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DOI: 10.1016/0005-2760(74)90101-5

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³¹P NUCLEAR MAGNETIC RESONANCE: APPLICATION TO THE STUDY OF HUMAN SERUM HIGH DENSITY LIPOPROTEINS

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(Received November 26th, 1973)

SUMMARY

Highly resolved ³¹P nuclear magnetic resonance (³¹P NMR) spectra were obtained from human serum high density lipoproteins. The spectral shifts obtained were interpreted as reflecting an influence of countercations on the polar phosphate head-groups of the phospholipids found in these lipoproteins. The results indicated that ³¹P NMR is a potentially useful technique in the study of the structure of serum lipoproteins.

The circulating serum lipoproteins are complexes of lipids and proteins the primary physiological function of which is the transport of lipids among tissues [1]. At present, the composition of these lipoproteins is reasonably well established [2]. However, the manner in which the lipid and protein are assembled into the lipoproteins is a subject on which much less information is available [3]. Very few experimental data are available on the location and microenvironment of the major lipid class, the phospholipids, in these complexes [4].

We have been using the technique of ³¹P nuclear magnetic resonance (³¹P NMR) to probe the structures of several substances of biological origin [5-8, and collaborative efforts with Khorana, H. G. and Barzilay, I., involving structure determinations of purified lipopolysaccharides]. This technique has proved to be a valuable analytical tool in these studies principally because of the simplicity of the spectra and because of the relative sensitivity of this 100% naturally abundant nuclide. The range of chemical shifts [19], approx. 760 ppm, is many times greater than for proton NMR, and thus may permit the resolution of signals from nearly identical chemical groups [6, 10, 11]. Further, the shifts of orthophosphate and its esters are sensitive to pH [9] and to the nature of the countercation(s) in solution [12].

Abbreviation: HDL₃, high-density lipoprotein fraction 3.

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In this study, we have applied the technique of ^{31}P NMR [13] to the analysis of human serum high-density lipoprotein fraction 3 (HDL₃). This lipoprotein contains 55% protein and 45% lipids [2]. Of the lipid content, 50% is in the form of phospholipids, which are comprised of 77% phosphatidylcholine, 9% sphingomyelin, and 5% lysophosphatidylcholine; the remaining 9% are represented by phosphatidylinositol, phosphatidylserine, phosphatidylethanolamine, and polyglycerophosphatides [2]. High density lipoprotein fraction 3 (HDL₃) is water-soluble, has a molecular weight of about 175000, and an average diameter of 65 Å [2].

The phosphorus resonance experiments were performed on a Bruker HFX-5 NMR spectrometer operating at 36.43 MHz for ^{31}P (^1H field, 90.0 MHz) and incorporating facilities for all modes of continuous-wave and broad-band decoupling between ^{31}P and ^1H , signal averaging of spectra, and the taking of Fourier-transform signal averaged spectra. Large-bore spinning sample tubes (10 or 13 mm) were employed. Details of the instrumentation have been described [5, 10]. In some instances, the samples contained 10% D₂O which served as an internal reference. The spectra, taken in the presence or absence of deuterium were identical. As is customary for ^{31}P NMR, positive chemical shifts were associated with increasing magnetic field strength; the reference was a sealed capillary tube containing 85% orthophosphoric acid.

The HDL₃ samples were isolated and purified from human serum by preparative ultracentrifugation as previously described [14]. Before spectral analysis, HDL₃ was extensively dialyzed for two days against the desired cation in buffer consisting of 0.15 M cation chloride, containing 0.05% cation EDTA, pH 7.2. The experiments which involved the alkaline earth cation, Mg²⁺, were performed by titration of HDL₃, in the presence of Na⁺ counterion, with a one-molar MgCl₂ solution at neutral pH. The phospholipid dispersions were also prepared in the presence of sodium ions as described previously [16].

In earlier studies involving isolated phospholipids we had determined that in a number of solvents phosphatidylcholine comes into resonance at a field about 1 ppm higher than that for any of the other phospholipids present in HDL₃. Moreover, smaller shift differences were found between sphingomyelin, phosphatidylethanolamine, lysophosphatidylcholine, phosphatidylinositol, and phosphatidylserine [8], the other phospholipid classes present in HDL₃. Thus, even though the lipoproteins are of high molecular weight, it appeared worth while to determine whether ^{31}P NMR could be applied to the study of the structural organization of phospholipids in HDL₃.

