Isolation and Characterization of Two Threonine-Poor Apolipoproteins of Human Plasma High Density Lipoproteins†

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ABSTRACT: Two apolipoproteins that are minor components normally of human plasma lipoproteins were discovered. They comprise up to 25% or more of the apolipoproteins in high density lipoproteins of some individuals with lipoprotein abnormalities associated with certain metabolic diseases and in individuals treated with amphotericin B for coccidiomycosis infection. The "new" apolipoproteins are distinctly different in amino acid composition, isoelectric point, and electrophoretic mobility from other known apolipoproteins. The "new" apolipoproteins are similar in amino acid composition. Both are unusually low in threonine, poor in valine and leucine, and rich in aspartic acid, glycine, alanine, and phenylalanine. They are relatively rich in arginine, although not as rich as the arginine-rich apolipoprotein, and have higher isoelectric points than other apolipoproteins except apoC-I. The more abundant of the two "new" apolipoproteins, of p$I \sim 6.5$, exists mainly as a dimeric protein of about 40 000 daltons and is dissociated by mercaptoethanol in the presence of sodium dodecyl sulfate. The less abundant one, of p$I \sim 6.0$, appears to be about 10 000 daltons. The threonine-poor apolipoproteins are associated mainly with plasma high density lipoproteins, particularly the denser HDL_3 subfraction, but small amounts were found in the very low and intermediate density lipoproteins. The HDL_3 with high content of threonine-poor apolipoproteins were indistinguishable from the normal HDL_3 by agarose or large pore polyacrylamide gel electrophoresis or by analytical ultracentrifugation. The lipid moiety of the abnormal HDL was richer in triglycerides and similar in phospholipid content to that of normal HDL.

At least nine distinct human plasma apolipoproteins, or even more if one considers the variant forms, have been isolated (Fredrickson, 1974; Morrisett et al., 1975). In addition to transporting insoluble lipids, the apolipoproteins are determinants of lipoprotein and lipid metabolism, by functioning specifically as enzyme cofactors (Fielding et al., 1972; Havel et al., 1973), as molecules for recognizing cell receptor sites (Brown & Goldstein, 1976), and as determinants of structure and physical properties of lipoproteins by their specific interactions with lipids.

Although each of the major density classes of plasma lipoproteins has a characteristically different complement of apolipoproteins, most apolipoproteins occur in more than one density class. The heterogeneity of apolipoproteins within a given class reflects in part the heterogeneity of the lipoproteins and the metabolic interconversions and exchange reactions among the lipoproteins. Apolipoprotein distribution within the lipoproteins classes as well as lipoprotein concentration in plasma is dependent upon diet (Shore et al., 1974a,b; Schonfeld et al., 1976), hormones (Shore et al., 1974b; Hillman et al., 1975; Schonfeld et al., 1974), genetic factors (Fredrickson & Levy, 1972; Havel & Kane, 1973; Alaupovic et al., 1974; Ferrans & Fredrickson, 1975), diseases affecting lipoprotein metabolism (Barclay, 1972), and other circumstances that perturb the synthetic, catabolic, or regulatory processes of the plasma lipoproteins.

Such alterations produced during treatment of two patients with amphotericin B for coccidiomycosis infection provided a favorable circumstance for isolation and characterization of two apolipoproteins that are present normally as minor components of the plasma high density lipoproteins and are selectively increased after treatment with the drug. The distribution of the newly discovered apolipoproteins among the various classes of lipoproteins and changes in lipoprotein distribution in plasma were investigated during drug therapy and in subjects with abnormalities in lipoprotein metabolism.

Materials and Methods

Isolation of Lipoproteins. Lipoproteins, the VLDL[1] ($d <$ 1.006 g/mL), IDL (d 1.006–1.019 g/mL), LDL (d 1.019–1.065 g/mL), and HDL subfractions HDL_2 (d 1.081–1.125 g/mL) and HDL_3 (d 1.125–1.200 g/mL), and d 1.200–1.250 g/mL lipoproteins were isolated from individual sera by centrifugation in media adjusted to the appropriate density with NaCl or $NaNO_3$ and D_2O (Shore, 1957). Serum was obtained from subjects who had fasted for 12 h, and lipoproteins were isolated in the presence of 0.0008 M EDTA, 0.01 M Tris-HCl (pH 7.4), and 0.01% sodium azide. Sera of two male patients were examined during treatment with am-B for coccidiomycosis infection and again 3 months after cessation of treatment, at which time the subjects were asymptomatic. The drug was administered essentially as described by Medoff (1976). One of the subjects was bled 6 weeks after initiation of treatment (9-8-1976), at the end of treatment (12-3-1976), and 3 months later (3-4-1977). The patient was asymptomatic for coccidiomycosis on 12-3-1976 and 3-4-1977. Isolated lipoproteins

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[1] Abbreviations used: VLDL, very low density lipoproteins; IDL, intermediate density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; EDTA, ethylenediaminetetraacetate; Tris, tris(hydroxymethyl)aminomethane; am-B, amphotericin B; DEAE, diethylaminoethyl.

