Natural Abundance $^{13}$C Nuclear Magnetic Resonance Studies of Human Plasma High Density Lipoproteins*

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High resolution proton-decoupled natural abundance $^{13}$C NMR spectra (at 15.18 MHz) of human plasma high density lipoproteins were recorded at 29°C, 35°C, and 43°C. Carbon 5 of unesterified cholesterol and the corresponding carbon of cholesteryl esters yield narrow, well resolved resonances. Values of nuclear Overhauser enhancements and integrated intensities for C-5 of free and esterified cholesterol, C-6 of cholesteryl esters, and the methyl groups on cationic nitrogen of phospholipids were measured. At 35°C, the ratio of integrated intensity of the C-5 resonance of cholesteryl ester to that of cholesterol, 5:1, is significantly different from the molar ratio of these species, 3:1, determined by chemical analysis. This result requires that at least two pools of cholesterol exist within the HDL particle and suggests that a substantial fraction of the cholesterol is sufficiently motionally restricted so as not to yield a detectable C-5 resonance. Comparison of integrated intensities of peaks assignable to cholesteryl esters and phospholipids suggests that all or a large fraction of the cholesteryl esters contribute to the high density lipoprotein spectrum. The integrated intensities of peaks reflecting cholesterol and cholesteryl esters do not change over the temperature range 29–43°C, suggesting that no major structural reorganization of these components involving phase changes or formation of highly ordered domains occurs over this temperature range.

The high density lipoproteins of human plasma are a heterogeneous collection of particles which are isolated by ultracentrifugal flotation over the density range 1.063 to 1.21 (1). The chemical composition, nature of the constituent polypeptides, chemical and enzymatic modification, immunological reactivity, and physical properties of HDL have been probed in considerable detail (1–4). The findings, together with results of spectroscopic studies of several kinds, have suggested a number of models for the structural organization of HDL (5–9); however, none of these can be considered to rest on firm foundations of experimental fact.

Among approaches to the understanding of the nature of lipid-lipid and lipid-protein interactions in HDL, which must determine the principal features of structural organization, are magnetic resonance studies of $^1$H (10–12), $^{31}$P (13–15), and $^{13}$C (9, 16, 17) nuclei. In addition, $^{13}$C NMR studies of certain reconstituted lipoprotein preparations have been reported (8, 9).

Previous efforts in the field of $^{13}$C NMR studies of human plasma HDL in this laboratory have (i) provided detailed, highly resolved spectra at 36–40°C; (ii) yielded assignments for most resolved resonances; and (iii) indicated spin-lattice relaxation times for a number of lipid carbon atoms and provided a semiquantitative analysis of these results in terms of segmental and rotational motions (16, 17). This work has provided little information concerning the effect of temperature on the molecular dynamics of the lipids of HDL as reflected in $^{13}$C NMR spectra and the relative contribution of different lipid classes to the spectra has not been determined. In an effort to provide information pertinent to these matters, as well as to extend previous observations, we have collected the following new information: (i) linewidths for several well-resolved lipid resonances at 29°C, 35°C, and 43°C; (ii) values of NOE for several lipid resonances at 35°C; and (iii) quantitation of the relative contributions of cholesterol, cholesteryl esters, and phospholipids to the $^{13}$C NMR spectrum at 35°C.

**EXPERIMENTAL PROCEDURES**

High density lipoproteins were isolated from pooled freshly collected serum of three healthy, fasting adults by ultracentrifugal flotation in KBr in the presence of $5 \times 10^{-4} \text{M} \text{EDTA}$ (18). HDL was obtained in the density range 1.063 to 1.210 g/cm$^3$ and was not subfractionated. HDL was further purified by increasing the density to 1.250 g/cm$^3$ with solid KBr, layering 1.5-ml portions under 25 ml of KBr solution of density 1.210 g/cm$^3$ and subsequently centrifuging for 12 h at 105,000 $\times g$. The upper 1-ml fractions containing the purified HDL were collected, pooled, and dialyzed for 48 h against 0.01 M potassium phosphate buffer, pH 6.0, containing 0.2 M KBr, 0.1% sodium azide, and 1% Na$_2$EDTA. Throughout HDL isolation and purification, 1 mm DTNB was present in order to inactivate lecithin:cholesterol acyltransferase.

