Preparation and properties of soluble, immunoreactive apoLDL

Lilian Socorro and Germán Camejo

Laboratorio de Lipoproteínas, Centro de Biofísica y Bioquímica,
Instituto Venezolano de Investigaciones Científicas (IVIC), Apartado 1827, Caracas-101, Venezuela

Abstract Immunoreactive apo-low density lipoprotein (LDL), soluble in mildly alkaline buffers of low ionic strength, was prepared by attaching LDL to a DEAE–Sepharose column and eluting the lipids with a 0–2% (w/v) gradient of nonionic detergents. Brij-36T, Nonidet P-40, and Triton X-100 gave similar results. After washing the detergent from the column, the bound apoLDL was eluted with 1 M NaCl, pH 7.4, with recoveries up to 85%. This apoLDL could be dialyzed extensively against low ionic strength solutions, and remained soluble as long as the pH was above 7. Spectrophotometric analysis showed that less than 0.1% (w/v) of cholesterol or phospholipids and less than 1% (w/v) of detergent remained associated with the protein. The apoLDL cross-reacted with LDL against antisera prepared vs. intact LDL. Pore-gradient polyacrylamide gel electrophoresis, with SDS and urea, showed that this preparation was less aggregated than organic solvent extracted apoLDL and appeared to be made of oligomers of two monomeric subunits, one with molecular weight around 22,700 and a smaller one of approximately 8000. Isoelectric focusing showed that there also was charge heterogeneity in the soluble apoLDL. —**Socorro, L., and G. Camejo.** Preparation and properties of soluble, immunoreactive apoLDL. *J. Lipid Res.* 1979. **20:** 631–638.

Supplementary key words oligomers · monomeric subunits · nonionic detergents

A major difficulty in the characterization of the protein moiety of plasma low density lipoprotein (LDL) has been the absence of procedures for the preparation of lipid-free apoLDL soluble in aqueous solutions without requiring ionic detergents or protein denaturants (1). The use of these protein-solubilizing agents precludes studies with sensitive immunological techniques or attempts to characterize the structure of apoLDL (apoB) in solutions of physiological composition.

Based on the previous work of Helenius and Simons (2), in which nonionic detergents and a combination of gel permeation columns were used to obtain detergent-soluble immunoreactive apoB, we have developed a procedure that gives good yields of an essentially lipid-free, detergent-free, soluble apoLDL that retains its immunoreactivity vs. anti-LDL antiserum. This apoLDL is heterogeneous in charge and appears to be made of oligomers from two subunits of approximately 22,700 and 8000 daltons.

MATERIALS AND METHODS

LDL preparation

Plasma was obtained from blood donors using conventional sterile procedures. As soon as the plasma was separated from blood cells we added 10 μl of a solution containing 1% Na_2EDTA, 0.1% NaN_3, and 1% chloramphenicol per ml of plasma to prevent bacterial growth. The plasma also contained 0.2 mM phenylmethyl sulfonyl fluoride (PMSF) to inhibit potential proteolytic activity. Solid KBr was used to adjust the desired background density, and LDL was obtained using two centrifugations for 20 hr (105,000 *g*) at 2°C at each of the 1.019 and the 1.063 g/ml density cuts in order to prepare the lipoprotein free of VLDL and HDL. In all runs, a 60 Ti rotor (Beckman Instruments, Palo Alto, CA) was used. The purified LDL was dialyzed for three 24-hr periods against 2 l of a buffer A made of 0.15 M NaCl, 5 mM Tris-HCl, pH 7.4, and 0.5 mM Na_2EDTA. All preparations were stored in this buffer at 4°C in the presence of 0.2 mM PMSF.

Preparation of soluble apoLDL

Aliquots of purified LDL solution containing 10–20 mg of lipoprotein (1–4 ml) were dialyzed for 24 hr against a buffer made of 50 mM Tris-HCl, pH 8.8 (buffer 1). This same buffer was used to equilibrate a

Abbreviations: LDL, low density lipoprotein, d 1.019–1.063 g/ml; VLDL, very low density lipoprotein, d < 1.019 g/ml; HDL, high density lipoprotein; apoLDL, the protein moiety of LDL; PMSF, phenylmethylsulfonyl fluoride; Na_2EDTA, disodium salt of ethylenediamine tetraacetic acid; SDS, sodium dodecyl sulfate; Brij-35, 23-polyoxyethylene lauryl ether; Brij-36T, 10-polyoxyethylene lauryl ether; Triton X-100 and Nonidet P-40, iso-octylphenoxypolyethoxyethanols; Tween-80, polyoxyethylene(2) sorbitan monooleate; dansyl chloride (DNS), 1-dimethylaminonaphthalene-5-sulfonylchloride; TEMED, tetramethylenediamine; AP, ammonium persulfate.

DEAE–Sepharose CL-6B column (Pharmacia Fine Chemicals, Uppsala). The dialyzed LDL was applied to the short column (1.5 × 15 cm) and immediately a gradient of the nonionic detergents Brij-36T (Sigma Chemical Co., St. Louis), Nonidet P-40 (Shell Chemicals), or Triton X-100 (Sigma) was started. The gradient was linear from 0–2% (w/v) detergent in buffer 1, with a total volume of 60 ml, and was pumped through the column at 20 ml/hr. After elution of LDL lipids with the detergent gradient, ten column volumes of buffer 1 were passed through to wash the detergent from the DEAE–Sepharose. The apoLDL was eluted with two column volumes of 1 M NaCl buffered to pH 7.4 with 50 mM Tris-HCl. ApoLDL was obtained as a single peak and the column was regenerated with 200 ml of buffer 1.

