Role of Apolipoprotein A-I in the Structure of Human Serum High Density Lipoproteins

RECONSTITUTION STUDIES*

MARY C. RITTER† AND ANGELO M. SCANU§

From the Departments of Medicine and Biochemistry, The University of Chicago Pritzker School of Medicine, and The Franklin McLean Memorial Research Institute, Chicago, Illinois 60637

For a better definition of the role of the human serum apolipoprotein A-I (apo A-I) in high density lipoprotein structure, a systematic investigation was carried out on factors influencing the in vitro association of this apoprotein with lipids obtained from the parent high density lipoprotein (HDL); these lipids include phospholipids, free cholesterol, cholesteryl esters, and triglycerides. Following equilibration, mixtures of apo A-I and lipids in varying stoichiometric amounts were fractionated by sequential flotation, CsCl density gradient ultracentrifugation, or gel-permeation chromatography, and the isolated complexes were characterized by physicochemical means. As defined by operational criteria (flotation at density 1.063 to 1.21 g/ml), only two types of HDL complexes were reassembled: one, reconstituted HDL, small with a radius of 31 Å and the other, reconstituted HDL, large with a radius of 39 Å. The two types incorporated all of the lipid constituents of native HDL and contained 2 and 3 mol of apo A-I, respectively. A maximal yield of reconstituted HDL (R-HDL) was observed at an initial protein concentration of 0.1 μM, where apo A-I is predominantly monomeric. At increasing protein concentrations, the amount of apo A-I recovered in R-HDL was found to be proportional to the initial concentration of monomer and dimer in solution. The composition and yield of the complexes were independent of ionic strength and pH within the ranges studied. Both simple incubation and co-sonication of apo A-I with HDL phospholipids produced complexes of identical composition, although the yield of complexes was higher with co-sonication. When the comparison of the same methods was extended to mixtures of apo A-I and whole HDL lipids, the results confirmed previous observations that co-sonication is essential for the incorporation of the neutral lipid into the R-HDL complexes.

The results indicate that (a) in vitro complexation of apo A-I with lipids is under kinetic control; (b) apo A-I can generate a lipid-protein complex with properties similar to those of the parent lipoprotein; (c) the process requires well defined experimental conditions and, most importantly, the presence in solution of monomers and dimers of apo A-I; (d) the number of apo A-I molecules incorporated into R-HDL determines the size and structure of the reassembled particle. All of these observations strongly support the essential role of apo A-I in the structure of human HDL.

Apolipoprotein A-I is the most abundant protein constituent of the human serum high density lipoprotein class. Thus, a definition of its structural role in the native complex would be of great interest. The lipid-binding capability of apo A-I has been examined in a number of laboratories (1, 2). The reported results have differed considerably as to the lipid-binding capacity of this apoprotein. In some cases, apo A-I was capable of binding only a small quantity of lipids (3-5); in other instances, it appeared to complex with relatively large amounts (6-8). Since the influence of protein concentration and medium conditions was not examined in these studies, the discrepancy in the reported results could be attributable to differences in experimental conditions, especially in concentrations. This possibility was suggested by previous observations from this (9) and other laboratories (10, 11) showing that the lipid-free apo A-I from human or other species self-associates in aqueous solutions, a fact that is suspected to influence the binding of this apolipoprotein for lipids. In the present work, we have systematically examined the interaction of human apo A-I and HDL lipids in vitro. The results obtained have defined the conditions for the maximal incorporation of apo A-I into complexes with properties similar to those of native HDL. They have also given an insight into the nature of the reassembly

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Isolation of Apo A-I from HDL

Human serum HDL was isolated from the blood of normal, healthy, fasting male donors (A) by ultracentrifugal flotation as previously described (12). The HDL preparations were dialyzed extensively against a solution of 0.15 M NaCl and 0.005% EDTA, pH 7.0, and delipidated at -10°C with 3/2 (v/v) ethanol/ethylether (13). The apoprotein of HDL (apo HDL) was first fractionated by Sephadex G-200 gel permeation chromatography in 8 M urea (14); Peak III was further fractionated by ion exchange chromatography on DEAE-Sephadex (Whatman DE52 microgranular) in 8 M urea (15). The apo A-I thus obtained migrated as a single band during polyacrylamide gel electrophoresis in 8 M urea (16) and during 0.1% sodium dodecyl sulfate-polycrylamide gel electrophoresis (17), and was indistinguishable from an authentic sample of apo A-I.

Isolation of HDL Lipids

The lipids obtained by ethanol/ether extraction of HDL (13) were concentrated by flash evaporation and stored in 9/1 (v/v) chloroform/methanol at -15°C. The phospholipids were separated from cholesterol esters, unesterified cholesterol, and triglycerides essentially according to the method of Rouser et al. (18) by chromatography on silicic acid (Unisil, 100 to 200 mesh, Clarkson Chemical, Williamsville, N. Y.).

Sonication of Lipids and Apo A-I

The sonication procedure was similar to that described previously (19). In a typical experiment, appropriate aliquots of lipid solutions were pipetted into the reaction vessel (Pyrex, 17.8 x 102 mm, Ivan Sorval, Inc., Newtown, Conn.) and dried down by blowing nitrogen gas. The residue was immediately suspended in 5.4 ml of 0.02 M EDTA, pH 8.6, by gentle stirring with a glass rod, and was subjected to sonic irradiation in a Branson sonifier model 1850 (Heat Systems-Ultrasonic, Inc., Plainville, L. I., N. Y.), with a standard microtip for 15 s at 75 watts and ambient temperature (about 27°C). The lipid dispersion obtained was then incubated at 41°C for 30 min to insure the gel-liquid crystalline transition of the hydrocarbon chains (20). Next, a 1.0-m1 solution of apo A-I dissolved in 0.02 M EDTA, pH 8.6, was added, and the resulting protein/lipid mixture was sonicated under nitrogen (75 watts, three 1-min bursts). Each sonic burst was sufficient to insure complete interaction between protein and lipid, and any additional sonication time did not influence the results.

