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### **Research Article**

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A new convenient method of preparation of soluble apoLDL, modified from a procedure previously described from this laboratory, is presented.

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# Evidence for the Identity of the Major Apoprotein in Low Density and Very Low Density Lipoproteins in Normal Subjects and Patients with Familial Hyperlipoproteinemia

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**ABSTRACT** The major apoprotein(s) from human plasma low density lipoproteins was isolated and compared with a major protein fraction (fraction I) from very low density lipoproteins (VLDL). Fraction I had been previously found to comprise approximately 40% of the total protein of VLDL. Fraction I from VLDL and apoLDL from normal subjects were indistinguishable in amino acid compositions and circular dichroic spectra. They yielded indistinguishable displacement curves of LDL-<sup>125</sup>I by radioimmunoassay and formed immunoprecipitin lines of complete identity. Fraction I from VLDL of normal subjects was compared with the fraction isolated from patients with familial types II, III, IV, and V hyperlipoproteinemia. There were no detectable differences between any of these fractions in amino acid compositions, circular dichroic spectra, and immunochemical properties. It was, therefore, concluded that short of peptide mapping or determination of amino acid sequences, fraction I from VLDL of each subject with familial hyperlipoproteinemia appears to be identical with fraction I and apoLDL from normal individuals.

A new convenient method of preparation of soluble apoLDL, modified from a procedure previously described from this laboratory, is presented.

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## INTRODUCTION

The major families of human plasma lipoproteins contain a number of proteins (apoproteins), some of which occur in more than one family. A compilation of current information about the apoproteins is shown in Table I. In terms of function in fat transport (11), one of the most important apoproteins appears to be apoLDL,<sup>1</sup> which may actually consist of more than one polypeptide (5, 9, 10). Although the bulk of apoLDL is found in low density lipoproteins (LDL) in the density range of 1.006–1.063 g/ml, evidence from qualitative immunoprecipitin reactions (7, 12, 13), electrophoresis (7, 12, 13), ultracentrifugation (12, 13), and gel filtration (7, 14) suggest the probable occurrence of apoLDL in the very low density lipoproteins (VLDL, d 0.950–1.006 g/ml). It was estimated from gel filtration experiments that apoLDL might comprise as much as 40% of the total apoprotein content of the VLDL (14). As such, it would represent the major apoprotein of VLDL, most of the remainder being divided among several smaller proteins (Table I). In a previous publication, the protein(s) in VLDL having immunochemical identity with apoLDL has been referred to as SF I (14); this fraction is here referred to as fraction I from VLDL.

In this communication we present chemical, optical, and further immunochemical studies of fraction I from

<sup>1</sup> *Abbreviations used in this paper:* apoLDL, the apoprotein of low density lipoprotein; CD, circular dichroic; HDL, high density lipoprotein; LDL, low density lipoprotein; m, mean residue rotation; MRW, mean residue weight; ORD, optical rotatory dispersion; VLDL, very low density lipoprotein;  $\theta$ , mean residue ellipticities.

TABLE I  
Nomenclature of the Plasma Apolipoproteins

Apolipoprotein	C-terminal system*	Alaupovic (1)	Scanu et al. (2)	Occurs in density range as major (M) or minor (m) constituent or is absent (a) or unknown (u)			
				Chylomicrons d < 0.95	VLDL d 95-1.006	LDL d 1.006-1.063	HDL d 1.063-1.21
ApoHDL-I	Gln‡ or Thr§	A	III	u	u	m	M
ApoHDL-II	Gln§	A	IV	u	u	u	M
ApoLDL	Ser	B	—	u	M	M	a
ApoVLDL-I	Ala	C	V	u	M	m	m
ApoVLDL-II	Ser¶	C	V	u	M	m	m
ApoVLDL-III	Glu	C	V	u	M	u	m

\* There is still uncertainty about the identification of several of these C-terminal amino acids as indicated in the following citations: ‡ Kostner and Alaupovic (3), § Shore and Shore (4, 5), and || Shore (6).

¶ Formerly thought to be C-terminal valine (7) but now believed to be serine (8). There is also a question of the occurrence of more than one polypeptide chain in apoLDL (5, 9, 10).

VLDL and apoLDL from low density lipoproteins. Fraction I, or apoLDL, present in both VLDL and LDL in plasma from normal subjects and patients representing familial types II, III, IV, and V hyperlipoproteinemia have been compared. The data indicate that short of peptide mapping or determination of amino acid sequence, fraction I from the VLDL of each subject appears to be identical with fraction I and apoLDL from normals. One of the great difficulties in the study of apoLDL is the insolubility of the protein in water when its complement of lipid is removed. We also present here a new convenient way to prepare soluble apoLDL, based on a modification of a previous method described from this laboratory (15).

