Fractionation of Human Serum High Density Lipoprotein in Urea Solutions. Evidence for Polypeptide Heterogeneity*

A. Scanu,† J. Toth, C. Edelstein,‡ S. Koga,§ and E. Stiller||

ABSTRACT: The lipid-free protein (apo-HDL_2) of immunochemically pure human serum HDL_2 (d 1.063–1.125) was separated by Sephadex G-200 chromatography in 8 M urea into four components: I, III, IV, and V with a per cent weight distribution of 5, 65, 22, and 8, and an apparent molecular weight of (10^3) 52.0–55.0, 25.8–28.0, 16.4–17.6, and 11.2–11.8, respectively (calibrated Sephadex columns, and disc gel electrophoresis in sodium dodecyl sulfate). Equilibrium ultracentrifugation analysis corroborated the molecular weight data obtained by chromatography and electrophoresis. However, under appropriate conditions of speed and protein concentration, III exhibited a smaller molecular weight component of approximately 17,000. Fraction II was minor and only occasionally seen. Fraction I appeared as an aggregate of the other components. Fractions III, IV, and V differed in immunological and spectral (circular dichroism and ultraviolet absorption spectroscopy) properties, electrophoretic mobility (disc gel electrophoresis), and amino acid composition.

These three fractions all showed heterogeneity by disc gel electrofocusing. The results suggest that apo-HDL_2 contains three distinct classes of polypeptides which are totally (IV and V) or partially (III) dissociated in urea and are probably made up of subcomponents. The observed similarity in band pattern (disc gel electrophoresis in the presence or absence of urea) between HDL_2 and apo-HDL_2 was taken to support the hypothesis that HDL_2 is made up of lipoprotein subspecies with distinct peptide moieties.

Previous studies from this laboratory on the kinetics of cleavage of the protein of human serum high density lipoprotein (apo-HDL)[1] by carboxypeptidase A led us to suspect the existence of at least two chemically distinct polypeptide chains (Scanu *et al.*, 1968). Additional experiments were therefore planned to give a firmer support to such an observation and, if necessary, to develop methods for the isolation of the heterologous components. We report here on the results obtained by gel filtration carried out in the presence of urea, an agent shown previously (Sanbar and Alaupovic, 1963; Scanu, 1965; Shore and Shore, 1962) to promote dissociation of apo-HDL in solution. Clear evidence was obtained to indicate that this protein is made up of chemically distinct components which also differ in physical and immunological properties. While work was in progress, papers from other laboratories (Rudman *et al.*, 1968; Shore and Shore, 1968a,b) on the heterogeneity of apo-HDL were published and those results will be discussed in relation to our current findings.

A preliminary account of this work has appeared (Scanu *et al.*, 1969b).

Materials and Methods

Materials. HDL_2 was separated from the sera of healthy male Caucasian donors, 20–24-years old, group A, Rh^+, by ultracentrifugal flotation (Scanu *et al.*, 1968). The purity of the preparations was checked by immunological means (Scanu *et al.*, 1968). The protein moiety, apo-HDL_2, was obtained by extraction with an ethanol–ether (3:2) mixture at −10° (Scanu, 1965) and dissolved, unless specified, in 10^{-2} M Tris buffer (pH 8.6)–10^{-3} EDTA. For gel filtrations, the apoprotein solutions (7–10 mg/ml) were dialyzed against the same Tris buffer brought to 8 M with urea at 27°. After preliminary experiments (see Results) a 48-hr dialysis time was selected. Apo-HDL_2 was also studied after reduction and alkylation (Scanu, 1967) in 8 M urea or after succinylation (Scanu *et al.*, 1968).

Gel Filtration Studies. After a series of pilot studies with various types of Sephadex (G-25, G-50 fine, G-100, and G-200, Pharmacia Fine Chemicals, Sweden) and column beds, the following conditions were found to be optimal for the fractionation of apo-HDL_2: type of Sephadex, G-200; bed dimensions, 2.5 × 90 cm; amount of apo-HDL_2 loaded, 20 mg; equilibrating and eluting buffer, 10^{-2} M Tris (pH 8.6)–10^{-3} M EDTA–8 M urea; direction of flow, ascending; flow rate, 8 ml/hr; temperature, 27°. Under these experimental conditions, each fractionation procedure lasted about 60 hr during which time the column effluent was continuously monitored at 280 mμ either in a Gilford 2000 recorder (Gilford Instruments Co., Oberlin, Ohio) attached to a Beckman DU spectrophotometer or in an ISCO recorder (Instrument Specialties Co., Lincoln, Neb.). Protein recoveries were in the

* From the Departments of Medicine and Biochemistry, The University of Chicago, and Argonne Cancer Research Hospital (operated by the University of Chicago for the U. S. Atomic Energy Commission), Chicago, Illinois. *Received March 27, 1969.* This investigation was supported by grants from the National Institutes of Health (HE-08727), Life Insurance Medical Research Fund (68-27), and the Chicago and Illinois Heart Association (RN 68-12).

† Recipient of Research Career Development Award HE-24,867.

‡ Present address: Department of Chemistry, Universite Libre, Bruxelles, Belgium.

§ Postdoctoral research fellow, Chicago and Illinois Heart Association.

|| Predoctoral research fellow, National Institutes of Health.

[1] Abbreviations used are: HDL_2, high density lipoprotein of d 1.063–1.125; apo-HDL_2, protein moiety of HDL_2 obtained by extraction with ethanol–ether; SDS, sodium dodecyl sulfate.

order of 80–90%. The fractions within each peak were pooled, dialyzed against 10^{-2} M Tris (pH 8.6)–10^{-3} EDTA to remove urea, and then concentrated by vacuum dialysis at 4°. In all of these steps the solutions remained clear.