Separation of Lipid-Protein Mixtures

In most experiments, the lipid/protein mixtures were first centrifuged at density 1.006 g/ml in a Beckman model L2-65B ultracentrifuge for 20 h at 11°C in a 40.3 rotor (Beckman Instruments, Palo Alto, Calif.) at 38,000 rpm. This step permitted the sedimentation of the unreacted apo A-I or any lipid-protein complex formed. Under conditions of lipid excess, the complete separation of the lipids not incorporated into the complex was obtained only by ultracentrifugation of the lipid-protein mixture at density 1.063 g/ml in a Beckman L2-66B ultracentrifuge for 24 h at 11°C in a 40.3 rotor at 38,000 rpm. The undernaturals of density 1.006 or 1.063 g/ml were further fractionated by isopycnic density gradient ultracentrifugation, ultracentrifugal flotation, or agarose column chromatography for separation of the lipid-free apo A-I from the lipid-protein complexes.

Protein and Lipid Assays

Protein Determination - Most protein determinations were performed by a modification of the method of Lowry et al. (21) with bovine serum albumin (Nutritional Biochemicals Corp., Cleveland, Ohio) used as a standard. In the density gradient experiments, to avoid interference by CaCl2, we first precipitated the protein with 10% trichloroacetic acid for 20 h and centrifuged it at 800 rpm for 27 min in the preparative SW-50 rotor (Beckman) at 1°C. The aqueous effluents were monitored for both protein and lipid. For the estimation of the apparent molecular weight of the lipid-protein complexes, the columns were calibrated with protein standards, including collagen, ovalbumin (Sigma), bovine serum albumin (Schwarz/Mann), lactic acid dehydrogenase from beef muscle (Sigma), yeast alcohol dehydrogenase (Sigma), human y-globulin, Fraction II (Schwarz/Mann), human serum HDL, catalase from beef liver (Sigma), horse apoferritin (Schwarz/Mann), agarose (Bio-Gel A-1.5m, 200 to 400 mesh; Bio-Rad Laboratories, Richmond, Calif.) or on columns (2.6 x 100 cm) of 4% agarose (Pharmacia Fine Chemicals, Uppsala, Sweden) of 8% agarose (Pharmacia). The eluting buffer was 0.15 M NaCl and 0.01% EDTA, pH 7.0, and was pumped at an upward flow rate of 10 ml/h for the 4% agarose column and 7 ml/h for the 8% agarose column. The eluate effluents were monitored for both protein and lipid. For the estimation of the apparent molecular weight of the lipid-protein complexes, the columns were calibrated with protein standards, including cytochrome c from horse heart (Sigma Chemical Co., St. Louis, Mo.), myoglobin from sperm whale (Schwarz/Mann, N. Y.), trypsin inhibitor (Sigma), ovalbumin (Sigma), bovine serum albumin (Schwarz/Mann), lactic acid dehydrogenase from beef muscle (Sigma), yeast alcohol dehydrogenase (Sigma), human y-globulin, Fraction II (Schwarz/Mann), human serum HDL, catalase from beef liver (Sigma), horse apoferritin (Schwarz/Mann), agarose (Bio-Gel A-1.5m, 200 to 400 mesh; Bio-Rad Laboratories, Richmond, Calif.) or on columns (2.6 x 100 cm) of 4% agarose (Pharmacia Fine Chemicals) and mercaptoethanol (Eastman Kodak Co., Rochester, N. Y.), respectively.

Lipid Analyses - The phosphorus content was determined by the method of Bartlett (22), and the quantity of phospholipids was calculated from the phosphorus determinations by use of a conversion factor of 25. Phospholipids from lipid-protein complexes were extracted with 2/1 (v/v) chloroform/methanol before analysis. The individual phospholipids were separated and identified by thin layer chromatography on precoated silica gel plates (Brinkmann Instruments, Inc., Westbury, N. Y.) with a standard mixture. The phospholipids were eluted with 9/1 (v/v) methanol/chloroform and then analyzed for phosphorus (22). The phosphorus content was determined by the method of Bartlett (22), and the quantity of phospholipids was calculated from the phosphorus determinations by use of a conversion factor of 25. Phospholipids from lipid-protein complexes were extracted with 2/1 (v/v) chloroform/methanol before analysis. The individual phospholipids were separated and identified by thin layer chromatography on precoated silica gel plates (Brinkmann Instruments, Inc., Westbury, N. Y.) with a standard mixture. The phospholipids were eluted with 9/1 (v/v) methanol/chloroform and then analyzed for phosphorus (22).

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cholesteryl linoleate and unesterified cholesterol, the molecular weights of cholesteryl esters, the amount of cholesteryl esters was calculated from the difference between total and free cholesteryl contents. Specific activities were determined from the known specific activity of the free cholesterol and cholesteryl oleate and from the concentration of the unlabeled material. For the calculation of the molar concentrations for cholesteryl esters, the molecular weights of cholesteryl linolate and unesterified cholesterol, 649 and 387, respectively, were used.

