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ISOLATION OF TURKEY SERUM LIPOPROTEINS AND PARTIAL CHARACTERIZATION OF THEIR APOLIPOPROTEINS

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CHAPTER I

INTRODUCTION

Serum lipoproteins are macromolecular complexes consisting of non-covalently bound protein, neutral lipids, and phospholipids. Lipoproteins exist as a polydisperse system which is heterogeneous with respect to size and hydrated density.

By the late 1920's, it was generally accepted that lipids were present in the euglobin fraction of serum proteins. The first lipoprotein preparation of constant chemical composition was isolated from horse serum by Macheboeuf in 1929 (1). Since this time, the lipoproteins of human serum have been studied in great detail. At the beginning of this study, only limited information on animal serum lipoproteins was found in the literature.

The purpose of this study was to investigate the plasma lipoprotein system of an animal other than human subjects that can develop atherosclerosis spontaneously and use it as a model for studying lipoprotein metabolism. Atherosclerosis occurs spontaneously in birds (2-13) to give lesions similar to those found in humans. Also, atherosclerosis can be induced in chickens (14-19), pigeons (20-22), Japanese quail (23),

and turkeys (24) by feeding diets containing various amounts of cholesterol. We chose the turkey as a representative of the avian species in which to study the serum lipoproteins and apolipoproteins.

Turkey serum lipoproteins were separated by ultracentrifugation into three density classes: VLDL, LDL, and HDL. These density classes were examined with respect to their lipid and protein composition, as well as their immunochemical properties. It was found that two lipoprotein families exist in turkey serum. Lipoprotein families A and B were purified and characterized by their immunochemical and chemical properties. This study represents the first systematic investigation of serum lipoproteins and apolipoproteins from male birds and should set a reference for changes which occur in lipoprotein metabolism when physiological parameters are changed.

CHAPTER II

LITERATURE REVIEW

Classification of Lipoproteins

The domestic chicken is the only representative of the avian species in which the serum lipoproteins have been studied in detail. Vanstone et al. (25) and Schjeide et al. (26) were first to study chicken serum lipoproteins by paper electrophoresis. Regions of the chromatogram corresponding to α- and β-globulins were stained with lipid stains. Because of their electrophoretic mobility, these bands were called α- and β-lipoproteins. The mobilities of chicken serum lipoproteins in paper electrophoresis are similar to those of human serum lipoproteins.

Similar to human serum lipoproteins, chicken serum lipoproteins can most easily be resolved by the use of ultracentrifugal techniques. In 1953, Schjeide and Dickinson (27) developed a partition cell for use in the preparative ultracentrifuge. Using this partition cell, they could separate (at a density of 1.063 g/ml) light and dense lipoproteins.

In 1954, De Lalla and Gofman (28) presented a method for ultracentrifugal isolation of human serum lipoproteins at various sequentially adjusted serum densities. Gofman's methodology resulted in a classification system for lipoproteins based on hydrated densities as unique

characteristics of lipoproteins. Gofman's method was modified by Freeman et al. (29) who also characterized the lipoprotein density classes according to their flotation and sedimentation coefficients. Figure 1a shows the density scale as well as the human serum lipoprotein profile characterized by flotation coefficients. Five density classes of lipoproteins were distinguished. Starting with the densest, they are VHDL, HDL, LDL, VLDL and chylomicrons. The nomenclature and abbreviations for serum lipoproteins are presented in the Appendix. The classification of lipoproteins based on their electrophoretic mobility or hydrated density are the two classification systems which are currently being used for avian serum lipoproteins.

Serum Lipoproteins of Male and Non-Laying Female Chickens

It appears that in the rooster, several lipoprotein density classes are missing (Figure 1b) (30). Chylomicrons and VLDL are not detectable in fasted serum by the use of the analytical ultracentrifuge. The LDL are present in small amounts and have a flotation coefficient of approximately S_f 8.0. The concentration of LDL in chicken serum is lower than that in human serum. HDL are the major lipoprotein density class found in roosters. There are two sedimenting peaks (at a density of 1.063 g/ml) in the HDL density range; one has a sedimentation coefficient of 4.5S and the other 7.0S. In a mature rooster, if the serum lipid falls below 300 mg/100 ml, the 7.0S peak will disappear leaving only the 4.5S peak. As we will see later, the lipoprotein profile of the laying chicken is more similar to the human serum lipoprotein profile than that of the rooster.

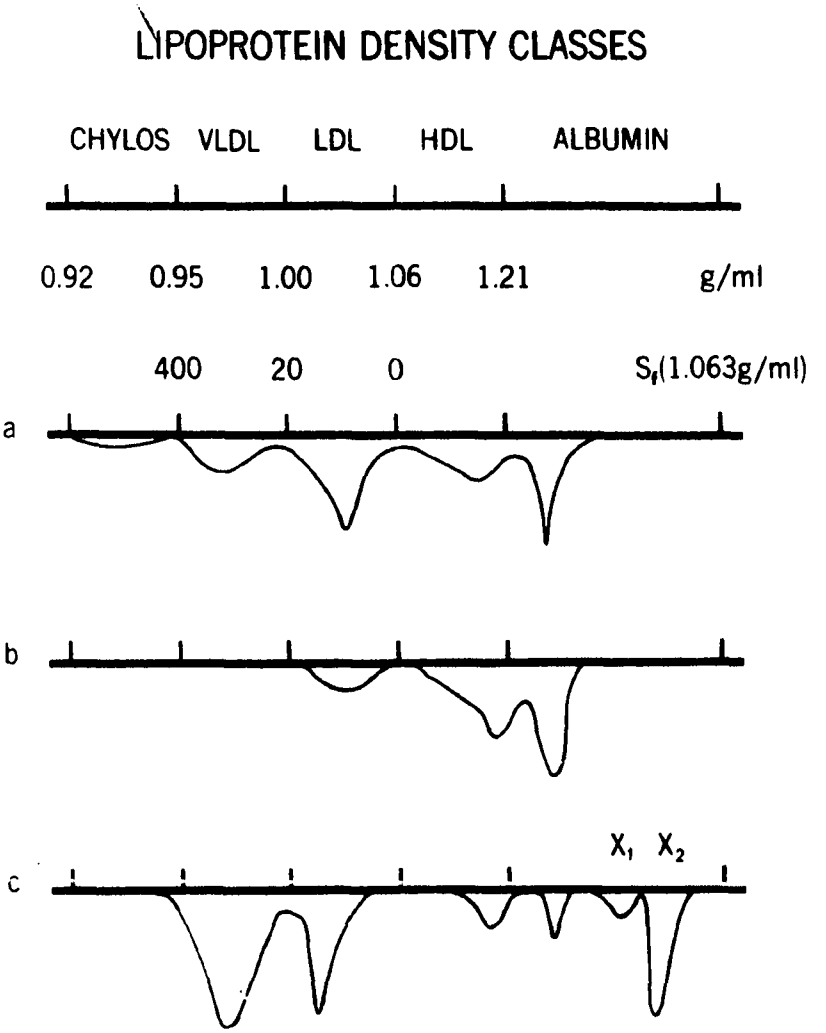

Figure 1. Appearance of serum proteins and lipoproteins in a composite ultracentrifuge pattern. a. Lipoprotein profile for human serum (ref. 29); b. lipoprotein profile for male chicken serum (ref. 30); c. lipoprotein profile for laying female chicken serum (ref. 30); X_1 = X_1-phosphoprotein; X_2 = X_2-lipoprotein.