When larger quantities of apo-HDL_2 (40–50 mg) were fractionated, Sephadex G-200 beds (5 × 90 cm) were employed in Pharmacia K-50 columns (5 × 100 cm) using the same Tris–urea buffer as above and an ascending flow rate of about 10 ml/hr. Under these conditions, the fractionation time was approximately 5 days.

Analyses of Sephadex Fractions. POLYACRYLAMIDE GEL ELECTROPHORESIS. The technique was essentially that of Davies (1964) with some modifications to allow for gel polymerization in the presence of urea (4, 6, and 8 M). Acrylamide (Eastman Kodak Co., Rochester, N. Y.) in monomer concentrations of 7.5% was used. Analyses were made either in Tris buffer (pH 8.3; Davies, 1964) or acetate buffer (pH 4.5; Reisfeld *et al.*, 1962) using a Buchler apparatus (Buchler Instruments Co., Fort Lee, N. J.). Electrophoresis was carried out at 4° for 50 min or 4 mA/tube (6 × 90 mm). The gels were fixed and stained for protein in 1% Amido Schwarz in 7% acetic acid (Davies, 1964), and for lipids by Oil Red O (Scanu, 1965). After destaining, some of the gels were scanned in a Canalco Mod F microdensitometer (Canal Industries, Canal, Bethesda, Md.).

MICROANALYTICAL ISOELECTRIC FOCUSING fractionation of apo-HDL_2 and derivatives was carried out in a polyacrylamide–urea bed (acrylamide–5% urea; urea at 6–8 M) containing carrier ampholytes (Ampholine, pH 3–10, LKB Produkter AB, Bromma, Sweden) using an adaptation of the method of Wrigley (1968). In the presence of urea, chemical polymerization (procedure B) was the preferred method. After electrophoretic separation, the protein zones were precipitated by 5% trichloroacetic acid and stained, without removal of the ampholytes, by coomasie brilliant blue (11–152B, Consolidated Laboratory, Inc., Chicago Heights, Ill.), according to Riley and Coleman (1968).

Molecular Weight Determinations. Three methods were used. GEL FILTRATION. The experiments were carried out in 2.5 × 90 cm G-200 Sephadex columns using 10^{-2} M Tris (pH 8.6)–10^{-3} M EDTA–8 M urea as the equilibrating and eluting buffer. β-Mercaptoethanol was omitted after determining that it did not affect the results. These columns were calibrated with nonenzymatic proteins of known molecular weight (Kit N 8109A, Mann Research Lab., New York, N. Y.). They were dissolved and dialyzed for 24 hr against the eluting buffer and then applied to the column either alone or as a mixture. The molecular weights of the unknowns were calculated from the calibration curve obtained by plotting the log of the molecular weight of each standard *vs.* its respective elution volume in milliliters (Andrews, 1964).

POLYACRYLAMIDE GEL ELECTROPHORESIS IN THE PRESENCE OF SDS. Acrylamide monomer concentrations of 5 and 10% were prepared in 0.1% according to Warner (1966). The electrophoretic conditions in 0.1 M phosphate buffer (pH 7.1) were those described by Shapiro *et al.* (1967). After migration, the gels were fixed with 20% sulfosalicylic acid and then stained with 0.025% coomasie brilliant blue. By the use of markers of known molecular weight, a calibration curve was obtained by plotting log *MW vs.* migration of each protein (Shapiro *et al.*, 1967).

SEDIMENTATION EQUILIBRIUM EXPERIMENTS. The studies were carried out in a Spinco Model E analytical ultracentrifuge using both interference and absorption optics. The high-speed meniscus depletion method (Yphantis, 1964) was used with the interference system employing a six-channel centerpiece of Yphantis and sapphire windows. The experimental details of this technique as applied to apo-HDL have been described (Scanu *et al.*, 1968) except that all studies were carried out with proteins that had been dialyzed against 10^{-2} M Tris buffer (pH 8.6)–10^{-3} M EDTA–8 M urea.

The studies by absorption optics were carried out with a unit equipped with an electronic speed control, photoelectric scanning, and multiplex system. Double-sector 12-mm cells were used fitted in a multicell Spinco analytical rotor to allow for the simultaneous analysis of unknowns with standards of known molecular weight (cytochrome *c* and chymotrypsinogen). The solvent and the solution sides of each cell contained 10 and 15 μl of Fluorochemical FC 306394 (Beckman Instrument Co., Palo Alto, Calif.). Proteins in the concentration of 0.2–0.3 optical density unit were studied in 10^{-2} M Tris buffer (pH 8.4)–8 M urea. Speeds from 20,000 to 40,000 rpm were used and equilibrium was assumed after successive scannings (280 mμ) taken at 3-hr intervals showed no change. All runs were carried out at 24°. The values of the apparent molecular weight, MW_{app}, were obtained from the relation

$$MW_{app} = \frac{2RT}{(1 - \bar{v}\rho)w^2} \frac{1}{\frac{d \ln c}{d(r^2)}}$$

where the term d ln c/d(r^2) was obtained from the slope of the ln OD *vs.* r^2 plot. All computations were carried out in a Model 300 Wang electronic calculator (Wang Laboratories Inc., Tewksburg, Mass.) using a specially designed program. Values of $\bar{v}$ were calculated from amino acid analysis (Cohn and Edsall, 1943). Corrections for the effect of 8 M urea on these $\bar{v}$ values were carried out by assuming that there was some binding of urea to the proteins and subtracting for the factor 0.02 (Hade and Tanford, 1967). Such assumptions proved valid for the markers (chymotrypsinogen and cytochrome *c*) which were studied in 8 M urea under ultracentrifugal conditions similar to those employed for the apo-HDL_2 fractions. Direct determinations of the $\bar{v}$ of the protein fractions in 8 M urea by pycnometry proved unsatisfactory.

