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A New Method for the Exchange of Lipid Classes of Human Serum High Density Lipoprotein

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Summary: Human serum high density lipoproteins ($d \approx 1.063-1.21$ g × cm^{-3}) exchange their phospholipid, cholesterol and cholesterol ester components when the same lipid classes are brought into micellar solution with sodium cholate. The stoichiometry of the lipid and apoprotein components remains constant when the lipid concentration in the micellar solution is that of the native HDL. We found with the corresponding radioactive lipid classes that an equilibrium at about 50% exchange is reached. Higher lipid and lipid cholate concentrations lead to an accumulation of lipid in the HDL particles. The procedure for lipid exchange described here yields the HDL preparation free of cholate.

Phosphatidylcholine and sphingomyelin are incorporated at up to 50% of the starting concentration in a single lipid exchange cycle at the expense of phosphatidylcholine of the native HDL. Phosphatidylethanolamine is completely incorporated in the HDL particles. The method also allows the cholesterol and cholesterol ester exchange of HDL particles.

Fluorescence spectroscopy and near ultraviolet circular dichroism measurements carried out before and during the lipid exchange process and with the final exchanged HDL preparation indicate that no conformational changes of the apoprotein components occur. The electron microscopic appearance of the HDL particles before and after the lipid exchange is identical.

The prospect of this lipid exchange for lipoprotein structural and metabolic studies is discussed.

Eine neue Methode für den Austausch der Lipidkomponenten des menschlichen Serum-High-Density-Lipoproteins

Zusammenfassung: Menschliche Serum-High-Density-Lipoproteine (HDL) ($d = 1.063-1.21$ g × cm^{-3}) tauschen ihre Phospholipide, ihr Cholesterin und ihre Cholesterinester gegen die gleichen Lipidkomponenten aus, wenn sich diese in micellarer Lösung befinden (mit Hilfe von Natriumcholat). Dabei bleibt die Stöchiometrie der Lipid- und Apoproteinkomponenten konstant, wenn die Lipidkonzentration in der micellaren Lösung der des nativen HDLs entspricht. Mit entsprechend radioaktiv markierten Lipiden fanden wir, daß bei Erreichen des Gleichgewichts etwa 50% von ihnen ausgetauscht waren. Höhere Lipid- bzw. Lipid- und Cholatkonzentrationen

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Abbreviations: HDL human serum high density lipoprotein, PC phosphatidylcholine, PE phosphatidylethanolamine, SPM sphingomyelin, C cholesterol, CE cholesterol ester, CD circular dichroism.

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führten zur Akkumulation des Lipids in den HDL-Partikeln.
Die hier beschriebene Austauschmethode ergibt eine HDL-Präparation, die frei von Cholat ist. In einem einzelnen Austauschzyklus werden sowohl rund 50% Phosphatidylcholin als auch Sphingomyelin gegen Phosphatidylcholin der nativen HDL-Partikel ausgetauscht. Phosphatidyläthanolamin wird vollständig in die HDL-Partikel aufgenommen. Die Methode erlaubt auch den Austausch des Cholesterins und der Cholesterinester des HDL. Die Konformationen der Apoproteine A-I und A-II bleiben unverändert wie Fluoreszenzspektroskopie- und Circulardichroismus-Messungen vor, während und nach dem Lipidaustauschprozess zeigen. Elektronenmikroskopisch (Form und Größe) verhalten sich die HDL-Partikel nach Negativ-Färbung vor und nach dem Lipid-Austausch gleich. Die Möglichkeiten, die diese Lipid-Austausch-Methode für Struktur- und Stoffwechseluntersuchungen an der HDL-Partikel eröffnet, werden diskutiert.

Key words: High density lipoprotein, micellar lipid, lipid exchange, purification and characterization of HDL.

It is often desirable to study the impact of particular lipid classes on membrane systems, lipid dependent enzymes and proteins and on lipoproteins with regard to their structural properties and relation to the respective protein functions. Delipidation and recombination of apoproteins with a specific lipid class often substituted by physical or chemical probes proved to be valuable tools in these studies on the lipid-protein interactions.