Circular Dichroism

Circular dichroic studies were performed as previously described (25) with a Cary model 6001 Spectropolarimeter provided with a circular dichroism attachment (Cary Instruments, Monrovia, Calif.) and calibrated with an aqueous solution of (d)-10-camphorsulfonic acid (Eastman).

Amino Acid Analyses

Lipid-free protein samples were hydrolyzed in 6 n HCl for 24 h at 110°C. Analyses were done in duplicate on a Beckman model 102 amino acid analyzer equipped with an automatic sample injector, scale expander, and integrator. Tryptophan was quantitated after hydrolysis in 4 n methanesulfonic acid (27).

Immunological Studies

Ouchterlony double diffusion experiments were carried out in 1% agarose in 0.05 M Veronal buffer, pH 8.6, at 4°C. The immunoprecipitation lines were compared with Amido black for proteins and were cleared with a carbamylation (31, 33).

Analytical Ultracentrifugation

For flotation analyses, we used a Beckman model k analytical ultracentrifuge equipped with electronic temperature and speed controls and a photometric scanning optical system. The reassembled HDL particle isolated by preparative ultracentrifugation was first dialyzed extensively against an NaCl/NaBr solution of density 1.21 g/ml and floated at this density. The analyses were conducted at 20°C, 44,000 rpm, with an AN-H rotor and a double sector cell. The reciprocal of the apparent sedimentation coefficients obtained at 44,000 rpm, with an AN-H rotor and a double sector cell. The reciprocal of the apparent sedimentation coefficients obtained at 44,000 rpm, with an AN-H rotor and a double sector cell. The reciprocal of the apparent sedimentation coefficients obtained at 44,000 rpm, with an AN-H rotor and a double sector cell.

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis in 8% urea (14) was a modification of the procedure described by Davies (16).

Agarose Gel Electrophoresis

Lipid-protein complexes and human serum were dialyzed extensively against 0.15 M NaCl and 0.01% EDTA, pH 7.0. Samples were then subjected to thin gel electrophoresis in agarose (28), by use of precast Agarose Universal Electrophoresis Film (C. A. B. 24,500 mmbar, and 0.05% EDTA, pH 8.6, according to the manufacturer’s instructions. The films were stained with Fast Red 7B for lipids and with Amido black for proteins, and were cleared with a solution of 1/1(v/v) absolute methanol/water.

Phospholipase Treatment

The reassembled complex was first dialyzed extensively against 0.15 M NaCl and 0.01% EDTA, pH 7.0. Enzymatic digestion by a phospholipase A, purified from Crotalus adamanteus venom (gift of Dr. Betty Shen, Department of Biochemistry, University of Chicago) proceeded under a stream of nitrogen in 1 mM borate buffer, pH 7.4, containing 7 mM CaCl2, in the absence of bovine serum albumin in a total volume of 2 ml, as described by Pattnaik et al. (29, 30).

Chemicals

The chemicals were reagent grade. Organic solvents were freshly distilled before use. Reagent grade urea (Malinkrodt, Inc., St. Louis, Mo.) was recrystallized from 95% ethanol and deionized through a mixed-bed ion exchange resin (Bio-Rad Laboratories, Richmond, Calif.). Fresh urea solutions were always utilized at 10°C to eliminate, or at least to minimize, cyanate formation and protein carbamylation (31–33).

RESULTS

In the initial phase of our work, we relied predominantly on the knowledge of the solution properties of apo A-I which we had gained from analytical ultracentrifugal studies (9), and we elected to investigate apo A-I at concentrations where this apoprotein had been shown to be predominantly monomeric. As a first approach, we utilized a 1:1 weight ratio of lipid to protein because early reports had indicated that this ratio might be suitable for reconstitution (34). Thereafter, the studies were planned in a systematic fashion for investigation of the effect on the reassembly process when changes were made in the parameters thought to influence the interaction between protein and lipids in vitro; these parameters included principally the apo A-I concentration, initial lipid/protein ratios, and medium conditions.

Formation and Separation of Lipid-Protein Complexes—Fractionation of lipid/protein mixtures of HDL lipids and apo A-I by CsCl density gradient ultracentrifugation, ultracentrifugal flotation, and gel permeation chromatography gave a good separation of the unreacted components from the lipid-protein complexes. We define a lipid-protein complex as the product containing both protein and lipid, and banding by ultracentrifugation or eluting on agarose columns in positions intermediate between those of the individual reactants. Typical profiles of lipid-free apo A-I, free lipid, and the lipid–apo A-I complex separated at an initial lipid/protein weight ratio of 1, as obtained by CsCl density gradient ultracentrifugation, are shown in Fig. 1. The lipid-free apo A-I band at a density of 1.28 g/ml (Fig. 1C) in the same position as that occupied by apo A-I in the absence of lipids (Fig. 1A). A band of protein-free lipid which floated to the top of the tube (Fig. 1, C and D) was identical with that of lipids alone (Fig. 1B). The lipid–apo A-I complex had a hydrated peak density of 1.13 g/ml. This complex was similar to that of native HDL in the broadness of its elution profile and, thus, behaved as a discrete particle with a distinct lipid and protein composition (Fig. 1, C and D). Varying the ionic strength of the medium between 0.5 mM and 0.5 M, or the pH between 6.6 and 8.6, had no effect on the yield or on the lipid/protein composition of the lipid–apo A-I complex.

The banding hydrated density of the lipid–protein complex formed varied with the initial lipid/apo A-I weight ratio (compare Fig. 1, C and D, with Fig. 3; also see Table VI). In all instances studied, however, the lipid–apo A-I complex floated at a density of 1.063 to 1.21 g/ml; thus it was similar to native HDL in operational terms. We will refer to this reassembled complex as R-HDL, using the subscript S (small) or L (large) to refer to the radius of the R-HDL particle (Table VI). Whereas R-HDL was formed predominantly at initial lipid/apo A-I weight ratios < 1, R-HDL was formed at initial weight ratios > 1.