Spectroscopic Studies. Circular dichroic spectra at 27° were recorded in a Cary Model 6001 spectropolarimeter equipped with a circular dichroism attachment. The experimental conditions described for the study of apo-HDL_3 (Scanu and Hirz, 1968) were applied to each apo-HDL_2 fraction. Spectra were also recorded as a function of temperature according to the technique previously reported (Scanu, 1969; Scanu *et al.*, 1969a). Protein solutions (0.5–1 mg/ml) in 0.01 M phosphate buffer (pH 8.4) were studied in 0.1-mm path-length cells (Pyrocell, Shoreham, Co., Inc., Winfield, N. J.) which were placed in a hollow, homemade, aluminum block through which water of desired temperature was circulated. Spectra were recorded after temperature equilibration was reached.

Ultraviolet absorption spectra were recorded in a Cary Model 14 spectrophotometer at 27° using 1-cm path-length quartz cells. The studies were carried out in the presence and absence of 8 M urea. The values were all corrected for scattering (Wetlaufer, 1962).

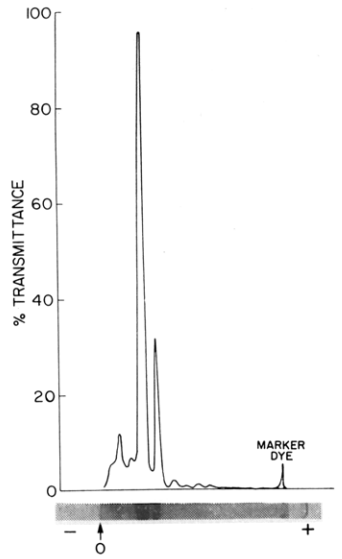

FIGURE 1: Polyacrylamide gel electrophoresis in 8 M urea of apo-HDL_2, pH 8.3. The upper panel indicates a densitometric scanning. For technical details, see text.

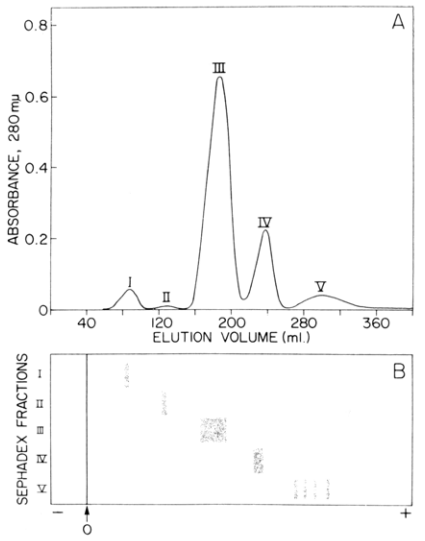

FIGURE 2: Gel filtration (Sephadex G-200 in 8 M urea) of apo-HDL_2. The lower panel indicates the behavior of the various fractions isolated from the column by polyacrylamide gel electrophoresis in 8 M urea.

Immunological Studies. Antisera were prepared by injecting male albino rabbits (2–3 kg) in their foot pads with 1.0–1.5 mg of apo-HDL_2 or fractions emulsified with equal volumes of complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.). After 3 weeks, the animals received a booster injection intramuscularly (2–4 mg of antigen suspended in the adjuvant) and 10 days later they were bled by heart puncture. After determination of their titers, the antisera were stored at −20° in 1:100,000 (w/v) merthiolate.

Immunoprecipitin reactions between antigens and specific antisera were conducted by the technique of double immunodiffusion in 1% agarose (Fisher Scientific Co., Chicago) as previously described (Scanu, 1965).

Amino Acid Analysis. Protein hydrolysates (6 N HCl, 21 hr, 110°) were separated in a fully automated Biocal 200 amino acid analyzer (Biocal Instrument Co., Richmond, Calif.). In the computations, corrections were made for amino acid losses during hydrolysis (Scanu, 1965). Cystine was determined as carboxymethylcysteine following S carboxymethylation of apo-HDL_2 or its fractions (Scanu, 1965). Tryptophan was determined according to Goodwin and Morton (1946).

Other Analyses. Total protein content was determined by the Lowry method (Lowry *et al.*, 1951) or as $N \times 6.25$ after nesslerization (Lange, 1958). Total cholesterol and phospholipids were determined as previously described (Scanu, 1965).

Reagents. Urea, reagent grade (J. T. Baker Chemical Co., Phillipsburg, N. J.), was recrystallized from ethanol. Solutions prepared with recrystallized urea gave comparable results with those filtered through a mixed-bed resin (AG-501-X8, 20–50 mesh, Bio-Rad, Richmond, Calif.).

Results

Fractionation of Apo-HDL_2 by Sephadex G-200; Analysis of the Fraction by Polyacrylamide Gel Electrophoresis and Disc Gel Electrofocusing. Apo-HDL_2 was fractionated by polyacrylamide gel electrophoresis in 8 M urea into eight components, two major (third and fourth bands from cathode) and six minor (Figure 1). From the densitometric scanning, the area corresponding to the third band was about 20% that of the second band. Analysis by disc gel electrophoresis in 6 M urea gave similar results; with 4 M urea the various bands were visible but their separation was less satisfactory.

In the absence of the dissociating agent the number of bands was increased and separation among them was less sharp than with urea.

Gel filtration of apo-HDL_2 through Sephadex G-200 in 8 M urea produced a clear separation of five peaks, labeled in order of elution as I, II, III, IV, and V (Figure 2). Peak II was a minor component not constantly seen, and will not be given consideration in these studies. When, after collection and concentration, peaks III–V were rerun in the same column either individually or as a mixture they emerged in the same elution volume without further resolution. On the other hand, rechromatography of peak I gave rise to peaks III and IV, although about 50% of the material still emerged as peak I.