The apoLDL was usually dialyzed for 48 hr against water or buffer 1 containing 0.2 mM PMSF. When concentrated solutions of apoLDL were required, the samples were dialyzed against water and lyophilized.

Preparation of solvent-extracted apoLDL

One-ml samples of LDL (up to 10 mg/ml) or detergent-delipidated apoLDL (1 mg of protein/ml) were added to 9 ml of methanol–chloroform 6:5 (v/v) in conical, Teflon-lined screw-capped glass tubes and kept at 4°C for 2 hr. The flocculated apoproteins were pelleted by centrifugation at 2500 rpm; the supernatant was decanted and the pellet was resuspended with a Vortex mixer in 5 ml of the same methanol–chloroform mixture. The suspension was centrifuged again and the supernatant solutions were combined. This solution, containing essentially all the lipids of LDL, or any detergent associated with the apoLDL prepared by the column procedure, could be used to measure lipids, after adjusting the solvents to the proportions used in the method of Folch, Lees, and Sloane Stanley (3). The remaining solvent associated with the pellet was evaporated under nitrogen and the quantitatively recovered apoproteins were dissolved in solutions containing 1% SDS for protein analyses, or in dissociating solutions used to prepare the sample for electrophoresis (4).

Electrophoresis

Agarose gel electrophoresis was carried out in 0.5% gels prepared in 0.25 M Tris–0.2 M glycine buffer, pH 8.4. Polyacrylamide gel electrophoresis was performed in a vertical slab cell (Hoeffer, Scientific Instruments, San Francisco). Three types of gels were used: *1*) 3–20% (w/v) polyacrylamide prepared in 0.25 M Tris–0.2 M glycine, pH 8.4, containing 0.2% (w/v) SDS; *2*) 3–20% (w/v) polyacrylamide in the same buffer as *1*, but 6 M in urea; and *3*) 4–40% (w/v) polyacrylamide, also with SDS and 6 M urea. For the three types of gels the electrode chambers contained the Tris–glycine buffer with 0.1% SDS. In all cases the proportion of acrylamide to bis-acrylamide was 30:1 and polymerization was induced by 0.5 μl of TEMED and 1 mg of AP per 10 ml of the 4–40% gel or 1 μl of TEMED and 2 mg of AP per 10 ml of the 3–20% gel. To improve the linearity of gradient formation, a 5–20% (w/v) sucrose gradient was superimposed on the acrylamide gradient. All the gel slabs were 3 mm thick and were prerun for 1 hr at 10 mA per slab and then run at this current for 24 hr.

ApoLDL samples and calibration proteins, obtained from Pierce Chemicals (Rockford, IL) were dissolved to 0.5 mg/ml in a freshly prepared dissociation solution made of 0.15 ml of 1 M Tris-HCl, pH 6.8, 10 mg of dithiothreitol, 2 g of sucrose, 3.6 g of urea, 5 ml of 10% SDS, and water to a volume of 10 ml. In each slot were placed 25–50 μg of protein. The gels were fixed and washed for 24 hr with 1 liter of isopropanol–acetic acid–water 25:10:65 (v/v), and stained for 6 hr in 0.2% (w/v) Coomasie brilliant blue R (Pierce Chemicals) dissolved in the fixing solvent. Destaining was achieved by several changes of isopropanol–acetic acid–water 10:10:80 (v/v).

Immunoelectrophoresis and immunodiffusion

Crossed-tandem immunoelectrophoresis was carried out as described by Axelsen, Krøll, and Weeke (5), using rabbit antisera prepared against pooled human plasma, pooled purified LDL, and pooled soluble apoLDL obtained with the DEAE–Sepharose detergent procedure. Double immunodiffusion was performed in agarose gels prepared in 0.2 M Tris–glycine buffer, pH 8.4. After washing with saline and water, and pressing, the dried gels were stained with Coomasie brilliant blue, as suggested by Krøll (5).

Isoelectric focusing

Electrofocusing was performed in a Multiphor apparatus (LKB Products, Bromma); the gel was 3% polyacrylamide and contained 2% ampholine, pH range 3.5–10. The gel was prerun for 1 hr at 2.5 watts and for 3 hr after application of the samples. Measurements with a surface electrode showed that a linear pH gradient had been achieved. The gels were washed, fixed, stained, and dried as described by Mayer (6).

Analytical molecular exclusion chromatography

To study the behavior of the soluble apoLDL in the presence of 6 M guanidinium-HCl or urea–SDS, Sephacryl S-200 gel was used (Pharmacia Fine Chemicals). The column (1.5 × 58 cm) was packed at 60 ml/hr and equilibrated with 6 M guanidinium-HCl in 50 mM Tris-HCl, pH 8.9, containing 0.1% (w/v) dithiothreitol or 8 M urea, and 0.1% SDS in the same

buffer. The calibrating proteins (Pierce Chemicals), 2 mg of each, and the soluble apoLDL (4 mg), obtained with the DEAE–Sepharose detergent procedure, were dissolved in the guanidinium-HCl buffer or urea–SDS and applied in 1-ml aliquots. The analytical runs were carried out at 20 ± 2°C with a flow of 2.12 ml/hr. The column effluent was monitored at 280 nm.

