Title

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DEGRADATION PRODUCTS FROM HUMAN SERUM HIGH DENSITY LIPOPROTEINS FOLLOWING DEHYDRATION BY ROTARY EVAPORATION AND SOLUBILIZATION

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RUNNING TITLE: DEHYDRATION-SOLUBILIZATION OF HIGH DENSITY LIPOPROTEINS

DISCLAIMER

Summary

1. Dehydration by rotary evaporation and solubilization at pH 8.6 of human serum high density lipoproteins resulted in the formation of a lipid-poor protein fraction ($d > 1.21$ g/ml) and a redistribution of the remaining lipid and protein components into three new ultracentrifugal fractions of varying composition and density.

2. The redistribution of lipid components was specific and resulted in a partitioning of the apolar cholesteryl esters and triglycerides predominantly into the $d < 1.006$ and d 1.006-1.063 g/ml product fractions. The polar phospholipids were major lipid components of the d 1.006-1.063 and d 1.063-1.21 g/ml product fractions.

3. The redistribution of the apoproteins of the treated high density lipoproteins was unique and suggested differences in their affinity for the lipids of the parent lipoprotein fraction. The minor apoprotein constituents of the high density lipoproteins, which are major constituents of normal very low density lipoproteins, predominated in the degradation products rich in apolar lipids. The predominant apoprotein (apoLP-gln I) of the parent fraction recombined with only a minimal amount of lipid and was confined almost exclusively to the lipid-poor $d > 1.21$ g/ml product fraction. The other major apoprotein constituent (apoLP-gln II) was found in products rich in phospholipids.

4. Electron microscopy of the different products showed the formation of discoidal particles in the phospholipid-rich d 1.063-1.21 g/ml product fraction; the particles in

the d 1.006-1.063 g/ml product fraction were spherical and resembled normal human low density lipoproteins, but were slightly smaller in size; the particles in the d < 1.006 g/ml product fraction were very diverse in size and, on the average, larger than normal very low density lipoproteins of human serum.

5. An immunoelectrophoretically slow-moving product was observed following dehydration and solubilization of high density lipoproteins and had immunochemical properties identical to the slow-moving lipid-poor component (αLP_B) obtained following exposure of high density lipoproteins to other mild degradative procedures; both corresponded to the lipid-poor d > 1.21 g/ml product fraction consisting predominantly of apoLP-gln I protein.

INTRODUCTION

The association between the protein and lipid moieties of human serum high density lipoproteins is subject to partial disruption by relatively mild procedures such as dehydration by rotary evaporation and solubilization at pH 8.6 (1), storage (2), freeze-thawing (2,3), repeated ultracentrifugation (2,3), bubble oxygenation (4), sonication (5), and exposure to detergents (3), ether (2,5,6) or urea (2). Under these conditions new lipid-rich and lipid-poor species are formed which differ from the parent high density lipoproteins. The physical and chemical properties of the new products suggest that some rather specific dissociations and reassociations between high density lipoprotein protein and lipid moieties occur during these disruptive perturbations (1-6).

Recent observations (7-9) indicate that the apoprotein moiety of the high density lipoproteins contains two major and a group of minor protein components. Detailed characterization of the products formed during disruption of high density lipoproteins may provide information on the affinities between these proteins and the lipid constituents of the high density lipoproteins and perhaps provide some insight into their organization in the parent macromolecules. In this report we describe some physical and chemical properties of the lipoprotein and protein products formed during disruption of high density lipoprotein structure by dehydration-solubilization procedures.

MATERIALS AND METHODS

Ultracentrifugal Preparation of Parent High Density Lipoprotein Fraction

Freshly prepared serum from healthy female subjects (ages 30 and 38) was raised to a salt background of d 1.063 g/ml by addition of NaBr. Six milliliter aliquots of this adjusted serum were subjected to preparative ultracentrifugation at 114,000 g, 16° for 48 h. After ultracentrifugation the top 2 ml fractions were separately removed and the remaining 4 ml fractions were stirred, pooled and subsequently raised to a salt background of d 1.21 g/ml by dialysis against a NaBr-NaCl solution (containing 0.1 mg/ml EDTA). Four milliliters of the dialyzed material were then layered below 2 ml of a NaBr-NaCl solution (d 1.21 g/ml, 0.1 mg/ml EDTA) and ultracentrifuged at 114,000 g, 16° for 48 h. The top 1 ml fractions containing the high density lipoproteins were separately removed by pipetting, adjusted to d 1.21 g/ml by dialysis, diluted, and subjected to preparative ultracentrifugation for 36 h at 114,000 g and 16°C. Following this final ultracentrifugation the top 1 ml fractions were removed by pipetting and pooled; this pool served as the parent high density lipoprotein fraction for subsequent dehydration, solubilization, and analytic procedures.

