Characterization of the Apolipoproteins of Rat Plasma Lipoproteins†

John B. Swaney,* Federico Braithwaite, and Howard A. Eder

ABSTRACT: Purified fractions of three major rat high-density lipoproteins (HDL) and one rat very low-density lipoprotein (VLDL) were isolated by Sephadex gel chromatography or preparative sodium dodecyl sulfate gel electrophoresis. These proteins were characterized by amino acid analysis, end-group analysis, molecular-weight determination, polyacrylamide gel electrophoresis, and circular dichroism. One of these rat proteins, of molecular weight 27 000, appears to be homologous with the human A-I protein. However, rat HDL possesses two additional major components not reported in human HDL—an arginine-rich protein of molecular weight 35 000, and a protein of molecular weight 46 000. The arginine-rich protein of the rat is similar in size and amino acid analysis to the arginine-rich protein reported in human VLDL. A major component of rat VLDL of 35 000 molecular weight appears similar or identical to the arginine-rich protein in rat HDL by every criterion employed for their characterization.

The rat has been demonstrated to be a useful animal model for studies on the metabolism of the plasma lipoproteins. Thus, the earliest studies on the turnover of the apolipoproteins of the lipoproteins were performed in the rat (Roheim et al., 1971). Windmueller et al. (1973) have studied biosynthesis of apolipoproteins by isolated perfused rat liver and intestine. It is possible to induce a variety of alterations in the apolipoprotein patterns by such techniques as feeding rats diets high in sucrose (Shiff et al., 1971) or cholesterol (Kuehl et al., 1974), as well as by the induction of diabetes (Bar-On et al., 1976). Recently, binding and interiorization of rat lipoproteins by cells in tissue culture have been studied (Bierman et al., 1974). In order to extend the usefulness of this animal model, we have undertaken a detailed characterization of the apolipoproteins of the rat. A preliminary report of our results has been published (Swaney et al., 1974).

The first studies on the rat apolipoproteins by Koga et al. (1971) and Bersot et al. (1970) showed the existence of multiple protein components of the rat serum lipoproteins, some of which appeared to be counterparts of apoproteins of the human lipoproteins. Herbert et al. (1974) have recently reported a detailed study of the low-molecular-weight apolipoprotein components of rat serum high-density lipoproteins. The present study deals with isolation and characterization of the middle-molecular-weight range (25 000–50 000) subunits which make up the bulk of the HDL[1] proteins, one of which is also an important component of VLDL.

Materials and Methods

Reagents. Carboxypeptidases A and B were obtained from Worthington Biochemicals, Freehold, N.J. Urea (ultra pure) was purchased from Schwarz/Mann, Orangeburg, N.Y. Methanesulfonic acid and dansyl chloride were purchased from Pierce Chemical Co., Rockford, Ill. Hydrazine (95%+) was obtained from Eastman Organic Chemical Co., Rochester, N.Y.

Lipoproteins. Rat serum was obtained from Sprague-Dawley rats maintained for 15–22 days on a semipurified high-sucrose diet, which contained (w/w): 20% vitamin-free casein, 60% sucrose, 5% lard, 1% vitamin mixture, 4% salt mixture, and 10% cellulose (obtained from General Biochemical Division, Chagrin Falls, Ohio). Serum from animals fed this diet contains elevated levels of VLDL and HDL (Shiff et al., 1971). Some of the studies were repeated with apolipoproteins obtained from chow-fed rats; no differences in results were observed. Ethylenediaminetetraacetic acid (EDTA), disodium salt, was added to the serum (1 mg/ml) before ultracentrifugation or directly to the blood after the animals were bled (1–2 drops of 20% EDTA/30 ml). Lipoprotein separation was carried out by the method of Havel et al. (1955) using the SW 41 rotor in the Beckman, Model L2-65B, ultracentrifuge at 15 °C. The serum was first centrifuged at 10 000 rpm for 30 min to remove chylomicrons. VLDL ($d < 1.006$) and LDL (d 1.006–1.063) were separated by 20 h of ultracentrifugation at 200 000g, while HDL (d 1.063–1.21) was separated by 40 h of ultracentrifugation at 200 000g. Densities above 1.006 were adjusted by addition of solid KBr.

The lipoprotein fractions were washed by resuspending them in the appropriate salt solution and repeating the centrifugation. That the HDL prepared by this method contains no LDL has been previously demonstrated in this laboratory (Lasser et al., 1973), and this was confirmed by electrophoresis which showed no B protein in the HDL. The washed lipoprotein solutions were dialyzed against 0.05% EDTA and were stored in the cold until used. Delipidated lipoprotein fractions were obtained by lyophilization, followed by extraction with ethanol–ether (3:1) by the procedure of Brown et al. (1969).

Apolipoprotein Isolation. Delipidated HDL or VLDL was dissolved in 0.2 M Tris-HCl, pH 7.5, 6 M urea (ultra pure), 2 mM decyl sulfate (buffer A) and applied to a 95 × 2.5 cm column of Sephadex G-200 equilibrated with buffer A. Some fractions were then pooled and dissolved in 0.2 M Tris-HCl, pH 7.5, 6 M urea (ultra pure), 0.1% sodium dodecyl sulfate (buffer B) and applied to a series of three tandem columns of Sephadex G-200 of 2.5-cm diameter and 270 cm in total length, which were equilibrated with buffer B. Column effluents were monitored at 280 nm with a Gilford, Model 240, recording spectrophotometer.

† From the Departments of Biochemistry and Medicine of the Albert Einstein College of Medicine, New York, New York 10461. *Received April 27, 1976.* Supported by Research Grants HL 02965 and HL 14236 from the National Institutes of Health, and Training Grant HL 5273 from the United States Public Health Service.