Analytical procedures

Protein was measured by the method of Lowry et al. (7) as modified by Schacterle and Pollack (8). Total cholesterol was determined according to Bowman and Wolf (9). Phospholipids were measured with the method of Beveridge and Johnson (10), and triglycerides according to Bigger, Erikson, and Moorehead (11). To evaluate the residual lipids remaining in apoLDL, 1-ml aliquots containing approximately 1 mg of protein were extracted by the procedure of Folch et al. (3), and measurements were carried out on samples of the chloroform phase that were evaporated under vacuum.

The amount of nonionic detergent remaining associated with the dialyzed, soluble apoLDL was established with the use of the strong absorption bands of the detergents: Brij-35 and Brij-36T at 220 nm, and Triton X-100 and Nonidet P-40 at 234 nm. One-ml aliquots of soluble apoLDL, containing 0.9–1.0 mg of protein, were added to 9 ml of methanol–chloroform 6:5 (v/v). As mentioned above, in this solvent the protein is quantitatively precipitated, and the amount of nonionic detergents, the only UV-absorbing species remaining in the supernatant after centrifugation, can be measured precisely with the aid of standard curves down to levels of less than 1 μg per ml of solvent, i.e., less than 1% of the weight of protein extracted.

N-terminal analysis of apoLDL obtained by direct methanol–chloroform extraction and of apoLDL prepared by the column procedure were carried out with the dansylchloride method described by Narita (12) in order to investigate eventual proteolysis during the DEAE–Sepharose column–detergent procedure. Thin-layer chromatography of the protein hydrolysates and of dansylated amino acid standards was used to establish the number and identity of the fluorescent amino acid derivatives.

RESULTS

Preparation of soluble apoLDL

An elution pattern of the LDL lipids and of the apoLDL is presented in **Fig. 1.** It can be observed that the lipids from the LDL bound to the DEAE–Sepharose column are removed, probably as a micellar suspension in combination with the nonionic detergent. Although only cholesterol determination was routinely used to follow lipid elution, the presence of the phospholipids and triglycerides in the first peak was also established by colorimetric procedures. The lipids in the first peak accounted for more than 95% of the LDL lipids. After washing the column for 10 hr with the detergent-free buffer 1, apoLDL was recovered as a narrow peak which did not contain measurable cholesterol or phospholipids. With the column size used (1.5 × 15 cm), we were able to load up to 20 mg of LDL and we recovered 70–80% of the LDL protein. The fractions containing the apoLDL were transparent and no appreciable change in their protein concentration was observed after aliquots were centrifuged for 1 hr at 105,000 *g*. Dialysis to remove NaCl and subsequent lyophilization led to loss of apoLDL, with an overall recovery of 50–65%. Once delipidated, the soluble apoLDL adhered strongly to surfaces, thus making it difficult to concentrate it by ultrafiltration on cellulose acetate membranes or by lyophilization.

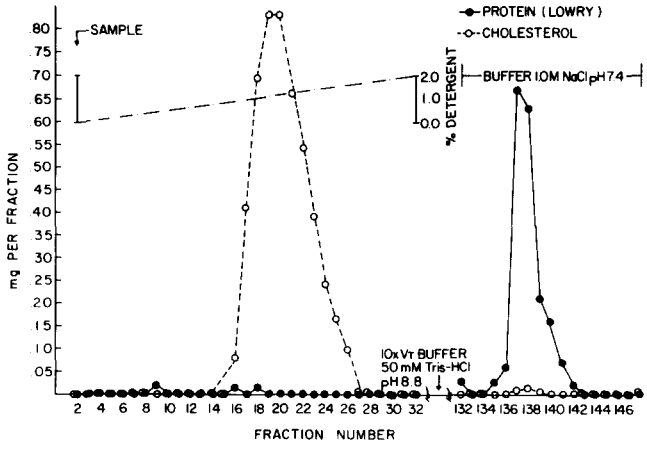

Fig. 1. Elution of LDL lipids and soluble apoLDL from a DEAE–Sepharose column. In this experiment 20 mg of LDL dissolved in 50 mM Tris-HCl buffer, pH 8.8, was applied to the column; the detergent used was Brij-36T. Flow was fixed at 20 ml/hr and 2-ml fractions were collected.

The elution pattern shown in Fig. 1 was obtained using the detergent Brij-36T, but very similar results were obtained with Nonidet P-40 or Triton X-100. On the other hand, Brij-35 or Tween-80, even at higher concentrations, gave only partially delipidated apoLDL. The procedure appears reproducible since similar results in terms of elution patterns and recoveries were obtained with six different preparations of LDL. With different batches of DEAE–Sepharose, we found that small changes in the molarity of the buffer used for loading the LDL on the column were required for optimal recoveries.

Using Brij-36T, Nonidet P-40, or Triton X-100, less than 5 μg of phospholipid and less than 5 μg of cholesterol per mg of apoLDL could be detected. Using

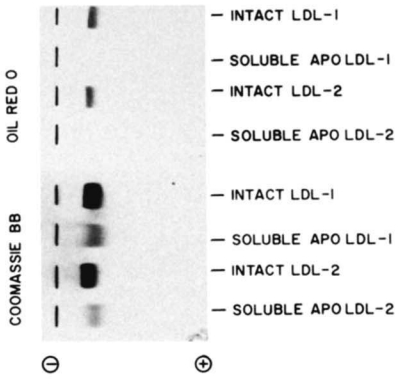

Fig. 2. Agarose gel electrophoresis of LDL and soluble apoLDL. Two preparations of LDL and their respective soluble apoLDL (10 μg of LDL protein or apoLDL) were applied to the slots. After electrophoresis for 1 hr at 8 V/cm, the slides first were stained with 0.2% oil red 0 in 60% ethanol, photographed, and then stained with 0.2% Coomasie brilliant blue R in isopropanol–acetic acid–water 45:10:45 (v/v).