Dehydration and Solubilization of Parent High Density Lipoprotein Fraction

Prior to dehydration, an aliquot of the parent fraction was dialyzed against a 0.95% ammonium acetate-ammonium

carbonate buffer (pH 7.4 and 4.7 mg/liter EDTA) and subsequently diluted with the same buffer to obtain a concentration of high density lipoproteins in the range of 15-20 mg lipoprotein/ml (determined refractometrically). Five milliliters of the adjusted solution were introduced into a 1000 ml round-bottomed flask and subjected to dehydration using a rotary flash evaporation apparatus (Rotary Film Evaporator, Labline, Inc., Chicago, Ill.). The flask, positioned at an angle, was rotated under vacuum at 40 rev./min, in a 37° water bath; vapor was collected in a dry ice-acetone trap. After 10-15 min of evaporation, a thin film of clear yellow material formed in the lower half of the flask; dehydration was continued for a total of 40 minutes.

Solubilization of the film material was achieved by gradual addition of 5 ml of 1.0 M Tris-HCl buffer (pH 8.6). After addition of the buffer, the flask was undisturbed for 10 min; subsequently, the flask was gently swirled to expose all of the film to the buffer. The final yellow solution exhibited some turbidity but was completely homogeneous and without any precipitated material (solubilization with double distilled water was substantial but incomplete; solubilization was complete with double distilled water containing sufficient NaOH to adjust pH to 8.6). Recovery of lipid and protein material was approximately 97%. The resulting solution was dialyzed against a d 1.006 g/ml NaCl solution (containing 0.1 mg/ml EDTA).

Ultracentrifugal Fractionation and Analysis of Products

The dehydrated-solubilized material was fractionated by sequential preparative ultracentrifugation (114,000 g, 16° and 24 h); first at d 1.006 g/ml, second at d 1.063 g/ml and third at d 1.21 g/ml. Lipoprotein fractions floating at the above densities as well as fractions containing sedimenting material of d > 1.21 g/ml were removed by pipetting (the bottom 1.5 ml of the material in the final ultracentrifugation will be designated: d > 1.21 g/ml fraction) and stored under N_2 for subsequent analysis. The procedures used for the analytic ultracentrifugation of the various fractions and the computer programs for graphic representation of the fully corrected schlieren patterns have been described elsewhere (10,11). Flotation rates described by S_f° values are rates in Svedberg units for lipoproteins in a medium of d 1.063 g/ml (NaCl, 26°, 52,640 rev./min). Flotation rates described by $F_{1.20}$ values are rates in Svedberg units for lipoproteins in a medium of d 1.20 g/ml (NaCl-NaBr, 26°, 52,640 rev./min). Both are corrected for concentration and Johnston-Ogston effects.

Analysis of Products

Chemical Analysis

Lipids in the ultracentrifugal and parent fractions were extracted, chromatographed on silicic acid, and quantified by infrared spectroscopy according to methods described earlier (12). Protein content of fractions was de-

termined by a modified Lowry method (13).

Protein Fractionation

Individual ultracentrifugal fractions were delipidated by two 12 h extractions with freshly prepared 3:1 (v/v) ethanol-diethyl ether at 4°, washed twice with diethyl ether and dried under N_2. The apoprotein residues dissolved completely in 0.2 M Tris-HCl, pH 8.2. Gel filtration was performed essentially as described by Scanu, et al. (8) with the minor modification that the column was equilibrated and eluted with 0.2 M Tris-HCl, 0.01% EDTA, 6 M urea, pH 8.2. To retard carbamylation of proteins (14), urea solutions were passed over a mixed bed ion-exchange resin (Rexyn I-300, Fisher Scientific Company, Philadelphia, Pa.). Buffers made with this deionized urea were stored at 4° and replenished every 24-48 h during experiments carried out at 25°. Column fractions were monitored for protein by their absorption at 280 nanometers, dialyzed against 0.01% EDTA, pH 8.2, using #18 cellulose casing (Union Carbide, Chicago, Ill.), and stored at -20°.