Apparently, no attempts have been made to fractionate chicken serum lipoproteins by the method of Cohn et al. (31). The chemical analysis of chicken serum lipoproteins has been done by Mills and Taylaur (32), Hillyard et al. (33, 34), Schjeide (35), and Kruski and Narayan (36). The per cent lipid composition is shown in Table 1. As the density of the lipoprotein class increases (VLDL to HDL), the relative per cent of phospholipid and protein increases. Also, the ratio of esterified cholesterol to free cholesterol increases with increasing density. HDL represent the major lipoprotein density class in normal fasting chicken serum (Table 2). Eighty per cent of chicken serum lipoproteins are HDL and only one per cent are VLDL.

By the use of the partition cell, Schjeide separated and determined the amount of carbohydrates in dense and light lipoproteins. The light lipoproteins ($S_f > 0$) had consistent amounts of hexosamine (0.015 mg), sialic acid (0.015 mg) and fucose (0.01 mg) per unit weight of lipoprotein (30). In a different approach, Vanstone et al. (25) found that lipoproteins separated by paper electrophoresis took up detectable amounts of tetracetate-Schiff stain.

No amino acid analyses of apolipoproteins of normal male chicken serum lipoproteins are available, but if they are similar to those of the estrogenized male or laying female, they can be derived from Table 5.

The next section will discuss the changes in serum lipoproteins that are effected at the onset of egg production or upon injection of estrogens.

TABLE 1

PER CENT COMPOSITION OF CHICKEN SERUM LIPOPROTEINS

Density Class	Density Range	Protein	Phospholipid	Cholesterol Free	Cholesterol Ester	Triglyceride	References
VLDL	d < 1.006	6.8	17.9	5.3	17.5	52.5	(32)
LDL	1.006-1.063	23.50	20.2	6.40	27.8	22.1	(32)
		15.55	19.11	6.66	18.22	40.44	(33), (34)
HDL_2	1.063-1.107	39.06	26.56	4.69	12.50	17.19	(33), (34)
HDL_3	1.107-1.21	49.14	24.57	2.35	10.64	13.30	(33), (34)
HDL	1.063-1.21	47.8	22.3	2.2	20.4	7.4	(32)
		50.0	30.1	1.8	14.8	2.3	(35)

TABLE 2

CONCENTRATION OF THE LIPOPROTEIN DENSITY CLASSES OF CHICKEN SERUM[a]

Density Class	Density Range	Lipoprotein	Total Lipid	Per Cent Protein
		mg/100 ml serum		
VLDL	d < 1.019	6.6 ± 3.3	4.4 ± 2.7	10.9
LDL	1.019-1.063	80.6 ± 10.3	57.0 ± 11.9	29.3
HDL	1.063-1.21	353.5 ± 37.3	155.1 ± 19.1	56.1

[a]Ref. 36.

Effects of Estrogen on Chicken Serum Proteins and Lipoproteins

Lorenz et al. (37-39) were the first to show that estrogens were responsible for the hyperlipidemia which occurred at the onset of egg production in chickens. Since that time, the lipid metabolism of birds injected with natural (40, 41) or synthetic estrogens such as diethylstilbestrol (DES) (42-49) has been studied. DES was first administered to birds because Lorenz suggested that it could increase weight gain in poultry (50). The hyperlipidemia in DES-treated birds is due mainly to increased de novo synthesis of triglycerides by the liver and not to mobilization of lipids from depot tissues (51). Hypercholesterolemia is another characteristic effect of estrogen treatment of birds (42, 47, 49, 52). The chick was recently suggested as a good model to study estrogen-induced hyperlipidemia (52).

The lipoprotein profile for the laying chicken (Figure 1C) more closely resembles that of the human than does the profile of the rooster. Upon administration of estrogens to the cockerel, rooster, or chick, the lipoprotein profile becomes the same as that of the laying hen. In the laying chicken, particles the size of chylomicrons are formed from exogeneous lipids. These large particles are not transported via the lymphatic system as in human, but enter the circulation via the portal vein (53, 54). Bensadoun and Rothfeld suggested that these particles should be called portomicrons since they are absorbed through the portal system (54).

VLDL are formed in the laying hen at the same time as portomicrons. The VLDL have a mean flotation rate of S_f 40 (55). The LDL are characterized by a decreased amount of lipoproteins with S_f 0-15 and a

mean flotation coefficient greater than S_f 15. The increase in flotation rate is presumably due to the increased amount of triglyceride in the LDL molecule. The HDL and other normal serum proteins are greatly decreased in the laying hen or estrogenized rooster. In addition to the changes in lipoproteins, two new proteins are found in the serum of the laying fowl: X_1-phosphoprotein and X_2-lipoprotein (25, 56, 64).

Serum X_1-phosphoprotein was called phosvitin by Common and Mok (56). They were the first to discover phosvitin as a constituent of hen's serum. When serum is diluted with nine volumes of water, the phosvitin precipitates from the serum (57, 58). Phosvitin was first isolated in good yield by Heald and McLachlan (59, 60) by precipitation with calcium salts and chromatography on DEAE cellulose. Phosvitin, as synthesized by the liver (61), contains from five to ten per cent phosphorus. The phosphorus is esterified mainly to serine (59). The molecular weight of phosvitin was determined to be 183,000 (62). Serum X_1-phosphoprotein is also a glycoprotein containing mannose, glactose, hexosamine, and sialic acid (63). Because of the highly negative charge, phosvitin moves faster than albumin when electrophoresed on paper. This protein is responsible for binding much of the calcium in the serum.

