Further Characterization of the Apolipoproteins of Rat Plasma Lipoproteins*

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ABSTRACT: Rat plasma lipoproteins of very low density (VLDL), low density (LDL), and high density (HDL) were isolated and their protein components (apolipoproteins) prepared by ethanol–ether delipidation. These were solubilized in the presence of decyl sulfate and urea and sequentially fractionated by gel filtration and DEAE-cellulose chromatography. LDL contained a single apolipoprotein. This LDL apolipoprotein represented approximately 25% of the apolipoproteins of VLDL. The remaining VLDL apolipoproteins consisted of a large protein (mol wt >25,000) unique to VLDL and a number of smaller proteins. One of the smaller proteins was separated by DEAE-cellulose chromatography into several immunochemically indistinguishable components. A major fraction (approximately 65%) of the HDL apolipoproteins appeared to consist of a single protein that was not present in the other lipoproteins. The other proteins in HDL included one or more smaller proteins also present in VLDL. Thus rat plasma lipoproteins contain at least four different apoproteins: one is common to LDL and VLDL, one is unique to VLDL, one is unique to HDL, plus a family of peptides shared by both very low density and high density lipoproteins.

The protein components (apolipoproteins) of the several families of plasma lipoproteins have been the subject of intensified study in the past few years. Most of the investigations have focused upon human lipoproteins. The existence of heterogeneity within the protein moiety of very low density lipoprotein (VLDL)[1] was suspected for many years on the basis of amino-terminal amino acid determinations (Shore, 1957). Immunochemical studies have demonstrated that the major proteins of both low density lipoprotein (LDL) and high density lipoprotein (HDL) also occur in human plasma VLDL (Levy and Fredrickson, 1966; Gustafson *et al.*, 1966; Granda and Scanu, 1966) and VLDL produced by perfused isolated rat livers (Windmueller and Levy, 1967). More recently, three other proteins have been isolated from human VLDL and partially characterized (Brown *et al.*, 1969, 1970a,-b; Shore and Shore, 1969). It has also been established that human HDL contains more than one apolipoprotein (Shore and Shore, 1968, 1969; Scanu *et al.*, 1969). To gain further insight into the degree of heterogeneity of the apolipoproteins and their interrelationships we have made a detailed study of the apoproteins of VLDL, LDL, and HDL in the rat. While these studies were in progress heterogeneity in the proteins of both VLDL and HDL of rat serum was reported (Koga *et al.*, 1969). We here report the isolation and further characterization of the major apoproteins of VLDL, LDL, and HDL in the rat.

Materials and Methods

Urea, Certified A. C. S. and Ultra Pure, was purchased from Fisher and Mann, respectively. Rexyn I-300, a mixed cation and anion-exchange resin, was obtained from Fisher and used to remove cyanate from urea solutions prior to preparation of buffers. The sources of all other materials have been previously described (Brown *et al.*, 1969).

Experimental Procedures

Source of Lipoproteins. Male Sprague–Dawley rats weighing 500–600 g were fed a standard laboratory chow *ad lib* and were not fasted prior to collection of blood by abdominal aortic puncture. Disodium EDTA was added to a final concentration of 1 mg/ml of blood. Plasma was recovered by centrifugation at 4° and all procedures thereafter were carried out at this temperature. Plasma was pooled and ultracentrifugation was begun within 24 hr.

Isolation of Lipoprotein Fractions. VLDL, LDL, and HDL were isolated by standard preparative ultracentrifugal techniques (Havel *et al.*, 1955) except that LDL was isolated between d 1.019–1.035, the density limits required in this species for preparation of pure LDL (Windmueller and Levy, 1967; Koga *et al.*, 1969). The VLDL fraction contained any chylomicrons that may have been present in the plasma. The $1.035 < d < 1.063$ fraction contained a mixture of LDL and HDL (Windmueller and Levy, 1967) and was discarded. All fractions were washed twice by ultracentrifugation at the respective maximum densities used for isolation.

Delipidation of Lipoproteins. After the final wash VLDL, LDL, and HDL were desalted by dialysis for 72 hr against

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[1] Abbreviations used are: VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; apo-VLDL, apo-LDL and apo-HDL, apolipoproteins of VLDL, LDL, and HDL obtained after solubilization of delipidated VLDL, LDL, and HDL.

TABLE I: Characterization of Antisera.