[1] Abbreviations used are: HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very low-density lipoprotein; CD, circular dichroism; ARP, arginine-rich protein; LMW, low molecular weight proteins of rat HDL; Glx, glutamic acid or glutamine; Asx, aspartic acid or asparagine; EDTA, ethylenediaminetetraacetic acid.

HDL apolipoproteins were also obtained by preparative sodium dodecyl sulfate gel electrophoresis, where 300–400 μg of apo-HDL were applied to cylindrical gels (7 mm i.d.). Electrophoresis was then carried out in 0.1 M sodium phosphate buffer (pH 6.9) with 0.1% sodium dodecyl sulfate by the procedure of Shapiro et al. (1967), as modified by Maizel (1971), except that the gels were cooled to 17 °C and electrophoresis was carried out for 16–18 h at reduced voltage. One of the 18 gels was stained with Coomassie blue and used as a template for cutting out the desired bands in the remaining gels. Initially, these gel sections were crushed either manually or with a polytron homogenizer and the proteins were eluted by diffusion. More recently, we have employed a modification of the method of Stevens (1975) in which the gel slices were reinserted into electrophoresis tubes and the components were electrophoresed into dialysis tubing, thus trapping the protein.

Analytical Sodium Dodecyl Sulfate Gel Electrophoresis. Analysis of apolipoprotein fractions obtained by these various methods was carried out with the sodium dodecyl sulfate–phosphate buffer system of Shapiro et al. (1967), using a slab gel apparatus or cylindrical gels. The column chromatographic fractions were analyzed with a slab containing a single concentration of acrylamide, but other analyses utilized the acrylamide gradient slab sodium dodecyl sulfate method of Swaney and Kuehl (1976). In this method, a linear gradient of acrylamide, ranging from 3% at the top to 27% at the bottom, is employed to maximize resolution of high- and low-molecular-weight proteins.

Amino Acid Analysis. Time course acid hydrolysis of purified apolipoproteins was carried out by hydrolysis in 5.6 N HCl for 18, 45, and 90 h, followed by analysis on a Beckman 120C amino acid analyzer with amplified range capacity. Analysis for tryptophan was performed by hydrolysis in 4 N methanesulfonic acid by the method of Liu, as reported by Moore (1972). Half-cystine and methionine were analyzed by acid hydrolysis following performic acid oxidation, as described by Moore (1963).

Dansylation. Purified proteins were reacted with dansyl chloride in the presence of 8 M urea and 1% sodium dodecyl sulfate solutions for 3 h at 37 °C. The dansylated proteins were separated from low-molecular-weight reactants and products by passage through Sephadex G-25 columns constructed from Pasteur pipettes. After lyophilization, the proteins were hydrolyzed in 5.6 N HCl and the dansylated amino acids were identified by chromatography on polyamide thin-layer plates, using the method of Weiner et al. (1972).

Hydrolysis with Carboxypeptidase. The carboxyl-terminal residues of A-I were determined by incubating the protein with carboxypeptidase A at a substrate–enzyme ratio of 25:1 at 37 °C for various periods of time; released amino acids were measured with a Beckman 120C amino acid analyzer.

Hydrazinolysis. In some cases, C-terminal amino acids in A-I and A-IV were determined by hydrazinolysis, using the method of Fraenkel-Conrat and Tsung (1967). A Beckman 120C amino acid analyzer was used for quantitation. Since only a few nanomoles of protein was hydrazinolyzed, amino acids were analyzed by direct application to the long column of the amino acid analyzer.

Double Immunodiffusion. Double immunodiffusion was carried out as described by Ouchterlony (1968). Antisera prepared against lipoprotein fractions and purified apolipoproteins were a gift of Dr. Paul Roheim.

Urea Gel–Sodium Dodecyl Sulfate Two-Dimensional Slab Gel Electrophoresis. Apo-HDL and apo-VLDL were electrophoresed on 0.3 × 10 cm gels in the urea gel system of Kane (1973), except that no tetramethylurea was used because the samples were already delipidated. One gel was stained with Coomassie brilliant blue and a duplicate gel was embedded horizontally in a 15% acrylamide slab gel. Electrophoresis was then carried out vertically into the slab using the sodium dodecyl sulfate–phosphate system (Shapiro et al., 1967). The slab gel was stained for 2 h in 0.1% Coomassie brilliant blue in 50% methanol and 9% acetic acid. Destaining was carried out by diffusion in 7% acetic acid and 5% methanol.

Quantitative Sodium Dodecyl Sulfate Gel Electrophoresis. Purified apolipoprotein samples, whose protein content was determined by amino acid analysis, were electrophoresed on 12% acrylamide cylindrical gels in the sodium dodecyl sulfate–phosphate system (Shapiro et al., 1967). Typically, four samples each of A-I, ARP, and A-IV, spanning a concentration range from 1–10 μg (in one instance, 2–25 μg were used) were quantitatively applied to gels and electrophoresed at the same time as samples of apo-HDL. Gels were then stained 6 h in 0.1% Coomassie brilliant blue and destained by diffusion in 7% acetic acid and 5% methanol. The stained bands were scanned at 498 or 530 nm with a Gilford spectrophotometer equipped with a linear transport device and an accessory for integration. From the scans, a value for area (in arbitrary units) per microgram of purified protein applied was obtained by averaging the results from each group of gels. This factor was then used to compute the amount of each apoprotein in apo-HDL from the area of the appropriate band obtained by scanning.

Circular Dichroic Measurements. CD studies were performed using a Cary, Model 60, spectropolarimeter with a 6001 CD attachment, which was calibrated at 280 nm with (+)-camphorsulfonic acid. The optical path length of the cell was 1.0 cm. Absorbancies of the solution were maintained to give readings below 0.6 kV dynode voltage at all wavelengths. A mean residue weight, based on amino acid analysis, of 114 was used for calculation of molar ellipticity for all samples except A-IV, for which 113 was used. Protein concentrations were determined by amino acid analysis. The percentages of the various forms of secondary structure were calculated by a computer analysis described by Listowsky et al. (1972).