When apo-HDL_2 was examined after succinylation, Sephadex G-200 chromatography showed a significant reduction of peak I (<1%), no peak II, and a relative distribution of peaks III–V similar to that of apo-HDL_2. Succinylation also produced an increase in the rate of anodic migration of each protein component.

The *S*-carboxymethylated product had a chromatographic behavior essentially similar to that of apo-HDL_2. By electrophoresis a more distinct separation was noted between the bands corresponding to peak III and IV due to an increased mobility of the latter.

By polyacrylamide gel electrophoresis in 8 M urea, a correlation was found between rates of elution from the Sephadex column and electrophoretic mobility. It may be noted that the broad peak V corresponds to the distinct bands by polyacrylamide gel electrophoresis. The percentage area distribution

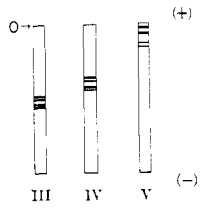

FIGURE 3: Disc gel electrofocusing in 6 M urea of apo-HDL_2 Sephadex fractions III–V. For experimental details, see text.

of peaks I, II, III, IV, and V, based upon protein determinations by the Lowry method, was 5, 2, 68, 20, 5, respectively, which is in close agreement with the densitometric data of the gel stained by Amido Schwarz.

Disc gel electrofocusing indicated differences in isoelectric point among Sephadex fractions III–V. Moreover, each of them exhibited a heterologous pattern characterized by four closely migrating bands stained with unequal intensity by coomasie blue (Figure 3). No significant differences were noted between gels containing 6 and 8 M urea.

Lipid Content of Sephadex Fractions. The Sephadex fractions showed no presence of either cholesterol of phospholipids, with the exception of peak I where quantitative thin-layer chromatographic analysis indicated about 5% lecithin by weight.

Molecular Weight Determination of the Various Sephadex Fractions. Peaks I, III, IV, and V appeared as a single band of different mobilities by polyacrylamide gel electrophoresis in 0.1% SDS. From the calibration curve using known molecular weight markers (see Methods), significant differences in the apparent molecular weights of peaks I, III, IV, and V were noted (Figure 4 and Table I). These figures compared well with those obtained from the calibration curve obtained from Sephadex G-200 chromatography in 8 M urea (Table I).

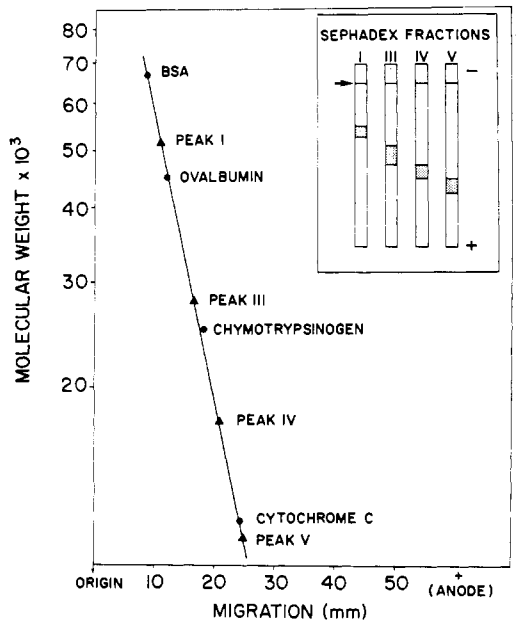

FIGURE 4: Plot of molecular weight *vs.* distance of markers and Sephadex fractions from apo-HDL_2 separated by polyacrylamide gel electrophoresis (0.1% SDS).

TABLE I: Apparent Molecular Weight Values of Apo-HDL_2 Sephadex Fractions Obtained by Three Independent Physical Methods.[a]

Apo-HDL_2 Fractions	Sephadex G-200, 8 M Urea	Disc Gel Electrophoresis, 0.1% SDS $\times 10^3$	Equilibrium Ultracentrifugation[b]
I	52.0–53.5	54.0–55.0	
III	25.8–27.0	27.5–28.2	16.6–17.0[c]
IV	16.4–17.2	17.0–17.4	17.2–17.6
V	11.2–12.1	10.6–11.4	11.0–11.8

[a] The values from two determinations are reported. [b] The values were calculated from the minimum slope near the meniscus. [c] This represented only a small portion of the samples. The major component had an apparent molecular weight of about 28,000.

By analytical ultracentrifugation a linear ln y/r^2 plot was obtained only with peak V both by Rayleigh interference and absorption optics. With all other products (peak II was not studied) the curve departed from linearity at the cell bottom probably relating to aggregation (Figure 5). The values of molecular weight obtained by ultracentrifugation as listed in Table I are those computed from the minimum slope near the meniscus. In peak III the clear evidence of a component with minimum molecular weight of about 17,400 was noted with the most concentrated specimen (1–2 mg/ml at 40,000 rpm). Other authors (Shore and Shore, 1968a,b) have suggested that apo-HDL or subfractions in urea exhibit preferential bound water. Should this be the case (and this, in our opinion, remains to be proven) the values listed in Table I may have to be reduced about 15%.

Immunological Studies. Peaks I, III, IV, and V all reacted against apo-HDL_2 antisera. Apo-HDL_2 and peak I gave a reaction of identity whereas with III, IV, and V, the reaction was only partial (Figure 6A). Sera against fraction III failed to react with fraction V (Figure 6B). The results obtained with peaks III and IV antisera are summarized in Table II. Antisera against peak V were not obtained. Differences in anti-

TABLE II: Summary of Immunological Results of Apo-HDL_2 and Its Sephadex Fractions.

	Antisera		
Antigen	Apo-HDL_2	III	IV
Apo-HDL_2	i	pi	pi
I	i[a]	pi[b]	pi
III	pi	i	pi
IV	pi	pi	i
V	pi	Neg[c]	Neg

[a] i = total identity. [b] pi = partial identity. [c] Neg = no reaction.

