Properties of human serum low density lipoproteins after modification by succinic anhydride

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ABSTRACT Human serum low density lipoprotein of d 1.019–1.063 (LDL_2) treated with succinic anhydride at pH 7.5–8.0 showed the same chemical composition, hydrodynamic properties (flotation and sedimentation coefficients, intrinsic viscosity) and optical properties (circular dichroism) as untreated LDL_2. However, in contrast to LDL_2, the succinylated product (s-LDL_2) failed to react with rabbit anti-LDL_2 antisera.

Extraction with ethanol–ether 3:1 yielded the succinylated apoprotein (s-apo-LDL_2), which was, unlike untreated apoprotein, soluble in aqueous buffers. Succinylated apoprotein, which was also immunologically unreactive, appeared to differ in structure from s-LDL_2, as assessed by the parameters of intrinsic viscosity and circular dichroism.

The molecular weights of both LDL_2 and s-LDL_2 obtained by the technique of sedimentation equilibrium were $2.1–2.3 \times 10^6$. By the same method, s-apo-LDL_2 gave an uncorrected figure of $3.95–4.15 \times 10^4$ and, after correction for succinyl functions, of $3.60–3.80 \times 10^4$. Because of the assumptions made in the computations, the latter figure was considered approximate.

The marked differences in molecular weight between s-apo-LDL_2 and whole apo-LDL_2 ($\sim 5 \times 10^5$) were taken to support the subunit structure of apo-LDL_2, which is envisaged as an aggregate of about 12 subunits which dissociate upon succinylation. Further, the large percentage (about 90%) of the free amino groups of LDL_2 found to react with succinic anhydride suggests that these groups are at the surface of the molecule.

KEY WORDS serum · low density lipoproteins · succinylated · apoprotein · antigenic reactivity · molecular weight · intrinsic viscosity · circular dichroism · subunit structure

IN THE COURSE OF STUDIES aimed at the elucidation of the nature of the reactive sites of human serum low density lipoprotein of d 1.019–1.063 (LDL_2) we observed that after modification by succinic anhydride this lipoprotein class can be deprived of essentially all its lipid complement to yield an apoprotein, apo-LDL_2, which is soluble in aqueous buffers. Previous attempts to prepare a water-soluble apo-LDL_2 under other experimental conditions had failed (1, 2) with the exception of a recently reported method employing sodium dodecyl sulfate, SDS (3). In the latter instance, however, the rather large amounts of surfactant needed for protein solubilization made interpretation of the hydrodynamic properties of the final product difficult. In view of these findings and the present poor understanding of the nature of apo-LDL_2 (4) studies were carried out on the properties of succinylated LDL_2 (s-LDL_2) before and after delipidation. The results obtained are the object of this report. Preliminary accounts have appeared (5, 6).

MATERIALS

Sera were obtained from the blood of healthy Caucasian male donors, 20–30 yr of age, group A, Rh positive, who had fasted overnight. 1 ml of 5% EDTA solution, neutralized to pH 7 with 0.1 N NaOH, was added to each 100 ml of serum. Chylomicrons were removed (Spinco 30.2 rotor, 9500 *g*, 10 min, 16°C) on the day of blood

Abbreviations: LDL_2, low density lipoproteins of d 1.019–1.063; apo-LDL_2, apoprotein of LDL_2 obtained by extraction of the lipids with ethanol–ether; s-LDL_2 and s-apo-LDL_2, succinylated LDL_2 and succinylated apo-LDL_2; SDS, sodium dodecyl sulfate.

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collection. Separation of lipoproteins from these chylomicron-free sera was begun either immediately or within 2 days of storage at 4°C.

METHODS

Separation of Lipoproteins

The aim of the present studies was the preparation of pure low density lipoprotein floating between density 1.019 and 1.063. Following the previously adopted nomenclature (3), we shall call this class LDL_2. In all the experiments, ultracentrifugation was carried out in the 30.2 rotor of a Spinco model L ultracentrifuge at 70,488 *g* at 16°C. Two methods of separation were initially compared.

Method A. Chylomicron-free sera were adjusted to d 1.019 by addition of recrystallized NaCl, and centrifuged for 22 hr. After removal of the top milliliter each tube was sliced in the middle clear zone, the bottom residues were collected, brought to d 1.063 with NaCl, and then centrifuged again for 22 hr. The top 1 ml fractions $(LDL)_2$ were centrifuged three more times at d 1.063 to eliminate possible contaminants and then dialyzed extensively against 0.15 M NaCl, 0.01% EDTA.

