Structure of Human Serum Lipoproteins: Nuclear Magnetic Resonance Supports a Micellar Model

Abstract. *High-resolution proton nuclear magnetic resonance spectra of low- and high-density lipoproteins from human serum closely resemble those of dispersions of lipoprotein lipids in water. Linewidths of hydrocarbon proton absorptions are not increased in the lipoproteins. In contrast, apolar binding of lysolecithin on serum albumin causes extensive line-broadening and an upfield chemical shift of the hydrocarbon proton resonances of lysolecithin. The results are consistent with a predominantly micellar structure for the lipoproteins rather than with extensive hydrophobic association of lipid and protein.*

Although serum lipoproteins have been studied for many years, the nature of lipid-protein interaction in these and many other lipoproteins remains unclear (*1*). Amphiphilic molecules such as the phospholipids are capable of both polar and apolar binding to proteins. In micellar systems the hydrocarbon chains of fatty acids mutually associate, and the interaction between lipid and protein is predominantly polar. With apolar binding the hydrocarbon chains of the lipids are associated with apolar amino acid residues on the apoprotein by dispersion forces and hydrophobic bonding. Polar and apolar interactions are molecularly quite different, but experimental approaches which distinguish between the two modes of attachment of lipids have been lacking.

High-resolution proton nuclear magnetic resonance (NMR) has been used to examine detergent micelles (*2*); this technique shows promise for the elucidation of the state of lipids in biological membranes (*3*). We report here a proton NMR study of the low-density (β) and high-density (α) lipoproteins from human serum.

The lipoproteins were isolated from fresh human serum and washed by flotation in solutions of NaBr, buffered at *p*H 7.4 with 0.01*M* phosphate, containing 0.005*M* EDTA. Centrifugations were carried out for 20 to 24 hours at 40,000 rev/min (Spinco 50 rotor). Both the low-density (1.006 to 1.070) and high-density (1.070 to 1.210) preparations appeared pure as judged by behavior in the analytical ultracentrifuge, and they moved as one band upon electrophoresis on cellulose acetate in barbital buffer, *p*H 8.6. The lipoproteins were concentrated by flotation in solutions (density 1.5) of NaBr in D_2O and were prepared for NMR by dialysis against three changes (10 volumes each) of 0.1*M* NaCl in D_2O. Lipids were extracted with mixtures of chloroform and methanol (2:1) and washed (*4*), then freeze-dried from benzene and dissolved in $CDCl_3$ or dispersed in D_2O by sonication for 5 to 10 minutes in an ice bath. Bovine serum albumin and lysolecithin were obtained commercially.

Spectra were obtained at 35°C in a temperature-controlled spectrometer (Varian A60A) with an external reference of sodium trimethylsilylpropane sulfonate in a coaxial tube. For comparison of peak areas in the lipoprotein and lipid extracted from the lipoprotein, the areas were referred to a common external coaxial reference of 10 percent benzene (by volume) in CCl_4 or of D_2O containing 30 percent H_2SO_4 and 1 percent $CuSO_4 \cdot 5H_2O$. Quantitative results were obtained by phosphorus (*5*) determinations. Spectral assignments were made according to Chapman (*6*) and by experiments on known lipids dissolved in $CDCl_3$ or sonically dispersed in D_2O. Chemical shifts are reported on the τ scale.

Nuclear magnetic resonance is a valuable technique for binding studies because linewidths of absorptions by individual groups of protons are functions of spatial constraints upon molecular motion. When molecules have freedom of motion, correlation times are short, local magnetic fields are time-averaged, and the absorption lines are narrow. When molecular motion is restricted, correlation times and linewidths increase. Broadened lines can result, for example, when solvent viscosity is very high or when molecular associations occur. Most globular proteins do not exhibit high-resolution spectra because the protons are constrained. Consequently, the linewidths of protons in small molecules bound to proteins are greatly increased to values characteristic of the macromolecule (*7*).