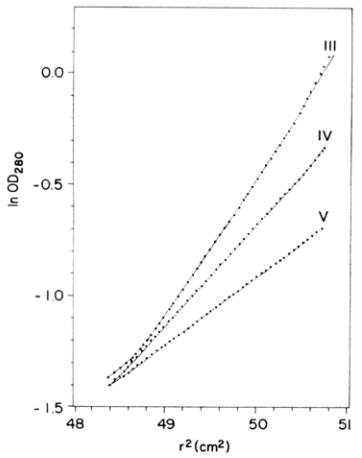

FIGURE 5: Equilibrium ultracentrifugation of apo-HDL_2 fractions III–V: ln y *vs.* r^2 plot.

genic properties were noted between peaks III, IV, and V. Particularly, fraction V did not share antigenic determinants with either III or IV.

Spectroscopy Studies. The circular dichroic spectra at 27° of apo-HDL and Sephadex fractions (Figure 7) indicated differences among the various products. All of these exhibited either a trough or a shoulder at 222 mμ and a band at 208 mμ with the exception of peak V where the minimum was at 205 mμ. Ellipticity values were in the following order: peak III > peak IV → peak V. Peak V also exhibited a shift of the crossover point to 198 mμ and a significant decrease of the ellipticity of the positive band with a maximum at 193 mμ. Peak I had a spectrum significantly different from the others and resembled closely that previously described for HDL_2 (Scanu and Hirz, 1968) with a predominance of the $[\theta]_{222}$ over the $[\theta]_{208}$ band.

In the temperature studies, all carried out in the absence of urea, no significant spectral changes were noted between 4 and 27°. Between 27 and 62° there was a marked thermal transition with maximal changes of the intensity of the 222- and 208-mμ bands of 60% (fraction III), 50% (fraction IV),

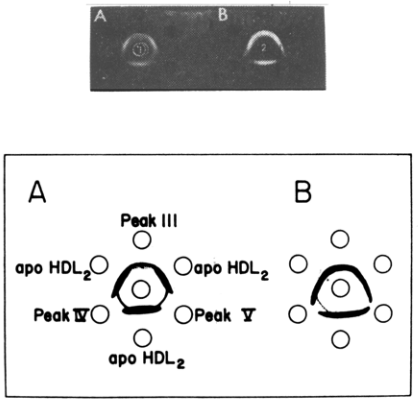

FIGURE 6: Patterns of immunoprecipitation (double-diffusion technique) of apo-HDL_2 fractions III–V. The same antigens were used for A and B. Central well 1: anti-apo-HDL_2; central well 2: anti-peak III.

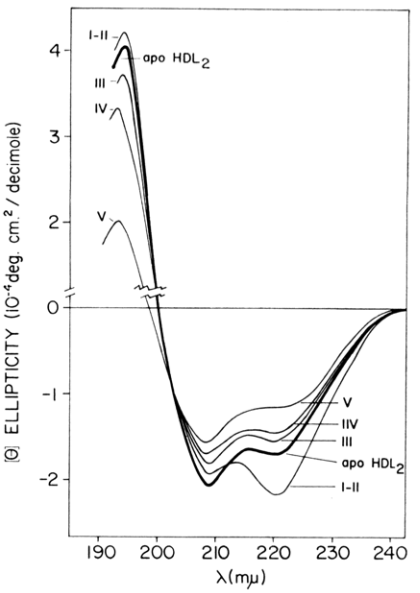

FIGURE 7: Circular dichroic spectra of apo-HDL_2 and Sephadex fractions.

and 40% (fraction V). Between 62 and 72° (the highest temperature studied) there was a tendency for a plateau. The spectral changes were compatible with an α-helix–random coil transition and were completely reversible upon cooling. Peaks III, IV, and V exhibited different titration curves (Figure 8) and distinct ultraviolet absorption spectra, and such differences were markedly accentuated in highly alkaline media up to pH 13.0 (Figure 9). The ultraviolet spectrum of fraction III at pH 8.5 in 8 M urea was characterized by a maximum at 278 mμ (276–280 mμ), a shoulder at 289 mμ, and a minimum at 252 mμ. At pH 13.0, two maxima were noted at 283 and 289 mμ (283 < 289 mμ) with a minimum at 272 mμ. Further, the intensity of the maximum at pH 13.0 was significantly higher than at pH 8.6.

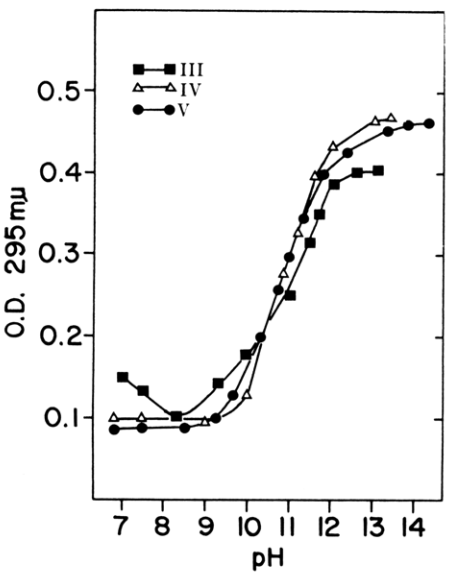

FIGURE 8: Titration curves of apo-HDL_2 Sephadex fractions III–V. (Protein concentration 0.425 mg/ml.)

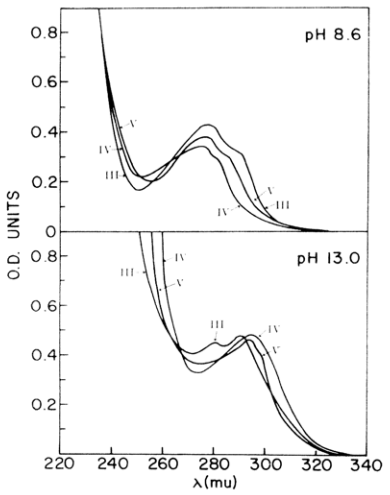

FIGURE 9: Ultraviolet absorption spectra of apo-HDL_2 Sephadex fractions III–V at pH 8.4 and 13.0. (Protein concentration 0.425 mg/ml.)