Antiserum	Reactivity						
	VLDL	Apo-VLDL	LDL	Apo-LDL	HDL	Apo-HDL	Albumin
Anti-apo-VLDL	+	+	+	+	+	+	−
Anti-apo-HDL	+	+	−	−	+	+	−

100 volumes of 0.01% disodium EDTA, pH 7.0. Following dialysis, the lipoproteins were lyophilized and totally delipidated. Each lyophilized lipoprotein fraction was delipidated with ethanol–diethyl ether (3:1) for 16 hr followed by two changes of ethanol–ether (3:1) for 4 hr each. After rinses of 1:1 ethanol–ether and 100% ether, the delipidated protein was dried under nitrogen (Brown *et al.*, 1969). Apo-VLDL contained 0.028% residual phosphorus by weight or 0.7% phospholipid. Apo-HDL contained 0.4% residual phospholipid.

Solubilization of Apoproteins. The delipidated VLDL and delipidated HDL were completely soluble in 0.2 M Tris-Cl–0.1 M sodium decyl sulfate, pH 8.2, with or without 6 M urea. Delipidated LDL was dissolved in Tris buffer containing decyl sulfate but no urea. The decyl sulfate concentration of apo-VLDL and apo-HDL was then reduced to 2 mM by dialysis.

Gel Filtration of Apoproteins. Gel filtration of solubilized protein was carried out using Sephadex G-150 equilibrated with 0.2 M Tris-Cl–2 mM sodium decyl sulfate, pH 8.2, with or without 6 M urea. Glass columns (2.5 × 150 cm) were used and 10-ml fractions were collected at a flow rate of 20 ml/hr. In all cases greater than 95% of the protein applied was recovered from the column.

DEAE-Cellulose Chromatography. Prior to DEAE-cellulose chromatography the various fractions obtained by Sephadex G-150 gel filtration were desalted by applying them to a 2.5 × 45 cm column of Bio Gel P-2 (100–200 mesh) and eluting them with the buffer (6 M urea titrated to pH 8.2 with a saturated solution of Tris-Cl) in which they were applied to the DEAE-cellulose columns. DEAE-cellulose was prepared as described previously (Brown *et al.*, 1969) except that the DEAE-cellulose was equilibrated with the loading buffer described above. Columns (0.9 × 22 cm) were used and elution was begun with 6 M urea solution in 0.02 M Tris-Cl. Conductivity of the loading buffer was less than 0.05 mmho per cm. Ionic strength was further increased over a linear gradient from this buffer to 6 M urea in 0.02 M Tris-Cl containing 0.15 M NaCl. All solutions were adjusted to pH 8.2. Fractions (5 ml) were collected and the flow rate was 20 ml/hr.

Protein and Phospholipid Determinations. Protein was measured as described previously (Lowry *et al.*, 1951). One unit of protein was defined as that amount of protein giving a color yield equal to 1 mg of bovine serum albumin (Brown *et al.*, 1969). Phospholipid phosphorous was determined by a previously described method (Bartlett, 1959) and phospholipid estimated by multiplying by the factor 25.

Immunochemical Analyses. Analyses by immunoelectrophoresis and Ouchterlony double diffusion were carried out as

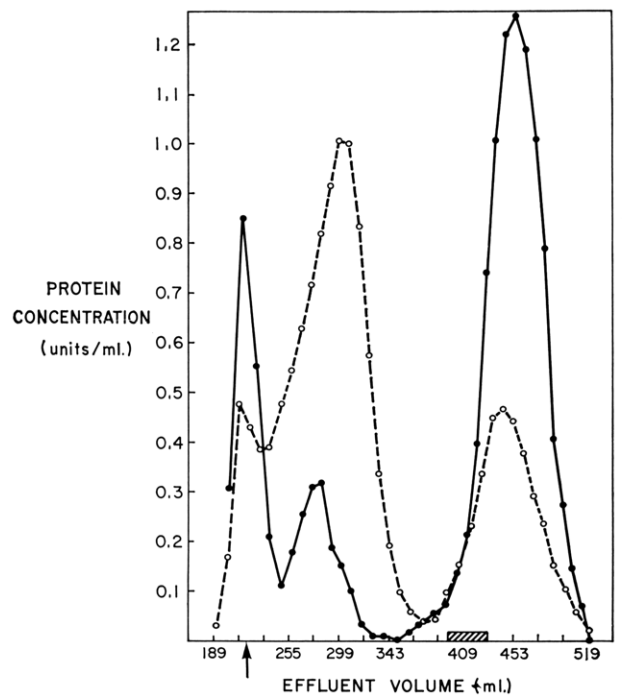

FIGURE 1: Sephadex G-150 gel filtration of apo-VLDL (solid line) and apo-HDL (broken line). From left to right the three apo-VLDL peaks are designated VS-1, VS-2, and VS-3, respectively. The three apo-HDL peaks are designated HS-1, HS-2, and HS-3, respectively. The samples were applied in and eluted with 0.2 M Tris, 6 M urea, and 2 mM decyl sulfate, pH 8.2. The elution volume of chymotrypsinogen A (mol wt 25,000) is indicated by the crosshatched bar and the void volume of the column is indicated by the arrow.

