A Comparative Study of the Effects of Chemical Modification on the Immunochemical and Optical Properties of Human Plasma Low-Density Lipoprotein(s) and Apoproteins

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1. The structure of human plasma low-density lipoprotein(s) [LD lipoprotein(s)] was investigated by several immunological and optical techniques. The effects of delipidation and of chemical modification by 3-carboxypropionylation, acetylation, diazotization and amidination were examined. A sensitive double-antibody radioimmunoassay for human LD lipoproteins is presented and is used to assess the extent of immunochemical modification. A computer best-fit analysis is used to analyse circular-dichroism (c.d.) spectra. These methods permit comparisons of the relative effects of chemical modification and delipidation of LD lipoprotein under similar experimental conditions. 2. 3-Carboxypropionylation, acetylation or diazotization produces qualitative and quantitative changes in the immunochemical properties of LD lipoprotein. Amidination causes minor changes detected by radioimmunoassay but not by double-diffusion experiments. In general, the order of effectiveness in displacing ^{125}I -labelled LD lipoprotein is amidinated > diazo or acetyl > 3-carboxypropionyl derivatives. 3. The order of the extent of conformational alteration induced in apoLD lipoprotein and LD lipoprotein, as judged by c.d. analyses, was 3-carboxypropionylation > diazotization > acetylation or amidination. 4. Delipidation of LD lipoprotein results in immunological alterations that are qualitatively detected by antisera to LD lipoprotein. Four of five antisera to apoLD lipoprotein form precipitin lines of identity between native LD lipoprotein and apoLD lipoprotein in double-diffusion experiments. An anti-(apoLD lipoprotein) serum that forms precipitin lines of complete identity between LD lipoprotein and apoLD lipoprotein reacts differently with these two antigens in radioimmunoassay. ApoLD lipoprotein is only one-fourth to one-half as effective as LD lipoprotein, on a protein basis, in the displacement of ^{125}I -labelled LD lipoprotein from this anti-(apoLD lipoprotein). 5. Conformational analysis indicates that apoLD lipoprotein retains a high proportion of the structural integrity of the native lipoprotein. Delipidation induces a small decrease in the content of β -structure and a small increase in disordered structure, without greatly affecting the α -helical content. Chemical modification produces more severe conformational changes of apoLD lipoprotein than of LD lipoprotein. Computer analysis of the c.d. spectra of apoLD lipoprotein indicates that addition of high concentrations of sodium decyl sulphate abolishes most of the β -conformation concomitant with increases in α -helical and disordered structure. 6. There is parallelism between the alteration of the charge of LD lipoprotein and apoLD lipoprotein and the extent of immunochemical and conformational changes.

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LD lipoprotein(s) and apoLD lipoprotein(s) are highly important components in the system of plasma

|| Abbreviations: LD lipoprotein(s), low-density lipoprotein(s) (plasma lipoproteins of density 1.025–1.05 g/ml); apoLD lipoprotein(s), lipid-free protein(s) of LD lipoproteins; HD lipoprotein(s), high-density lipoprotein(s) (plasma lipoproteins of density 1.063–1.210 g/ml); VLD lipoprotein(s), very-low-density lipoprotein(s) (plasma

lipid transport. In the inherited disease abetalipoproteinaemia, the selective absence of apoLD lipoprotein in plasma is associated with complete failure of chylomicrons, VLD lipoprotein and LD lipoprotein to appear in plasma (Gotto *et al.*, 1971). Immunochemically, the LD lipoprotein is heterogeneous (Albers *et al.*, 1972) and there is some evidence that apoLD lipoprotein may consist of more than one polypeptide (Shore & Shore, 1969; Kane *et al.*, 1970). At the present time there is no convenient way of fractionating the protein components of LD lipoprotein when they are associated with lipids as in the native state. Various soluble preparations of lipid-free LD lipoprotein or apoLD lipoprotein have been compared with native LD lipoprotein and shown qualitatively to possess certain immunological and optical similarities (Gotto *et al.*, 1968a,b,c; Scanu *et al.*, 1969). The relationship between the state of lipidation and structure of LD lipoprotein is of considerable importance to the understanding of lipid transport. Dearborn & Wetlaufer (1969) have shown that the conformation of LD lipoprotein is dependent on the degree of lipidation. The more heavily lipidated species of LD lipoprotein appeared to contain relatively more pleated sheet (β -conformation) and less helical and disordered structure than did the species with a smaller complement of lipid.

In part because of the difficulties in studying the protein moiety of LD lipoprotein several investigators have used chemical modification of the protein to investigate its structural properties (Kane *et al.*, 1970; Margolis & Langdon, 1966a,b; Lees, 1967; Gotto *et al.*, 1968a,b; Scanu *et al.*, 1968). Lees (1967) has suggested that acetylation and diazotization of LD lipoprotein may alter the conformation of the protein so that it resembles that of apoLD lipoprotein. Several investigators have observed that chemical modification may alter the immunological reactions of LD lipoprotein while the optical properties may not be detectably changed (Gotto *et al.*, 1968a,d; Scanu *et al.*, 1968).

In none of the many experiments with chemical modification or with delipidation have quantitative methods been employed to assess immunological and optical parameters. Although we realized the potential hazards of interpretation of experiments of this kind when applied to heterogeneous families of lipoproteins and detergent-solubilized apoproteins, it nonetheless seemed worthwhile to undertake a comparison of the effects of chemical modification of LD lipoprotein isolated over a narrow density range (1.025–1.050g/ml) and of the lipid-free protein(s) derived therefrom. No attempt was made to fractionate the apoprotein(s) since the lipidated species could not be fractionated for comparison.

|| (footnote continued): lipoproteins of density approx. 0.95–1.006g/ml; *d* 1.21 supernatant, plasma fraction of density less than 1.210g/ml; *d* 1.21 infranatant, plasma fraction of density greater than 1.210g/ml; c.d., circular dichroism; o.r.d., optical rotatory dispersion.

nate the apoprotein(s) since the lipidated species could not be fractionated for comparison.

In the present paper we have studied the effects of 3-carboxypropionylation, acetylation, diazotization or amidination on the structure of native and delipidated LD lipoprotein with several qualitative and quantitative immunochemical and optical analyses. We describe a double-antibody radioimmunoassay for human plasma LD lipoprotein that appears quite sensitive to structural alteration. In c.d. experiments we employ three models of computer best-fit analysis to obtain a more quantitative evaluation of conformation changes (Greenfield & Fasman, 1969; Saxena & Wetlaufer, 1971). Within the experimental and theoretical limitations inherent in the methods employed, our studies permit relative comparisons of the effects of chemical modification on the immunochemical and optical properties of LD lipoprotein and apoLD lipoprotein under similar experimental conditions.