## METHODS

### Patient selection

Lipid and lipoprotein analyses were performed on fasting samples. "Normal" subjects had normal lipoprotein patterns on paper electrophoresis and concentrations of plasma lipoproteins (adjusted for age and sex) that did not exceed the upper 5% cutoff (16). Plasma was also obtained from four adult patients, one with each of familial types II, III, IV, and V hyperlipoproteinemia. The diagnoses were based on the following: (a) a qualitative assessment of the paper electrophoretic pattern of plasma and of the fractions of density <1.006 and >1.006 g/ml, (b) quantitative measurements of the plasma lipoproteins by cholesterol determination in conjunction with heparin-manganese precipitation and ultracentrifugation (17), (c) exclusion of potential secondary disorders which might produce hyperlipoproteinemia, including hypothyroidism, diabetes mellitus, alcoholism, pancreatitis, dysglobulinemia, nephrosis, and renal disease, and (d) family screening. All but the patient with type III also had at least one first degree relative with the same abnormal lipoprotein pattern.

### Materials

Recrystallized sodium decyl sulfate was purchased from Mann Research Laboratories, Inc., New York and was

>99% pure by thin-layer chromatographic analysis. Diethyl ether was analytical reagent grade. All other chemicals were of the highest purity commercially available.

### Preparation of VLDL and LDL

All the plasma used for this study was obtained by plasmapheresis from subjects after a 12-14 hr fast. Plasma was collected in 0.01% EDTA, pH 7.0, and stored at 4°C unless otherwise indicated. Fractionation was begun within 2 wk of collection. The VLDL were isolated ultracentrifugally as previously described (7). LDL were isolated by ultracentrifugal flotation (18), usually between densities 1.019 and 1.063 g/ml. For certain comparisons indicated in the text the more narrow range of 1.025-1.050 g/ml was employed. Each preparation of VLDL or LDL was washed two to three times by layering a salt solution of the appropriate density over the lipoproteins and recentrifuging. Before delipidation, VLDL preparations formed precipitin lines with antisera to whole plasma or LDL but not with anti-high density lipoproteins (HDL). All LDL preparations were tested with rabbit antisera to whole plasma, LDL, HDL, and albumin. They reacted only with antisera to LDL.

### Delipidation and preparation of soluble apoproteins

**VLDL.** VLDL were delipidated as earlier described (7) except for the omission of insoluble potato starch. The VLDL apoproteins were solubilized in 200 mM sodium tris-HCl buffer, pH 8.2, containing 100 mM sodium decyl sulfate (7), chromatographed on Sephadex G-150 (14), and eluted with 200 mM sodium tris-HCl, pH 8.2, containing 2 mM sodium decyl sulfate. The initial protein fraction eluted from the column, previously designated SF I (14), is here referred to as fraction I from VLDL. This fraction was concentrated by ultrafiltration and dialyzed against the tris buffer containing either 2 or 0.5 mM sodium decyl sulfate. These preparations were stored at protein concentrations of 0.5-2.0 mg/ml at 4°C and were used for chemical, optical, and immunological studies within 1-7 days.

**LDL.** The following procedure reproducibly allowed recovery of >95% of the total protein of LDL (apoLDL) in a soluble form. LDL was dialyzed for 24 hr against 0.01%

sodium EDTA, pH 7.0, and then lyophilized. 5 mg of the lyophilized lipoprotein were extracted in 50 ml, conical centrifuge tubes with diethyl ether:ethanol (3:1). The tube was filled to within about 0.5 ml of the top, stoppered, and shaken vigorously with a mechanical shaker for 12 hr at 4°C. After centrifugation, the solvent was removed by decanting, fresh solvent was added and the extraction was repeated for a further 12 hr. The residue was dried at 4°C under nitrogen and was completely solubilized by incubation at 4°C for 4 hr in 130 mM sodium tris-HCl, pH 8.2 containing 100 mM sodium decyl sulfate and 0.1% EDTA. The solubilized apoprotein did not sediment when centrifuged at 10,000 *g* for 30 min. After solubilization, the concentration of detergent was reduced by dialysis to 0.5–2.0 mM without loss of solubility. At concentrations of detergents <0.5 mM, visible aggregation occurred within a period of several hours. This aggregation was immediately reversed by increasing the concentration of sodium decyl sulfate.

### Gel filtration

Sephadex gels were equilibrated for 48 hr at room temperature with the buffer to be used for elution. The gel was poured into glass columns, 2.5 × 150 cm, with stirring and equilibrated another 24 hr with the solvent used for eluting. A protein load of 5–10 mg was applied in the eluting solvent. Columns were developed at 25°C and the effluent monitored for absorbancy at 280 mμ, protein content, and immunochemical reactivity against specific rabbit and sheep antisera for apoLDL. The void volume was determined with dextran blue. Ovalbumin (mol wt, 37,000), serum albumin (mol wt, 67,000), and α-globulin (mol wt, 116,000) were used to calibrate the column. We noted, as have others,<sup>9</sup> that Sephadex gels exhibited a significant reduction in exclusion limits in the presence of relatively high concentrations; i.e., > 20 mM, of sodium decyl or dodecyl sulfate.

### Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was performed in 8 M urea at pH 9.4 or 2.8 (19). Gels were prepared with 10% acrylamide and were stained with 0.05% coomassie blue.

### Chemical analyses

Apoproteins were extracted with chloroform-methanol (2:1, v/v) for measurements of triglyceride (20), cholesterol (21), and phospholipid (22), and for analysis by thin-layer chromatography with both polar and nonpolar solvents (23). Protein was determined by the method of Lowry, Rosebrough, Farr, and Randell (24), sodium decyl sulfate by a procedure previously used for sodium dodecyl sulfate (25) and total reducing sugar according to Clamp, Dawson, and Hough (26).

### Amino acid analyses

Apoprotein samples were dried at room temperature over P<sub>2</sub>O<sub>5</sub> in an evacuated desiccator. After adding oxygen-free, constant-boiling (5.7 N) HCl, the samples were placed in a desiccator, and alternately flushed and evacuated (27) with oxygen-free nitrogen at least six times before hydrolysis for 22 hr at 110°C. After hydrolysis, the samples were lyophilized, dissolved in 0.2 N citrate buffer, pH 2.2, and analyzed on a Beckman model 120B amino acid analyzer (Beckman Instruments, Inc., Fullerton, Calif.) by the

<sup>9</sup> Eugene P. Kennedy, personal communication.

method of Moore, Spackman, and Stein (28) with an accelerated system (29). Each analysis was performed in triplicate. Cysteic acid was measured in duplicate analyses on a sample subjected to oxidation with performic acid before acid hydrolysis (30).

### Circular dichroism

Circular dichroic (CD) spectra were measured in 50 mM phosphate buffer, pH 8.2, (containing 0.01% EDTA) at two protein concentrations (0.1–1.0 mg/ml), and at two light paths (0.1 and 0.5 mm). The temperature was 24 ± 1°C. Calibrations were done with D-10-camphorsulfonic acid. All measurements were made in duplicate and were reproducible within 3% to 210 mμ. After each run, a blank was obtained by repeating the measurements with the omission of the protein. The blank contained the buffer and sodium decyl sulfate at the same concentration present in the apoprotein solution. The ellipticity angle,  $\theta^\circ$ , was measured directly with the Cary 60 Spectropolarimeter (Cary Instruments, Monrovia, Calif.), model 6001 CD accessory. The mean residue ellipticity,  $[\theta]$ , in degrees square centimeters/decimole was calculated from

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

where MRW is the mean residue weight (115 from the amino acid composition), *l* the optical path of the cell in centimeters and *c* the concentration of protein in grams/milliliter. Optical rotatory dispersion (ORD) measurements in the far ultraviolet range were made under similar conditions and were used to calculate the mean residue rotation,  $[m]$ , in degrees square centimeters/decimole.

### Immunological techniques

Double diffusion experiments were performed with plates made with 1% agarose (Marine Colloids, Inc., Springfield, N. J.) in 150 mM NaCl. Plates were developed at 4°C and were observed for 7 days. The immunoelectrophoretic techniques used have been previously described (31, 32). Rabbit antisera to LDL or apoLDL were produced by two injections into the foot pads of white New Zealand rabbits of 1.25 ml of protein solution (6–8 mg of protein) mixed with 0.75 ml of complete Freund's adjuvant. Sheep antisera to apoLDL were prepared by subcutaneous injection of 1.5 ml of antigen (7.5 mg protein), mixed with 1.5 ml of complete Freund's adjuvant, in each quarter of a female Chevoit sheep. After 22 days, the series of injections was repeated. 11 days later, the antiserum was harvested by intracardiac puncture and was collected in 0.01% EDTA, buffered at pH 7.0. These antisera reacted strongly with LDL and apoLDL. The sheep but not the rabbit antisera also reacted very faintly with albumin. The anti-albumin activity was easily removed by absorption with albumin. The antisera did not react with HDL or other plasma proteins.

For quantitative comparisons a specific radioimmunoassay was developed, which will be described in detail in another communication.<sup>8</sup> Sheep antibodies prepared against apoLDL were precipitated with sodium sulfate, dialyzed against 150 mM NaCl and bound to bromoacetylcellulose

<sup>8</sup> Birnbaumer, M. E., R. I. Levy, and A. M. Gotto. Development of a specific radioimmunoassay for the protein moiety of human low density lipoproteins. In preparation.