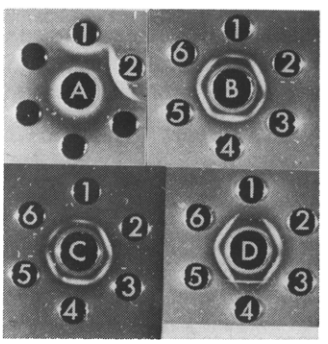

FIGURE 2: Ouchterlony immunodiffusion of the various isolated apoproteins of apo-VLDL and apo-HDL. Anti-apo-VLDL was placed in the center wells of plates A and B. Anti-apo-HDL was placed in the center wells of plates C and D: antigens, plate A, 1 = fraction VS-1, 2 = plasma LDL; plates B and C, 1 = V3-3, 2 = H3-3, 3 = V3-4, 4 = H3-4, 5 = V3-5, 6 = H3-3; plate D, 1 = V2-1, 2 = V3-3, 3 = H3-4, 4 = H2-1, 5 = V3-4, 6 = H3-3.

TABLE II: Immunochemical Reactivity of Apoproteins.

	Reactivity								
Antiserum	apo-LDL[a] (a)	VS-1 (a)	V2-1	V3-1 V3-2	V3-3 V3-4 V3-5 (b)	HS-1 (c)	H2-1 (c)	H3-1 H3-2	H3-3 H3-4 (b)
Anti-apo-VLDL	+	+	+	−	+	−	−	−	+
Anti-apo-HDL	−	−	−	−	+	+	+	−	+

[a] Indicates immunochemical identity between antigens labeled with "a". Same for b and c.

previously described (Levy and Fredrickson, 1965). Separate antisera to apo-VLDL and apo-HDL were prepared by mixing 6 units of the solubilized, lipid-free proteins with complete Freund's adjuvant 3:1 (v/v) and injecting the mixture into the footpads of New Zealand white rabbits. After 21 days each rabbit received a booster injection of 5 units of protein in adjuvant; the antisera were harvested 7 days later. The antiserum to apo-VLDL reacted with VLDL, LDL, HDL, apo-VLDL, apo-LDL, and apo-HDL, but not with LDL or albumin (Table I). The antiserum to apo-HDL reacted with VLDL, HDL, apo-VLDL, and apo-HDL, but not with LDL, apo-LDL, or albumin.

Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis was performed at pH 8.9 in 8 M urea using 15% acrylamide (Reisfeld and Small, 1966).

Results

VLDL Apolipoproteins. GEL FILTRATION CHROMATOGRAPHY. Gel filtration of apo-VLDL through Sephadex G-150 in 0.2 M Tris-Cl containing 2 mM decyl sulfate permitted the elution of two major fractions and a third incompletely separated fraction eluting between the two. When 6 M urea was added to the buffer, three distinct fractions were obtained (Figure 1). These fractions, hereafter referred to as VS-1, VS-2, and VS-3,[2] respectively, contained approximately 23, 13, and 64% of the total apo-VLDL applied.

CHARACTERIZATION OF THE SEPHADEX G-150 FRACTIONS. Fraction VS-1 contained 0.068% residual phosphorous or 1.7% phospholipid. It formed a single line of identity with LDL when both were reacted against the antiserum to apo-VLDL (Figure 2A and Table II). No other apoproteins were detected in this fraction with either anti-apo-VLDL or anti-apo-HDL. Fraction VS-1 remained in the 2.5% stacking gel after polyacrylamide gel electrophoresis (Figure 3).

Fraction VS-2 was separated into a major band, V2-1,[3] and two bands of greater mobility by polyacrylamide gel electrophoresis (Figure 3). V2-1 was isolated from these two other minor bands by DEAE-cellulose chromatography. V2-1 reacted with anti-apo-VLDL but not with anti-apo-HDL (Figure 2D and Table II).