Experimental

Materials

Succinic anhydride and *p*-arsanilic acid were purchased from Eastman-Kodak Distillation Products Industries, Rochester, N.Y., U.S.A. Sodium decyl sulphate, recrystallized from ethanol, was a generous gift from Dr. Ralph Reisfeld (National Institutes of Health, Bethesda, Md., U.S.A.) or was purchased from Schwartz-Mann Research Laboratories (Orangeburg, N.J., U.S.A.) and was 99% pure by t.l.c. Methanolic HCl was prepared by bubbling HCl gas (1.1–1.4 mol) through 40 ml of methanol, determined to be free of water by examination of its n.m.r. spectrum. All other chemicals were of the highest purity commercially available.

Preparation of LD lipoprotein, apoLD lipoprotein and their chemical derivatives

Preparation of LD lipoprotein. Plasma, obtained by plasmapheresis from normal subjects after an overnight fast, was collected in disodium EDTA, pH 7.0 (0.1 g/l), and stored at 4°C. Isolation of LD lipoprotein from pooled plasma was begun with 2 weeks of collection. LD lipoprotein was prepared by ultracentrifugal flotation (Havel *et al.*, 1955) between densities 1.025 and 1.050g/ml. Within the present limits of immunological detection, all preparations of LD lipoprotein used for the present study were pure and did not react with antisera to albumin, HD lipoprotein or other plasma constituents. Mean sedimentation flotation values, S_f^0 (de Lalla & Gofman, 1954), were determined to be 6.1 and 6.2 for two of the preparations. [S_f^0 is the flotation rate in Svedberg units (10^{-13} cm/s per dyn per g), corrected for concentration dependence and to standard conditions of

26°C and 1.744M-NaCl ($\rho_{26} = 1.063$ g/ml).] The lipoprotein solutions used for immunological studies contained 0.1% sodium EDTA, pH7.0, and either 0.15M-NaCl or 0.13M-Tris-HCl buffer, pH8.0. Immunochemical reactions of LD lipoprotein in 0.15M-NaCl and Tris-HCl solutions were indistinguishable.

Preparation of apoLD lipoprotein. Preparation of apoLD lipoprotein was begun immediately upon isolation of LD lipoprotein. LD lipoprotein was dialysed for 24h against 0.01% disodium EDTA, pH7.0, and then freeze-dried. A portion of the LD lipoprotein (5mg as protein) was twice extracted for 16h at 4°C with 50ml of diethyl ether-ethanol (3:1, v/v) and the precipitate dried under N₂. ApoLD lipoprotein (5mg) prepared in this way was solubilized by incubation for 4h at 5°C with 1ml of 0.13M-Tris-HCl buffer, pH8.2, containing 100mM-sodium decyl sulphate and 0.1% EDTA. Once the apoprotein was obtained in solution, the concentration of sodium decyl sulphate was lowered by dialysis to 0.5mM. The apoprotein prepared in this way was not sedimented by centrifugation for 30min at 10000g. The residual detergent was determined by chemical analysis (Karush & Sonenberg, 1950) and was assumed to be bound to the apoprotein since it was not detectable in the final diffusate. In certain experiments, as indicated in the text, apoLD lipoprotein was prepared by treatment of LD lipoprotein with 0.10M-sodium decyl sulphate as previously described (Gotto *et al.*, 1969) and the soluble apoprotein-detergent complex was isolated by ultracentrifugation or gel filtration or both (Simons & Helenius, 1970). ApoLD lipoprotein solutions used in these studies contained 0.1% disodium EDTA, 0.13M-Tris-HCl buffer (pH8.2) and 0.5mM-sodium decyl sulphate unless otherwise indicated.

Preparation of chemical derivatives of LD lipoprotein and apoLD lipoprotein. Chemical modification was performed within 24h of isolation of LD lipoprotein. All of the chemical modification reactions with LD lipoprotein were carried out at protein concentrations of 10–20mg/ml. 3-Carboxypropionyl LD lipoprotein was prepared by the addition of solid succinic anhydride to LD lipoprotein at pH7.5–8.0 (Haas, 1964), by using a 60:1 molar ratio of the anhydride to lysine. LD lipoprotein was acetylated with acetic anhydride at 4°C, pH7.5–8.0, with a 50:1 molar ratio of reagent to lysine. The procedure of Riordan & Vallee (1964) was followed, except that liquid acetic anhydride was used. The reagent was added in small increments with a micropipette and constant stirring. The acylation procedures were performed with a pH-stat and were followed by the ninhydrin reaction (Moore & Stein, 1948) until >99% of the initial free amino groups had disappeared. The pH was maintained at 7.5–8.0 during the course of addition of acylating reagent, by titration

with 2.0M-NaOH. Amidinated LD lipoprotein was prepared by treating LD lipoprotein at 4°C and pH8.5 with methyl acetimidate-HCl (Wofsy & Singer, 1963) by using a 100:1 molar ratio of reagent to lysine. Amino acid analysis for Dnp-lysine after reaction with fluorodinitrobenzene and acid hydrolysis (Wofsy & Singer, 1963) indicated that more than 90% of the ϵ -amino groups of lysine had reacted with methyl acetimidate. LD lipoprotein was diazotized with diazobenzene-arsenate at pH9.1 by the procedure of Tabachnick & Sobotka (1960) as modified by Margolis & Langdon (1966b). Spectral analysis at alkaline pH values (Tabachnick & Sobotka, 1960) showed that 10% of the histidine and 26% of the tyrosine residues disappeared after the coupling reaction.

Chemical derivatives of LD lipoproteins were separated from excess of reagent by dialysis for 24h against 0.15M-NaCl containing 0.01% sodium EDTA, pH7.0. The buffer was changed four times. Delipidation of derivatives of LD lipoprotein was carried out immediately after dialysis at protein concentrations of 4–5mg/ml as described for the delipidation of LD lipoprotein. Although 3-carboxypropionyl LD lipoprotein was soluble in the absence of detergent, the solubilization of each apoprotein derivative was carried out in an identical way so that comparisons of immunochemical and optical properties would not reflect differences of preparation.

The immunological reactions of each derivative of LD lipoprotein and apoLD lipoprotein were tested by immunoelectrophoresis, Ouchterlony plate experiments and radioimmunoassay and the optical properties by c.d. within 24h of preparation. These derivatives were generally stable to storage for up to 7 days at 4°C. The preparations were thereafter discarded. Occasionally, visible aggregation occurred earlier than 1 week, in which case any precipitant material was removed by centrifugation for 30min at 10000rev./min. The resultant supernatant solutions were found to be indistinguishable in their immunological and optical properties from the solutions of derivatives tested immediately after chemical modification.