Thus NMR can show which lipid protons are immobilized by binding to proteins. This principle is illustrated in Fig. 1 for the binding of lysolecithin by bovine serum albumin. The lines for the methyl and methylene protons of the hydrocarbon chains and the methyl protons of the quaternary amine of the choline moiety are of particular interest because they reflect the binding of the apolar and polar portions of the molecules. In the absence of serum albumin, the narrow lines indicate rather unrestricted molecular motion for all three sets of protons in lysolecithin micelles; with protein present, the hydrocarbon lines are selectively broadened. Added protein does not affect the chemical shift (6.72 ppm), the linewidth (2 hz), or the peak area of the amine protons. However, the linewidths for the absorption of methylene and methyl protons progressively broaden with increasing concentration of protein until at high concentrations of protein no discrete lines can be observed. Line-broadening is accompanied by an upfield shift of 5 to 10 hz from

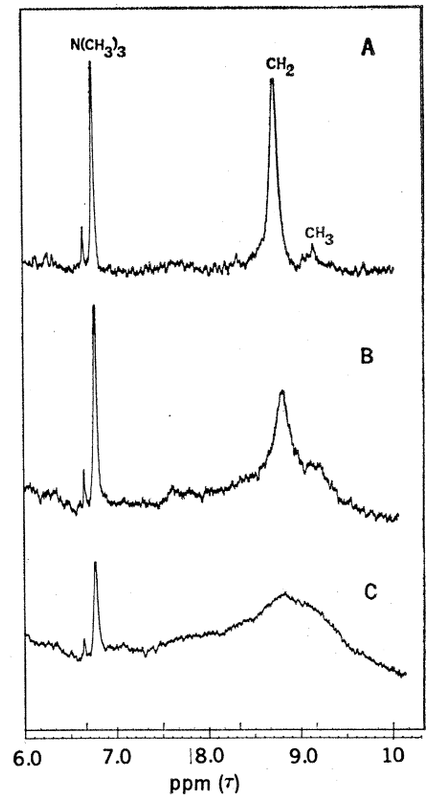

Fig. 1. The effect of bovine serum albumin upon the NMR spectrum of 0.5 percent (weight-volume) lysolecithin in 0.1*M* NaCl D_2O: (A) No protein added; (B) 3 percent (weight-volume) protein present; (C) 10 percent protein present. In (C) the amplitude setting of the spectrometer was half that of (A) and (B). All the solutions were optically clear with no evidence of precipitation.

the position for micelles without protein (8.70 ppm). Shifts of this magnitude could arise from ring currents if some of the fatty acid chains were bound near residues of aromatic amino acids.

We believe that the selective broadening of the absorption lines of the hydrocarbon protons in the lysolecithin–serum albumin system indicates that the fatty acid chains of the lysolecithin molecules are bound by apolar association and are consequently constrained, whereas the polar ends are unbound and unconstrained. Thus the binding of lysolecithin to serum albumin appears to be hydrophobic.

In order to interpret the serum lipoprotein results, we investigated the NMR spectra of low-density lipoprotein lipids dispersed in D_2O by sonication (Fig. 2). Lipids from the high-density lipoproteins give essentially the same results. Although in organic solvents, such as a mixture of $CDCl_3$ and CD_3OD, the broad bands in the region from 7.0 to 8.2 ppm are partially resolved into their individual components, intermolecular association in water increases linewidth so that only the envelopes of these minor absorptions are observed. However, the lines of major interest are well resolved and reasonably narrow. These are the methylene protons at 8.69 ppm, the olefinic protons at 4.66 ppm from the hydrocarbon chains, and the methyl protons of the quaternary ammonium group at 6.74 ppm from the choline moiety of lecithin. Absorptions from cholesterol, cholesterol esters, and the terminal methyl groups of hydrocarbon chains occur in the neighborhood of 9.1 ppm. Linewidths of phospholipids in water are not as narrow as those of the lysolecithin spectrum (Fig. 1). This result is expected, since the behavior of lysolecithin in water is similar to that of a detergent, whereas most diacyl phospholipids do not have measurable critical micellar concentrations and usually produce a bilayer structure in water (*8*). When compared to those of conventional detergent micelles, the broader linewidths observed with sonicated phospholipids suggest that the molecules are slightly more constrained but are still quite mobile.