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were dialyzed at 4 °C on rapid dialyzers to reduce the salt concentration to about 0.05 M before delipidation and further analysis of the protein moieties. Lipoproteins were delipidated as described previously (Shore & Shore, 1973). Protein concentration was determined by the method of Lowry et al. (1951).

Polyacrylamide Gel Electrophoresis and Isoelectric Focusing of Apolipoproteins. Apolipoprotein compositions of all the lipoproteins of normal, hyperlipoproteinemic, and drug-treated subjects were compared by polyacrylamide gel electrophoresis and isoelectric focusing. Discontinuous polyacrylamide gel electrophoresis was carried out essentially as described by Davis (1964) in 8% gels (5 × 90 mm) containing 7 M urea. Delipidated samples in a volume of 60 μL were preincubated for 1 h with 7 M urea. Electrophoresis was carried out at 2.5 mA/tube for a period of 2–2.5 h. Gels were fixed and stained for 2 h in 0.5% Coomassie blue R-250 in 12.5% trichloroacetic acid and destained in 10% trichloroacetic acid.

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was carried out in 10% gels (5 × 100 mm) by the method of Weber & Osborn (1969). Samples in 75 μL were preincubated for 2 h at 37 °C in the presence of 1% sodium dodecyl sulfate, 1.9 M glycerol, and 0.05 M sodium phosphate buffer, pH 7.2, with and without 1.25% mercaptoethanol. Protein standards of known molecular weight were electrophoresed simultaneously. Gels were fixed and stained for 3 h in 20-mL tubes containing 0.2% Coomassie blue R-250 in 50 methanol:10 acetic acid:40 water (vol %). The gels were destained in a mixture of 25 methanol:10 acetic acid:65 water (vol %), which was changed several times over a period of up to 24 h.

Polyacrylamide gel isoelectric focusing was carried out over the pH ranges 3.5 to 10, 4 to 8, and 5 to 7 in 6% gels, 5 × 93 mm, containing 6 M urea as described by Wrigley (1968). The apparatus was similar to that of Righetti & Drysdale (1974). Electrolysis was started at a constant current of 1 mA/gel until the voltage reached 400 V and then continued at a constant voltage of 400 V. The anodal and cathodal chambers contained 0.2 vol % H_2SO_4 and 0.4 vol % ethanolamine, respectively. Water at 10 °C was circulated around the gels before and during electrolysis. Apolipoprotein samples in a volume of 60 μL were preincubated for 1 h at room temperature with 6 M urea and 2% ampholyte, with and without 1.25% mercaptoethanol, and applied at the cathodal end of the gel. The gels were stained overnight in a solution containing 0.02% Coomassie blue R-250, 5% trichloroacetic acid, 5% sulfosalicylic acid, and 25% methanol. Staining was continued for 8 h in a solution containing 0.05% Coomassie blue R-250, 25% isopropyl alcohol, 10% acetic acid, and 0.1% $CuSO_4 \cdot 7H_2O$. Gels were destained in a solution containing 0.01% Coomassie blue R-250, 25% isopropyl alcohol, 10% acetic acid, and 0.1% $CuSO_4 \cdot 7H_2O$. The fixed and stained gel patterns were photographed and scanned densitometrically at 635 nm with a recording spectrophotometer equipped with a linear transport system. The pH gradient in the gels was determined by measuring the pH of water (CO_2-free) extracts of serial slices 2-mm thick of gel after electrolysis.

Polyacrylamide gel isoelectric focusing was also used to isolate HDL apolipoproteins for analysis by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and determination of amino acid composition. For this purpose, 250 μg of apoHDL/gel was separated by isoelectric focusing. The gel regions corresponding to p*I*s 6.0 and 6.5, apoAI, and apoAII were excised and the proteins eluted by shaking the gel slices in 0.05 M ammonium bicarbonate solution containing 0.1% sodium dodecyl sulfate. The extracted proteins were lyophilized and then washed with a mixture of acetone:H_2O (9:1). In some experiments, the dried proteins were washed briefly with 10% trichloroacetic acid at 0 °C. After removal of the trichloroacetic acid solution from the protein precipitate, 1 mL of cold water was added, and the frozen samples were again lyophilized to remove any remaining trichloroacetic acid.