General analytical methods

Protein concentrations of LD lipoprotein, amidinated LD lipoprotein, acetyl LD lipoprotein and 3-carboxypropionyl LD lipoprotein were measured by the method of Lowry *et al.* (1951), by using bovine serum albumin for standards and employing a correction for differences in absorption (Margolis & Langdon, 1966a). Measurements with acetyl LD lipoprotein and 3-carboxypropionyl LD lipoprotein were made after complete deacylation of the acyl-tyrosine residues at pH7.5 as determined by changes in E_{278} (Riordan & Vallee, 1964). The protein concentration of diazo LD lipoprotein was determined by

the ninhydrin reaction (Moore & Stein, 1948) based on the known lysine content of the protein (Margolis & Langdon, 1966a).

Immunochemical techniques

Preparation of antisera. For each experiment, identical concentrations of the various antigens were used. Antisera to apoLD lipoprotein (A, B, T, U,) 3-carboxypropionyl LD lipoprotein (Q), 3-carboxypropionyl apoLD lipoprotein (R, S), acetyl LD lipoprotein (M, N), diazo LD lipoprotein (O, P) and amidinated LD lipoprotein (J, L) were prepared by one (B, N, P and L), two (A, M, O, J, R and S) or three (Q, T, U) injections into the foot-pads of white New Zealand rabbits of 1.25 ml of the antigen (5–8 mg of protein) mixed with 0.75 ml of complete Freund's adjuvant. Antisera were harvested 10–14 days after the last injection (Levy & Fredrickson, 1965; Levy *et al.*, 1966a).

Sheep antisera to apoLD lipoprotein, designated SA₁ and SB₁, were prepared by subcutaneous injection of 1.5 ml of antigen (7.5 mg of protein), mixed with an equal volume of complete Freund's adjuvant, in each fore- and hind-quarter of female Cheviot sheep weighing approx. 80 kg. Antisera were harvested by intracardiac puncture (under pentobarbital anaesthesia) and collected in disodium EDTA (final concentration 0.01%, pH 7.0). Erythrocytes were removed by centrifugation. Antiserum SB₁ was collected 22 days after the initial injection. Sheep SA₁ was given a second injection on day 22 and antiserum SA₁ was collected 11 days later. Sheep antisera to the plasma d 1.21 supernatant from two normal animals (SD₁, SD₂) were prepared in an identical manner to antiserum SA₁.

Antisera were concentrated in dialysis bags by exposure to dry Sephadex G-50 at 4°C. All antisera were stored at 4°C. A sheep antiserum to apoLD lipoprotein (SA₁) was purified and shown to be associated with the immunoglobulins G by chromatography on DEAE-cellulose (Vaerman *et al.*, 1963) and by immunoelectrophoresis with specific antisera to sheep IgG.

Classification of antisera. The classification of the antisera used in this investigation is based on reactions observed with immunoelectrophoresis and on Ouchterlony plates. A specific antigen-antibody reaction was considered negative only if no precipitin line was observed over a ratio of antigen (at 4.0 mg of protein/ml) to antibody (at serum concentration obtaining in the donor) of 64:1 to 1:64 (v/v). If no precipitin line was observed over this range, the donor's serum was concentrated threefold and the experiment repeated. The specificity of the anti-(apoLD lipoprotein) serum, SA₁, was confirmed with an IgG fraction purified from this antiserum. In earlier publications the rabbit antiserum Q had been called SDB₁

(Gotto *et al.*, 1968b). The antisera S₁, S₂, S_{1A}, R₂ and R₄, prepared against HD lipoprotein, have been described previously (Levy *et al.*, 1966b).

Agarose diffusion. Ouchterlony plates were made with 1% agarose obtained from Marine Colloid, Springfield, N.J., U.S.A., in 0.15 M-NaCl. Plates with wells requiring 5–10 µl or 40–50 µl of antigen or antibody were used. They were allowed to develop at 4°C and observed for at least 1 week. Photographs of immunoprecipitin lines were usually taken daily.

Immuno-electrophoresis. A standard procedure for immunoelectrophoresis (Levy & Fredrickson, 1965; Levy *et al.*, 1966a) was employed, by using agarose. Because of their increased mobility, electrophoresis of the 3-carboxypropionylated, acetylated and diazotized lipoprotein and apoprotein was carried out for only 30 min. The antigen was then allowed to diffuse against 50–60 µl of antiserum (at the serum concentration in the donor unless otherwise indicated) for 24–72 h at 23°C in a high-humidity chamber. The method of development, staining and photographing of the immunoelectrophoresis plates has been described (Levy & Fredrickson, 1965; Levy *et al.*, 1966a).

Radioimmunoassay. The protein moiety of LD lipoprotein was measured quantitatively by a double-antibody radioimmunoassay similar to a procedure for the measurement of plasma insulin (Morgan & Lazarow, 1963) and subsequently applied to the assay of rat LD lipoprotein (Eaton & Kipnis, 1969). ¹²⁵I-labelled LD lipoprotein was prepared from freshly prepared LD lipoprotein by the iodine monochloride procedure (MacFarlane, 1964), and was generally stable for at least 7 days at 4°C as tested by radioimmunoassay. Fresh solutions of ¹²⁵I-labelled LD lipoprotein were prepared weekly. Under the labelling conditions employed, over 95% of the radioactivity was in the protein moiety of LD lipoprotein, as shown by extraction with chloroform-methanol (2:1, v/v). An antiserum prepared against the lipid-free protein moiety of LD lipoprotein (antiserum A) was used as the first antibody. Incubation conditions were selected to minimize non-specific precipitation of ¹²⁵I-labelled LD lipoprotein when the first antibody was omitted from the system. The use of silicone-treated tubes and of buffer containing 3% human serum albumin (Pentex Research Products Division, Miles Laboratories Inc., Kanakee, Ill., U.S.A.) decreased non-specific precipitation to only 2–3% of the total label added for the assay. The second antibody was a sheep antiserum to rabbit γ-globulin. To obtain a workable precipitate it was necessary to add carrier rabbit serum before addition of the second antibody. For initial incubation, 200 µl of antiserum A (diluted 1:100 000) was mixed with 100 µl of ¹²⁵I-labelled LD lipoprotein (8 ng containing 3000 c.p.m.), 500 µl of 150 mM-NaCl containing 0.01% disodium EDTA and 100 µl of 50 mM-sodium borate buffer, pH 8.2, containing 3% albumin. All dilutions were

EXPLANATION OF PLATE I

Immunodiffusion patterns of chemical derivatives of LD lipoproteins

Centre wells contained antiserum Hy [anti-(LD lipoprotein)]. Outer wells 1 and 2 contained LD lipoprotein in the upper panel and 3-carboxypropionyl LD lipoprotein in the lower panel. Outer wells 3 and 4 contained 3-carboxypropionyl LD lipoprotein in the left upper panel, amidinated LD lipoprotein in the right upper panel and LD lipoprotein in the lower panel. The volume of antigen solution was $10\mu\text{l}$ per well and the protein concentration was 4 mg/ml. All precipitin lines were unstained in this and subsequent plates.

