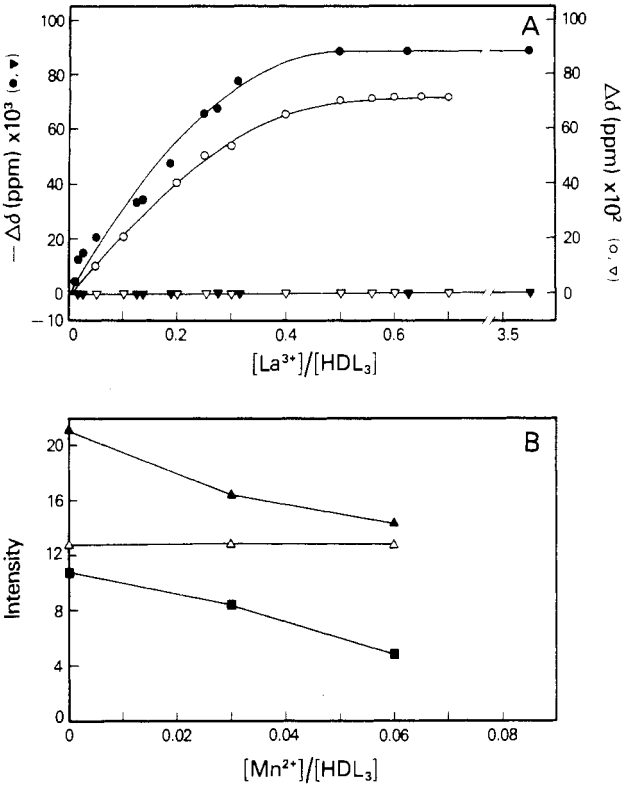

FIGURE 2: Chemical shifts and relaxation enhancements produced by paramagnetic ions in the ^{13}C resonances of the phospholipid $-N-(CH_3)_3$ groups and the surface [4-^{13}C]cholesterol molecules in human HDL_3. The abscissas give the molar ratios of paramagnetic ion to HDL_3 phospholipid (M_r 787). (A) Yb^{3+} gives upfield shifts, and Pr^{3+} gives downfield shifts. $N(CH_3)_3$ resonances: (○) Yb^{3+}; (●) Pr^{3+}. Surface [4-^{13}C]cholesterol resonances: (▽) Yb^{3+}; (▼) Pr^{3+}. (B) Effect on the signal intensity of the addition of Mn^{2+}: (■) $N(CH_3)_3$; (△) core [4-^{13}C]cholesterol; (▲) surface [4-^{13}C]cholesterol. The intensity was measured as peak height and is expressed in arbitrary units.

are in agreement with published values [cf. Hamilton et al. (1974), Stoffel et al. (1974), Brainard et al. (1980), Brainard & Cordes (1981), and Yeagle (1981)], and it is apparent that the nuclei in question relax similarly in all three systems. The T_1 values for the HDL [4-^{13}C]cholesterol nuclei which resonate at δ 42.2 reflect the relatively rapid molecular motion which also gives rise to the low $\Delta\nu_{1/2}$ noted earlier. Thus, for HDL_3, the T_1 values for the resonances at δ 41.7 and 42.2 are 140 and 450 ms, respectively; the equivalent numbers for HDL_2 are 150 and 460 ms, respectively. These values can be compared with T_1 = 160 and 500 ms for [4-^{13}C]cholesterol solubilized in the bilayer of egg PC unilamellar vesicles and $CDCl_3$, respectively.

Paramagnetic Ions as Structural Probes. (A) Shift Reagents. The addition of paramagnetic $YbCl_3$ and $PrCl_3$ to native and reconstituted HDL_2 and HDL_3 produces changes ($\Delta\delta$) in the ^{13}C chemical shifts of the polar group resonances. Of the $N(CH_3)_3$ signal, 100% is shifted upfield or downfield, respectively, indicating that all the $N(CH_3)_3$ groups (including those of sphingomyelin) are present at the surface of the HDL particle and readily accessible to the cations in the aqueous phase [cf. Finer et al. (1975) and Hauser (1975)]. Figure 2A depicts the $\Delta\delta$ values of the $N(CH_3)_3$ signal of reconstituted HDL_3 as a function of the molar ratio of $YbCl_3$ or $PrCl_3$ to phospholipid. No change in the chemical shift of the resonance from [4-^{13}C]cholesterol molecules located on the surface (δ 41.7) was observed up to mole ratios of lanthanide to phospholipid of 0.7 and 4.0 for $YbCl_3$ and $PrCl_3$, respectively. At higher mole ratios, both cations caused visible precipitation of HDL.

