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LIPID TRANSPORT IN THE AVIAN SPECIES

PART 1. ISOLATION AND CHARACTERIZATION OF APOLIPOPROTEINS AND MAJOR LIPOPROTEIN DENSITY CLASSES OF MALE TURKEY SERUM

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Summary

(1) Lipoproteins from the serum of male turkeys maintained on a normal diet were separated by sequential preparative ultracentrifugation into VLDL ($d < 1.006$ g/ml), LDL ($d = 1.006$—1.063 g/ml), HDL ($d = 1.063$—1.21 g/ml) and VHDL ($d > 1.21$ g/ml). Lipoprotein density classes were characterized by analytical ultracentrifugation, agarose electrophoresis, immunodiffusion and immunoelectrophoresis, and by quantitative determination of protein, lipids and individual phosphatides.

(2) HDL were the major density class representing 75% of the total lipoprotein content, LDL accounted for approximately 20% and VLDL for only 3—5% of the total lipoproteins.

(3) VLDL were characterized by a relatively low content of glyceride (34%). Cholesterol esters were the major lipid (38%) of LDL, and the phospholipids (26%) of HDL. Glycerides of all major density classes consisted of equal amounts of triglycerides and diglycerides.

(4) Phosphatidylcholine was the major phosphatide in all density classes. The composition of phosphatides was very similar in the VLDL and LDL, but it was different in the HDL. The ratio of phosphatidylcholine/sphingomyelin was higher in HDL than in VLDL and LDL.

(5) Immunological and electrophoretic studies showed that all three major density classes consisted of two lipoprotein families designated, in analogy to

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the human serum lipoprotein system [1], as LP-A and LP-B. The exception was HDL_3 (d = 1.125—1.21 g/ml) which contained only LP-A.

(6) ApoB was insoluble in aqueous buffers but could be solubilized after reduction and carboxymethylation. No C- or N-terminal amino acids were released by the usual chemical methods. The carbohydrate moiety of ApoB contained mannose, galactose and galactosamine.

(7) ApoA consisted of a non-identical polypeptides designated in analogy to the human polypeptides as A-I and A-II. A-I was the major ApoA polypeptide and had a molecular weight of about 27,000. This polypetide contained no half cystine, and the aspartic acid as the N-terminal and alanine as the C-terminal amino acids. A-II had a molecular weight of about 10,000, contained no half cystine and had alanine as the C-terminal amino acid. A-II showed no N-terminal amino acid by either dansylation, dinitrophenylation or Edman's procedure. Neither A-I nor A-II contained neutral sugars or hexosamines.

(8) Concentrations of polypetides analogous to human ApoC, ApoD and "arginine-rich" polypeptide, if present, were too low for their unequivocal chemical characterization.

Key words: *Apolipoprotein A — Apolipoprotein B — Lipoprotein A — Lipoprotein B — Lipoprotein density classes — Turkey serum*

Introduction

There are some remarkable differences in metabolism and transport of lipids between the avian and mammalian species. In contrast to most mammals, birds synthesize 90 to 95% of their fatty acids *de novo* in the liver rather than in the adipose tissue [2—4]. The absorption of exogenous long-chain fatty acids in the avian species occurs exclusively by way of the portal vein [5,6]. These fatty acids are transported from the intestine into the systemic circulation as triglycerides of VLDL. It has been suggested that, by analogy to mammalian chylomicrons, these avian lipoproteins rich in dietary lipids be named portomicrons [6].

Since relatively little is known about the nature of the avian serum lipoprotein system in general and lipid carriers in particular, we have initiated a systematic study of the physical, chemical and immunological properties of the turkey serum lipoprotein system. The turkey was chosen as a representative of avian species for the following reasons. The apolipoproteins and corresponding lipoprotein families of turkey serum have not yet been characterized; due to their size, mature turkeys are a source of relatively large volumes of blood necessary for extensive lipoprotein studies; turkeys have been used frequently as a model for the study of naturally occurring as well as experimentally induced atherosclerosis [7,8]; their plasma lipids and lipoproteins are easily amenable to dietary and hormonal manipulations. Compared to man, turkeys are considered hypertensive with average systolic pressures of 192 to 251 mm Hg [9]. Since hypertension and elevated serum lipids are important risk factors for coronary heart diseases in man [10,11], the turkey represents an excellent model for studying the effect of normal and abnormal lipid transport processes on the develop-

ment of atherosclerotic lesions in the presence of hypertension.

This paper reports the isolation and characterization of apolipoproteins and major lipoprotein density classes of male turkeys maintained on a normal diet.

Materials and methods

Source of serum

Serum from Broad Breasted White male turkeys was purchased from Colorado Serum Company Laboratories, Denver, Colorado, and from Pel-freeze Biologicals, Inc., from Rogers, Arkansas. Serum was obtained from mature male turkeys (19—30 wks of age) after an overnight fast. Serum samples were pooled from several birds. The birds were fed Purina Poultry Chow containing 14% protein and 1—2% fat and were maintained under normal management conditions.

Isolation of lipoprotein density classes

Three major lipoprotein density classes were separated from turkey serum by preparative ultracentrifugation carried out in the Ti 60 rotor of the Spinco L-2 or L2-65-B ultracentrifuges at 5° C. Very low density lipoproteins (VLDL, $d <$ 1.006 g/ml) were prepared by layering 25 ml of serum under 10 ml of NaCl (d = 1.006 g/ml) containing 0.1% EDTA and centrifuging at 105,000 × g, for 22 h. The top layer containing the VLDL was removed by using a Spinco tube slicer and the infranatant portion was used for the isolation of lipoproteins at higher densities. Low density lipoproteins (LDL, d = 1.006—1.063 g/ml) and high density lipoproteins (HDL, d = 1.063—1.21 g/ml) were separated sequentially by adjusting the density of the infranatant solution with solid KBr to 1.063 and 1.21 g/ml, respectively. A portion of the HDL was subfractionated into high density lipoprotein$_2$ (HDL_2, d = 1.063—1.125 g/ml) and high density lipoprotein$_3$ (HDL_3, d = 1.125—1.21 g/ml) subfractions. Very high density lipoproteins (VHDL) included all lipoporteins with $d >$ 1.21 g/ml. All lipoprotein density classes except VHDL were recentrifuged at appropriate solution densities (1.006, 1.125 and 1.21 g/ml) until no albumin was detected by immunodiffusion analysis. Albumin was isolated and purified from the VHDL fraction of turkey serum by precipitation with trichloroacetic acid and extraction with ethanol [12].

Delipidization of lipoproteins

Lipoproteins were totally delipidized according to a modification of the procedure of Scanu and Edelstein [13]. Lipoprotein preparations were dialysed exhaustively against distilled water at 4° C and lyophilized. The lyophilized lipoproteins were delipidized at 4° C by 5—8 successive extractions with absolute ethanol—diethyl ether (3 : 1, v/v) followed by 3—5 extractions with anhydrous diethyl ether. The residues were allowed to dry at room temperature. Lipoproteins delipidized by this procedure contained less than 1% phospholipid. The ethanol—diethyl ether extracts were pooled and evaporated to dryness on a rotatory evaporator in preparation for lipid analyses.