Amino Acid Analysis. The dried protein was hydrolyzed in vacuo for 40 h at 110 °C with 250 μL of 4 N methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole. The trichloroacetic acid-soluble material was also subjected to amino acid analysis after dilution and lyophilization. Amino acid analysis was carried out by the procedure of Liu & Chang (1972), in which a 20-cm column is used for analysis of basic amino acids, including tryptophan, and amino sugars.

Lipid Analyses. Lipids extracted from the HDL were determined gravimetrically. Samples were analyzed for phosphorus by the method of Chen et al. (1956). The lipids were separated as described by Nelson (1967) by one-dimensional, thin-layer chromatography on a glass plate coated with silica gel. The solvent was petroleum ether:diethyl ether:acetic acid (85:15:2, v:v:v). This solvent was also used for a preliminary washing of the plate, which was then air-dried, activated for 30 min at 120 °C, and cooled in air before the samples were applied. After chromatographic separation of the lipids, the plates were air-dried, sprayed with 18 N H_2SO_4, and then heated on a hot plate to char the lipids.

Electrophoretic and Ultracentrifugal Analyses of Lipoproteins. Serum lipoproteins during and after treatment of patients with amphotericin B and of normal individuals were compared by agarose electrophoresis (Hatch et al., 1973) and by polyacrylamide gel electrophoresis of sera and isolated lipoproteins with a lipid-binding stain amido Schwarz 10 B (Naito et al., 1973). Since these methods indicated the serum HDL concentration was abnormally low during amphotericin B treatment, the lipoprotein distribution and concentrations were determined by an analytical ultracentrifugation technique by Dr. Frank Lindgren of Donner Laboratory at the University of California at Berkeley. The method, described elsewhere by Lindgren et al. (1972), involves a computerized analysis of the schlieren data on lipoprotein distributions during centrifugation.

Results

Polyacrylamide Gel Isoelectric Focusing and Polyacrylamide Gel Electrophoresis of Apolipoproteins. The serum lipoproteins of four normal individuals, two males and two females, were analyzed simultaneously with those of am-B-treated patients. Because the plasma HDL concentrations were decreased and LDL elevated moderately during am-B treatment, the lipoproteins of two nephrotic patients and two each of types IIa, III, and IV hyperlipoproteinemic subjects whose plasma HDL concentrations were lower than normal were examined. The HDL subfractions of five diabetic patients, some with low plasma HDL, were also examined.

In patients treated with am-B, the HDL contained two apolipoproteins that have not been noted previously in the lipoproteins of humans or animals. The two proteins are not evident except possibly as trace components in the polyacrylamide gel electrophoresis patterns of normal HDL_2 and HDL_3 (gels 1 and 2 of Figure 1). In gels 3 to 6 and 10 to 13 of Figure 1, the two proteins are seen in the less acidic region of pH 4 to 8 gels. The HDL_2 (patterns 3 and 5) contain less of the "new" proteins relative to apoA-I than the HDL_3 (patterns 4 and 6). The *d* 1.20–1.25 fraction (pattern 7), which accounted for only a very small amount of the am-B plasma lipoproteins, con-

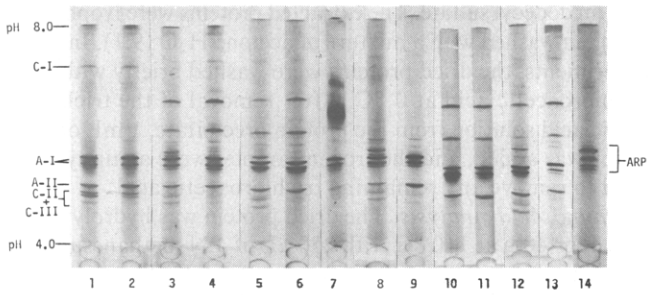

FIGURE 1: Polyacrylamide gel isoelectric focusing between pH 4 and 8 of HDL apolipoproteins (100 μg/gel) in 6% gels containing 6 M urea. (1 and 2) Normal HDL_2 and HDL_3, respectively; (3 and 4) am-B HDL_2 and HDL_3, respectively, after 6 weeks of treatment; (5, 6, and 7) am-B HDL_2, HDL_3, and *d* 1.20–1.25 fraction (225 μg of protein), respectively, at end of treatment; (8 and 9) HDL_2 and HDL_3, respectively, 3 months after ending treatment; (10 and 11) am-B HDL_3 with and without mercaptoethanol, respectively; (12) nephrotic HDL_3; (13) diabetic HDL_3; and (14) isolated arginine-rich apolipoprotein.