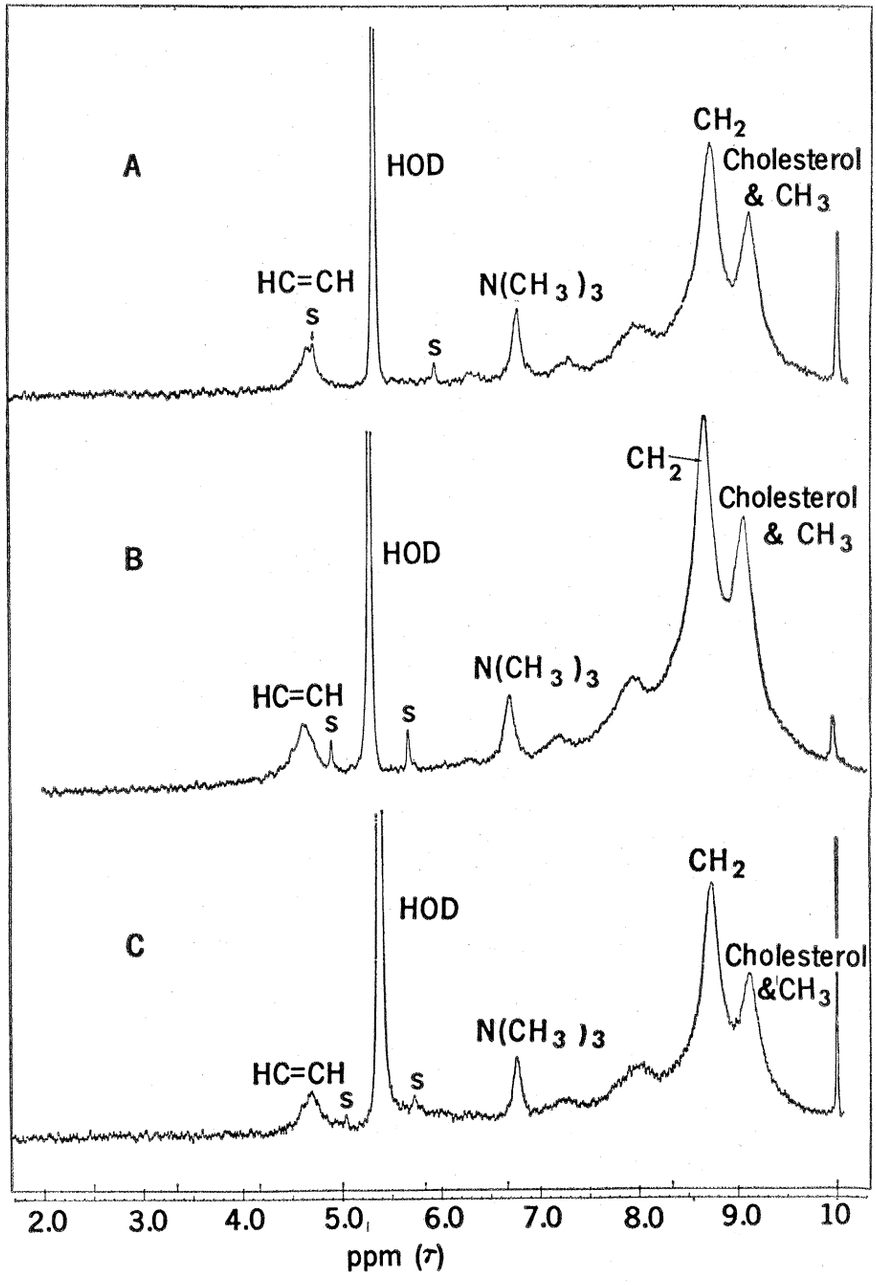

Fig. 2. The NMR spectra of (A) low-density lipoprotein lipids dispersed in D_2O by sonication, 512 μg of phosphorus per milliliter; (B) low-density serum lipoproteins in 0.1*M* NaCl-D_2O, 625 μg of lipid phosphorus per milliliter; (C) high-density serum lipoproteins in 0.1*M* NaCl-D_2O, 832 μg of lipid phosphorus per milliliter. The spectrum for high-density lipoprotein lipids dispersed in D_2O was essentially identical to (A). Spinning side bands are labeled *S*.

Because of unsaturation, the hydrocarbon chains in water dispersions of most naturally occurring phospholipids at room temperature are in a liquid-like state, well above a liquid-solid transition temperature (*9*). Dispersions of synthetic saturated phospholipids such as L-α-dipalmitoyl lecithin show such phase transitions (*10*). Resonance lines, present above the transition temperature, vanish below the transition temperature. We therefore conclude that the high-resolution spectra obtained from the lipoprotein lipids in water indicate that both the polar and apolar ends of the molecules are mobile. The steroids are also sufficiently mobile to give rise to absorption and apparently are solubilized in the liquid-like interior of the phospholipid micelles.