Fractionation of apolipoproteins

A-I and A-II polypeptides were fractionated by gel filtration of apoHDL$_3$ on a column of Bio-Gel A-5m with 6 *M* guanidine HCl as the elution solvent. Due to high cost of ultra-pure guanidine HCl, we developed a procedure for the preparation of a colorless solution of 6*M* guanidine HCl from a commercially available practical grade of this chemical. The amount of guanidine HCl (practical grade, Sigma, St. Louis, Missouri) necessary for preparing 4 l of a 6 *M* solution was dissolved in 3 l of distilled water, filtered through a filter paper and passed through a column (5 × 50 cm) of activated charcoal (6—14 mesh, Matheson, Coleman and Bell, Norwood, Ohio). The guanidine HCl solution was repeatedly passed through the column until the optical density at 280 nm was less than 0.080. This solution was then made 0.01 *M* with respect to Tris and 0.05% with respect to EDTA by adding 40 ml of a stock solution of 1 *M* Tris in 5% EDTA. The pH was adjusted to 7.2 and the volume to 4 l. Twenty-five to fifty mg of apoHDL$_3$ were dissolved in 3 ml of the 6 *M* guanidine HCl solution, pH 7.2, and applied onto a column (2.5 × 120 cm) of Bio-Gel A-5m, 100—200 mesh (Bio-Rad Laboratories, Richmond, California), equilibrated with the same solution. Three-ml fractions were collected at a flow rate of 20—30 ml/h and the elution pattern was continuously monitored for protein concentration by absorbancy at 280 nm using a Buchler Uviscan (Buchler Instruments, Fort Lee, New Jersey). Since polyacrylamide gel electrophoresis or any other analytical procedure cannot be performed in the presence of guanidine HCl, the column fractions were pooled according to their optical density at 280 nm, dialyzed exhaustively against distilled water and lyophilized.

Reduction and carboxymethylation of apolipoprotein B (ApoB) was carried out by the method of Hirs [14]. The reduced and carboxymethylated ApoB was chromatographed on a column (2.5 × 100 cm) of Bio-Gel A-5m equilibrated with 6 *M* guanidine HCl buffer, pH 7.2.

Immunological methods

Double immunodiffusion [15] and immunoelectrophoresis [16] were carried out on glass slides (25 × 75 mm) coated with 1% agarose (Bio-Rad Agarose, Richmond, Calif.) employing Veronal buffer, pH 8.6, ionic strength 0.05. Immunoelectrophoresis was carried out at 6.5 V/cm for 60 min. The precipitin patterns were allowed to develop for 48—72 h at room temperature. Developed plates were washed overnight with distilled water and dried under a strip of lintless paper. They were stained for protein with Amido Black 10B or for lipid with Oil Red O.

Preparation of antibodies

The lipoprotein density classes, A-II polypeptide and albumin to be used as antigens were dialyzed against 0.15 *M* NaCl containing 0.1% EDTA. Equal volumes of antigens (protein concentrations varied between 0.5 and 10 mg/ml) and Freund's complete adjuvant were homogenized and then injected intraperitoneally into white New Zealand rabbits. One or two additional injections at intervals of 10 days were sufficient to elicit antibody titers adequate for our studies. Blood was drawn from rabbits by cardiac puncture 2—3 weeks after the

initial immunization. Blood samples (30—40 ml) were collected thereafter at weekly intervals. The antisera were stored at — 20° C with sodium azide (1 mg/ml antiserum) as a preservative.

Agarose electrophoresis

Agarose electrophoresis was carried out as described for immunoelectrophoresis except that the lipoprotein samples (20—30 μl) were mixed with equal volumes of 2% agarose cooled to 50° C and placed into a slot (1.0 × 0.1 cm) cut in the gel. After electrophoresis, the slides were fixed in 10% trichloroacetic acid for 1 h, then washed in distilled water for 2 h with several changes of distilled water. Washed slides were covered with lintless paper and allowed to dry overnight. Staining for protein and lipids was the same as described for immunoelectrophoretic patterns.

Polyacrylamide gel electrophoresis

Analytical discontinuous polyacrylamide gel electrophoresis (PAGE) was done according to the procedure described by Davis [17] with the exception that the electrophoretic separation was carried out with 8 *M* urea in the separating gels and 4 *M* urea in the stacking and sample gels. Electrophoresis was carried out at a constant current of 4 mA/tube employing Tris—glycine buffer, pH 8.2. The acrylamide monomer concentration was 7% (w/v). Electrophoresis was terminated when the tracking dye (Bromophenol Blue) had migrated 4.0 cm into the separating gel. The gels were fixed and stained simultaneously with a solution of 0.12% Coomassie Brilliant Blue R-250 in 10% trichloroacetic acid (0.25 : 10.0, v/v).

Sodium dodecyl sulfate—polyacrylamide gel electrophoresis (SDS—PAGE) was performed on 10% acrylamide gels as described by Weber and Osborn [18]. Molecular weights of apolipoproteins were estimated by plotting log molecular weight versus distance of migration of the polypeptides into sodium dodecyl sulfate containing gels. Human serum albumin, ovalbumin, myoglobin, lysozyme and cytochrome c were used as reference proteins.

Amino acid analysis

Samples of apolipoproteins were dried under vacuum in the presence of P_2O_5 and hydrolyzed in constant boiling HCl in an evacuated tube at 110° C for 24 and 72 h. The hydrolyzate was filtered through a fine frit sintered glass filter, then analyzed in a Beckman Model 120C amino acid analyzer according to the accelerated procedure of Benson and Patterson [19]. Half cystine and methionine were determined as cysteic acid and methionine sulfoxide after oxidation with performic acid [20].

Analysis of terminal amino acids

The N-terminal amino acids were determined by dansylation [21], dinitrophenylation [22], and by the Edman procedure [23] using a Beckman Model 890C sequenator.

The C-terminal amino acids were determined by enzymatic digestion of apolipoproteins with carboxypeptidases A and B as described by Ambler [24]. Protein samples were dissolved in 0.1 *M* N-ethylmorpholine, pH 8.5, and the

enzymes were added in a ratio of 1 part enzyme to 80 parts protein. The reaction mixture was incubated at 37° C, and the aliquots were taken at various time intervals. The reaction was stopped by adding 10% trichloroacetic acid. After removal of the precipitated protein, the supernate was evaporated to dryness, redissolved in 0.2 *N* sodium citrate and analyzed on a Beckman Model 120C amino acid analyzer The carboxyl terminal amino acids were also determined by hydrazinolysis according to the procedure of Braun and Schroeder [25].

Lipid and protein analyses

Lipid analyses were performed on the ethanol—diethyl ether extracts of lipoprotein preparations. After evaporating the extract to dryness, the residues were dissolved in chloroform and transferred to volumetric flasks. Neutral lipids were separated on precoated thin-layer plates (Silica Gel G, Schwarz/Mann, Orangeburg, N.Y.) using a solvent system containing 113 ml hexane, 35 ml diethyl ether, and 3 ml glacial acetic acid. The lipid spots were visualized by exposing the plates to iodine vapor and scraped into tubes for quantitative analysis.