(Pentex Biochemical, Kankakee, Ill.) as described by Jagendorf, Patchornik, and Sela (33). LDL were isolated ultracentrifugally between densities 1.025 and 1.050 g/ml and labeled with  $^{125}\text{I}$  by the iodine monochloride procedure (34). The bound antibody could remove from solution >95% of added LDL- $^{125}\text{I}$ . More than 90% of the antibody-bound LDL- $^{125}\text{I}$  could be displaced by cold LDL. The assay was run, and all dilutions were made, with 50 mM sodium borate buffer, pH 8.5, containing 3% human albumin. In each experiment, the ability of antigen to displace LDL- $^{125}\text{I}$  was compared with the displacement by cold LDL. After centrifugation, the supernatant solution was removed. The precipitates (containing bound tracer) were counted. These data were used to calculate  $\ln ([B/\text{Bo}]/[1-(B/\text{Bo})])$ , where Bo are the number of counts per minute bound to the antibody in the absence of cold antigen and B are the number of counts per minute bound to the antibody in the presence of cold antigen. Values for  $\ln ([B/\text{Bo}]/[1-(B/\text{Bo})])$  were plotted against the logarithm of the quantity of cold antigen added (in nanograms of protein).

## RESULTS

*Solubilization of apoLDL; comparison of solubility and aggregation with fraction I from VLDL.* Reproducible solubilization of apoLDL was greatly facilitated by dialysis against a solution of low ionic strength (0.01% disodium EDTA, pH 7.0) followed by lyophilization before delipidation. These two steps were critical and represented a modification of a procedure we previously reported for the preparation of soluble apoLDL (15). With the current technique, incubation of delipidated LDL for 4 hr at 4°C with 100 mM sodium decyl sulfate resulted in complete solubilization. The apoprotein retained its solubility when the concentration of detergent was reduced by dialysis to 0.5 mM. There was visible turbidity if the concentration was further reduced. Although the apoprotein was in an aggregated state in 0.5 mM sodium decyl sulfate, the particle size was sufficiently small to permit passage through a 0.1  $\mu$  millipore filter.

Fraction I from VLDL exhibited similar characteristics of solubility. It was not significantly soluble in aqueous buffers between pH 5 and 9 in the absence of detergent, was readily solubilized by 100 mM sodium decyl sulfate at 4°C and maintained its solubility when the concentration of detergent was reduced to the same extent as with apoLDL.

When subjected to chromatography on Sephadex G-100, G-150, or G-200 in 0.5, 2, or 100 mM sodium decyl sulfate, both apoLDL and fraction I eluted at the void volume of the column in an apparently highly aggregated state.

*Composition of apoLDL and fraction I from VLDL.* ApoLDL and fraction I from VLDL contained 94–96% protein by dry weight from Lowry determinations and total recovery after amino acid analysis. The remainder was phospholipid (0.9–1.5%), sodium decyl sulfate

(0.1–0.5% as determined by colorimetric analysis [25] after exhaustive dialysis of the apoprotein against water) and carbohydrate (approximately 3% in apoLDL based on content of reducing sugar, not determined in fraction I). The phospholipids were predominantly phosphatidyl choline and phosphatidyl ethanolamine as determined qualitatively by thin-layer chromatography. ApoLDL and fraction I from VLDL contained less than 1% each of cholesterol, cholesteryl esters, or triglycerides as determined by chemical analysis or thin-layer chromatography. Small quantities of the other major apoproteins of VLDL were found in LDL isolated between densities 1.019–1.063 g/ml or in fraction I corroborating the findings of Lee and Alaupovic with apoLDL (10). These apoproteins were identified by polyacrylamide gel electrophoresis and by immunoprecipitation lines in double diffusion experiments. A semiquantitative estimate of these other VLDL-apoproteins was obtained by serially diluting apoLDL and determining the maximal dilution at which the proteins could be immunochemically detected. By comparisons with standard solutions of the VLDL apoproteins, designated as “C” proteins, it was estimated that they comprised <5% of the protein isolated from the lipoproteins of density 1.019–1.063 g/ml. The proportion of these proteins was greatly reduced by restricting LDL preparations to the density range of 1.025–1.050 g/ml. As judged by polyacrylamide gel electrophoresis, the “C” proteins were completely removed by chromatography of apoLDL or by rechromatography of fraction I on Sephadex G-150 in 2 mM sodium decyl sulfate.

*Amino acid composition of apoLDL and fraction I from VLDL of normals and patients with types II to V hyperlipoproteinemia.* The amino acid composition of three preparations of apoLDL were compared with values obtained from other laboratories (Table II). Whereas there was some variation from one laboratory to another, the over-all agreement was quite good between this study and earlier ones. Similarities between the over-all amino acid compositions of apoVLDL and apoLDL have been previously noted (12, 36). The amino acid composition of apoLDL was virtually indistinguishable from that of fraction I from four normal subjects (subjects A, B, and a pool of C and D, Table III) and from the average composition of all fractions I from both normal and hyperlipoproteinemic subjects (Table III). In order to eliminate possible contributions of the “C” proteins, amino acid analyses were performed on preparations purified by chromatography or rechromatography on Sephadex G-150 as described in Methods. Again the composition of apoLDL and of fraction I were within experimental error (Table IV). It is not known whether the small decrease in content of glutamic acid in the purified preparations was