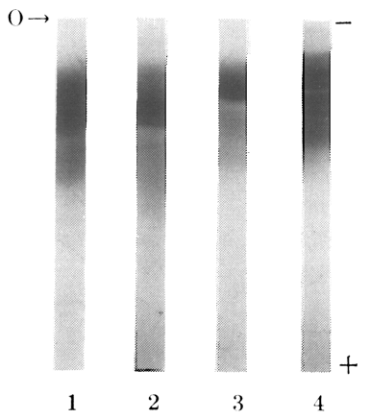

FIGURE 10: Polyacrylamide gel electrophoresis of HDL_2 in the absence of urea (1), 4 M urea (2), 6 M urea (3), and 8 M urea (4).

Fraction IV, at pH 8.6 in 8 M urea, had a maximum at 276 mμ with a small shoulder at 281 mμ and a minimum at 250 mμ. At pH 13.0 the peak maximum at 292 mμ (tyrosine ionization) was increased in intensity over the 276-mμ maximum seen at pH 8.6. In addition, there was no second maximum attributable to tryptophan.

Fraction V in 8 M urea exhibited at pH 13.0 one maximum at 292 mμ and a small shoulder at 298 mμ. The results of all of the above experiments were essentially the same in the absence of urea, except for some difference of the relative intensity of the 283- and 289-mμ bands.

Amino Acid Analysis. The results (Table III) indicated marked differences in amino acid composition among the various fractions and between them and apo-HDL_2. Peak I had a rather similar composition to apo-HDL_2 although some differences were observed. Peaks III and IV differed markedly from each other; the former contained no cystine and very small amounts of isoleucine, the latter had little histidine and arginine and no tryptophan. The amino acid composition of peak V was distinct from that of III and IV.

Studies on HDL_2 (Figure 10). By disc gel electrophoresis, HDL_2 was fractionated into several bands with distribution similar to that seen in apo-HDL_2. This was particularly evident in the absence and in the presence of 4 and 6 M urea, with some decrease in mobility induced by the denaturing agent (Figure 9). In 8 M urea, the separation among bands was less distinct although heterogeneity was still detected (Figure 10). All of the bands were stained by both Amido Schwarz and Oil Red O.

Discussion

Our experiments indicate that gel filtration in 8 M urea of the essentially lipid-free protein of human serum HDL_2 affords the separation of four components (a fifth component, fraction II, was only occasionally seen) differing in molecular weight, amino acid composition, and immunological and spectral properties. The heaviest component, designated as fraction I, which contained about 5% phospholipid by weight, probably represented an aggregate of the other fractions on the basis of (1) similarity of its amino acid composition with the starting product; (2) appearance of components of lower molecular weight upon rechromatography; (3) its marked decrease in apo-HDL_2 preparations examined after succinylation. We cannot rule out the possibility, however, that the aggregate may be associated with a distinct large molecular weight species, occurring in apo-HDL_2 in rather low concentration, since a small amount of fraction I was detected by gel filtration in all experimental conditions employed and was also seen by polyacrylamide gel electrophoresis in 8 M urea.

Fraction III was the most abundant component of apo-HDL_2, about 70% of the total. It had an apparent molecular weight of approximately 27,000 (evidence for a lower molecular weight component of about 17,000 was obtained by ultracentrifugation) by three independent physical methods (ultracentrifugation, gel filtration, and disc gel electrophoresis, all in 8 M urea) and had a characteristic amino acid composition showing trace or low amounts of isoleucine and no cystine. In terms of molecular weight, it is of interest to point out the similarity between the values for the largest portion of fraction III obtained in these studies and those previously reported for succinylated apo-HDL_2 (Scanu *et al.*, 1969a). The latter exhibited homology by equilibrium ultracentrifugation which had also been the case for apo-HDL_3 studied in guanidine hydrochloride (Cox and Tanford, 1968), and for apo-HDL_2 and apo-HDL_3 in sodium dodecyl sulfate (Scanu, 1967; Shore and Shore, 1967). To reconcile the previously suggested molecular weight homology of apo-HDL with the evidence for heterology obtained in the present experiments (see data on fractions IV and V) one must assume that either the lower molecular weight components were overlooked, or that under the given experimental conditions, they formed aggregates of the same size as fraction III. Concerning the 17,000 molecular weight component noted in fraction III under appropriate ultracentrifugal conditions, the question arose as to whether it could represent a small contamination by fraction IV which had a similar molecular weight. However, this was found not the case, since identical results were obtained with fraction III filtered two or three times through Sephadex and no evidence of peak IV

TABLE III: Amino Acid Composition of Apo-HDL_2 and Sephadex Fractions.