Free cholesterol and cholesterol esters were determined by using the Hycel Cholesterol Kit (Hycel, Inc., Houston, Texas) or the Oxford Tri-Chol Kit (Oxford Laboratories, San Mateo, Calif.). Cholesterol esters were calculated as cholesterol oleate.

Diglyceride and triglyceride contents were determined by the method of Van Handel and Zilversmit [26] using triolein as the reference. Diglycerides were calculated as diolein.

Individual phosphatides were separated on pre-coated thin-layer plates (Silica Gel G, Schwarz/Mann) using a solvent system containing 97.5 ml chloroform, 37.5 ml methanol, 6 ml distilled water, and 12 ml glacial acetic acid. The spots visualized by exposing the plates to iodine vapor were scraped into tubes for phosphorus determination. Phosphorus content was measured by the method of Gerlach and Deutiche [27] in the presence of silica gel.

Protein content of lipoprotein solutions was determined by the method of Lowry et al. [28] using human serum albumin as the standard. The amount of protein in totally delipidized lipoproteins was quantitated gravimetrically after drying to a constant weight under vacuum in the presence of P_2O_5.

Carbohydrate analysis

Quantitative determination of carbohydrates was carried out by a modification [29] of the gas—liquid chromatographic procedure of Griggs et al. [30]. Neutral sugars were analyzed after hydrolysis of protein samples with 1 *N* HCl for 4 h at 100° C, and the amino sugars after hydrolysis with 4 *N* for 6 h at 100° C.

Analytical ultracentrifugation

Ultracentrifugal analysis was carried out in a Spinco Model E analytical ultracentrifuge equipped with schlieren and interference optical systems, and an electronic speed control. The photographic plates were measured on a Nippon microcomparator (Nikon Co., Japan). Samples for the determination of flota-

tion coefficients were dialyzed against NaCl at 1.063 or KBr at 1.21 g/ml. Flotation rates were calculated as described by De Lalla and Gofman [31].

Results

Chemical composition of lipoprotein density classes

The HDL of turkey serum represented over 75% of the total lipoprotein content. The LDL accounted for approximately 20% and the VLDL for only 3—5% of the lipoproteins (Table 1). Since HDL were the major lipoprotein density class, they contained most of the glycerides, free cholesterol and phospholipds. LDL contained about half of the total serum cholesterol in the form of esterified cholesterol.

The protein and phospholipid contents of lipoprotein density classes increased with increasing densities (Table 2). The composition of VLDL was characterized by a relatively low glyceride content (34%). Cholesterol esters were the major lipid class in LDL, whereas phospholipids were the major lipids in HDL. Both LDL and HDL had a relatively low content of glycerides. In all density classes, the diglycerides represented about half of the total glycerides.

The phospholipid composition of lipoprotein density classes is shown in Table 3. Phosphatidylcholine was the major phosphatide found in all density classes. The distribution of phosphatides was very similar in VLDL and LDL, but was different from the distribution in HDL. The HDL contained relatively more phosphatidylcholine and less lysophosphatidylcholine and sphingomyelin than VLDL and LDL.

Analytical ultracentrifugation of lipoprotein density classes

VLDL displayed a single peak with a flotation coefficient, S_f, of 33. LDL showed a single asymmetrical boundary with a flotation coefficient, S_f, of 7.4. HDL exhibited a single, symmetrical peak with a flotation coefficient of 4.2 at $d = 1.21$ g/ml.

Electrophoretic characterization of lipoprotein density classes

Figure 1 shows the electrophoretic patterns of whole serum and lipoprotein density classes in 1% agarose. VLDL had a mobility (pre-β) slightly faster than LDL. The LDL migrated mainly in the β-globulin and HDL migrated in the α-globulin positions. Based on the electrophoretic mobility, the major components of LDL were β-lipoproteins and the major components of HDL were α-lipoproteins. The electrophoretograms showed, however, that LDL also contained small amounts of α-lipoproteins, and HDL contained detectable amounts of β-lipoproteins. These results indicate that the turkey serum lipoprotein system consists of at least two distinct lipoprotein families: lipoprotein A (LP-A) characterized by apolipoprotein A (ApoA) and α-mobility on agarose, and lipoprotein B (LP-B) characterized by apolipoprotein B (ApoB) and β-mobility on agarose [32].

Immunological characterization of lipoprotein density classes

The characterization of rabbit antisera to the major density classes examined by immunodiffusion and immunoelectrophoresis is shown in Table 4. Antise-

TABLE 1

CONCENTRATION OF APOLIPOPROTEINS AND INDIVIDUAL LIPIDS OF TURKEY SERUM LIPOPROTEIN DENSITY CLASSES

Density class	Protein	Phospholipid	Cholesterol		Triclyceride	Diglyceride	Lipoprotein
			Free	Ester			
	(mg/100 ml)						
VLDL (n = 4)	1.20 ± 0.02 [a]	1.89 ± 0.25	0.32 ± 0.13	2.32 ± 0.37	1.52 ± 0.26	1.46 ± 0.34	8.71 [b]
LDL (n = 4)	16.07 ± 4.15	16.19 ± 5.12	3.18 ± 0.64	23.57 ± 2.35	1.22 ± 0.32	1.94 ± 0.85	61.69
HDL (n = 6)	131.87 ± 35.11	59.40 ± 5.75	6.47 ± 2.25	19.57 ± 3.00	4.85 ± 2.16	3.09 ± 0.52	225.25

[a] Mean ± standard deviation.

[b] Recovery of lipids after repeated ultracentrifugation was 70%.

TABLE 2

CHEMICAL COMPOSITION OF TURKEY SERUM LIPOPROTEIN DENSITY CLASSES

Density class	Protein	Phospholipid	Cholesterol		Triglyceride	Diglyceride
			Free	Ester		
	(%)					
VLDL (n = 4)	13.78	21.70	3.67	26.64	17.45	16.76
LDL (n = 4)	26.05	26.24	5.15	38.21	1.98	3.14
HDL (n = 6)	58.54	26.37	2.87	8.69	2.15	1.37

TABLE 3

COMPOSITION OF PHOSPHOLIPIDS OF TURKEY SERUM LIPOPROTEIN DENSITY CLASSES

Density class	Phosphatidyl-ethanolamine	Phosphatidyl-choline	Sphingomyelin	Lysophosphatidyl-choline
	(%)			
VLDL (n = 4)	8.80 [a] (6.37—12.47) [b]	58.87 (48.93—73.63)	21.59 (15.60—27.00)	13.37 (11.90—15.45)
LDL (n = 4)	6.60 (5.39—7.72)	59.65 (50.51—72.92)	27.52 (21.62—34.85)	12.83 (8.99—20.20)
HDL (n = 6)	8.58 (6.75—11.22)	71.78 (60.31—74.18)	12.83 (8.99—20.20)	6.51 (5.61—12.74)

[a] Mean.
[b] Range.