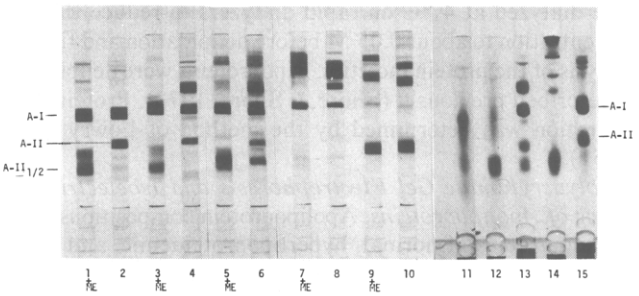

FIGURE 3: Sodium dodecyl sulfate gel electrophoresis of apolipoproteins with and without mercaptoethanol (ME). (1 and 2) Normal HDL_3 (30 μg); (3 and 4) am-B HDL_3 (30 μg) at end of treatment; (5 and 6) am-B HDL_3 (30 μg) after 6 weeks of treatment; (7 and 8) *d* 1.20–1.25 fraction (75 μg) at end of treatment; (9 and 10) standard mixture of bovine serum albumin, ovalbumin, and cytochrome *c;* (11 and 12) bands 1 (p*I* ~ 6.5) and 2 (p*I* ~ 6.0) from polyacrylamide gel isoelectric focusing; (13 and 14) am-B HDL_3 and VLDL after 6 weeks of treatment; and (15) normal HDL_3.

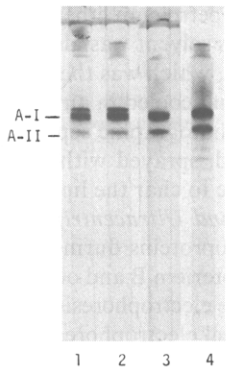

FIGURE 2: Polyacrylamide gel electrophoresis of apoHDL_3 in 8% gels containing 7 M urea. (1 and 2) am-B HDL_3, 20 and 40 μg, respectively; (3 and 4) normal HDL_3, 20 and 40 μg, respectively.

tained a small amount of the two apolipoproteins and apoA-I in addition to albumin. The relative amounts of the two proteins and their isoelectric points, about 6.5 and 6.0 in 6 M urea, are unchanged by reduction of the apoHDL_3 with mercaptoethanol (patterns 10 and 11 of Figure 1). Of the known apolipoproteins only apoC-I has a higher isoelectric point. Proportionately smaller amounts of the two proteins are found in HDL isolated from the am-B-treated patients 3 months after treatment was stopped (patterns 8 and 9) and patients with primary hyperlipoproteinemias. The two proteins are evident in polyacrylamide gel electrophoresis or isoelectric focusing patterns of apoHDL of normal individuals only occasionally in small amounts unless the gel is overloaded with protein, e.g., $\geqq$ 200 μg in polyacrylamide gel isoelectric focusing. The two new proteins could be detected in polyacrylamide gel isoelectric focusing patterns of 100 μg of apoHDL in all of the abnormal HDL subfractions, with greater amounts in HDL_3 than in HDL_2. The HDL of the nephrotic patients (pattern 12 of Figure 1) and one of the type IIa subjects contained a moderate amount. The HDL of diabetic patients (all treated with insulin), with one exception, contained small amounts. The exception, HDL from an 18-year-old male who had been treated with insulin for 1 week, contained considerable amounts (pattern 13 of Figure 1).

Densitometric scans of the stained polyacrylamide gel isoelectric focusing gels allow only semiquantitative estimates of the relative amounts of the two "new" apolipoproteins in the HDL_3 because not all proteins bind Coomassie blue to the same extent. However, the scans indicate the two "new" apolipoproteins comprise a considerable fraction, possibly 25–30% or more, of the total proteins in the am-B HDL_3. The ratios of apoA-I to apoA-II are similar in HDL_3 during and after am-B treatment and higher than in most normal HDL. ApoA-I and apoA-II bands were identified by polyacrylamide gel isoelectric focusing of apolipoproteins isolated by DEAE-cellulose chromatography (Shore & Shore, 1973) and from the amino acid composition of the protein eluted from gel slices after polyacrylamide gel isoelectric focusing. The protein of p*I* ~ 6.5 often is more abundant than the protein of p*I* ~ 6.0.