Fraction VS-3 contained five bands separable by polyacrylamide gel electrophoresis. These are referred to as V3-1, V3-2, V3-3, V3-4, and V3-5 (Figure 3). V3-1 and V3-2 did not bind to the DEAE-cellulose and were thus eluted together when fraction VS-3 was loaded onto the anion-exchange column (Figure 4). V3-3, V3-4, and V3-5 were eluted as separate peaks (Figure 4). Of the total protein applied to the DEAE-cellulose, 40% was recovered. The mixture of V3-1 and V3-2 accounted for about 25% of the recovered protein, V3-3 and V3-4 for greater than 70%, and V3-5 for less than 5%. V3-1 and V3-2 did not react with antisera to either apo-VLDL or apo-HDL. V3-3, V3-4, and V3-5 formed a line of identity when reacted against either anti-apo-VLDL or anti-apo-HDL (Figure 2B,C and Table II); all three of these proteins migrated with α_1 mobility upon immunoelectrophoresis (Figure 5B).

LDL Apolipoprotein. On Sephadex G-150, apo-LDL eluted in the void volume. Apo-LDL remained in the 2.5% stacking gel after polyacrylamide gel electrophoresis. Neither LDL nor apo-LDL reacted with anti-apo-HDL but both formed a single precipitin line with anti-apo-VLDL (Figure 5A and Table II). As mentioned above, LDL and the protein of fraction VS-1 were identical (Figure 2A).

HDL Apolipoproteins. GEL FILTRATION CHROMATOGRAPHY. On Sephadex G-150, apo-HDL in 0.2 M Tris-Cl, containing

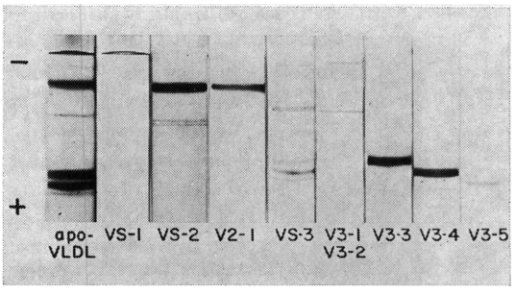

FIGURE 3: Polyacrylamide gel electrophoresis of apo-VLDL, the three fractions obtained by gel filtration of apo-VLDL, and the isolated apoproteins obtained by DEAE-cellulose chromatography of fractions VS-2 and VS-3. The procedure was performed at pH 8.9 in 8 M urea on 15% acrylamide.

[2] Abbreviations used for fractions obtained by Sephadex G-150 gel filtration are: VS-1, VS-2, VS-3; HS-1, HS-2, HS-3; where V refers to apo-VLDL and H to apo-HDL.

[3] Abbreviations used for subfractions of the apolipoproteins are: V2-1; V3-1, V3-2, V3-3, V3-4, V3-5; H2-1; H3-1, H3-2, H3-3, and H3-4. V2 and V3 refer to Sephadex fractions VS-2 and VS-3, and H2 and H3 to Sephadex fractions HS-2 and HS-3, respectively. The second digit of each abbreviation was derived by numbering each band (starting with the one nearest the cathode) obtained on polyacrylamide gel electrophoresis of fractions VS-2, VS-3, HS-2, and HS-3.

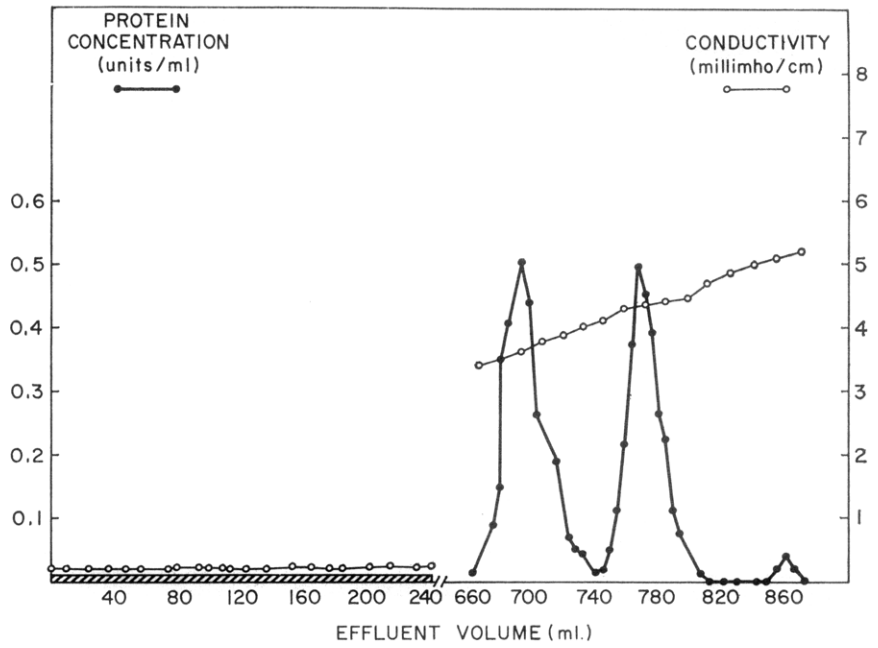

FIGURE 4: DEAE-cellulose chromatography of fraction VS-3. Protein concentration is indicated by the line formed by the solid circles and conductivity of the buffer by the line formed by the open circles. The crosshatched bar represents the volume which V3-1 and V3-2 eluted in while the column was being loaded. The peaks are, in order of elution, V3-3, V3-4, and V3-5.

