Proc. Nat. Acad. Sci. USA
Vol. 71, No. 9, pp. 3696–3700, September 1974

^{13}C Nuclear Magnetic Resonance Spectroscopic Evidence for Hydrophobic Lipid–Protein Interactions in Human High Density Lipoproteins

(reassembling of [^{13}C]lipids and high density apolipoproteins/spin lattice relaxation)

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Communicated by E. H. Ahrens, Jr., May 31, 1974

ABSTRACT **Phosphatidylcholines, sphingomyelins, cholesterol, and cholesterol esters were enriched with ^{13}C by chemical synthesis in specific positions of their hydrophilic groups and aliphatic chains. Their spin-lattice relaxation times were determined in organic solvents. The substances were organized as liposomes and recombined with total human high density apolipoproteins and the two separated main components, apolipoprotein A-I (apoLp-Gln-I) and apolipoprotein A-II (apoLp-Gln-II).**

These ^{13}C nuclear magnetic resonance data established that in reassembled high density lipoproteins the phospholipid molecules bind to the apoprotein moieties with their hydrophobic fatty acid chains and not with their hydrophilic zwitterionic groups. Apolipoprotein A-I preferentially binds phosphatidylcholine, although its lipid-binding capacity is smaller than that of apolipoprotein A-II. Apolipoprotein A-II avidly reassembles with sphingomyelin by hydrophobic interactions. A model of the molecular organization of the high density lipoprotein particle has been derived.

^{13}C nuclear magnetic resonance (NMR) has many advantages over several physical methods for the study of lipid–lipid and lipid–protein interactions in lipoproteins and membranes since the ^{13}C atoms are internal probes with no perturbation of the structure. This probe can be exactly localized in complex structures, and its sharp resonance lines with well-separated chemical shifts can be reliably assigned. Furthermore, the spin-lattice relaxation rates (T_1) of the individual carbon atoms give insight into their mobility, depending on the molecular geometry, segmental molecular motion, viscosity of the surroundings (solvents), and noncovalent interactions.

The draw-back of the low natural abundance (1.1%) can be overcome by the chemical synthesis of simple and complex lipid molecules in which the ^{13}C isotope is enriched in the carbon atoms of choice. Suitable methods have been elaborated in this laboratory for the synthesis of the predominant lipid components of lipoproteins and membranes: [*26*-^{13}C]cholesterol, [*N*-$^{13}CH_3$]phosphatidylcholine, and [*N*-$^{13}CH_3$]-sphingomyelin (1). The most common mono- and polyunsaturated fatty acids were labeled in distinct carbon atoms of their alkyl chains and introduced into the 2-position of phosphatidylcholine, into sphingomyelin, and into cholesterol esters (2).

Abbreviations: HDL, high density serum lipoproteins; apo-HDL, delipidated proteins of HDL; apoA-I (apoLp-Gln-I) and apoA-II (apoLp-Gln-II), two main protein components of HDL; PC, phosphatidylcholine; SPM, sphingomyelin; NMR, nuclear magnetic resonance.

The enrichment of ^{13}C in the hydrophilic and hydrophobic moiety of these lipids increases the sensitivity of the method and allows the determination of T_1 times of special regions of these molecules in reasonable periods of time. It should give insight into hydrophilic and hydrophobic interactions of lipid–protein complexes.

A readily accessible system for the study of lipid–lipid and lipid–protein interactions in their natural molecular organization is the high density lipoprotein (HDL) fraction of human serum. Detailed information is available on the lipid and protein composition of these particles. Two apoproteins, apoA-I and apoA-II, comprise almost 90% of the molecule, with a high degree of α-helical structure (61–70%) (3–9). The amino-acid sequence of the latter has been elucidated (10). The apoproteins are assembled with an approximately equal amount of lipids. The lipid components are mainly phosphatidylcholine, sphingomyelin, cholesterol esters, and cholesterol. The acyl residues of the ester lipids are predominantly unsaturated fatty acids (oleic and linoleic acid).

The molecular organization of the molecules in the HDL particle relates closely to other important questions concerning the structure and interactions of lipids and proteins in more complex lipoprotein aggregates, such as biological membranes.