In disc electrophoresis with pH 8.3 buffer in 8% resolving gels containing 7 M urea, only one additional apolipoprotein band, slightly lower in mobility than apoA-I, is seen in patterns of am-B HDL_3 (Figure 2). This band does not correspond to human albumin, a possible contaminant of HDL that migrates slightly faster than apoA-I. In 10% gels containing sodium dodecyl sulfate, the am-B HDL (patterns 4, 6, and 13 of Figure 3) contains a major protein with an apparent molecular weight of about 40 000 which is not present in the mercaptoethanol-treated apoHDL (3 and 5 of Figure 3) or in normal HDL (patterns 1 and 2). ApoA-II also is reduced by mercaptoethanol to the monomeric species. In samples treated with mercaptoethanol (e.g., pattern 3 of Figure 3), the apoA-I band is much larger than in the sample before reduction (pattern 4). Apparently the 40 000 component was reduced to subunits similar in effective size to apoA-I. Although it appears that a subunit of about 10 000 daltons (patterns 6 and 12) is present in the "new" proteins of am-B HDL without mercaptoethanol, it was not formed except possibly in small amount on reduction of the 40 000 species (compare patterns 3 and 4 and 7 and 8). Densitometric scanning of the gels (Figure 4) confirmed the increase in stained protein in the apoA-I region after reduction with mercaptoethanol.

The two "new" proteins separated by polyacrylamide gel isoelectric focusing were eluted from gel slices and electrophoresed in sodium dodecyl sulfate gels (patterns 11 and 12 of Figure 3). The sodium dodecyl sulfate–polyacrylamide gel electrophoresis pattern of the less acidic (p*I* about 6.5) component showed a major species with an apparent molecular weight of about 25 000 (slightly smaller than apoA-I) and smaller amounts of species with higher and lower molecular weights; the more acidic (p*I* about 6.0) component yielded mainly species with molecular weight of about 10 000–12 000 and small amounts of higher molecular weight species. The sodium dodecyl sulfate–polyacrylamide gel electrophoresis patterns of am-B apoHDL with and without mercaptoethanol

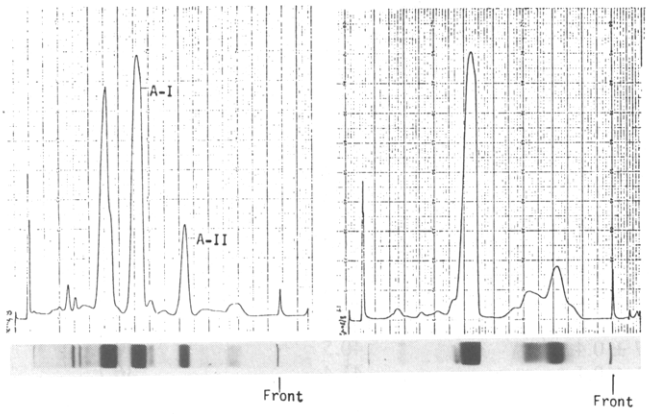

FIGURE 4: Densitometric scans of sodium dodecyl sulfate–polyacrylamide gel electrophoresis patterns of am-B apoHDL$_3$ (30 μg/gel) without (on left) and with (on right) mercaptoethanol treatment.

and of the proteins isolated by polyacrylamide gel isoelectric focusing suggest an associating–dissociating system involving sulfhydryl–disulfide interconversion in the "new" apolipoproteins.

In am-B serum, very little of the two "new" proteins was found in lipoprotein fractions other than in the HDL. Small amounts of the proteins were present in the VLDL and IDL but were not detectable in the LDL. None of the lipoprotein fractions isolated from normal serum contained more than very small amounts of the "new" proteins. The essentially lipoprotein-free fraction ($d > 1.25$ g/mL) of serum was not examined for content of the apolipoproteins. Among the lipoproteins of am-B serum, the HDL$_3$ fraction contains by far the largest amount of the "new" apolipoproteins because HDL$_3$ are much more abundant than the HDL$_2$ and the d 1.20–1.25 lipoproteins (shown in a later section) and the amount of "new" proteins relative to other proteins is greatest in the HDL$_3$.