Immunochemical Techniques

Immunoelectrophoresis and immunodouble diffusion were performed in 1% agarose (SeaKem, Bausch and Lomb Inc., Rochester, N.Y.) using standard techniques. Antisera were prepared in sheep and rabbits as previously described (2). The reactivities of antisera used in this study are presented in Table I. The difficulty in preparing high density lipoprotein antisera free of reactivity to low density

lipoproteins and human serum albumin has been noted previously (2). The apoVLDL-S3 fraction (15) used to prepare the anti-apoLP-ser* and anti-apoLP-ala antisera was also contaminated with trace amounts of low density lipoprotein antigen. Because none of the antisera were monospecific, immunodiffusion experiments were performed by placing an antiserum in the center well and surrounding it with a delipidated, chromatographically-purified, known antigen, alternating with the delipidated unknown fractions. The identity of an unknown fraction could be established if it showed a reaction of identity with the purified neighboring antigen. Immunoprecipitin lines formed by low density lipoprotein, albumin, apoLP-gln I, apoLP-gln II, apoLP-ser and either apoLP-ala_1 or apoLP-ala_2 were nonidentical with each other. ApoLP-ala_1 and apoLP-ala_2 gave indistinguishable immunochemical reactions. Equivalence titers to purified

*Constituent apoproteins of the high density lipoproteins will be referred to as follows: major apoproteins, apoLP-gln I (corresponding to Apo A-I (16), R-thr (7), Fraction III (8) and Band C (9)) and apoLP-gln II (corresponding to Apo A-II (16), R-gln (7), Fraction IV (8), and Band D (9)) with carboxyterminal glutamine (16); and minor apoproteins, apoLP-ser, apoLP-glu, apoLP-ala_1 and apoLP-ala_2, with carboxyterminal serine, glutamic acid, alanine and alanine, respectively (17,18). Antisera to individual apoproteins will be designated by above terminology.

high density lipoprotein apoproteins were determined semi-quantitatively using the technique of Piazzi (19) and were used to estimate appropriate proportions of the reactants in double diffusion experiments.

Electron Microscopy

Negative staining procedures and electron microscopy of lipoprotein samples have been described elsewhere (20).

Other Techniques

Polyacrylamide gel electrophoresis was performed in 0.6 x 8.0 cm tubes. Gels containing 10% acrylamide were run in 8 M urea at pH 9.4 using the buffer systems of Reisfeld and Small (21) and stained with 0.05% Coomassie blue (Colab Laboratories Inc., Chicago Heights, Ill.) as described by Chrambach et al. (22). Twenty to 50 µg of protein were applied to each tube. Procedures for paper electrophoresis of lipoproteins have been described previously (23). The paper strips were stained with bromphenol blue for proteins and Oil Red O for lipids.

RESULTS

Physical Appearance of Ultracentrifugal Fractions Isolated from Dehydrated-Solubilized Parent High Density Lipoprotein Fraction

The ultracentrifugal $d < 1.006$ g/ml product fraction was pale yellow in color and highly turbid, indicating the presence of very large and probably lipid-rich particles. The light scattering properties of this fraction were comparable to those of normal very low density lipoproteins of

d < 1.006 g/ml. The d 1.006-1.063 g/ml product fraction was also pale yellow but showed appreciably less turbidity than the d < 1.006 g/ml product fraction. Except for its color, this product fraction was comparable in appearance to normal low density lipoproteins of d 1.006-1.063 g/ml. The d 1.063-1.21 g/ml product fraction was pale yellow and almost translucent, indicating the presence of particles of predominantly small size. The appearance of this product fraction differed from normal high density lipoprotein primarily in its slightly greater light scattering property. The ultracentrifugal d > 1.21 g/ml product fraction was colorless and transparent; on pipetting, this fraction indicated the presence of soluble protein material by its tendency to froth and its increased viscosity compared to the background salt solution.

Distribution of Lipid and Protein among Ultracentrifugal Fractions

Representative data showing the ultracentrifugal distribution of the lipid and protein moieties of the high density lipoproteins after dehydration-solubilization are presented in Table II. Lipids were primarily distributed among the three major flotation fractions resulting in a 77% reduction in the amount of lipid initially present in the parent fraction. The d < 1.006 and d 1.006-1.063 g/ml product fractions contained approximately 27 and 31%, respectively, of the lipid material initially present in the parent high density lipoprotein fraction. The reduction in

the protein content of the parent fraction was approximately 82%, with the bulk of the protein shifting into the lipid-poor d > 1.21 g/ml product fraction.

Lipid Composition of Ultracentrifugal Fractions

Analysis of the different fractions from two separate preparations indicated a marked redistribution of lipids among the newly formed lipoprotein species (Table III). There was a pronounced shift of cholesteryl esters and phospholipids from the parent high density lipoproteins into the d < 1.006 and d 1.006-1.063 g/ml product fractions. The d < 1.006 g/ml product fraction was highest in cholesteryl ester (64%) and triglyceride (7%) content and lowest in protein (4%) content. The major lipid components of the d 1.006-1.063 g/ml product fraction were cholesteryl esters (20%) and phospholipids (54%); the average protein content was 15%. The composition of the lipoprotein material remaining in the d 1.063-1.21 g/ml product fraction was considerably different than that of the parent and, relative to the parent fraction, contained a reduced amount of cholesteryl esters and an increased amount of phospholipids. The material in the d > 1.21 g/ml product fraction was almost exclusively protein (99%); the lipid moiety (1%) was predominantly phospholipid. Based on the above observations, the effects of dehydration-solubilization on high density lipoproteins appeared to include: (1) a dissociation of a substantial amount of lipid-poor protein material and (2) a redistribution of the lipid and remaining protein components into lipid-rich lipoprotein complexes.