Delipidation often leads to denaturation of the apoproteins, their aggregation is due to strong hydrophobic forces and insufficient restoration of their native conformation by the recombination methods applied. These hazards can be circumvented by leaving the apoprotein permanently in a lipid environment, their native lipid surroundings being exchanged against a lipid class added to the system in the presence of the ionic detergent sodium cholate. We described this approach for the successful substitution of phosphatidylcholine of rabbit-sarcoplasmic $Ca^{2\oplus}$-ATPase by ^{13}C-labelled lecithin species with no residual detergent in the final purified enzyme-lipid complex, which still retained its full enzymic activity[1].

In this communication we describe the adaptation of this method to the human serum high density lipoprotein class. Lecithin, cholesterol and cholesterol esters have been exchanged against different phospholipid classes (phosphatidylcholine, sphingomyelin, phosphatidylethanolamine), cholesterol and cholesterol esters in the presence of sodium cholate. These HDL particles with the exchanged lipid classes differ in no way from native HDL as proven by their density, electron microscopy, circular dichroism, fluorescence, chromatographic properties, agarose and dodecylsulfate polyacrylamide gel electrophoresis.

Materials and Methods

Lipids

[1,2-$^3H_2$]Cholesterol (specif. radioact. 40–60 Ci/mmol) and sodium-[*carboxyl*-^{14}C]cholate (specif. radioact. 50 mCi/mmol) were purchased from Amersham-Buchler (Braunschweig). The other substrates used [*N-methyl*-^{14}C]1,2-dioleoyl-3-*sn*-glycerophosphocholine, [9,10,12,13-$^3H_4$]1,2-dilinoleoyl-3-*sn*-glycerophosphocholine, cholesteryl-[9,10-$^3H_2$]oleate and 1-[9,10-$^3H_2$]-stearoyl-2-[1-^{14}C]oleoyl-3-*sn*-glycerophosphoethanolamine were synthesized in this laboratory by established procedures[2,3].

Methods

Human serum high density lipoprotein (d = 1.063–1.21 g × cm^{-3}) was isolated by the sedimentation-flotation procedure[4,5]. The density of the HDL particles was determined by CsCl gradient (11–20%) centrifugation as described before[6]. Size, homogeneity and shape of the HDL preparations were assessed by electron microscopy of the samples negatively stained with 2% phosphotungstic acid. A Philips EM 300 was used. Samples were concentrated by ultrafiltration using Amicon UM 2 filters.

Sodium cholate was separated from high density lipoprotein fractions and excess lipids by passing the concentrated samples over a Sephadex G-25 column (2.5 × 50 cm). 0.02M Tris/EDTA buffer pH 8.2, (0.02M NaCl, 0.02M EDTA, 0.02% NaN_3) was used as eluent during the chromatographic steps.

Phospholipids or mixtures of phospholipids/cholesterol/cholesterol ester in molar ratios of 80:24:53 were dispersed with sodium cholate in the above buffer by sonication in an atmosphere of argon with cooling in ice water (75 W, 30 min, Branson sonifier microtip).

Excess lipid was separated from the HDL fraction by Sepharose 4B column chromatography (1.5 × 100 cm). Chromatography was followed by determination of the 280 nm absorption and the distribution of the radioactive lipids.

Thin-layer agarose gels were used for lipoprotein electrophoresis in sodium barbiturate 0.05M, 0.035% EDTA buffer pH 8.6 using the Corning ACI Casette Electrophoresis cell (Palo Alto, Cal.) with a constant power supply of 90 V. For fixation and staining the instructions of the manufacturer were followed. Polyacrylamide gel electrophoresis of the apoproteins according to Weber and Osborn[7], was combined with densitometry of the gels.

Comparative measurements of circular dichroism of control samples and of HDL preparations with exchanged lipid classes were carried out at ambient temperature with a Jasco 41 A spectropolarimeter and fluorescence spectra were recorded with a Perkin Elmer fluorescence spectrometer, Model MPF-3.

Radioactive samples were counted in a Packard Tricarb Scintillation counter Model 544. For aqueous samples the scintillator Soluene 350 was used.