Agarose and Polyacrylamide Gel Electrophoresis of Lipoproteins. Electrophoresis of isolated lipoproteins and whole serum in agarose or in large pore polyacrylamide gels gave no evidence of abnormality in electrophoretic behavior or heterogeneity resulting from the presence of abnormal lipoprotein species containing the "new" apolipoproteins and less or no apoA-I or apoA-II. In large pore polyacrylamide gels (Figure 5), the d 1.081–1.125 g/mL fraction contains some LDL-like (β mobility) lipoproteins as well as the usual HDL$_2$ molecules (pattern 1); the am-B HDL$_3$ (pattern 2) and normal HDL$_3$ (pattern 3) are very similar and the mixture of the two gives one band (pattern 4). The LDL concentration in serum was somewhat higher and the HDL lower during am-B treatment than at 3 months after termination of treatment. This was observed by densitometric scanning of the large pore gels of whole serum and in agarose electrophoresis patterns of whole sera that were stained for lipids after electrophoresis.

Analytical Ultracentrifugation of Serum Lipoproteins from Patients during Amphotericin B Treatment. Ultracentrifugal analysis of serum lipoproteins (Figure 6) as described by Lindgren et al. (1972) indicated that the concentration of HDL was lower and of LDL higher at the end of am-B treatment than normal; otherwise the distribution of lipoproteins within the various density classes was similar to that in normal males 35–50 years of age (Lindren et al., 1972). The HDL was predominantly HDL$_3$ as in normal male humans, and the $S_f°$ rate of the LDL, 6.95, was within the normal range (6.20 ± 0.96). The concentrations of HDL$_3$ (162 mg/dL) and of HDL$_2$ (28 mg/mL) were lower than the averages of 221.9 ± 30.8 and 53.4 ± 48.3 mg/dL, respectively, in age-matched normal

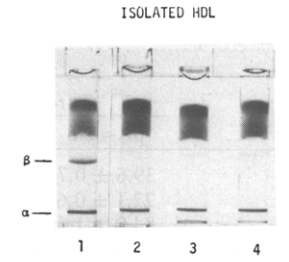

FIGURE 5: Large pore polyacrylamide gel electrophoresis of isolated HDL subfractions. (1 and 2) HDL$_2$ and HDL$_3$ (125 μg of each) isolated from am-B patient after 6 weeks of treatment; (3) normal HDL$_3$ (125 μg); (4) am-B HDL$_3$ (62.5 μg) + normal HDL$_3$ (62.5 μg).

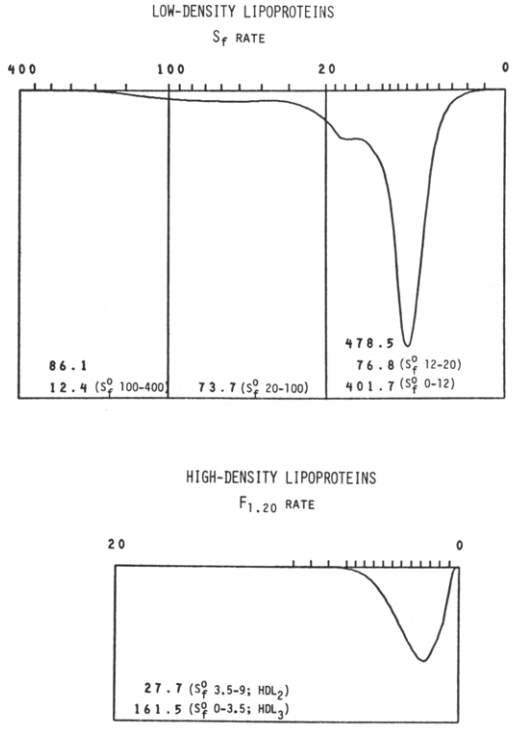

FIGURE 6: Computer-reconstructed diagrams of the schlieren patterns of serum lipoproteins of patient at end of treatment with am-B. Details of the computer program which generates the diagrams are given by Lindgren et al. (1972). The concentrations of the lipoprotein classes ($S_f°$ 0–12, etc.) are given in mg/100 mL serum.

males; the concentrations of LDL (402 mg/dL) and IDL (77 mg/dL) were higher than the average of 367.9 ± 80 and 38.3 ± 22.4 mg/dL, respectively, in age-matched normal males (Lindgren et al., 1972).