Taking advantage of this similarity, in operational terms, to native HDL, we isolated R-HDL and R-HDL in large
Interaction of Apo A-I with HDL Lipids

Fig. 1. Profiles of apo A-I, lipid, and lipid-apo A-I complexes obtained by density gradient ultracentrifugation. The gradient extends from density 1.45 approximately to density 1.05 g/ml (from left to right). Apo A-I and/or HDL lipids were co-sonicated in a total volume of 6.4 ml and centrifuged at 11" for 24 h at 38,000 rpm in a Spinco 40.3 rotor, followed by isopycnic density gradient ultracentrifugation as described under "Materials and Methods." The number above each band is the average density in grams/ml. A, 1.5 mg of sonicated apo A-I without lipid (the dotted line denotes a typical density gradient observed after centrifugation); B, 1.2 mg of sonicated HDL lipids without apo A-I; C, 1.2 mg of apo A-I and HDL lipids at an initial lipid/protein weight ratio of 1; D, 21 μg of apo A-I and HDL lipids at an initial lipid/protein weight ratio of 1.

amounts by the quick and convenient method of ultracentrifugal flotation. Utilizing this method, we observed no differences in the yield or in the lipid/protein composition of the R-HDL particles compared to those obtained after separation by CsCl density gradient ultracentrifugation.

The fractionation of the sonicated lipid/apo A-I mixtures by agarose column chromatography also afforded a good separation of the reacted and unreacted components. The lipid-free protein and the free lipids eluted near the inclusion volume and the void volume of the column, respectively, whereas the lipid-protein complex eluted as a broad peak between these two peaks.

To establish conditions for maximal complexation between HDL lipids and apo A-I, we examined the effect of incubation versus co-sonication on the interaction of apo A-I with either HDL phospholipids or the whole of the HDL lipids (Table I). With mixtures of apo A-I and HDL phospholipids, no differences were found in the composition of the complex formed either by incubation or co-sonication, although the yields were higher with co-sonication. When apo a-I, in turn, interacted with the total HDL lipids, co-sonication not only produced an actual increase of apo A-I and phospholipid incorporated into the lipid-protein complex, but also promoted the incorporation of both unesterified and esterified cholesterol. Under all conditions, the specific activity of the cholesteryl esters was the same as that of the starting material, indicating that the complex formation does not discriminate between the cholesteryl esters isolated from HDL and radioisotopic cholesteryl oleate. Sonication of apo A-I in the absence of lipids did not result in structural changes of the apoprotein, as determined by circular dichroic measurements, immunodiffusion against rabbit anti-apo A-I antibody, electrophoretic behavior in polyacrylamide gel electrophoresis in the presence of urea or in 0.1% sodium dodecyl sulfate, and amino acid analysis. In addition, the apo A-I recovered in the lipid-protein complex after sonication, when subjected to a quantitative double antibody radioimmunoassay analysis (35), was indistinguishable from native apo A-I.

Effect of Apo A-I Concentration—To determine the influence of protein concentration on yield and on the lipid-binding capacity of apo A-I, we next studied the reassembly of apo A-I at different apo A-I concentrations, but at a constant ratio of protein to lipid. As shown in Table II and Fig. 1D, at the low concentration of 0.1 μM apo A-I, the incorporation of the apoprotein into R-HDL reached almost 100%. The parallel increase in the concentration of the reactants caused a progressive decrease in the percentage of lipid and protein recovered in the complex (Table II; compare also Fig. 1, C with D). In spite of the low recoveries at high apo A-I concentrations, however, the lipid distribution of the complex was unaffected (Table II).

The above experiments suggested a possible relationship between the state of association of the protein and its lipid-binding capacity. To investigate further the dependence of the lipid-binding capacity of apo A-I on protein oligomerization, we first determined the state of aggregation of apo A-I under our experimental conditions. For this purpose, we passed lipid-free apo A-I, in varying concentrations, through agarose columns calibrated with proteins of known molecular weight and estimated the apparent molecular weight of the eluted material. At low concentrations (0.1 μM), apo A-I eluted predominantly as a monomer (Fig. 2A), whereas oligomer forms (dimers and tetramers) prevailed at higher protein concentrations (1 μM, Fig. 2B, and 6.5 μM, Fig. 2C).

Previous work in this laboratory by frontal elution-gel filtration chromatography (9) showed that the multiple species of apo A-I in solution attain equilibrium rather rapidly at 20°C. Our gel filtration studies suggest, however, that the establishment of the oligomerization equilibrium at 8°C is much slower than the time required for the experiment. Indeed, monomer-oligomer peaks were symmetrical and clearly resolved as shown in Fig. 2. In addition, the amount of each oligomeric species seemed to be independent of the time required for passage of the solution through the column. For these reasons, one can assume as a first approximation that, at 8°C, little interconversion of the oligomeric forms of apo A-I occurred during gel filtration. With this assumption, the self-association of apo A-I can then be assessed from our gel filtration studies. The concentration of each oligomeric species, as determined by graphical integration from the gel filtration elution diagram (Fig. 2), is shown in Table III (Columns 2 to 4) for various initial apo A-I concentrations. Column 5 of Table III lists the concentrations at which apo A-I is incorporated into the complex, as calculated from the results in the second row of Table II. From these data, it is evident that the amount of apo A-I incorporated in R-HDL, correlated best with the sum of the amount of this apoprotein present in solution as monomer and dimer (Column 4); only at an initial apo A-I concentration of 0.1 μM was there a good correlation with the monomer of apo A-I in solution. At higher concentrations, no
Interaction of Apo A-I with HDL Lipids

Apo A-I (1.2 mg) was either co-sonicated with dispersed HDL lipids or phospholipids (from HDL lipids) at an initial weight ratio of 1 as described under "Materials and Methods" or incubated with the lipids at 4°C for 24 h under nitrogen in a total volume of 6.4 ml. The resulting mixtures were then centrifuged and analyzed as outlined in the legend of Fig. 1. Lipid-free apo A-I and the lipid- apo A-I complex are the fractions shown in Fig. 1.