Serum X_2-lipoprotein is also called lipovitellin by Schjeide and Urist (64). The X_2-lipoprotein contains only twenty per cent lipid which consists mainly of phospholipid. Serum lipovitellin has a sedimentation coefficient of 16.5S. By paper electrophoresis, serum X_2-lipoprotein shows several bands in the post β-globulin region (25). The X_1-phosphoprotein and X_2-lipoprotein form coprecipitates when the serum is diluted with water. Since these two complexes have different sedimentation co-

efficients, they can be separated from one another in the ultracentrifuge. The per cent composition of the X_2-lipoprotein present in serum from laying hens or estrogenized roosters is as follows: protein, 76.5; sterol esters, 1.6; triglycerides, 4.4; sterols, 1.5; and phospholipids, 16.0 (64). Serum X_2-lipoprotein is also a glycoprotein containing about 0.034 mg hexose, 0.0025 mg fucose, 0.01 mg sialic acid and 0.013 mg hexosamine per mg of polypeptide (30). Serum X_1-phosphoprotein and X_2-lipoprotein have similar counterparts in egg yolk.

The chemical composition of lipoproteins from estrogenized chickens has been determined by Hillyard et al. (34). The concentration of serum apolipoproteins and lipids from the lipoprotein density classes are shown in Table 3. In contrast to the rooster, where HDL are the major density class, it is found that at the onset of egg production or upon administration of estrogens, the VLDL become the major lipoprotein density class. The increase in VLDL is due in part to the increase in the synthesis of triglycerides and phospholipids by the liver (52). In VLDL, the triglycerides increase to over 10,000 mg/100 ml of serum. The VLDL in the study also include particles the size of chylomicrons which are very rich in triglycerides. Also, the LDL are increased, but not to the extent that the VLDL are increased. The HDL_2 and HDL_3 are decreased by about four-fold. Serum cholesterol is greatly increased in estrogen-treated birds. Most of the cholesterol is found in the VLDL.

Not only are the levels of the density classes different in estrogenized birds, but also the per cent composition of the individual density classes is changed (Table 4). Triglycerides increase in VLDL and HDL. The phospholipid content of the density classes is slightly

TABLE 3

CONCENTRATIONS OF LIPIDS AND APOLIPOPROTEINS FROM ESTROGENIZED CHICKENS[a]

Density Class	Density Range	Protein	Phospholipid	Cholesterol		Triglycerides
				Free	Ester	
		mg/100 ml serum				
VLDL	d < 1.006	1,952	3,197	485	718	10,797
LDL	1.006-1.063	260	316	44	55	649
HDL_2	1.063-1.107	31	10	2	3	25
HDL_3	1.107-1.210	85	24	2	5	23

[a]Ref. 34.

TABLE 4

PER CENT COMPOSITION OF SERUM LIPOPROTEINS FROM ESTROGENIZED CHICKENS[a]

Density Class	Density Range	Protein	Phospholipid	Cholesterol		Triglycerides
				Free	Ester	
VLDL	d < 1.006	11.38	18.64	2.83	4.19	62.96
LDL	1.006-1.063	19.64	23.87	3.32	4.15	49.02
HDL_2	1.063-1.107	43.66	14.08	2.84	4.23	35.21
HDL_3	1.107-1.210	61.15	17.27	1.44	3.60	16.55

[a]Ref. 34.

elevated, but remains constant over the entire density range. In the HDL_3 density class, the relative content of protein increases from fifty per cent to sixty per cent. The most obvious change in lipids upon the injection of DES is the marked increase of triglycerides and cholesterol in the VLDL.

The apolipoproteins of the density classes from laying hens have been characterized by Hillyard et al. (55). Also, the amino acid composition of chylomicrons and lipoproteins $S_f > 15$ were studied by Schjeide (30). Chylomicrons and lipoproteins $S_f > 15$ have similar amino acid compositions (Table 5). On the basis of similar amino acid composition, Schjeide inferred that the protein moiety of the two density classes was the same. In a more classical separation of serum lipoproteins into VLDL, LDL, and HDL, Hillyard et al. (55) characterized the apolipoproteins according to their amino acid composition and immunological properties. ApoVLDL and apoLDL had similar amino acid compositions which were significantly different from the amino acid composition of apoHDL. Relatively large amounts of aspartic acid, glutamic acid, and leucine are characteristic of apoLDL and apoVLDL. ApoLDL differs from apoHDL in the amounts of the following amino acids: histidine, arginine, threonine, serine, glutamic acid, glycine, half cystine, methionine, isoleucine, leucine and phenylalanine. ApoLDL has a greater amount of phenylalanine than tyrosine. Also, the ratio of serine to threonine is greater than unity. In apoHDL, the ratio of tyrosine to phenylalanine is always greater than unity and the ratio of serine to threonine is less than unity. The amino acid composition of serum X_1-phosphoprotein (phosvitin) has a characteristic amount of serine which occurs mainly as phosphoser-

TABLE 5

AMINO ACID COMPOSITION OF SERUM APOLIPOPROTEINS, ALBUMIN, X_1-PHOSPHOPROTEIN AND X_2-LIPOPROTEIN FROM LAYING HENS[a]

	Chylomicrons[b]	S_f 15[b]	VLDL[c]	LDL[c]	HDL[c]	X_1[d]	X_2[b]	Albumin[b]
Lysine	8.4	8.8	7.02	7.46	8.92	7.56	7.6	7.9
Histidine	1.6	1.8	1.12	1.43	0.77	3.66	2.9	1.9
Arginine	2.5	4.4	4.58	4.40	7.51	7.33	6.3	4.8
Aspartic acid	10.9	10.7	10.9	10.5	8.54	9.58	8.8	10.0
Threonine	5.8	5.5	6.26	6.31	4.57	4.52	5.0	4.5
Serine	5.6	5.5	7.09	7.75	4.29	10.57	9.0	5.7
Glutamic acid	12.6	12.4	11.3	13.1	20.5	11.44	10.6	13.6
Proline	4.4	4.2	3.62	3.54	4.35	4.54	5.8	5.7
Glycine	5.5	4.8	4.44	5.07	1.96	3.22	5.7	5.3
Alanine	9.6	7.3	7.65	7.36	9.19	5.92	7.9	7.4
Half cystine	0	0	0.99	0.80	0.23	2.00	0	6.3
Valine	5.4	7.4	6.60	6.22	5.34	6.68	7.4	5.7
Methionine	2.0	2.3	2.02	1.72	2.33	2.73	2.3	2.3
Isoleucine	7.2	6.0	6.54	5.55	2.51	5.60	6.0	3.8
Leucine	10.5	10.9	10.4	10.3	13.0	8.46	8.4	7.5
Tyrosine	3.9	3.6	3.41	2.39	2.55	5.29	3.1	3.1
Phenylalanine	4.2	4.4	4.25	4.40	2.07	5.15	3.2	4.5
Tryptophane	N.D.	N.D.	1.77	1.66	1.38	3.31	N.D.	N.D.

[a]Values in moles/100 moles amino acids in sample.

[b]Ref. 30.

[c]Ref. 55.

[d]Ref. 63.

ine (63). Serum X_2-lipoprotein (lipovitellin) shows an amino acid composition different from other serum lipoproteins. Serum albumin is also shown in Table 5 for comparison with the other serum lipoproteins. Albumin has significantly more half cystine than any of the other serum lipoproteins or estrogen-induced proteins.

