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# $^{31}$P Nuclear Magnetic Resonance Spectroscopy of Native and Recombined Lipoproteins

**(lipid–protein binding/high- and low-density lipoproteins/europium-lipoprotein interactions/ sphingomyelin-phosphorus ratio of lipoproteins/chemical shift and relaxation times of lipoprotein constituents)**

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**ABSTRACT** **Native and recombined lipoproteins have been studied by $^{31}$P nuclear magnetic resonance spectroscopy. Very low-, low-, and high-density lipoproteins exhibited characteristic spectra. The main resonances were assigned to phosphatidylcholine and sphingomyelin. Relaxation times for these phospholipids were separately measured in low-density lipoproteins and high-density lipoproteins. The effect of paramagnetic ions ($Eu^{+++}$) on the nuclear magnetic resonance spectrum of high-density lipoproteins is reported.**

The use of nuclear magnetic resonance (NMR) has emerged as a useful tool for examining lipid–lipid and lipid–protein interactions. Recently, human serum lipoproteins have been examined by proton and $^{13}$C NMR spectroscopy (1–6). To date, however, $^{31}$P studies of human lipoproteins have not been reported. Different groups of phospholipids, each defined according to their polar head group moiety, are present to a different extent in lipoprotein classes. $^{31}$P nuclear magnetic resonance spectroscopy might contribute to the understanding of the arrangement and spatial organization of the molecules within these particles. The purpose of this study was to provide data on the $^{31}$P NMR spectra (Fourier transform technique) of human very low-density lipoproteins (VLDL), low-density lipoproteins (LDL), high-density lipoproteins (HDL) and *in vitro* reconstituted lipoproteins. Various resonances have been assigned to the appropriate P atoms, and spin lattice relaxation times ($T_1$) of phosphatidylcholine and sphingomyelin in LDL and HDL have been separately measured. The effect of paramagnetic ions ($Eu^{+++}$) on the NMR spectrum of HDL is reported.

## MATERIALS AND METHODS

*NMR Spectroscopy.* All NMR spectra were obtained with a Varian XL-100-15 spectrometer operating at 40.48 MHz modified for Fourier transform by Digilab, Inc. The pulse modulator/amplifier supplied 30-$\mu$sec pulses at 1.3-sec intervals into a cross coil probe. This arrangement gave a tipping angle of about 56°. Field/frequency stabilization was obtained from the deuterium resonance of the solvent. Proton decoupling was accomplished with a broad band modulator and amplifier of Varian design. Free induction decay data were averaged with a Nova computer using 16 k of data storage and a 12 kHz digitation rate corresponding to 6 kHz sweep width. Thus, the transformed spectra had maximum resolution of 0.7 Hz. In general, between 2 and 10 thousand transients were averaged before multiplication of the accumulated free induction decay by an exponentially decaying function. The chemical shifts reported are believed accurate to within ±0.1 ppm. $T_1$'s are considered accurate to within 10%. In experiments employing $Eu^{+++}$, small volumes of Tris buffer, pD = 7, I = 0.01, containing appropriate amounts of $Eu(NO_3)_3$ (Merck, Sharp & Dohme), were added to the HDL solution, which had previously been exhaustively dialyzed against the above buffer. The sample was vortexed gently during the addition of the paramagnetic ions in order to obtain rapid mixing.

*Isolation of Lipoprotein and Apolipoprotein Fractions.* Plasma was obtained from fasting normal volunteers by plasmapheresis. The plasma was collected in 0.01% disodium EDTA, pH 7.5, and used within 24 hr for the isolation of lipoprotein fractions. VLDL were isolated at plasma density (1.006) by ultracentrifugal flotation at 5°, LDL and HDL were isolated by ultracentrifugal flotation between KBr densities 1.019 and 1.063 and 1.063–1.21 g/ml, respectively. All fractions were washed twice by ultracentrifugation at the respective densities used for isolation. After the final wash, VLDL, LDL, and HDL were desalted by dialysis for 72 hr against 100 volumes of 0.01% disodium EDTA, pH 8. The concentration of some preparations was increased by ultrafiltration to a maximum of 5–10 mg of protein per ml. Prior to NMR analysis, samples were dialyzed against 0.05 M $NH_4HCO_3$ in $D_2O$, pD 8.6. HDL was delipidated with chloroform–methanol as previously described (7). ApoA-II, one of the major peptides of HDL, was isolated according to Shore and Shore (8). ApoHDL or apoA-II were solubilized in 0.1 M $NH_4HCO_3$ buffer, pH 8.6, and used in a concentration of 5 mg/ml for recombination experiments.