(B) Relaxation Enhancement Reagent. Figure 2B shows the changes in $\Delta\nu_{1/2}$ of the $N(CH_3)_3$ signal of reconstituted HDL_3 as a function of the Mn^{2+}/phospholipid molar ratio. The addition of increasing quantities of $MnCl_2$ to native and reconstituted HDL caused broadening of the line widths of the polar group resonances, whereby the total signal intensities were affected. This is consistent with the shift reagent experiments, indicating that all the phospholipid polar groups are exposed to the aqueous phase. A similar effect was observed in the $\Delta\nu_{1/2}$ of [4-^{13}C]cholesterol molecules resonating at δ 41.7 (Figure 2B). No change in the line width of the [4-^{13}C]cholesterol resonance at δ 42.2 was observed up to a mole ratio of Mn^{2+} to phospholipid of 0.2. At higher mole ratios, $MnCl_2$ caused visible precipitation of HDL.

(C) Relaxation Enhancement with $MnCl_2$. The spin–lattice relaxation enhancement induced by paramagnetic ions such as Mn^{2+} is a function of the Mn^{2+}–^{13}C distance and has been used to obtain distance information in PC bilayers (Weinstein et al., 1980) and to map enzyme active sites [e.g., see Raushel & Villafranca (1980)]. The observed spin–lattice relaxation rate enhancement attributed to the influence of the bound Mn^{2+} ($1/\Delta T_1$) is given by

$$\frac{1}{\Delta T_1} = \frac{1}{T_1(Mn^{2+})} - \frac{1}{T_1(Mg^{2+})} \qquad (1)$$

where $T_1(Mn^{2+})$ and $T_1(Mg^{2+})$ are values measured in the presence of the same concentrations of these two cations. Assuming fast exchange and low occupancy of the phosphate binding sites of the PC bilayers, $1/\Delta T_1$ is related to r, the Mn^{2+}(unpaired electron)–^{13}C nucleus distance, by the simplified Solomon–Bloembergen equation:

$$r = C\left(\Delta T_1 \frac{3\tau_c}{1 + \omega^2\tau_c^2}\right)^{1/6} \qquad (2)$$

In eq 2, C is a collection of constants whose value depends on the spin of the paramagnetic ion and the gyromagnetic ratio of the nucleus. ω is the Larmor precession frequency, and τ_c is the correlation time of the Mn^{2+}–^{13}C nuclear interaction. Using eq 1 and 2, we can use the relative relaxation enhancements of the [4-^{13}C]cholesterol and $N(CH_3)_3$ nuclei to obtain relative distances of the Mn^{2+} ion from these nuclei because

$$\frac{r_{Mn\text{-chol}}}{r_{Mn\text{-}N(CH_3)_3}} = \left(\frac{\Delta T_{1,chol}}{\Delta T_{1,N(CH_3)_3}}\right)^{1/6} \qquad (3)$$

Knowing that Mn^{2+} and other paramagnetic cations bind to the phosphate group in PC systems (Hauser & Phillips, 1979), and having distance ratios from the cation to various glycerylphosphorylcholine protons (Hauser et al., 1976), we can deduce the location of the [4-^{13}C]cholesterol nucleus with respect to the PC molecules in the surface.

If it is assumed that the conformation of the phosphocholine group and the binding of Mn^{2+} are the same for HDL_2, HDL_3, and PC/cholesterol vesicles, then $r_{Mn\text{-}N(CH_3)_3}$ should be the same in all three systems. Consequently, eq 3 gives a measure of the immersion of the cholesterol molecules at the surfaces of HDL_2, HDL_3, and egg PC bilayers. The T_1 values in the absence and presence of Mn^{2+} for selected ^{13}C resonances in the PC molecules and for [4-^{13}C]cholesterol are listed in Table II. The relaxation enhancements induced by the addition of Mn^{2+} at Mn^{2+}/phospholipid = 0.06 mol/mol are similar for the [4-^{13}C]cholesterol and $N(CH_3)_3$ resonances from HDL and egg PC vesicles. The polar group T_1 values are significantly reduced while the hydrocarbon chain $(CH_2)_n$ resonances