EXPLANATION OF PLATE 2(A)

(A) Immunodiffusion patterns of LD lipoprotein, apoLD lipoprotein and their chemical derivatives

(a) The centre well contained antiserum SA₁ [anti-(apoLD lipoprotein)] and the outer wells contained apoLD lipoprotein (1 and 4), acetyl apoLD lipoprotein (2) and diazo apoLD lipoprotein (3). (b) The centre well contained antiserum SC₁ [anti-(LD lipoprotein)] and the outer wells contained LD lipoprotein (1 and 4), acetyl LD lipoprotein (2) and diazo LD lipoprotein (3). The volume of antigen solution placed in each outer well was 10 μ l at a protein concentration of 4 mg/ml.

EXPLANATION OF PLATE 2(B)

(B) Immunodiffusion patterns of LD lipoprotein and of apoLD lipoprotein delipidated with ether-ethanol or with 100 mM-sodium decyl sulphate

The outer wells contained LD lipoprotein (1 and 2), apoLD lipoprotein delipidated with ether-ethanol and solubilized with sodium decyl sulphate (4, 6 and 8) and apoLD lipoprotein delipidated and solubilized by 100 mM-sodium decyl sulphate without exposure to ether-ethanol (3, 5 and 7). The centre well contained anti-(LD lipoprotein) serum Hy. The volume of antigen solution placed in each outer well was 10 μ l at a protein concentration of 4 mg/ml.

made with an 8% (w/v) solution of albumin in 50mM-sodium borate buffer, pH 8.0. The contents of the tubes were well mixed, stoppered and incubated at 4°C for 72h. In blank samples, either the first or second antibody or both were omitted. After incubation for 72h, 10 μ l of carrier rabbit serum and 200 μ l of the second antibody (goat antiserum to a rabbit IgG fraction) were added. After further incubation for 16h at 4°C, the precipitate was separated by centrifugation. These conditions were shown to be sufficient to precipitate over 90% of the total radioactivity of 125 I-labelled LD lipoprotein in the absence of unlabelled antigen. At least 95% of the 125 I-labelled LD lipoprotein could be displaced by the addition of unlabelled antigen. Both the supernatant and precipitate were counted separately for radioactivity in a Packard Autogamma counter at an efficiency of 40%. Results were expressed as the ratio of bound (radioactivity in the precipitate) to free (radioactivity in the supernatant) LD lipoprotein protein. This ratio (B/F) was determined for each set of duplicate tubes. The assay detected as little as 10ng of LD lipoprotein protein. A standard curve with unlabelled LD lipoprotein was determined with each assay. Standard deviations, based on differences between duplicate analyses, averaged 10ng. Duplicates differing by more than 20% were run again.

Circular dichroism and optical rotatory dispersion

C.d. spectra were measured in duplicate at two different light-paths (0.1 and 0.5mm) and at two different protein concentrations (0.2 and 1.0mg/ml). All spectra were recorded at 23°C. Three different preparations of each derivative of LD lipoprotein and apoLD lipoprotein were studied. The duplicate measurements varied less than 3% from 240 to 210nm and less than 5% between 210 and 208nm. The solvent used for LD lipoprotein and its derivatives was 50mM-sodium phosphate buffer, pH 8.2, containing 0.01% EDTA. The same buffer was used for c.d. measurements of apoLD lipoprotein and its derivatives, except that the buffer contained 0.5mM-sodium decyl sulphate. After each run a blank was obtained by repeating the spectrum with the omission of protein. Spectra were recorded with a Cary 60 Spectropolarimeter, by using a model 6001 circular-dichroic accessory. The mean residue ellipticity $[\theta]$, was calculated from the expression:

$$[\theta] = \frac{\theta^\circ}{10} \times \frac{\text{MRW}}{lc}$$

where MRW is the mean residue weight (115), l is the optical path of the cell in cm, c is concentration of protein in g/ml and θ° is the measured ellipticity angle. O.r.d. spectra in the far-u.v. range were made under the same conditions as described for the c.d. studies and were used to calculate the mean residue of

rotation $[m]$. The units for $[\theta]$ and $[m]$ are in $\text{deg} \cdot \text{cm}^2/\text{dmol}$.

C.d. spectra were analysed over the range 208–240nm with a computer best-fit technique and with three different model systems (Shrager, 1970). The computed fractions of the α -helical, β -structure and disordered conformations were constrained so that they were greater than or equal to 0 and so that the sum of the three fractions was 1. The values used for the mean residue ellipticities of the α -helical, β -structure and disordered modes were those previously determined for poly-L-lysine in solution in model 1 (Greenfield & Fasman, 1969), poly-L-lysine in films for disordered structure and in solution for α -helical and β -conformation in model 2 (Fasman *et al.*, 1970), and those obtained by Saxena & Wetlaufer (1971) by combining experimental c.d. values with X-ray-diffraction parameters in model 3. Error function is expressed as root mean square of the difference between observed and computed values.

Results

Immunoelectrophoresis and double-diffusion experiments of LD lipoprotein and derivatives

LD lipoprotein and its chemically modified derivatives were evaluated with a variety of antisera. As judged qualitatively by double-diffusion experiments, the chemical procedures, with the exception of amidination, produced definite changes in the immunological reactions of LD lipoprotein. Antibodies prepared against either whole serum or LD lipoprotein formed precipitin lines of only partial identity between LD lipoprotein and 3-carboxypropionyl LD lipoprotein, acetyl LD lipoprotein, or diazo LD lipoprotein (Plates 1 and 2A). These antisera formed precipitin lines of identity between LD lipoprotein and amidinated LD lipoprotein (Plate 1). Despite the induction of antigenic alterations by chemical substitution, LD lipoprotein, LD lipoprotein derivatives and their antisera all exhibited cross-reactivity (Plates 1 and 2A). For example, antibodies to whole serum, to LD lipoprotein and to LD lipoprotein derivatives reacted with LD lipoprotein and all of its derivatives. Although not all of these reactions were measured quantitatively, it is possible to generalize that the antiserum made to a particular derivative formed a heavier precipitin line to that derivative than to others. Antisera to 3-carboxypropionyl or acetyl LD lipoprotein formed only weak precipitin lines with native LD lipoprotein.

