Determination of the molecular weight of apoprotein subunits from low density lipoprotein by gel filtration

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ABSTRACT Another method has been developed for obtaining a soluble apoprotein from the low density lipoprotein (LDL) of human plasma in the density class $1.019 < d < 1.063$. The approximate molecular weight of the apoprotein subunit from this lipoprotein density class was determined by gel filtration on Sephadex G-200 to be about 80,000. Both on gel filtration and analytical ultracentrifugation the soluble apoprotein showed one peak, but on cellulose acetate electrophoresis it showed two bands, which suggests two differently charged components. Because of the nature of the determination, the value of 80,000 probably represents an upper limit to the molecular weight of the LDL subunits.

SUPPLEMENTARY KEY WORDS delipidation · subunit heterogeneity · amylopectin sulfate · sodium dodecyl sulfate · human plasma

COMPARATIVELY LITTLE is known about the protein moiety of the low density lipoprotein (1–3) from human plasma. Only recently was a method reported for obtaining a soluble apoprotein from the LDL (4). Earlier values proposed for the molecular weight of apoprotein subunits were obtained by the indirect methods of *N*-terminal amino acid analysis (4, 5) and percentage protein composition of the intact LDL molecule (6, 7). There is much variation in these indirect values, which range from 70,000 to 500,000. Recently, direct determination of the subunit weight of apo-LDL has been made from sedimentation equilibrium measurements on apo-LDL dissolved in 8 M urea (8) and on a succinylated apo-LDL preparation (9, 10). Subunit weights of 64,000 and 36–38,000, respectively, were reported.

The homogeneity of the LDL apoprotein has not been accurately assessed. Upon ultracentrifugation of phospholipid–protein residues of LDL, as major and a faster-moving minor boundary have been observed (11). After complete delipidation of LDL, a major and a faster-moving minor boundary were found on ultracentrifugation and a major and minor component were observed on agar gel and immunoelectrophoresis (4). Thus, there is strong evidence for at least two peptide components in the LDL molecule. The present studies were undertaken to ascertain a value for the molecular weight of the protein subunits by gel filtration and to obtain some index of the number of different peptide chains contained in the LDL molecule.

EXPERIMENTAL PROCEDURES

Isolation and Delipidation of LDL

LDL was precipitated from pooled, outdated acid citrate dextrose (ACD) plasma (obtained from the Louisville Regional Blood Center of the American Red Cross) with amylopectin sulfate according to the method of Levy, Lynch, McGee, and Mehl (12). The LDL–amylopectin sulfate complex was dissolved in 3.5 M NaCl containing 5% $BaCl_2$. The resultant lipoprotein solution was layered under an equal volume of 0.5 M NaCl and centrifuged for 1.2×10^8 *g*-min at 4°C in a Beckman Spinco model LHV preparative ultracentrifuge with a fixed angle rotor. Under these conditions the VLDL and LDL floated to the top of the tube to give the appearance of a fatty layer. This layer was removed with a tube slicer,

Abbreviations: LDL, low density lipoprotein; VLDL, very low density lipoprotein; SDS, sodium dodecyl sulfate.

its density was adjusted to 1.019 with crystalline NaCl (for density determinations see *Ultracentrifugation*), and the fraction was centrifuged as before. The VLDL floated to the top of the tube; the LDL sedimented to form a yellow layer in the bottom of the tube which was removed, readjusted to a density of 1.019, and centrifuged as before. Any remaining VLDL floated to the top of the tube. Again, the LDL formed a yellow layer at the bottom of the tube. The LDL was removed, adjusted to a density of 1.063, and centrifuged as before. Now the LDL floated to the top of the tube and the concentrated yellow band thus formed was removed and stored at −20°C until used.

The lipoprotein was delipidated by a modification of the method of Shore and Shore (13). 50–100 mg of LDL in 10 ml of 0.1 M Tris buffer was shaken vigorously at room temperature with 10 ml of diethyl ether–ethanol 3:2 and centrifuged at 1,500 *g* for 10 min. The yellow ether layer was poured off and the remaining solution was extracted twice with 10-ml portions of diethyl ether–ethanol 3:1, each time with vigorous shaking in order to disperse the protein precipitate, and centrifuged. After the last centrifugation the protein cake was removed, blotted, and immediately placed in 2–5 ml of a solubilization mixture consisting of 8 M urea, 0.2 M sodium dodecyl sulfate (SDS), and 0.1 M Tris buffer (pH 9.8). The protein cake was broken up and allowed to stand overnight whereupon most of the protein was solubilized. The solution was then extruded several times through a 20 gauge needle and then dialyzed for 24 hr against two changes each of the following solutions: 4 M urea, 0.05 M Tris; 1 M urea, 0.05 M Tris, 0.05% SDS; and 0.05 M Tris, 0.05% SDS. The resulting solution contained 5–10 mg of LDL apoprotein per ml.