Analytic Ultracentrifugation of Fractions

Graphic presentations of analytic ultracentrifugal patterns of a parent fraction and the lipoprotein product fractions isolated following dehydration-solubilization are shown in Figure 1. In the parent high density lipoprotein fraction, lipoprotein material was present strictly within the $F_{1.20}$ 0-9.0 flotation interval with a major peak at $F_{1.20}$ 2.3. This pattern also showed the presence of a substantial concentration of lipoprotein species in the $F_{1.20}$ 3.5-9.0 (HDL_2) range. The ultracentrifugal pattern of the d 1.063-1.21 g/ml fraction isolated from dehydrated-solubilized high density lipoproteins was considerably broader (flotation range included material in the $F_{1.20}$ 0-28.0) and indicated a more heterogeneous distribution of lipoprotein macromolecules. The presence of material in the $F_{1.20}$ 9.0-28.0 range in the d 1.063-1.21 g/ml product fraction suggested the formation of lipoprotein species of larger size than the parent lipoproteins. Analytic ultracentrifugation of each of the low density product fractions (d < 1.006 and d 1.006-1.063 g/ml fractions) showed the presence of significant concentrations of material with broad ranges of flotation rates. The ultracentrifugal pattern for the d < 1.006 g/ml product fraction showed a distribution ranging from S_f° 20 to S_f° 400. At the concentration used for this analysis there was little indication of material of flotation rates greater than S_f° 400. The d 1.006-1.063 g/ml product fraction contained material in the S_f° 0-50.0 flotation interval with a major peak at approximately S_f° 3.5.

The analytic ultracentrifugal pattern of the d > 1.21 g/ml product fraction showed (Fig. 2) one major sedimenting peak (in d 1.006 g/ml NaCl, at 52,640 rev./min and 26°). A faster sedimenting minor peak was occasionally observed.

Electron Microscopy of Ultracentrifugal Fractions

Electron microscopy of the parent fraction (Fig. 3a) showed particles with an average overall diameter of 85 Å apparently composed of subunits (20). Some larger structures were also observed in the parent high density lipoprotein fraction suggesting aggregation of these lipoproteins during preparative procedures for electron microscopy.

At least two species of particles were apparent in the ultracentrifugal d 1.063-1.21 g/ml fraction (Fig. 3b) isolated from dehydrated-solubilized material. One species was similar in size and shape to normal high density lipoproteins; the other species consisted of apparently discoidal particles which varied between 140-350 Å in diameter and stacked in aggregates with a 50 Å periodicity. The stacking was more extensive at elevated concentrations, suggesting that it was an artifactual phenomenon occurring during drying in the negative stain.

Electron microscopy of the d 1.006-1.063 g/ml product fraction (Fig. 3c) showed primarily spherical particles which were very heterogeneous in size. The diameters of the majority of the particles were in the range between 140-200 Å. These particles resembled normal low density lipoproteins in configuration but were smaller in size (the

normal
diameter of/low density lipoproteinsis approximately 210-250 Å). Very large spheroidal particles (300-1000 Å) were also observed in these preparations.

Electron micrographs of the d < 1.006 g/ml product fraction (Fig. 3d) showed particles exhibiting a greater diversity in size (240 up to 20,000 Å) than normal very low density lipoproteins (230-5000 Å). Most particles, however, were 1000 Å and greater. The very large particles often had a mottled appearance and frequently were aggregated. Like very low density lipoproteins these particles were very electron-transparent and showed evidence of deformation when in contact with other particles.

Paper Electrophoresis of Ultracentrifugal Fractions

The electrophoretic mobility of the ultracentrifugal fractions isolated from the dehydrated-solubilized parent high density lipoprotein fraction is presented in Fig. 4. All of the fractions migrated slower than the parent lipoprotein fraction. Most of the d < 1.006 g/ml product fraction remained at the origin; the remainder spread from the origin to the pre-β position. The other three product fractions had similar pre-β mobilities. The intensities of the protein and lipid stains were in qualitative agreement with the composition data for each fraction, as shown in Table III.