2 mM decyl sulfate and 6 M urea, pH 8.2, was separated into three fractions, HS-1, HS-2, and HS-3 (Figure 1). These fractions contained approximately 14, 59, and 27%, respectively, of the apo-HDL.

CHARACTERIZATION OF SEPHADEX G-150 FRACTIONS OF APO-HDL. Most of the protein in fraction HS-1 remained in the stacking gel on polyacrylamide gel electrophoresis, but several slowly migrating bands also appeared (Figure 6). HS-1 protein contained 1.7% residual phospholipid.

Fraction HS-2 separated into one major band and two faster migrating bands on polyacrylamide gel electrophoresis (Figure 6). The major band, designated H2-1, was separated by DEAE-cellulose chromatography from the minor ones which were not present in quantities sufficient for further characterization. When reacted against anti-apo-HDL, fractions HS-1 and H2-1 formed a line of identity (Table II). Neither HS-1 nor H2-1 reacted with anti-apo-VLDL.

The protein of fraction VS-1 that was identical with apo-LDL did not react with anti-apo-HDL and thus was not identical with apoprotein H2-1. Similarly, V2-1 did not react with anti-apo-HDL (Figure 2D) whereas H2-1 did.

Fraction HS-3 was separated into four bands by polyacrylamide gel electrophoresis (Figure 6). DEAE-cellulose chromatography of fraction HS-3 separated all four of these proteins (Figure 7) referred to as H3-1, H3-2, H3-3, and H3-4. About 50% of the protein applied to the DEAE-cellulose was recovered and of this, H3-3 and H3-4 together amounted to about 60%, and H3-1 and H3-2 about 20% each. Neither H3-1 nor H3-2 reacted with the antisera made to apo-VLDL or apo-HDL. H3-3 and H3-4 reacted with antisera to both apo-VLDL and apo-HDL, migrated with α_1 mobility upon immunoelectrophoresis (Figure 5C), and appeared identical with each other on Ouchterlony immunodiffusion (Figure 2B,C and Table II).

When V3-3, V3-4, V3-5, H3-3, and H3-4 were reacted against antisera to either apo-VLDL or apo-HDL, a line

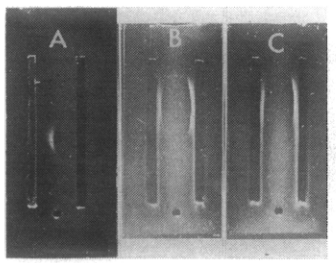

FIGURE 5: Immunoelectrophoresis of isolated apoproteins and plasma LDL. Anti-apo-VLDL was placed in the trough on the left and anti-apo-HDL in the trough on the right of each slide. The antigens placed in the wells prior to electrophoresis were A, plasma LDL; B, V3-4; C, H3-4.

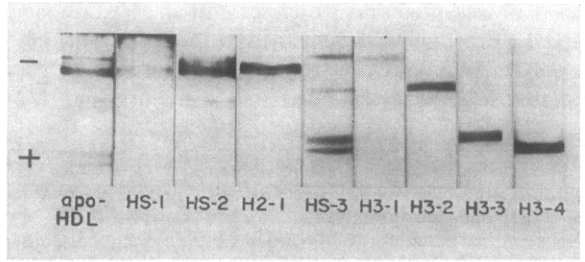

FIGURE 6: Polyacrylamide gel electrophoresis of apo-HDL, the three fractions obtained by gel filtration of apo-HDL, and the isolated apoproteins obtained by DEAE-cellulose chromatography of fractions HS-2 and HS-3. The electrophoresis was carried out in 8 M urea on 15% acrylamide at pH 8.9.