Lipids of Serum and High Density Lipoproteins. The plasma cholesterol and triglyceride concentrations were quite similar during and after treatment, despite the changes in lipoprotein distribution. Total cholesterol was 190 mg/dL at the end of treatment and 214 mg/dL 3 months after treatment was stopped; triglycerides were 165 and 153 mg/dL, respectively.

The lipid composition of the HDL during and after treatment was compared with that of normal HDL. The HDL$_3$ preparations isolated during and after treatment were 49.4 and 51.8% by weight lipid, respectively, and the corresponding HDL$_2$ preparations were 62.7 and 62.0% lipid. Normal HDL$_3$ preparations were 46–47% lipid. The lipid moieties of HDL during and after treatment were similar in phospholipid content: 42.3 and 42.7% by weight, respectively, for HDL$_3$ lipids, and 40.2 and 39.6%, respectively, for HDL$_2$ lipids. The values

TABLE I: Amino Acid Composition (Moles/10^3 Moles of Amino Acids) of Apolipoproteins Isolated from HDL_3 of Amphotericin B Treated Patient.

	Threonine-poor apolipoproteins		Apo A-I	
	$pI \approx 6.5$	$pI \approx 6.0$	Obsd	Expected
Lys	39.6 ± 0.7	44.8 ± 1.8	84.7	81.6
His	22.1 ± 0.6	22.9 ± 1.0	19.0	20.4
Arg	84.0 ± 1.5	77.7 ± 1.4	63.1	65.3
Asp	140.5 ± 0.3	129.9 ± 0.3	85.9	85.7
Thr	5.3 ± 0.4	10.3 ± 0.1	38.7	40.8
Ser	83.2 ± 0.5	85.8 ± 0.2	64.2	69.4
Glu	97.4 ± 1.5	111.7 ± 1.0	191.5	187.8
Pro	40.4 ± 0.4	38.7 ± 0.4	40.5	40.8
Gly	107.3 ± 1.3	101.1 ± 0.1	41.4	36.7
Ala	149.9 ± 0.7	137.0 ± 1.3	78.1	77.6
Val	9.8 ± 0.6	18.9 ± 1.0	51.0	53.1
Met	10.8 ± 0.7	18.5 ± 0.6	12.1	12.3
Ile	21.8 ± 0.2	20.7 ± 0.3	0	0
Leu	40.5 ± 1.0	44.6 ± 0.7	159.1	163.3
Tyr	42.2 ± 0.9	42.9 ± 1.2	26.2	24.5
Phe	73.2 ± 0.8	67.0 ± 1.0	24.3	24.5
Trp	31.8 ± 1.0	28.4 ± 1.4	16.0	16.3

for the lipids extracted from two normal HDL_3 samples were 45.8 and 44.5% phospholipid. Thin-layer chromatography of the lipids of HDL_2 and HDL_3 indicated that both fractions were richer in triglycerides and poorer in cholesterol and cholesteryl esters during am-B treatment than at 3 months after treatment was stopped. The lipids of normal HDL were also poorer in triglycerides than the am-B HDL.

Amino Acid Composition of the Apolipoproteins. Quantitative amino acid data (Table I) on the two "new" proteins isolated from am-B apoHDL_3 by polyacrylamide gel isoelectric focusing indicate that they are similar to one another but possibly not identical and are distinctly different from previously characterized apolipoproteins. Each protein was analyzed three times: on two isolations after 6 weeks of treatment (once from pH 4 to 8 gels and once from pH 5 to 7 gels) and one isolation from pH 4 to 8 gels at the end of treatment. ApoA-I components isolated at the same times were analyzed as a control. The two "new" apolipoproteins contain much less threonine, in moles percent, than do other apolipoproteins. They are relatively poor in valine and leucine and relatively rich in aspartic acid, glycine, alanine, phenylalanine, and arginine. On the basis of one residue of threonine per mole, the minimum molecular weight for the less acidic component with an apparent pI of about 6.5 is about 22 000 and that of the more acidic component with an apparent pI of about 6.0 about 11 000. These values of minimum molecular weight correspond reasonably well to those estimated from sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the proteins eluted from gel slices after polyacrylamide gel isoelectric focusing.