TABLE II  
Amino Acid Composition of apoLDL

Amino acid	Present study	Margolis and Langdon (35)	Levy et al. (36)	Granda and Scanu (12)	Pinon and Laudat (37)
<i>moles 100,000 g protein*</i>					
Asp	96	97	94	95	97
Thr	59	58	57	57	58
Ser	78	74	76	76	76
Glu	109	113	106	102	110
Pro	42	34	37	29	34
Gly	43	44	43	38	43
Ala	56	56	54	54	56
Val	45	56	48	43	48
Met	15	17	11	12	14
Ile	48	56	50	46	51
Leu	107	109	98	98	98
Tyr	30	27	29	30	30
Phe	45	48	45	42	49
Lys	71	61	73	70	72
His	18	23	22	18	20
Arg	27	29	31	25	30
Half Cys	7	6	6	6	—

\* Rounded to the nearest integer.

of significance. The differences between the composition of fraction I from normal subjects and from individual patients with types II, III, IV, and V hyper-

TABLE III  
Comparison of Amino Acid Composition of apoLDL and Fraction I from VLDL of Normal Subjects

Amino acid	ApoLDL	Fraction I			Pool of subjects C and D
		All subjects‡	Subject A	Subject B	
		<i>moles/100,000 g protein*</i>			
Asp	95.9±0.4	95.8±1.5	97.2±1.2	93.4±3.4	96.1±2.3
Thr	58.9±0.2	58.9±0.5	59.4±0.7	58.3±0.7	58.9±0.6
Ser	78.5±0.2	79.5±1.0	79.2±1.0	79.9±1.8	79.3±0.6
Glu	108.9±0.7	110.3±1.1	110.4±0.6	110.4±2.3	110.7±0.6
Pro	41.9±0.5	41.0±1.5	41.8±0.9	43.4±4.6	41.9±1.5
Gly	43.5±0.3	45.7±1.2	44.6±0.4	47.0±0.5	46.4±1.6
Ala	55.7±0.4	58.6±1.5	57.0±0.4	60.1±0.4	58.4±1.6
Val	45.5±0.3	45.5±0.4	45.1±0.5	45.3±0.5	45.2±0.1
Met	14.9±0.1	15.2±0.5	14.9±0.4	14.3±0.3	14.7±0.4
Ile	48.2±0.2	46.1±1.1	46.7±0.6	44.9±0.3	46.3±1.2
Leu	106.9±0.3	105.9±0.6	106.5±0.5	106.0±0.4	105.8±0.9
Tyr	29.7±0.6	29.7±0.7	30.2±0.7	28.7±1.4	29.4±0.8
Phe	44.5±0.2	44.0±0.7	44.1±0.6	43.4±1.9	44.3±0.4
Lys	71.3±0.3	69.4±0.9	69.9±2.5	69.9±1.6	69.3±1.0
Hist	18.5±0.3	18.1±0.4	17.8±1.0	17.6±0.7	17.9±0.4
Arg	26.9±0.3	29.1±0.9	28.0±0.5	30.3±1.2	28.9±1.2
Half Cys§	7.4 —	— —	— —	— —	7.9 —

\* ±SD of triplicate analyses.

‡ Includes values from both normal subjects (this table) and hyperlipoproteinemic patients from Table V.

§ Mean of duplicate analyses from performic acid oxidized protein.

TABLE IV  
Comparison of Amino Acid Composition of Chromatographically Purified apoLDL and Fraction I from VLDL of Normal Subjects\*

Amino acid	ApoLDL	Fraction I from VLDL
<i>moles/100,000 g protein‡</i>		
Asp	96.2	96.1
Thr	59.6	64.2
Ser	77.0	76.7
Glu	94.9	97.0
Pro	37.2	35.8
Gly	48.8	46.6
Ala	61.5	58.4
Val	49.9	46.3
Met	15.6	14.4
Ile	44.1	47.6
Leu	108.2	111.8
Tyr	28.9	29.9
Phe	43.0	46.6
Lys	70.9	70.9
Hist	19.2	21.7
Arg	27.8	27.8

\* ApoLDL was purified by chromatography and fraction I from VLDL by rechromatography on Sephadex G-150 as described in the text. The small "C" proteins were undetectable on polyacrylamide gel electrophoresis.