Dry Weight Determinations. Dry weight and protein determinations were made on aliquots of the native LDL and solubilized apo-LDL solutions dialyzed against distilled water. During delipidation the organic phase obtained after each extraction was evaporated to dryness under nitrogen, and the dry weight of the lipid material was determined after the samples had been dried overnight with phosphorus pentoxide in the Abderhalden drying apparatus. Control determinations also were made on dialysates and organic solvents.

Gel Filtration

Gel filtration on Sephadex G-200 was performed by a modification of the method of Andrews (14). Sephadex G-200 was allowed to swell in buffer (0.05 M Tris, 0.05% SDS saturated with $CHCl_3$) for three days; the 2.5 × 50 cm column that was then poured had a flow rate of 10 ml/hr. Samples of ovalbumin, bovine serum albumin, and human γ-globulins (10 mg sample in 1 ml of buffer) were chromatographed in duplicate to give a calibration curve for the column. Next, 5–10 mg of delipidated LDL or 25–50 mg of native LDL, each added in 1 ml of buffer, were chromatographed. 2-ml fractions were collected in an automatic fraction collector and the amount of protein in each tube was determined (15) at 540 mμ. Elution volumes (V_e) could be determined accurately to the nearest milliliter.

Electrophoresis and Ultracentrifugation

Electrophoresis on paper was performed with a Beckman Spinco model R electrophoresis apparatus in 0.05 M Veronal buffer (Mallinckrodt Chemical Works, St. Louis, Mo.), pH 8.6, at a constant current of 1.5 ma for 18 hr. Strips were stained with bromophenol blue and Oil Red O.

Electrophoresis on cellulose acetate (Millipore Celotate strips) was performed with the Beckman Spinco electrophoresis apparatus in 0.07 M Veronal buffer, pH 8.6, at a constant voltage of 250 v for 4 hr. Strips were stained for protein with Ponceau S.

Immunoelectrophoresis in agar gel was performed by the method of Scheidegger (16) in the LKB 6800 A immunoelectrophoresis apparatus.

Analytical ultracentrifugation was performed in a Beckman Spinco model E analytical ultracentrifuge equipped with schlieren optics. Speeds of 52,640 and 59,780 rpm were used for flotation and sedimentation experiments, respectively. All experiments were performed at 20°C with a bar angle of 70°. Viscosity determinations were made with a standard Ostwald viscometer. Density determinations were made with a "Speegrav" specific gravity instrument (Biological Research, Inc., Bridgeton, Mo.).

Chemical Analyses and Materials

After digestion with 5 N sulfuric acid, phosphorus was determined by the Gomori (17) modification of the Fiske–Subbarow (18) method.

Bovine serum albumin, fraction V powder, ovalbumin, γ-globulins, human fraction II, and goat and sheep antisera to human LDL were obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio. Rabbit anti-human antisera were obtained from Hoechst Pharmaceuticals, Inc., Kansas City, Mo. Sephadex G-200 was purchased from Pharmacia, Uppsala, Sweden. All chemicals used in this study were reagent grade.

RESULTS

Homogeneity of LDL

Paper electrophoresis of the native LDL showed a single sharp band migrating with the β-globulins and staining for both protein and lipid. Flotation in the analytical ultracentrifuge at d_{20} = 1.063 showed a single peak with

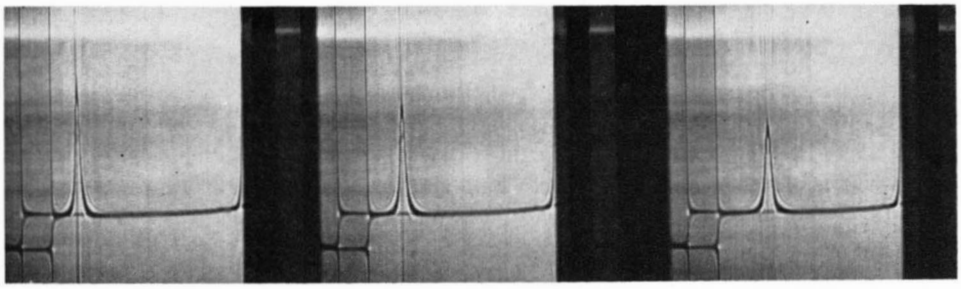

FIG. 1. Ultracentrifugal schlieren pattern of the soluble LDL apoprotein. Experimental conditions: 59,780 rpm, 20°C, 0.05 M Tris, 0.05% SDS. Pictures taken at 30, 40, and 60 min after steady speed had been reached.