Results

By elution from sodium dodecyl sulfate gels, three principal fractions were obtained: the A-I, ARP (arginine-rich protein), and the A-IV proteins (Swaney et al., 1974), as well as a heterogeneous low-molecular-weight fraction which corresponds to the HS-III Sephadex fraction of Bersot et al. (1970). Re-electrophoresis of these fractions on a sodium dodecyl sulfate slab gel (Figure 1a) shows that these proteins retain their electrophoretic properties. An exception to this are the minor bands of molecular weight 55 000–70 000 seen in the apo-HDL, which can also be seen faintly in purified A-I and ARP, suggesting that these bands might be aggregated forms of these proteins which are not completely dissociated in the detergent.

It should be noted that on occasional gels, the ARP band in HDL and the corresponding band in VLDL appeared to be resolved into two closely spaced zones, suggesting possible heterogeneity of this component.

Because the gel-extraction method yields only limited quantities of purified proteins, Sephadex gel filtration was used to prepare larger quantities of apolipoproteins. Chromatography of apo-VLDL on a 2.5 × 90 cm column of Sephadex G-200 yielded three fractions (VS-I, VS-II, and VS-III), as

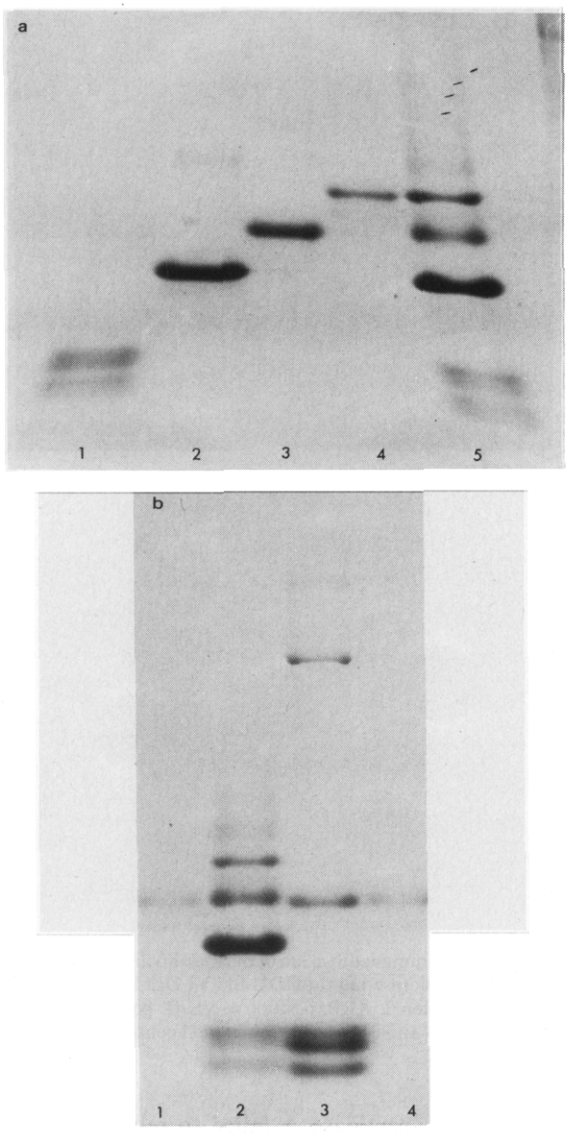

FIGURE 1: Sodium dodecyl sulfate gel electrophoresis of delipidated rat lipoproteins and purified apolipoprotein fractions on 3–27% acrylamide gradient gels. (a) Position 1, fraction LMW; position 2, purified A-I; position 3, purified ARP; position 4, purified A-IV; position 5, apo-HDL. (b) Position 1, VS-II; position 2, apo-HDL; position 3, apo-VLDL; position 4, VS-II.

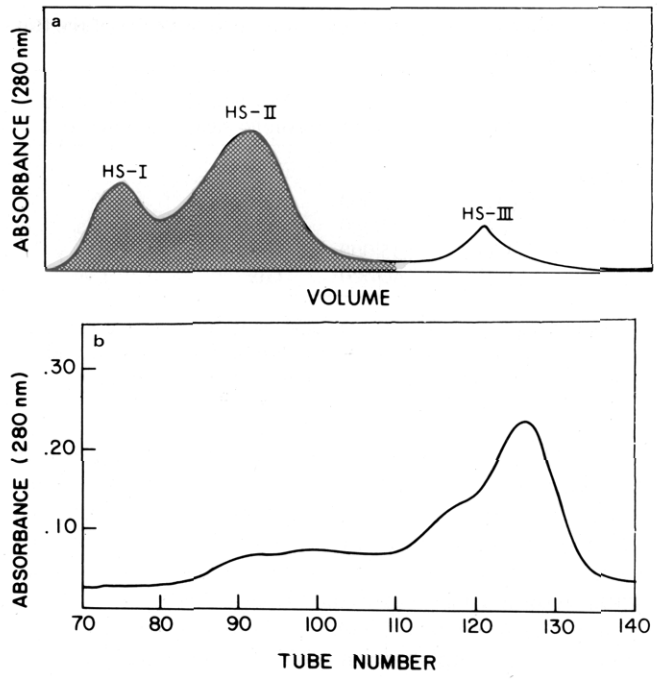

FIGURE 2: Sephadex gel permeation chromatography of rat apo-HDL. (a) Chromatography of rat apo-HDL on a 2.5 × 90 cm column of Sephadex G-200 in 7 M urea buffer with sodium dodecyl sulfate. The fractions in the cross-hatched area were pooled, concentrated, and applied to a 2.5 × 270 cm column of Sephadex G-200 in 7 M urea, 0.1%, which yielded the elution tracing shown (b).