10 column volumes to wash the detergent and dialysis for 48 hr against low ionic strength, neutral, or alkaline buffers, less than 10 μg of detergent per mg of apoLDL (less than 1% w/w) could be detected associated with the protein.

Agarose electrophoresis and isoelectric focusing

The availability of soluble apoLDL allowed us to compare its behavior in agarose gel electrophoresis to that of its parent LDL. **Fig. 2** shows the patterns obtained using two lipoprotein preparations and their respective soluble apoLDL. It is clear that most of the electrophoretic behavior in this support medium is a function of the protein moiety of LDL that moved as a single band, slightly more anodic than apoLDL, inasmuch as there was no material stainable with oil red 0. When the gels were overloaded, we observed a faint anodic component in apoLDL, indicating some heterogeneity in net charge; this is also suggested by the results of isoelectric focusing experiments presented in **Fig. 3.**

Polyacrylamide gel electrophoresis

Using calibrating proteins, it was possible to establish good linear correlations between the logarithm of protein molecular weight and migration distance for the pore-gradient gels. The 4–40% gradient allowed better resolution of low molecular weight proteins, whereas the 3–20% gels showed better resolution for polypeptides above 100,000 daltons. **Fig. 4** presents the relationships established for the gels used with their respective linear equations and correlation coefficients.

In the gels containing only 0.1% SDS (**Fig. 5**), the apoLDL obtained by direct organic solvent extraction showed two major bands with molecular weights of 394,000 and 444,600, although the soluble apoB showed a complex pattern with bands of molecular weights ranging from 58,700 to 444,600. The pattern of the soluble apoB was similar even when treated with methanol–chloroform after being prepared by the column procedure. Using 4–40% polyacrylamide gradients on gels containing 6 M urea and 0.1% SDS, the solvent-extracted apoLDL showed a major band above 200,000 molecular weight, whereas the soluble apoB showed components with apparent molecular weight as low as 44,200 (**Fig. 6**). The increments in molecular weight from band to band are suggestive of an oligomeric series of two subunits, one of average molecular weight 22,700 ± 3200 and one, less abundant and smaller, probably with a molecular weight around 8000.

Although the conditions used for isolation of LDL and apoLDL were unlikely to induce proteolysis, we explored this possibility by examining the pattern of dansyl-amino acids obtained from a preparation of soluble apoLDL obtained by the column procedure and the parent LDL. The patterns obtained were indistinguishable, both in the number of spots and their R_f values. The two samples, in two solvent systems, showed one spot that migrated as dansyl-glutamic, and two other very faint spots corresponding to didansyl lysine and ϵ-dansyl lysine. These results indicate that delipidation with nonionic detergents in the DEAE–Sepharose column did not lead to fragmentation of the major polypeptides of apoLDL.

Analytical molecular exclusion chromatography

The availability of Sephacryl S-200 superfine provides a new tool for studies of association–dissociation of polymers due to its high resolution (**Fig. 7**). We used

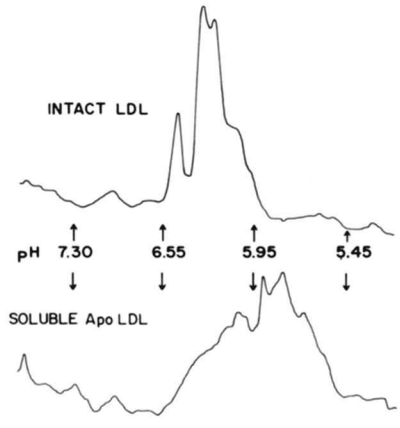

Fig. 3. Densitometric tracings from isoelectric focusing of intact LDL and soluble apoLDL. Twenty μg of LDL protein or apoLDL were applied to the 3% (w/v) polyacrylamide gel containing 2% ampholine (v/v) (pH 3.5–10.0).