Amino and carboxyl terminal amino acids of the density classes from laying chickens are summarized in Table 6 (55). Lysine was the only N-terminal amino acid detected by the FDNB method for the apolipoproteins of VLDL and LDL. Aspartic acid was the major N-terminal amino acid found in apoHDL. By hydrazinolysis, tyrosine was the major C-terminal amino acid found in both apoVLDL and apoLDL. In addition to tyrosine, valine and leucine were released by treatment of apoVLDL with carboxypeptidase A (CPA). Detection of alanine and leucine after hydrazinolysis of apoHDL indicated the presence of more than one polypeptide. Alanine, valine, and serine were the only amino acids released by the treatment of apoHDL with CPA at pH 6.0, 6.7 and 8.2. At a pH of 8.9, leucine, glutamine and lysine were released by CPA from apoHDL. Three C-terminal amino acids and only two N-terminal amino acids were found for apolipoproteins of chicken serum.

The major polypeptide of VLDL had a molecular weight of 21,800 (55). Upon reduction and carboxymethylation, the apoprotein of VLDL had a molecular weight by disc gel electrophoresis of 9,000. The major apoprotein of LDL had a molecular weight of 21,300. In addition to the major band in apoVLDL and LDL, minor bands of higher molecular weights were found. The major band on polyacrylamide gel electrophoresis (PAGE) from apoHDL had a molecular weight of 30,100. A minor band of molecular

TABLE 6

AMINO AND CARBOXYL TERMINAL AMINO ACIDS OF THE LIPOPROTEIN DENSITY CLASSES FROM LAYING CHICKENS

Density Class	Density Range	Terminal Amino Acid		
		Amino[a]	Carboxyl[b]	Carboxyl[c]
VLDL	d < 1.006	Lys	Tyr	Tyr, Val, Leu
LDL	1.006-1.063	Lys	Tyr	Tyr
HDL	1.063-1.21	Asp	Ala, Leu	Ala, Val, Ser

[a]By DNP.

[b]By hydrazinolysis.

[c]By CPA.

weight of 20,000 to 24,000 was seen in some preparations. Other bands in apoHDL had higher molecular weights. ApoLDL was soluble in a buffer containing SDS. ApoHDL formed aggregates which could not be dissociated by urea, guanidine, SDS, triton X-100, or any other dissociating agents tested.

Two non-identical antigenic determinants were found when chicken serum lipoproteins were injected into rabbits. One determinant was characteristic of LDL and VLDL, and the other of HDL. It was found that the major determinant of HDL could be detected in LDL. Also, the major determinant of LDL could be found in HDL. On immunoelectrophoresis, apoHDL gave two precipitin lines with antibodies to apoHDL indicating antigenic heterogeneity of HDL.

Because of such polypeptide heterogeneity of the density classes, a chemical classification system for human serum lipoproteins based on the apolipoproteins was proposed for the differentiation of lipoprotein families (66). Lipoprotein families were defined as "polydisperse systems of lipid-protein complexes characterized by the presence of a single distinct apolipoprotein or its constitutive polypeptides" (67). The chemical classification of lipoproteins currently recognizes three distinct lipoprotein families: lipoprotein family LP-A, characterized by the presence of apolipoprotein A; lipoprotein family LP-B, by apolipoprotein B; and lipoprotein family LP-C, by apolipoprotein C. The chemical classification system for human serum lipoprotein families is presented in Table 7.

TABEL 7

CHEMICAL CLASSIFICATION OF HUMAN PLASMA LIPOPROTEINS

Lipoprotein Family	Apolipoproteins	Constitutive Polypeptides	N-Terminal Amino Acid	C-Terminal Amino Acid	Missing Amino Acids
LP-A	ApoA	A-I	Aspartic acid (67-69)	Glutamine (70)	Ile, 1/2 Cys (71)
		A-II	Pyrollidone Carboxyllic acid (72)	Glutamine (70, 71)	His, Arg, Try (71, 73, 74)
LP-B	ApoB	B	(Glutamic acid) (67, 75-78)	(Serine) (67)	None (79, 80)
LP-C	ApoC	C-I	Threonine (81, 82)	Serine (81, 82)	His, Tyr, 1/2 Cys (83)
		C-II	Threonine (84)	(Glutamic acid) (84)	His, 1/2 Cys (85)
		C-III-1[a]	Serine (83)	Alanine (83)	Ile, 1/2 Cys (83)
		C-III-2[a]	Serine (83)	Alanine (83)	Ile, 1/2 Cys (83)

[a]C-III-1 and C-III-2 are polymorphic forms of C-III peptide. Roman numerals represent the parent polypeptide, while Arabic numbers designate polymorphic forms.

Lipoproteins of Chicken Egg Yolk

With the discovery that estrogens induce the formation of certain serum proteins rich in phosphorus (37, 57, 86), it was only a short time before these serum proteins were linked to similar proteins found in egg yolk. In 1956, Flickenger and Rounds (86) and Schjeide and Urist (64), independently concluded that the unique serum proteins were specific precursors of certain proteins found in avian egg yolk.

When the hen reaches an age where she is physiologically and anatomically ready to reproduce, there occurs an increased estrogen in the circulation. Estrogens induce the production of two specific serum proteins: X_1-phosphoprotein (phosvitin) and X_2-lipoprotein (lipovitellin). A three-fold increase in the synthesis of the protein moiety of β-lipoproteins (87) results in an increased amount of VLDL and LDL. The estrogen-induced serum proteins and VLDL pass via the circulation to the ovary where they cross the capillary and egg yolk membranes to be deposited as part of the egg yolk. VLDL seems to be removed intact from the serum (88). The deposition of serum proteins in the egg yolk occurs over a period of several days. Evidence for the sequence of events in protein deposition was given by Flickinger and Rounds (86). Radioactive phosphorus was injected into laying hens. The label was first concentrated in the liver for several hours, then was observed in the serum proteins. After one or two days, the radioactivity was highest in the yolks of developing eggs.

The nomenclature and definition of several yolk lipoproteins has become confusing due to the fact that different investigators have used different terms for the same component. In order to clarify this confu-

sion, a description of several of the egg yolk components is given below.

Lipovitellin is usually used to designate the high density lipoprotein of avian yolk. It is also used to designate the corresponding fraction from other species. In the high density fraction of egg yolk, there are two components called α-lipovitellin and β-lipovitellin. The lipovitellins usually are associated with lipid-free phosvitin as inclusion bodies called granules.

Vitellin is the lipid-free protein moiety of lipovitellin.