Method B. The chylomicron-free sera were brought to d 1.063 with solid NaCl and centrifuged for 22 hr. The top 1 ml fractions containing the entire LDL class were then adjusted to d 1.006 by dilution with deionized water and centrifuged again for 18 hr. The top milliliter ($d < 1.006$) was discarded, the tubes were sliced, and the bottom residues (containing lipoproteins of density 1.006–1.019 and 1.019–1.063) were centrifuged at d 1.019. After the top milliliters (d 1.006–1.019) had been collected, the desired lipoprotein class of d 1.019–1.063 (LDL_2) was floated by centrifugation at d 1.063 and washed again three times at d 1.063 to remove possible contaminants.

Further Purification Steps

To eliminate possible low molecular weight contaminants, we passed some specimens of LDL_2, separated and purified by either method A or B, through Sephadex G-25 columns (1 × 25 cm) equilibrated with 0.15 M NaCl, 0.05% EDTA. We avoided excessive dilution by collecting 0.5 ml aliquots. The fractions containing yellow pigmented LDL_2 were easily identified by visual inspection and by absorbancy reading at 280 mμ.

Tests of Purity

The techniques of starch gel electrophoresis and immunoelectrophoresis were those previously described (7) except that agarose (Fisher Scientific Company, Chicago, Ill.) replaced agar in the immunodiffusion experiments. The specific anti-human LDL antisera were those previously prepared in the rabbit by Granda and Scanu (3). LDL_2 appeared as a single band in both starch gel and agarose and exhibited a single arc of precipitation after immunodiffusion against anti-LDL antisera. It failed to react with either anti-high density lipoprotein or anti-albumin antisera.

LDL_2 prepared by either method A or B showed unchanged electrophoretic and immunochemical behavior after gel filtration. On the basis of these results, procedure B was adopted because it was best suited for the laboratory. Such preparations had an average S_f of 6.4 (range 5.4–7.4) and a protein content of approximately 23% by weight.

Succinylation

This was essentially the procedure described by Hass for the enzyme aldolase (8). Solid succinic anhydride (Eastman Chemical Products, Inc., Rochester, N.Y.) was added in small increments to solutions of LDL_2 (5–10 mg of protein/ml) to a final molar ratio of the reagent to lysine (from amino acid analysis) of 60:1, the pH being maintained between 7.5 and 8 by addition of 0.1 N NaOH. The reaction, which was at first monitored manually with the aid of a single glass electrode, was later carried out automatically in a TTA-31 titrator (Radiometer Co., Copenhagen, Denmark). The specimens were then extensively dialyzed against 0.15 M NaCl, 0.01% EDTA. The same procedure was applied for the succinylation of apo-LDL_2 obtained by the SDS procedure of Granda and Scanu (3). The extent of succinylation was determined by the ninhydrin reaction (9).

The dinitrophenylation procedure of Levy (10) was used to determine the number of succinyllysine residues formed, as suggested by Gounaris and Perlmann (11). In such a procedure the dinitrophenylated apo-LDL_2 was hydrolyzed with 6 N HCl and the number of free lysines released from the succinyllysine complexes was determined by quantitative amino acid analysis.

O-succinylation of tyrosine was determined by the Lowry reaction according to Habeeb, Cassidy, and Singer (12). *O*-succinylation of hydroxy amino acids was determined by the alkaline hydroxylamine reaction as described by Gounaris and Perlmann (11).

Delipidation Procedures

In the case of LDL_2, lipids were removed, in the presence of SDS according to Granda and Scanu (3) except that a 3:1 ethanol–ether mixture replaced the 1:3 mixture earlier employed. The larger percentage of ethanol

produced a finer protein precipitate. Gentle fragmentation of the precipitate by means of a glass rod insured greater contact between protein and solvent. Unbound SDS was removed by anion-exchange chromatography (Dowex 1 X 8, mesh 100–200), instead of by dialysis (3), with Tris buffer pH 8.6 ionic strength 0.1, as eluent. After passage through the column the protein solution became slightly turbid because of the suspended fine particles. These preparations cleared promptly upon succinylation. Their SDS content (3) was about 1% by weight.

The conditions of delipidation for s-LDL_2 were the same as for LDL_2 with the important exception that *SDS was omitted* from the system. The product obtained was readily soluble in Tris buffer pH 8.6, ionic strength 0.1, and was dialyzed against it or other buffers (see Results) to remove the excess of succinic anhydride.

In either procedure the recovery of delipidated apo-LDL_2 was about 90%. Removal of lipid from LDL_2 not treated with either SDS or succinic anhydride resulted in a product that was either insoluble or had limited solubility in aqueous buffers in the pH range 3–11.

