Removal of Lipids from Human Plasma Low-Density Lipoprotein by Detergents*

Ari Helenius and Kai Simons†

ABSTRACT: Four different detergents have been found to remove all major lipids from human plasma low-density lipoprotein (LDL). The detergents used were a bile salt–sodium deoxycholate, a neutral detergent–Nonidet P40, an anionic detergent–sodium dodecyl sulfate, and a cationic detergent–cetyltrimethylammonium bromide. High concentrations of these detergents were added to aqueous solution of LDL. The protein moiety could then be separated from the mixed lipid–detergent micelles by gel filtration in the presence of micellar concentrations of the detergent. The lipid-free protein obtained with Nonidet P40 or with sodium deoxycholate retained the immunological properties of the native LDL as shown by double diffusion in agarose gels against anti-LDL–sera. The lipid-free protein obtained with sodium dodecyl sulfate or cetyltrimethylammonium bromide had altered immunological properties.

Characterization of the lipid-free protein moiety (apo-LDL)[1] of human plasma low-density lipoprotein (LDL) has been hindered by its insolubility. Recently preparations of soluble apo-LDL have been obtained in a number of laboratories, but agreement as to the size and identity of the polypeptide chains in the LDL molecule remains elusive (Shore and Shore, 1967, 1969; Day and Levy, 1968; Scanu *et al.*, 1968; Pollard *et al.*, 1969; Kane *et al.*, 1970). The difficulties are reminiscent of the situation in the membrane protein field where, for instance, the molecular weight estimates for the major protein component of the erythrocyte membrane vary from about 5000 to about 200,000 depending on the methods used (Laico *et al.*, 1970; Gwynne and Tanford, 1970).

Previous procedures for preparing soluble apo-LDL depend on delipidation of LDL by extraction with organic solvents. We have studied the effects of detergents on LDL, since it is known that these dissociate the lipids from membranes (Engelman *et al.*, 1967; Salton and Schmitt, 1967; Jones and Kennedy, 1969; Razin and Barash, 1969; Bont *et al.*, 1969; Lenard, 1970). Triton WR-1339 and sodium decyl sulfate have been shown to partially remove lipid from LDL (Scanu and Oriente, 1960; Gotto *et al.*, 1969). We recently showed that sodium dodecyl sulfate (SDS) displaced all the lipid from LDL, and soluble apo-LDL could be isolated by gel filtration as a SDS complex (Simons and Helenius, 1970). In the present study three other detergents: sodium deoxycholate (NaDOC), Nonidet P40, and cetyltrimethylammonium bromide (CTAB) have been tested as well as SDS. All these detergents removed the lipids from LDL. The immunological properties of the soluble apo-LDL preparations were compared with native LDL by double diffusion in agarose gels. We have also developed methods of removing detergent from apo-LDL.

Materials and Methods

Isolation and Purity of LDL. The density fraction 1.019–1.050 g/cm^3 was prepared from individual sera of healthy male donors by ultracentrifugation as described by Havel *et al.* (1955). EDTA (1.3 mM) was added to all solutions and the pH adjusted to 7.0. The solution densities were checked with an areometer. Centrifugation at 1.050 g/cm^3 was repeated at least once and the final LDL solution dialyzed overnight against 0.15 M NaCl–1.3 mM EDTA (pH 7) at 4°. The LDL solutions were stored at 0° under nitrogen for not longer than 2 weeks.

The purity of the LDL was tested immunologically, as described elsewhere (Simons and Helenius, 1969). The only contaminants were high-density lipoproteins (less than 0.5%). Lipid analysis, amino acid analysis, and electron micrographs of a typical preparation have been published (Simons *et al.*, 1970). Of the total lipid, 56% was cholesterol ester, 27% phospholipid, 13% free cholesterol, and 5% triglyceride. The lipid–protein weight ratio was 3.75.

Detergents. The purity of Nonidet P40 (polyoxyethyleneoctylphenol with a mean of 9.0 ethylene oxide units/molecule, Shell) was determined by thin-layer chromatography (Nadeau and Waszeciak, 1967). After charring the polydispersity in the hydrophilic parts of the Nonidet P40 molecules was seen as a regular pattern of 12–14 spots. The pattern was indistinguishable from that given by Triton X-100, another polyoxyethyleneoctylphenol (with a mean of 9–10 ethylene oxide units/molecule, Rohm & Haas). Contamination by other organic material was not detected. NaDOC (Mann) contained less than 2% other bile salts when analyzed by gas–liquid chromatography (see Chemical Analysis). SDS (Fluka) was recrystallized three times from ethanol and was pure on thin-layer chromatography (silica gel G (Merck), chloroform–methanol–7 N NH_4OH, 65:25:4, v/v). CTAB (Merck) was pure on thin-layer chromatography without prior purification (chloroform–methanol–formic acid–H_2O, 65:25:2:2, v/v).

Gel Filtration. Swelling, equilibration, and packing of Sephadex and Sepharose gels (Pharmacia) were done as

* From the Department of Serology and Bacteriology, University of Helsinki, Haartmaninkatu 3, Helsinki 29, Finland. *Received January 28, 1971.* Supported by grants from the Sigrid Jusélius Foundation, the National Research Council for Medical Sciences and Finska Vetenskaps-Societeten, Helsinki, Finland.