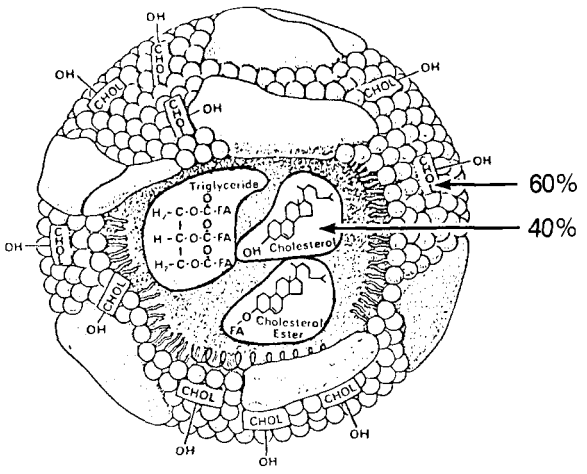

FIGURE 3: Oil-drop model of human plasma high-density lipoprotein. The surface layer of protein and amphiphilic lipid molecules encapsulate a nonpolar core of cholesterol ester, triglyceride, and cholesterol molecules [after Brewer (1981)]. The polar groups of the phospholipid molecules are all exposed to the aqueous phase; 40% of the unesterified cholesterol molecules are solubilized in the apolar core while the remaining 60% are associated with phospholipid molecules in the surface. Cholesterol molecules exchange between the surface and core locations in the time scale 10 ms–300 s.

are not affected by the presence of Mn^{2+} ions in the aqueous phase. At a mole ratio of 0.06, neither $MnCl_2$ nor $MgCl_2$, the latter was used as a control for nonspecific ion binding effects, caused visible precipitation of HDL or vesicles.

Substitution of the T_1 values for [4-^{13}C]cholesterol and $N(CH_3)_3$ in HDL_2, HDL_3, and egg PC vesicles from Table II into eq 1 and 3 gives the distance ratio $r_{Mn\text{-}chol}/r_{Mn\text{-}N(CH_3)_3}$ in all three systems. The distance ratio has the same value of 1.0 for HDL_2 (0.97 ± 0.05, error is expressed as a range), HDL_3 (1.03 ± 0.07), and 10 mol % cholesterol/egg PC vesicles (0.99 ± 0.07).

Discussion

Current models of HDL structure feature an ~90-Å diameter particle in which a surface layer of amphiphilic molecules encapsulate a core of nonpolar cholesterol ester and triglyceride molecules [e.g., see Edelstein et al. (1979) and Brewer (1981)]. Figure 3 presents a cartoon of an HDL particle, and it is apparent that our Yb^{3+} and Pr^{3+} chemical shift data in Figure 2A are consistent with this in that all the PC and sphingomyelin $N(CH_3)_3$ groups are exposed to the aqueous phase. The secondary structure of the apolipoproteins is known to contain some 60–70% amphiphilic α-helix [for a review, see Morrisett et al. (1977)]. The phospholipids, cholesterol esters, and triglycerides in such HDL particles are known to be in a disordered, fluid state, and this gives rise to high-resolution NMR spectra such as those depicted in Figure 1 [for a review of NMR studies of lipoproteins, see Keim (1979)]. However, relatively little is known about the location and motion of cholesterol molecules in HDL. Models of HDL tend to have the cholesterol located exclusively among the phospholipid molecules in the surface although recent magnetic resonance studies have indicated that some cholesterol is dissolved in the neutral lipid core (Avila et al., 1978; Hauser & Kostner, 1979; Lund-Katz & Phillips, 1981). The present ^{13}C NMR data give a detailed description of the physical state of unesterified cholesterol molecules in HDL.