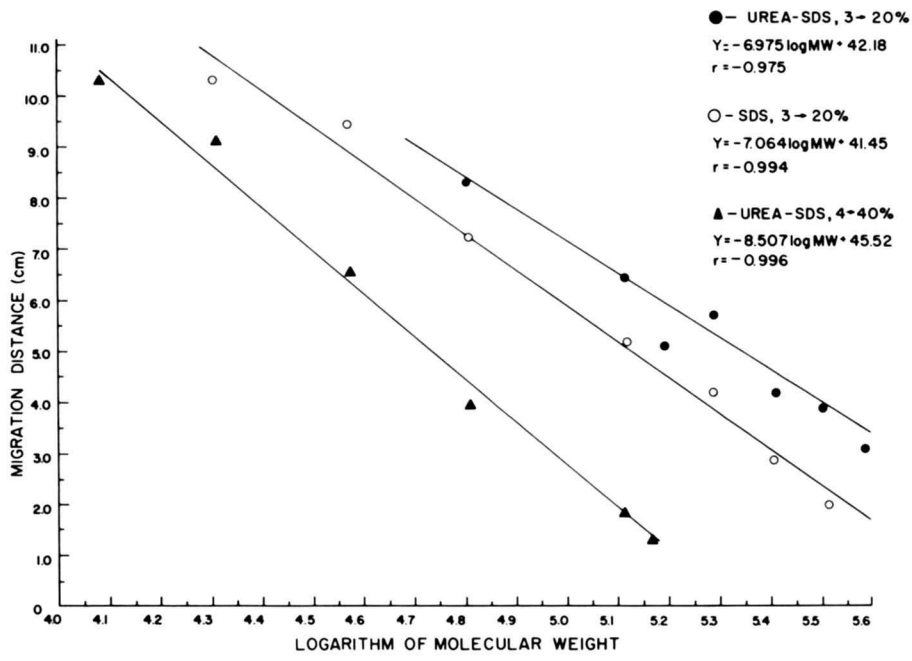

Fig. 4. Linear relationships between logarithm of molecular weight and migration distance established for calibration proteins in pore-gradient polyacrylamide gel electrophoresis. The following proteins were used: cytochrome *c*, 12,500; soybean trypsin inhibitor, 21,500; E. coli RNA polymerase (three chains), 39,000, 155,000, 165,000; and bovine serum albumin which in these conditions give bands corresponding to the monomer 68,000; dimer, 136,000; trimer 204,000; tetramer 272,000; pentamer 340,000; and hexamer 408,000.

this gel in the presence of 6 M guanidinium-HCl and dithiothreitol or with 8 M urea in 0.1% SDS. Unexpectedly, in the presence of 6 M guanidinium-HCl, the soluble apoLDL behaved as solvent-extracted apoLDL, appearing as a major component (73%) with a molecular weight above 250,000, and two minor ones of 22,000 (12%) and 10,000 (15%). However, the same column equilibrated with 8 M urea in 0.1% SDS fractionated the soluble apoLDL into three major peaks, one with an apparent molecular weight above 300,000 (31%), a second one of 250,000 (25%), and a third of 22,000 (44%). These results suggest that 6 M guanidinium-HCl is not as effective a dissociating agent for apoLDL as the mixtures of SDS and 8 M urea or even SDS alone.

Immunoreactivity of soluble apoLDL

In these experiments anti-human plasma, anti-LDL, and anti-soluble apoLDL antisera were used to study cross-reactivity and retention of immunoreactivity in

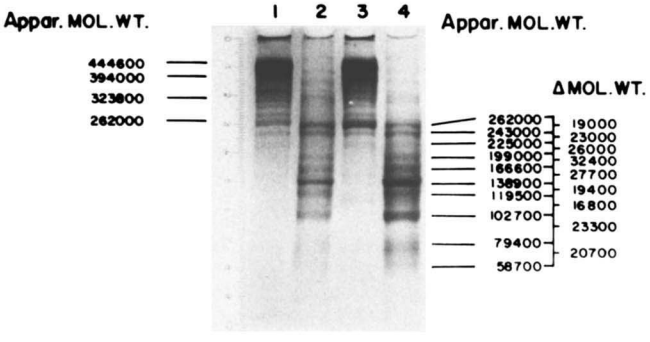

Fig. 5. Pore-gradient polyacrylamide gel electrophoresis. Gradient, 3–20% in the presence of 0.1% SDS (w/v). Gels *1* and *3*, methanol–chloroform-extracted apoLDL; *2* and *4*, the corresponding soluble, detergent-extracted apoLDL. Apparent molecular weights calculated from relationships in Fig. 4 and △ mol. wt. is the difference between consecutive, well-defined bands.

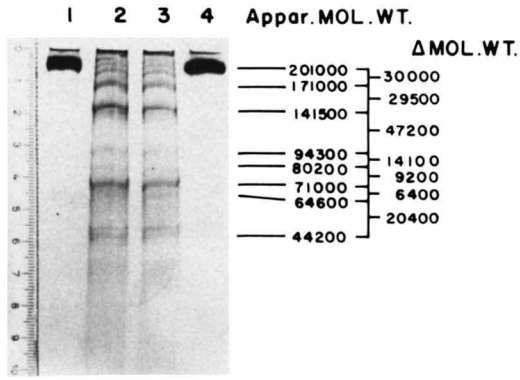

Fig. 6. Pore-gradient polyacrylamide gel electrophoresis. Gradient, 4–40% in the presence of 0.1% SDS (w/v) and 6 M urea. Gels *1* and *4*, methanol–chloroform-extracted apoLDL; *2* and *3*, corresponding soluble DEAE-column detergent-extracted apoLDL.

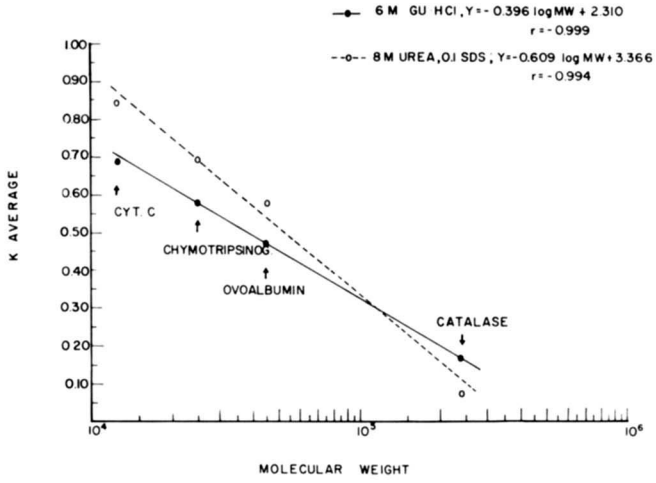

Fig. 7. Relationship between K_d and logarithm of molecular weight for the Sephacryl S-200 superfine column (1.5 × 58 cm), flow 2.12 ml/hr., equilibrated with 6 M guanidinium-HCl (–○–) and 8 M urea, 0.1% SDS (––○––).

soluble apoLDL. Tandem-crossed immunoelectrophoresis was selected to compare the reactivity of LDL and its soluble apoLDL. This method gives both qualitative and quantitative information when used for comparison of antigens of similar electrophoretic mobility and related immunogenic properties. Results from selected experiments are shown in **Fig. 8.** Fig. 8*A* corresponds to the pattern obtained using anti-total human plasma antiserum and clearly shows that the major determinant(s) is(are) shared by LDL and its soluble apoLDL, but that a fast-moving component, not evident in LDL, is made visible by the delipidation.