Reduction and Alkylation of s-Apo-LDL_2

s-Apo-LDL_2 dissolved in 8 M urea was reduced with 0.1 M β-mercaptoethanol at pH 7.4 for 2 hr at room temperature and then alkylated with 0.11 M iodoacetate at 7.4 for 3 hr at 0°C. Urea was removed either by dialysis or by gel filtration (Sephadex G-10). The completeness of the reaction was assessed quantitatively by amino acid analysis (Technicon Co., Ardsley, N.Y.) of reduced and alkylated s-apo-LDL_2 after hydrolysis with 6 N HCl for 22 hr under conditions described previously (7). Carboxymethyl cysteine was used as a standard.

N-Terminal Amino Acid Analysis

This analysis of s-apo-LDL_2 was conducted by the dinitrophenylation technique as outlined by Fraenkel-Conrat, Harris, and Levy (13).

C-Terminal Amino Acid Analysis

This analysis was carried out by enzymatic hydrolysis with carboxypeptidase A (Worthington Corporation, Freehold, N.J.). The reaction was carried out with a 1:50 molar ratio of enzyme to substrate at pH 8.4 37°C, for various time intervals. The reaction was stopped by addition of trichloroacetic acid to a final concentration of 5%. The amino acids in the supernatant fraction were identified by paper high voltage electrophoresis (Gilson Medical Electronics, Middleton, Wis.) and in a Technicon amino acid analyzer. Conditions for electrophoresis were: buffer, pyridine–acetic acid–water 1:10:289, pH 3.70; 3000 v, 200 ma, 1 hr.

Filtration of s-apo-LDL_2 through Agarose Columns

Experiments were carried out in 2 × 40 cm columns of Sepharose 4B (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden, concentration of agarose 4%, particle size in the swollen state, 40–190 μ) equilibrated with Tris–HCl buffer pH 8.6, ionic strength 0.01. 20–30 mg of s-apo-LDL_2 was applied to each colum. The flow rate was 0.4 ml/min. Absorbancy at 280 mμ was continuously monitored in a Gilford (Oberlin, Ohio) recording spectrophotometer. The fractions corresponding to each elution peak were pooled, concentrated by pervaporation, and then dialyzed against the desired buffer before analysis.

Ultracentrifugal Studies

The experiments by the high-speed sedimentation equilibrium technique of Yphantis (14) were carried out at 20°C in a Spinco model E analytical ultracentrifuge equipped with Rayleigh interference optics using a 6-channel Epon high-strength epoxy centerpiece to allow for the simultaneous interferometric observation of three solution–solvent pairs. The solutions analyzed had a protein concentration of 0.01–0.04% in the following buffers: Tris–HCl pH 8.6; carbonate pH 10 and 11; borate pH 11.4. All buffers had an ionic strength of 0.1 and contained 0.1–0.5 M KCl. No evidence for peptide bond cleavage at pH 11.4 was obtained by comparative ninhydrin reaction of the two products at pH 8.6 and 11.4 and from the search of small molecular weight peptides by high voltage electrophoresis.

LDL_2 and s-LDL_2 were analyzed at 12,000 rpm for 24 hr; s-apo-LDL_2, at 25,000 rpm for 48 hr. Attainment of equilibrium was indicated by the constancy of the sedimentation pattern in serial photographs taken at 1-hr intervals. Fringe displacements were measured as a function of the radial position in the cell (r) by means of a Nikon two-dimensional microcomparator. Corrections for window distortion were made according to Yphantis (14). The values of molecular weight (M) were obtained from the relation (14):

$$M = \frac{2RT}{(1 - \bar{v}d)\omega^2} \cdot \frac{d \ln c}{d(r^2)}$$

where the term $d \ln c/dr^2$ was obtained from the slope of the $\ln y$ vs. r^2 plot. In this case of the succinylated product, the values were corrected for the number of succinyl functions estimated to be bound to the lysine groups and for the cations attached to the succinyl residues and uncompensated glutamyl and aspartyl residues (15).

Sedimentation velocity was determined for proteins in dilute solution in a 12 mm single sector cell by means of interference optics and analyzed according to Richards and Schachman (16). For concentration ranges between 1 and 5 mg/ml schlieren optics were used.

Determination of Partial Specific Volume, $\bar{v}$

For LDL_2 the value of 0.964 was the reciprocal of the hydrated density obtained from ηF versus ρ plots (17), where η and ρ were relative viscosity and density of the solvent, respectively, and F the flotation rate of the lipoprotein. The same value of $\bar{v}$ was assumed for s-LDL_2.