Discussion

The two threonine-poor apolipoproteins of HDL appear to be variants of the same protein. They are very similar in amino acid composition and in general both, not just one, are increased relative to apoA-I and apoA-II in abnormal circumstances that favor their accumulation. However, the size of the subunit is difficult to reconcile with this hypothesis. There is a small subunit with a molecular weight of about 10 000. Yet the major component, after reduction by mecaptoethanol, yields mainly, if not entirely, a subunit with a molecular weight of about 25 000. The molecular weight of the dimeric form, which appears to be smaller than ovalbumin, cannot be estimated accurately from sodium dodecyl sulfate–polyacrylamide gel electrophoresis because of a possible effect of disulfide cross-links on Stokes radius (note the effect of mercaptoethanol on the mobility of serum albumin and ovalbumin in patterns 9 and 10 of Figure 3). If the dimeric form comprised two subunits differing in amino acid composition and molecular weight, the isoelectric focusing pattern should have been altered by pretreatment of the protein with mercaptoethanol. This was not the case (patterns 10 and 11 of Figure 1). Further studies involving partial or complete amino acid sequencing and "fingerprinting" of peptides from proteolysis or treatment with group-specific reagents such as cyanogen bromide are needed to determine the structural homology between the two proteins. Such studies along with carbohydrate analyses would also establish the molecular basis of their difference in isoelectric points, which is considerably larger than the differences among variants of apoA-I, apoC-III, or the arginine-rich protein.

In studies of lipoproteins, urea–polyacrylamide gel electrophoresis or particularly sodium dodecyl sulfate–polyacrylamide gel electrophoresis is frequently used as the main or sole means of identifying apolipoproteins in isolated lipoproteins or in fractions of apolipoproteins separated by chromatography. The patterns of Figures 2 and 3 show that both methods can be inadequate for this purpose. The threonine-poor apolipoproteins would not be separated from apoA-I under less favorable circumstances in urea–polyacrylamide gel electrophoresis or by sodium dodecyl sulfate–polyacrylamide gel electrophoresis of mercaptoethanol-treated samples. In sodium dodecyl sulfate–polyacrylamide gel electrophoresis, the smaller subunit could not be distinguished from apoC-II and apoC-III, and without mercaptoethanol the larger one could be mistaken for arginine-rich apolipoprotein or apoA-IV, the rat apolipoprotein, which has a subunit size of 46 000 with or without mercaptoethanol and is very unlike the threonine-poor apolipoproteins in amino acid composition and isoelectric point (Swaney et al., 1974; Gidez et al., 1977).

The physiological and biochemical mechanisms responsible for enrichment of the threonine-poor apolipoproteins in plasma HDL during am-B treatment and in diabetic and nephrotic patients are not yet evident. These patients had in common low concentrations of plasma HDL and possibly acidosis. The two proteins were also found in HDL of rabbits and monkeys, particularly those that hyporespond to cholesterol feeding (unpublished observations). Thus, the am-B effect might be useful in animal studies in elucidate factors regulating HDL

composition and the functions of the threonine-poor apolipoproteins in the transport and metabolism of lipids. Am-B forms complexes with cholesterol in cell membranes (Norman et al., 1972) and lipoproteins (Klimov & Nikiforova, 1973). Membrane properties and functions are altered, and in humans renal tubular acidosis of the distal tubule type, probably related to the hypokalemic and nephrocalcinosis-producing effects of the drug, is a common toxic effect (Medoff, 1976). However, at peak concentrations of 1 to 4 μg/mL plasma, am-B would be present in only a small fraction of plasma HDL particles.

No doubt it is of some metabolic significance that the threonine-poor apolipoproteins are concentrated in the HDL_3, rather than in the HDL_2 subfraction, and are found in smaller proportion in the VLDL. Most of the minor apolipoproteins of HDL, apoC-I, apoC-II, apoC-III, and arginine-rich apolipoproteins are more abundant in HDL_2 than in HDL_3, and are even more concentrated (% apolipoprotein) in the VLDL than in the HDL. These apolipoproteins, or their complexes with lipids, cycle between VLDL and HDL. However, the metabolic relationships among the HDL_3 and HDL_2 are not well understood. Possibly the HDL_3 are more closely related to lipoprotein intermediates formed by lecithin:cholesterol acyl transferase action on nascent HDL. The threonine-poor apolipoproteins might be components of nascent HDL that are lost during conversion of the predominant HDL species in plasma or, alternatively, of normally minor species of lipoproteins that are accumulated in plasma under certain abnormal metabolic conditions or as a consequence of drug action. The electrophoretic and ultracentrifugal analyses of am-B HDL did not provide evidence for lipoprotein species that contain the "new" apolipoproteins but not apoA-I or apoA-II, but the possibility cannot yet be ruled out.

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