Results

Method for exchanging genuine lipid species of HDL particles against externally added lipids

The phospholipid exchange method, which we developed for the substitution of sarcoplasmic $Ca^{2\oplus}$-ATPase lipids by particular ^{13}C-labelled phospholipid species, was adapted to the problem of modifying the lipid composition of human serum high density lipoprotein particles without dissociation of their lipids and apoproteins. Therefore the lipid species phospholipid, cholesterol or cholesterol ester were dispersed, at the same stoichiometry as the native HDL, with sodium cholate. The micellar solution was then agitated with the native HDL preparation for exchange of the respective lipid class. One of the lipid classes may be either ^{13}C- or azido-labelled. This exchange procedure was followed by the complete separation of the bile salt and purification of the HDL particles from excess lipids. The flow sheet summarizes these steps (Fig. 1).

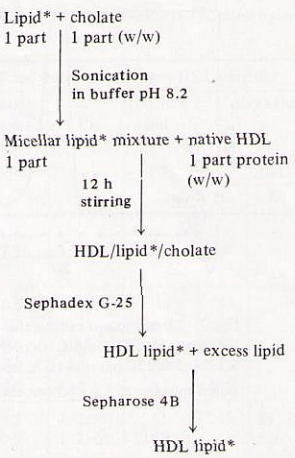

Fig. 1. Exchange of lipids of serum high density lipoprotein.

In the first chromatographic step bile salts were completely separated from the lipoprotein fraction by Sephadex G-25 chromatography. Using ^{14}C-labelled cholate we could demonstrate that all the radioactivity eluted quite separately from the lipoprotein-lipid band in the cholate peak (Fig. 2). The HDL band eluted together with excess lipids in the void volume. The pooled fractions were concentrated by ultrafiltration and the HDL particles were separated from excess lipid on a Sepharose 4B column, Fig. 3. This fraction contained a population of spherical HDL particles very homogeneous in size with an average diameter of 100–120 Å as determined by negative staining and electron microscopy. The size of the HDL particles was retained in all cases in which the ratio of the three main lipid classes of HDL phosphatidylcholine, cholesterol and cholesterol esters in micellar solution reflected that of native HDL (80 mol phosphatidylcholine, 24 mol cholesterol and 53 mol cholesterol ester per mol apoHDL (M_r 100000)). It is possible to increase the size of the HDL particles in diameter by either using only phosphatidylcholine:cholate 400:140 mol/mol per mol apoHDL or by raising simultaneously the concentration of sodium cholate 400:700 mol/mol per mol apoHDL.

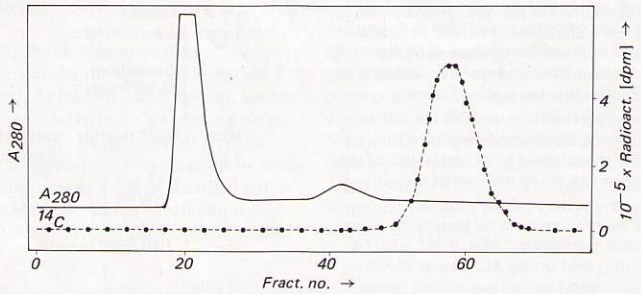

Fig. 2. Separation of radioactive cholate from HDL previously equilibrated with a micellar solution of dioleoyllecithin/cholesterol/cholesteryl oleate/[^{14}C]cholate (molar ratio 80:24:53:230 per mol HDL lipoprotein) on Sephadex G-25.

Bed dimensions: 2.5 x 50 cm; eluant: 0.050M phosphate buffer, pH 7.4.

Exchange of phospholipid classes

Phosphatidylcholine, phosphatidylethanolamine and sphingomyelin present as lipid components in HDL in a molar ratio of approximately 80:6:12 per mol HDL can be exchanged against lipids of the native particle. But, in addition, cholesterol and cholesterol ester exchange as well. The experimental proof is given in Table 1 which summarizes four sets of experiments. In the first experiment the only lipid class [N-*methyl*-^{14}C]-1,2-dioleoylglycerophosphocholine was used in increasing concentrations and at a constant cholate to HDL ratio. In the second series of exchange experiments ^{14}C-labelled dioleoyllecithin, [1,2-$^{3}H_2$]cholesterol and cholesteryl-[9,10-$^{3}H_2$]oleate in the molar ratio 80:24:53 per mol HDL and in the third experiment 1-[1-^{14}C]-stearoyl-2-[9,10-$^{3}H_2$]oleoylglycerophosphoethanolamine substituted for the cholesterol ester in the artificial lipid mixture of the exchange method. The following conclusions can be drawn from the data of Table 1.