Immunoelectrophoresis of apoLD lipoprotein and derivatives

Since the apoprotein preparations contained 0.5mM-sodium decyl sulphate, this detergent was studied for its potential effects at low concentrations

Table 1. *Immunochemical reactivity of apoLD lipoprotein and its chemical derivatives*

Immunochemical reactivity was determined by immunoelectrophoresis of apoLD lipoprotein or its chemical derivatives against the specified antiserum as described in the Experimental section. A strong and consistent precipitin line is indicated by +. When no precipitin line was observed, the antiserum was concentrated twofold. The complete absence of a precipitin line at both concentrations of donor's antiserum is indicated by -. The presence of a precipitin line only after the antiserum was concentrated twofold is indicated by $\pm$. n.d., Not determined.

Source of antisera (as immunizing antigen)	Immunochemical reactivity (with reactant antigen)				
	ApoLD lipoprotein	Amidinated apoLD lipoprotein	Acetyl apoLD lipoprotein	Diazo apoLD lipoprotein	3-Carboxypropionyl apoLD lipoprotein
ApoLD lipoprotein (A)	+	+	+	+	+
ApoLD lipoprotein (B)	+	+	$\pm$	$\pm$	-
Amidinated LD lipoprotein (J, L)	+	+	-	-	-
Acetyl LD lipoprotein (M, N)	-	-	+	+	-
Diazo LD lipoprotein (O, P)	-	-	-	+	-
3-Carboxypropionyl LD lipo- protein (Q)	-	-	-	-	+
3-Carboxypropionyl apoLD lipoprotein (R, S)	-	n.d.	-	-	+
LD lipoprotein (Hy)	+	+	-	-	-
ApoLD lipoprotein (SA ₁)	+	+	+	+	+
ApoLD lipoprotein (SB ₁)	+	+	$\pm$	$\pm$	n.d.
LD lipoprotein (SC ₁)	-	-	-	-	-
Normal plasma <i>d</i> 1.21 infranatant (SD ₁)	+	+	-	-	-
Normal plasma <i>d</i> 1.21 infranatant (SD ₂)	+	+	-	-	-
Whole serum (H ₃)	+	+	-	-	-

on the immunological reactions. Except for a slight increase in mobility on immunoelectrophoresis, the immunoprecipitin reactions of LD lipoprotein were unchanged at a detergent concentration of 0.5 mM. Soluble apoLD lipoprotein prepared by delipidation with 100 mM-sodium decyl sulphate and dialysis without exposure to organic solvents formed precipitin lines of complete identity with apoLD lipoprotein delipidated by ether-ethanol as described in the Experimental section (Plate 2B). For purposes of comparison, apoLD lipoprotein was solubilized in the absence of detergent by incubation at pH 11.5 as described by Scanu *et al.* (1969) and was shown to form precipitin lines of identity with detergent-solubilized preparations when tested with anti-(apoLD lipoprotein) sera (A, SA₁, T, U).

Antisera to apoLD lipoprotein formed precipitin lines of only partial immunochemical identity between apoLD lipoprotein and acetyl, diazo (Plate 2A) and 3-carboxypropionyl apoLD lipoprotein, but formed lines of complete identity between apoLD lipoprotein and amidinated apoLD lipoprotein. Anti(3-carboxypropionyl apoLD lipoprotein) sera

formed precipitin lines with native and modified LD lipoprotein, but out of the delipidated lipoproteins or derivatives with only 3-carboxypropionyl apoLD lipoprotein (Table 1).

Six of seven antisera made in rabbits, sheep or horses to whole serum (H₃), the *d* 1.21 supernatant fractions (SD₁, SD₂), LD lipoprotein (Hy) or amidinated LD lipoprotein (J, L), formed precipitin lines with apoLD lipoprotein (Table 1). None of these antisera formed precipitin lines with 3-carboxypropionyl, acetyl or diazo apoLD lipoprotein over a wide range of antigen/antibody concentrations (Table 1). They did react with amidinated apoLD lipoprotein (Table 1) and formed precipitin lines of identity between this derivative and apoLD lipoprotein. Antibodies to any one chemical derivative of LD lipoprotein reacted with that derivative after delipidation but generally did not form detectable lines with the other delipidated derivatives (Table 1). Of all the antisera to the chemical derivatives of LD lipoprotein, only anti-(amidinated LD lipoprotein) (antiserum J) reacted with apoLD lipoprotein, as judged by precipitin lines (Table 1).

The six antisera referred to above that reacted with apoLD lipoprotein did, however, recognize differences between the native lipoprotein and apolipoprotein, forming lines of only partial immunochemical identity between these antigens [see Plate 1 for reaction with anti-(LD lipoprotein), antiserum Hy]. One sheep antiserum to LD lipoprotein (SC₁) formed precipitin lines with LD lipoprotein and its derivatives but not with apoLD lipoprotein or with any delipidated derivative (Table 1). In addition, several antisera (S₁, S₂, S_{1A}, R₂ and R₄) that were prepared to HD lipoprotein, but reacted with both HD lipoprotein and LD lipoprotein (Levy *et al.*, 1966b), failed to react with apoLD lipoprotein and its derivatives.

In contrast with antisera to LD lipoprotein, four of the antisera prepared against apoLD lipoprotein detected no qualitative antigenic differences between LD lipoprotein and apoLD lipoprotein. Three rabbit (A, T, U) and one sheep (SB₁) antisera to apoLD lipoprotein formed lines of complete identity between the apoprotein and native LD lipoprotein. Another sheep antiserum (SA₁) formed small spur lines between these two antigens.