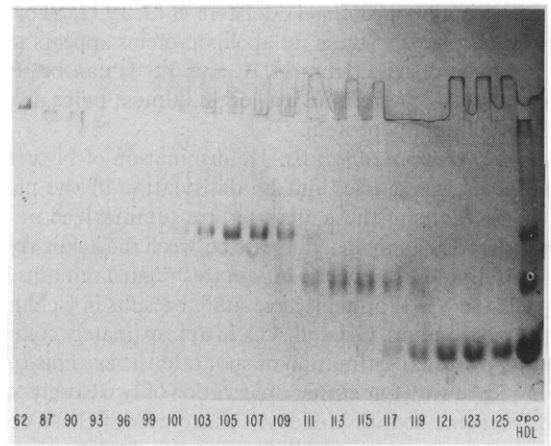

FIGURE 3: Sodium dodecyl sulfate gel electrophoresis on 10% polyacrylamide slabs of aliquots from individual tubes obtained in the elution of apoproteins from a column of Sephadex G-200 (Figure 2b). The numbering in the figure corresponds to the tube numbers shown in Figure 2b. The gel on the right shows the simultaneous electrophoresis of a sample of rat apo-HDL.

reported by Bersot et al. (1970) and the VS-II fraction was used for subsequent characterization studies. Electrophoresis of this fraction is shown in Figure 1b. It can be seen to have the same mobility, and, hence, molecular weight, as ARP from HDL.

With these columns, separation of the apoproteins of HDL was incomplete. Of the three peaks observed, the greater part of the first peak (HS-I, Figure 2a) consisted of large molecular species, which on sodium dodecyl sulfate gel electrophoresis dissociated into A-I, ARP, and A-IV, indicating that this fraction contained aggregates of these proteins. HS-II contained principally A-I, but also ARP and A-IV. The major portion of A-IV was found in the trough between HS-I and HS-II.

In order to disaggregate these complexes and achieve better resolution of the protein components, the HS-I and HS-II fractions shown in Figure 2a (cross-hatching) were pooled for rechromatography. They were concentrated and dialyzed against a different buffer which contained 0.1% sodium dodecyl sulfate in place of the decyl sulfate, and the samples were subjected to gel filtration on Sephadex G-200 using three columns 90-cm long in tandem. Figure 2b shows the elution tracing which resulted. Selected tubes were run on sodium dodecyl sulfate gel electrophoresis (Figure 3) and it was apparent that, although there was some overlapping of bands, there was substantial improvement in resolution: The A-IV protein was found in tubes 100–111, ARP in tubes 109–119, and A-I in tubes 117–132; by pooling tubes in the middle of these ranges purified fractions of these proteins could be obtained for subsequent studies.

Molecular Weight Analysis. Molecular weight estimates were obtained by sodium dodecyl sulfate electrophoresis in 10%

TABLE I: Middle-Molecular-Weight Range Apoproteins of the Rat Plasma Lipoproteins.

	Protein	Mol Wt[a]	N-terminal Amino Acid[b]	C-terminal Amino Acid[c]
VLDL	VS-II (ARP)	35 000	Glx, Asx	*d*
HDL	A-I	27 000	Asx	Ala
	ARP	35 000	Glx, Asx	*d*
	A-IV	46 000	Glx	Lys

[a] Determined by sodium dodecyl sulfate gel electrophoresis. [b] Determined by dansylation. [c] Determined by carboxypeptidase hydrolysis and hydrazinolysis. [d] Not determined (see text).

acrylamide gels. A linear calibration curve was obtained with molecular-weight standards, and this was used to calculate the molecular-weight averages, which are presented in Table I and which are derived from six experiments. The values obtained agreed to within 6%, but a more reasonable estimate of potential error is 10% (Weber and Osborn, 1969).

Amino Acid Analysis. Each of the purified proteins was subjected to a complete amino acid analysis, which included a time-course hydrolysis for which there were two analyses for each hydrolysis period. Separate analyses for tryptophan were carried out, as well as performic acid oxidation, followed by acid hydrolysis, for half-cystine and methionine. The results obtained are summarized in Table II, which presents the data both in weight percentages and in moles of amino acids per mole protein, based on estimated molecular weights. Unlike the human apolipoproteins (Edelstein et al. 1972; Shore and Shore, 1968), none of these rat apolipoproteins appears to lack any amino acid. ARP from HDL and VS-II can be seen to possess about 13% arginine by weight, almost twice as much as any other apolipoprotein.

Amino-Terminal Analysis. Determination of N-terminal amino acids was carried out by dansylation of the purified proteins. Results of these analyses are summarized in Table I. This method cannot discriminate between the acidic residues and their amides, so uncertainty in this regard remains. Both the ARP and VS-II proteins gave similar results in yielding two N-terminal residues, Glx and Asx, in approximately equivalent amounts by visual estimation of spot intensities. This finding was in accord with our earlier observation of two closely-spaced bands on some sodium dodecyl sulfate gels of these components, and confirms the possibility that these purified fractions contain more than one protein. Analysis of each protein was carried out on at least two samples.

Carboxyl-Terminal Analysis. Analysis of the carboxyl-terminal amino acid of the A-I protein was carried out by hydrolysis with carboxypeptidase A and by hydrazinolysis. Carboxypeptidase A digestion of the A-I protein indicated alanine to be the carboxyl-terminal residue (Swaney et al. 1974); no amino acids were released by digestion with carboxypeptidase B. This result was confirmed by hydrazinolysis, which showed alanine to be the only amino acid found in significant quantities after treatment with anhydrous hydrazine. Carboxypeptidase B failed to release any amino acids from ARP, but did release lysine from the A-IV protein. A time-course hydrolysis of A-IV with carboxypeptidase B released lysine, which rose to 0.87 mol/mol of protein by 5-h hydrolysis at room temperature with an enzyme–substrate ratio of 1:10. Carboxypeptidase B did, however, appear to release comparable amounts of aspartic acid and alanine under these conditions, even after subtraction of enzyme and substrate blanks.