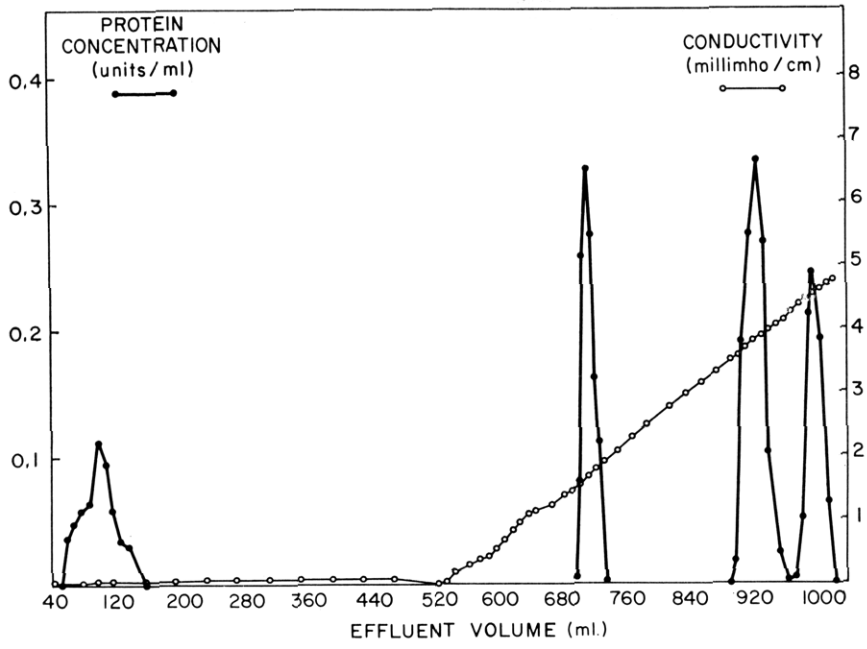

FIGURE 7: DEAE-cellulose chromatography of fraction HS-3. Protein concentration is indicated by the line formed from the solid circles and conductivity of the buffer by the line formed by the open circles. The peaks are in order of elution, H3-1, H3-2, H3-3, and H3-4.

of identity formed between all of these proteins (Figure 2B,-C and Table II). Furthermore, when V3-3 and H3-3 were mixed and electrophoresed on polyacrylamide gel, a single band appeared (Figure 8A). Similarly, a single band appeared when V3-4 and H3-4 were mixed and electrophoresed (Figure 8B).

In order to show that the apoprotein occurring as H3-3, H3-4, V3-3, and V3-4 was nonidentical with V2-1 and H2-1, all of these apoproteins were reacted against anti-apo-HDL by Ouchterlony double diffusion. V2-1 did not react but H2-1 did, forming a precipitin line of nonidentity with H3-3, H3-4, V3-3, and V3-4 (Figure 2D).

Discussion

The terminology employed to identify the apolipoproteins separated in these studies is summarized in Figure 9. The proteins were identified primarily on the basis of their elution from gel filtration and ion-exchange chromatography. Polyacrylamide gel electrophoresis and immunochemical methods were used to judge the identity or nonidentity of the apolipoproteins isolated from the several lipoprotein families. More specific identification of the proteins will evolve from analyses of primary structure now in progress.

The VLDL apolipoproteins (apo-VLDL) were separated into at least three, and possibly as many as five different proteins. Approximately one-quarter of the total protein was immunochemically identical with the apparently single apolipoprotein present in LDL (apo-LDL). This protein was present in the void volume of the gel filteration column; it did not react with anti-apo-HDL sera, but formed a single precipitin line with anti-apo-VLDL sera.

The second protein in apo-VLDL was eluted as a single peak on gel filtration. This protein (V2-1) was separated by DEAE-cellulose chromatography from two other trace components observed on polyacrylamide gel. Protein V2-1 reacted only with anti-apo-VLDL sera. It accounted for less than 13% of apo-VLDL. A comparison of the pattern of elution from Sephadex of this protein with that of chymotrypsinogen suggested that V2-1 has a molecular weight exceeding 25,000. Gel filtration of the apolipoproteins was performed in buffers containing 6 M urea. Nevertheless, aggregation could not be entirely excluded as the reason why V2-1 appeared to have such a relatively high molecular weight. It is noteworthy that in human apo-VLDL, all of the proteins, other than the apo-LDL that is present, have appeared to

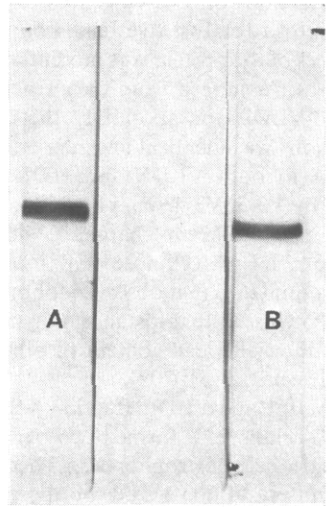

FIGURE 8: Coelectrophoresis of apoproteins on 15% polyacrylamide gel in 8 M urea at pH 8.9. (A) Single band obtained when V3-3 and H3-3 were mixed prior to electrophoresis. (B) Single band obtained when V3-4 and H3-4 were mixed prior to electrophoresis.