**TABLE I**

Comparison of incubation and co-sonication of apo A-I with HDL lipids

| Analytical results | Apo A-I + HDL lipids | Apo A-I + phospholipids |
|-------------------|----------------------|------------------------|
|                   | No sonication | Sonication | No sonication | Sonication |
| Lipid-free apo A-I, % weight | 85 | 60 | 80 | 66 |
| Apo A-I in complex, % weight | 15 | 40 | 20 | 34 |
| Lipid/apo A-I weight ratio in complex | 0.20 | 0.51 | 0.60 | 0.57 |
| Cholesteryl esters- apo A-I in complex, molar ratio | 4.7 | 2.2 |
| Free cholesterol- apo A-I in complex, molar ratio | 8.7 | 15.2 | 22.8 | 22.0 |

- Peak hydrated density of 1.13 g/ml.
- Peak hydrated density of 1.14 g/ml.
- Too low to be detected.

**TABLE II**

Effect of apo A-I concentrations on yield and composition of lipid-apo A-I complex isolated by CsCl density gradient ultracentrifugation

The solutions of apo A-I of varying concentrations were equilibrated in 4 ml of 0.02 M EDTA, pH 8.6, for 2 h at 4°C, and then co-sonicated with HDL lipids, when present, at an initial lipid/protein weight ratio of 1 in a total volume of 6.4 ml under "Materials and Methods." The sonicated mixtures were centrifuged at 11°C for 24 h at a density of 1.006 g/ml, followed by CsCl density gradient ultracentrifugation (see "Materials and Methods"). Lipid-free apo A-I and the lipid-apo A-I complex are the fractions shown in Fig. 1.

**TABLE III**

Relationship between oligomeric forms of apo A-I and amount of apo A-I incorporated in R-HDL at different initial apo A-I concentrations at lipid/protein weight ratio of 1

The data in the subheading and from the second row of Table II are the molar concentrations in the 1st and 5th columns of this table. The data in the 2nd and 3rd columns were calculated from elution profiles of apo A-I on agarose columns at each specified concentration. The area corresponding to the dimeric or monomeric forms of apo A-I was divided by the total area of the oligomeric forms of apo A-I, and the quotient was multiplied by the amount of apo A-I in the initial mixture. The data in the 4th column give the sum of data in Columns 2 and 3. The conditions for the fractionation of apo A-I by agarose column chromatography were those described in the legend of Fig. 2. The molarity of apo A-I is expressed in terms of the molecular weight of the monomer.

- From agarose column experiments.
- Based on yield of cholesteryl esters and free cholesterol.

**FIG. 2**

Elution profiles of apo A-I, at different concentrations, from agarose columns. Apo A-I was equilibrated in 0.02 M EDTA, pH 8.6 (4 ml, A and B; 6.2 ml, C), for 4 h at 4°C, centrifuged, and followed by 8% agarose column chromatography as described under "Materials and Methods." Vv refers to the column void volume and Vc to the total column volume (void plus internal volume). A, 12 µg or 0.1 µM apo A-I; B, 112 pg or 1 PM apo A-I; C, 1200 pg or 6.5 µM apo A-I.
Comparison of properties of two R-HDL$_2$ particles and native HDL$_1$

Apo A-I was co-sonicated at an initial concentration of 0.5 μM at an initial lipid/protein weight ratio of either 0.5 or 1.0 and centrifuged at a density of 1.063 g/ml as described under "Materials and Methods." The density 1.063 g/ml undernatants were then fractionated by CsCl density gradient ultracentrifugation, ultracentrifugal flotation at density 1.91 g/ml, or gel permeation chromatography as described under "Materials and Methods." The properties of R-HDL$_{1.16}$ and R-HDL$_{1.13}$ formed at an initial lipid/protein weight ratio of 0.5 and 1.0, respectively, are listed in Columns 1 and 2, respectively. For comparison, properties of native human serum HDL$_2$ are given in the last column.

|                  | R-HDL$_{1.16}$ | R-HDL$_{1.13}$ | HDL$_1$  |
|------------------|----------------|----------------|----------|
| Physical parameters |                |                |          |
| Hydrated density, g/ml | 1.18           | 1.13           | 1.18*    |
| $S_{V,1.21}$, Svedberg units | 3.30*          | 3.30          | 2.00*    |
| $[\Theta]_{222, m} \times 10^{16}$ | 2.08           | 2.30          | 2.20     |
| Apparent molecular weight $\times 10^6$ | 0.92-1.15      | 1.15          | 1.75*    |
| Electrophoretic mobility (aga $\beta$, cm$^2$·g/mol) | $\sim \alpha_1$ | $\alpha_1$ | $\alpha_2$ |
| Weight per cent |                |                |          |
| Protein          | 70             | 60             | 57*      |
| Total phospholipids | 21             | 28             | 26*      |
| Phosphatidylcholine | 17.6*          | 23.2*          | 20.8*    |
| Sphingomyelin     | 1.5            | 2.8            | 2.3*     |
| Phosphatidylethanolamine | 1.9           | 2.0            | 2.1*     |
| Lyso phosphatidylcholine | 2             | 3              | 2*       |
| Free cholesterol  | 7              | 9              | 11*      |
| Cholesterol esters | 9              | 11             | 11*      |
| * Ref. 34.       |                |                |          |
| ** Major component; a minor component was also present.|
| * Degrees $\cdot$cm$^2$·g/mol.|
| * Ref. 36.       |                |                |          |
| * May also represent small amounts of phosphatidylserine and phosphatidylinositol.|
| * Includes 2.4% phosphatidylinositol and 0.6% phosphatidylserine.|
| * Ref. 24.       |                |                |          |