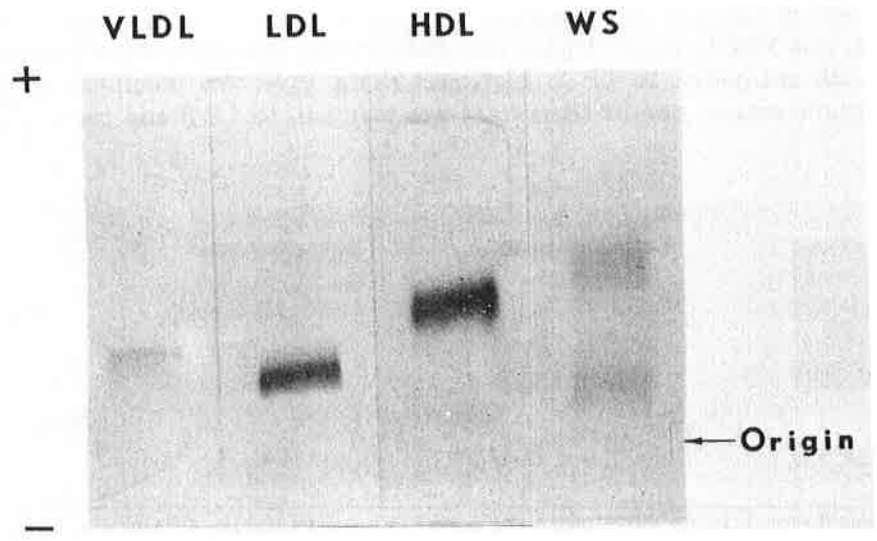

Fig. 1. Agarose gel electrophoresis of turkey serum lipoprotein density classes. The patterns were stained for lipid with Oil Red O.

TABLE 4

CHARACTERIZATION OF ANTISERA TO TURKEY SERUM LIPOPROTEIN DENSITY CLASSES

Designation of antisera	Host animal	Total protein injected	A-I	A-II	Lipo-protein B	Albumin	Comment
Anti-VLDL	Rabbit	3—8 mg	—	—	+	—	
Anti-LDL	Rabbit	5—10 mg	+	+	+	—	Antibodies to A-I and A-II were removed by adding HDL_3 [a]
Anti-HDL	Rabbit	10—20 mg	+	+	+	—	Antibodies to LP-B were removed by adding VLDL [b]
Anti-A-II	Rabbit	10—20 mg	—	+	—	—	Prepared from injection of fraction 5 from chromatography of apoHDL_3 in Bio-Gel A-5m

[a] After absorption of anti-LDL serum with HDL_3 this antiserum was designated anti-LP-B.
[b] After absorption of anti-HDL serum with VLDL this antiserum was designated anti-LP-A.

rum to VLDL contained antibodies only to LP-B. The antisera to LDL and HDL contained antibodies to both LP-A (A-I and A-II polypetides) and LP-B. The antibodies to LP-B which were detected in the antiserum to HDL could be removed by adding VLDL to the antiserum. Likewise, the antibodies to LP-A found in antiserum to LDL could be absorbed by treating the antiserum with HDL_3. The antiserum to LDL was designated anti-LP-B after absorption with HDL_3 and the antiserum to HDL was designated anti-LP-A after absorption with VLDL.

The availability of antisera to LP-A and LP-B permitted the immunological identification of these two lipoprotein families in the major density classes. The results of double diffusion experiments are presented in Fig. 2. VLDL, LDL and HDL_2 gave a single line of identity with antiserum to LP-B. A single broad precipitin line of identity was also seen when antiserum to LP-A reacted with LDL, HDL and VHDL. Some VLDL preparations also showed a weak precipitin line with antibodies to LP-A. LDL and HDL_2 gave two precipitin lines with anti-whole serum: one of these lines was identical to LP-B and the other

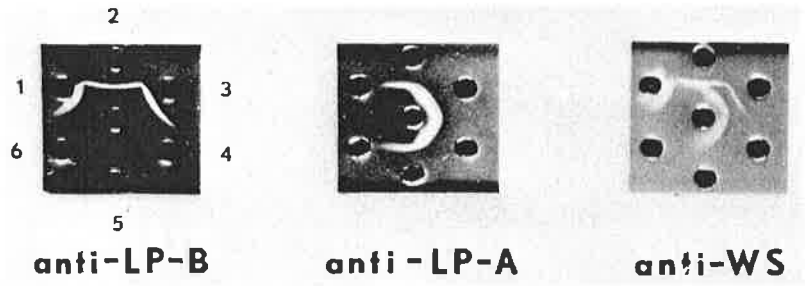

Fig. 2. Immunodiffusion or turkey serum lipoprotein density classes in 1% agarose. Antigens were placed in the outer wells and the antisera in the inner wells. Anti-LP-B, anti-lipoprotein B; anti-LP-A, anti-lipoprotein A; anti-WS, anti-whole serum; 1, very low density lipoprotein, 2, low density lipoproteins, 3, high density lipoproteins$_2$, 4, high density lipoproteins$_3$, 5, very high density lipoproteins.

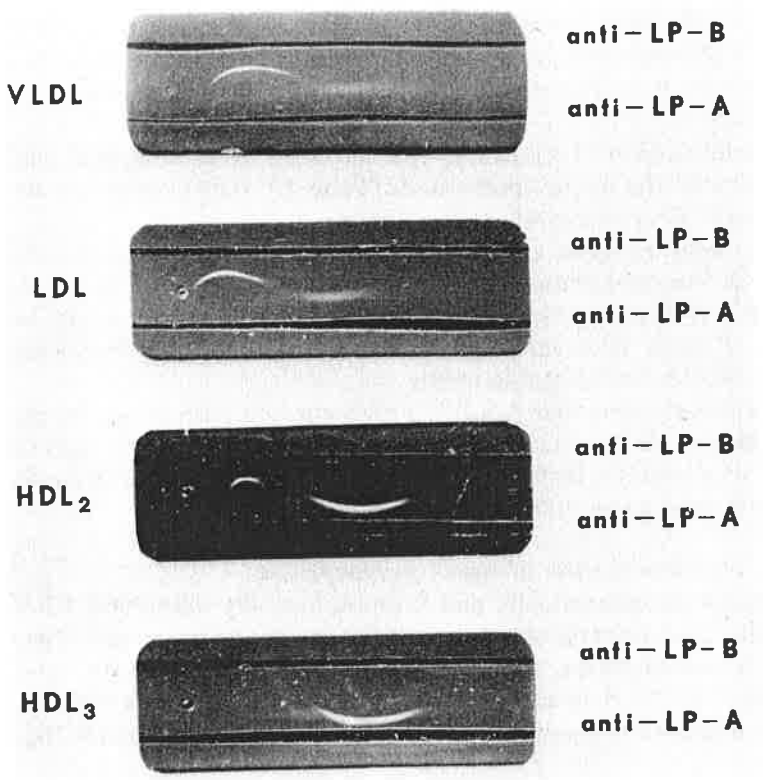

Fig. 3. Immunoelectrophoresis of turkey serum lipoprotein density classes in 1% agarose. Antigens were placed in the center wells and the appropriate antisera in the troughs. The anode is on the right and the cathode on the left. Anti-LP-A, antilipoprotein A; anti-LP-B, anti-lipoprotein B.

to LP-A. None of the purified lipoprotein density classes reacted with antiserum to albumin.