As can be seen from the data presented in this report, ^{31}P NMR spectra of high quality can be obtained from HDL₃, and these provide a good resolution of the phospholipid resonances. Fig. 1A shows the ^{31}P NMR spectrum obtained from an aqueous phospholipid dispersion composed of one part phosphatidylcholine and one part sphingomyelin. The spectrum is typical for such dispersions in that the signals, although resolved, are broad and lack any informative detail [17]. On the other hand, the spectra from HDL₃ (Fig. 1B-E), taken under similar experimental conditions and presented at the same sweep width, are highly resolved and exhibit considerable detail. In all cases, the large signal at 0.9 ppm is attributed to phosphatidylcholine. This interpretation agrees with the known shift position of phosphatidylcholine in a number of solvents [8], and also with the known amount present in HDL₃ [2].

The spectrum of HDL₃ in the presence of Na⁺ countercation (Fig. 1B) exhibited an additional smaller resonance, most likely arising from the remaining phos-

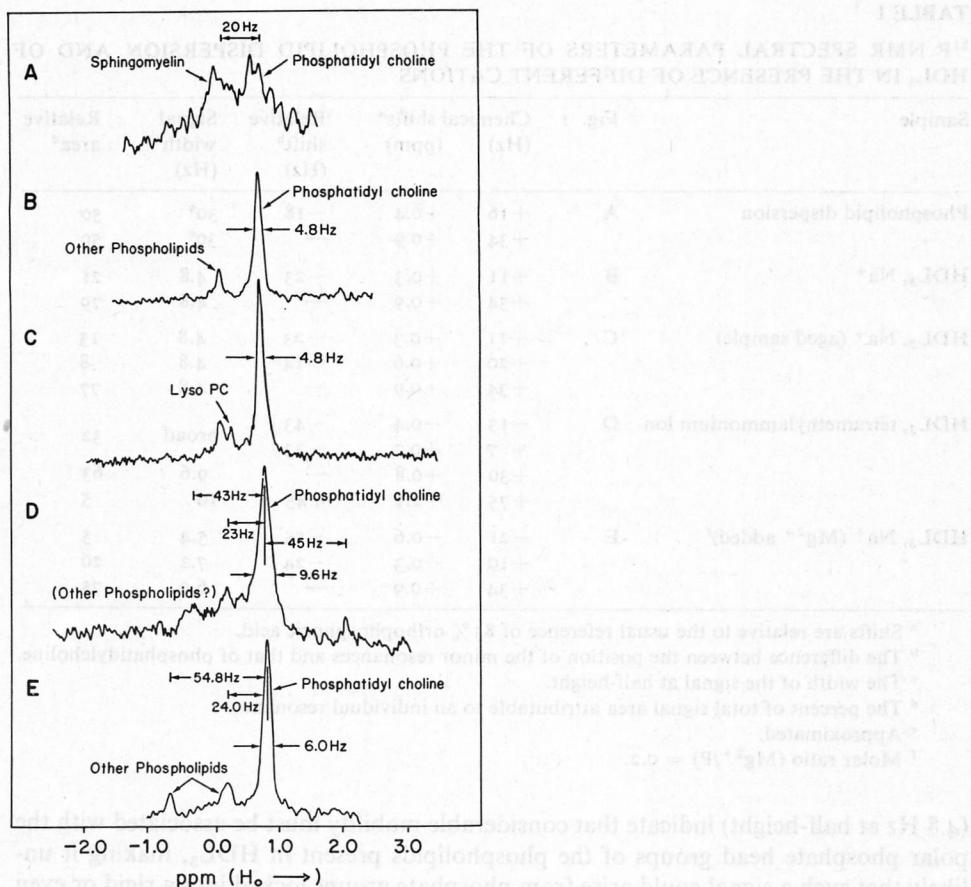


Fig. 1. ^{31}P NMR spectra from a phospholipid dispersion and from samples of human serum lipoprotein HDL_3 (about 15 mg/ml) in the presence of various metal ions: A, spectrum from a phospholipid dispersion composed of phosphatidylcholine and sphingomyelin (1:1, wt/wt) with sodium as the cation; B, HDL_3 in the presence of Na^+ countercation; C, aged HDL_3 in the presence of Na^+ countercation; D, HDL_3 in the presence of tetramethylammonium countercation; E, HDL_3 in the presence of Na^+ countercation to which MgCl_2 has been added (ratio of Mg^{2+} to P is 1:5). Spectra A, B, D, and E are signal-averaged continuous-wave recordings taken during broad-band decoupling of the protons. A sealed capillary containing C_6F_6 served as the coaxial reference (lock) signal; 2–4 days of signal-averaging time were required. Sweep width, 4 Hz/cm (120 or 240 Hz); sweep time, 3.7 Hz/s. Spectrum C is a ^{31}P Fourier-transform spectrum also taken during broad-band decoupling of the protons. The reference signal in this case was the deuterium signal from the HDO in the water solvent. One half day was required for signal averaging; a Nicolet 1085 (16 K) computer was employed. Pulse width, 4.5 μs ; delay time, 238 μs ; sweep width, 2100 Hz (238 μs per data point, 16384 data points); cycling time, 4 s. All spectra are displayed with the same horizontal scale. Spectral parameters are presented in Table I.

pholipids. Two aspects of this spectrum are worth noting. One is the narrowness of both resonance signals; the other is the fact that the smaller signal comes into resonance at one position even though the phospholipids contributing to that resonance exhibit a significant shift difference when the individual dispersions are studied in other solvents [8]. The narrow line widths of HDL_3 in the presence of Na^+ counterions