† To whom to address correspondence.

[1] Abbreviations used are: LDL, low-density lipoprotein; apo-LDL, protein moiety of LDL; NaDOC, sodium deoxycholate; SDS, sodium dodecyl sulfate; CTAB, cetyltrimethylammonium bromide; CMC, critical micellar concentration.

recommended by the manufacturer. To increase the flow rate of Sephadex G-200, Sepharose 4B, and Sepharose 6B, swollen gel beads smaller than 149 microns in diameter were removed by sieving. The columns were fitted with flow adaptors, and run upward with the aid of a peristaltic pump connected to the inlet tube. The sample volumes were below 2% of the total volume of the column. Filtration was performed at room temperature unless otherwise indicated in the text. Blue Dextran 2000 (Pharmacia) and 2-mercaptoethanol (Fluka) were used to calibrate the columns.

Chemical Analysis. Protein was determined by the method of Lowry *et al.* (1951) using bovine albumin (Armour) as a standard. SDS (0.1%) was added to the reaction mixture to avoid lipid opalescence. Quantitative amino acid analyses after hydrolysis with 6 N HCl (110°, 22 hr) with a Beckman 120C amino acid analyzer were occasionally performed to check the protein determinations. The LDL concentration was calculated by multiplying the protein concentration by 4.75.

Phosphorus was measured directly from the eluent fractions according to Bartlett (1959), and the amount of phospholipid calculated by multiplying the amount of phosphorus by 25. Total cholesterol was assayed by the method of Abell *et al.* (1952).

For qualitative analysis of neutral lipids and for quantitative determination of NaDOC and Nonidet P40 samples were extracted by the procedure of Folch *et al.* (1957) (chloroform–methanol–water, 8:4:3, v/v) as specified by Renkonen *et al.* (1963). Samples that contained NaDOC were made 1 N with HCl so that the bile salt would be extracted in its protonated water-insoluble form. Before thin-layer chromatography of the neutral lipids excess quantities of SDS, CTAB, or NaDOC in the lipid extracts were removed using 1 g of silicic acid (Unisil 100–200 mesh, Clarkson Chemical Co.) columns. The neutral lipids were eluted with 10 ml of chloroform whereas the detergents and phospholipids were retained in the column. The neutral lipid fraction was then subjected to thin-layer chromatography fractionation as described elsewhere (Simons *et al.*, 1970).

For Nonidet P40 determinations the lipid extracts were evaporated to dryness under nitrogen and dissolved in methanol. The absorbance at 276 nm was measured from the ultraviolet spectrum obtained with a Hitachi recording spectrophotometer, and the amount of Nonidet P40 calculated from standard solutions (Nadeau and Siggia, 1967). Deoxycholic acid was determined from the lipid extracts by gas–liquid chromatography according to Grundy *et al.* (1965), except that a column of 1% neopentyl glycol succinate (Applied Science Laboratories) was used. SDS was determined according to the method of Karush and Sonenberg (1950) as modified by Pitt-Rivers and Impiombato (1968).

Approximate critical micellar concentrations (CMC) for SDS, CTAB, and NaDOC in the buffers used were determined by the dye solubilization method of Williams *et al.* (1955) using 4-dimethylaminoazobenzene (Merck). Since the CMC of neutral detergents is known to be very low (<0.5 mM) (Becher, 1967), the CMC of Nonidet P40 was not determined.

Immunological Techniques. Antisera to LDL were prepared in rabbits. Each rabbit received a total of 14 mg of LDL in Freund's complete adjuvant subcutaneously in three injections at 2-week intervals. The rabbits were bled 10 days after the last injection. Double diffusion was done by the Ouchterlony (1958) method in 1% agarose gels (L'Industrie Biologique Francaise) containing 0.9% NaCl and buffered with 0.02 M phosphate (pH 7.0). The gels were stained for protein [thiazin red (1.0 g), Amido Black (1.0 g), light green SF (1.0 g), mercuric chloride (1.0 g), and acetic acid (20 ml) diluted to 1000 ml with water]. Intrawell absorption (Berg and Bearn, 1966) was used for immunoabsorption studies.