Protein Composition of Ultracentrifugal Fractions

The proteins in each fraction were characterized by polyacrylamide gel electrophoresis (Fig. 5) and immunodiffusion (Table IV). Sufficient protein was present in

the parent and the d 1.063-1.21 and d > 1.21 g/ml product fractions to permit quantification of the major and minor apoprotein components using Sephadex G-200 chromatography (Fig. 6 and Table V). The terminology used to designate the Sephadex fractions corresponds to that proposed by Scanu, et al. (8). The distribution of these Sephadex fractions in the parent high density lipoproteins was in good agreement with results from other laboratories (8,9). In general, fraction I contained 2-10%, fraction II 0-3%, fraction III 65-75%, fraction IV 20-25%, and fraction V 5-10% of the total high density lipoprotein protein (24). Fractions I (Table II) and II contained material which reacted strongly with anti-apoLP-gln I and weakly with anti-apoLP-gln II sera (24). Their elution in the Sephadex G-200 void volume, appearance on polyacrylamide gels (multiple smeared bands of slower mobility than apoLP-gln I) and tendency to decrease on redelipidation or prolonged urea exposure suggested that they were aggregated or incompletely delipidated apoproteins (24). Further purification of fractions III, IV, and V from the parent high density lipoprotein fraction using DEAE chromatography by the method of Shore and Shore (7) and characterization of the electrophoretic mobility, amino acid composition, molecular weights, end-terminal amino acids, and immunochemical reactivity (24) confirmed and related previous fractions (7-9). Fractions III and IV consisted of apoLP-gln I and apoLP-gln II, respectively. Fraction V contained a mixture of components, principally apoLP-ser, apoLP-glu, apoLP-ala_1 and apoLP-ala_2.

Remarkable differences in the distribution of these proteins in the products were evident (Figs.5 and 6, Tables IV and V). The minor protein constituents of the high density lipoproteins, apoLP-ser, apoLP-glu, apoLP-ala$_1$ and apoLP-ala$_2$, predominated in the d < 1.006 and d 1.006-1.063 g/ml product/ fractions (Fig. 5 and Table IV). As determined by polyacrylamide gel electrophoresis, apoLP-glu was almost entirely confined in the d < 1.006 g/ml product fraction. The apoLP-ala bands were most prominent in this fraction, but significant amounts were found in the d 1.006-1.063 and d 1.063-1.21 g/ml product fractions as well. This distribution was confirmed by the immunochemical studies outlined in Table IV. ApoLP-ser was not resolved on the polyacrylamide gels*, but was detected immunochemically, in both the d < 1.006 and d 1.006-1.063 g/ml product fractions. The d 1.063-1.21 g/ml product fraction differed from the parent high density lipoproteins in that apoLP-gln II was the major constituent (76.7%) with only small amounts of apoLP-gln I (4.0%) and fraction V proteins (9.6%). The remaining 9.7% (fraction I) contained both apoLP-gln I and apoLP-gln II.

*Purified preparations of apoLP-ser migrate slowly in this gel system and produce diffuse bands just beyond the boundary of the stacking and running gels (15). The presence of apoLP-ser of this mobility in polyacrylamide gels of normal high density lipoproteins can be demonstrated immunochemically, but its appearance is partially obscured by proteins in fraction I which also migrate in this position.

At least 92.5% of the protein moiety of the d > 1.21 g/ml product fraction was apoLP-gln I (Fig. 6 and Table V). In addition, only apoLP-gln I was detectable in the fraction I material obtained on chromatography of the d > 1.21 g/ml product fraction. A trace of apoLP-gln II (0.8%) was also present by gel filtration chromatography and could be seen on heavily loaded polyacrylamide gels. Based on amounts of apoLP-gln I and apoLP-gln II determined in fractions III and IV, one can calculate that at least 66% of the original apoLP-gln II was in the d 1.063-1.21 g/ml product fraction, and 82% of the original apoLP-gln I was in the d > 1.21 g/ml product fraction. These estimates were not corrected for the apoLP-gln I and apoLP-gln II present in fraction I or in the discarded intermediate ultracentrifugal fractions (Table II). Most of the remaining apoLP-gln II appeared to be in the d 1.006-1.063 g/ml fraction (Fig. 5).

Effect of Dehydration-Solubilization on Immunoelectrophoresis of High Density Lipoproteins

Previous studies had shown (2) only a single rapidly-moving form (designated αLP_A) on immunoelectrophoresis of fresh plasma against anti-high density lipoprotein sera. Following ultracentrifugal isolation of high density lipoproteins, an additional slower-moving lipid-poor form (αLP_B) appeared. As shown in Fig. 7, αLP_A was present in both HDL_2 (d 1.063-1.125 g/ml) and HDL_3 (d 1.125-1.21 g/ml) and reacted with both anti-apoLP-gln I and anti-apoLP-gln II. The αLP_B, absent in HDL_2 but present in HDL_3, was produced

during dehydration-solubilization of HDL_2, and reacted only with anti-apoLP-gln I. The d > 1.21 g/ml product fraction from dehydrated-solubilized high density lipoproteins contained only αLP_B (not shown). These experiments indicated that αLP_B is a lipid-poor form of apoLP-gln I which is produced during dehydration and solubilization of HDL_2. In corollary experiments αLP_B was also produced during dehydration-solubilization, sonication or ether-extraction of normal fresh plasma.