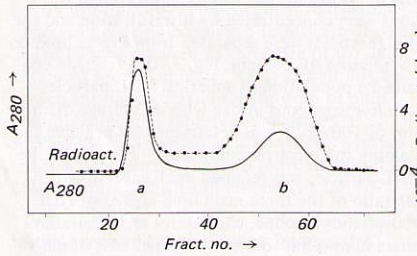

Fig. 3. Separation of HDL from excess lipid on Sepharose 4B.

Bed dimensions: 1.5 x 100 cm. The lipid mixture used for the exchange consisted of [*N-methyl*-^{14}C]dioleoyllecithin, [1,2-$^{3}H_2$]cholesterol and cholesteryl-[9,10-$^{3}H_2$]-oleate 80:24:53 (mol/mol) per mol HDL. 2-m*l* fractions were collected; only the ^{3}H activity was plotted.
a Excess lipid; b HDL.

The lipid exchange method allows the exchange of each lipid class against e.g. its radioactive, ^{13}C- or azido-labelled form. If only lecithin/cholate micelles are applied a considerable loss of cholesterol from the HDL particles can be observed. When a 400 molar excess of lecithin over HDL is used, the particle accumulates the phospholipid up to three times the amount of the native HDL. A micellar solution of phosphatidylcholine, cholesterol and cholesterol ester in a molar ratio 80:24:53, which is that found in the high density lipoprotein fraction, exchanges each lipid class without alteration of the stoichiometry of the lipid in the native particles. Phosphatidylethanolamine, although a minor constituent of HDL, can be quantitatively incorporated into HDL by the method described here.

Table 1. Lipid exchange between native serum high density lipoprotein (HDL) and [N-*methyl*-^{14}C]dioleoyllecithin, [1,2-$^{3}H_2$]cholesterol and/or cholesteryl-[9,10-$^{3}H_2$]oleate solubilized with sodium cholate.

Stoichiometry of cholate/lipid mixed micelles						Analytical data of exchanged HDL particles						
	Lipid classes [μmol]			Cholate [μmol]	HDL [μmol]	Total phosphorus [μmol]	Total cholesterol [μmol]	C/CE	Protein yield [%]	Exchange [%]		
	PC	C	CE							PC	C	CE
Native HDL (control)					0.1	9.3	9.1	1:2.62	65	–	–	–
I	2.66			14	0.1	9.6	6.0	1:10	41	53		
	5.33			14	0.1	10.5	6.2	–	60	58		
	16			14	0.1	12.4	6.5	1:15.6	53	83		
	40			14	0.1	17.1	7.3	–	58	133		
	40			70	0.1	28.3	8.6	–	91	306		
II	8	2.4	5.3	23	0.1	9.4	9.1	1:2.65	65	45	55	
	8	2.4	5.3	23	0.1	9.6	9.2	1:2:67	53	40	n.d.	35
III[a]	8	2.4	4.8 PE[a]	23	0.1	11.3	n.d.	–	37	100	PE	
IV[b]	7[b] SPM	2.5	5	23	0.1	6.93	n.d.	–	60	52	SPM	

[a] 4.8 μmol 1-[1-^{14}C]stearoyl-2-[9,10-$^{3}H_2$]oleoylphosphatidylethanolamine instead of cholesteryl oleate.
[b] 7 μmol [9,10-$^{3}H_2$]*N*-oleoylsphingenine-1-phosphocholine (*N*-oleoylsphingomyelin) instead of PC.

The lipid exchange method has also been applied twice consecutively to a native HDL preparation. A cholate/dioleoylphosphatidylcholine/cholesterol/cholesteryl-oleate micellar solution was used and a recovery of 65% of HDL protein was achieved. The enrichment of oleic acid in the phosphatidylcholine fraction has been determined by gas liquid chromatography of the fatty acid methyl esters of the PC fraction isolated by preparative thin-layer chromatography. Table 2 presents the fatty acid analysis of the phosphatidylcholine fraction after the first and second passage of native HDL through the exchange procedure.

55 and 67% of the lecithin species have been exchanged by the repetition of the procedure. In the fourth experiment [*N-methyl*-^{14}C]sphingomyelin was used instead of phosphatidylcholine in the mixed micellar solution which then consisted of labelled sphingomyelin/cholesterol/

Table 2. Enrichment of dioleoyllecithin in HDL by the repetitive application of the lipid exchange method. Native HDL (30 mg protein, 0.3 μmol) was equilibrated with a micellar solution of 20 μmol dioleoyllecithin, 13 μmol cholesteryl oleate, 6.2 μmol cholesterol and 70 μmol sodium cholate as described under Methods.