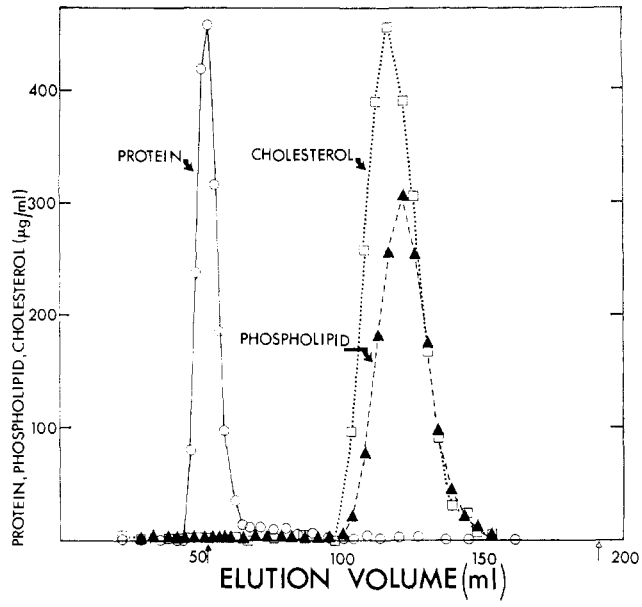

FIGURE 1: Separation of apo-LDL and LDL lipids by gel filtration in the presence of NaDOC. Column: Sephadex G-200 (2.5 × 30 cm). Buffer: 10 mM NaDOC–0.05 M NaCl–0.05 M sodium carbonate (pH 10). Sample: 27 mg of LDL in 1.5 ml of 0.05 M NaCl–0.05 M sodium carbonate (pH 10) with 310 mg of NaDOC added. Pumping rate 8.8 ml/hr, fractions 2.2 ml. Elution volume of Blue Dextran indicated with solid arrow and 2-mercaptoethanol with empty arrow. (○) Protein, (▲) phospholipid, and (□) total cholesterol.

Dialysis and Ultrafiltration. The dialysis tubing (Union Carbide) was soaked in 1% $NaHCO_3$ and 0.15% EDTA and brought to 80°. The tubing was then throughly rinsed with distilled water.

Results

Lipid Removal Using NaDOC. Samples of LDL containing 10–35 mg of LDL/ml were dialyzed against 0.05 M NaCl–0.05 M sodium carbonate (pH 10) overnight at 4°, and 11.5 mg of solid NaDOC was then added per mg of LDL. The clear yellow solution was applied to a Sephadex G-200 column equilibrated with 10 mM NaDOC–0.05 M NaCl–0.05 M sodium carbonate (pH 10). The CMC of NaDOC in this buffer was 2–3 mM. The protein eluted in the void volume as a clear solution (Figure 1). The protein recovery was 84% (mean of five experiments). The protein fraction contained no detectable phospholipids or cholesterol (less than 1% by weight), and no other neutral lipids could be shown by thin-layer chromatography of lipid extracts of the protein peak. If 5 mg of NaDOC/mg of LDL were added to the sample instead of 11.5 mg, the bulk of the cholesterol esters and triglycerides eluted in the void volume with the protein. The phospholipids, the free cholesterol, and some cholesterol esters eluted later in a separate peak as in Figure 1.

When tested against anti-LDL serum the lipid-free apo-LDL gave a precipitin line which fused with that given by native LDL (Figure 2a). Intrawell absorption experiments showed that all anti-LDL activity could be removed from the antiserum by adding apo-LDL.

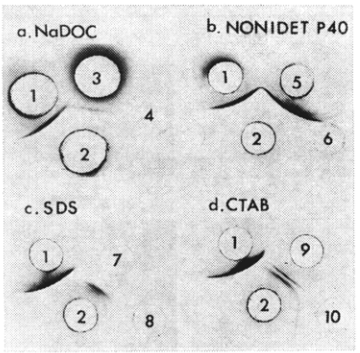

FIGURE 2: Immunodiffusion of various apo-LDL preparations in agarose gel. Buffer: 0.02 M sodium phosphate (pH 7.0)–0.9% NaCl. (1) LDL (2.4 mg/ml); (2) rabbit anti-LDL serum; (3) apo-LDL prepared with NaDOC (top fraction of protein peak in Figure 1); (4) 1% NaDOC in eluent buffer (see Figure 1); (5) apo-LDL prepared with Nonidet P40 (top fraction of protein peak in Figure 4); (6) 1% Nonidet P40 in 0.1 N Tris (pH 7.7); (7) apo-LDL prepared with SDS (top fraction of the protein in Figure 6); (8) 1% SDS in 0.1 M Tris (pH 7.7); (9) apo-LDL prepared with CTAB (top fraction of pool 1 in Figure 7); (10) 1% CTAB in 0.1 M sodium citrate (pH 5.5). The gels were stained for protein.

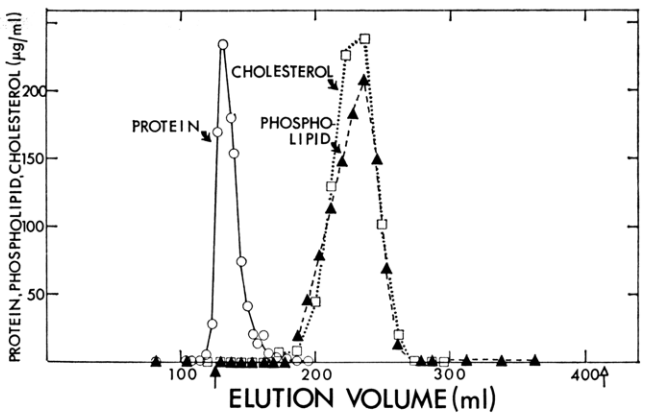

FIGURE 4: Separation of apo-LDL and LDL lipid by gel filtration in the presence of Nonidet P40 at 4°. Column: Sephadex G-200 (2.5 × 90 cm). Buffer: 0.3% Nonidet P40 (w/v), 0.03 M Tris (pH 7.7–0.02% sodium azide. Sample: 46 mg of LDL in 6 ml of 0.03 M Tris (pH 7.7)–0.02% sodium azide with 520 mg of Nonidet P40 added. Pumping rate 8.4 ml/hr, fractions 4.2 ml. Elution volumes of Blue Dextran and 2-mercaptoethanol are indicated with a solid and an empty arrow. (○) Protein, (▲) phospholipid, and (□) total cholesterol.