Low density fraction (LDF) of egg yolk is that portion that floats on centrifugation. The LDF contains two components designated LDF_1 and LDF_2, both of which have a hydrated density less than 1.0 g/ml. Some authors call this fraction VLDL because it floats when centrifuged at a density of 1.006 g/ml (89, 90). Others call it low density lipoprotein because it has a chemical composition similar to serum LDL (91, 92). Low density material that forms part of the egg yolk granule is called LDFG.

Lipovitellenin is derived from LDF by extraction with diethyl ether (93). It is sparingly soluble at lipid contents less than fifty per cent. Sometimes lipovitellenin is not distinguished from the parent LDF.

Vitellenin is the lipid-free protein moiety of LDF or lipovitellenin. Egg yolk lipoproteins have been reviewed recently by Cook and and Martin (94).

Low Density Fraction of Egg Yolk

Egg yolk consists of the fluid portion and granules. Lipovitellin and phosvitin are found only in the granules, while lipovitellenin

is located in the egg yolk fluid. The LDF can be separated from the HDF by centrifuging at a density of 1.00 to 1.20 g/ml (94). The LDF has a chemical composition as follows: protein (11-17 per cent), phospholipid (22-28 per cent), and neutral lipid (59-67 per cent) (95-100). Lipid accounts for 83-89 per cent of the lipoprotein molecule. The LDF, which is found in avian egg yolk, is not found in the eggs of other species (94).

In 1958, Turner and Cook established that lipovitellenin was derived from the LDF, and a lipid content of at least 50 per cent was necessary for it to remain soluble in aqueous solutions (97). The LDF can be separated into two fractions: LDF_1 and LDF_2. LDF_1 contains 3-4 per cent more lipid than LDF_2 (99, 100). Both LDF_1 and LDF_2 are heterogeneous with respect to particle size and lipid content (99). When partially delipidized, LDF_1 and LDF_2 yield four or five components which differ in size and lipid content (90, 93, 100).

The major N-terminal amino acids of unfractionated LDF were reported to be arginine and lysine (101). The major C-terminal was glutamic acid (101). Smith and Turner found lysine to be the major N-terminal amino acid of the proteins of the LDF (102). In 1968, Augustyniak and Martin isolated two glycopeptides from the LDF of egg yolk and found aspartic acid to be the N-terminal amino acid of both peptides (103). In 1972, Hillyard et al. (55) reported that lysine was the N-terminal and tyrosine the C-terminal amino acid of egg yolk lipovitellenin. No arginine was found as the N-terminal amino acid and glutamic acid was not found to be the C-terminal amino acid.

It appears that there are several polypeptides in the LDF or lipovitellenin of egg yolk. Lysine is probably the N-terminal of the

major polypeptide. Another glycoprotein is present which has aspartic acid at the N-terminus. Hillyard et al. (55) showed that the lipovitellenin of egg yolk was immunochemically identical to the major apolipoprotein of serum VLDL. Serum apoVLDL has lysine as the major N-terminal and tyrosine as the C-terminal amino acids.

An apoprotein from the LDF of Emu egg yolk has been isolated by Burley (104). It had lysine as the N-terminal residue and contained no histidine, cystine, sulfhydryl groups or phosphate.

The particles found in the LDF are essentially spherical in shape and increase in size with increased lipid content (105). The available experimental evidence suggests that the LDF particle consists of a triglyceride core surrounded with phospholipids, cholesterol and protein on the surface (90-92, 103, 106, 107).

The lipoproteins found in egg yolk granules will be discussed in the next section.

High Density Fraction of Egg Yolk

The high density lipoproteins (lipovitellins) of avian egg yolk are found exclusively in the granular portion of the yolk. About 4 per cent of the granules consist of the protein moiety of the LDF. The granules can be easily separated from the yolk fluid by centrifugation, but lipovitellin was usually prepared by dissolving egg yolk in 0.4 M magnesium sulfate and diluting to 0.2 M (108). Lipovitellin having 16-22 per cent lipid could be separated from egg yolk by chromatography on columns of hydroxylapatite (109) or TEAE cellulose (110).

By electrophoresis, it was shown that lipovitellin contained two components with different mobilities. These two components were termed

α- and β-lipovitellin (111). The two lipovitellins could be separated from one another on columns of hydroxylapatite (109) or TEAE cellulose (110). Both lipovitellins contained about 40 per cent neutral lipid and 60 per cent phospholipid (98). Four per cent of the neutral lipid was cholesterol, which was mainly in the free form. Lecithin accounts for 75 per cent of the total phospholipids.

It was found by several investigators that α- and β-lipovitellin dissociate reversibly into subunits when exposed to aqueous solvents of differing pH and ionic strength (113-117). The polypeptide subunits of lipovitellin were found to have molecular weights of 29-32,000 and 42-45,000 daltons (118, 119).

The amino acid compositions of the lipovitellins are almost identical, but differ from lipovitellenin (Table 8) (112). Both α- and β-lipovitellin were reported by Neelin and Cook to have lysine and arginine as the major N-terminal amino acids (Table 9) (101).

Lipid-free phosvitin was also found in the HDF of egg yolk. The molecular weight of phosvitin was 30,000 at a pH of 4.0 and contains about 10 per cent phosphorus by weight (120). Phosvitin was found to be heterogeneous by Clark (121). Two components were resolved by chromatography on Sephadex G-100. The major polypeptide had a molecular weight of 34,000 and alanine as the N-terminal residue. The minor component had a molecular weight of about 28,000 and had lysine as the N-terminal residue. Glucosamine, methionine, and leucine were absent in the minor component. Two phosvitins were also found in yolks from the ostrich, duck and turkey (122).

Purified phosvitin contains 6.5 per cent carbohydrate. Shainkin

TABLE 8

AMINO ACID COMPOSITION OF EGG YOLK LIPOPROTEINS[a]

	Vitellenin[b]	Vitellin[b]	Vitellin[b]	Phosvitin[c]
Lysine	8.12	5.65	6.02	7.66
Histidine	1.49	2.15	2.05	4.83
Arginine	4.78	5.42	5.68	5.38
Aspartic acid	10.88	9.60	9.43	6.35
Threonine	5.41	5.31	5.68	2.14
Serine	6.90	9.04	9.32	55.09
Glutamic acid	10.83	11.53	11.93	6.00
Proline	3.51	5.42	5.45	1.42
Glycine	4.87	4.86	4.77	2.19
Alanine	7.31	7.91	7.73	3.28
Half cystine	0.54	1.81	2.05	0
Valine	6.63	6.21	6.59	1.44
Methionine	2.03	2.60	2.73	0.45
Isoleucine	6.36	5.65	6.25	0.75
Leucine	10.69	9.27	9.09	1.09
Tyrosine	3.52	3.39	3.18	0.44
Phenylalanine	4.33	3.28	3.30	0.86
Tryptophane	1.76	0.90	0.80	0.66

[a]Moles amino acid/100 moles recovered.