Radioimmunoassay

Since antibodies to apoLD lipoprotein reacted with all derivatives of LD lipoprotein and apoLD lipoprotein and qualitatively detected identical or very

Fig. 1. Radioimmunoassay of LD lipoprotein and its chemical derivatives

Values for the quantity of non-radioactive antigen added (in ng of protein) are plotted against the observed bound/free ratio for the corresponding sample. For experimental details see the text. (a) Displacement of ¹²⁵I-labelled LD lipoprotein from anti-(apoLD lipoprotein) serum A by LD lipoprotein (●), amidinated LD lipoprotein (○) and acetyl LD lipoprotein (▲). (b) Displacement of ¹²⁵I-labelled LD lipoprotein from anti-(apoLD lipoprotein) serum A by LD lipoprotein (●), diazo LD lipoprotein (▲) and 3-carboxypropionyl LD lipoprotein (○). (c) Displacement of ¹²⁵I-labelled LD lipoprotein from anti-(apoLD lipoprotein) serum A by LD lipoprotein (●), apoLD lipoprotein (○) and diazo apoLD lipoprotein (▲). (d) Displacement of ¹²⁵I-labelled LD lipoprotein from anti-(apoLD lipoprotein) serum A by LD lipoprotein (●) and amidinated apoLD lipoprotein (○).

similar immunochemical determinants in the native and delipidated molecules, a radioimmunoassay was employed to obtain a more quantitative estimate of these reactions. A standard curve for the displacement of ^{125}I -labelled LD lipoprotein by unlabelled LD lipoprotein is shown in Fig. 1(a). Antiserum A to apoLD lipoprotein was used for these experiments, since antisera to native LD lipoprotein did not form precipitin lines with chemical derivatives of apoLD lipoprotein (Table 1). The curve was usually sigmoid on a semilogarithmic plot and its most sensitive portion was between 10 and 100 ng of protein. Some variation in the appearance of the displacement curves was noted for different preparations of ^{125}I -labelled LD lipoprotein. The explanation for these differences is not known. The order of effectiveness of displacement of ^{125}I -labelled LD lipoprotein by the various chemical derivatives was consistent, however, in all of the experiments. The qualitative conclusions described above were borne out in that amidinated LD lipoprotein resembled LD lipoprotein more closely than the other derivatives in the ability to displace ^{125}I -labelled LD lipoprotein (Figs. 1a and 1b). The small differences between the displacement curves of LD lipoprotein and amidinated LD lipoprotein were reproducible, but the structural significance of the differences is unknown. The order of effectiveness in displacing ^{125}I -labelled LD lipoprotein was amidinated LD lipoprotein > diazo LD lipoprotein > acetyl LD lipoprotein > 3-carboxypropionyl LD lipoprotein. Similarly, amidinated apoLD lipoprotein

gave a displacement curve more nearly resembling that of apoLD lipoprotein than did the other apoprotein derivatives. Typical experiments are shown in Figs. 1(c) and 1(d). Diazo apoLD lipoprotein (Fig. 1c) and acetyl apoLD lipoprotein were more effective in displacement than 3-carboxypropionyl apoLD lipoprotein but were less effective than the amidinated derivative. It should be noted that all of the LD lipoprotein and apoLD lipoprotein derivatives formed detectable precipitin lines with antiserum A in the qualitative immunochemical assay (Table 1). As estimated from the displacement curves, apoLD lipoprotein displaced approximately one-quarter to one-half as much ^{125}I -labelled LD lipoprotein as did the same quantity of native LD lipoprotein protein.

Effects of chemical modification on the c.d. spectra of LD lipoprotein

We examined the c.d. spectra of derivatives of LD lipoprotein and apoLD lipoprotein by computer best-fit analysis, with the use of data from model systems (Greenfield & Fasman, 1969; Fasman *et al.*, 1970; Saxena & Wetlaufer, 1971) to aid in the interpretation. We have used three models to describe the parameters of α -helical, disordered and β -structures. The accuracy of c.d. analysis by computer best-fit techniques will undoubtedly improve as superior reference systems for each structural mode are developed. Despite the obvious limitations of the

Table 2. *Effects of chemical modification on the c.d. spectrum of LD lipoprotein*

C.d. spectra were analysed from 208 to 240 nm with a computer best-fit technique as described in the Experimental section (Shrager, 1970). The percentages of the α -helical, β -structure and disordered conformations were computed for the observed spectra, previously determined values for the mean residue ellipticities for these three conformations being used. In model 1, the values for the three conformations were those of poly-L-lysine in solution; in model 2, the values for β -structure and α -helix were those of poly-L-lysine in solution and for disordered structure were those of poly-L-lysine in films; in model 3, experimental c.d. values were combined with X-ray-diffraction parameters (Saxena & Wetlaufer, 1971). Error function is expressed as the root mean square of the difference between observed and computed values.

Preparation		Composition of secondary structure or conformation (%)											
											Error function		
		α -Helix			β -Structure			Disordered structure					
Model no.	...	1	2	3	1	2	3	1	2	3	1	2	3
LD lipoprotein		24	24	31	44	35	32	32	41	37	612	657	1150
Amidinated LD lipoprotein		22	21	29	45	35	33	33	44	38	428	537	1140
3-Carboxypropionyl LD lipoprotein		27	25	33	35	25	27	38	50	40	423	433	860
Diazo LD lipoprotein		22	21	28	39	28	31	39	51	41	386	301	760
Acetyl LD lipoprotein		23	22	30	47	38	34	30	40	36	750	791	1270

Table 3. *Effects of chemical modification on circular dichroism of apoLD lipoprotein*

See Table 2 for an explanation of the computer analysis of the c.d. spectra.

Preparation		Composition of secondary structure or conformation (%)											
											Error function		
		α -Helix			β -Structure			Disordered structure					
Model no.	...	1	2	3	1	2	3	1	2	3	1	2	3
LD lipoprotein		24	24	31	44	35	32	32	41	37	612	657	1150
ApoLD lipoprotein		22	21	28	37	26	30	41	54	42	358	284	762
ApoLD lipoprotein (preparation ultrafiltered)		24	22	29	36	25	28	40	53	42	655	536	882
Amidinated apoLD lipoprotein		22	21	28	40	29	31	38	50	41	347	300	866
Acetyl apoLD lipoprotein		24	23	29	34	22	28	42	55	43	423	349	706
Diazo apoLD lipoprotein		22	21	27	31	18	28	47	61	45	393	280	552
3-Carboxypropionyl apoLD lipoprotein		26	24	30	17	0	19	57	76	51	583	363	376
ApoLD lipoprotein plus sodium decyl sulphate (100mM)		35	34	40	14	0	13	51	66	47	1510	1370	1490

approach we employ, it is the one that at the present time gives the closest approximation to X-ray analysis for proteins with large amounts of ordered structure.

Although we have presented the computer-generated data in Tables 2 and 3, we do not wish to stress quantitative interpretations. We view these analyses only as approximations, but they may indicate changes that would not be otherwise detected.