To remove the bile salt the protein peak was concentrated by ultrafiltration and subjected to gel filtration in detergent-free medium. The protein eluted in the void volume of Sephadex G-75 and the bile salt eluted close to the total volume of the column (Figure 3a). No NaDOC was detected in lipid extracts of the protein fraction by gas–liquid chromatography or thin-layer chromatography. Lipid was also extracted after hydrolysis of the protein (2 N HCl, 7 hr, 110°) to be certain that all detergent was extracted. The fractions containing the detergent-free protein had no visible precipitate, but ultracentrifugation at 100,000*g* for 1 hr pelleted the protein and none was found in the supernatant.

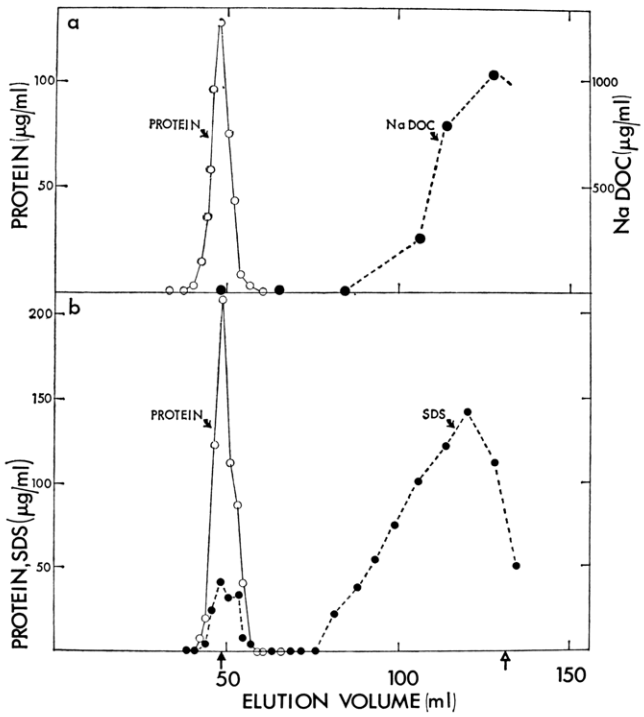

FIGURE 3: Removal of NaDOC and SDS from apo-LDL preparations. Column: Sephadex G-75 (2.5 × 22.5 cm). Buffer: 0.1 M Tris (pH 9.0). (a) 0.9 mg of apo-LDL in 2.0 ml of 10 mM NaDOC, 0.05 mM NaCl, and 0.05 M sodium carbonate (pH 10). (b) 1.5 mg of apo-LDL in 1.2 ml of 0.1% SDS–0.1 M Tris (pH 7.7) was dialyzed for 18 hr at 4° against 0.1 M Tris (pH 9.0) and applied to the column. Pumping rate and fractions as in Figure 1. Elution volumes of Blue Dextran and 2-mercaptoethanol are indicated with a solid and an empty arrow. (○) Protein and (●) detergent.

Lipid Removal Using Nonidet P40. Samples of LDL containing 30–50 mg of LDL/ml were dialyzed against 0.03 M Tris (pH 7.7)–0.02% sodium azide (w/v) overnight at 4°. Eleven milligrams of Nonidet P40 per milligram of LDL was added and the clear solution applied to a Sephadex G-200 column equilibrated with 0.3% Nonidet P40 (w/v), 0.03 M Tris (pH 7.7), and 0.02% sodium azide at 4°. The protein eluted close to the void volume as a clear solution with a recovery of 60% (mean of four experiments) (Figure 4). The protein was free from detectable phospholipids and cholesterol, and no other neutral lipids could be detected by thin-layer chromatography of lipid extracts. Low ionic strength and low temperature were essential for good separation of protein from lipid in the column.

In double diffusion against anti-LDL serum the apo-LDL gave one major precipitin line which fused with the line of native LDL (Figure 2b).

The apo-LDL prepared with Nonidet P40 was recovered virtually detergent free when subjected to gel filtration at 4° in Sepharose 4B equilibrated with a low ionic strength Tris buffer (0.02 M Tris, pH 9.0). A substantial amount of the protein eluted in the void volume which indictated that it was highly aggregated (Figure 5). The bulk of the Nonidet P40 eluted later as shown by the absorbance at 276 nm. The protein pool (pool a in Figure 5) was lyophilized, extracted for lipid, and the amount of Nonidet P40 in the extract determined. The amount of protein in the pool was assayed by quantitative amino acid analysis. Only 0.02 mg of Nonidet P40 was found per mg protein in two experiments.