[b]Ref. 112.

[c]Ref. 123.

TABLE 9

TERMINAL AMINO ACIDS OF EGG YOLK LIPOPROTEINS

Egg yolk fraction	N-terminal		C-terminal		References
	Major	Minor	Major	Minor	
LDF	Arg, Lys	Asp, Ala, Ser	Glu	Ala, Ser	(101)
LDF	Lys	Asp, Ser, Ala	N.D.[a]		(102)
LDF	Lys	-	N.D.		(55)
LDF (glyco-peptides)	Asp	-	N.D.		(103)
HDF					
α-Vitellin	Arg, Lys	Ser, Tyr	N.D.		(101)
β-Vitellin	Arg, Lys	Tyr, Ser	N.D.		(101)
Phosvitin	Ala	Lys	N.D.		(121)

[a]Not determined.

and Perlmann (126) isolated a glycopeptide from a pronase digest of phosvitin which contained 6 residues of hexose, 5 of glucosamine, and 2 of sialic acid. The carbohydrate was attached to an asparigine residue of the glycopeptide.

The amino acid composition of egg yolk phosvitin is shown in Table 8. Phosvitin has very low amounts of methionine, isoleucine, tyrosine, phenylalanine and tryptophane. Half cystine is missing from phosvitin. The predominant amino acid is serine. The phosvitin in egg yolk is thought to be derived from the lipophosphoglycoprotein complex found in hen's serum. In the yolk, phosvitin is found free of lipid; thus some drastic changes have been made upon deposition in the egg yolk. The major electrophoretic component from phosvitin in hen's serum is not seen in the egg yolk; thus a change in composition of the phosvitin complex occurs when it is deposited in the egg yolk (124, 125).

The next section will discuss the incidence of atherosclerosis in exotic birds.

Atherosclerosis in Exotic Birds

Atherosclerotic lesions of birds resemble those found in man (13, 127). Fatty streaks are the most common lesion, but plaque formation and advanced necrotic lesions are detectable in older birds. Advanced lesions are more prevalent in older birds, but they are also observed in very young birds.

Finlayson has studied the lesions of exotic birds which died at the London Zoological Gardens (3). There are wide differences among birds in the susceptibility to the development of atherosclerosis. Carniverous birds are extremely likely to develop atherosclerotic lesions.

Listed in decreasing order of susceptibility are: birds of prey, doves and pigeons, chickens and turkeys, ostriches and parrots, ducks and geese, cranes and herons, crows and lovebirds, sea birds, and hummingbirds and penguins. Different breeds of pigeons also show differences in susceptibility to atherosclerosis. The Show Racer and Racing Homer have a lower incidence of atherosclerosis than the Autosexing King, Silver King or White Carneau (127, 128).

Birds, in general, have a higher median cholesterol than man (Table 10) (3). Serum cholesterol is variable in birds, but usually increases with increasing age. Serum cholesterol in exotic birds varies from 50 mg/100 ml in the Carolina Duck to 860 mg/100 ml in the Grey Parrot. High serum cholesterol values cannot be directly correlated with the incidence of atherosclerosis.

TABLE 10

SERUM CHOLESTEROL VALUES FOR EXOTIC BIRDS[a]

	Sex	Duration in Captivity	Serum Cholesterol (mg/100 cc)
Whooper swan	F	14 yrs.	775
Mute swan	M		567
Carolina duck	M		50
Common teal			190
Night heron	M	12 yrs.	100
Sacred Ibis	F		280
Undulated hornbill	F	7 months	140
Black kite	M	12 yrs.	179
Southern hooded vulture		3 months	177
Nepal kalij pheasant	F	12 yrs.	210
Eastern sarus crane	M		135
White pelican			185
Grey parrot	M		860
Emu	M	4 yrs.	115
Humboldts' penguin		2 yrs.	390
Black-footed penguin	F	5 yrs.	780
Black-footed penguin	F	8 yrs.	167

[a]Ref. 3.

CHAPTER III

MATERIALS AND METHODS

Materials

Turkey Serum

Fasting serum from Broad Breasted White (BBW) male turkeys was obtained from Colorado Serum Company Laboratories, Denver, Colorado. Serum from a mixed population of male and nonlaying female turkeys was obtained from Pel-Freeze Biologicals, Inc., Rogers, Arkansas. The birds were fed a standard Purina Poultry Chow.

Chemicals

Ammonium carbonate, ammonium sulfate, glacial acetic acid, calcium chloride, hydrochloric acid, magnesium chloride, manganous chloride, 2-mercaptoethanol, phosphoric acid, phosphorus pentoxide, potassium hydroxide, pyridine, sodium bromide, sucrose, trichloroacetic acid, urea, acetone, chloroform, methanol, petroleum ether and sec-butyl alcohol were purchased from J. T. Baker Chemical Co., Phillipsburg, New Jersey; barbital, formic acid, manganese sulfate, potassium bromide, sodium phosphate (mono- and di-basic), and sodium sulfate from Fisher Scientific Co., Fair Lawn, New Jersey; ammonium molybdate, 1-amino-2-naphthol-4-sulfonic acid, cupric sulfate, disodium salt of EDTA, sodium barbital, sodium bicarbo-

nate, sodium carbonate and sodium tartrate from Mallinckrodt Chemical Works, St. Louis, Missouri; ferric chloride, 1-fluoro-2-4-dinitrobenzene, guanidine hydrochloride, and sodium sulfite from Matheson, Coleman and Bell, Norwood, Ohio; Amido Black 10B, bromophenol blue, sodium azide, sodium dodecyl sulfate and sodium azide from K & K Laboratories, Inc., Hollywood, California; cholesterol, 2-amino-(hydroxy-methyl)-1, 3-propanediol (Tris), 8-anilino-1-naphthalene-sulfonic acid, glycine and N, N'-methylenebisacrylamide (Bis) from Eastman Organic Chemicals, Rochester, New York; acrylamide ammonium persulfate, riboflavine, and N, N, N', N'-tetramethylethylenediamine (TEMED) from Canalco, Rockville, Maryland; Oil Red O from Allied Chemical Corp., New York, New York; absolute and 95% ethanol from U. S. Industrial Chemicals Co., New York, New York; special Agar Noble and Freund's complete adjuvant from Difco Lab., Detroit, Michigan; agarose, Biogel A-5m, and Cellex-D from Bio-Rad Lab., Richmond, California; anhydrous hydrazine from Pierce Chemical Co., Rockford, Illinois; 1-dimethylaminonaphthalene-5-sulfonyl chloride (DNS-Cl), and Cleland's reagent from Calbiochem, Los Angeles, California; DNS-amino acid standards from Mann Research Lab, New York, New York; DNP amino acid standards from Supelco, Inc., Bellefonte, Pennsylvania; carboxypeptidase A, carboxypeptidase B, and trypsin from Worthington Biochemical Corp., Freehold, New Jersey; human albumin from Certified Blood Donor Service, Woodbury, New York; Coomassie Brillant Blue R, α-methyl-D-glucoside, lysozyme, cytochrome c, ribonuclease, and ovalbumin from Sigma Chemical Co., St. Louis, Missouri; Blue Dextran 2000, concanavalin A-Sepharose, Sephadex (G-25, G-75, G-100, G-200), and LH-20 from Pharmacia Fine Chemicals, Inc., Pitscataway, New Jersey; ultra pure urea from Schwarz/Mann,

Orangeburg, New York; and No-Jax cellulose casing, size 18, from Union Carbide, Chicago, Illinois.