TABLE I

³¹P NMR SPECTRAL PARAMETERS OF THE PHOSPHOLIPID DISPERSION AND OF HDL₃ IN THE PRESENCE OF DIFFERENT CATIONS

Sample	Fig. 1	Chemical shifts ^a (Hz)	Chemical shifts ^a (ppm)	Relative shift ^b (Hz)	Signal width ^c (Hz)	Relative area ^d
Phospholipid dispersion	A	+16 +34	+0.4 +0.9	-18 —	30 ^e 30 ^e	50 50
HDL ₃ , Na ⁺	B	+11 +34	+0.3 +0.9	-23 —	4.8 4.8	21 79
HDL ₃ , Na ⁺ (aged sample)	C	+11 +20 +34	+0.3 +0.6 +0.9	-23 -14 —	4.8 4.8 4.8	15 8 77
HDL ₃ , tetramethylammonium ion	D	-13 +7 +30 +75	-0.4 +0.2 +0.8 +2.1	-43 -23 — +45	broad 9.6 10	32 63 5
HDL ₃ , Na ⁺ (Mg ²⁺ added) ^f	E	-21 +10 +34	-0.6 +0.3 +0.9	-55 -24 —	5.4 7.2 6.0	5 20 75

^a Shifts are relative to the usual reference of 85% orthophosphoric acid.

^b The difference between the position of the minor resonances and that of phosphatidylcholine.

^c The width of the signal at half-height.

^d The percent of total signal area attributable to an individual resonance.

^e Approximated.

^f Molar ratio (Mg²⁺/P) = 0.2.

(4.8 Hz at half-height) indicate that considerable mobility must be associated with the polar phosphate head groups of the phospholipids present in HDL₃, making it unlikely that such a signal could arise from phosphate groups locked into a rigid or even semirigid lattice network. More information on this aspect is expected from relaxation time studies currently under study, which are aimed at an evaluation of the degree of mobility of the polar head groups in HDL₃.

The single resonance signal (0.3 ppm) generated by the remaining phospholipids indicated that their phosphorus atoms are in equivalent magnetic environments; the significance of these findings in regard to the HDL₃ structure remains undetermined, however.

One interesting example of the usefulness of ³¹P NMR in the study of HDL₃ is shown in Fig. 1C. The HDL₃ in this example (in the presence of Na⁺ countercation) was stored at 6 °C for a period greater than six months and exhibited rotational and circular dichroic properties similar to those of fresh preparations of HDL₃. The ³¹P NMR spectrum obtained from the aged HDL₃, however, showed two resonances at a field lower than that of phosphatidylcholine. By chemical analysis, the additional resonance (0.6 ppm) was found to correspond to lysophosphatidylcholine, which is a known artifact found in aged samples [15]. In this instance, therefore, ³¹P NMR proved to be a sensitive probe which indicated chemical changes in the intact HDL₃ that would not be detectable by other nondestructive techniques (e.g. analytical ultracentrifugation; circular dichroic techniques).