Fig. 8*B* is from an experiment using anti-LDL antiserum, and it shows that after delipidation the soluble apoLDL retained its reactivity towards an antiserum prepared against intact LDL. However, this serum did not detect the fast-moving component present in soluble apoLDL.

Fig. 8*C* presents the pattern obtained using anti-soluble apoLDL antiserum; here also LDL and its soluble apoLDL cross-reacted, but again the heterogeneity of the soluble apoLDL was evident.

Fig. 9 presents the results of double immunodiffusion experiments using anti-soluble apoLDL serum in rosettes *A, B,* and *C* and anti-intact LDL in *D, E,* and *F*. In *A* and *D*, aliquots of intact LDL containing 1, 2, 3, 4, 5, and 6 μg of LDL protein were placed in outer holes 1 to 6, respectively. With both antisera a single precipitin line is visible. In *B* and *E*, 1, 2, 3, 4, 5, and 6 μg of soluble apoLDL were placed in the external holes 1 to 6, respectively. It can be observed that both antisera react with soluble apoLDL, but that anti-apoLDL detects heterogeneity in the apoLDL (rosette *B*). In *C* and *F*, LDL (3 μg of protein) was placed in holes 1, 3, and 5, and in holes 2, 4, and 6 were placed 3 μg of soluble apoLDL. It is obvious that both antisera show antigenic identity of the major determinant(s) of LDL and soluble apoLDL.

DISCUSSION

The protein moiety of LDL is the least known of the apolipoproteins and there have been conflicting reports concerning its size and heterogeneity. Shore and Shore (13) reported a subunit of 64,000, obtained after ether–ethanol extraction and treatment with iodoacetamide and sodium borohydride. This subunit was soluble in 8 M urea. Day and Levy (14), also using ether–ethanol extraction and solubilization in 8 M urea in 0.2 M SDS, reported an apoLDL subunit that behaved as a molecule of 80,000 molecular weight on gel filtration. Scanu, Pollard, and Reader (15) prepared apoLDL subunits of 36,000–38,000 after succinylation and ether–ethanol extraction. No immunological properties were studied in the above preparations.

Gotto, Levy, and Fredrickson (16) also used succinylation and delipidation with ether–ethanol to prepare an apoLDL soluble in decylsulphate solutions, which retained some immunological reactivity, although no patterns of identity with LDL were presented.

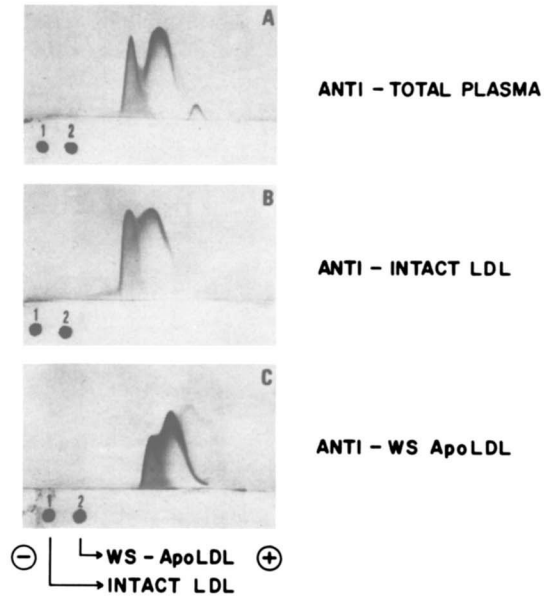

Fig. 8. Tandem-crossed immunoelectrophoresis of intact LDL and soluble apoLDL. In the sample holes were placed 10 μg of LDL protein and soluble apoLDL for the run in the first dimension. In the second dimension, the gel contained 5% of (*A*) anti-human plasma antiserum, (*B*) anti-intact human LDL, and (*C*) anti-soluble apoLDL delipidated by the described column procedure.

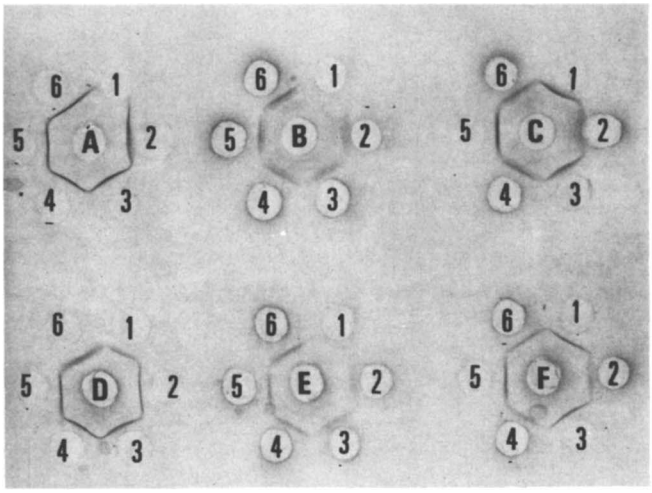

Fig. 9. Double immunodiffusion patterns using anti-soluble apoLDL antiserum (*A*, *B*, and *C*) and anti-intact LDL (*D*, *E*, and *F*). In the external holes (1 to 6) of *A* and *D* were placed LDL samples containing 1, 2, 3, 4, 5, and 6 μg of protein. In *B* and *E*, 1, 2, 3, 4, 5, and 6 μg of soluble apoLDL were in holes 1–6, respectively. In *C* and *F*, LDL containing 3 μg of protein was placed in holes 1, 3, and 5, and the same amount of soluble apoLDL was put into holes 2, 4, and 6.