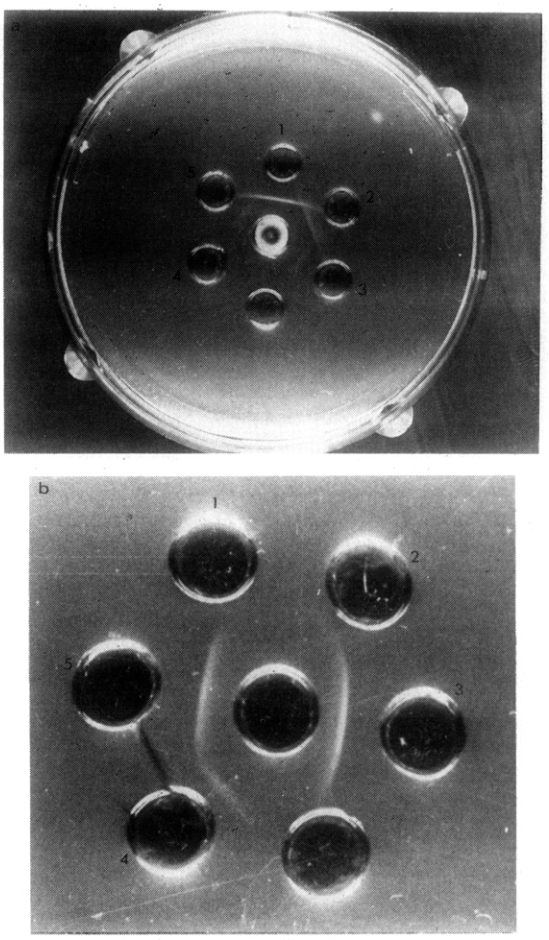

FIGURE 4: Double immunodiffusion of purified apolipoproteins reacted against antisera to sucrose-fed rat HDL and VLDL. Position 1, A-I; position 2, A-IV; position 3, ARP; position 4, VS-II; position 5, ARP. (a) Center well contains anti-HDL. (b) Center well contains anti-VLDL.

However, carboxypeptidase A failed to release more than background levels of any amino acid, and upon hydrazinolysis lysine was the predominant amino acid released, with no significant release of aspartic acid or alanine. These data suggest that lysine is the C-terminal amino acid of A-IV. Carboxypeptidase A released no significant amounts of amino acids from ARP and there was insufficient material to complete the study.

Blank runs with either carboxypeptidase A or B yielded only trace amounts of serine and glycine under the most rigorous conditions employed with the apolipoproteins. Amounts of these apolipoproteins were adjusted to yield between 1 and 4 nm of the released C-terminal amino acid.

Immunochemical Analysis. Purified components were reacted against antisera to rat HDL and rat VLDL by the method of Ouchterlony (1968) to determine immunological relatedness of the proteins (Figure 4a). It was found that the precipitin lines for the HDL components A-I, ARP, and A-IV did not coalesce and those for A-I and A-IV clearly intersected.

The ARP line was distinct from the A-I and A-IV lines. Antiserum prepared against ARP did not cross-react with A-I or A-IV, nor did ARP react with anti-A-I or anti-A-IV. Immunologic identity of ARP, prepared from HDL and VLDL, is demonstrated in Figure 4b, where ARP from HDL and from VLDL formed precipitin lines with anti-VLDL that coalesced. On the basis of these immunochemical data, as well as amino

TABLE II: Amino Acid Analysis of Rat Apolipoproteins.[a]

	A-I mol/ 27 000	Range	A-IV mol/ 46 000	Range	ARP mol/ 35 000	Range	VS-II (ARP) mol/ 35 000	Range
Lys	20.7 (9.8%)	19–24	27.4 (7.6%)	25–29	14.6 (5.4%)	14–15	11.5 (4.3%)	8–14
His	4.7 (2.4%)	4–6	6.3 (1.9%)	6–8	2.1 (0.8%)	1–4	1.7 (0.7%)	1–2
Arg	12.2 (7.0%)	8–15	16.4 (5.6%)	15–18	29.1 (13.0%)	24–33	30.3 (13.7%)	29–33
Asp	31.4 (13.4%)	31–32	50.2 (12.6%)	45–52	27.7 (9.1%)	27–28	26.3 (8.7%)	25–28
Thr	11.2 (4.2%)	10.9–11.4	21.2 (4.7%)	20.9–21.3	15.3 (4.4%)	15–16	14.7 (4.3%)	13–16
Ser[b]	10.1 (3.3%)		22.1 (4.2%)		14.2 (3.5%)		15.1 (3.8%)	
Glu	41.9 (20.0%)	41–42	82.5 (23.2%)	70–88	69.6 (25.7%)	63–74	72.6 (27.1%)	72–74
Pro	6.7 (2.4%)	6–7	16.8 (3.6%)	16–19	6.6 (1.8%)	4–10	5.5 (1.5%)	4–9
Gly	11.2 (2.4%)	11–12	19.6 (2.4%)	18–23	17.2 (2.8%)	17–18	20.8 (3.4%)	18–26
Ala	18.1 (4.8%)	17.9–18.4	26.6 (4.1%)	26–28	27.9 (5.7%)	27–29	28.8 (5.9%)	28–30
Half-Cys[c]	1.1 (0.4%)		2.2 (0.5%)		ND[d]		ND[d]	
Val	12.6 (4.6%)	11–14	26.2 (5.7%)	23–30	17.9 (5.1%)	16–19	18.9 (5.4%)	16–22
Met	5.9 (2.8%)		9.4 (2.7%)		12.0 (4.5%)		10.8 (4.1%)	
Ile	4.2 (1.8%)	4–5	9.8 (2.4%)	8–12	7.0 (2.3%)	6–8	7.4 (2.4%)	7–9
Leu	30.1 (12.6%)	30–31	49.8 (12.3%)	49–53	34.6 (11.2%)	34–36	33.0 (10.8%)	32–33
Tyr	4.9 (2.9%)	4–5	5.8 (2.1%)	5–7	3.8 (1.8%)	3–4	3.4 (1.6%)	2–4
Phe	6.7 (3.7%)	6–7	13.4 (4.3%)	11–16	4.6 (1.9%)	4–6	3.7 (1.6%)	3–5
Trp[e]	2.2 (1.5%)		1.3 (0.5%)		1.8 (1.0%)		1.4 (1.4%)	