Several models of the HDL structure have been proposed (7, 11–20). There is no general agreement on any one of these structures, most likely because of insufficient knowledge of the lipid–protein interactions and the tertiary structure of the proteins.

The objective of our ^{13}C NMR studies was to provide a deeper understanding of lipid–protein interactions in general and of the molecular organization of a complex structure like HDL in particular.

MATERIALS AND METHODS

^{13}C-Labeled Lipids. [*26*-^{13}C]Cholesterol, [*N*-$^{13}CH_3$]phosphatidylcholines, and [*N*-$^{13}CH_3$]sphingomyelins were synthesized as described (21, 1). [*N*-$^{13}CH_3$]Lysophosphatidylcholine, obtained by phospholipase A_2 (*Crotalus adamanteus*) treatment of [*N*-$^{13}CH_3$]phosphatidylcholine, was catalytically reduced and then acylated with the acyl chlorides of the following ^{13}C-labeled fatty acids: [*1*-^{13}C]-, [*3*-^{13}C]-, [*8*-^{13}C]-, and [*11*-^{13}C]oleic acid and [*1*-^{13}C]-, [*3*-^{13}C]-, and [*14*-^{13}C]-linoleic acid. Sphingosylphosphorylcholine (22) was acylated with the following fatty acids: [*1*-^{13}C]palmitic acid and [*3*-^{13}C]- and [*11*-^{13}C]oleic acid (W. Stoffel and F. Bolkenius, *Hoppe-*

Seyler's Z. physiol. Chem., in preparation); [*26*-^{13}C]cholesteryl, [*1*-^{13}C]- and [*11*-^{13}C]oleate esters were prepared by acylation of free cholesterol with the corresponding fatty acylchlorides. All lipid samples were carefully stored in the dark under purified nitrogen or argon at −24° as benzene solutions. For quantitative evaluation of the reassembling studies, the corresponding ^{14}C-labeled phosphatidylcholines (*1*-[^{3}H]stearoyl-2-[*1*-^{14}C]oleoyl- or linoloyl-3-glycerophosphorylcholine, [*1*-^{14}C]palmitoyl- and [*1*-^{14}C]oleoyl sphingomyelins, [*26*-^{14}C]-cholesterol, and cholesteryl [*1*-^{14}C]oleate) were added in trace amounts (^{13}C/^{14}C = 200:1).

Apolipoproteins. HDL and apoHDL were obtained by established procedures (23, 24). The two main fractions, apoA-I and apoA-II, were isolated by DEAE–cellulose chromatography (25) in a homogeneous form, as shown by polyacrylamide gel electrophoresis (26) and by immunoprecipitation (antisera were kindly provided by Dr. Greten, Medizinische Klinik, Heidelberg). The amino-acid composition of the two proteins compared well with those published (24, 27).

Recombination of Lipoprotein Complexes. Phosphatidylcholine and sphingomyelin liposomes with and without cholesterol and cholesterol esters in the proportion present in native HDL particles were prepared above the transition temperature of their monomolecular films (28) by ultrasonication (29). These lipid dispersions were used for ^{13}C-NMR measurements and subsequently for reassembling studies with apoHDL, apoA-I, and apoA-II. This procedure will be described elsewhere (30). The lipid composition and the ratios of the individual lipids were analyzed quantitatively by thin-layer chromatography of aliquots in suitable solvent systems for phospholipids (31) and for cholesterol and cholesteryl esters (32). No decomposition of any of the lipids occurred during the sonication and recombination procedures. The Lowry procedure (33) was used for protein determination and the Langmuir technique for the force-area isotherms of the purified individual lipid classes (phosphatidylcholine, sphingomyelin, and cholesterol esters) of HDL (28).