Fatty acid	Fatty acid composition of purified phosphatidylcholine fractions [% of total fatty acid mixture]						
	16:0	16:1	18:0	18:1	18:2	20:3	20:4
Native HDL	32.8	1.5	20.7	14.3	21.8	1.8	7.2
After 1. exchange	19.2	–	10.4	55	15.7	–	–
After 2. exchange	15.2	–	6.7	67	11.5	–	–

cholesterol ester/sodium cholate in a molar ratio of 70:25:50:230 per mol HDL. The concentration of radioactive sphingomyelin in the exchanged HDL particles proved that about 50% of the ^{14}C label had moved from the mixed micellar form into the particles. Sphingomyelin must have been incorporated from the mixed sphingomyelin containing micelles with substitution of a comparable number of phosphatidylcholine molecules keeping the ratio of phosphocholine polar head groups to apoproteins constant.

Characterization of HDL particles after passage through the lipid exchange procedure

The HDL preparation, carried through the lipid exchange procedure with equilibration against the micellar solution of labelled dioleoyllecithin, cholesterol and cholesterol esters, sedimented at a density between 1.063–1.21 g x cm^{-3}.

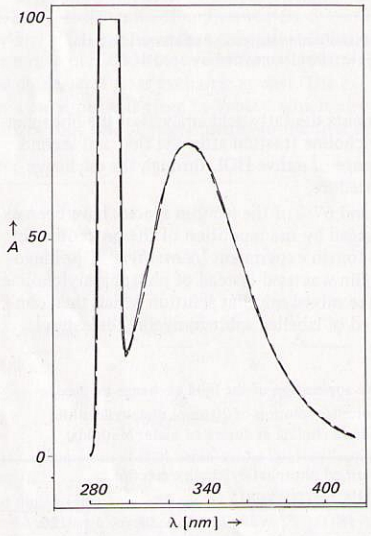

Fig. 4. Fluorescence spectroscopy of native and lipid exchanged HDL.
The two spectra coincided exactly.

Fluorescence spectroscopy and circular dichroism

Intrinsic tryptophan fluorescence and circular dichroism properties of HDL apolipoproteins were measured during the lipid exchange process and following purification of the exchanged HDL particles. These two methods, which are sensitive to conformational changes of the apoprotein structures, could also lend insight into the molecular events during the lipid exchange. The fluorescence spectra of native and exchanged HDL

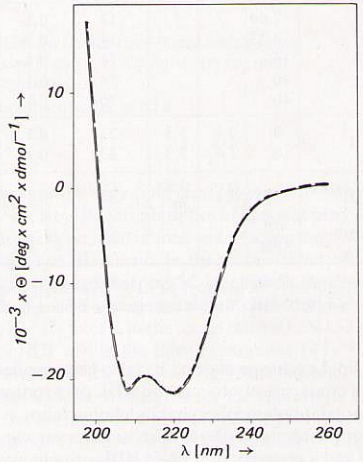

Fig. 5. Near ultraviolet CD spectra of native and lipid exchanged HDL.
The corrected spectra of the two HDL preparations were identical.

coincide when corrected for identical protein concentrations. No shift occured in the maximum or the intensity of the fluorescence when HDL particles and the micellar solution of the lipid-cholate mixture were subjected to the exchange process, Fig. 4. Also the CD spectra of HDL before (native), during and after the lipid exchange exhibited identical ellipticities in the ultraviolet region (Fig. 5), which indicates that the α helicity and β structure are not perturbed during and by this procedure.

Agarose and dodecylsulfate-polyacrylamide gel electrophoresis

Native HDL and the HDL preparations in which the lipid classes had been exchanged were compared by agarose chromatography, Fig. 6.

The protein and lipid positive band coincided exactly proving the same electrophoretic mobility of native and lipid exchanged HDL.

Dodecylsulfate-polyacrylamide gel electrophoresis of the apoproteins of both HDL preparations revealed the same pattern of their component apoproteins apoLp A-I and apoLp A-II, with identical intensities of the protein bands stained with Coomassie blue, Fig. 7.