When solutions containing 6.5 μM apo A-I were co-sonicated with HDL lipids at an initial lipid/protein weight ratio less than 1, the hydrated density and lipid composition of the lipid-protein complexes formed were found to differ from those obtained at a weight ratio of 1. For example, at an initial lipid/protein weight ratio of 0.2, a dominant lipid-protein complex with a peak density of 1.21 g/ml was observed together with a small amount of complex of a peak density of 1.15 g/ml (Fig. 3). Approximately 55% of apo A-I remained lipid-free and peaked at density 1.28 g/ml, and the protein-free lipids banded at the top of the tube. As the initial lipid/protein weight ratio was increased above 0.2, a decrease in the amount of lipid-protein complex of density 1.21 g/ml occurred concomitant with an increase in the amount of a complex with smaller particle size. Compared to native HDL$_1$, the lipid-apo A-I complexes which formed below a weight ratio of 1 were impoverished in lipid (see Table VI). One of the R-HDL$_2$, particles, R-HDL$_{1.16}$ (the subscript 1.16 again referring to the hydrated density of the particle), at an initial lipid/apo A-I weight ratio of 0.5, was partially characterized; its properties are summarized in Table IV, Column 1.

At initial lipid/apo A-I weight ratios above 1, CsCl density gradient ultracentrifugation poorly resolved a broad component with general properties similar to R-HDL$_2$ (see Table VI), and a light component which floated near the top of the tube and contained mostly lipid with some protein. The latter particle will be referred to as the lipid-rich particle. For example, at an initial lipid/apo A-I weight ratio of 4 and an initial apo A-I concentration of 6.5 μM, approximately 30% of apo A-I remained lipid-free, whereas about 20% and 50% were recovered in R-HDL$_1$ and the lipid rich particle, respectively. When the initial lipid/protein weight ratio was increased above 6, both R-HDL$_1$ and the lipid-free apo A-I disappeared, whereas the lipid-rich particle became the predominant component.

Gel permeation chromatographic studies corroborated the density gradient ultracentrifugation observations. As the initial lipid/protein weight ratio of the co-sonicated mixtures increased above 1, a significant increase in the components of higher particle size was obtained. For example, at our initial lipid/protein weight ratio of 4 and an initial apo A-I concentration 6.5 μM, approximately 40% of the eluted apo A-I was lipid-free, and 50% was part of a heterogeneous lipid-protein complex with an apparent particle weight of 15.1 to 17.5 x 10^4. Agarose columns gave poor resolution of the R-HDL$_2$ and lipid-rich particles observed by CsCl density gradient ultracentrifugation. In addition, a small amount of the lipid-rich particle now eluted in the void volume.

The lipid-rich particle, formed at an initial lipid/protein weight ratio of 4 and an initial concentration of 6.5 μM apo A-I, floated at density 1.063 g/ml. Its apparent weight ranged from 17.5 x 10^4 to 2.8 x 10^5, as estimated by gel permeation chromatography. Its weight per cent composition was protein, 11%; phospholipid, 68%; free cholesterol, 9%; cholesteryl esters, 12%. An ultracentrifugal analysis of this lipid-rich component indicated a heterogeneous particle having components of $S_{V,1.063}$ between 2.0 and 48 at 20°.

The results of the compositional analyses parallel the observed shift of the lipid-apo A-I complex from a peak density of 1.21 g/ml to smaller densities (see Table VI). In particular, increasing the initial lipid/apo A-I weight ratio entailed a steady increase in the phospholipid content of the particle. In contrast, the content of free cholesterol and cholesteryl esters increased until the lipid/protein weight ratio reached approximately 1; further increases in this ratio did not produce any increase in the cholesterol content of the particle. At the initial lipid/apo A-I weight ratio of 0.2, the limiting factor appears to be the phospholipid content, since all of the initial phospholipid in the reaction mixture was incorporated into the complex. At higher weight ratios, however, the sphingomyelin/phosphatidylcholine ratios increased almost by a factor of 2 (see Table IV), indicating that, when excess phospholipid is present, the complex is preferentially enriched in sphingomyelin. On the other hand, the phospholipid/cholesteryl ester ratio was virtually constant up to an initial lipid/apo A-I weight ratio of 1.
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ratio of $z$, whereas the cholesteryl ester/free cholesterol ratio decreased steadily.