Lipid Removal Using SDS. We have previously described a method of separating the LDL lipids from the apo-LDL by SDS treatment and gel filtration (Simons and Helenius, 1970). We now have a simplified method. Samples of LDL containing 50–80 mg of LDL/ml were dialyzed against 0.1

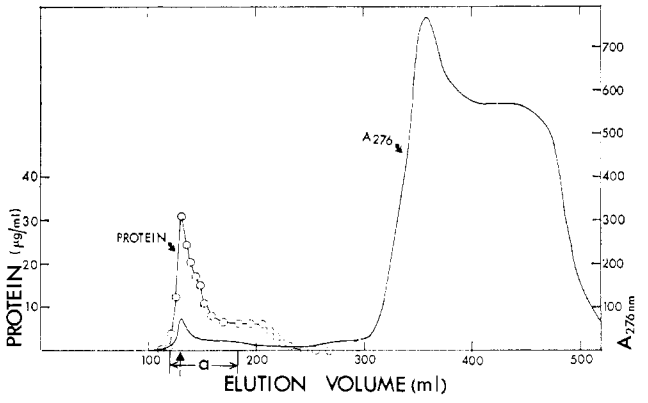

FIGURE 5: Removal of Nonidet P40 from apo-LDL. Column: Sepharose 4B (2.5 × 90 cm). Buffer 0.02 M Tris (pH 9.0) at 4°. Sample: 1.6 mg of apo-LDL in 4.2 ml of 0.03 M Tris (pH 7.7)–0.02% sodium azide containing 50 mg of Nonidet P40. Pumping rate 8.4 ml/hr, fractions 4.2 ml. The elution volume of Blue Dextran is indicated with a solid arrow. (○) Protein and (—) $A_{276\ nm}$. Nonidet P40 and protein was assayed from pool a.

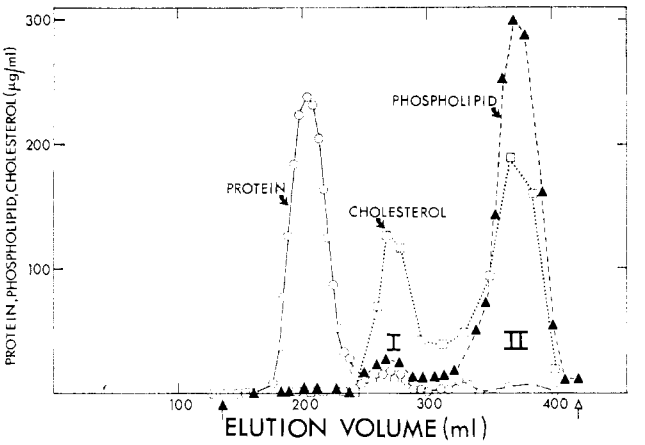

FIGURE 6: Separation of apo-LDL and LDL lipid by gel filtration in the presence of SDS. Column: Sepharose 4B (2.5 × 90 cm). Buffer: 0.1% SDS0.1 M Tris (pH 7.7). Sample: 71 mg of LDL in 5 ml of 0.1 M Tris (pH 7.7) with 430 mg of SDS added. Pumping rate 8.8 ml/hr, fractions 4.4 ml. Elution volumes of Blue Dextran and 2-mercaptoethanol are indicated with a solid and an empty arrow. (○) Protein, (▲) phospholipid, and (□) total cholesterol.

M Tris (pH 7.7) overnight at 4°. Six milligrams of solid SDS per milligram of LDL was added and the clear solution applied to a column of Sepharose 4B equilibrated with 0.1 M Tris (pH 7.7)–0.1% SDS (3.5 mM). The CMC of SDS in this buffer is below 1 mM. The protein eluted virtually free of cholesterol and phospholipid with a yield of 76% (mean of three experiments) (Figure 6). No other neutral lipids were detected by thin-layer chromatography. Two separate lipid peaks appeared of which one (peak I) was rich in neutral lipids and the other (peak II) in phospholipids.

In double diffusion the apo-LDL obtained by SDS treatment formed two precipitin lines against anti-LDL serum, neither of which fused with the line produced by native LDL (Figure 2c). These results showed that SDS altered the immunological reactivity of apo-LDL. Only part of the SDS bound to apo-LDL could be removed gel filtration in detergent free media. The protein eluted from Sephadex G-75 in the void volume separated from the bulk of SDS (Figure 3b). The protein contained 0.25 mg of bound SDS/mg of protein.

Lipid Removal Using CTAB. Samples of LDL were dialyzed against 0.1 M sodium citrate (pH 5.5) overnight at 4°. Four milligrams of solid CTAB was added per milligram of LDL and the clear solution applied to a Sepharose 6B column equilibrated with 0.1 M sodium citrate (pH 5.5)–0.2% CTAB (5.5 mM). The CMC of CTAB in this buffer was below 1 mM. The column did not completely separate protein from phospholipids and total cholesterol (Figure 7a). The front half of the protein peak (pool 1) contained no lipid as shown by phosphorus and cholesterol analyses, and thin-layer chromatography. When the lipid-containing pool 2 was concentrated and rerun through the same column protein and phospholipid eluted in the same volumes as in the first run showing that CTAB separated the lipid from the protein as did the other detergents tested.