[a] Results are reported as moles of amino acid per mole of protein and are followed by the range of values observed in the four to six determinations used to determine each average value. Underneath this value is a weight percentage for each amino acid in the sample. [b] Determined by extrapolating values for hydrolysis at 17, 45, and 90 h to zero time. [c] Determined as cysteic acid and methionine sulfone following performic acid oxidation (single determination). [d] ND, not determined. [e] Determined by hydrolysis with 3 N methanesulfonic acid (single determination).

acid analysis and molecular weight data, we conclude that the ARP of HDL and VS-II of VLDL share common components and both will be referred to as ARP.[2]

Although distinct precipitin bands were not seen when A-I and A-IV are reacted with anti-VLDL, the precipitin band for ARP (Figure 4b, position 3) does arc into the A-IV region. This could be due to slight contamination of A-IV with ARP.

Urea Gel Electrophoresis. It has been common practice for workers in this field to utilize electrophoresis in urea-containing gels for the identification of apolipoprotein components. In order to correlate these previous results with our present understanding of the rat apolipoproteins, we have utilized the two-dimensional procedure to provide this information. Samples of apo-HDL (Figure 5a) and apo-VLDL (Figure 5b) were electrophoresed on cylindrical gels containing urea. Replicate gels were stained and a schematic drawing of such a gel is shown in the upper portion of Figure 5a, whereas the actual replicate gel is shown in Figure 5b. It can be seen in Figure 5a that at the interface between the spacer and resolving gels there accumulate aggregates of the major apolipoproteins. This points out the difficulty in assuming that material in this region is a homogeneous entity, such as a "B" protein. This result is also in agreement with our Sephadex column separations, which showed aggregates in the early peak (HS-I) when apo-HDL was separated in a urea-containing buffer without sodium dodecyl sulfate.

In the diagram, in Figure 5a, are three closely spaced bands of relatively low mobility. In order of increasing mobility, these are the A-IV, ARP, and A-I proteins. These particular gels gave excellent resolution of these components; oftentimes only two bands are observed. The low-molecular-weight proteins (A-II, C-II, C-III-0, and C-III-3) have greater mobilities and have been identified by electrophoresis of purified individual components (Swaney and Gidez, unpublished observations).

Figure 5b shows a similar two-dimensional analysis of rat apo-VLDL. The apo-B present between the spacer and the resolving gel does not migrate in the 15% acrylamide slab. The ARP band can readily be recognized as the single band with slower mobility on the urea–polyacrylamide gel and as the spot

[2] Reaction of purified ARP or VS-II against anti-VS-II yields one major precipitin band and one faint additional band.

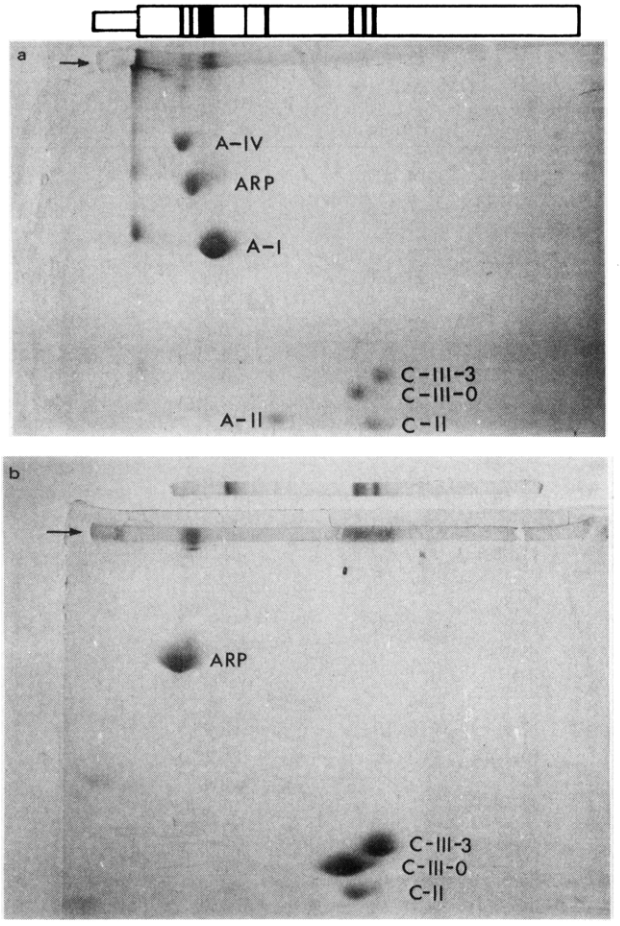

FIGURE 5: Two-dimensional separation of apo-HDL (a) and apo-VLDL (b). Apolipoproteins were first electrophoresed in urea-containing cylindrical gels and an unstained gel was positioned in the slab gel electrophoresis apparatus at the position shown by the arrow. A 15% acrylamide sodium dodecyl sulfate gel was then poured around it and electrophoresis was carried out in the vertical direction. Above the slab in gel A is shown a schematic diagram of a stained urea gel, while in gel B is shown an actual urea gel above the slab. Bands in this slab are spread out in the horizontal direction, relative to the cylindrical gel, because of elongation resulting from compression of the cylindrical gel between the glass plates of the slab apparatus.