DISCUSSION

Dehydration by rotary evaporation and solubilization at pH 8.6 of human serum high density lipoproteins resulted in a highly specific and reproducible redistribution of the lipid and protein components of the parent material. The composition of the ultracentrifugal d < 1.006 g/ml product fraction was most interesting because of its high content of cholesteryl esters and its apoprotein composition, which consisted predominantly of the minor apoproteins of high density lipoproteins. These proteins are major apoprotein constituents of normal very low density lipoproteins in human serum. The density range, size and shape, aspects of protein composition, and the content of apolar lipid of this fraction were comparable to those of normal very low density lipoproteins. Unlike very low density lipoproteins, however, the major apolar "core" lipid of this fraction was cholesteryl ester instead of triglyceride. Also, unlike very low density lipoproteins, the d < 1.006 g/ml product fraction

was lacking in apoLDL which is the major protein of the very low density lipoproteins and is physiologically essential for their production (25). The presence of the minor apoproteins in association with this cholesteryl ester-rich fraction indicated a particular affinity of these proteins for apolar lipids.

The ultracentrifugal d 1.006-1.063 g/ml product fraction contained approximately 30% of the apoLP-gln II protein of the parent high density lipoprotein fraction. This fraction also accounted for approximately 25% of the total recovered cholesteryl esters. Under electron microscopy, the majority of these lipoprotein particles was smaller in size than normal low density lipoproteins, but was similar to the d 1.006-1.063 g/ml product formed following exposure of high density lipoproteins to diethyl ether (26). Unlike the dehydration-solubilization product, however, the ether degradation product was very similar in lipid composition to the parent high density lipoprotein and differed from the parent only in protein content (parent protein, 52%, ether-product protein, 12%) (6). Since the lipid and protein contents of the degradation products obtained by both methods were very comparable, major structural determinants of particles in this fraction may be principally the lipid-protein proportions and the type of protein present. Studies by Scanu, et al., (5) on products formed from extensive ether degradation of high density lipoproteins and on reassembly products formed during sonication of mixtures of apoproteins

and lipids of the high density lipoproteins, also indicated preferential incorporation of apoLP-gln II into the d < 1.063 g/ml species. The trace content of apoLP-gln II in the d < 1.006 g/ml product fraction and its presence in the phospholipid-rich d 1.006-1.063 and d 1.063-1.21 g/ml product fractions suggest a selective affinity of this protein for polar lipids (phospholipid).

The protein moiety of the d 1.063-1.21 g/ml product fraction consisted almost exclusively of apoLP-gln II. This product showed a higher phospholipid and lower cholesteryl ester percentage content than the parent high density lipoproteins; the phospholipid/cholesteryl ester weight ratio increased from 1.4 (parent) to 3.7 (d 1.063-1.21 g/ml product fraction). Under electron microscopy, the particles of this fraction exhibited a wide range of sizes and shapes; however, a frequent observation was the appearance of disc-like units which were aligned in stacks. Such aggregates have previously been observed under electron microscopy of the d 1.063-1.21 g/ml fraction isolated from the plasma of patients deficient in lecithin-cholesterol acyltransferase (27). In these patients the cholesteryl ester content of the total high density lipoprotein fraction was extremely low but high in both unesterified cholesterol and phospholipid (phosphatidyl choline + sphingomyelin/cholesteryl ester average weight ratio approximately 19) (28). Furthermore, work with model systems has shown that sonication of either apoLP-gln I or apoLP-gln II with mixtures of lecithin and unesterified

cholesterol also produces comparable discoidal-shaped particles and stacks as observed under electron microscopy (29). When cholesteryl esters, in amounts present in normal high density lipoproteins, were incorporated into the above sonication mixtures, the discs were transformed into spherical particles. Considering its wide diversity of particle size and shape, the d 1.063-1.21 g/ml product fraction probably consists of a distribution of particles containing primarily apoLP-gln II and phospholipid but with varying amounts of apolar lipids. Particles with negligible to low amounts of apolar lipid (primarily cholesteryl esters) are probably discoidal, while particles containing substantial amounts of apolar lipid appear spherical.