‡ Mean of duplicate analyses.

lipoproteinemia were small (Table V) and probably of no significance in view of the standard deviations of the original determinations (Table III). In a previous study (37), no differences were found in the

TABLE V  
Comparison of the Amino Acid Composition of Fraction I from the VLDL of Normal and Hyperlipoproteinemic Subjects

Amino acid	Normals (mean from subjects of Table III)	Type II	Type III	Type IV	Type V
<i>moles/100,000 g protein*</i>					
Asp	95.6±2.0	94.7±0.3	96.6±0.4	96.4±1.1	94.9±0.5
Thr	58.9±0.6	58.7±0.4	59.3±0.2	59.0±0.7	58.2±0.2
Ser	79.5±0.4	78.3±0.3	81.5±1.5	79.6±0.5	78.9±0.2
Glu	110.5±0.2	110.8±2.8	108.5±0.8	109. ±0.3	111.4±0.4
Pro	42.4±0.9	38.4±0.4	40.8±0.3	40.6±0.6	41.5±0.5
Gly	46.0±1.2	44.9±0.4	45.7±1.2	45.4±0.3	44.6±0.1
Ala	58.5±1.6	59.8±0.3	58.2±0.4	57.6±0.4	59.4±0.9
Val	45.2±0.1	46.3±0.3	45.8±0.3	45.8±0.3	45.4±0.8
Met	14.6±0.3	16.0±0.3	15.2±0.2	15.6±0.4	15.4±0.2
Ile	46.0±0.9	45.4±0.9	46.8±0.6	46.2±0.8	45.5±0.3
Leu	106.1±0.4	106.2±0.2	105.7±0.6	105.8±0.6	106.5±1.5
Tyr	29.4±0.8	30.0±0.4	30.3±0.8	30.3±0.6	29.8±1.0
Phe	43.9±0.5	42.7±0.3	44.5±0.8	44.5±1.4	44.8±1.8
Lys	69.7±0.4	68.7±1.4	69.8±1.1	69.1±0.8	70.1±1.3
Hist	17.8±0.2	18.1±0.5	17.9±0.3	18.7±0.9	18.0±0.6
Arg	29.1±1.2	29.8±1.9	28.3±0.4	29.4±0.5	29.6±0.4

\* ±SD.

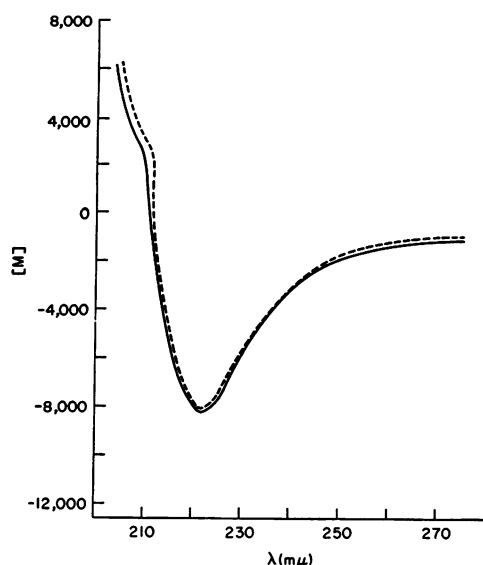


FIGURE 1 Optical rotatory dispersion curves of apoLDL (solid line) and of fraction I from VLDL (dashed line).

amino acid composition of apoLDL from normal and type II hyperlipoproteinemic subjects.

*Optical rotatory dispersion (ORD) and circular dichroism (CD) of apoLDL and fraction I from VLDL.* The ORD curves of apoLDL and fraction I from VLDL did not significantly differ in the far ultraviolet range (Fig. 1). Respective values of  $[M]$  were  $-8100 \pm 300$  and  $-8000 \pm 450$ . In its CD spectrum, apoLDL exhibited a negative trough at 216–218  $m\mu$ , similar to previous studies (38), with  $[\theta]_{217.5}$  of  $-11,503 \pm 625$  (Fig. 2, Table VI). A slight shoulder was noted at 208–210  $m\mu$ , also consistent with earlier reports (38). In the far ultraviolet range, the CD spectra of apoLDL and of fraction I from VLDL were virtually indistinguishable, (Fig. 2, Table VI). Purification by chro-

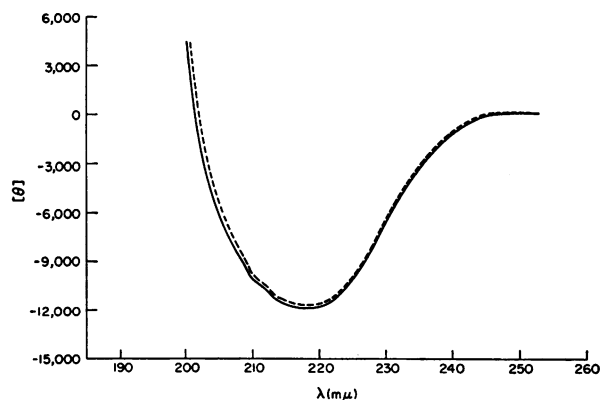


FIGURE 2 Circular dichroic spectra of apoLDL (solid line) and a fraction I from VLDL (dashed line).