The purity of the isolated HDL fraction was determined by electrophoresis in 1% agarose in barbital/barbiturate buffer, ionic strength 0.05, pH 8.6, and by immunodiffusion in 1% agarose gels at 25°C according to the method of Ouchterlony (19) using rabbit antibodies produced against human plasma HDL and LDL. Preparations used in all experiments were electrophoretically homogene-
ous and reacted with anti-HDL but not with anti-LDL antiserum. Immunodiffusion reaction of HDL with anti-total human serum yielded a single precipitin line, further indicating immunochromatographic purity of the sample.

Total protein was determined by the method of Lowry et al. (20), employing crystalline bovine serum albumin as reference protein.

The concentration of lipid was determined by dry weight. Aliquots of the lipid extract were analyzed for neutral lipids by thin layer chromatography. Samples were applied to precoated Silica Gel G plates (20 x 20 cm) (Sil G-25 HR, Brinkmann Instruments, Inc.). The solvent system was petroleum ether-diethyl ether-acetic acid (volume ratio, 85:15:1). Plates were developed by exposure of standards and one column of HDL lipids to iodine vapor, the remaining portion of the plate being covered with aluminum foil. The lipids were identified by comparison of the Rf values with those for appropriate standards. The unexposed regions corresponding to unesterified cholesterol and cholesteryl esters were scraped off the plates and their concentrations were determined quantitatively by chemical analysis, using the method of Bowman and Wolf (21).

Prior to NMR measurements, lipoprotein samples were concentrated to 140 mg/ml by dialysis against Sephadex G-200 (Pharmacia Fine Chemicals). Fully proton-decoupled $^{13}$C NMR spectra were obtained essentially as described (16, 17, 22). Nuclear Overhauser enhancement measurements were made by the gated decoupling method (23, 24). The delay period of 2.7 s with no proton decoupling was chosen to be at least 4 times the value of T1 for the carbon resonances of interest, based on T1 values obtained previously (16) and on estimated upper limits for the T1 values of C-5 of the cholesterol and cholesteryl esters (based on intensities in spectra recorded with the use of recycle times of 1.1 s and 2.2 s). Chemical shifts were measured as described (16). They are reported in parts per million downfield from Me$_4$Si.

**RESULTS**

Proton-decoupled natural abundance $^{13}$C NMR spectra of human plasma HDL (140 mg/ml) were recorded at 29°C, 35°C, and 43°C, at 15.18 MHz; the 35°C spectrum recorded using a recycle time of 1.1 s is shown in Fig. 1. A spectrum recorded at the same temperature but using a recycle time of 2.2 s revealed no features additional to those obtained using the shorter recycle time. When 2 carbon atoms have different spin-lattice relaxation times (T1), their resonances may have different intensities if the recycle time is not much longer than the T1 values (25). This spectrum, as well as those recorded at 29°C and 43°C, is similar to the ones previously reported at 35°C (16, 17) but reveals additional detail. In addition to those previously assigned, there are several new observable resonances, as a consequence of higher signal/noise ratio.

The most significant new resonance is that at 141.3 ppm downfield from Me$_4$Si, Peak 3 in Fig. 1, which in previous spectra (16) was barely above signal/noise level. This peak is assigned to the C-5 ring carbon of unesterified cholesterol, while the more intense C-5 resonance (Peak 4) is assigned to esterified cholesterol exclusively. Chemical shift data for these assignments are presented in Table I, and the complete rationale for these assignments is presented later (see "Discussion"). The sharp carbonyl resonances previously attributed to lipid carbonyl atoms (Peaks 1 and 2 in Fig. 1) are assigned specifically to phospholipid carbonyl (Peak 1) and cholesteryl ester and triglyceride carbonyl (Peak 2), based on chemical shift data obtained with aqueous and anhydrous model systems and on the relative intensities of the two peaks in the $^{13}$C spectra of VLDL, LDL, and HDL (16), which have markedly differing phospholipid/cholesteryl ester/triglyceride ratios (2). The assignments for phospholipid carbonyl and cholesteryl ester carbonyl are in accord with assignments made from the $^{13}$C enrichment techniques of Assmann et al. (9).