Dehydration-solubilization resulted in marked dissociation of lipid-poor protein, specifically apoLP-gln I, from the parent high density lipoproteins. This material was found almost exclusively in the ultracentrifugal d > 1.21 g/ml product fraction and accounted for most of the original apoLP-gln I of the parent lipoproteins. This lipid-poor protein fraction has immunoelectrophoretic properties identical to αLP_B which had earlier been described as a degradation product resulting from exposure of high density lipoproteins to mild physical and chemical procedures (2). The frequent observations of the ease of dissociation of apoLP-gln I from high density lipoproteins suggest a labile bonding of this apoprotein to lipoprotein structures in the

presence of the total lipids and other apoproteins of high density lipoproteins. The formation of the cholesteryl ester-rich d < 1.006 g/ml fraction during dehydration-solubilization may be a consequence of the almost complete dissociation of apoLP-gln I. Under ether treatment where the dissociation of apoLP-gln I does not appear as extensive, the new lipoprotein species formed were found only in the d 1.006-1.063 g/ml fraction and had the same lipid composition as the parent high density lipoproteins.

It is interesting that the dehydration-solubilization products qualitatively exhibit the same pattern of distribution of polar and apolar lipids as observed in normal serum lipoproteins of corresponding densities. Recently, a sonication procedure for reassembly of apoproteins and lipids of high density lipoproteins has been described by Hirz and Scanu (30). In a study (5) utilizing this procedure, products reassembled by sonication were ultracentrifugally isolated and analyzed for their lipid and protein composition. The distribution of lipids and specific apoproteins among the different fractions was highly comparable to that observed in the present report.

Changes in the state (freezing) and amount (evaporation) of water in the lipoprotein environment appear to strongly influence lipoprotein structure (31,32,33). In the present work, the perturbation of lipoprotein structure resulted during dehydration and solubilization of high density lipoproteins. Such treatment may disrupt hydrophobic

as well as electrostatic interactions in lipoprotein structure. The formation of new lipoprotein species following solubilization of high density lipoproteins dehydrated by rotary evaporation may include such processes as recombination of dissociated lipid and protein moieties, as well as recombination of dissociated lipid-protein and protein-protein subunit complexes.

Figure 1

Graphic computer presentations of analytic ultracentrifugal patterns of preparative fractions obtained from dehydrated-solubilized high density lipoproteins; (Upper) comparison of d 1.063-1.21 g/ml product fraction with parent HDL fraction; (Lower) flotation patterns of d < 1.006 and d 1.006-1.063 g/ml product fractions. To facilitate comparison of shapes and flotation ranges of the products with the parent material, the product distributions were plotted to correspond with the concentration value for the parent total high density lipoprotein fraction.

Figure 2

Ultracentrifugal schlieren pattern (tracing) of the d > 1.21 g/ml product fraction obtained at 26°, 52,640 rev./min in a medium of d 1.006 g/ml (NaCl). Pattern represents distribution of material after 64 minutes of ultracentrifugation at 52,640 rev./min.

Figure 3

Electron micrographs of negatively stained preparations of parent material and ultracentrifugal fractions obtained following dehydration-solubilization of parent high density lipoprotein fraction: (a) parent high density lipoprotein fraction; (b) d 1.063-1.21 g/ml product fraction; (c) d 1.006-1.063 g/ml product fraction; (d) d < 1.006 g/ml product fraction. All micrographs are magnified 212,000 X.

Figure 4

Paper electrophoresis of the parent high density lipoprotein fraction and each of the ultracentrifugal fractions from dehydrated-solubilized high density lipoprotein fraction stained for protein (P) and lipid (L).

Figure 5

Polyacrylamide gel electrophoresis of the protein components of the parent high density lipoprotein fraction and each of the ultracentrifugal product fractions.

Figure 6

Sephadex G-200 chromatography of the parent high density lipoprotein fraction and the d 1.063-1.21 and d > 1.21 g/ml product fractions from dehydrated-solubilized high density lipoproteins. Twenty milligrams of each fraction were applied to a column equilibrated in 0.2 M Tris-HCl, 0.01% EDTA, 6 M urea, pH 8.2 and eluted with the same buffer. The void volume is indicated by the arrow. The Roman numerals used to designate the eluted fractions correspond to those used by Scanu, et al. (8) and are further identified in Table V and in the test. Fraction II, intermittently found by those investigators, was not identified in these fractions. It is very similar to fraction I in composition (23).

Figure 7

Immunoelectrophoresis of HDL_2, HDL_3 and dehydrated-solubilized HDL_2. Antigens: 1, HDL_2; 2, HDL_3; 3, dehydrated-solubilized HDL_2. Antibodies: GII, anti-apoLP-gln II; GI, anti-apoLP-gln I. Reactivities corresponding to αLP_A and αLP_B are designated.