Apo-LDL obtained by CTAB treatment gave two precipitin lines against anti-LDL serum in double diffusion. Neither of these fused with the line given by native LDL (Figure 2d). One of the lines could have been caused by unspecific precipitation of some serum component, judging by the fact that this line fused with the line obtained with the 1% CTAB control in the peripheral well.

Since CTAB altered the immunochemical reactivity of apo-LDL, we did not study whether it could be removed from apo-LDL by gel filtration in detergent-free medium.

Discussion

Four detergents with highly different structures removed all major lipids from LDL. We did not study the minor lipid components of LDL amounting to less than one per cent of the total lipids: the sphingoglycolipids (Simons *et al.*, 1970) and possible covalently bound lipids (Fisher and Gurin, 1964). The detergents were simply added to aqueous LDL solutions. High concentrations of detergent were needed to obtain

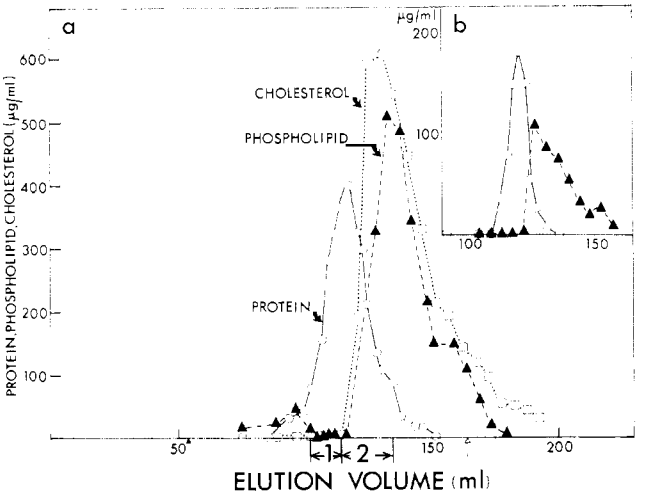

FIGURE 7: Separation of apo-LDL and LDL lipids by gel filtration in the presence of CTAB. Column: Sepharose 6B (2.5 × 31 cm). Buffer: 0.2% CTAB–0.1 M sodium citrate (pH 5.5). (a) 58 mg of LDL in 1.5 ml (0.1 M sodium citrate (pH 5.5) with 220 mg of CTAB added. (b) Pool 2 was concentrated by ultrafiltration and a 1.5-ml sample containing 2.0 mg of apo-LDL with 70 mg of CTAB added rerun. Pumping rate 10.5 ml/hr, 2.6-ml fractions. Elution volumes of Blue Dextran and 2-mercaptoethanol are indicated with a solid and an empty arrow. (○) Protein, (▲) phospholipid, and (□) total cholesterol.

conditions in which all the lipid of LDL could be solubilized by the detergent micelles. The protein was separated from the mixed micelles, formed by detergent and lipids, by gel filtration in the presence of micellar concentrations of detergent. The size of the micelles depends on a number of factors such as the nature of the detergent, temperature, ionic strength, pH, and the nature, composition, and amount of lipid solubilized (Elworthy *et al.*, 1968). Bile salts are known to form small mixed micelles with lipids such as cholesterol (Woodford, 1969), phospholipids (Shankland, 1970), and glycerides (Feldman and Borgström, 1966). The best separation was therefore obtained with NaDOC. Longer columns and rerunning were needed to obtain satisfactory separation with the other detergents.

All the previous procedures for preparing lipid-free apo-LDL involved organic solvent extraction to remove lipids. The lipid-free protein was obtained as a precipitate which could be solubilized in high concentrations of SDS (Granda and Scanu, 1966; Shore and Shore, 1967), or its homolog sodium decyl sulfate (Gotto *et al.*, 1968a). If apo-LDL were succinylated, it dissolved in pH 11.5 buffers even in the absence of detergent (Scanu *et al.*, 1968). Recently another method, based on interphasal extraction with ether in the presence of guanidine hydrochloride and Tween 20, has been reported (Kane *et al.*, 1970). All these methods denatured the protein moiety, as shown by changes in its optical rotatory dispersion and circular dichroic spectra when compared to native LDL (Gotto *et al.*, 1968b; Scanu *et al.*, 1969). The denaturation was also reflected by changes in the immunological properties of the resulting apo-LDL (Granda and Scanu, 1966; Gotto, 1969).