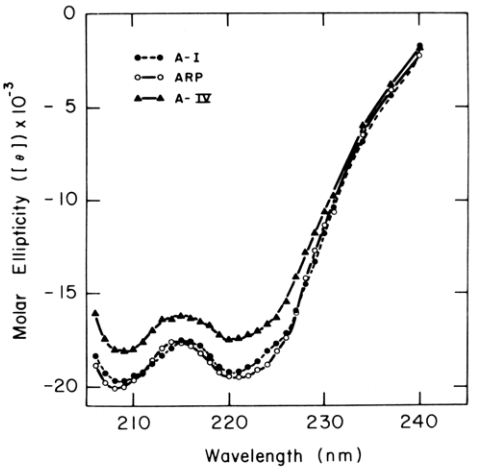

FIGURE 6: Circular dichroism of purified apolipoproteins. The curve shown for ARP was obtained from a sample dialyzed against 0.002 M Tris-HCl, pH 8.5. The tracing for the A-IV protein was obtained from a sample dialyzed exhaustively against urea, to more effectively remove sodium dodecyl sulfate, followed by dialysis against 0.002 M Tris-HCl, pH 8.5. The curve shown for A-I was obtained for samples treated by either of the above procedures.

in the upper left of the slab. The rat VLDL does not possess an A-II band, and the low-molecular-weight components form a group in the lower right of the slab gel and which are identified by analogy with the comparable HDL components.

Circular Dichroism. Purified apolipoproteins, obtained from preparative sodium dodecyl sulfate gels, were used for studies of secondary structure by circular dichroism. Since residual amounts of sodium dodecyl sulfate could alter the secondary structure (Visser and Blout, 1971), efforts were made to remove the detergent by the method of Weber and Kuter (1971). Samples of A-I and A-IV so treated were used to obtain CD spectra (Figure 6), but low recoveries of ARP, presumably bound to the ion-exchange resin used, prevented us from using ARP so treated for these experiments. As an alternative, a preparation of ARP was dialyzed exhaustively to remove the detergent, and the curve shown in Figure 6 is for such a sample. Samples of A-I and A-IV were also treated in this fashion. This A-I sample generated a spectrum virtually superimposable with the sample from which sodium dodecyl sulfate was more rigorously removed by ion exchange, whereas the A-IV gave a slightly more negative trough at 222 nm (−17 400 deg cm^2/dmol) than the previous sample, which is consistent with the promotion of helix by residual detergent. The curves shown in Figure 6 were used to calculate the peptide bond structures of the protein. The amounts of α helix and β structure observed, respectively, were as follows: A-I: 58% α, 9% β; A-IV: 52% α, 11% β; ARP: 58% α, 8% β. Since the complete removal of sodium dodecyl sulfate from these samples was not checked, these values must be regarded as only approximate.

Quantitation of Apoproteins in HDL. In order to ascertain the relative quantities of the apolipoproteins in the HDL molecule, we employed the procedure of Wright and Olins (1975), in which the proteins are separated by electrophoresis and the relative staining for each component is measured. The use of Coomassie brilliant blue was necessitated by the low quantities of protein available; the work of Bennett and Scott (1971) indicated that this dye could be used for quantitation if a spectrophotometer having a narrow spectral bandwidth was employed.

A linear relationship between area and weight of protein applied to the gel was found over the range of 1–25 μg applied to the gel in each of three separate experiments. The slope obtained by averaging the data was used to calculate the amount of each protein in apo-HDL samples, which were electrophoresed simultaneously. The slopes obtained for a given protein were found to vary with the age of the staining solution, length of time of staining, etc. Therefore, results were only computed for pure apoproteins and apo-HDL samples, which were treated identically; the amounts of apo-HDL components applied to the gel were in the range of linearity found for the purified apolipoproteins.

The intensity of staining per unit weight is higher for A-I than for ARP or A-IV. Thus, if such data were not utilized, estimates of the amounts of A-I would be erroneously high. The weight ratios of the major protein components of chow-fed and sucrose-fed rat HDL, relative to A-I, are given in Table III.

Since apo HDL was used for obtaining the relative weight ratios of A-IV, ARP, and A-I, the question arises whether selective losses of these components occur during the solvent delipidation procedure. To test this possibility, sodium dodecyl sulfate gel electrophoresis was carried out on five aliquots of an HDL fraction and of apo-HDL prepared from this same fraction. Optical density scanning of these gels revealed that

TABLE III: Relative Weight Ratios ± SD.

Lipoprotein/ Apolipoprotein	A-I	ARP	A-IV
Chow-fed rat HDL	1.0	0.35 ± 0.06	0.44 ± 0.15
Sucrose-fed rat HDL	1.0	0.57 ± 0.09	0.68 ± 0.12

the relative areas of different apoprotein bands for delipidated and undelipidated HDL agreed to within 8%. While there may be a slight preferential loss of A-I which results from solvent delipidation, the differences are quite small relative to the estimated error of the quantitation procedure itself. Consequently, the molar ratios estimated for apo-HDL are applicable to intact rat HDL, as well as apo-HDL.