^{13}C NMR Spectra. All proton noise-decoupled ^{13}C NMR spectra were obtained at 22.63 MHz with a Bruker WH-90 pulse spectrometer operating in the Fourier transform mode. A pulse sequence (180°-t-90°) for T_1 measurements was used (34). An internal deuterium field/frequency lock was used. The FID signals were obtained with a 20-μsec pulse. Sample temperature was controlled by a Bruker ST 100/700 variable temperature controller with an accuracy better than ±1°. Sample tubes (10 mm) constricted to about 1-mm inner diameter below the liquid surface minimized the liquid–vapor interchange (35). All samples were flushed thoroughly with purified argon gas.

RESULTS

In order to get insight into the lipid–lipid and lipid–protein interactions in human high density lipoproteins, the spin lattice relaxation times of the main lipids participating in the molecular organization of human high density lipoproteins, namely phosphatidylcholine (PC), sphingomyelin (SPM), cholesterol, and cholesterol esters, were measured (*a*) in $CDCl_3$ and (*b*) as aqueous lipid dispersion of uniform particle size (liposomes) reassembled with (*c*) total apoHDL and (*d*) the separated main components apoA-I (apoLp-Gln-I) and apoA-II (apoLp-Gln-II).

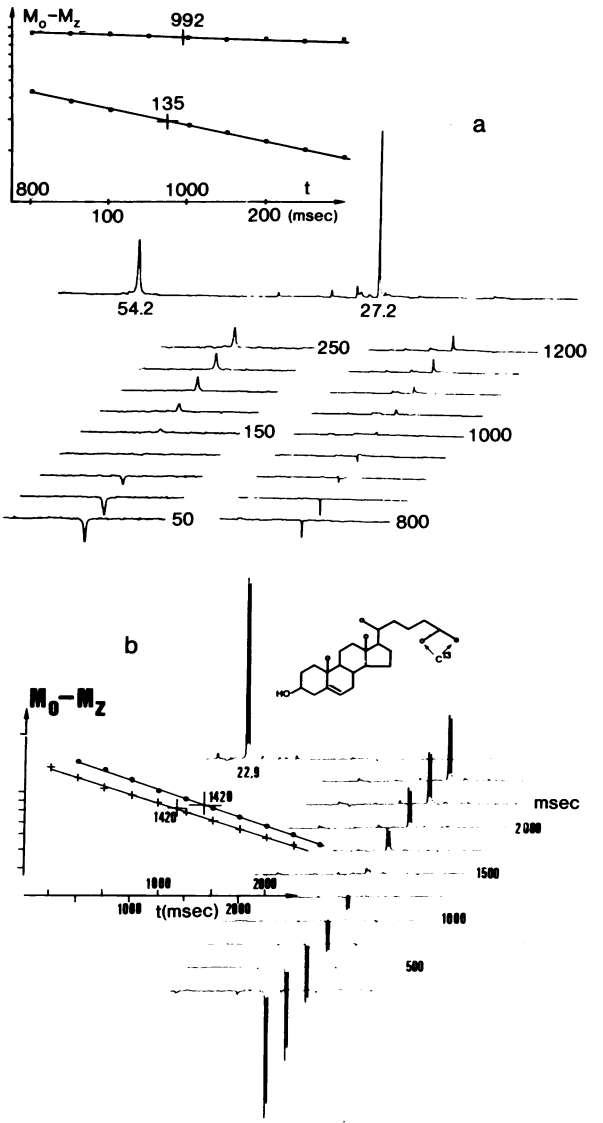

FIG. 1. PRFT spectral set for the determination of spin-lattice relaxation times (T_1) of ^{13}C-enriched carbon atoms in (*a*) 1-stearoyl-2-[*11*-^{13}C]oleoyl-3-glycerophosphoryl[*N*-^{13}CH$_3$]choline ([*N*-^{13}CH$_3$]18:0/[*11*-^{13}C]18:1 phosphatidylcholine) and (*b*) [*26*,*27*-^{13}C]cholesterol. Solvent: $CDCl_3$, 37°. Fourier transformation of 50 accumulated transients.