Protein incorporation was also studied as a function of the initial lipid/protein weight ratio at a constant concentration of apo A-I (Table V). For this purpose, we determined the distribution of apo A-I between the lipid-free form, R-HDL, and the lipid-rich particle. These data, expressed in molarities at equilibrium, are presented in Columns 2 to 4 of Table V. At initial lipid/apo A-I ratios between 0.2 and 2.0 (Column 1), the amount of apo A-I incorporated into the complex was constant within the experimental error, 2.4 to 3.3 $\mu M$ with a mean $\pm$ standard deviation of 2.8 $\pm$ 0.4 $\mu M$ (bracketed data, Column 3). These results were in agreement with those obtained under conditions of varying protein concentration at a constant lipid/protein weight ratio of 1 (compare with bracketed data in Table III, Columns 4 and 5). At lipid/protein weight ratios above 2, a significant decrease occurred in the apo A-I incorporated both in R-HDL and in the lipid-free fraction, accompanied by a countervailing increase of apo A-I in the lipid-rich particle. It is possible that, under those conditions, the amount of R-HDL formed was underestimated, if it was entrapped in the floating lipid-rich particle and had not separated under our experimental conditions. Our results as a whole confirm that the generation of R-HDL is not a true equilibrium process.

Data Analyses – The data presented in the previous sections allowed us to answer the question as to how many different classes of R-HDL particles were formed under our conditions of reconstitution, and whether the lipid composition of the particles was a reflection of apoprotein content. To this end, we used the results obtained at varying initial lipid concentration while keeping the initial concentration of apo A-I constant (Table VI). Using the apparent molecular weight and hydrated density obtained from the peak fraction of the lipid-rich particle, we calculated the radius of the individual particles from the relationship

$$M \times 10^{14} = \frac{4\pi r^2}{3} \times 6 \times 10^{15}$$

Table V

Amount of apo A-I incorporated in lipid-free form and in R-HDL complex and lipid-rich particle as function of varying lipid/apo A-I weight ratios at constant apo A-I concentration

The conditions for the co-sonication of apo A-I at an initial concentration of 5.5 $\mu M$ with different amounts of HDL lipids, centrifugation at density 1.063 g/ml, and analysis of the density 1.063 g/ml fraction of native HDL particles are similar to those of HDL3, indicating a good correlation between phospholipid content and particle size within the classes. On the other hand, both cholesteryl esters and free cholesterol increased significantly in the 31 A class, but remained constant in the 39 A class. A comparison of these data with those for native HDL indicates that R-HDL forms at an initial lipid/protein weight ratio above 2, contains amounts of protein, phospholipid, and free cholesterol comparable to those of HDL, but only about half as many cholesteryl esters. The results also show that R-HDL, formed at an initial lipid/protein weight ratio above 2, contains more phospholipid than expected for an HDL particle. On the other hand, the lipid-rich particle obtained at an initial lipid/protein weight ratio of 4 appears to be not a lipoprotein, but most probably a lipid vesicle with apo A-I adsorbed to it.

DISCUSSION

The present studies have shown that, when lipid-free apo A-I from human serum HDL is re-exposed in vitro to HDL lipids, it can form well-defined complexes which are similar in operational terms to a native HDL. The behavior of the reassembled HDL particles is similar to that of native HDL in terms of physical parameters, namely, electrophoresis, ultracentrifugation, and circular dichroism, as well as in enzymatic digestion by snake venom phospholipase A2. The formation of the two R-HDL particles, one small and containing 2 mol of apo A-I, the other large and containing 3 mol of apo A-I, is kinetically driven and the process does not reach a thermodynamic equilibrium. Our results have also shown that the reassembly process requires the presence in solution of monomer-dimer forms of apo A-I which, once incorporated into the reassembled particles, determine the size and chemical composition of the latter. A size-limiting role of apo A-I in HDL has been suggested before (37) and is validated by the current work. These results confirm preliminary observations made in this laboratory (38) and further support the concept that the state of association of apo A-I has a marked influence on its ability to bind lipids, presumably because the sites involved in protein-protein interactions are also those participating in the association with lipids (9). The correlation between protein concentration and complex formation is in agreement with the results recently obtained in this laboratory, showing that Macacus rhesus apo A-I, which is predominantly monomeric even at high concentrations (up to 1 mg/ml), is incorporated into lipid-protein complexes at a high yield under all conditions (39). We feel that the reason...
for the absence of interaction between the higher oligomers of human apo A-I and lipids lies in the relatively slow dissociation of these protein aggregates. Since lipid-protein complex formation is irreversible under our experimental conditions, and since oligomerization of apo A-I is a reversible process (9), it is safe to surmise that, given a long enough interaction time, all protein would ultimately end up in the lipid-protein complex. According to this hypothesis, it is possible that only monomers could enter into the complex if the rate of dissociation of the dimer were rapid enough. Thus, the solution properties of apo A-I should be determined before reassembly studies are undertaken. This parameter was not always carefully examined in previous work (3-8), and this may account for the conflicting data reported in literature on the in vitro binding of apo A-I with lipids.

The above results confirm that co-sonication induces no detectable structural alterations of either lipids or apoprotein; and it is, in fact, indispensable for the in vitro incorporation of cholesteryl esters into the complex (20, 25). In addition, co-sonication accelerates the rate of interaction of phospholipids with the apoprotein.