In the case of s-apo-LDL_2 the $\bar{v}$ value was calculated from the relation: $\bar{v} = 1/d - 1/c\,[(d - d_0)/d_0]$ where d and d_0 were the densities of solution and solvent, respectively, and c the protein concentration in g/100 ml. Density measurements were obtained by the use of a special jacketed density cell (Cahn Instrument Co., Paramount, Calif.) described by Elgert and Cammann (18), attached to a Cahn RG electrical microbalance provided with an automatic recorder. Determinations of $\bar{v}$ were also carried out according to Edelstein and Schachman (19) by differential sedimentation equilibrium of s-apo-LDL_2 in H_2O and D_2O using the linear portion of the $\ln y$ vs. r^2 plot. In the computation, a K value of 1.0155 (19) was assumed. This method offered the advantage of requiring protein concentrations of less than 1 mg/ml and permitted simultaneous determinations of v and molecular weight (19). As assessed from the study of known protein standards, this procedure proved the most reliable and was ultimately the only one employed for $\bar{v}$ determinations. The $\bar{v}$ for s-apo-LDL_2 was 0.716. The value for apo-LDL_2 could not be determined because of its lack of solubility in aqueous media. Its computation from amino acid analysis data (3) according to Schachman (20) gave a value of 0.728.

Circular Dichroism

These measurements in the spectral region between 300 and 185 mμ were carried out in a Cary model 6001 spectropolarimeter at 27°C with 0.1 mm pathlength cells (Pyrocel, Shoreham Mfg. Co., Inc., Winfield, N.J.) and protein concentrations of 1–2 mg/ml in phosphate buffer, pH 8.4, 0.1 M. The value of molar ellipticity $[\theta]$ was obtained from the relation: $[\theta] = \theta/10 \times (MRW/lc)$ where θ = observed ellipticity, MRW (mean residue weight) = 112, l = cell path length in cm, and c = concentration of solute in g/ml. The optical activity of the lipid moiety, measured in hexane, was found to be negligible and was not taken into account in the calculation of the molar ellipticity of the protein moiety.

Other Analyses

Protein was determined by the method of Lowry, Rosebrough, Farr, and Randall (21). In 2:1 chloroform–methanol extracts (22) we determined lipid phosphorus (23), total cholesterol (24), and triglycerides (25). Viscosity was measured at 20°C in a capillary Ostwald Folins viscometer as described previously (26).

RESULTS

Extent of Succinylation of Various LDL Products and Nature of the Reactive Sites

As indicated by the ninhydrin reaction, the extent of succinylation (about 90%) in LDL_2 was similar to that of delipidated apo-LDL_2 prepared according to Granda and Scanu (3). In the pH range employed (7.5–8.0), the extent of succinylation of either of the two products was not significantly changed when the succinic anhydride:lysine ratio was increased above 60:1, or when the succinylated product was resuccinylated after removal of lipids. Of the potential reactive sites, succinylation affected predominantly, but not exclusively, the ϵ-amino groups of lysine (Table 1). The possible reactivity of the quanidyl residues was not studied.

Effect of Succinylation on the Chemical Composition of LDL_2 (Table 2)

The composition of LDL_2 was not significantly affected by succinylation. The same was true for the products after delipidation.

Electrophoretic and Immunological Properties

As expected from the replacement of the positively-charged-NH_3^+ groups of lysine with negatively charged succinyl functions, the electrophoretic mobility of

TABLE 1 POTENTIAL ACTIVE SITES OF APO-LDL_2 FOR REACTION WITH SUCCINIC ANHYDRIDE AT pH 8

	Extent of Reaction
	%
N-terminal α-amino	100
Lysine ϵ-amino	92
Tyrosine	10
Serine, Threonine	10
Cystine	0
Guanidine	n.d.

n.d., not determined.

TABLE 2 CHEMICAL (PERCENTAGE) COMPOSITION OF LDL_2 AND APO-LDL_2 BEFORE AND AFTER SUCCINYLATION

Lipoprotein Class	Protein	Phospholipids	Cholesterol	Triglycerides
Before delipidation		%		
LDL_2	23.7	25.3	44.0	7.0
s-LDL_2	22.0	25.0	46.0	7.0
After delipidation				
Apo-LDL_2*	99	<1.0	—	—
s-Apo-LDL_2†	99	<1.0	—	—

* The material was prepared by delipidation in the presence of SDS (3).