*Lipoprotein Phospholipid Analysis.* Phosphorus in LDL and HDL and recombined lipoproteins was determined on the total lipid extract by the technique of Bartlett (10). The relative amounts of phosphatidylcholine and sphingomyelin were determined in duplicate on a separate aliquot of the original total lipid extract by two dimensional thin-layer chromatography on plates of silica gel F-254 using chloroform–methanol–ammonium hydroxide–water, 180:105:75:7.5, in the first dimension and chloroform–methanol–am-

Abbreviations: NMR, nuclear magnetic resonance; VLDL, very low-density lipoproteins; LDL, low-density lipoproteins; HDL, high-density lipoproteins; PC, phosphatidylcholine; SPM, sphingomyelin; PG, phosphatidylglycerol.

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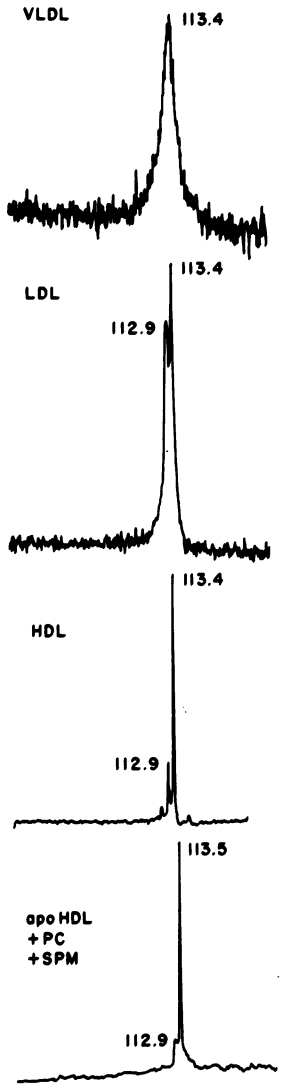


FIG. 1. Phosphorus NMR spectra of native and recombined lipoproteins. Chemical shifts were assigned to phosphatidylcholine (113.5) and sphingomyelin (112.9) as indicated in *Results*. The protein concentration of individual samples was 5–10 mg/ml. Reassembly experiments were done as outlined in *Methods*.

monium hydroxide–water, 120:160:5.5, in the second dimension (11). To visualize the lipids, the plates were stained with iodine vapor; appropriate sectors were scraped off, eluted with chloroform–methanol–water, 100:50:10, and phosphorus determined.

*Glycerophosphate Esters.* Diacyl phosphoglycerides (phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol) were selectively hydrolyzed using methanolic sodium hydroxide which preferentially removes the fatty acids, and leaves the phosphate ester linkages intact (12). The resulting sodium salts of the phosphodiesters were passed through a column of Dowex 50 hydrogen ion-exchange resin using deionized water. The aqueous glycerophosphate ester solutions recovered from the resin were concentrated by lyophilization. The glycerophosphate esters were analyzed as their trimethylsilyl (TMS) derivatives using the direct inlet systems of an LKB 9000 mass spectrometer (13). Sphingosinephosphorylcholine was obtained by acid hydrolysis of sphingomyelin as described earlier (14). Sphingosine bases

TABLE 1. *$^{31}P$ chemical shift data for native lipoproteins, recombined lipoproteins, and selected reference compounds*

| Compound | Chemical shift, ppm from $P_4O_6$* |
|---|---|
| 85% $H_3PO_4$ | 112.5 |
| Phosphorylcholine | 109.4 |
| L-α-Glycerylmonophosphate | 108.3 |
| L-β-Glycerylmonophosphate | 108.6 |
| L-α-Glyceryldiphosphate | 108.5 |
| | 109.0 |
| L-α-Glycerylphosphorylcholine | 112.8 |
| L-α-Glycerylphosphorylethanolamine | 112.1 |
| L-α-Glycerylphosphorylinositol | 112.6 |
| L-α-Glycerylphosphorylserine | 112.9 |
| D-erythro-sphingosinephosphorylcholine | 113.0 |
| | 113.2 |
| L-α-Lysolecithin in $D_2O$ | 113.0 |
| L-α-Lecithin in $D_2O$ | 113.5 |
| Sphingomyelin in $D_2O$ | 112.9 |
| ApoHDL + L-α-lecithin | 113.5 |
| ApoHDL + sphingomyelin | 112.9 |
| ApoA-II + L-α-lecithin | 113.5 |
| ApoA-II + sphingomyelin | 112.9 |
| HDL | 112.9 |
| | 113.5 |
| LDL | 112.9 |
| | 113.5 |