The results of immunoelectrophoretic analysis of the density classes were similar to those obtained by double diffusion. Immunoelectrophoresis clearly showed that VLDL, LDL, and HDL_2 contained LP-A and LP-B families (Fig. 3). HDL_3 reacted only with antiserum to LP-A.

Isolation and partial characterization of ApoB

Since the electrophoretic and immunologic testing of LDL indicated the presence of LP-B as the major and LP-A as the minor lipoproteins, the totally delipidized LDL were extracted with 0.1 *M* Tris, pH 8.0, to remove ApoA or its constitutive polypeptides. Insoluble ApoB was recovered by low speed centrifugation. Reduced and carboxymethylated ApoB was solubilized by dialysis against either 0.01 *M* Tris—8 *M* urea or 0.01 *M* Tris alone. When chromatographed on a column of Bio-Gel A-5m equilibrated with 6 *M* guanidine HCl, most of the carboxymethylated ApoB was eluted at the void volume. This result indicates a marked tendency of even carboxymethylated ApoB to aggregate in a dissociating solvent. Although on the urea-PAGE most of the carboxymethylated ApoB remained at the junction of the stacking and separating gels, a small portion mi-

grated slowly as a doublet into the separating gel (Fig. 4). A negative result of immunologic testing of these two bands with antibodies to LP-A excluded the possible presence of ApoA polypeptides. Fast migrating bands were not detectable by urea—PAGE.

The amino acid composition of ApoB was characterized by aspartic acid, glutamic acid and leucine as the major amino acids (Table 5). Half cystine was determined as cysteic acid after performic acid oxidation.

N-terminal amino acid analysis of ApoB or carboxymethylated ApoB by either dansylation, dinitrophenylation or the automated Edman reaction gave negative results. This indicated that the amino terminus of ApoB might be blocked. Digestion of ApoB with carboxypeptidases A and B failed to release measurable amounts of C-terminal amino acids.

Carbohydrate analysis showed that ApoB is a glycoprotein with mannose, galactose and galactosamine as the sugar constituents occuring in the amounts of 12, 5 and 6 moles/10^5 g protein, respectively. No sialic acid was found in ApoB but these experiments need to be verified.

Isolation and partial characterization of ApoA polypeptides

The absence of an electrophoretically and immunologically detectable LP-B rendered HDL_3 as the most suitable starting material for the isolation and characterization of ApoA polypeptides. Totally delipidized HDL_3 was equally soluble in 0.01 *M* Tris (pH 8.01), 8 *M* urea or 6 *M* guanidine HCl. On urea—PAGE, apoHDL_3 showed a major slow moving band and a faster migrating band (Fig.

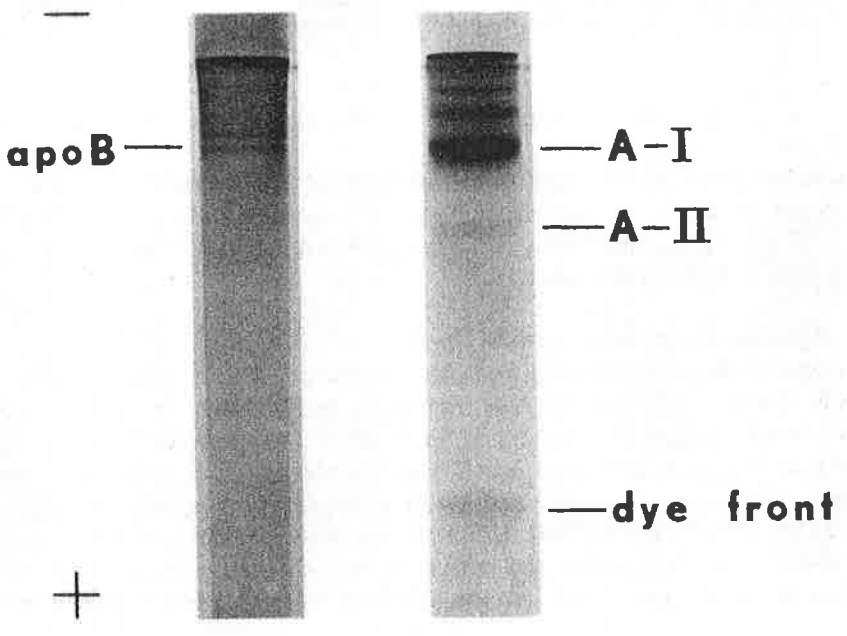

Fig. 4. Urea—polyacrylamide gel electrophoresis of reduced carboxymethylated ApoB (left) and apoHDL_3 (right). The gels were 7% acrylamide and were stained with Coomassie Brilliant Blue R-250.

4). The two bands which migrated slower than the major band were shown to be immunochemically identical to the major band. This result suggests that these bands represent polymeric forms of the major band. Whereas HDL_3 gave a single precipitin line with antibodies to LP-A, the totally delipidized HDL_3 showed two distinct immunoprecipitin lines when reacted with the same antiserum (Fig. 5). These results suggested that $apoHDL_3$ contained two antigenic components.

$ApoHDL_3$ was separated by column chromatography on Bio-Gel A-5m into five fractions (Fig. 6). When fractions 1—4 were examined by immunodiffusion, two precipitin lines characteristic of $apoHDL_3$ were observed, whereas fraction 5 showed only the precipitin line closer to the antibody well (Fig. 5). On SDS—PAGE, the protein present in fraction 1 remained at the top of the gel (Fig. 7). This protein probably represents an aggregated form of the major polypeptide. Fraction 2 displayed a major band migrating to the middle part of the gel and a minor band near the dye front. Fraction 3, which contained most of the protein of $apoHDL_3$, showed the same bands as fraction 2. Fraction 4 contained equal amounts of the two polypeptide bands, whereas fraction 5 consisted only of the faster migrating polypeptide. Rechromatography of fraction 3 resulted in a preparation which displayed a single protein band with a molecular weight of 27,000 daltons. This major polypeptide of $apoHDL_3$ was designated as A-I (Fig. 8). The single fast moving polypeptide of fraction 5 with a molecular weight of 10,000 was designated as A-II (Fig. 8).

The immunodiffusion pattern in Fig. 5 shows that $apoHDL_3$ gave two precipitin lines with anti-LP-A serum. The faster diffusing precipitin line of $apoHDL_3$ gave an identity reaction with the immunoprecipitin line formed between