† The material was prepared by delipidation of s-LDL_2.

s-LDL_2 in agarose media was markedly greater than that of LDL_2 (Fig. 1). The mobility of s-apo-LDL_2 was intermediate between those of LDL_2 and s-LDL_2. In contrast to LDL_2, both s-LDL_2 and s-apo-LDL_2 failed to react with rabbit antiserum to normal LDL.

Studies by Circular Dichroism (Fig. 2)

The ultraviolet and far-ultraviolet circular dichroism spectra of LDL_2 were indistinguishable from that of s-LDL_2. They were characterized by two negative Cotton effects at 222 and 208 mμ, a crossover at 200 mμ, and a positive Cotton effect at 194 mμ. The markedly different spectrum of s-apo-LDL_2 was characterized by a shift of the optically active electronic transitions toward lower wave lengths. The $n \rightarrow \pi^*$ transition was shallow, and deflection maxima of the $\pi \rightarrow \pi^*$ parallel and $\pi \rightarrow \pi^*$ perpendicular were at 205 and 192 mμ, respectively.

Ultracentrifugal and Viscosity Studies

LDL_2 and s-LDL_2 had the same flotation (S_f 6.4) property. Sedimentation equilibrium showed that they were monodisperse, as indicated by the linearity of the plot of ln y vs. r^2 (Fig. 3). Values near the meniscus were too low to be accurate. The molecular weight of the two products was 2.1–2.3 × 10^6 (Table 3). No corrections were made for the presence of succinyl residues in s-LDL_2. s-apo-

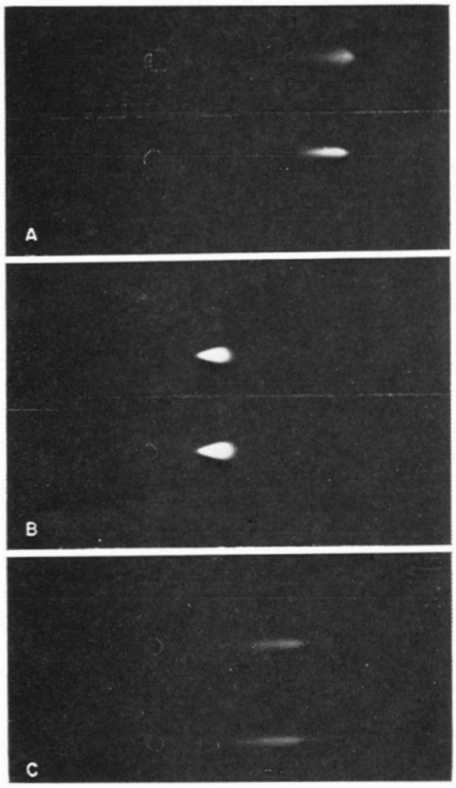

FIG. 1. Agarose electrophoresis of s-LDL_2 (A), LDL_2 (B), and s-apo-LDL_2 (C). Veronal buffer pH 8.6, 6 v/cm, 40 ma, 27°C, 1 hr. Staining: Amido schwartz.

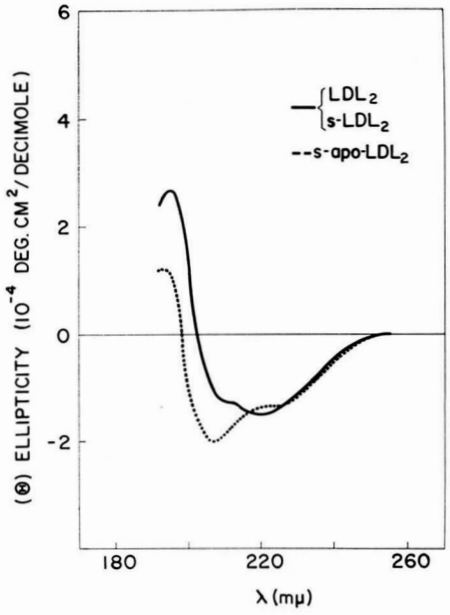

FIG. 2. Ultraviolet and far-ultraviolet circular dichroism spectra of LDL_2, s-LDL_2, and s-apo-LDL_2.