Microenvironments of Cholesterol Molecules in HDL. Since well-defined resonances from [4-^{13}C]cholesterol molecules in HDL are apparent in the high-resolution spectra (Figure 1), these molecules are in a fluid state and not packed in a rigid lattice, as they would be in a crystal. Furthermore, the fact that there are two resonances, 0.5 ppm apart, indicates that the cholesterol molecules occupy two magnetically and physically distinct environments. Examination of the chemical shift values suggests that the downfield resonance at 42.2 ppm arises from cholesterol molecules in the neutral lipid core of the HDL particle while the 41.7 ppm peak is due to cholesterol molecules associated with phospholipid molecules in the surface. This assignment is corroborated by the fact that when discoidal HDL particles lacking cholesterol ester and triglyceride are reconstituted, the resonance from core cholesterol molecules disappears (Figure 1C). The cholesterol molecules (δ 41.7) in the surface of the HDL particle are exposed to the aqueous phase as indicated by the line broadening observed when Mn^{2+} ions are added to the water (Figure 2B). As expected, the core cholesterol molecules are too far removed to be influenced by paramagnetic ions in the water.

The relative proportions of cholesterol molecules in the surface and core are derived from the integrals of the 41.7 and 42.2 ppm resonances, and as shown in Figure 3, the cores of HDL_2 and HDL_3 contain 40% of the cholesterol molecules with the remainder in the surface. If all the cholesterol molecules were in the surface, the cholesterol/phospholipid mole ratios of the surface monolayer (Figure 3) for HDL_3 and HDL_2 would be 0.26 and 0.36, respectively. Studies of the reconstitution of apoA-I with cholesterol/PC mixtures (Jonas & Krajnovich, 1978; Tall & Lange, 1978; Pownall et al., 1979) indicate that as the cholesterol content is increased above a cholesterol/PC mole ratio of about 0.25, the lipid/protein interaction is inhibited. However, the present quantitative description of the distribution of cholesterol molecules between the surface and core of HDL shows that in fact the cholesterol/phospholipid mole ratios for the surface monolayers of HDL_3 and HDL_2 are only 0.16 and 0.22, respectively. This is consistent with a facile interaction of the amphiphilic helices of apoA-I with the PC matrix in the surface of HDL. Since amphiphilic helices and cholesterol molecules apparently compete for interaction with phospholipid molecules, the presence of apolipoprotein molecules in the surface of HDL may enhance the partitioning of cholesterol molecules into the core. The triglyceride/cholesterol ester cores of HDL_3 and HDL_2 contain 8% and 11% w/w cholesterol, respectively. It is known that cholesterol is soluble in anhydrous, liquid cholesterol ester or triglyceride with the solubility increasing as the fraction of cholesterol ester is raised (Miller & Small, 1983; Small, 1970); for instance, a total of 12% w/w cholesterol can be dissolved in anhydrous liquid cholesterol oleate/triolein mixture which is fairly high in cholesterol oleate (Small, 1970). However, it is not possible to easily relate such bulk-phase solubility data to the core of HDL. The small size of the core (e.g., HDL_3 contains 32 cholesterol ester, 10 triglyceride, and ~5 cholesterol molecules) prevents the cooperative molecular interactions normally present in a bulk liquid of the same composition [cf. Tall et al. (1977)] so that the solvent characteristics are likely to be modified. Furthermore, the presence of water is known to decrease the solubility of cholesterol in such nonpolar oils (Jandacek et al., 1977), and quantitative information on these effects is lacking. Obviously, since cholesterol can be accommodated in both the core and surface, the potential of HDL to solubilize and transport cholesterol is increased. Indeed, HDL can solubilize significant amounts of extra cholesterol (Jonas et al., 1978; Lundberg et al., 1982), and such additional cholesterol molecules are situated in both the core and surface (S. Lund-Katz and M. C. Phillips, unpublished results), indicating that in normal circulating human

HDL_2 and HDL_3 neither location is saturated with cholesterol.