TABLE VI  
Comparison of Mean Residue Ellipticities  $[\theta]$  of apoLDL and Fraction I from Normal and Hyperlipoproteinemic Subjects

Protein source*	$[\theta]$ in deg $cm^2/dmole$ ( $\pm$ SD or mean of duplicate analyses)
apoLDL A	$-11,503 \pm 625$
apoLDL B†	$-12,188$
Subject A (normal)	$-10,478$
Subject B (normal)	$-10,350$
Subjects C and D pool (normals)	$-11,883$
Subject E‡	$-11,555$
Type II	$-10,626$
Type III	$-10,542$
Type IV	$-10,494$
Type V	$-10,373$
All subjects, normals and hyperlipoproteinemic	$-10,999 \pm 703$

\* All are fractions I from VLDL except for apoLDL.

† Purified by chromatography on Sephadex G-150 as described in the text.

‡ Purified by rechromatography on Sephadex G-150 as described in the text.

matography or rechromatography on Sephadex G-150 did not alter the CD in the far ultraviolet range. Within the limits of experimental error and variability, there were no significant differences in the CD spectra of fraction I from the VLDL of normal subjects or patients with types II, III, IV, and V hyperlipoproteinemia.

*Immunological properties of apoLDL; comparison with fraction I from the VLDL of normal and hyperlipoproteinemic subjects.* Antisera prepared against LDL reacted with LDL and apoLDL but formed precipitin lines of only partial identity between them (Fig. 3). In contrast, antisera prepared to apoLDL gave precipitin lines of complete identity between LDL and the apoprotein. Antibodies to either LDL or apoLDL yielded lines of complete identity between apoLDL and

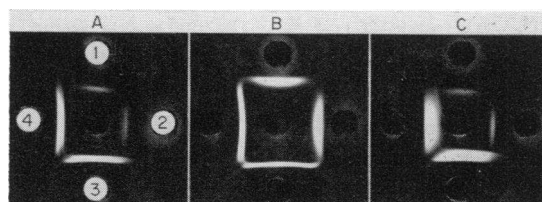


FIGURE 3 Immunodiffusion patterns of LDL and apoLDL. Center wells contained rabbit antisera to LDL (A and C) or to apoLDL (B). Outer wells 1 and 2 contained apoLDL and 3 and 4 contained native LDL. The volume of antigen solution used was 10  $\mu$ l/well and the protein concentration was 4 mg/ml.

fraction I from the VLDL of normal subjects or patients with types II, III, IV, or V hyperlipoproteinemia (Fig. 4).

These qualitative comparisons between LDL, apoLDL, and fraction I from VLDL were complemented by the use of a specific radioimmunoassay. Quantitative differences between the ability of LDL and apoLDL to displace LDL- $^{125}$ I from the antigen-antibody complexes were demonstrated. The displacement curves of apoLDL and fraction I from VLDL were indistinguishable (Fig. 5).

## DISCUSSION

The comparison of the properties of apoLDL and of fraction I of VLDL was greatly facilitated by the procedure for solubilization used. It represents a modification of a procedure earlier reported from this laboratory (15). The critical new points in the present methodology were the dialysis against EDTA solutions of low ionic strength and the lyophilization before delipida-

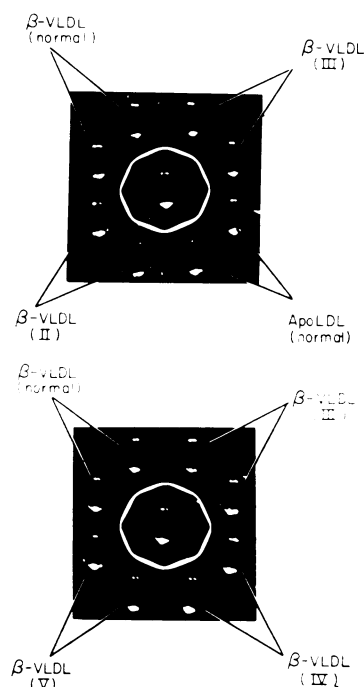


FIGURE 4 Immunodiffusion patterns of apoLDL and of fraction I from VLDL from normal subjects and from patients with familial hyperlipoproteinemia. In each panel, the center well contained rabbit antisera to apoLDL. Fraction I from VLDL (indicated as  $\beta$ -VLDL) or apoLDL was placed in the outer wells as indicated on the figure. 10  $\mu$ l of each were used at a protein concentration of 4 mg/ml. The source of each apoprotein is indicated on the figure as either normal (from a normal subject) or by II through V (referring to a particular type of familial hyperlipoproteinemia).