* $P_4O_6$ was used as external standard instead of 85% $H_3PO_4$ to eliminate overlap of resonance bands. Chemical shifts of glycero-mono- and diesters were measured at pD 8; shifts of native and recombined lipoproteins as well as phosphatidylcholine (lecithin) and sphingomyelin were measured at pD 8.6.

liberated during acid hydrolysis (15) were isolated by thin-layer chromatography (chloroform–methanol–water, 65:25:4) and analyzed as their TMS-N-Acetyl-derivatives by gas-chromatography (16). More than 95% of the base mixture consisted of erythro-C18:1 sphingosine. Glycerylmono- and diphosphate were obtained commercially (Pierce Chemicals). Glycerophosphate esters and sphingosinephosphorylcholine were taken up in 100 mM $NH_4HCO_3$ in $D_2O$, pH 8.

*Reassembly Experiments.* [*methyl*-$^3$H]Phosphatidylcholine (specific activity $3.5 \times 10^6$ cpm/μmol) and [*methyl*-$^{14}$C]-sphingomyelin (specific activity, $2.8 \times 10^5$ cpm/μmol) were prepared according to the method of Stoffel *et al.* (17). The purity of the lipid fractions was ascertained as previously described (9). Phosphatidylcholine (PC) (60 mM) and sphingomyelin (SPM) (15 mM) were sonicated in 50 mM $NH_4HCO_3$ buffer, pH 8.6, and yielded clear dispersions on sonication for 15–30 min. Sonicated PC and SPM vesicles were combined, vortexed gently, and immediately added dropwise to a solution of apoHDL in 4 ml of 0.1 M $NH_4HCO_3$ buffer (5 mg of protein per ml). After incubation for 60 min at room temperature in a Dubnoff shaking water bath, the protein–lipid solution was adjusted to 1.063 g/ml with solid KBr and spun for 48 hr in a 40.3 rotor at 39,000 rpm. The infranatant fractions (3 ml) were adjusted to 1.25 g/ml and spun for 48 hr at 39,000 rpm. The lipid–protein complex was found in the supernatant while small amounts of residual unbound protein were in the infranatant. The supernatant fraction was analyzed for protein by the procedure of Lowry *et al.* (18) and for phospholipid

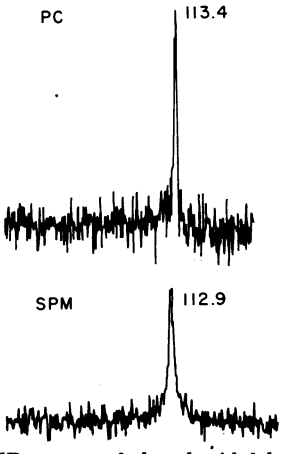


FIG. 2. $^{31}$P NMR spectra of phosphatidylcholine (50 mM) and sphingomyelin (50 mM), each sonicated in $D_2O$ for 5 min at 4°.

by counting the radioactivity individually present in PC and SPM. Separation and isolation of both lipids was obtained by thin-layer chromatography as described above. In similar recombination experiments, apoA-II was added to either sonicated PC or sonicated SPM solutions in equal amounts (w/w) and the recombined particles isolated by flotation between KBr densities 1.063–1.25 g/ml. Before analysis by $^{31}$P NMR spectroscopy, all samples were exhaustively dialyzed against 0.05 M $NH_4HCO_3$ buffer, pD 8.6.