Discussion

Recent investigations have revealed ever-increasing complexities of subunit structure in the lipoproteins. Studies by Koga et al. (1971) and Bersot et al. (1970) indicated rat HDL to be even more complex in its protein moiety than human HDL. Interpretation of turnover data and understanding of the effects of the metabolic perturbations on lipoprotein structure give special importance to the characterization of apolipoprotein components. Studies by Herbert et al. (1974) have already facilitated understanding of the low-molecular-weight components of rat HDL, and in the present study attention has been primarily directed at the proteins of rat HDL and VLDL of molecular weights between 25 000 and 50 000.

Rat apo-VLDL was resolved by Sephadex chromatography, yielding the three fractions described by Bersot et al. (1970): VS-I, VS-II, and VS-III. VS-I is the high-molecular-weight component also found in LDL. The VS-II fraction was intermediate in molecular weight (Figure 1b) and was used as such for analysis. The VS-III fraction contains the very low-molecular-weight VLDL components. Column chromatography was less useful for rat HDL because of the existence of several poorly resolved components in the 25 000–80 000 molecular weight range and because of residual aggregation, despite the presence of urea. Preparative sodium dodecyl sulfate gels proved most suitable for isolation of limited amounts of pure components, especially when electrophoretic elution of bands was employed for protein recovery.

The HDL of all species studied thus far (e.g., Shore and Shore, 1968; Cox and Tanford, 1968; Jonas, 1972; Edelstein et al., 1973) contains a component of molecular weight of 27 000, which constitutes a majority of the protein; this component is generally referred to as the A-I protein. The rat also possesses such a component (Table I), and so we have termed this the A-I protein. The amino-terminal residue of rat A-I (Asp or Asn) is probably identical to human A-I (Asp), but the carboxyl-terminal amino acid is Ala in the rat and Gln in the human. The amino acid composition of rat A-I is generally similar to human A-I (Baker et al., 1974). However, rat A-I possesses 4 mol of isoleucine/mol of protein, while human A-I has none. Rat A-I is also higher in aspartic acid and lower in arginine and leucine than the human counterpart. The other major component of human HDL, the A-II protein, is seen in Figure 5a to be only a minor component of rat HDL and could not be found in bovine HDL (Jonas, 1972).

Rat HDL also contains, in addition to several low-molecular-weight proteins, two additional major components, with molecular weights of 35 000 and 46 000. A 35 000 molecular weight component was also found in VLDL, and these proteins from both HDL and VLDL were found to be identical immunochemically and gave very similar results by N-terminal analysis (Table I) and by amino acid analysis (Table II). These components have been termed ARP, based on their relatively high arginine content. They are similar in molecular weight and amino acid analysis to the arginine-rich protein reported in human VLDL (Shore and Shore, 1973). The only substantial differences in amino acid composition between ARP of rat and human are large amounts of aspartic acid, glutamic acid, and methionine, and lower levels of proline and tryptophan in rat ARP (Shelburne and Quarfordt, 1974). It is of interest that in man the ARP is found chiefly in the VLDL, with only trace amounts present in HDL.

The 46 000 molecular weight protein, which we have designated as A-IV, is a new apolipoprotein not reported before in any species. Immunological studies (Figure 4a), as well as data on molecular weight and the amino terminal residue (Table I), have shown that this protein is distinct from the other apolipoproteins of HDL, rather than representing an aggregated form. The functional role of this protein is not known, but it is of interest that this protein is not found in HDL molecules which have been newly secreted from perfused livers, suggesting that this subunit is added to the HDL molecule after its secretion from the liver (Marsh, 1976).

Human HDL contains extensive amounts of α helix, as do the apolipoproteins, and it has been postulated that the helix structure is an essential feature of the lipid–protein interaction (Segrest et al., 1974). Koga et al. (1969) showed that rat HDL and apo-HDL also show CD spectra consistent with extensive amounts of α helix (50–60%). Our studies on the isolated apolipoproteins are consistent with these data, with A-I and ARP possessing around 60% α helix and A-IV about 50% α helix (Figure 6). In fact, the secondary structure observed for rat apo-A-I of approximately 58% α helix, 9% β structure, and 34% random coil is very similar to that of human apo-A-I, which contains 55% α helix, 8% β structure, and 37% disordered structure by CD (Lux et al., 1972), providing additional evidence that these proteins are homologous. The relatively high level of α helix in all the rat apolipoproteins might suggest that this structure is important in the lipid–protein interactions in rat lipoproteins.

In addition to studying the properties of the isolated apolipoproteins, it was of interest to determine their proportions in the intact HDL molecule. Since sodium dodecyl sulfate gel electrophoresis is the only method we have found to resolve adequately the HDL components in the 25 000–80 000 molecular weight range, we utilized quantitative scanning methods to estimate the amounts of the different components (Table III). From the data in Table III, we can calculate that the molar ratio of A-I–ARP–A-IV is approximately 3.7:1.0:0.96 for chow-fed rat HDL and 2.3:1.0:0.91 for sucrose-fed rat HDL if all of these components were present in a single molecular species. Should rat HDL be composed of two or more families (Alaupovic, 1971), these ratios would then correspond to average values. The fact that these ratios approximate whole numbers is consistent with the existence of a homogeneous population of HDL, although not proof for it.

These studies of the rat apolipoproteins have made it possible to study in depth the effects of certain metabolic perturbations on apolipoprotein composition and distribution. Kuehl et al. (1974) have found that in rats fed high cholesterol diets the HDL is deficient in the ARP, while the VLDL and intermediate density fraction (d 1.006–1.03) contain proteins char-

acteristic of HDL (A-I and A-IV) in addition to the usual VLDL apoproteins. In diabetic rats (Bar-On et al., 1976), the HDL contains decreased amounts of ARP and A-IV, despite the fact that the concentration of HDL in serum is increased. In addition, A-IV was found in the VLDL and in appreciable amounts in the $d > 1.21$ fraction. Further studies of apolipoprotein patterns in other types of experimental hyperlipoproteinemia are being undertaken.

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