Discussion

Reconstitution investigations of lipoproteins and membrane protein (enzyme)-lipid complexes are very valuable tools for the study of lipid-protein interactions on a molecular level. This is particularly feasible, when lipid components which serve as physical or chemical probes are being recombined with the protein component. However one prerequisite is the isolation and purification of the protein, which is accompanied by delipidation. In the case of serum lipoprotein apoproteins, myelin basic protein and myelin proteolipid dramatic changes of the conformation occur when the lipid environment is removed.

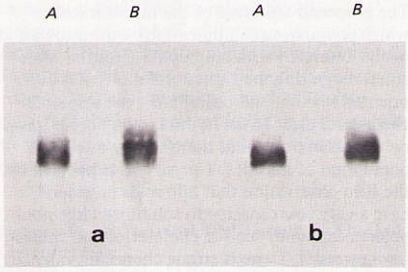

Fig. 6. Lipoprotein agarose electrophoresis of A, native HDL and B, lipid exchanged HDL.
a, amido black staining; b, fat-red 7B staining. 0.05M sodium barbital buffer, pH 8.6 with 0.035% EDTA; 90 V for 35 min. An ACI Casette electrophoresis cell was used.

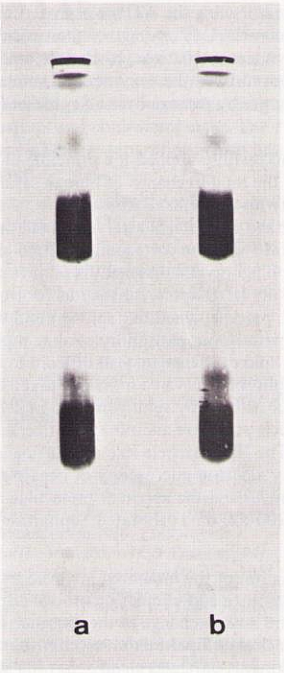

Fig. 7. Dodecylsulfate-polyacrylamide gel electrophoresis (15% acrylamide) of
a, native HDL and b, HDL after lipid exchange procedure.

Sarcoplasmic ATPase loses its enzymic activity when the phospholipid halo is removed. The consequent approach to the problem is therefore to exchange the lipid species of the genuine protein-lipid complex against those which may serve as probes of the lipid-protein interactions. This has been demonstrated for sarcoplasmic ATPase. In previous studies on the lipid-protein interactions in rabbit sarcoplasmic ATPase we developed a phospholipid exchange method by forming mixed micelles consisting of ^{13}C-labelled phosphatidylcholine-sodium cholate (3 α, 7 α, 12 α-trihydroxycholanic acid)[1]. The advantages of this approach are a) the rapid exchange of the phospholipids between micelle and membrane

phospholipids leaving the ATPase activity unaltered, whereas the $Ca^{2\oplus}$ storage of the vesicular structure remains unchanged, b) cholate can be simply removed from the enzyme/lipid/cholate-micelle mixture by passage over a Sephadex G-25 column.

We have successfully applied the principle of this method to the lipid exchange of human serum high density lipoproteins. Radioactive lecithin, cholesterol and cholesterol esters in mixed micelles equilibrate with the corresponding lipid classes in HDL. Sphingomyelin, normally a minor constituent of the HDL lipids, substitutes for lecithin in the HDL particle, when this sphingolipid with the same zwitterionic phosphorylcholine moiety is used for lipid exchange instead of lecithin. The total lipid phosphorus value indicates that lecithin has been substituted by sphingomyelin molecules in the particle structure and no apposition has occurred. The HDL particle size remains constant as shown by electron microscopy of negatively stained samples and the apparent molecular weight of 200000 on a calibrated Sepharose 4B column.

The observation of the unaltered intrinsic tryptophan fluorescence and circular dichroism during and after the lipid exchange in the presence of the sodium cholate/lipid mixed micellar solution is very striking. It indicates clearly that the apoprotein components A-I and A-II retain their conformation completely, also that the integrity of the particles is preserved and that the cholate/lipid micelles interact only with the lipid areas in the HDL particle surface. The HDL model which we proposed[8] demands the orientation of all polar head groups of phospholipids and the 3-hydroxy group of cholesterol toward the particle surface, the cholesterol esters being embedded in the hydrophobic interior of the HDL particle.