(*a*) Fig. 1 gives representative examples of T_1-determinations of 1-stearoyl-2-[*11*-^{13}C]oleoyl-3-glycerophosphoryl [*N*-^{13}CH$_3$]choline (Fig. 1a) and [*26*-^{13}C]cholesterol (Fig. 1b) in deuterochloroform. The spin lattice relaxation times (T_1) of the different phosphatidylcholine and sphingomyelin species used in this study are summarized in Table 1. It becomes apparent that the mobility of the alkyl chain of the free fatty acids is remarkably reduced after incorporation into the phospholipid molecule. The T_1-times of the methyl group of the choline head groups of PC and SPM in $CDCl_3$ are low compared with those after these molecules are arranged in bilayers of liposomes.

(*b*) Liposomes prepared from these phospholipids by ultrasonication (36) had an outer diameter of 250 Å, as estimated by electron microscopy after negative staining. A representative example for the T_1-measurement of enriched 1-stearoyl-2-[*11*-^{13}C]oleoyl-3-glycerophosphoryl[*N*-^{13}CH$_3$]choline in liposomes is given in Fig. 2.

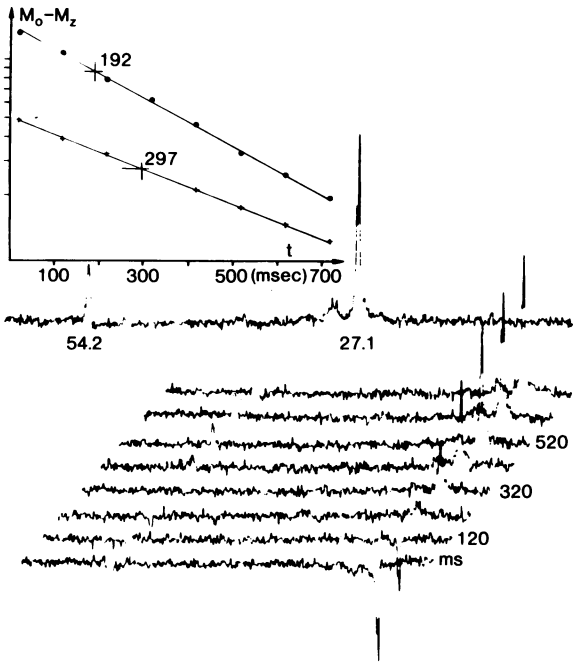

FIG. 2. PRFT spectral set for the determination of T_1 of ^{13}C-enriched carbon atoms in 1-stearoyl-2-[*11*-^{13}C]oleoyl-3-glycerophosphoryl[*N*-$^{13}CH_3$]choline in liposomes. Conditions were the same as under Fig. 1. Solvent: D_2O. Number of accumulated signals: 3000.

(*c*) Total apoHDL and (*d*) the separated apoA-I and apoA-II were reassembled with mixtures of well-defined ^{14}C- and ^{13}C-labeled species of phosphatidylcholine, sphingomyelin, and cholesterol ester and cholesterol. Table 2 summarizes the stoichiometry of the lipid molecules reassembled with apoA-I, apoA-II, and apoHDL present in native HDL. ApoHDL binds PC and SPM very like the native HDL (ratio 6:1), except that only one-half of cholesterol and one-third of cholesterol esters are incorporated in the particles. ApoA-I reassembles preferentially with PC (PC/SPM, 12:1), whereas apoA-II avidly associates with sphingomyelin (SPM/PC, 4:1). An example of the T_1-measurements of reassembled HDL with 1-stearoyl-2-[*14*-^{13}C]linoloyl-3-glycerophosphorylcholine is given in Fig. 3. The results of the T_1-determinations of all other recombined lipoproteins in comparison with those of the same lipids in aqueous dispersions are summarized in Table 3. The reconstituted and ^{13}C-labeled HDL, LpA-I, and LpA-II complexes (30) showed the following striking changes: (*1*) The T_1-times of the polar head groups increase compared to those of the liposome bilayer lipids. (*2*) [*14*-^{13}C]Linoleic acid in phosphatidylcholine and [*11*-^{13}C]oleic acid in sphingomyelin exhibit a dramatic reduction of their T_1-times when recombined with apoHDL and apoA-II. The mobility of cholesterol [*14*-^{13}C]linoleate (T_1 = 305 msec) of reassembled HDL is considerably reduced compared to that of the corresponding PC (T_1 = 440 msec) but slightly higher than that in mixed vesicles.