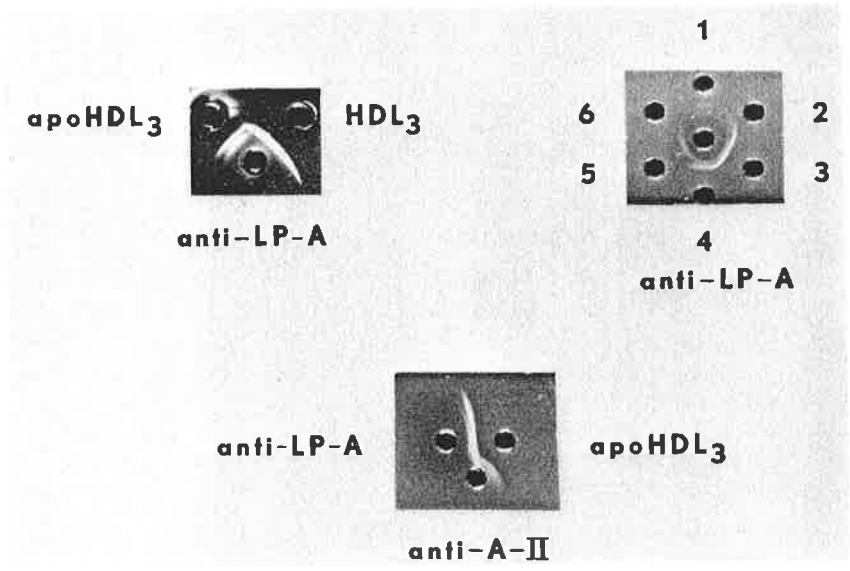

Fig. 5. Immunodiffusion of lipoprotein A and apolipoprotein A in 1% agarose. The top left pattern shows the reaction of intact and delipidized HDL_3 against anti-lipoprotein A. The top right pattern shows the reaction of five fractions, obtained by chromatography of $apoHDL_3$ on Bio-Gel A-5m, against anti-lipoprotein A. The bottom pattern shows the non-identity reaction of the A-I and A-II polypeptides.

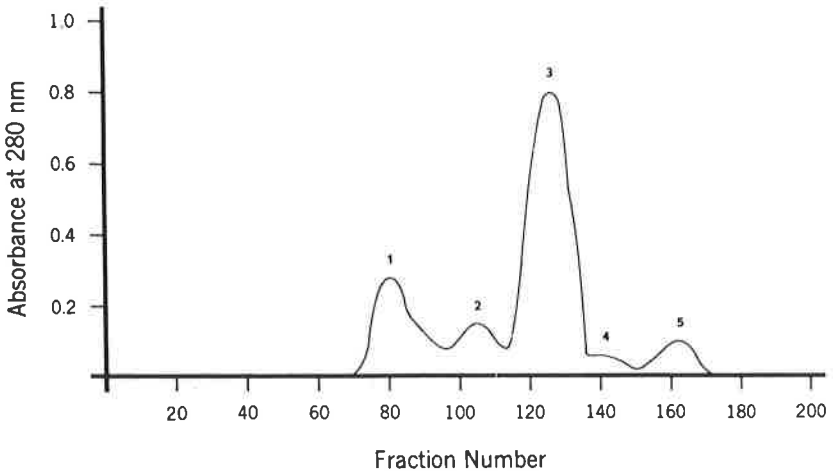

Fig. 6. Elution profile of apoHDL$_3$ on a column of Bio-Gel A-5m equilibrated with 6 *M* guanidine—HCl. Twenty-five mg of sample were applied and 3 ml fractions were collected at a flow rate of 20—30 ml/h.

apoHDL$_3$ and monospecific antibodies to A-II. These results identified the precipitin line closer to the antigen well as that of A-I and the precipitin line closer to the antibody well as that of A-II. Antiserum to A-II precipitated both A-I and A-II polypeptides from intact HDL$_3$. This finding indicated that A-I and A-II are present in the same lipoprotein molecule.

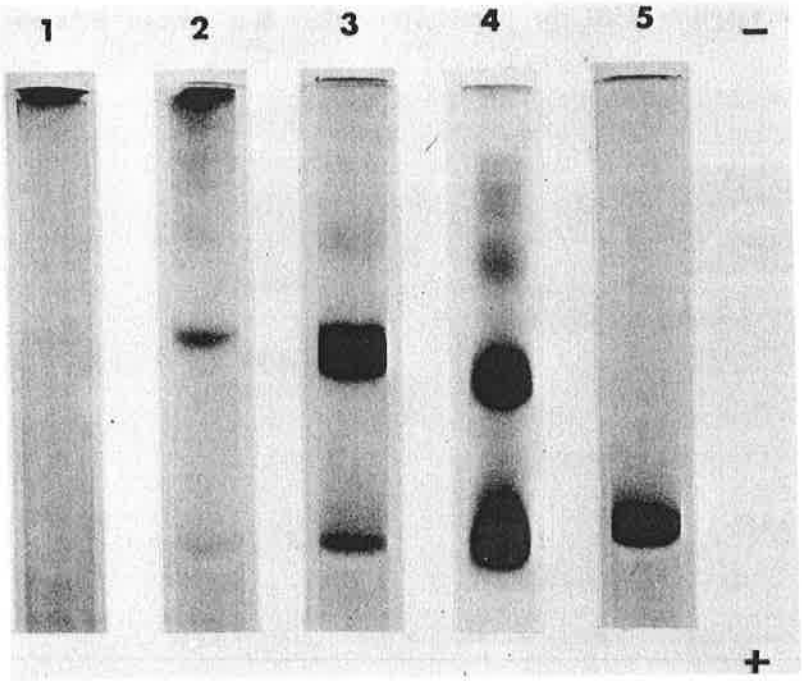

Fig. 7. SDS-polyacrylamide gel electrophoresis of fractions obtained by chromatography of apoHDL$_3$ on a column of Bio-Gel A-5m equilibrated with 6 *M* guanidine HCl. Gels were stained with Coomassie Brilliant Blue R-250.

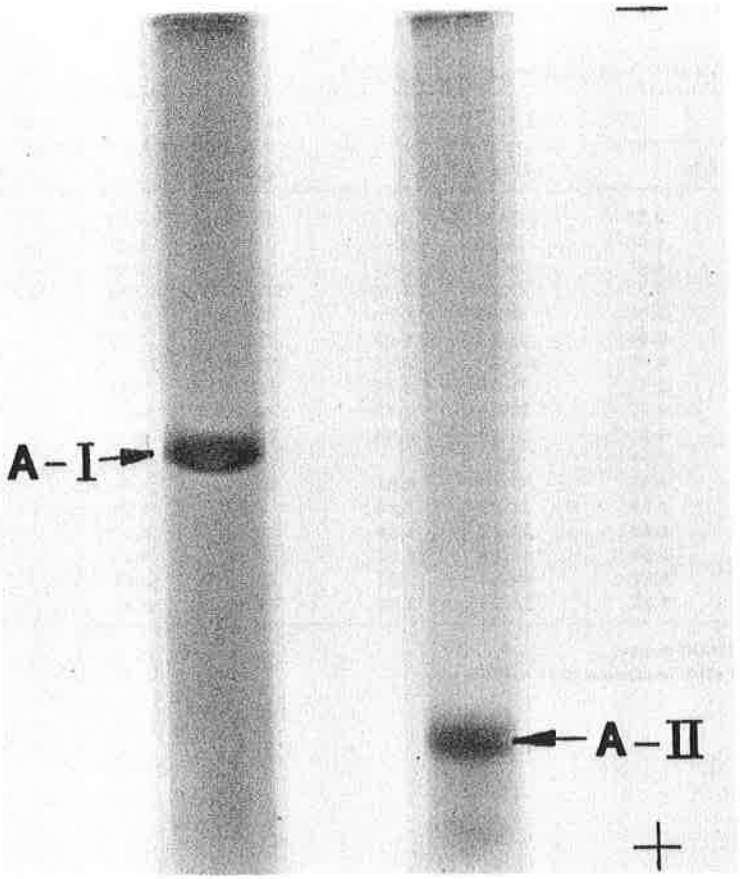

Fig. 8. SDS—polyacrylamide gel electrophoresis of purified A-I and A-II polypeptides. Approximately 50 μg of protein were applied to each gel. Gels were stained with Coomassie Brilliant Blue R-250.