Methods

Separation Techniques

Ultracentrifugation. For analytical purposes, 7 ml of serum were overlayered with 4 ml of 0.15 M NaCl in 0.1 per cent EDTA and centrifuged in a Type Ti 50 rotor of the Model L preparative ultracentrifuge (Beckman Instruments, Inc., Palo Alto, California) at 105,000 x g for 22 hours at 4°C. After 22 hours, the top 2 ml were removed by a tube-slicing technique to yield VLDL (129). The LDL (1.006-1.063 g/ml), HDL_2 (1.063-1.125 g/ml), and HDL_3 (1.125-1.21 g/ml) were isolated sequentially by adjusting the solution density of the infranate with solid KBr to 1.063, 1.125, and 1.21 g/ml and centrifuging at 105,000 x g for 22 hours (129). A scheme for the isolation of lipoproteins is shown in Figure 2.

In some cases, the HDL_2 and HDL_3 were isolated as one density class, HDL (1.063-1.21 g/ml). All fractions were recentrifuged at the appropriate density (1.006, 1.063, 1.125, 1.21 g/ml) to remove contaminating serum albumin.

When a larger preparation of lipoproteins was needed, the serum was first centrifuged at serum density (1.015-1.017 g/ml) in a Type Ti 60 rotor of the L2-50 or L2-65-B preparative ultracentrifuges (Beckman Instruments, Inc., Palo Alto, California) for 22 hours at 105,000 x g. After 22 hours, the top 4 ml were removed by a tube-slicing technique. LDL, HDL_2 and HDL_3 were separated sequentially by adjusting the solution

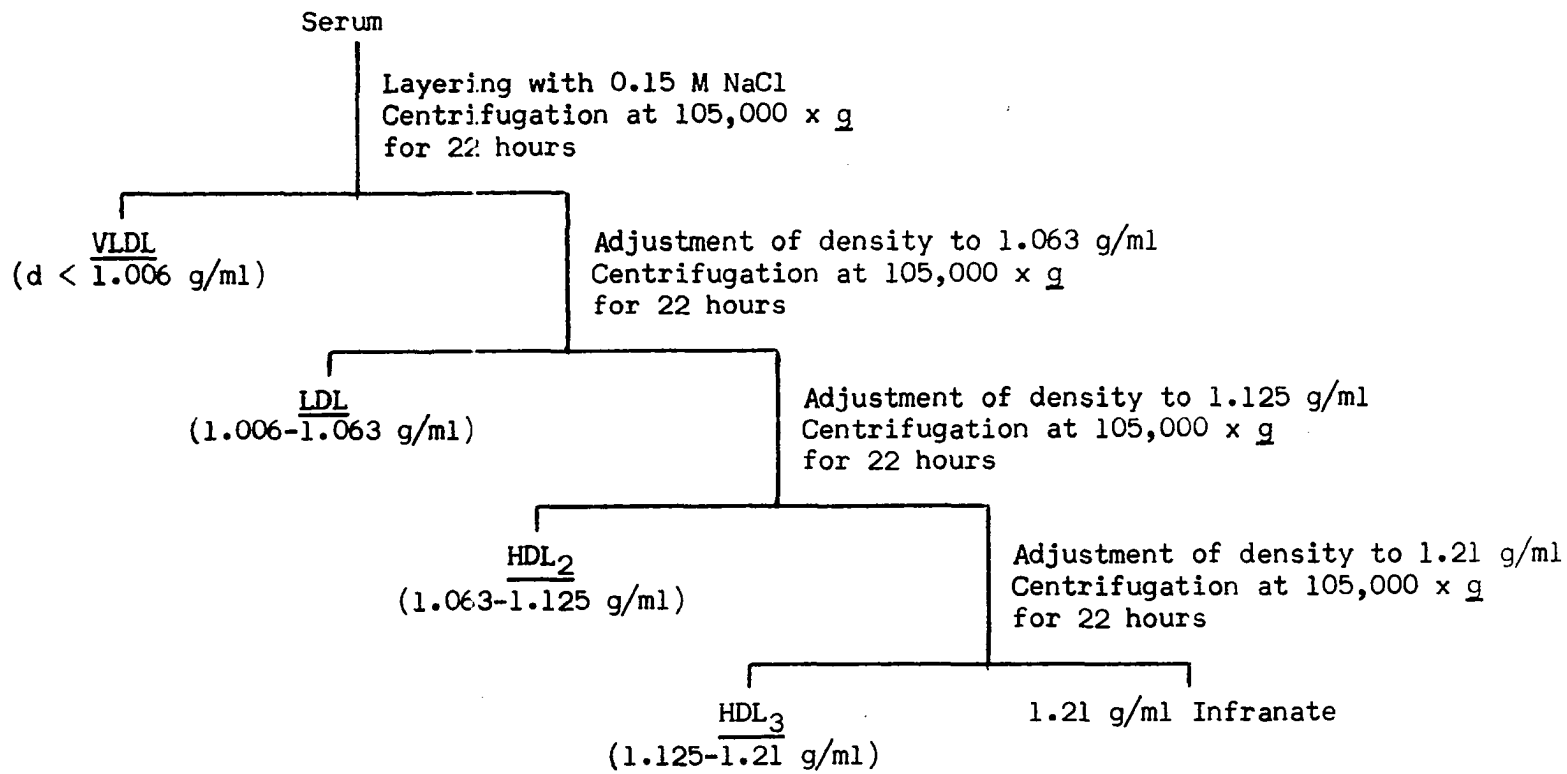

Figure 2. Ultracentrifugal isolation of lipoproteins.

density as described previously.