TABLE 1. *Spin-lattice relaxation times (T_1) in msec of specifically labeled species of phosphatidylcholine, sphingomyelin, cholesterol esters, and cholesterol in $CDCl_3$*

Carbon atoms	T_1 (msec) *N*-$^{13}CH_3$ 18:0/^{13}C 18:1 PC	Oleic acid	Carbon atoms	T_1 (msec) *N*-$^{13}CH_3$ 18:0/^{13}C 18:2 PC	Linoleic acid
N-CH_3	196		*N*-CH_3	196	
C 3	430	2450	C 1	4780	8770
C 8	912	1960	C 3	515	1290
C 11	1435	2060	C 14	3150	3560
^{13}C 16:0 SPM			^{13}C 18:1 SPM		
N-CH_3	92		*N*-CH_3	105	
C 1	471		C 11	1123	
[*26,27*-^{13}C]Cholesteryl-[^{13}C]oleate			Cholesteryl[^{13}C]-linoleate		
C 26,27	1940		C 1	8475	
C 11	2030		C 3	740	
			C 14	3630	
[*26,27*-^{13}C]Cholesterol					
C 26,27	2060				

DISCUSSION

In the present study lipid–lipid and lipid–protein interactions in HDL have been investigated with phosphatidylcholines, sphingomyelins, cholesterol esters, and cholesterol 90% enriched with ^{13}C in defined single carbon atoms either of the hydrophilic group or along the chains of their acyl residue palmitic, oleic, and linoleic acids. The comparison of the relaxation times (T_1) of identical C-atoms of the free fatty acids and after incorporation into phosphatidylcholine, sphingomyelin, and cholesterol esters in $CDCl_3$ solution (Table 1) proves the immobilization of the carboxy-terminal end of the fatty acid in lecithin and sphingomyelin due to van der Waal's forces between the adjacent saturated segments of chains fixed by the ester linkage. This observation corresponds to that reported by Levine *et al.* (37). The spin lattice relaxation times of C-atoms of the unsaturated fatty acyl chains of the free acid or incorporated in phospholipids in $CDCl_3$ do not increase linearly from the COOH-group to the terminal CH_3-group. Our measurements demonstrate that segments of acyl chains have very different mobility, particularly beyond the olefinic bond most distant from the carboxylic group, as exemplified for oleic and linoleic acid labeled at C-11 and 14, respectively. This is due to an enhanced rotation about the C-C single bond adjacent to the double bond (38) and trans-gauche (t-g) and

TABLE 2. *Number of individual lipid molecules bound in reassembled lipoproteins*

Lipids	Native HDL*	Protein component apoA-I†	apoA-II‡	apoHDL§
PC	46	12	6	58 (42)
SPM	8	1	24	8 (10)
Cholesterol	21	—	1	9 (17)
CE	46	1	3	15 (17)

* Contains 3 molecules of apoA-I and 1 molecule of apoA-II.

† 5.2 mg of apoA-I bound 2.7 mg of lipids [2.4 mg of PC, 0.2 mg. of SPM, 0.1 mg of cholesterol ester (CE)].

‡ 11 mg of apoA-II bound 16.3 mg of lipids (3.4 mg of PC, 11.5 mg of SPM, 1.0 mg of CE, and 0.1 mg of cholesterol).

§ 15.1 (50.0) mg of apoHDL were associated with 9.5 (28.0) mg of lipids [6.8 (16) mg of PC, 0.8 (3.4) mg of SPM, 1.4 (5.5) mg of CE, and 0.5 (3.1) mg of cholesterol].

g^+-t-g^- conformational changes with kink formations (39). The different segmental mobility of the unsaturated fatty acid is observed in $CDCl_3$ solution and in aqueous micellar solution as well.