The studies cited suggest that apoLDL consists of subunits in different extents of association and/or aggregation (17) and that, unless side-chain charge modifications are introduced and denaturants are added, once the lipids are removed by organic solvents, a strong tendency toward protein–protein interaction is present.

ApoLDL has been prepared without the use of organic solvents with combinations of detergents and protein denaturants. Kane, Richards, and Havel (18) obtained apoLDL using Tween-20 and 6 M guanidinium-HCl, with a molecular weight of 26,000 for the smallest subunit. Lipp and Wiegandt (19), using only SDS treatment, showed the presence of many bands in polyacrylamide gel electrophoresis of apoLDL; the smallest component had an apparent molecular weight of 10,000. However, in a subsequent study (20) in which the LDL was extracted with methanol–chloroform and the apoLDL was solubilized with 5% SDS, they found that the "primary pattern" in SDS–polyacrylamide gel electrophoresis corresponded to a single subunit of 250,000 and that the "secondary pattern" of multiple bands was due to endogenous proteolysis that occurred during storage of LDL. Chapman and Kane (21), on the other hand, indicated that if provisions are taken against bacterial growth, the apoLDL obtained showed only a "primary pattern" in SDS–polyacrylamide electrophoresis with two major bands of 318,000 and 276,000 molecular weight; this apoLDL was also obtained with ether–ethanol extraction. These results appear to be in agreement with those of Smith, Dawson, and Tanford (22), who used ethanol–ether extraction and solubilization in 7 M guanidinium-HCl to prepare an apoLDL with a molecular weight between 250,000 and 276,000.

Chen and Aladjem (23), using delipidation with sodium deoxycholate and no solvent extraction, obtained an apoLDL that on SDS–polyacrylamide gel electrophoresis gave a complex pattern interpreted as an oligomeric series of two main subunits of 9,500 and 13,000 molecular weight. Recently, these authors concluded that apoLDL is made of similar subunits of 10,000 daltons in different degrees of aggregation (24). Mateu et al. (25) have interpreted their small-angle x-ray scattering pattern of intact LDL as suggestive also of a small apoLDL subunit (8000 daltons), located at the surface of the lipoprotein particle.

Within so many apparently conflicting reports, it is, however, possible to draw some conclusions. When apoLDL is prepared by side-chain modifications and organic solvent extraction, intermediate molecular weight subunits (26,000–80,000) are detectable in the presence of ionic detergents and/or urea (13–16, 18). When only organic solvents are used, the apoLDL obtained is associated and has molecular weights ranging from 250,000 to 318,000 (21–23, 26). Finally, when detergents are used in the absence of organic solvents, or in studies of intact LDL, the results suggest the presence of small subunits with molecular weights between 8,000 and 10,000 (21, 23–25).

The method described in this paper allows the preparation of an apoLDL that is soluble in mildly alkaline buffers, and that has not been treated with organic solvents and is essentially free of detergents. Although this apoLDL is probably highly self-associated in the absence of dissociating agents, it can penetrate agarose and soft acrylamide gels, and it retains the immunological properties present in intact LDL. Our preliminary results indicate that this preparation is made of oligomeric series of at least two small subunits of 22,700 and 8,000 daltons. The immunoelectrophoresis, the immunodiffusion, and the isoelectric focusing also suggest that apoLDL is heterogeneous.

The results obtained with molecular exclusion chromatography in the presence of 6 M guanidinium-HCl, where a large proportion of apoLDL showed a molecular weight above 250,000, might shed light on the apparent controversy in the cited literature. These data suggest that 6 M guanidinium-HCl does not efficiently dissociate the apoLDL prepared using organic solvents (20–22) or by the present procedures. Our results indicate that the presence of 8 M urea and SDS leads to a more dissociated apoLDL, as suggested by Bradley et al. (27).

Presently, there is much interest in the interaction of

LDL with cells and the role it may play in regulation of cholesterol metabolism and atherogenesis (1, 28). These interactions appear to be mediated through the protein moiety of LDL, as recently suggested by Shireman, Kilgore, and Fisher (29), but the experiments with purified apoLDL have been hampered by the denaturing agents required to solubilized delipidated apoLDL. It appears that the soluble apoLDL, whose preparation is described here, probably retaining its native structure, could be useful in studies concerning the functions of the protein moiety of LDL.

We thank Dr. Elba M. Avila and Dr. Manuel Rieber for suggestions and comments. Also we thank the Consejo Venezolano de Investigaciones Científicas y Tecnológicas (CONICIT) for partial support of this project. We are grateful for the secretarial help received from Mrs. Anita Istok.

Received 2 February 1978 and in revised form 4 October 1978; accepted 15 January 1979.