The general features of the c.d. spectra of LD lipoprotein and its 3-carboxypropionyl, acetyl, diazo and amidinated derivatives were quite similar (Figs. 2 and 3). No significant changes were detected in the α -helical content of LD lipoprotein as a consequence of chemical modification (Table 2). All models detected a decrease in β -structure and a concomitant increase in disordered structure in 3-carboxypropionyl LD lipoprotein and diazo LD lipoprotein as compared with the native lipoprotein. The magnitude of the changes, however, were relatively small and with model 3 were not significant. Spectral analysis of acetyl LD lipoprotein or amidinated LD lipoprotein yielded values practically indistinguishable from those for LD lipoprotein (Table 2). The fits between observed and calculated data were poorer for model 3 than for models 1 and 2 (Table 2 and Fig. 4).

Effects of chemical modification on the optical properties of apoLD lipoproteins

Comparison of the c.d. spectrum of apoLD lipoprotein (Figs. 5 and 6) with that of LD lipoprotein showed that delipidation resulted in a significant

decrease in the magnitude of the negative ellipticity at 216–218nm. The spectrum of the diazo derivative was flattened between 208 and 220nm, whereas those of amidinated apoLD lipoprotein and acetyl apoLD lipoprotein were very similar to that of apoLD lipoprotein (Fig. 5). The spectrum of 3-carboxypropionyl apoLD lipoprotein was markedly different (Fig. 6). There was a decrease in the magnitude of the negative ellipticity at 215–220nm and a new, deep negative trough at 205–208nm. 3-Carboxypropionylation also induced significant changes in the o.r.d. spectrum of apoLD lipoprotein (Fig. 7). In corroboration of the c.d. results, the o.r.d. spectrum of 3-carboxypropionyl apoLD lipoprotein exhibited a significant decrease in $[\eta]_{233}$ compared with apoLD lipoprotein. These results with 3-carboxypropionyl apoLD lipoprotein are consistent with a decrease in the content of ordered structure, either β -structure or α -helical, and an increase in the content of disordered structure. The qualitative impressions were borne out by the analyses shown in Table 3. In altering the conformation of apoLD lipoprotein, 3-carboxypropionylation produced the greatest change, amidination and acetylation the smallest, with diazotization being intermediate in its effect. None of the models detected a significant change in the α -helical content when LD lipoprotein was delipidated or apoLD lipoprotein was chemically substituted. Consistently, model 3 predicted conformation changes of smaller magnitude and gave poorer fits between observed and calculated spectra than did models 1 and 2. Except for 3-carboxypropionyl apoLD lipoprotein, model 3 did not detect significant differences

Fig. 2. Circular-dichroism spectra

●, LD lipoprotein; ○, amidinated LD lipoprotein; ▲, diazo LD lipoprotein. The buffer used was 50mM-sodium phosphate, pH8.2 (with 0.01% EDTA); 0.1 mm path length at 23°C. For experimental details see the text.

between apoLD lipoprotein and any of its derivatives.

Addition of sodium decyl sulphate at a concentration of 100mM to apoLD lipoprotein resulted in the appearance of a new deep negative trough between 205 and 210 nm (Fig. 8). All three models predicted a sharp decline in β -structure with increases in disordered structure and α -helical conformation as a consequence of adding detergent (Table 3). The error functions were relatively large. The computer models attempted to fit the observed data by generating spectra with negative troughs at 208–210 and 220–222 nm. Ultrafiltration of apoLD lipoprotein did not alter its c.d. spectrum.

Discussion

The use of radioimmunoassay and of computer analysis of c.d. data allow us to make comparisons

Fig. 3. Circular-dichroism spectra

●, LD lipoprotein; ○, acetyl LD lipoprotein; ▲, 3-carboxypropionyl LD lipoprotein. The buffer used was 50mM-sodium phosphate, pH8.2 (with 0.01% EDTA); 0.1 mm path length at 23°C. For experimental details see the text.

between the relative effects of chemical modification and delipidation of LD lipoprotein on its immunological and optical properties. First, we will consider the effects of chemical modification apart from the question of lipidation. Chemical substitution on the protein moiety of LD lipoprotein usually led to changes in immunochemical reactivity that could be detected by both qualitative and quantitative tests. This interpretation is consistent with previous qualitative experiments with acetyl and diazo LD lipoprotein (Lees, 1967). Antisera prepared against LD lipoprotein and its chemical derivatives detected antigenic differences between the native and modified lipoprotein even with qualitative methods, i.e. double diffusion and electrophoretic experiments. These antisera nonetheless exhibited reactivity with all of

Fig. 4. Circular-dichroism spectrum of LD lipoprotein

Observed c.d. spectra (○) are compared with spectra calculated (●) by the computer best-fit technique employing the three models described in the Experimental section. The percentages of the α -helical, β -structure and disordered conformations were computed for the observed spectra, previously determined values for the mean residue ellipticities for these three conformations being used. (a) Model 1: the values for the three conformations were those of poly-L-lysine in solution. (b) Model 2: the values for β -structure and α -helix were those of poly-L-lysine in solution and for disordered structure were those of poly-L-lysine in films. (c) Model 3: experimental c.d. values were combined with X-ray-diffraction parameters (Saxena & Wetlaufer, 1971). Conditions for recording the observed spectra were: 50mm-sodium phosphate buffer, pH8.2 (with 0.01 % EDTA); 0.1 mm path length at 23°C. For further experimental details see the text.

the lipoprotein derivatives. Amidinated LD lipoprotein was an exception to these generalizations; in qualitative immunochemical reactions it was indistinguishable from LD lipoprotein. We employed an antiserum to apoLD lipoprotein to examine by radioimmunoassay the effects of chemical substitution of LD lipoprotein. Consistent with the qualitative experiments, amidinated LD lipoprotein was very similar to LD lipoprotein in the displacement of ^{125}I -labelled LD lipoprotein. However, there were small, reproducible differences between the displacement curves of native and amidinated LD lipoprotein, indicating apparent immunochemical differences not detected by double-diffusion experiments. 3-Carboxypropionyl LD lipoprotein was the least effective of the derivatives in displacing the iodinated LD lipo-

protein. Acetyl and diazo LD lipoprotein were more effective than 3-carboxypropionyl LD lipoprotein and less effective than amidinated LD lipoprotein. Our findings raised the possibility that the degree of immunochemical change is related to the extent of the charge of the molecule.

The use of computer analysis and radioimmunoassay permitted comparisons to be made between the conformational and immunological characteristics of LD lipoprotein, apoLD lipoprotein and their derivatives. A notable parallel is seen between the effects of chemical modification on the immunochemical reaction and on the c.d. spectra of LD lipoprotein (and of apoLD lipoprotein). The relative potency of chemical modification in altering the immunochemical properties of LD lipoprotein and apoLD

Fig. 5. Circular dichroism spectra

●, ApoLD lipoprotein; ○, amidated apoLD lipoprotein; △, diazo apoLD lipoprotein. The buffer used was 50mM-sodium phosphate, pH 8.2 (with 0.01% EDTA); 0.1mm path length at 23°C. For experimental details see the text.