S_f = 3.4. Immunoelectrophoresis in agar gel using anti-human antisera showed a single precipitin band. Gel filtration on Sephadex G-200 showed a single symmetrical peak with no tailing.

According to dry weight determinations of the lipids extracted, delipidation was almost 100% complete and no detectable amount of protein was lost during the extraction. The amount of SDS bound to apo-LDL was 20% (w/w) of the protein as calculated from dry weight measurements and protein determinations, corrections being made for the phospholipid (2%, see below) and carbohydrate (5% [19]) still bound to the protein.

The LDL apoprotein, recovered after delipidation, sedimented in the analytical ultracentrifuge as a single symmetrical peak with S = 3.2 (Fig. 1). Cellulose acetate electrophoresis revealed a major and a minor band (Fig. 2). A typical gel filtration pattern for the protein from delipidated LDL is shown in Fig. 3. A single peak with slight tailing is observed. Phosphorus analysis of the delipidated protein revealed a phospholipid content of 2%, assuming all phosphorus was present as phospholipid.

Molecular Weight of the LDL Apoprotein Subunit

The molecular weight calibration curve shown in Fig. 4 was determined by plotting elution volumes (V_e) against the logarithms of the molecular weights of the proteins used for calibration. Values of 45,000, 67,000, and 160,000 were used for the molecular weights of ovalbumin,

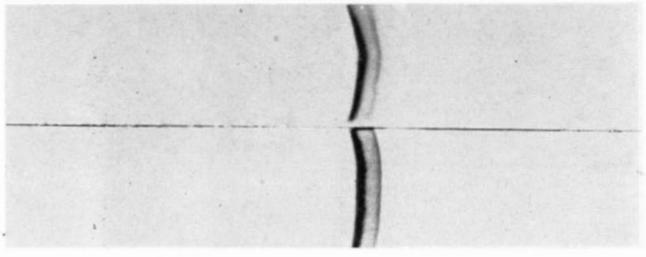

FIG. 2. Cellulose acetate electrophoresis of apo-LDL. Experimental conditions: 0.07 M Veronal buffer, pH 8.6, 250 v for 4 hr. Strips stained with 0.2% Ponceau S in 3% trichloroacetic acid. Anode toward the right.

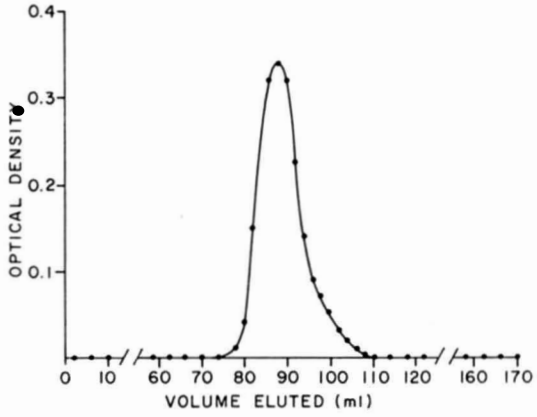

FIG. 3. A typical gel filtration pattern of the apoprotein of LDL on Sephadex G-200; 0.05 M Tris with 0.05% SDS was the eluting buffer. Average elution volume was 87.5 ml.

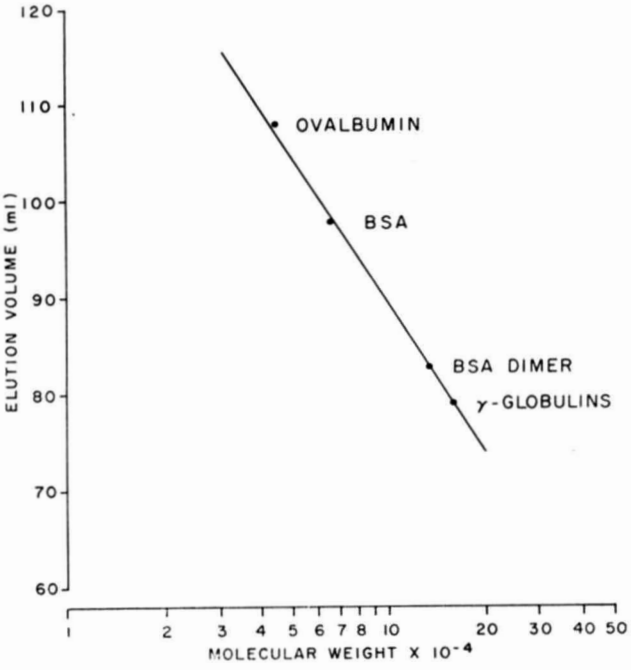

FIG. 4. Calibration curve for the Sephadex G-200 column. Molecular weights of 45,000, 67,000, and 160,000 were used for ovalbumin, bovine serum albumin (BSA), and γ-globulins, respectively.