The NMR spectra of both the high- and low-density serum lipoproteins are quite similar, and both closely resemble the spectra of the lipoprotein lipids sonically dispersed in water. All the peaks resolved in the lipoproteins are accounted for by lipid protons. Line-

widths of the methylene, olefin, and steroid absorptions observed in the protein-free lipids in D_2O are not broadened in the native lipoproteins. We conclude that in the lipoproteins, unlike the lysolecithin–serum albumin system, the lipid hydrocarbons are not constrained in motion by association with protein. In addition, unlike the lysolecithin–serum albumin system, the methylene protons in the lipoproteins show no upfield chemical shift. As with the lipoprotein lipids dispersed in D_2O, the cholesterol molecules appear to be mobile and are consequently solubilized by inclusion in the phospholipid micelles.

Since only a small fraction of the lipid molecules in the lipoproteins may be sufficiently unconstrained to produce motional narrowing of resonance lines, proton counting by comparison of peak areas in the lipoproteins and the protein-free lipid is essential. This operation was carried out for both classes of lipoproteins by integration of the total area under the peaks assigned to steroids and fatty acid hydrocarbons. In both the high- and low-density lipoproteins, about 95 percent of the proton absorption in the hydrocarbon region can be accounted for by lipids. Because of inaccuracies inherent in the integration procedure and because protein would contribute a small amount of absorption in the region integrated, a small fraction of the lipid molecules, possibly 5 or 10 percent, may be constrained in motion, and hence bound by apolar association. However, apolar binding does not appear to be the major mode of lipid-protein association.

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Boll Weevil Found in Pre-Columbian Cotton from Mexico

Abstract. *A well-preserved, teneral adult female boll weevil,* Anthonomus grandis *Boheman (broad sense), was discovered in fragments of a cultivated cotton boll found in Guila Nacquitz Cave, Level A, dated about A.D. 900, near Mitla, Oaxaca, Mexico. This find antedates any previously known association of the boll weevil with cultivated cotton by about 900 years and negates the contention that this association began in the 18th century. The specimen is intermediate in form between* Anthonomus grandis grandis *Boheman and the thurberia weevil,* Anthonomus grandis thurberiae *Pierce.*

Archeological excavations made under the direction of Flannery (*1*) in the Oaxaca Valley of Mexico furnished us with numerous samples of cotton fragments including boll segments, seeds, and fiber. Although size of the boll is small, the fragments can be readily identified as *Gossypium hirsutum* L. All fragments studied to date are from Guila Nacquitz Cave (near Mitla, Oaxaca), Level A, which is dated about A.D. 900 (Monte Alban IV cultural horizon).

While dissecting an intact lock, Stephens found a well-preserved adult boll weevil, *Anthonomus grandis* Boheman (broad sense), that had failed to emerge from the pupal chamber within the cotton seed (*2*). This lock included five seeds, two of them empty shells. Also the boll wall immediately adjacent to the pupal chamber containing the weevil had a small (2.0-mm diameter) circular perforation, apparently the emergence hole of another weevil or beetle that had occupied that pupal chamber. A piece of cast skin with a setiferous tubercle was found in a second chamber in the lock (Fig. 1).

As currently understood, the species *Anthonomus grandis* Boheman (*3*) includes three infraspecific forms, two treated as subspecies, *Anthonomus grandis grandis* Boheman, the economically important common boll weevil and *Anthonomus grandis thurberiae* Pierce, which is normally associated with the wild cotton *Gossypium thurberi* Todaro and is not of significant economic importance. The third form includes intergrades between the two subspecies. Such intergrades have been collected in cultivated cotton and in wild cotton other than *G. thurberi.*

Three taxonomic characters (*3*) are used by taxonomists to distinguish adults of infraspecific forms of *Anthonomus grandis,* namely, the curvature of the setae of the pronotum, the sculpture of the metepisternum, and the shape and sculpture of the scutellum. By resort to these characters, it is possi-

Fig. 1. Dissected cotton lock in which boll weevil was found. Specimen is shown at right beneath millimeter scale.