## RESULTS

Table 1 gives the $^{31}$P chemical shifts for some reference materials and recombined lipid-apoprotein systems together with shifts for native VLDL, LDL, and HDL. In each of the two native lipoproteins, two main resonances were observed together with several minor resonances. The two major resonances had a reproducible relationship in all HDL and LDL samples examined (five each). Fig. 1 shows the $^{31}$P spectra of native and recombined lipoprotein fractions. The major HDL and LDL phospholipids are PC and SPM; the chemical shifts of these major resonances do, indeed, correspond to these lipids when they are either sonicated in $D_2O$ buffer (Fig. 2)

TABLE 2. *Ratio of PC and SPM in native lipoproteins*

| Sample | Relative peak height of PC/SPM resonances | PC/SPM phosphorus ratio* |
|---|---|---|
| HDL† I | 4.2 | 4.3 |
| II | 4.3 | 4.6 |
| III | 4.2 | 4.2 |
| IV | 4.2 | 4.4 |
| V | 4.2 | 4.2 |
| LDL† I | 1.4 | 1.6 |
| II | 1.3 | 1.7 |
| III | 1.3 | 1.3 |
| IV | 1.4 | 1.2 |
| V | 1.3 | 1.5 |

* $\delta P_4O_6$ was used as reference instead of 85% $H_3PO_4$ to eliminate overlap of resonance bands.

† Human LDL and HDL were isolated as described in *Methods* and used for NMR analysis with a protein concentration of 5–10 mg/ml.

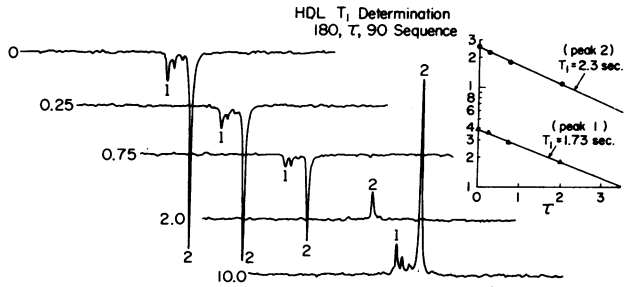


FIG. 3. Determination of relaxation time, $T_1$, for phosphatidylcholine (peak 2) and sphingomyelin (peak 1) in human HDL. Numbers (0, 0.25, 0.75, and 1.0) indicate T values employed.

or recombined individually with apoHDL or apoA-II (Table 1). Criteria used to establish lipid–protein interaction are presented in the accompanying paper (9). The SPM versus PC phosphorus shift difference is on the order of 0.5–0.6 ppm. No integrations were obtained, but if one compares the peak heights (the half-band widths were approximately the same), a 4.2:1 PC to SPM ratio is obtained for the HDL and 1.3:1 for the LDL; these are consistent with chemical determinations (Table 2).

Strictly on the basis of chemical shift criteria, it appears that the magnetic environment of the phosphorylcholine head groups of PC and SPM are not significantly disturbed by the lipid–protein interaction. However, it is well known from phosphorus magnetic shift theory (19) that nearest-neighbor electronic interactions dominate. With long-range effects and bond distortions having only minor contributions to the phosphate chemical shift, it should be expected that the diester would determine the chemical shift. This does not rule out the use of shift criteria in the future for lipid–protein binding since increased accuracy and multiple determination may, in fact, show a consistent, if small, shift pattern in lipid–protein interaction.

Spin lattice relaxation times ($T_1$) were obtained for native HDL (Fig. 3, Table 1). Systematic determinations of $T_1$'s obtained by comparing sonicated lipid vesicles and recombined lipid–protein systems, will be reported elsewhere (20).

Half band width ($\nu_{1/2}$) measurements on sonicated lipids versus native HDL, and recombined lipid–apoprotein systems suggested that protein binding is concomitant with narrower phosphorus resonances. One interpretation of this might be that the protein has preference for vesicles of specific diameter. This concept was supported by the uniform particle size of the recombined lipoprotein systems studied (9).

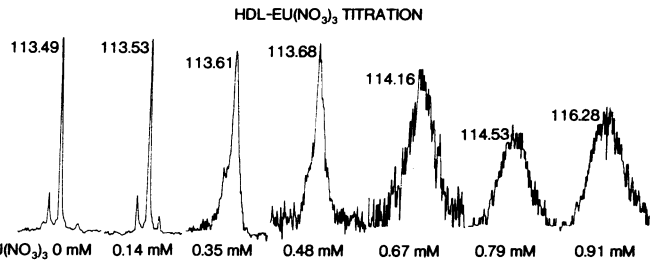


FIG. 4. Effect of $Eu^{+++}(NO_3)_3$ concentration on HDL phosphatidylcholine and sphingomyelin phosphorus resonances. Chemical shift data are given in ppm.