The results presented in this paper show that the lipid and protein composition of the reconstituted particles is determined primarily by the lipid/protein ratio in the reaction mixture during sonication. This fact, together with the irreversibility of R-HDL formation, could point to the important role played by protein-lipid interactions preceding the actual formation of the lipoprotein particles. One could hypothesize that the apo A-I monomer and dimer in solution are initially adsorbed at the surface of the large lipid emulsions. Such an adsorption should lower the interfacial tension, thereby facilitating the formation of local budding and eventually leading to the separation of the R-HDL particles from the bulk lipid phase. If such a mechanism is indeed operative, the surface concentration of the adsorbed apo A-I should play a determining role as to the size of the R-HDL particle formed, since interfacial tension controls the formation of small and large particles; for example, a very low interfacial tension, obtained with a high surface concentration of adsorbed apo A-I, is necessary for the formation of small R-HDL particles. This mechanism would explain why low lipid/protein ratios lead to small R-HDL particles and why an increase of the lipid/protein ratio is accompanied by a steady increase in the particle size. Such a mechanism would also account for the fact that sonication accelerates the formation of lipoprotein particles without having any effect on their size and composition, since the energy supplied by sonication would increase the frequency of formation of buds whereas the size of the R-HDL particles would still be controlled by the interfacial tension. Finally, this mechanism of generation of lipoproteins through protein adsorption implies that the lipid composition of the lipoprotein should reflect the lipid composition close to the surface of the bulk lipid phase and not that of the interior lipid phase. One would expect an enrichment in polar lipids close to the lipid-water interface which would account for the decrease of the nonpolar lipids into lipoproteins of decreasing size. An important conclusion which we reached from these studies is that the formation of reassembled HDL is controlled by the interaction of apo A-I with the whole lipid phase and it is not the result of stepwise addition of individual lipid molecules to the apoprotein.

It is now established that human HDL contains, besides apo A-I, another important apoprotein, apo A-II, the structural role of which is not yet clearly defined. On the other hand in animal species like the cow (40), pig (41), or dog (42), HDL contains little, if any, apo A-II. Therefore, the possibility arises that human HDL could be a mixture of two lipoproteins containing either apo A-I or apo A-II, each of the two

### Table VI

| Particles formed at initial lipid/apo A-I weight ratio | Physical parameters | Chemical composition |
|------------------------------------------------------|---------------------|---------------------|
|                                                      | Apparent molecular weight | Hydrated density | Radius | Protein (mol/particle (%)) | Phospholipid | Cholesteryl esters (mol/particle (%)) | Free cholesterol (mol/particle) |
|                                                      | g/ml     | Å                |                     |                     |                     |                     |
|           | 0.2      | 0.74             | 1.21               | 29.0               | 2.0 (76.1)          | 13.6 (14.4)       | 4.3 (3.8)          | 1.3 (0.7) |
|           | 0.50     | 0.92             | 1.16               | 31.6               | 2.2 (66.6)          | 23.7 (20.0)       | 9.5 (6.7)         | 4.5 (1.9) |
|           | 0.67     | 1.00             | 1.14               | 32.6               | 2.2 (60.2)          | 34.5 (28.7)       | 10.5 (6.2)        | 5.2 (2.0) |
|           | 1.00     | 1.15             | 1.13               | 34.3               | 2.3 (57)           | 39.5 (26.6)       | 15.0 (8.5)        | 8.5 (9.9) |
| R-HDL<sub>s</sub> | 2.00     | 1.43             | 1.12               | 37.0               | 2.8 (55.5)          | 55.2 (29.9)       | 15.6 (7.1)        | 10.9 (8.0) |
|           | 4.00     | 1.75             | 1.11               | 39.7               | 2.9 (45.7)          | 92.9 (42.5)       | 19.9 (6.2)        | 9.5 (2.1) |
|           | 6.00     | 1.90             | 1.11               | 40.1               | 3.1 (44.4)          | 105.1 (44.0)      | 16.4 (5.1)        | 9.8 (2.0) |
| Lipid-rich particle | 4.00     | 1.90             | <1.063             | 42.3               | 0.7 (10.9)          | 156.0 (63.7)      | 33.4 (11.4)       | 4.2 (8.6) |
| Control   |                     | 1.75<sup>c</sup> | 1.15<sup>c</sup>   | 39.2               | 3.4 (55)<sup>d</sup>| 51.9 (22.5)<sup>d</sup>| 32.0 (11.7)<sup>d</sup>| 13.0 (9.9)<sup>d</sup> |

<sup>a</sup> Assuming a hydrated density of 1.00.
<sup>b</sup> Ref. 34.
<sup>c</sup> Ref. 35.
<sup>d</sup> Ref. 44; the protein content of HDL<sub>s</sub> was considered to be the equivalent weight of apo A-I.
species being difficult to separate from the other because of their similar properties. In fact, a human HDL containing predominantly apo A-I has been reported in literature (43), but its artificial product cannot be ruled out. On the other hand, a naturally occurring HDL containing apo A-II as the sole protein has not yet been reported. Although apo A-II exhibits a higher affinity for cholesteryl esters than does apo A-I (44), further a naturally occurring HDL containing apo A-II has been reported in literature (43), but its artifactual production cannot be ruled out. On the other hand, a naturally occurring HDL containing apo A-II as the sole protein has not yet been reported. Although apo A-II exhibits a higher affinity for cholesteryl esters than does apo A-I (44), further a naturally occurring HDL containing apo A-II has been reported in literature (43), but its artifactual production cannot be ruled out. On the

More information is obviously needed concerning the relative contribution of apo A-I and apo A-II to the structure of HDL. Studies in this direction are now in progress in our laboratory. In the conduct of these studies, we recognize that results from reassembly work may not be sufficient to explain all of the structural features of native HDL. On the other hand, if the information obtained by this method is carefully analyzed and duly complemented by other methods, it is very valuable. In this context, we have already shown that the kinetics of hydrolysis of an R-HDL particle by phospholipase A₂ is identical with that previously described for native HDL (29, 30). Work is also being carried out in this laboratory on intact HDL particles after chemical or enzymatic treatment, and on the surface topography of assembled and native HDL.

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