Sodium cholate forms mixed micelles with phosphatidylcholine, the size of which are dependent on a number of parameters such as pH, ionic strength and lecithin to sodium cholate ratio. Since lecithin has a much lower cmc (critical micellar concentration) and therefore an extremely low monomer concentration the insoluble amphiphilic phospholipid is concentrated in the micelle. A molecular arrangement of lecithin and sodium cholate in the micelle has been proposed according to which lecithins form small bilayers to the hydrocarbon region of which cholic acid molecules are attached by their hydrophobic portions and their hydrophilic, the α-hydroxy groups and the negatively charged side chain bearing portions, are aligned in the surface of the micelle. The molecular weight of the micelles is dependent largely on the ratio of insoluble amphiphile to sodium cholate. As a consequence the interaction of these micelles with the HDL particles leads to an increased lecithin content of HDL (Table 1). Cholate/lecithin micelles form clear solutions. The admixture of cholesterol and cholesterol esters causes turbid micellar solutions which are stable over days. This indicates that cholesterol and cholesterol esters, together with lecithin and cholate, form entities which must have a very high micelle weight.

It is well known that sodium cholate poorly solubilizes cholesterol; about 30 to 100 bile salt molecules are needed for one cholesterol molecule[9]. We used a stoichiometry of cholate/lecithin/cholesterol/cholesterol esters of 230:80:24:53 for the lipid exchange which is 1.5 mol cholate per mol lipid. The stability of the turbid micellar solution requires the interdigitation of cholesterol in the lecithin bilayer and the solubilization of the cholesterol ester in the hydrophobic medium of the phospholipid bilayer. A very likely arrangement of the lipid/cholate micelle is visualized in Fig. 8.

The proposed structure of the mixed micelle which postulates a solubilization by the association of the detergent with amphiphilic lecithin molecules integrating the cholesterol molecules in an oriented manner and assimilating the insoluble cholesterol ester in the hydrocarbon core is based on the assumption that there is only one gross population of micelles. This view is supported by the firm observation that bile acids in general have a very low capacity to solubilize large nonpolar compounds such as cholesterol and cholesterol esters[9]. There is strong chemical evidence that the HDL particle is largely stabilized by apoprotein-apoprotein interactions*,**.

* Stoffel, W. & Därr, W., this J., in preparation.
** Stoffel, W., Etti, H. & Cremer, P., this J., in preparation.

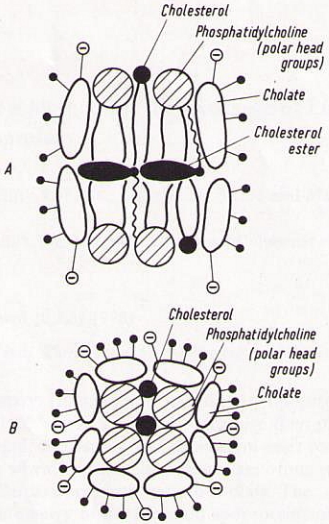

Fig. 8. Model of mixed cholate/lecithin/cholesterol/cholesterol ester micelle.
A, cross-section; B, top view.

In our proposed HDL model the amphiphilic phospholipids are aligned in patches in the surface of the particle. These are prone to fusion with easily dissociable cholate-mixed micelles. The integration of one half of the micellar sandwich into the HDL particles by which lecithin molecules serve as vehicles for cholesterol and cholesterol ester molecules requires the simultaneous exclusion of the lipid present in the particle in order to explain the equilibrium reached in this lipid exchange procedure.

This mechanism is more attractive than assuming a carrier function of single cholate molecules for each lipid class because of the aforementioned favourable micelle formation with lecithin but unfavourable micelle formation of cholate with cholesterol and cholesterol ester. The result would be a phase separation which has not been observed.

The method described here has been used for the introduction of ^{13}C-labelled phospholipids, cholesterol and cholesterol esters* and radioactive azidolabelled phospholipids in order to further studies on the lipid-protein interactions and the structure of serum lipoprotein**.

The electron microscopic control of the HDL preparation by Dipl. Biol. H. Scheefers and the skilful technical assistance of Mrs. R. Blau is gratefully acknowledged. This work was supported by the *Deutsche Forschungsgemeinschaft.*

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