Experimental values of integrated intensities from spectra recorded with full proton-decoupling and gated proton-decoupling are collected in Table II. Values of NOE were calculated as the ratio of integrated intensity of the fully decoupled resonance to the value obtained with gated decoupling. The NOE is an increase in the intensity of a $^{13}$C resonance as a result of proton decoupling. Different carbon atoms may have different values of the NOE. Therefore, knowledge of the NOE is required before one can use the relative intensities of $^{13}$C resonances in spectra with proton-decoupling for quantitative analytical purposes (25). Relative integrated intensities under conditions of gated decoupling, which eliminates NOE contribution to peak intensities, are compared with those expected on the basis of the chemical composition of HDL. The relative integrated intensities for cholesteryl esters and choline-containing phospholipids are in good agreement with analytical chemical data. In contrast, the measured intensity of the C-5 resonance of unesterified cholesterol is <0.5 of that anticipated on the basis of the cholesterol content of HDL. Chemical determination of the ratio of cholesteryl esters to unesterified cholesterol in the HDL sample used in this work was carried out immediately following isolation of the HDL sample and again following the NMR experiments to ensure that possible enzymatic activity had not altered this ratio. A ratio of 2.9 ± 0.2 was obtained. Comparing this value with that for the relative corrected integrated intensities (Table III) suggests that a significant fraction of the HDL cholesterol is not observed in the NMR experiment.

Linewidths for several resonances of lipids of HDL were measured at 29°C, 36°C, and 43°C. Those for unesterified cholesterol C-5, the methyl groups on cationic nitrogen of choline-containing phospholipids, and the terminal methyl group of fatty acyl chains are in the range 5 to 7 Hz and are independent of temperature over the range studied. We estimate that the instrumental contributions (field inhomogeneity, field drift, incomplete proton decoupling, digital broadening) are near 2 to 3 Hz. In addition, chemical shift heterogeneity may contribute to observed linewidths. Therefore, it is not feasible to use the observed linewidths for these reso-

<table>
<thead>
<tr>
<th>Assignment</th>
<th>Peak No. in Fig. 1</th>
<th>HDL</th>
<th>Cholesteryl olate and free cholesterol in triolein</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-5 of free cholesterol</td>
<td>3</td>
<td>141.2</td>
<td>141.2</td>
</tr>
<tr>
<td>C-5 of cholesteryl ester</td>
<td>4</td>
<td>139.7</td>
<td>139.7</td>
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<tr>
<td>C-6 of cholesteryl ester</td>
<td>5</td>
<td>122.3</td>
<td>122.3</td>
</tr>
<tr>
<td>C-4 of free cholesterol</td>
<td>6</td>
<td>---</td>
<td>120.0</td>
</tr>
<tr>
<td>C-3 of cholesteryl ester</td>
<td>7</td>
<td>73.1</td>
<td>73.1</td>
</tr>
<tr>
<td>C-3 of free cholesterol</td>
<td>8</td>
<td>71.0</td>
<td>---</td>
</tr>
</tbody>
</table>

1 For a 3/1 mole ratio of ester/free cholesterol dissolved in excess triolein to form an isotropic solution.
2 May contribute to Peak 5.
3 Probably contributes to Peak 6.
4 May contribute to Peak 6.

These assignments are consistent with two distinct environments for lipid carbonyl carbon atoms, an aqueous (phospholipid) and nonaqueous (cholesterol ester and triglyceride) environment. These chemical shift differences may be a result of hydrogen-bonding to phospholipid carbonyls and a lack of such bonding to triglyceride and cholesteryl ester carbonyls (26).

J. A. Hamilton and N. J. Oppenheimer, unpublished observations.

### Table I

<table>
<thead>
<tr>
<th>Assignment</th>
<th>Peak No. in Fig. 1</th>
<th>HDL</th>
<th>Cholesteryl olate and free cholesterol in triolein</th>
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</thead>
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<tr>
<td>C-5 of free cholesterol</td>
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<td>C-6 of cholesteryl ester</td>
<td>5</td>
<td>122.3</td>
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<tr>
<td>C-4 of free cholesterol</td>
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<td>C-3 of cholesteryl ester</td>
<td>7</td>
<td>71.0</td>
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<td>8</td>
<td>71.0</td>
<td>---</td>
</tr>
</tbody>
</table>

For a 3/1 mole ratio of ester/free cholesterol dissolved in excess triolein to form an isotropic solution.

May contribute to Peak 5.

Probably contributes to Peak 6.

May contribute to Peak 6.
Fig. 1. Proton-decoupled natural abundance $^{13}$C NMR spectrum of human plasma HDL (140 mg/ml, at 35°C, pH 8.0), recorded at 15.18 MHz, with a recycle time of 1.105 s and 131,072 accumulations. Other conditions were essentially as in Ref. 16.