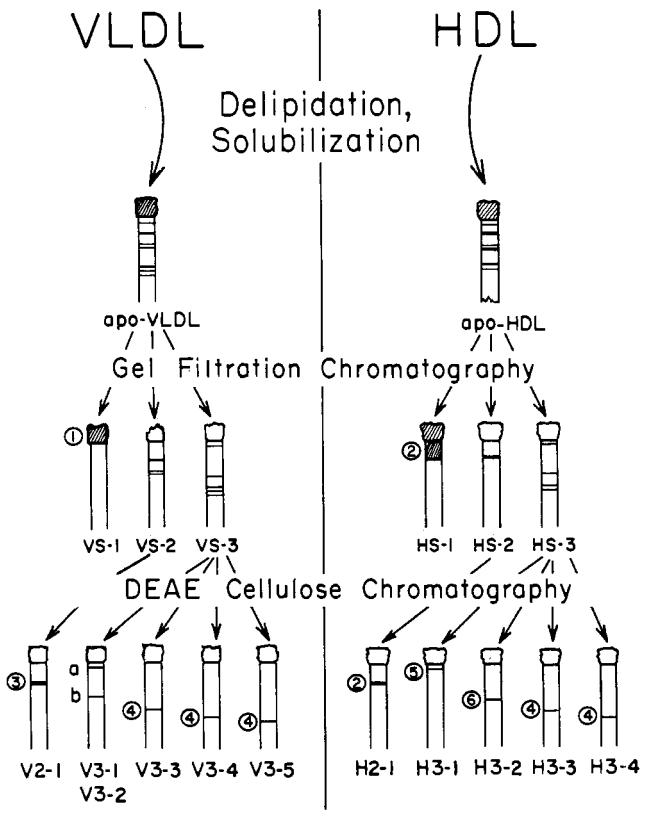

FIGURE 9: Summary diagram of procedures and polyacrylamide gel electrophoresis of fractions and isolated apoproteins. The encircled numbers 1–6 each represent immunochemically distinct apoproteins; 5 and 5 may be identical with a and b (see text for further explanation).

have molecular weights of 14,000 or less (Brown *et al.*, 1969). More recent studies indicate they are below mol wt 10,000 (Brown *et al.*, 1970b).

The remaining 65% of the rat apo-VLDL emerged from the Sephadex column in a third peak. This was eventually separated into four fractions on DEAE-cellulose. These fractions were separable into a total of five bands on polyacrylamide gel. Three-quarters of the protein was present in three fractions (V3-3, V3-4, V3-5). As judged from their reactions with antisera to both apo-VLDL and apo-HDL, the protein in these latter three fractions was identical and represented an antigen normally present in both VLDL and HDL. The apparent paradox created by V3-3, V3-4, V3-5 having immunochemical identity despite their different charge, as demonstrated by both elution from DEAE-cellulose and mobility on polyacrylamide gel, is similar to that observed for one of the human VLDL apolipoproteins. In that instance, polymorphism is due to differences in the sialic acid content of otherwise identical protein units (Brown *et al.*, 1970b).

The rest of the rat apo-VLDL fraction VS-3 was not absorbed by DEAE-cellulose. It formed two bands (V3-1, V3-2) on polyacrylamide gel electrophoresis. These proteins did not react with antisera to apo-VLDL or apo-HDL.

The apolipoproteins in the rat HDL did not include any apo-LDL. About 20% of the total apo-HDL was represented by fractions identified as H3-3 and H3-4. These proteins had the same mobility on polyacrylamide gel as the proteins V3-3 and V3-4 found in apo-VLDL. All of these bands proved to be immunochemically identical. Two other minor components of apo-HDL (H3-1, H3-2) had mobilities on polyacrylamide gel that were very similar to the apo-VLDL proteins V3-1 and V3-2. Like the latter two proteins, H3-1 and H3-2 did not react with any of the available antisera to apo-VLDL, apo-HDL, or apo-LDL.

About 65% of the apo-HDL appeared to be a single protein (H2-1). On polyacrylamide gel and immunoelectrophoresis, H2-1 formed a single band or precipitin line. It reacted only with anti-apo-HDL sera. On Sephadex, protein H2-1 eluted before chymotrysinogen suggesting that the molecular weight of H2-1 was greater than 25,000. As was the case with the VLDL apolipoprotein V2-1, aggregation of H2-1, even in 6 M urea, may have contributed an erroneous impression of molecular weight.