Motions and Packing of Cholesterol Molecules in HDL. (*A*) *Core Cholesterol.* Since the [4-^{13}C]cholesterol nuclei dissolved in the core of HDL_2 and HDL_3 have similar T_1 and $\Delta\nu_{1/2}$ values to those observed for cholesterol in liquid organic solvents such as $CDCl_3$, it follows that the molecules must be undergoing similar isotropic tumbling motions in all systems. This is consistent with earlier reports that the core of HDL is in a fluid disordered state (Hamilton & Cordes, 1978). It is apparent from Table II that T_1 values for the 4-^{13}C atom of cholesterol in the cores of HDL_2 and HDL_3 are the same while $\Delta\nu_{1/2}$ is somewhat higher in HDL_3 (Table I). Since the T_1 values in these systems are sensitive to fast molecular motions, it follows that these motions are the same for cholesterol molecules in HDL_2 and HDL_3. The apparent T_2 $[=(\pi\Delta\nu_{1/2})^{-1}]$ is affected principally by slower motions involving reorientation of local segments (Brainard & Cordes, 1981; Brainard & Szabo, 1981; Yeagle, 1981) so the data in Table I suggest that these motions of core cholesterol molecules are somewhat slower and/or more restricted in HDL_3 compared to HDL_2. The cholesterol molecules in the cores of both HDL particles are more restricted than when dissolved in bulk organic solvents, as reflected by the narrower [4-^{13}C]cholesterol resonances observed in systems such as liquid triolein.

(*B*) *Surface Cholesterol.* In contrast, both the fast and slow motions of cholesterol molecules in the surfaces of HDL_3 and HDL_2 are the same as reflected by the spin–spin and spin–lattice relaxation of the [4-^{13}C]cholesterol nuclei (Tables I and II). It is of interest to compare the T_1 and $\Delta\nu_{1/2}$ values for cholesterol molecules in the surface of HDL_2 and HDL_3 to those in egg PC small unilamellar vesicles because the anisotropic motions of cholesterol molecules in the latter system have been studied extensively. The T_1 of about 150 ms for the 4-^{13}C atom of cholesterol in all three systems (Table II) is consistent with previous results showing that the correlation time for fast axial rotation of cholesterol molecules in liquid-crystalline bilayers is about 10^{-10} s (Yeagle, 1981; Brainard & Cordes, 1981; Taylor et al., 1982). In contrast to T_1 values, T_2 ($\Delta\nu_{1/2}$) values are sensitive to particle size because the tumbling motions of the whole particle can contribute to line narrowing. However, the $\Delta\nu_{1/2}$ of cholesterol in vesicles seems to be differentially broadened in that its value is larger in vesicles whereas the $N(CH_3)_3$ and fatty acyl terminal CH_3 resonances have the same line widths in HDL and vesicles, despite the differences in particle size (Table I). This suggests that the line width of 73 Hz for vesicles compared to about 55 Hz for HDL_2 and HDL_3 is a reflection of more restricted and/or slower cholesterol oscillations in the PC bilayer system. This variation in the slow motions of cholesterol molecules in the surface of HDL compared to vesicles may be a reflection of looser molecular packing due to the greater curvature of the surface of the former particles. The presence of apolipoprotein molecules apparently does not significantly reduce the fast axial rotations of surface cholesterol molecules in HDL because the T_1 values are the same in HDL and PC vesicles (Table II). This lack of an effect on T_1, and the observation of a narrower C-4 cholesterol resonance in HDL compared to vesicles, suggests that there is not a strong direct interaction between the cholesterol and apolipoprotein molecules in the surface of HDL; filipin binding studies imply that apoA-I in HDL is in closer proximity to unesterified cholesterol than apoA-II (Blau et al., 1982).

The Mn^{2+} relaxation enhancement experiments summarized in Figure 2B and Table II, coupled with the shift reagent data in Figure 2A, permit a more detail examination of the exact location of cholesterol molecules in the surfaces of egg PC bilayer vesicles and HDL particles. The chemical shifts induced by Pr^{3+} and Yb^{3+} (Figure 2A) are not accompanied by significant line broadening and are of pseudocontact origin (Knowles et al., 1976). They are proportional to r_i^{-3} where r_i is the distance between the metal ion and the ^{13}C nucleus of interest. In addition, the shifts are sensitive to orientation in that they are a function of θ, the angle between the vector r_i and the principal symmetry axis of the complex formed by lanthanide ion binding near the ^{13}C nucleus, averaged over motions rapid on the NMR time scale. In contrast, the line broadening and relaxation enhancement caused by Mn^{2+} ions (Figure 2B, Table II) give only distance information over a shorter range because these effects are proportional to r_i^{-6} and are not affected by orientation (eq 2; Knowles et al., 1976).