Carbon atoms 11 and 14 of oleic and linoleic acid in the phospholipids again have a pronounced free mobility (Table 3). The incorporation of cholesterol into the phospholipid bilayer, however, distinctly inhibits the mobility of C-11 of oleic acid. The rigid nucleus of cholesterol intercalated between the phospholipid molecules reaches up to this region of the bilayer. Here we give additional experimental evidence for the well-known "tightening" effect of cholesterol in membranes. The T_1-time of C-14 of linoleic acid in PC, however, is distinctly increased. The cholesterol nucleus provides additional space for motions of the terminal segment of linoleic acid. This consideration may be relevant for cholesterol and polyunsaturated fatty acids containing phospholipids associated in membranes.

The small T_1-times of the [*N*-$^{13}CH_3$]choline residues of PC and SPM in $CDCl_3$ refer to their low mobility due to the zwitterionic interaction of the hydrophilic groups in the interior of the inverted micelles. In liposomes, however, their polar groups orient to the inner and outer surface, which allows greater mobility. This is reflected in their increased T_1-times.

The T_1-times of the ^{13}C label in the polar head groups of PC and SPM in the reconstituted lipoprotein complexes remain unchanged or even increase slightly compared to their T_1-times in liposomes. The small but real increase in T_1-times of [*N*-$^{13}CH_3$]choline carbon in the surface of the 80- to 100-Å diameter particle compared to the vesicles may be due to a curvature effect (40). This is strong evidence against ionic interactions of phospholipids with the apolipoproteins.

The significant decrease of T_1 of C-14 of linoleic acid in phosphatidylcholine and C-11 of oleic acid in sphingomyelin when recombined with apoHDL and apoA-II, respectively, gives direct evidence for the hydrophobic interaction between these apolipoproteins and the fatty acid chains of the associated phospholipids. T_1 values of C-3 of these fatty acids are not strikingly changed because this part of the acyl chain is already tightly packed. At least the terminal segments of the oleic and linoleic acid residues, measured here, are involved in the hydrophobic lipid–protein interactions. The very obvious immobilization of the alkane chain of [*14*-^{13}C]linoleate of the cholesterol esters is interpreted as hydrophobic interaction with the apoprotein and the lipid monolayer, as shown in Fig. 4 *A*. These reassembling studies give strong evidence for the nonrandom distribution of phosphatidylcholine and sphingomyelin in HDL. The rather selective assembling of sphingomyelin molecules with apoA-II and of phosphatidylcholine with apoA-I and the lower lipid-binding capacity of apoA-I compared with apoA-II require further studies.

The amino-acid sequence of apoA-II (10) is missing clusters of aliphatic amino-acid residues. This is no prerequisite for the formation of hydrophobic sites. The sideness of pitches with aliphatic and aromatic side chains could provide the sites of lipid–protein interactions. These areas have to be defined. The results of these studies prove the hydrophobic binding of the fatty acyl residues of phospholipids and sphingomyelin to apoproteins of HDL but exclude ionic interactions of these molecules. Incorporating the present analytical and physical data (41–43, 11–20) we propose a model of HDL given in Fig. 4. Hydrated polar head groups of phospholipids and the hydrated hydrophilic areas of the apolipoproteins form the surface of the HDL sphere which is more than half covered by the apolipoproteins. The length of the polar group of dipalmitoyllecithin has been determined to be 11 Å by x-ray (12, 13, 44).