Fig. 6. Circular dichroism spectra

●, ApoLD lipoprotein; ○, acetyl apoLD lipoprotein; △, 3-carboxypropionyl apoLD lipoprotein. The buffer used was 50mM-sodium phosphate, pH 8.2 (with 0.01% EDTA); 0.1mm path length at 23°C. For experimental details see the text.

lipoprotein was 3-carboxypropionylation > diazotization or acetylation > amidation, and in changing the secondary structure was 3-carboxypropionylation > diazotization > acetylation or amidation. It is possible that both the changes in immunological and conformational characteristics are in part related to the alteration in charge of LD lipoprotein and its apoprotein. 3-Carboxypropionylation causes the greatest alteration in charge of the modifiers employed, whereas amidination modifies lysine residues without affecting the charge. Acetylation decreases the net positive charge, whereas diazotization introduces additional negative charges. Our findings suggest that some of the sites associated with the immunological activity of the protein moieties of lipoproteins exhibit a dependence on the charge or conformation or both of the molecule and are not solely determined by specific linear sequences of amino acids.

A second major focus of this investigation is the effect of lipidation on the properties of LD lipo-

protein. Several immunological differences were noted between native and delipidated LD lipoprotein. Qualitative antigenic differences between LD lipoprotein and apoLD lipoprotein were detected by antisera to LD lipoprotein but not by four out of five antisera to apoLD lipoprotein. Granda & Scanu (1966) have previously shown that antisera to LD lipoprotein also react with apoLD lipoprotein. By radioimmunoassay with antiserum A it was determined that apoLD lipoprotein was one-half to one-quarter as effective as LD lipoprotein (on a protein basis) in displacing ^{125}I -labelled LD lipoprotein, although this antiserum formed precipitin lines of complete identity between the native lipoprotein and the apolipoprotein. Chemical derivatives of apoLD lipoprotein showed decreased activity in the radioimmunoassay, similar to that of the LD lipoprotein derivatives, with amidated apoLD lipoprotein being the least affected. The decreased effectiveness of apoLD lipoprotein as compared with LD lipoprotein in the radioimmunoassay must at present remain

Fig. 7. Optical rotatory dispersion spectra

Spectra for apoLD lipoprotein (—) and 3-carboxypropionyl apoLD lipoprotein (····) are shown. The buffer was sodium phosphate, pH 8.2 (with 0.01% EDTA); 0.1 mm path length at 23°C. For experimental details see the text.

speculative. Since antiserum A was prepared against apoLD lipoprotein, both the native and delipidated lipoproteins should contain the same number of antigenic sites and exhibit the same displacement curves. It is possible that the antigenic sites are more exposed on the surface of LD lipoprotein than on the surface of apoLD lipoprotein where protein-protein interactions are most likely to predominate. ApoLD lipoprotein is known to exist in a highly aggregated state.

In earlier experiments delipidation of LD lipoprotein was shown to lead to significant c.d. spectral changes although the soluble apoprotein retained much of the β -structure of the native lipoprotein (Gotto *et al.*, 1968a,b,c,d; Scanu *et al.*, 1969). These qualitative impressions are consistent with the results of computer analysis. Models 1 and 2 indicated that delipidation of LD lipoprotein leads to a decrease in β -conformation and an increase in disordered structure, but that apoLD lipoprotein retains much of the secondary structural characteristics of LD lipoprotein. LD lipoprotein contained 32–44% β -structure and apoLD lipoprotein contained 26–37% by the three models. The computer analysis detected large conformational changes after 3-carboxypro-

Fig. 8. Circular dichroism of apoLD lipoprotein

Measurements were made in 0.5 mM (●) and 100 mM (○) sodium decyl sulphate. The buffer used was 50 mM-sodium phosphate, pH 8.2 (with 0.01% EDTA); 0.1 mm path length at 23°C. For experimental details see the text.

pionylation of apoLD lipoprotein, small changes after diazotization and little or no significant changes after acetylation or amidination.

The question might be raised of the appropriateness of the apoprotein preparations used for the immunological and optical comparisons in this study. Each of the preparations contained 0.5 mM-sodium decyl sulphate for purposes of comparability. Hence it might be argued that the immunochemical and optical similarities of apoLD lipoprotein and LD lipoprotein were due to a small residue of detergent bound to the apoprotein and causing it to resemble a lipoprotein. For this reason we compared detergent-solubilized and alkali-solubilized apoLD lipoprotein and showed that they reacted with antiserum A forming precipitin lines of identity. Scanu *et al.*

(1969) have reported that the alkali-solubilized apoLD lipoprotein has a c.d. spectrum qualitatively similar to the one shown here for detergent-solubilized preparation. Our intention is not to imply that alkali-solubilized apoLD lipoprotein is necessarily a good control for the detergent-solubilized protein but to show that antigenic and conformational properties of the lipid-free protein could be demonstrated in the complete absence of detergent. Helenius & Simons (1971) have reported that apoLD lipoprotein solubilized with the neutral detergent Nonidet P.40 or with sodium deoxycholate retained the immunochemical properties of LD lipoprotein in double-diffusion experiments. ApoLD lipoprotein solubilized with sodium dodecyl sulphate or cetyltrimethylammonium bromide exhibited immunochemical differences. The apoprotein used for their experiments contained bound detergent, ranging from 0.02mg of Nonidet to 0.25mg of sodium dodecyl sulphate/mg of protein, compared with approx. 0.03mg of detergent/mg of protein in the present investigation. Helenius & Simons (1971) concluded from their study that apoLD lipoprotein obtained with Nonidet P.40 or sodium deoxycholate has all the major immunochemical determinants of native LD lipoprotein and these have not undergone detectable conformational changes. Although this is a perfectly acceptable qualitative interpretation, the results described in the present paper illustrate that more sensitive techniques may demonstrate differences when Ouchterlony experiments do not. ApoLD lipoprotein and LD lipoprotein formed precipitin lines of identity with antiserum A. However, this same antiserum detected significant differences between LD lipoprotein and apoLD lipoprotein in the double-antibody radioimmunoassay.

A different question is that of the effect of high concentrations of sodium decyl sulphate or dodecyl sulphate on the properties of apoLD lipoprotein. The computer analyses in the present study support the previous impressions (Gotto *et al.*, 1968c; Scanu *et al.*, 1969) that high concentrations of detergent induce an increase in the content of α -helix with an associated decrease in content of β -conformation and an increase in disordered structure.

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