Cations such as Pr^{3+}, Yb^{3+}, and Mn^{2+} bind to the phosphate groups of phospholipid molecules in bilayers and serum lipoproteins and affect both nearby ^{13}C and the ^{31}P phosphate group resonances [e.g., see Shapiro et al. (1975), Sears et al. (1976), and Henderson et al. (1975)]. The cholesterol molecules in the core of HDL particles and lipid hydrocarbon chain methylene groups are too far removed from the cation binding site to be influenced (Table II, Figure 2B). The spectral characteristics of surface cholesterol and phospholipid molecules are altered when Mn^{2+} ions bind to the particle as indicated by the decrease in intensity (Figure 2B) and the reductions in T_1 values (Table II). The fact that the resonances from surface cholesterol molecules in HDL are not shifted by Pr^{3+} and Yb^{3+}, even when these ions are inducing maximal shifts in the $N(CH_3)_3$ ^{13}C resonances (Figure 2A), must be due to the value of θ because the effect on the chemical shift induced by these cations is of longer range than the relaxation enhancement effect of Mn^{2+} ions. The zero shifts for surface cholesterol molecules reported in Figure 2A can be explained if θ is close to half the tetrahedral angle (i.e., θ = the "magic" angle) so that the dipolar interaction between the unpaired electron on the shift reagent and the [4-^{13}C]-cholesterol nucleus is zero.

The Mn^{2+} relaxation enhancement experiments (Tables II) give further information on the location of surface cholesterol molecules because assuming that Mn^{2+} is bound to the phospholipid phosphate groups in all cases, it follows that the distance $r_{Mn\text{-}N(CH_3)_3}$ is the same in egg PC vesicles, HDL_2, and HDL_3; the glycerophosphocholine conformation is the same in all systems (Reijngoud et al., 1982). Consequently, the distance ratio in eq 3 gives a measure of the immersion of the cholesterol molecules, and its value of unity shows that the Mn^{2+} is essentially equidistant from the $N(CH_3)_3$ carbon atoms and the C-4 atom of surface-located cholesterol molecules. Comparison with the distance ratios of protons in the PC polar group from Gd^{3+} bound to the phosphate group [see Table III of Hauser et al. (1976)] indicates that protons in the $N(CH_3)_3$ group and the region of the first carbon atom of the acyl chains are approximately equidistant from the paramagnetic ion. It follows that to a first approximation in both PC bilayers and HDL the 4-^{13}C atom of cholesterol is located in the region of the phospholid acylcarboxyl group. This is in agreement with earlier neutron diffraction and ^{13}C NMR studies of PC/cholesterol bilayers which showed that the cholesterol C-3 atom is at the position of the glycerol/fatty acid ester bonds (Worcester & Franks, 1976) and the cholesterol C-4 is localized in the ester bond region of the bilayer (De Kruijff, 1978).

(*C*) *Surface/Core Exchange.* When the cholesterol and phospholipid molecules are juxtaposed in HDL as described above, the cholesterol hydroxyl groups are exposed to the aqueous phase, and the cholesterol molecules can transfer to other lipoprotein particles by desorption into the aqueous phase. Previous studies from our laboratory (Lund-Katz et al., 1982) have established the kinetics of this exchange process for the HDL/LDL system. At least 94% of the cholesterol molecules in HDL_3 are in a single kinetic pool for exchange, indicating that intraparticle movement of cholesterol between the core and surface microenvironments is more rapid than the desorption from the surface into the aqueous phase. It follows that the residence times of cholesterol molecules in HDL_2 and HDL_3 particles, about 360 and 240 s, respectively (Lund-Katz et al., 1982), are upper limits for the residence times in either the surface or the core location.

The fact that surface and core [4-^{13}C]cholesterols resonate 0.5 ppm apart (Figure 1A,B) rather than giving a single resonance at an average chemical shift means that the cholesterol molecules are undergoing slow exchange on the NMR time scale, and a lower limit for the residence time in either pool can be derived. Under these conditions, the lifetime (τ) of a nucleus in one or an other of the states leading to the two chemically shifted resonances is given by eq 4 (Abraham &

$$\tau \geq 2^{1/2}/(\pi\Delta\nu) \qquad (4)$$

Loftus, 1978). τ is given in seconds when the difference in chemical shift ($\Delta\nu$) between the two resonances is expressed in hertz. Under the spectral conditions of Figure 1, $\Delta\nu \simeq 45$ Hz for the surface and core cholesterol molecules so that from eq 4, $\tau \geq 10$ ms.