Fig. 4 shows spectra of HDL to which increasing amounts of $Eu^{+++}$ were added. The phosphorus peaks broadened and shifted with each increment of paramagnetic species added. No residual peak was observed at the original chemical shift, suggesting that all of the lipid was available for binding.

## DISCUSSION

The structural organization and modes of interaction between the major lipid and protein components of the various lipoprotein classes obtained from human serum are poorly understood. Spectroscopic techniques, including optical rotary disperson (21), circular dichroism (22–25), nuclear magnetic resonance (1, 2), electron spin resonance (26–29), infrared- (25, 30) and fluorescence (31) studies have been applied to native and reconstituted high-density lipoproteins and have provided valuable information on the conformation, environment, and interaction of individual components. The detailed description of the overall structure of HDL, however, has so far depended on electron microscopy and x-ray scattering studies. Unfortunately, these techniques have come to controversial conclusions. Based on electron microscopy data, a subunit structure of HDL, with an electron-dense central core surrounded by 4 to 5 subunits, each with its own complement of protein, phospholipid, and neutral lipids, has been proposed (32, 33). On the other hand, data derived from x-ray techniques indicate that HDL has an organized symmetrical structure with a relatively electron-poor central (nonpolar) region and a relatively electron-rich (polar) outer-region (34). Although there is suggestive evidence, primarily derived from enzymatic studies (35, 36), that the majority of phospholipids are located on the surface of native HDL particles, firm conclusions could not be drawn. No information has thus far been obtained on the location of sphingomyelin, one of the most abundant phospholipids in HDL. We have demonstrated with $^{31}P$ NMR spectroscopy that HDL and LDL exhibit spectra characterized by two major resonances, corresponding to PC (high-field signal) and SPM (low-field signal). The high-field resonance accounted for 80 and 60% of the total signal area, and the low-field resonance for 20 and 40%, in HDL and LDL, respectively. The ratios of the peak heights corresponded to the ratios of PC to SPM ascertained by chemical analysis. The assignment of the resonances was based on the spectroscopic behavior of PC and SPM, which were studied individually as sonicated vesicles or in recombination experiments with apolipoproteins. Thus, the relative ratio of PC and SPM, when recombined in different ratios with apoHDL, could be determined. Moreover, the clear separation of these resonances allowed the individual determination of $T_1$'s.

It has been repeatedly demonstrated that paramagnetic ions (shift reagents) (37) permit differentiation of the internal and external surfaces of membranes without their impairment (38–40). In aqueous PC dispersions containing paramagnetic ions, the $N^+$ $(CH_3)_3$ signal of the PC molecules in the lipid bilayer consists of two components. One component corresponds to internal PC molecules which are in contact with the ion free internal aqueous phase. The other component is shifted to a high field and corresponds to molecules at the external surface contact with the bulk salt solution (38). A differential effect of $Eu^{+++}$ on sonicated phosphatidylglycerol (PG) and sonicated PC vesicles has been recently observed by phosphorus magnetic resonance spectroscopy (40). By contrast with PC, the addition of $Eu^{+++}$ to sonicated PG dispersions did not significantly shift but rather broadened the phosphorus peak. This finding was attributed to the fact that $Eu^{+++}$ binds more strongly to PG than PC. We have established that gradual increase of $Eu^{+++}$ concentration in a solution of HDL results in both broadening and shifting of the phosphorus resonances. Because of the considerable broadening effect, SPM and PC phosphorus resonances are no longer separated above 0.5 mM $Eu^{+++}$ concentration. Physical characteristics of HDL in the presence of the highest $Eu^{+++}$ concentrations (1 mM) employed appear to be unchanged, as ascertained by ultracentrifugal flotation (1.063–1.21) and circular dichroism (9), indicating that no significant structural perturbation of HDL had occurred. Since, as in the case of sonicated PC vesicles, no unshifted component corresponding to the original peak is left, we conclude that, essentially all phospholipid phosphorus is located at the outer surface of the HDL particles.


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