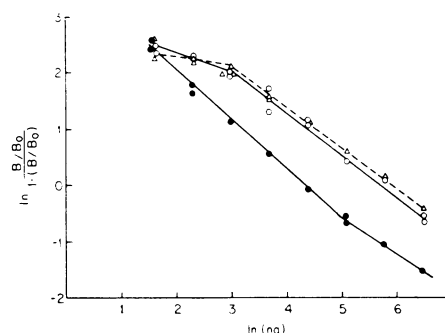


FIGURE 5 Radioimmunoassay of LDL ( $\bullet$ — $\bullet$ ), apoLDL ( $\circ$ — $\circ$ ), and a fraction I from VLDL ( $\Delta$ — $\Delta$ ). These curves show the displacement of LDL- $^{125}$ I from sheep anti-apoLDL serum by cold LDL, apoLDL, and fraction I from VLDL. The results are plotted as  $\ln \{ [B/Bo] / [1 - (B/Bo)] \}$  vs. the logarithm of the quantity of the cold antigen added (in nanograms protein), where  $Bo$  are the counts per minute bound to the antibody in the absence of cold antigen and  $B$  are the counts per minute bound to the antibody in the presence of cold antigen.

tion. It has recently been shown that aggregation of LDL is minimized by low ionic strength, neutral pH, and by the presence of EDTA (39). Lyophilization under such conditions might be expected to decrease protein-protein interactions. Similarly, removal of water by the lyophilization procedure might weaken intrachain and interchain hydrogen bonding of the apoprotein and result in an enhanced interaction between the protein and the detergent. Lyophilization of LDL before delipidation has also been used by Rudman, Garcia, Abell, and Akgun (40) in the preparation of apoLDL but these workers also required treatment with trypsin in order to achieve solubilization. ApoLDL was rapidly and reproducibly solubilized with the procedure herein described.

After reduction of the concentration of sodium decyl sulfate to 0.5 mM, the protein exhibited many of the optical and immunological characteristics of the native lipoprotein. Early attempts to remove the lipid from LDL usually resulted in the formation of an insoluble gel (41). Successful solubilization of lipid-free preparations have involved the use of ionic (12, 15, 42, 43) and nonionic (9) detergents, urea (42, 43), guanidine (9), alkalization in the presence of urea (44), chemical modification by succinylation (45–47), or maleylation (9), or reduction with alkylation, and of careful phase extraction so as to avoid precipitation of the protein at any stage of delipidation (5).

Each method has its relative advantages and drawbacks. It should be emphasized that none of the methods currently available for the solubilization and dispersion of apoLDL are able to achieve a satisfactory dissociation of the protein into its monomeric subunits.



The method described in this manuscript results in a soluble preparation of apoLDL which contains a small residue of bound detergent. We have earlier shown that high concentrations of sodium decyl sulfate can displace the lipid from LDL (48). While high concentrations of sodium decyl sulfate have been shown to produce large changes in the CD spectrum of apoLDL, these changes were reversed when the concentration of detergent was reduced to 0.5 mM (38). The effect of the small residue of detergent on the immunological and conformational properties of apoLDL cannot now be completely assessed. The detergent solubilized apoprotein also exhibited a CD spectrum similar to the native lipoprotein but in which the quantity of the beta structure apparently had been reduced (38). Scanu, Pollard, Hirz, and Kothary (44) have shown that apoLDL solubilized by alkali and urea in the absence of detergent has a CD spectrum very similar to, although not identical with, native LDL. Detergents have been found to produce varied conformational effects in different proteins, including an increase in  $\alpha$ -helical content (49), a conversion of helical to beta structure (50), or an increase in random coil (51). The findings in this manuscript are similar to those of Corry and Stone (52) who solubilized the stromal proteins from red blood cells in 1% sodium dodecyl sulfate and performed immunological studies after dialysis to reduce the concentration of detergent. It was concluded by these workers that the stromal proteins were completely solubilized, and were not denatured by this treatment. Immunological integrity was judged to be intact.

On the basis of solubilization, amino acid composition, circular dichroism, and immunological reactions, the major apoproteins of LDL and fraction I of VLDL apoproteins appear to be identical, corroborating conclusions from earlier investigations (7, 12-14). This finding provides a rational basis for the absence of VLDL as well as LDL in the inherited disease of abetalipoproteinemia (53). It has recently been shown that although apoLDL is absent from plasma in this disorder, the other major apoproteins of VLDL are present in plasma, presumably as part of the HDL complex (11). Thus, there is strong evidence of structural, genetic, and functional linkage between apoLDL and fraction I of VLDL, likely through the catabolism of VLDL to LDL.

There was no evidence found to indicate a protein structural defect in the fraction I from the VLDL of the subjects studied with types II, III, IV, and V hyperlipoproteinemia. Subtle differences, such as those involving the substitution of one or more amino acid residues, were not necessarily excluded by the present study. There is also no proof that all patients with any

of the types of familial hyperlipoproteinemia now recognizable represent genetically homogeneous disorders. Hence abnormalities in apoLDL, in either or both LDL and VLDL, may later be discovered in some patients.

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