REFERENCES

1. Goldstein, J. L., and M. S. Brown. 1977. The low density lipoprotein pathway and its relation to atherosclerosis. *Ann. Rev. Biochem.* **46:** 897–930.
2. Helenius, A., and K. Simons. 1971. Removal of lipids from human plasma low density lipoproteins by detergents. *Biochemistry.* **10:** 2542–2547.
3. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation of total lipids from animal tissues. *J. Biol. Chem.* **226:** 497–509.
4. Camejo, G., L. Mateu, F. Lalaguna, R. Padron, S. Waich, H. Acquatella, and H. Vegas. 1976. Structural individuality of human serum LDL associated with a differential affinity for a macromolecular component of the arterial wall. *Artery.* **2:** 79–91.
5. Krøll, J. 1973. Tandem-crossed immunoelectrophoresis. *In* A Manual of Quantitative Immunoelectrophoresis. N. H. Axelsen, J. Krøll, and B. Weeke, editors. Universitetsförlaget. Oslo. 57–59.
6. Mayer, J. W. 1976. Simple and rapid methods for drying polyacrylamide gels after isoelectric focusing. *Anal. Biochem.* **76:** 369–373.
7. Lowry, O., N. Rosebrough, A. Farr, and R. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
8. Schacterle, G., and R. Pollack. 1973. A simplified method for the quantitative assay of small amounts of protein in biological material. *Anal. Biochem.* **51:** 654–655.
9. Bowman, R., and R. Wolf. 1963. A rapid and specific ultramicromethod for total serum cholesterol. *Clin. Chem.* **8:** 302–309.
10. Beveridge, J., and S. Johnson. 1949. The determination of phospholipid phosphorus. *Can. J. Res. (Sec. E).* **27:** 159–163.
11. Bigger, H. G., J. M. Erikson, and W. R. Moorehead. 1975. A manual colorimetric assay of triglycerides in serum. *Clin. Chem.* **21:** 437–447.
12. Narita, K. 1970. End group determination. *In* Protein Sequence Determination. S. B. Needleman, editor. Springer-Verlag, N.Y. 25–90.
13. Shore, B., and V. Shore. 1967. The protein moiety of human serum β-lipoproteins. *Biochem. Biophys. Res. Commun.* **28:** 1003–1007.
14. Day, C. E., and R. S. Levy. 1968. Determination of the molecular weight of apoprotein subunits from low density lipoprotein by gel filtration. *J. Lipid Res.* **9:** 789–793.
15. Scanu, A., H. Pollard, and W. Reader. 1968. Properties of human serum low density lipoproteins after modification by succinic anhydride. *J. Lipid Res.* **9:** 342–349.
16. Gotto, A. M., R. I. Levy, and D. S. Fredrickson. 1968. Human serum beta-lipoprotein: Preparation and properties of a delipidated soluble derivative. *Biochem. Biophys. Res. Commun.* **31:** 151–157.
17. Osborne, J. C., and B. Brewer. 1977. The plasma lipoproteins. *Adv. Protein Chem.* **31:** 253–337.
18. Kane, J. P., E. G. Richards, and R. J. Havel. 1970. Subunit heterogeneity in human serum beta-lipoprotein. *Proc. Natl. Acad. Sci. USA* **66:** 1075–1082.
19. Lipp, K., and H. Wiegandt. 1973. Studies on the B-protein of human serum β-lipoprotein using SDS–polyacrylamide gel electrophoresis. *Hoppe Seyler's Z. Physiol. Chem.* **334:** 262–266.
20. Krishnaia, K. V., and H. Wiegandt. 1974. Demonstration of a protease-like activity in human serum low density lipoprotein. *FEBS Lett.* **40:** 265–268.
21. Chapman, M. S., and J. P. Kane. 1975. Stability of the apoprotein of human serum low density lipoprotein: absence of endogenous endopeptidase activity. *Biochem. Biophys. Res. Commun.* **66:** 1030–1036.
22. Smith, R., J. R. Dawson, and C. Tanford. 1972. The size and number of polypeptide chains in human serum low density lipoprotein. *J. Biol. Chem.* **247:** 3376–3381.
23. Chen, C. H., and F. Aladjem. 1974. Subunit structure of the apoprotein of human serum low density lipoproteins. *Biochem. Biophys. Res. Commun.* **60:** 549–554.
24. Chen, C. H., and F. Aladjem. 1978. Further studies on the subunit structure of human serum low density lipoproteins. *Biochem. Med.* **19:** 178–187.
25. Mateu, L., A. Tardieu, V. Luzzati, L. Aggerbeck, and A. M. Scanu. 1972. On the structure of human serum low density lipoprotein. *J. Mol. Biol.* **70:** 105–116.
26. Kostner, G. M., E. Polz, and A. Holaseck. 1977. Studies on the structure of lipoprotein B: preparation of a soluble apolipoprotein B. *In* Lipid–Protein Complexes. H. Peeters and J. P. Massue, editors. European Press, Ghent, Belgium. 5–13.
27. Bradley, W. A., M. F. Rohde, A. M. Gotto, and R. L. Jackson. 1978. The cyanogen bromide peptides of the apoprotein of low density lipoprotein (apoB): its molecular weight from a chemical view. *Biochem. Biophys. Res. Commun.* **81:** 928–935.
28. Ross, R., and L. Harker. 1976. Hyperlipidemia and atherosclerosis. *Science.* **193:** 1096–1100.
29. Shireman, R., L. L. Kilgore, and W. R. Fisher. 1977. Solubilization of apolipoprotein B and its specific binding by the cellular receptor for low density lipoprotein. *Proc. Natl. Acad. Sci. USA* **74:** 5150–5154.