bovine serum albumin, and γ-globulins, respectively (14). Three independent determinations of different preparations of LDL protein gave elution volumes of 88.5, 88.0, and 86.0 ml, corresponding to values of 105,000, 107,000 and 118,000 for its molecular weight. Thus, the LDL apoprotein subunit we isolated has a molecular weight near 110,000. If the 5% carbohydrate content (19), the 2% phospholipid content determined in these studies, and the 20% SDS which is attached to the protein are taken into account, the above value of 110,000 is reduced to 80,000 as the molecular weight for the peptide portion of the LDL apoprotein subunit.

The elution volume in gel filtration correlates best with Stokes' radius of the protein. The Sephadex column was calibrated with globular proteins, and the molecular weight determination for the apo-LDL subunit is accurate only if it is assumed also to be spherical. However, after delipidation and solubilization in urea and SDS, it is possible that apo-LDL could be distended and in a random coil arrangement. To the extent that this is true our value will be proportionately high. Thus, the value of 80,000 represents an upper limit for the molecular weight of the apo-LDL subunit.

DISCUSSION

We have developed another method for obtaining a soluble apoprotein from LDL. This procedure is similar to that of Granda and Scanu (4) in that we carried out extraction under alkaline conditions using both ethanol and diethyl ether and employing somewhere in the process the anionic detergent, SDS. However, it is unlike their method in that no prior incubation with SDS is required, and delipidation is performed at room temperature instead of −10°C and in a matter of minutes instead of hours.

The molecular weight of the LDL apoprotein subunit has been determined by chromatography on Sephadex G-200. This gel filtration procedure gave reproducible results and probably was accurate within 10%. A value of 80,000 was obtained for the molecular weight of the peptide portion of the LDL apoprotein subunit devoid of phospholipid, carbohydrate, and SDS. If 2.5×10^6 is taken as the best estimate of the molecular weight of the LDL (1, 8, 10) of which 20% is protein, then on the basis of our data there could be no less than six protein subunits per LDL molecule. Margolis and Langdon (2) treated native LDL with iodoacetate-^{14}C and found an incorporation of isotope equivalent to 14% of the total half-cystine content, as determined by amino acid analysis (1). They concluded from this indirect evidence that there was one sulfhydryl group per 10^5 g of LDL apoprotein and proposed that the LDL has five peptide chains with a molecular weight of about 100,000 each. Shore and Shore (8), using sedimentation equilibrium, determined the molecular weight of LDL subunits in 8 M urea to be 64,000. These authors pointed out that if a correction factor for preferentially bound water is applied to their data, this value is reduced to 42,000. They suggested that the LDL molecule contains about 9–10 subunits that are identical or very similar in molecular weight. More recently Scanu, Pollard, and Reader (10) used sedimentation equilibrium to study succinylated apo-LDL and obtained a molecular weight for LDL subunits of 36–38,000. These workers suggested that the LDL molecule contains about 12 subunits of homologous weight. If we wish to correlate our subunit molecular weight values with those from these last two groups, our data would indicate either a distended peptide chain or, possibly, dimer formation between subunits.

Contrary to the concept that the LDL apoprotein is homogeneous (1, 20), our electrophoretic data lead to the same conclusion reached by other investigators, who have demonstrated more than one LDL peptide chain (4, 11). An indication of some degree of aggregation has been noted both in the urea preparations of apo-LDL of Shore and Shore (8) and in the succinylated preparations of Scanu et al. (10). While it is possible that what is conceived of as heterogeneity may represent aggregation, in our preparations we did not detect such aggregation in the analytical ultracentrifuge or during gel filtration. In our sample the heterogeneity was detected during electrophoresis, which indicates a charge difference between peptides either because of a difference in their constituent amino acids or because of a difference in their binding affinity for SDS. The isolation and characterization of these two electrophoretic components will be necessary before the question of aggregation and (or) heterogeneity of the LDL peptides can finally be settled.

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