The amino acid composition of A-I was characterized by the absence of half cystine (Table 5). Glutamic acid, leucine and lysine were the major and histidine, methionine and phenylalanine the minor amino acids. Digestion of A-I with a mixture of carboxypeptidases A and B (Fig. 9) indicated alanine as the C-terminal amino acid. This finding was verified by hydrazinolysis which released alanine as the only amino acid. The N-terminal amino acid of A-I was aspartic acid and the terminal sequence was H_2N-Asp-Asp-Asn-Gln-Thr-Pro-Leu-Asn-Glu-Ileu-R. Carbohydrate analysis of A-I indicated the absence of sugar constituents.

The main characteristic of the amino acid composition of A-II was the absence of half cystine (Table 5). Glutamic acid, aspartic acid and leucine were the major and histidine, methionine and phenylalanine the minor amino acids. Alanine was found to be the C-terminal amino acid by digestion with carboxypeptidases A and B (Fig. 10) and by hydrazinolysis. Dansylation, dinitrophenylation and Edman degradation of A-II failed to release an N-terminal amino acid residue. A-II contained no neutral sugars or hexosamine. The A-II preparations did not activate human or turkey post-heparin lipoprotein lipases.

TABLE 5

AMINO ACID COMPOSITION OF APOLIPOPROTEINS A AND B [a]

	ApoB		A-I		A-II	
	Mean ± S.D.		Mean ± S.D.		Mean ± S.D.	
Lysine	71.81	2.86	114.61	4.17	74.43	4.97
Histidine	2.44	1.38	6.85	0.35	6.04	1.17
Arginine	41.58	4.41	85.51	8.87	58.28	1.83
Aspartic acid	107.34	3.71	83.68	8.59	123.27	15.54
Threonine	70.89	3.41	59.09	6.30	72.65	2.66
Serine	89.01	9.24	31.82	1.63	55.20	0.75
Glutamic acid	129.45	6.27	206.20	9.82	175.39	20.21
Proline	45.33	2.21	32.28	6.32	53.06	7.31
Glycine	60.62	6.11	27.71	2.43	44.09	4.46
Alanine	74.75	6.21	94.90	6.89	87.51	6.14
Half cystine [b]	15.61	0.91	0		0	
Valine	58.94	4.83	51.69	3.61	63.97	1.65
Methionine	6.43	2.72	11.09	1.90	6.04	1.01
Isoleucine	48.65	4.42	25.13	1.89	25.46	6.77
Leucine	103.47	1.87	141.29	12.25	118.62	5.97
Tyrosine	31.11	2.37	24.20	1.87	21.39	6.61
Phenylalanine	43.84	6.22	16.98	2.32	16.39	6.44

[a] Values expressed as moles/1000 moles.
[b] Determined as cysteic acid after performic acid oxidation.

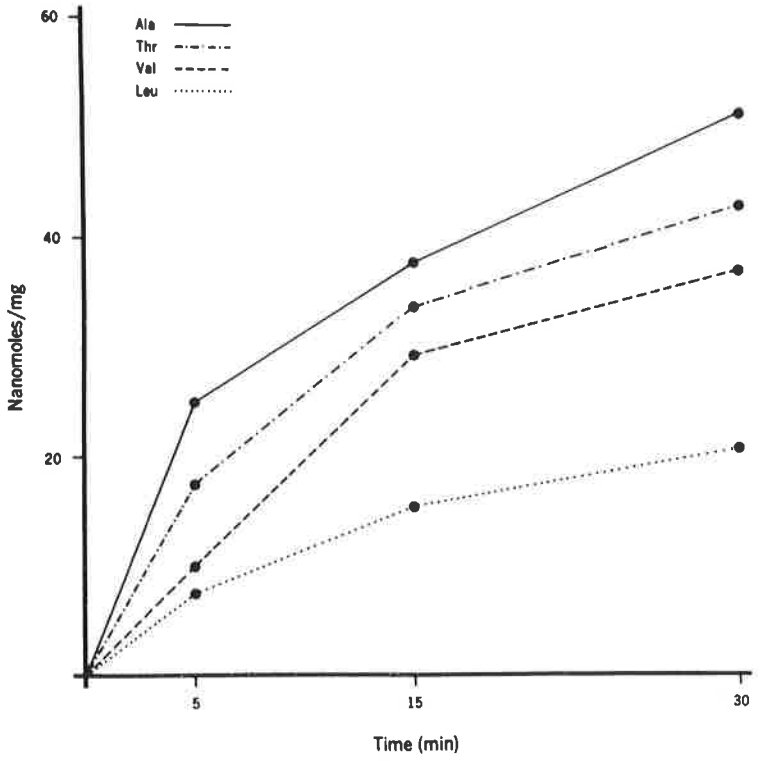

Fig. 9. Release of amino acids from the A-I polypeptide by carboxypeptidase A and B at 37° C and pH 8.5. The ratio of enzyme to polypeptide was 1 : 80.

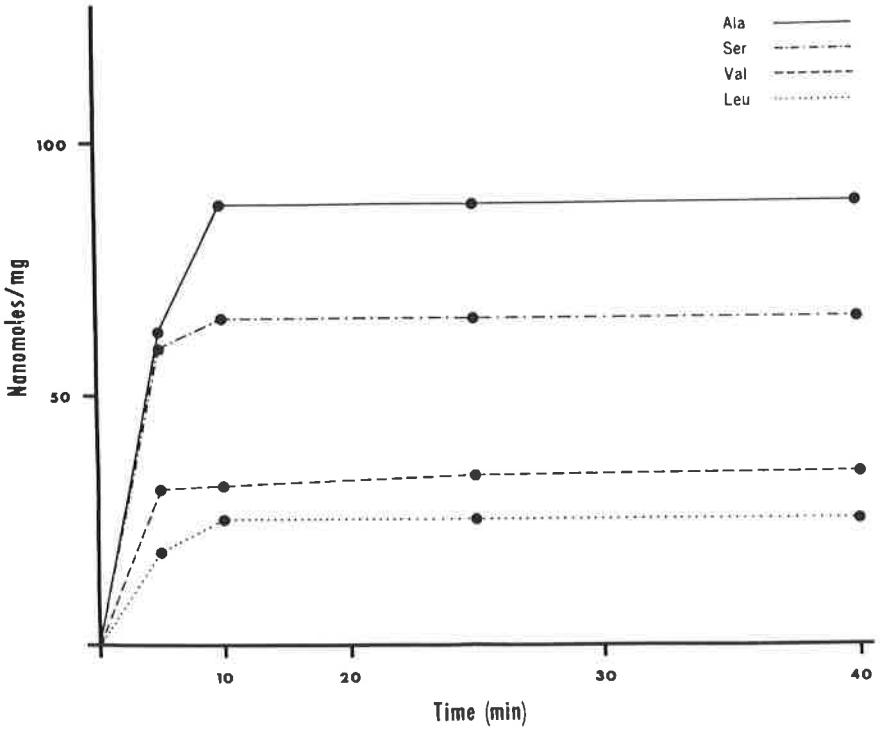

Fig. 10. Release of amino acids from the A-II polypeptide by carboxypeptidase A and B at 37°C and pH 8.5. The ratio of enzymes to polypeptide was 1 : 80.