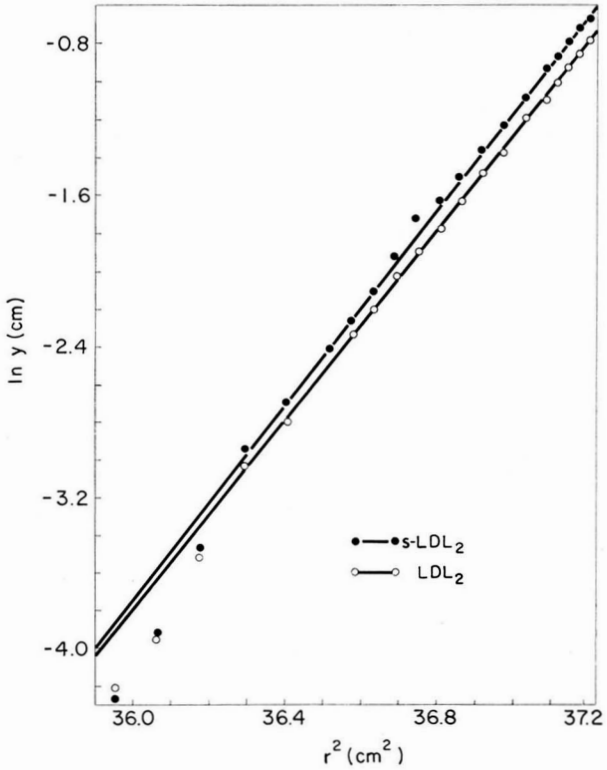

FIG. 3. Sedimentation equilibrium of LDL_2 and s-LDL_2. Logarithm of fringe displacement at equilibrium plotted against r^2.

LDL_2, obtained either by delipidation of s-LDL_2 or by succinylation of delipidated apo-LDL_2, was clearly heterogeneous. In the ultracentrifuge it exhibited a single sedimenting component ($S_{20,w}$ = 2.15) and formed a gel at the bottom of the liquid columns. By sedimentation

TABLE 3 VALUES OF MOLECULAR WEIGHT CALCULATED FROM DATA ON SEDIMENTATION EQUILIBRIUM

Materials	Buffer	Number of Observations*	Ranges of mol wt†
LDL_2	Tris, pH 8.6, 0.01 M	4	$2.2–2.3 \times 10^6$
s-LDL_2	Tris, pH 8.6, 0.01 M	4	$2.1–2.2 \times 10^6$
s-Apo-LDL_2‡	Carbonate, pH 10, 0.01 M	10	$3.60–3.80 \times 10^4$
s-Apo-LDL_2§	Carbonate, pH 10, 0.01 M	6	$3.68–3.80 \times 10^4$

* Separate analyses of different preparations.

† The data for s-LDL_2 were uncorrected for the presence of succinyl functions. Corrections were applied for s-apo-LDL_2 products. For details, see text.

‡ Prepared by delipidation of s-LDL_2

§ Prepared by succinylation of apo-LDL_2.

equilibrium, the ln y vs. r^2 plot of s-apo-LDL_2 indicated a heterologous system (Fig. 4, I). At pH 8.6, linearity was observed only in about 40% of the liquid column. At pH 11.6 the linearity increased up to 70%. In all the experiments, the molecular weight calculated from the slope of this linear component was $3.9–4.0 \times 10^4$. After correction (see Methods) the figure ranged between 3.6 and 3.8×10^4 (Table 3). Figures of molecular weight of the same order of magnitude were obtained with s-apo-LDL_2 after reduction and alkylation.

The intrinsic viscosity of s-LDL_2, 0.072 deciliters/g was markedly increased upon delipidation: s-apo-LDL_2 = 0.182. This latter value was significantly different from that for nonsuccinylated apo-LDL_2 prepared according to Granda and Scanu (3): 0.124.

Properties of s-Apo-LDL_2 after Filtration through Agarose Columns

Of the two fractions obtained, one (fraction I) was in the void volume; the other (fraction II) was retained. When after concentration, each of the fractions was filtered again through agarose, the two components were again observed, although in different relative proportions (Fig. 5). Fractions I and II had amino acid compositions similar to that of apo-LDL_2 (3), and the same *C*-terminal serine. None of the succinylated product had a detectable *N*-terminal amino acid, probably because of its replacement by a succinyl function. By ultracentrifugation, both fractions I and II showed a component with an $S_{20,w}$ value near 2.15. A gel at the bottom of the cell was clearly observed only in fraction I. In the sedimentation