Amino Acid	Apo-HDL_2	Moles of Amino Acid/100,000 g of Protein			
		I	III	IV	V
Aspartic	59.4 ± 0.9[a]	64.4 ± 1.0	70.1 ± 1.20	30.0 ± 0.42	64.0 ± 1.0
Threonine	37.0 ± 0.48	36.2 ± 0.5	36.2 ± 0.50	48.0 ± 0.71	45.4 ± 0.70
Serine	48.0 ± 0.52	44.2 ± 0.56	46.0 ± 0.60	47.0 ± 0.70	68.8 ± 0.84
Glutamic	134.2 ± 1.80	130.1 ± 1.7	138.6 ± 1.8	125.2 ± 1.50	136.9 ± 1.60
Proline	36.8 ± 0.40	37.1 ± 0.40	42.0 ± 0.5	23.7 ± 0.32	22.5 ± 0.40
Glycine	33.2 ± 0.38	38.4 ± 0.40	39.2 ± 0.50	22.3 ± 0.36	34.8 ± 0.38
Alanine	55.4 ± 0.60	53.1 ± 0.62	64.2 ± 0.70	39.0 ± 0.54	58.0 ± 0.70
Half-cystine	5.7 ± 0.06	3.8 ± 0.04	Trace	16.0 ± 0.02	Trace
Valine	48.2 ± 0.52	44.2 ± 0.58	49.0 ± 0.60	42.0 ± 0.62	43.5 ± 0.64
Methionine	7.8 ± 0.09	6.3 ± 0.08	7.0 ± 0.08	10.0 ± 0.02	8.0 ± 0.08
Isoleucine	5.2 ± 0.08	8.0 ± 0.09	1.8 ± 0.02	12.0 ± 0.02	6.6 ± 0.08
Leucine	105.2 ± 1.3	103.4 ± 1.40	141.2 ± 1.60	62.0 ± 0.76	70.0 ± 0.88
Tyrosine	27.6 ± 0.34	15.6 ± 0.20	28.1 ± 0.4	23.8 ± 0.32	24.3 ± 0.36
Phenylalanine	26.0 ± 0.34	24.8 ± 0.34	26.4 ± 0.36	24.1 ± 0.36	34.5 ± 0.46
Lysine	68.8 ± 0.85	63.9 ± 0.82	63.4 ± 0.82	78.2 ± 0.89	49.2 ± 0.67
Histidine	10.9 ± 0.10	8.9 ± 0.10	18.2 ± 0.2	1.6 ± 0.02	5.8 ± 0.07
Arginine	34.2 ± 0.41	30.1 ± 0.42	55.8 ± 0.7	1.0 ± 0.01	21.1 ± 0.32
Tryptophan	15.0 ± 0.02	12.6 ± 0.14	20.0 ± 0.3		4.0 ± 0.06

[a] Values represent average of four determinations plus and minus the standard error of the mean.

was obtained in these products by either polyacrylamide gel electrophoresis or disc gel electrofocusing.

In terms of concentration, fraction IV was the second largest component of apo-HDL_2. It had a molecular weight of about 17,000, trace amounts of histidine and arginine, and no tryptophan, was relatively rich in cystine, and had distinct immunological properties from either fraction III or V. Although homogeneous by disc gel electrophoresis in SDS, it was heterogeneous in urea gels or by disc gel electrophoresis, suggesting the presence of subcomponents differing in charge density but with identical or very similar molecular weight. The presence of cystine in this fraction (in contrast with fraction III) was also indicated by the disc gel electrophoresis of *S*-carboxymethylated apo-HDL_2 which showed an increased anodic mobility of the band corressponding to Sephadex fraction IV.

Fraction V was the most difficult to work with, because of its low content in all preparations used. This fraction had the lowest molecular weight, a different amino acid composition from fractions III and IV, and no antigenic determinant in common with the other components. More clearly than the other fractions it showed heterogeneity by disc gel electrophoresis in 8 M urea, and this was corroborated by disc gel electrofocusing which suggested chemical heterogeneity, and a very similar or identical molecular weight (see data on disc gel electrophoresis in SDS and equilibrium ultracentrifugation).

Differences among the various apo-HDL_2 components were also shown by circular dichroism studies where a progressive increase of disorder, indicated by the decrease of the amplitude of the 222-mμ band, was noted from fractions III to V. Whether such differences were merely due to differences in amino acid composition among these components or whether there was an added effect by aggregation was not established. A similarity was noted between the spectrum of fraction I and that previously reported for whole HDL_2 (Scanu and Hirz, 1968). It may be pointed out that this fraction more closely resembled the starting lipoprotein in that it contained some lipid (about 5%) and probably all of its protein units, as based upon amino acid analysis.

While this work was in progress, other authors obtained evidence of the chemical heterogeneity of apo-HDL. Rudman *et al.* (1968) used preparations from lyophilized human serum HDL (*d* 1.063–1.21) extracted with ethanol–acetone (1:1). This lipid-free product was resolved into three fractions on Sephadex G-200 columns equilibrated and eluted with 1 N acetic acid. The amino acid analyses of these fractions after purification were taken to be compatible with the presence of two polypeptide chains, A and B, having the tendency to form mixed molecular aggregates. A molecular weight for the two subunits of 20,000–25,200 was estimated by gel filtration in the presence of SDS.

Shore and Shore (1968b), who fractionated the protein moieties of human serum HDL of *d* 1.083–1.124 and 1.126–1.195 on DEAE-cellulose in 8 M urea, also obtained evidence for two distinct polypeptide chains, although their properties differed from the fractions prepared by Rudman *et al.* Sedimentation equilibrium studies in 8 M urea and 7 M guanidine hydrochloride of the protein moiety of HDL of *d* 1.126–1.195, together with quantitative C-terminal analysis, indicated that the two peptides (R-Thr and R-Gln) probably occur in equimolar amounts and have a molecular weight of about 15,000 (Scanu *et al.*, 1969b).