We studied the immunochemical properties of our apo-LDL preparations by double diffusion in agarose gels. The apo-LDL prepared by SDS or CTAB treatment reacted with antiserum against native LDL, but the precipitin lines did not fuse with the line obtained with native LDL. Most antigenic determinants are known to depend on the conformation of the protein (Sela, 1970). It is therefore possible that removal of LDL lipids using SDS or CTAB denatures the protein moiety. An alternate possibility is that the change in immunologic properties may be due to altered charge caused by binding of these detergents to the protein. In contrast, apo-LDL prepared with Nonidet P40 gave one major precipitin line with anti-LDL which fused with the line obtained with native LDL. So did apo-LDL prepared with NaDOC, but the resulting precipitin line was weaker than with identical amounts of Nonidet P40 apo-LDL. These qualitative studies indicate that the apo-LDL obtained with Nonidet P40 or NaDOC carries all the major immunological determinants of the intact LDL molecule, and that these have not undergone conformational changes resulting in detectable immunochemical alterations. These results are in agreement with what is known about the effects of various detergents on proteins. The neutral detergents and bile salts are generally mild in their effects (Swanson *et al.*, 1964; Soltysiak and Kaniuga, 1970), whereas SDS and CTAB are known to inactivate enzymes and denature proteins (Tanford, 1968).

The Nonidet P40 and NaDOC delipidation methods therefore appear to be mild procedures with potential usefulness in the analysis and separation of lipids from proteins also in other lipoprotein structures. We have successfully used the Nonidet P40 procedure to remove the lipid from Semliki Forest virus membrane protein without loss of the hemagglutinating activity of the protein (K. Simons and L. Kääriäinen, unpublished results). With SDS treatment this activity was lost (Simons and Kääriäinen, 1970).

In all procedures described the lipid-free apo-LDL was first obtained in a detergent milieu. Gel filtration in detergent-free media was used to remove the detergent from the apo-LDL. NaDOC could be removed completely, Nonidet P40 almost completely (0.02 mg of apo-LDL) and, in agreement with earlier dialysis experiments (Simons and Helenius, 1970), 0.25 mg of SDS remained bound per mg of apo-LDL. Ultracentrifugation and gel filtration experiments (Figure 5) indicated that the apo-LDL was aggregated when NaDOC and Nonidet P40 was removed, although the solutions had no visible precipitate. The apo-LDL preparations in the presence and absence of detergents are now being further characterized.

Acknowledgments

We thank Professor Ossi Renkonen for his help and expert advice, and Dr. T. A. Miettinen for the quantitative bile acid determinations. The skillful technical assistance of Miss Hilkka Virta and Mrs. Satu Cankar is acknowledged.

References

Abell, L. L., Levy, B. B., Brodie, B. B., and Kendall, F. E. (1952), *J. Biol. Chem. 195*, 357.

Bartlett, G. R. (1959), *J. Biol. Chem. 234*, 466.

Becher, P. (1967), *in* Nonionic Surfactants, Schick, M. J., Ed., New York, N. Y., Marcel Dekker, p 478.

Berg, K., and Bearn, A. G. (1966), *J. Exp. Med. 123*, 379.

Bont, W. S., Emmelot, P., and Vaz Diaz, H. (1969), *Biochim. Biophys. Acta 173*, 389.

Day, C. E., and Levy, R. S. (1968), *J. Lipid Res. 9*, 789.

Elworthy, P. H., Florence, A. T., and Macfarlane, C. B. (1968), Solubilization by Surface-active Agents, London, Chapman.

Engelman, D. M., Terry, T. M., and Morowitz, H. J. (1967), *Biochim. Biophys. Acta 133*, 381.

Feldman, E. B., and Borgström, B. (1966), *Lipids 1*, 430.

Fisher, W. R., and Gurin, S. (1964), *Science 143*, 362.

Folch, J., Lees, M., and Sloane-Stanley, G. H. (1957), *J. Biol. Chem. 226*, 497.

Gotto, A. M. (1969), *Proc. Nat. Acad. Sci. U.S. 64*, 1119.

Gotto, A. M., Levy, R. I., and Fredrikson, D. S. (1968a), *Biochem. Biophys. Res. Commun. 31*, 151.

Gotto, A. M., Levy, R. I., and Fredrikson, D. S. (1968b), *Proc. Nat. Acad. Sci. U. S. 60*, 1436.

Gotto, A. M., Levy, R. I., Lindgren, F. T., and Fredrikson, D. S. (1969), *Biochim. Biophys. Acta 176*, 667.

Granda, J. L., and Scanu, A. (1966), *Biochemistry 5*, 3301.

Grundy, S. M., Ahrens, E. H., and Miettinen, T. A. (1965), *J. Lipid Res. 6*, 397.

Gwynne, J. T., and Tanford, C. (1970), *J. Biol. Chem. 245*, 3269.

Havel, R. J., Eder, H. A., and Bragdon, J. H. (1955), *J. Clin. Invest. 34*, 1345.

Jones, T. H. D., and Kennedy, E. P. (1969), *J. Biol. Chem. 244*, 5981.

Kane, J. P., Richards, E. G., and Havel, R. J. (1970), *Proc. Nat. Acad. Sci. U. S. 66*, 1075.

Karush, F., and Sonenberg, M. (1950), *Anal. Chem. 22*, 175.

Laico, M. T., Ruoslahti, E. I., Papermaster, D. S., and Dreyer, W. J. (1970), *Proc. Nat. Acad. Sci. U. S. 67*, 120.