<table>
<thead>
<tr>
<th>Carbon atom</th>
<th>Integrated intensity</th>
<th>NOE$^a$</th>
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<tbody>
<tr>
<td></td>
<td>Decoupled</td>
<td>Gated decoupled</td>
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<tr>
<td>Cholesterol C-5</td>
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<td>2.0</td>
</tr>
<tr>
<td>Cholesteryl C-5</td>
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<td>10.2</td>
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<td>Cholesterol C-6 (total)</td>
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<td>10.5</td>
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<td>Choline (CH$_3$)$_2$N$^+$</td>
<td>103.1</td>
<td>39.6</td>
</tr>
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</table>

$^a$ Ratio of intensities with full proton decoupling and gated proton decoupling.

Values of integrated intensities, nuclear Overhauser enhancements, and linewidths for selected $^{13}$C resonances of human plasma HDL at 35°C

NMR spectra were recorded on a sample containing 140 mg/ml of human plasma HDL at pH 8.0. For fully proton-decoupled spectra, a recycle time of 1.1 s was employed; spectra were recorded following acquisition of 131,072 transients. Gated decoupled spectra were obtained with a 3.8 s recycle time.

Integrated intensities in spectra recorded with full proton decoupling were obtained for the unesterified cholesterol C-5 resonance (Peak 3), the esterified cholesterol C-5 resonance (Peak 4), the total cholesterol C-6 resonance (Peak 5), the choline N$^{+}$(CH$_3$)$_2$ resonance (Peak 8), and the fatty acyl CH$_3$ resonance (Peak 9) at 29°C and 43°C under the accumulation conditions described in Fig. 1. The estimated maximum error for the intensity measurements is ±15%; except for the unesterified cholesterol C-5 and the total cholesterol C-6 (±20%). The estimated error limits, except for the choline N$^{+}$(CH$_3$)$_2$ and CH$_3$ which showed increases at low temperature, just outside the estimated error of measurement. Such changes may reflect a small increase in T$_1$ values at higher temperatures, since the recycle time employed was <3.5 × T$_1$ for these resonances.

DISCUSSION

In the present study we have used concentrated solutions of HDL (140 mg of lipoprotein/ml) and longer recycle times than previously employed (16) to reveal additional features of the $^{13}$C NMR spectrum and to make possible the quantification of certain NMR parameters not previously reported for HDL. The concentration independence of $^{13}$C NMR spectra of solutions of HDL has been established previously (16). Except for improved signal to noise ratios, the $^{13}$C spectrum at 35°C is essentially identical with that presented in Ref. 16.

The assignment of distinctive $^{13}$C resonances to unesterified and esterified cholesterol and the quantification of these species is a crucial step in understanding the details of the rather complex NMR spectrum of HDL. Several carbon atoms of cholesterol and cholesteryl acetate in organic solvents have distinctive $^{13}$C chemical shifts for corresponding carbon atoms (28). When cholesterol and cholesteryl oleate are dissolved in triolein, the only corresponding carbon atoms which yield distinctive peaks assignable specifically to the esterified and unesterified species are the resonances for the C-3, C-5, and C-6 carbon atoms of the cholesterol ring (Table I). The unesterified cholesterol C-3 resonance falls at 71.0 ppm, which is a crowded region in HDL spectra (Fig. 1). Thus, the unambiguous assignment of cholesterol resonances to the unesterified and esterified form is easiest for C-6 and C-5.

On the basis of the chemical shift data (Table I), the weak resonance at 141.2 ppm in Fig. 1 (Peak 3) is assigned to the sterol ring carbon C-5 of unesterified cholesterol and the more intense resonance at 139.7 ppm (Peak 4), to C-5 of...
cholesterol esters. Based on the relative intensities of the C-5 resonances for free and esterified cholesterol, the remaining peaks assigned to cholesterol carbon atoms (17) reflect cholesterol esters, primarily. The question of whether the C-6 of unesterified cholesterol contributes to the C-6 ester resonance at 122.3 ppm is not answerable on the basis of these data alone. Because the C-6 resonance is broader, a weak resonance for unesterified cholesterol might not be clearly resolved but could contribute to the observed asymmetry of the peak. Recently, however, a narrow resonance at 120.7 ppm has been observed in spectra of HDL obtained at high magnetic field (63.4 kG), suggesting that unesterified cholesterol makes some contribution to the 122.3 ppm resonance in the lowfield spectra (29).