The present studies are consonant with prior evidence that some apolipoproteins are shared by several families of rat plasma lipoproteins (Windmueller and Levy, 1968; Ockner *et al.*, 1968; Koga *et al.*, 1969). They also confirm the heterogeneity in rat apo-VLDL and apo-HDL described by Koga *et al.* The observations of the latter have here been extended to isolation of the apolipoproteins, and, in turn, their separate quantification and immunochemical examination.

Our investigations of rat plasma lipoproteins have been conducted using methods developed first for the study of human VLDL apolipoproteins (Brown *et al.*, 1969, 1970a). The rat apolipoproteins isolated in the third fraction from gel filtration thus correspond in size to a number of relatively small proteins in human apo-VLDL, at least three of which have already been characterized (Brown *et al.*, 1969, 1970a,b). The rat apolipoproteins have similar mobility on polyacrylamide gel, but further studies are in progress to determine the comparability of the terminal residues, amino acid composition, and other properties of these proteins isolated from rat and man.

A major difference in distribution of these apo-VLDL proteins is already apparent between the two species. In this regard, it should first be noted that neither our experiments or those of Koga *et al.*, convincingly demonstrated the presence of two major HDL apolipoproteins such as have been established in man (Shore and Shore, 1969). It is thus difficult to compare the degree to which smaller apolipoproteins (as distinguished from the major and larger proteins that make up apo-LDL and apo-HDL) are shared by HDL and VLDL. In man, less than 5% of the total HDL apolipoproteins are also found in apo-VLDL.

Both the rat and human VLDL apolipoproteins include the relatively large protein which composes apo-LDL. In man, this latter protein makes up nearly 40% of apo-VLDL. The present studies confirm immunochemical data (Eaton and Kipnis, 1969) showing that the contribution of apo-LDL to VLDL apolipoproteins is only about half as great in the rat as it is in man.

Specific functions cannot yet be assigned to all of the apolipoproteins isolated thus far in man or the rat. Each of the apolipoproteins described here has been isolated in lipoproteins from hepatic perfusates (Mahley *et al.*, 1970). With the exception of the minor apo-VLDL proteins (V3-1 and V3-2), they have also been demonstrated in preparations from rat liver Golgi apparatus (Mahley *et al.*, 1970). The evidence that they are associated with lipoproteins at their sites of origin

in tissue is an indication that these proteins are not contaminants acquired in lymph or plasma. It may be assumed that they have some relationship to lipoprotein formation and fat transport. The proof of this will require more investigation.

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Terminal Labeling of High Molecular Weight Ribonucleic Acid with Dimedone*

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ABSTRACT: The reaction of dimedone (5,5-dimethyl-1,3-cyclohexanedione) with dialdehydes resulting from periodate oxidation of the 3′-hydroxyl terminus of RNA has been studied as a terminal-labeling technique in polynucleotide sequence analysis. One molar equivalent of dimedone-2-^{14}C is incorporated per free 3′ terminus of oxidized AMP, tRNA, rRNA, or bacteriophage f2 RNA. Little or no dimedone is associated with unoxidized RNA. The labeling process does not result in appreciable nonspecific incorporation or detectable polynucleotide degradation. The terminal label is sufficiently stable at neutral pH, except in the presence of amines, to permit identification of only predicted terminally labeled oligonucleotide fragments from RNA upon nuclease digestion followed by DEAE-cellulose chromatography.

End-labeling techniques have played an important role in the development of protein chemistry. By analogy, similar techniques in RNA chemistry have been valuable in the identification of terminal mono- and oligonucleotides of RNA molecules. Such information is useful in sequence work as well as for the determination of heterogeneity in RNA preparations (RajBhandary, 1968).

A number of end-labeling techniques have been developed which exploit the periodate oxidation possible only at the 3′ terminus (also called the 5′-linked or right-hand end) of a polyribonucleotide. Generally the procedures have involved first the oxidation of RNA by periodate and then derivatization of the resulting uniquely reactive dialdehyde. The dialdehyde generated by periodate oxidation has been treated with tritiated isonicotinoylhydrazine (Hunt, 1965), tritiated 2-phenylethylammonium chloride (Wimmer and Reichmann, 1969), ^{14}C-labeled semicarbazide (Steinschneider and Fraenkel-Conrat, 1966a), and tritiated sodium borohydride (RajBhandary, 1968; DeWachter and Fiers, 1967; Glitz *et al.*, 1968).

None of the methods of derivatization has been generally employed, indicating a lack of clear superiority of any of the

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