Lenard, J. (1970), *Biochemistry 9*, 1129.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Nadeau, H. G., and Siggia, S. (1967), *in* Nonionic Surfactants, Schick, M. J., Ed., New York, N. Y., Marcel Dekker, p 863.
Nadeau, H. G., and Waszeciak, P. H. (1967), *in* Nonionic Surfactants, Schick, M. J., Ed., New York, N. Y., Marcel Dekker, p 904.
Ouchterlony, Ö. (1958), *Progr. Allergy 5*, 1.
Pitt-Rivers, R., and Impiombato, F. S. A. (1968), *Biochem. J. 109*, 825.
Pollard, H., Scanu, A. M., and Taylor, E. W. (1969), *Proc. Nat. Acad. Sci. U. S. 64*, 304.
Razin, S., and Barash, V. (1969), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 3*, 217.
Renkonen, O., Kosunen, T. U., and Renkonen, O.-V. (1963), *Ann. Med. Exp. Biol. Fenn. 41*, 375.
Salton, M. R. J., and Schmitt, M. D. (1967), *Biochem. Biophys. Res. Commun. 27*, 529.
Scanu, A., and Oriente, P. (1960), *J. Exp. Med. 113*, 735.
Scanu, A., Pollard, H., Hirz, R., and Kothary, K. (1969), *Proc. Nat. Acad. Sci. U. S. 62*, 171.
Scanu, A., Pollard, H., and Reader, W. (1968), *J. Lipid Res. 9*, 342.
Sela, M. (1970), *Ann. N. Y. Acad. Sci. 169*, 23.
Shankland, W. (1970), *Chem. Phys. Lipids 4*, 109.
Shore, B., and Shore, V. (1967), *Biochem. Biophys. Res. Commun. 28*, 1003.
Shore, B., and Shore, V. (1969), *Biochemistry 8*, 4510.
Simons, K., Ehnholm, C., Renkonen, O., and Bloth, B. (1970), *Acta Pathol. Microbiol. Scand. 78*, 459.
Simons, K., and Helenius, A. (1969), *Ann. Med. Exp. Biol. Fenn. 47*, 48.
Simons, K., and Helenius, A. (1970), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 7*, 59.
Simons, K., and Kääriäinen, L. (1970), *Biochem. Biophys. Res. Commun. 38*, 981.
Soltysiak, D., and Kaniuga, Z. (1970), *Eur. J. Biochem. 17*, 70.
Swanson, P. D., Bradford, H. F., and McIlwain, H. (1964), *Biochem. J. 92*, 235.
Tanford, C. (1968), *Advan. Protein Chem. 23*, 211.
Williams, R. J., Phillips, J. N., and Mysels, K. J. (1955), *Trans. Faraday Soc. 51*, 728.
Woodford, F. P. (1969), *J. Lipid Res. 10*, 539.

Steryl Glucosides in *Phaseolus aureus*. Use of Gas–Liquid Chromatography and Mass Spectrometry for Structural Identification*

Roger A. Laine† and Alan D. Elbein

ABSTRACT: Chloroform–methanol extracts of a membrane-containing particulate enzyme fraction from *Phaseolus aureus* were concentrated to a small volume and subjected to thin layer chromatography in three solvent systems. An α-naphthol–H_2SO_4-staining band was purified and analyzed by gas–liquid chromatography and by combined gas–liquid chromatography–mass spectrometry. Two major components were identified as stigmasteryl and β-sitosteryl glucosides by mass spectra of the intact molecules as trimethylsilyl derivatives.

During studies on the biosynthesis of cell wall polysaccharides in mung bean seedlings, we found that a particulate enzyme preparation incorporated radioactivity from UDP-D-glucose-^{14}C into chloroform–methanol-soluble products. Since some types of glycolipids have been shown to be intermediates in bacterial cell wall synthesis (Anderson *et al.*, 1965; Dankert *et al.*, 1969; Lennarz and Talamo, 1966), we undertook to examine the endogenous glycolipids in the mung bean particulate enzyme fraction to determine whether similar intermediates occur in the synthesis of the plant cell wall.

This report concerns the identification of components in one lipid band isolated by thin-layer chromatography of crude lipid extracts. During the isolation, radioactivity associated with the lipid bands was monitored for related enzymatic investigations not considered here. Although only 400 μg of the lipid band was obtained for analysis, its constituents were easily identified as a series of steryl glucosides by the use of combined gas–liquid chromatography–mass spectrometry. The methods employed in this inquiry should be generally applicable to some types of glycolipids and may be useful in the investigation of membrane components.

Experimental Section

Preparation of the Membrane Particulate Fraction. A particulate enzyme fraction was prepared by grinding mung bean shoots with sand (Elbein, 1969). Material sedimenting between 1000*g* and 30,000*g* was resuspended in buffer and used as the enzyme fraction. Large-scale incubation mixtures

* From the Department of Biology, Rice University, Houston, Texas, and the Department of Biochemistry, The University of Texas Medical School, San Antonio, Texas 78229. *Received September 25, 1970.* This work was supported by a research grant from the Robert A. Welch Foundation. A preliminary report of this work was presented at the 29th Annual Meeting of the American Society of Biological Chemists, Atlantic City, N. J. (1970).

† Present address: Department of Biochemistry, Michigan State University, East Lansing, Mich. 48823.