The ratio of the integrated intensity of the cholesteryl ester C-5 resonance to that for the cholesterol C-5 resonance (Table III) is clearly greater than the chemically determined molar ratio for our sample. Two explanations can be advanced to explain this result: (a) a portion of the unesterified cholesterol has a C-5 resonance whose chemical shift corresponds to that of the ester and hence contributes to the peak at 139.7 ppm; (b) a portion of the unesterified cholesterol has a relatively broad (undetectable) resonance. In view of the small variance in chemical shifts for these resonances in a variety of lipid systems, we feel that the latter explanation is the more likely. However, it is important to note that the casuistic conclusion derived from the experimental observation, that there is more than one pool of cholesterol in human plasma HDL, holds regardless of which explanation one chooses.

The ratio of intensities for the choline N+(CH3)3 resonance and the cholesteryl ester C-5 resonance in human plasma HDL corrected for measured differences in NOE (Table II) is in close agreement with the corresponding ratio based on chemical analysis. In spectra of pig serum HDL, about 93% of the N+(CH3)3 groups contribute to the narrow proton NMR resonance (12). If it is assumed that the same holds true for the 13C NMR resonance in human HDL, the choline resonance provides an internal standard. Our data suggest that most or all cholesteryl ester molecules contribute to the C-5 resonance for cholesteryl esters in human HDL while about 50% or less of the unesterified cholesterol molecules contribute to the narrow cholesterol C-5 resonance. Should less than 90 to 100% of the choline N+(CH3)3 groups in phospholipids of HDL contribute to the narrow choline resonance, the absolute amount of cholesteryl esters and cholesterol contributing to the C-5 resonances would be correspondingly smaller.

The observation that a substantial fraction of unesterified cholesterol molecules does not contribute to the 13C NMR spectrum of human HDL contrasts with interpretations of integrated area measurements in proton NMR spectra of human HDL (11) and pig HDL (12), which led to the conclusion that virtually all of both forms of cholesterol molecules contribute to these spectra. However, no resolved resonances specifically attributable to cholesterol or cholesteryl esters were observed in these studies and the fractional decrease in the regions which were integrated in the proton spectra would be too small to detect a loss of 50% of the cholesterol molecules.

The integrated intensity of the unesterified cholesterol C-5 resonance did not change between 29°C and 43°C, showing that the sizes of the two cholesterol domains did not change over this temperature range. Since nascent HDL is a site of esterification of cholesterol in the plasma by the enzyme lecithin:cholesterol acyltransferase (30), the observation of two domains of cholesterol may have metabolic significance.

We have previously demonstrated that the cholesterol ring moiety occupies a region within the HDL particle that is of much higher microviscosity than that of the surrounding aqueous medium but similar to the viscosity of triolein (16). This conclusion applies to the cholesteryl esters, and not to unesterified cholesterol, since it is based on data for the C-6 resonance at 122.3 ppm, which represents cholesteryl esters primarily. The principal additional finding which resulted from work reported herein is that more than one pool of cholesterol is present in HDL. In addition, the results of this study extend our previous conclusions (16, 17) in three significant respects: (a) both the measured NOE and linewidth of the cholesteryl ester C-6 resonance are in disagreement with theoretical values of these parameters calculated from the measured T1 value (16) using a mathematical model which assumes isotropic reorientation; this discrepancy is probably a reflection of the anisotropic reorientation of the steroid ring of cholesteryl esters in HDL (29); (b) the C-6 linewidth exhibits a temperature dependence which is similar to the temperature dependence found for concentrated cholesteryl ester-triglyceride solutions which do not undergo liquid/liquid crystalline transitions (31); (c) the size of the liquid cholesteryl ester domain does not change significantly between 43°C and 29°C, since the integrated intensities of the cholesteryl C-5 resonance and the total cholesterol C-6 resonance are unchanged between these two temperatures.

Recent small angle x-ray scattering studies of HDL are consistent with a spherical particle containing an outer layer of protein and polar groups of phospholipids (and probably unesterified cholesterol) and an inner hydrocarbon core of neutral lipids (32). Our observation of at least two pools of cholesterol is consistent with a model in which a fraction of cholesterol is dissolved in the neutral lipid core with the remainder relatively immobilized, possibly formed in a complex with polar lipids, proteins, or both.

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Natural abundance 13C nuclear magnetic resonance studies of human plasma high density lipoproteins.
E M Avila, J A Hamilton, J A Harmony, A Allerhand and E H Cordes


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