Discussion

Results of this study show that HDL represent the major circulating lipoprotein density class in plasma of male turkeys. The occurrence of HDL as the major density class has also been established in chicken, goose and pigeon [33, 34], and in several mammals such as horse, sheep, pig and dog [35]. Like in the immature hen or cockerel [36], serum from male turkeys maintained on a normal diet contained no chylomicron and only a small amount of VLDL. It has been suggested that the low concentration of VLDL can be ascribed to an apparently very efficient tissue lipolytic system which is capable of preventing their accumulation in the serum of non-laying birds [36]. In contrast to our results, Mills and Taylour [34] found a high concentration of VLDL (S_f 100-400) in turkey serum. Whether this discrepancy is due to a difference in sex, dietary regimen or an impairment in the removal mechanism of VLDL is not known.

The chemical composition of lipoprotein density classes is very similar in the turkey, chicken and other avian species [33,34,37].

Immunochemical studies have revealed that the male turkey serum lipoprotein system consists of at least two distinct lipoprotein families designated, in analogy to the human serum lipoprotein system [32], as LP-A and LP-B. VLDL, LDL and HDL_2 contain both LP-A and LP-B. In contrast, HDL_3 contains only LP-A. The LP-B from VLDL, LDL and HDL_2 showed a reaction of complete

identity when examined by double diffusion. Likewise, the LP-A from LDL, HDL_2, HDL_3 and VHDL showed a reaction of identity. The distribution of these two lipoprotein families over a relatively wide range of hydrated densities clearly indicates their polydisperse character and thus explains the antigenic heterogeneity of the major density classes.

Serum lipoproteins from male turkeys contain two major apolipoproteins designated, in analogy with human apolipoproteins, as ApoA and ApoB. As the protein moiety of LP-B, ApoB occurs mainly in VLDL and LDL. The physical and chemical properties of turkey ApoB are very similar to those of human apoB [38—40]. Turkey apoB is insoluble in aqueous buffers, but can be solubilized after reduction and carboxymethylation in dissociating agents such as 6 *M* guanidine HCl. The amino acid composition is characterized by half cystine, and by glutamic acid, aspartic acid and leucine as the major amino acids. The nature of both the N-terminal and C-terminal amino acids is at best uncertain. ApoB contains a carbohydrate moiety consisting of mannose, galactose and galactosamine. The amino acid composition of turkey ApoB is very similar to that of chicken apoLDL [36]. Hillyard et al. [36] found lysine as the N-terminal and tyrosine as the C-terminal amino acids of chicken serum apoVLDL and apoLDL. So far, we have not been able to confirm these amino acids as the terminal amino acids of turkey apoB.

The second major apolipoprotein of male turkey lipoproteins is ApoA which, as the protein moiety of LP-A, occurs mainly in HDL. ApoA is the only major apolipoprotein of HDL_3 and consists of 2 non-identical polypeptides analogous to human A-I and A-II. Most of the physical and chemical properties of turkey A-I and A-II are very similar to those of human A-I and A-II [41—43]. Some of the physical and chemical similarities between turkey and human A-I include (a) molecular weight of 27,000 daltons, (b) electrophoretic mobility in urea- and SDS-PAGE, (c) tendency to form aggregates, (d) amino acid composition characterized by leucine, glutamic acid and lysine as the major amino acids by the absence of half cystine, (e) aspartic acid as the N-terminal amino acid, (f) homology of the N-terminal sequence, and (g) absence of a carbohydrate moiety. The major difference is the substitution of alanine for glutamine as the C-terminal amino acid of turkey A-I. Although A-I preparations from human [44], non-human primates [45,46] and pig [47] all contain glutamine at the C-terminus, the A-I polypeptide from the rat [48] and the major apolipoprotein from chicken HDL [36] have alanine as the C-terminal amino acid. In contrast to human A-I, turkey A-I contains a small amount of isoleucine. The chemical similarity between the human and turkey A-II polypeptides includes (a) glutamic acid, leucine and lysine as the major amino acids, (b) blocked N-terminal amino acid and (c) absence of a carbohydrate moiety. The A-II polypeptides from these two species differ, however, in the molecular form, and C-terminal amino acid. Turkey A-II, like those of rhesus [45] and rat [49] but unlike those of human [44] and chimpanzee [46], exists in a monomeric form. The A-II polypeptide from the turkey lacks half cystine and its molecular weight is approximately that of human A-II. In contrast to the human polypeptide, the amino composition of turkey A-II is characterized by the presence of histidine and arginine. Because of a reduced size, turkey A-II migrates in the electric field faster than human A-II. The migration rate of turkey A-II in urea—PAGE

is slower than those of either rat [49] or human [50] ApoC-polypeptides. Like rat A-II [49], turkey A-II contains alanine as the C-terminal amino acid. It should be pointed out that alanine was the only amino acid released by hydrazinolysis of the turkey A-II polypeptide.

Immunologic studies with a monospecific antiserum to A-II showed that A-I and A-II polypeptides constitute the protein moiety (apoA) of LP-A. Results of a study on the isolation and characterization of turkey LP-A and LP-B will be presented in a separate communication.

The heterogeneity of density classes is not confined to the turkey lipoprotein system. It has been demonstrated that human plasma lipoproteins consist of at least four lipoprotein families referred to as lipoproteins A, B, C [32,50] and D [51]. Although it has recently been demonstrated that ApoC-polypeptides and the socalled "arginine-rich" polypeptide occur in mammalian species [52,53] other than man, we have not been able to detect and/or to isolate these polypeptides from male turkey lipoproteins in quantities necessary for their unequivocal identification and chemical characterization. Their presence, however, in microgram quantities cannot be excluded on the basis of experiments described in this paper. In fact, we have recently established (unpublished experiments) that turkey serum activates both lipoprotein lipase C-I and C-II [54] from human post-heparin plasma. Although A-I, A-II and ApoB were shown to be inactive, a later HDL_3 fraction eluted from the Bio-Gel A-5m column activated both human serum lipoprotein lipases. If ApoC-polypeptides are the only specific activators of serum and tissue lipoproteins lipases, it appears on the basis of present experiments that their presence in some animal species may be detected only by biological tests.

In summary, the plasma lipoprotein system of male turkey consists of LP-A and LP-B as the distinct lipoprotein families. The LP-A is the major lipoprotein family of HDL and the LP-B the major lipoprotein family of LDL and VLDL. The protein moiety of LP-A consists of two non-identical polypeptides designated, in analogy with human apoliproteins, as A-I and A-II. The third major apolipoprotein is ApoB, the protein moiety of LP-B. The concentrations of ApoC, ApoD and "arginine-rich" polypeptide, if present, are too low for their unequivocal chemical characterization.

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