The spectrum obtained from HDL₃ in the presence of tetramethylammonium countercation shows a still greater divergence in the observed resonance signals (Fig. 1D). Relative area measurements of this spectrum indicate that at least 10% of the minor resonances arise from phosphatidylcholine phosphates. Thus, in this case there are differences in the local magnetic fields around some of the phosphatidylcholine residues, suggesting that these residues may be either located in a modified environment or oriented in HDL₃ in a manner which differs from that of the bulk of the phosphatidylcholine molecules. Spectrum 1E was taken at a point in the titration of HDL₃ (in the presence of Na⁺ countercation with Mg²⁺ ion) which yielded the greatest amount of spectral information (the ratio of Mg²⁺ to total phosphorus was 1:5, mole/mole). Area measurements suggest that all of the phosphatidylcholine resonances reside in the major signal. In consequence, the low field signals arise from the remaining phospholipids in HDL₃. The signal at -0.6 ppm, not present in the sample prior to addition of Mg²⁺ (Fig. 1B), corresponds to the position obtained with an aqueous sphingomyelin dispersion in the presence of Na⁺ countercation; it remains to be established, however, whether this resonance can be assigned to the sphingomyelin of HDL₃ in the presence of Mg²⁺.

As the concentration of Mg²⁺ was increased above a ratio of Mg²⁺ to total phosphorus of 1:5 (mole/mole), the resonance bands coalesced into broad signals (20–30 Hz) showing little or no fine structure. In fact, the signals could be obliterated altogether by the addition of sufficient alkaline earth metal ion. This apparent loss of signal is the result of extensive broadening which distributes the signal area over an extended frequency band so that it cannot be detected above the spectral noise level.

TABLE II

EFFECT OF DIFFERENT CATIONS ON THE FLOTATIONAL AND CIRCULAR DICHROIC PROPERTIES OF HDL₃

The s_{20} values are uncorrected to water or infinite sample dilution. The HDL₃ concentration was 15 mg/ml. Both flotational and circular dichroic parameters are for solution of 0.15 M cation chloride, 0.05% cation EDTA, pH 7.2.

Cation	$s_{20} \times 10^{13}$ (cm · s ⁻¹)	Ellipticity (θ) (10^{-4} degree · cm · dmole ⁻¹)	
		285 nm band	222 nm band
Na ⁺	4.55	-2.45	-2.37
Tetramethylammonium ion	4.61	-2.64	-2.42
Mg ²⁺	4.57	-2.54	-2.45

The flotational properties of HDL₃ in the analytical ultracentrifuge and its circular dichroic spectral properties in the presence of the different countercations suggest no gross structural changes in the HDL₃ particle with any of these ions (Table II).

From the current studies it is clear that ³¹P NMR can be an effective tool for the study of the properties of the phospholipids of HDL₃, and probably of the other serum lipoproteins*. The method appears sensitive to subtle perturbations of the environ-

* This result is corroborated by ³¹P NMR studies, currently under investigation, with human serum low density lipoproteins and high-density lipoprotein fraction 2.

ment of these lipoproteins, and thus allows for their study under conditions close to their native state. The judicious use of countercations can lead to the selective shifting of phospholipid resonances, and it should be possible to translate this, in turn, into information on fine structural detail. Further, the information derived from the spectra does not need to reflect only the disposition of the phospholipids. Perturbations of other lipoprotein components, e.g., the proteins, cholesterol, cholesterol esters, triglycerides, or the solvent water itself, which are transmitted to the phospholipids, will be reflected in the phospholipid spectra.

The narrowness of the phospholipid ^{31}P signal in HDL₃, which allows for the resolution of the separate phospholipid resonance bands, is a fortunate experimental fact, but one that does not lend itself to a straight-forward interpretation. The narrow signal means that the magnetic field gradients about the phosphorus atoms are being effectively averaged. Precisely how this is accomplished by a complex with a molecular weight of about 175000 is not clear. The solution of this problem could shed considerable light on the structure of HDL₃ as well as of lipoproteins in general, and on the dynamic relationships operating between their lipid and protein components.

ACKNOWLEDGMENTS

Supported by a grant from the General Research Support Grant of the College of Medicine of the University of Illinois, Grants U.S.P.H.S.-NS09345, U.S.P.H.S.-11702 (T.G. and T.O.H.); Grants No. HL 08727 and HL 06481 from the U.S.P.H.S., the Illinois and Chicago Heart Association (A72-6), and the Atomic Energy Commission (A.M.S.). A.M. Scanu is a recipient of U.S.P.H.S. Career Development Award No. HL 24867. A.W. Kruski is a recipient of U.S.P.H.S. Post-Doctoral Fellowship No. HL 52970. T.O. Henderson gratefully acknowledges the helpful suggestion of Dr Ronald H. Hirz in the initial phases of this research.

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