It follows that in the exchange between the surface and core of HDL, the cholesterol molecules at either location have residence times in the range 10 ms–ca. 300 s. This rapid exchange of cholesterol between the surface and core of HDL means that there are no large activation energy barriers that have to be surmounted. Since the cholesterol partitions almost equally between the surface and core of HDL, the free energy of cholesterol must be similar in both locations. The equilibrium constant is 0.67 so that the free energy of transfer of cholesterol molecules from the surface to the core is 0.24 kcal/mol.

Physiological Significance. HDL particles are thought to be involved in cellular cholesterol homeostasis in that they are acceptors for cholesterol molecules leaving a cell and are also involved in the conversion of cholesterol to cholesterol ester by the enzyme lecithin:cholesterol acyltransferase (LCAT) in the plasma (Glomset, 1968). The present findings show that although cholesterol molecules are in two physically distinct states in HDL particles, on the biological time scale they act as one pool. Thus, cholesterol molecules entering the surface of HDL by transfer from a cell or another lipoprotein particle will rapidly equilibrate with the pool of cholesterol molecules in the core. Conversely, as LCAT in association with apoA-I converts surface-located cholesterol molecules into cholesterol ester, additional substrate molecules from the core can enter the surface in the time scale 10 ms–300 s.

Human HDL_2 and HDL_3 have essentially the same structure with respect to the physical state of cholesterol molecules. Consequently, any differences in the metabolic roles of these two particles cannot be attributed directly to the behavior of the cholesterol molecules therein. Neither the surface nor the core microenvironments of HDL particles are saturated with cholesterol which is consistent with the role of HDL as the putative acceptor in the reverse transport of cholesterol from peripheral tissue to the liver [for reviews, see Tall & Small (1980) and Scanu et al. (1983)].

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Magnetic Resonance of a Monoclonal Anti-Spin-Label Antibody†

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ABSTRACT: The nuclear magnetic resonance spectra of monoclonal Fab antibody fragments have been recorded in the absence and presence of a specific spin-label dinitrophenyl hapten. The difference spectra reveal the presence of 11–12 aromatic amino acids in the region of the combining site. By selective deuteration of this hybridoma antibody, these amino acids have been identified as three tryptophans, six or seven tyrosines, one phenylalanine, and one histidine. Difference spectra have also been recorded that depend on ring-current chemical shifts. The latter difference spectra show that among the 11–12 amino acids there are two tyrosines and one tryptophan in close proximity to the dinitrophenyl (DNP) ring. On the basis of ultraviolet absorption measurements, it is concluded that this tryptophan is stacked against the DNP ring. Selective deuteration of hybridoma antibodies directed against a paramagnetic hapten provides a powerful new approach for the study of the structural basis of antibody diversity and specificity.

Antibody molecules play a crucial role in defense against infection. When confronted by almost any foreign molecule, the immune system is able to produce antibody proteins of high affinity and exquisite specificity. The structural basis of antibody specificity has been one of the major concerns of immunochemistry for many years. Statistical analysis of antibody sequences shows that this specificity is determined by relatively short segments, the hypervariable regions (Wu & Kabat, 1970). Crystallographic studies reveal that the combining sites are formed by these hypervariable regions [for recent reviews, see Amzel & Poljak (1979) and Pecht (1982)]. Most of the amino acids in the nonhypervariable region form a rigid, relatively invariant framework, termed the immunoglobulin fold. This framework consists of two layers of antiparallel β-pleated sheets held together by a disulfide bond and enclosing a hydrophobic interior.

Complexes formed between haptens and specifically elicited anti-hapten antibodies have not yet been investigated by X-ray crystallography or nuclear magnetic resonance (NMR)[1]

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[1] Abbreviations: Fab, antigen binding fragment of antibody; SL, spin-label (see Materials and Methods for structure); DNP, dinitrophenyl; DNP-Gly, (2,4-dinitrophenyl)glycine; EDTA, ethylenediaminetetraacetic acid; NO, nitroxide; Fv, variable-region fragment of antibody; FabSL and FabDNP-Gly, complexes of antibody with indicated antigen; Tris, tris(hydroxymethyl)aminomethane. D(amino acid) means protein was grown with indicated amino acids deuterated; H(amino acid) means that the spectral features are mainly from the indicated amino acid.

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