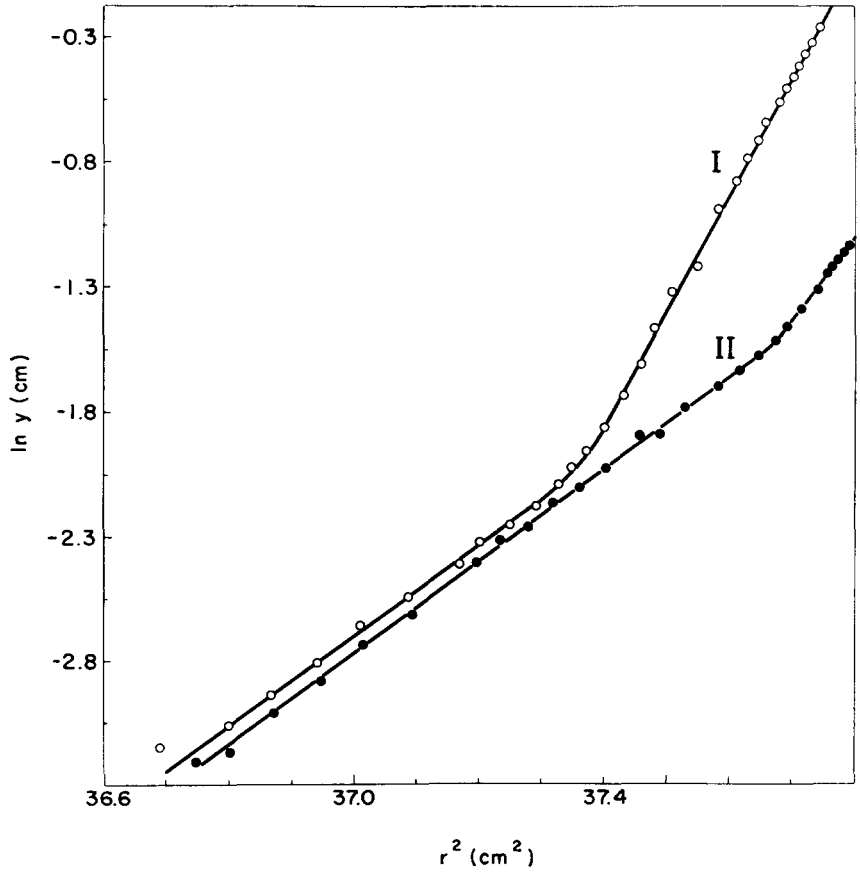

FIG. 4. Sedimentation equilibrium of s-apo-LDL_2 before (I) and after (II) gel filtration. Logarithm of fringe displacement at equilibrium plotted against r^2.

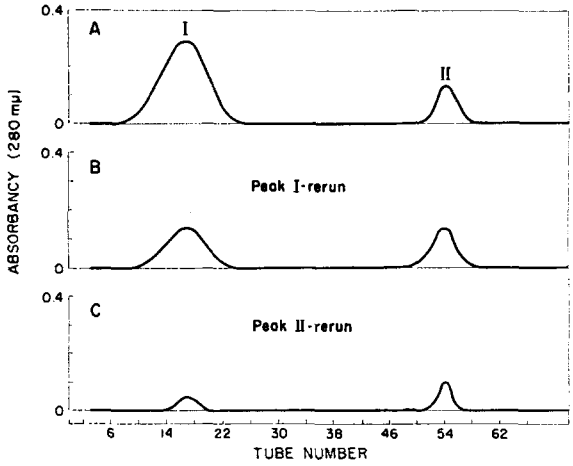

FIG. 5. Separation of s-apo-LDL_2 by gel filtration (agarose). Column dimensions: 40 x 2 cm; eluting buffer, Tris–HCl, pH 8.6, 0.01 M; flow rate, 0.4 ml/min; volume, 2 ml/tube.

equilibrium experiments fraction I exhibited a nonlinear $\ln y$ vs. r^2 plot. In turn, the plot of fraction II was largely linear (Fig. 4, II). The molecular weight estimated from this slope was $3.95–4.15 \times 10^4$, and after correction, $3.60–3.80 \times 10^4$.