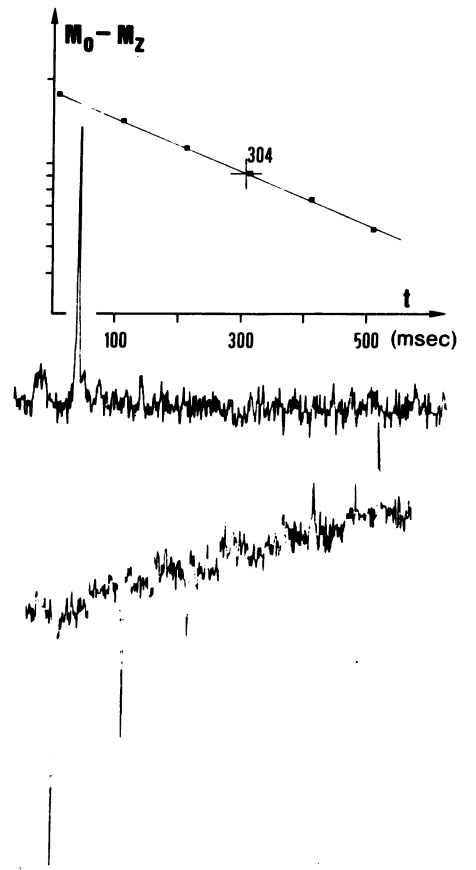

FIG. 3. PRFT spectra for the determination of T_1 of 1-stearoyl-2-[*14*-^{13}C]linoloyl-3-glycerophosphorylcholine after reassembling with apoHDL. Solvent: 0.1 M NH_4HCO_3, pH 7.4. Number of accumulated signals: 8000.

TABLE 3. *^{13}C spin lattice relaxation times (T_1) in msec of ^{13}C-enriched phosphatidylcholine, sphingomyelin, and cholesterol ester organized in liposomes and reassembled with total apoHDL, apoA-I, and apoA-II.*

Lipid	Liposomes	Mixed liposomes	HDL	apoA-II
N-^{13}C 18:0; ^{13}C 18:1 PC				
N-CH_3	428	430	485	—
C 11	278	210	—	—
N-$^{13}CH_3$ 18:0; ^{13}C 18:2 PC				
N-CH_3	428	430	485	560
C 3	122	134	116	120
C 14	638	705	440	—
1-^{13}C 16:0 SPM		660	675	
N-^{13}C ^{13}C 18:1 SPM				
N-CH_3		424	485	560
C 11		242	200	144
Cholesteryl[*14*-^{13}C] 18:2		250	305	—
Cholesteryl[*11*-^{13}C] 18:2		177	—	—

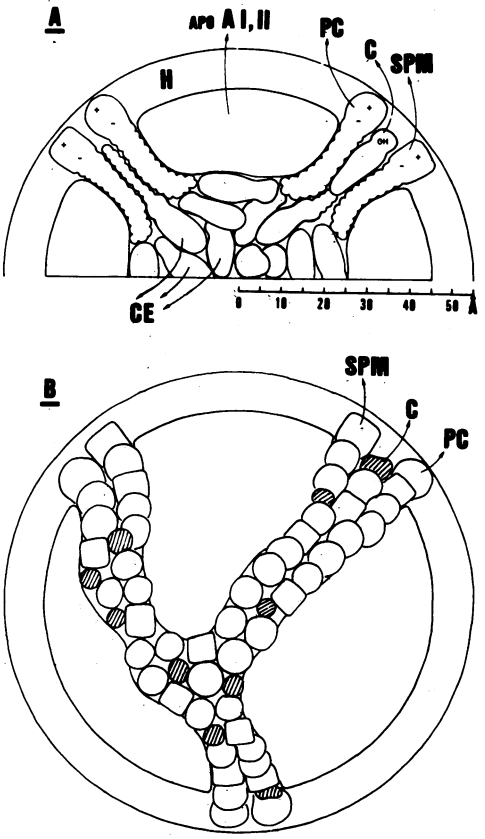

FIG. 4. Schematic model of human HDL, (*A*) Cross-section, (*B*) top view. H = hydration shell, PC = phosphatidylcholine, SPM = sphingomyelin, C = cholesterol, CE = cholesterol esters, apoA-I, II = apolipoprotein I,II.

The phospholipids must be organized as a monolayer in which the polar head groups of about 100–120 phospholipid molecules and cholesterol cover the rest of the surface of the particle. Their predominantly unsaturated long-chain fatty acyl residues (about 25–30 Å) are bound to the apolipoproteins. We suggest further, from our ^{13}C NMR studies with ^{13}C-labeled cholesterol and its linoleate ester (W. Stoffel, O. Zierenberg, B. Tunggal, and J. B. Greter, manuscript in preparation), that free cholesterol intercalates with the phospholipid monolayer, but the cholesterol nucleus of the esters resides in the center of the sphere with their fatty acid residues interacting with the apoprotein and the phospholipid monolayer.

This project is supported by the Deutsche Forschungsgemeinschaft (SFB 74).

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