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TABLE I

CHARACTERIZATION OF ANTISERA

Antibody	Immunizing Antigen	Reactivity							
		apoLP-ser	apoLP-glu	apoLP-ala$_1$	apoLP-ala$_2$	LDL*	apoLP-gln I	apoLP-gln II	HSA
anti-apoLP-ser	apoVLDL-S3**	+	-	-	-	+	-	-	-
anti-apoLP-ala	apoVLDL-S3	-	-	+	+	+	-	-	-
anti-HDL	HDL	+	-	-	-	+	+	+	+
anti-apoLP-gln I***		+	-	-	-	+	+	-	+
anti-apoLP-gln II***		+	-	-	-	+	-	+	+

* Abbreviations in this table and subsequent tables and figures: HDL, high density lipoproteins (d 1.063-1.21 g/ml); LDL, low density lipoproteins (d 1.006-1.063 g/ml); VLDL, very low density lipoproteins (d < 1.006 g/ml); HSA, human serum albumin. For nomenclature on apoproteins see text.

** ApoVLDL-S3 refers to the third fraction obtained from the chromatography of detergent-solubilized apo-VLDL on Sephadex G150 (15). Two rabbits, injected with the same preparation, gave antisera with different specificities, as shown.

*** These antisera were made by absorption of anti-HDL with a five-fold excess of chromatographically purified apoLP-gln I (anti-apoLP-gln II) and apoLP-gln II (anti-apoLP-gln I).

TABLE II

ULTRACENTRIFUGAL DISTRIBUTION OF LIPID AND PROTEIN MOIETIES OF PARENT HIGH DENSITY LIPOPROTEIN FRACTION AFTER DEHYDRATION-SOLUBILIZATION

Ultracentrifugal Fraction	Lipid (%)*	Protein (%)*	Lipid + Protein (%)*
d < 1.006 g/ml	27	1	15
d 1.006-1.063 g/ml	31	7	20
d 1.063-1.21 g/ml	23	18	20
d > 1.21 g/ml**	***	63	30
Parent HDL	100	100	100

*Tabulated values are percentages of lipid and protein moieties in ultracentrifugal fractions calculated relative to initial amounts of these moieties in parent HDL fraction. Percentages do not total up to 100% due to omission of lipid and protein material in intermediate fractions collected during preparative ultracentrifugal procedures and to small unavoidable losses encountered during fractionation.

**See text for definition of this fraction.

***A very small amount of lipid (< 1% of total lipid in parent fraction) was recovered in this fraction. The lipid was primarily phospholipid.

TABLE III

COMPOSITION OF ULTRACENTRIFUGAL FRACTIONS ISOLATED FROM DEHYDRATED-SOLUBILIZED PARENT HIGH DENSITY LIPOPROTEIN FRACTION

Ultracentrifugal Fraction	Protein*		Lipid*							
			Cholesteryl esters		Phospholipids		Unesterified cholesterol		Triglycerides	
	A**	B	A	B	A	B	A	B	A	B
d < 1.006 g/ml	4	4	64	64	18	20	6	6	8	6
d 1.006-1.063 g/ml	17	13	23	17	52	56	4	10	4	4
d 1.063-1.21 g/ml	41	35	12	11	40	46	4	5	3	3
d > 1.21 g/ml	99	99	--	--	1	1	--	--	-	--
Parent HDL	48	44	21	18	25	30	3	5	3	3

*Tabulated values are percentages by weight.

**Columns A and B designate results of two separate experiments using two different parent high density lipoprotein fractions.

TABLE IV

IMMUNOREACTIVITY OF ULTRACENTRIFUGAL FRACTIONS

Ultracentrifugal Fraction	Reactivity*			
	Anti-apoLP-gln I	Anti-apoLP-gln II	Anti-apoLP-ser	Anti-apoLP-ala
d < 1.006 g/ml	+	+	+++	+++
d 1.006-1.063 g/ml	+	++	++	+++
d 1.063-1.21 g/ml	+	++++	0	+
d > 1.21 g/ml	++++	0	0	0

*Reactivities were graded, according to intensity and position of the immunoprecipitin lines, from 0 (no reaction) to ++++ (strong reaction). The specificity of the reaction between the unknown fraction and an antiserum was confirmed by its identity with the immunoprecipitin line formed by the antiserum and a purified antigen of the same name.

TABLE V

SEPHADEX G-200 CHROMATOGRAPHY OF ULTRACENTRIFUGAL FRACTIONS

Sephadex Fraction	Proteins*	Percent Composition		
		Parent HDL	d 1.063-1.21 g/ml	d > 1.21 g/ml
I	**	1.5	9.7	6.7
III	apoLP-gln I	71.0	4.0	92.5
IV	apoLP-gln II	21.0	76.7	0.8
V	apoLP-ser, apoLP-glu, apoLP-ala$_1$, apoLP-ala$_2$	6.5	9.6	0.0

*Proteins in Sephadex G-200 fractions I-V.

**The composition of this fraction was variable.

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