DISCUSSION

The present findings indicate that if human serum LDL_2 (low density lipoprotein of d 1.019–1.063) is freed from its lipid complement by treatment with cold ethanol-ether, the resulting apoprotein, containing less than 1% lipid, becomes insoluble in aqueous buffers. On the other hand, if LDL_2 is treated with succinic anhydride before lipid extraction, the succinylated apoprotein, which also contains about 1% lipid, is soluble in water. By the procedure described the yield is approximately 95% of the initial lipoprotein protein. In a previous report (3) data were presented to suggest that apo-LDL_2 is made of apoprotein subunits probably held by lipid. The lack of solubility of apo-LDL_2 in aqueous media was considered to be due to the association of subunits favored by the removal of lipids. The results of the current studies appear to corroborate such a view. The solubility in water of the succinylated products can be attributed to the large percentage of the free amino groups that are accessible in LDL_2 to covalent modification by succinic anhydride (Table 1), so that when lipids are removed, each of the apoprotein subunits will have an increase in their net negative charge, a condition favoring their dissociation through electrostatic repulsions. Interestingly, s-apo-LDL_2 prepared by delipidation of s-LDL_2 was similar in behavior to the products prepared by direct succinylation of delipidated apo-LDL_2 solubilized in the presence of sodium dodecyl sulfate (3). Thus, in the preparation of water-soluble s-apo-LDL_2, the detergent may be omitted provided that succinylation precedes the step of lipid extraction; when protein aggregation was allowed to occur by omission of the detergent, succinylation of apo-LDL_2 produced solubilization of only 40–50% of the starting material. Another important conclusion from these studies is that most of the lysines in LDL_2 are probably at the surface of the molecule, in agreement with the findings by Margolis and Langdon (27).

The significant role of lipids in the overall makeup of LDL_2 is clearly indicated by the comparative studies on the hydrodynamic and optical properties of succinylated and nonsuccinylated LDL_2. In spite of the extensive chemical modification, s-LDL_2 had the same properties as LDL_2 in terms of ultracentrifugal behavior (flotation and sedimentation equilibrium) and the parameters of intrinsic viscosity and circular dichroism. This contrasted markedly with the differences noted when these products were studied after delipidation. In the latter instance, only s-apo-LDL_2 was found to be water-soluble and this product in turn appeared to be different in conformation (see data on circular dichroism and intrinsic viscosity) from s-LDL_2. Previous (28) and current results, together with very recent studies in this laboratory (manuscript in preparation) on the optical properties of s-LDL_2 and s-apo-LDL_2 under various conditions of solvent perturbation, seem to favor the hypothesis that the secondary and tertiary structure of the LDL_2 apoprotein, which according to present knowledge (29) may be considered as a mixture of α-helix, random chain, and perhaps some β-structure, is to a large extent stabilized by lipids. This is indicated by the failure of LDL_2 to undergo spectral changes, in contrast to the marked shifts noted under similar conditions with the delipidated product, apo-LDL_2.

Further support for a subunit structure of LDL_2 apoprotein is derived from current ultracentrifugal studies, in which definition of the molecular weight of apo-LDL_2 has been attempted through the high-speed sedimentation equilibrium technique of Yphantis (14). As indicated by the analysis of the fringe patterns obtained, dissociation of s-apo-LDL_2 was incomplete within the pH range of 8.6–11.4, although at pH 11.4 only about 70% of the preparation was monodisperse. These findings may be taken to indicate that the experimental conditions employed were insufficient to overcome all stabilizing noncovalent interactions among subunits, probably represented by residual electrostatic forces between guanidyl groups of arginine and carboxyl groups of aspartic–glutamic residues. In favor of this interpretation, and against the alternative possibility that apo-LDL_2 is made of subunits of different molecular weight, are the results of the chromatographic studies indicating that s-apo-LDL_2 aggregates readily and that

aggregated and dissociated forms have similar chemical compositions. If one assumes that apo-LDL_2 is made of subunits of homologous weight and takes the molecular weight figures of LDL_2 and s-apo-LDL_2 in Table 3 and the data on the chemical composition of LDL_2 in Table 2, one concludes that apo-LDL_2 (mol wt $\sim 5 \times 10^5$) has an average of 12 subunits (mol wt $\sim 4 \times 10^4$). However, this estimate may not be an accurate one, because of the number of assumptions to be made in the calculation of the molecular weight of s-apo-LDL_2 and the difficulty in obtaining an exact figure for its partial specific volume.

While this manuscript was in its final stage of preparation, a mol wt of 6.4×10^4 was reported by Shore and Shore (30) for the reduced and alkylated protein moiety of LDL of d 1.029–1.039 dissolved in SDS and urea. Calculations from the data on sedimentation equilibrium were carried out according to the method of Schachman and Edelstein (31) assuming in 8 M urea a rather large contribution of preferentially bound water (0.51 g of water per g of protein). Recently the validity of such a method has been seriously questioned on both theoretical and experimental grounds by Kirby Hade and Tanford (32). This and the experimental limitations encountered by Shore and Shore in obtaining exact values for the partial specific volume of LDL apoprotein in urea and water justify a reservation in accepting their molecular weight figure. It may be pointed out, although this is merely coincidental, that when no preferential interaction of the protein with water was assumed, Shore and Shore obtained a mol wt of 4.2×10^4, a figure in the range of that reported here for s-apo-LDL_2.

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