When compared with those by Rudman *et al.* and Shore and Shore, our results provide evidence that apo-HDL has a structural complexity greater than previously suspected; such a

conclusion also is applicable to HDL_3 (unpublished data). It may be pointed out that Rudman *et al.* had not claimed complete purity of the "monomeric" unit, whereas Shore and Shore left about 30% of the protein unfractionated. It is possible therefore that peptides like those in our peak V may have been overlooked, and that the relative proportions of the two polypeptide chains described by each author may have been incorrectly estimated. In fact, the amino acid data given by Rudman *et al.* are more compatible with a 3:1 weight ratio for peptides A and B than the 1:1 ratio suggested by these authors. Inconsistencies can also be found in the data by Shore and Shore (1968b) who assumed equimolar amounts of R-Thr and R-Gln peptides in one of their major DEAE chromatography fractions (peak V) which, on the other hand, by polyacrylamide gel electrophoresis showed two bands with a marked difference of staining intensity (Amido Schwarz). Comparisons of our amino acid data with those by Shore and Shore (1968b) indicate that the R-Thr and R-Gln peptides of those authors closely resemble those of our fractions III and IV, especially in terms of almost total absence of cystine and isoleucine (fraction III or R-Thr), or histidine, arginine, and tryptophan (fraction IV or R-Gln). Since in one case gel filtration and in the other case ion-exchange chromatography were used, it is evident that these peptides are different both in size and charge. According to Shore and Shore (1968b), who estimated a molecular weight of about 15,000 for both subunits, the R-Thr peptide would be present in 8 M urea as a dimer, in contrast to the R-Gln peptide which would be present in a monomeric form. Our molecular weight determinations appear to support the concept that the various apo-HDL_2 components exhibit a different degree of aggregation in urea solutions, and give minimum molecular weight values (see Table I) for fractions III and IV that are near but not identical with R-Thr and R-Gln, respectively. Our figures would become closer to those of Shore and Shore if we should assume, like those authors, that there is a marked preferential binding of water when apo-HDL is dissolved in 8 M urea. In the absence of definitive experimental data for the $\bar{v}$ of our peptides, we are unable to choose between this possibility and the alternate (used in our computations) favoring the interactions between protein and urea. Utilization of the amino acid data to validate the molecular weight figures may not be appropriate in our case, since we suspect, on the basis of the heterogeneity shown by the three Sephadex fractions by disc gel electrofocusing and also by DEAE-chromatography (unpublished), that they may each contain heterologous chains. Thus, values reported in Table II are likely to represent only average amino acid compositions.

In view of the demonstration of peptide heterogeneity in apo-HDL_2, one is led to ask whether HDL_2 represents a single lipoprotein species having distinct polypeptide chains or whether HDL_2, as we prepare it in the ultracentrifuge, is a mixture of HDL species each with distinct peptide moieties. No definitive answer to this question can be provided by the present studies. Our inclination to favor the second hypothesis comes from the result of the analysis of HDL_2 by polyacrylamide gel electrophoresis (Figure 10) which indicates a close similarity in the patterns of this lipoprotein and its delipidated product either in the presence or absence of urea. Should this be expressive of true HDL_2 heterogeneity, it may be possible to isolate these lipoprotein components on a preparative scale and to define whether their protein moieties are of the same nature as those obtained from apo-HDL_2 in our current studies. Work in this direction is now in progress in this laboratory.

Acknowledgments

The authors thank Dr. C. Goodheart from the American Medical Association Research Institute, Chicago, Ill., for carrying out the experiments with a Spinco Model E analytical ultracentrifuge equipped with photoelectric scanning and multiplex systems.

References

Andrews, P. (1964), *Biochem. J. 91*, 222.
Cohn, E. J., and Edsall, J. T. (1943), *in* Proteins, Amino Acids and Peptides as Ions and Dipolar Ions, Cohn, E. J., and Edsall J. T., New York, N. Y., Reinhold, p 375.
Cox, A. C., and Tanford, C. (1968), *J. Biol. Chem. 243*, 3083.
Davies, B. J. (1964), *Ann. N. Y. Acad. Sci. 121*, 404.
Goodwin, T. V., and Morton, R. A. (1946), *Biochem. J. 40*, 628.
Hade, E. P. K., and Tanford, C. (1967), *J. Am. Chem. Soc. 89*, 5034.
Lange, C. A. (1958), *Anal. Chem. 30*, 1692.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Reisfeld, R. A., Lewis, U. J., and Williams, D. E. (1962), *Nature 195*, 281.
Riley, R. F., and Coleman, M. K. (1968), *J. Lab. Clin. Med. 72*, 714.
Rudman, D., Garcia, L. A., Abell, L. I., and Akgun, S. (1968), *Biochemistry 7*, 3136.
Sanbar, S. S., and Alaupovic, R. (1963), *Biochim. Biophys. Acta 71*, 235.
Scanu, A. (1965), *J. Lipid Res. 7*, 295.
Scanu, A. (1967), *J. Biol. Chem. 242*, 711.
Scanu, A. (1969), *Biochim. Biophys. Acta* (in press).
Scanu, A., and Hirz, R. (1968), *Proc. Natl. Acad. Sci. U. S. 59*, 890.
Scanu, A., Pollard, H., Hirz, R., and Kothary, K. (1969a), *Proc. Natl. Acad. Sci. U. S.* (in press).
Scanu, A., Reader, W., and Edelstein, C. (1968), *Biochim. Biophys. Acta 160*, 32.
Scanu, A., Toth, J., Edelstein, C., and Stiller, E. (1969b), *Fed. Proc. 28*, 894.
Shapiro, A. L., Vinuela, E., and Maizel, J. V. H. (1967), *Biochem. Biophys. Res. Commun. 28*, 815.
Shore, B., and Shore, V. (1968a), *Biochemistry 7*, 2773.
Shore, V., and Shore, B. (1962), *Biochem. Biophys. Res. Commun. 9*, 455.
Shore, V., and Shore, B. (1967), *Biochemistry 6*, 1962.
Shore, V., and Shore, B. (1968b), *Biochemistry 7*, 3396.
Warner, J. R. (1966), *J. Mol. Biol. 19*, 383.
Wetlaufer, D. B. (1962), *Advan. Protein Chem. 17*, 304.
Wrigley, C. (1968), *Sci. Tools 15*, 17.
Yphantis, D. A. (1964), *Biochemistry 3*, 297.