Delipidization. Total delipidization was accomplished by two methods. The first method was carried out essentially as described by Scanu et al. (130). The samples were first dialyzed exhaustively against distilled water at 4°C, then lyophilized. The delipidization procedure was begun by treating the lyophilized lipoproteins with absolute ethanol-diethyl ether (3:1, v/v) on a rotator (15 rpm for 30 minutes) with a total of 5-8 extractions, followed by low speed centrifugation to remove the solvent. In the final step, the protein was extracted overnight with absolute ethanol-diethyl ether (2:1, v/v) followed by 3-5 extractions with diethyl ether. All extractions were carried out at 4°C. The residue was then allowed to dry at room temperature. Protein delipidized by the above procedure usually contained less than 1 per cent phospholipid. The ethanol-diethyl ether extracts were pooled and evaporated to dryness on a rotary evaporator in preparation for lipid analysis.

The second method of delipidization was described by Rudman et al. (74) for delipidization of human α-lipoproteins. Turkey HDL were chromatographed on a column of Sephadex LH-20 (Pharmacia). The gel particles were swollen in a solvent mixture consisting of 2-butanol:acetic acid:water (4:1:5, v/v/v), and packed in a 2.5 x 100 cm column (Glenco Scientific Inc., Houston, Texas). A sample of HDL containing 40-70 mg protein was dissolved in the elution buffer and applied to the column. Sometimes the cholesterol precipitated when the buffer was added to a solution of HDL. In that case, cholesterol was removed by filtration. The flow rate was 25-30 ml/hour. If the flow rate was less than 25 ml/hour, the column was repacked. Column fractions of 5 ml were collected

using a Buchler Fractomette 200 fraction collector (Buchler Instruments, Inc., Fort Lee, New Jersey) and monitored either by a Buchler Uviscan or a Gilford Model 2000 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio).

Isolation of albumin. The 1.21 g/ml infranate was used as the starting material for the isolation of serum albumin. This fraction was first dialyzed exhaustively against distilled water. To 100 ml of the 1.21 g/ml infranate, an equal volume of 10 per cent TCA was added (131). Most of the proteins precipitated when the TCA was added. The precipitate was recovered by centrifuging in an SS34 rotor of the Sorvall centrifuge (Ivan Sorvall Inc., Norwalk, Connecticut) at 10,000 rpm for 30 minutes at 4°C. The precipitate was washed 3 times with 5 per cent TCA and recovered each time by centrifugation. The supernates were discarded. The washed 5 per cent precipitate was then suspended in 80 ml of 95 per cent ethanol and stirred at room temperature for 30 minutes. The precipitate was removed by centrifugation and the supernate, which contained mainly albumin, was saved. The supernate was dialyzed against water to remove the ethanol. During dialysis, some precipitate formed and was removed by low speed centrifugation. Since the volume had increased by about four-fold during dialysis, the albumin was concentrated by lyophilization. Purity of the albumin preparation was evaluated by polyacrylamide gel electrophoresis (PAGE) and by immunodiffusion against anti-WS.

Column chromatography. Sephadex G-25, G-75, G-100, and G-200 (Pharmacia Fine Chemicals, Inc., Pitscataway, New Jersey) were either equilibrated with the appropriate buffer for several days or placed in a boiling water bath to swell the gel, then equilibrated with buffer.

When urea was to be used in the equilibrating buffer, it was best to first swell the gel in water then equilibrate with the urea buffer. Sephadex G-75 and G-100 columns (2.5 x 100 cm) were used mainly for fractionating apolipoproteins. Sephadex G-25 columns (2.5 x 60 cm) were used for desalting.

Bio-gel A-5m was used to separate the two non-identical polypeptides of ApoA. The gel was equilibrated with 6 M guanidine hydrochloride, adjusted to pH 6.5, and poured into a 2.5 x 100 cm column. From 30 to 50 mg of apoHDL$_3$ were dissolved in 3 ml of the guanidine HCl buffer. Three milliliter fractions were collected at a rate of 30 ml/hour. If the flow rate decreased significantly, the column was repacked. During the first few fractionations, the column "bled", thus adding a significant amount of non-dialyzable polysaccharide to the column effluent. Every second tube was monitored at 280 nm on a Gilford Model 240 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). Since PAGE cannot be run in the presence of guanidine HCl, the fractions were pooled according to their optical density at 280 nm. The pooled fractions were dialyzed exhaustively against distilled water and lyophilized. Polypeptides prepared in this manner were readily soluble in aqueous buffers.

Diethylaminoethyl cellulose (Bio-Rad Laboratories, Richmond, California) (0.40 and 0.70 meq/g dry weight) was used for ion exchange chromatography of apolipoproteins. One-hundred grams of DEAE-cellulose powder were washed successively with 0.5 N NaOH, distilled water, 0.5 N HCl, and distilled water. The last wash was checked with silver nitrate until no chloride was detected, then the slurry of cellulose was adjusted to a pH of 7.2. After equilibrating with the appropriate buffer, the

DEAE-cellulose was poured into the column (1.5 x 30 cm) and further equilibrated with the buffer. If 8 M urea was used in the buffer, it was added after the column had been packed. The pH of the effluent was checked before the addition of a sample. Flow rate varied, but was usually 20-30 ml/hour. A linear gradient of NaCl was used to elute the proteins from the column (132).

Hydroxylapatite (Bio-Rad Laboratories, Richmond, California) was washed first with 1 M phosphate buffer, distilled water, then equilibrated with 0.01 M phosphate buffer, pH 8.0. A linear gradient of 0.01-1.0 M phosphate was used to elute the proteins.

Concanavalin A-Sepharose affinity chromatography was used to separate lipoprotein families A and B. The binding site of concanavalin A is specific for α-D-mannosyl, α-D-glucosyl, and sterically similar residues. Con-A-Sepharose (Pharmacia) was first equilibrated with a buffer containing 0.02 M Tris (pH 7.2), 1.0 M NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, then poured into a 2 x 30 cm column (Ace Glass, Inc., Louisville, Kentucky) fitted with a fritted glass filter. The lipoprotein solutions were dialyzed overnight against this equilibration buffer. The flow rate was adjusted to about 30 ml/hour and the sample was applied. The lipoproteins which were retained by Con-A-Sepharose were eluted with the equilibration buffer which contained 0.20 M α-D-methyl-glucoside. Five milliliter fractions were collected and monitored for protein concentration at 280 nm on a Gilford Model 240 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). The fractions were also checked by immunodiffusion with antisera to LP-A and LP-B.

Preparative polyacrylamide gel electrophoresis. Preparative

electrophoresis was performed using the Buchler Fractrophorator (Buchler Instruments, Inc., Fort Lee, New Jersey). The separating gel was about 5 cm long and sample was polymerized in a 3 per cent sample gel. All gels had the same composition as those used for analytical PAGE. A Tris-glycine buffer, pH 8.3, was used as the elution buffer as well as the electrode buffer. The separating gel was pre-electrophoresed for 30 minutes at a constant current of 12 milliamperes, then the sample was applied and polymerized. The sample gel was overlayered with buffer containing several drops of tracking dye (0.05 per cent Bromophenol Blue). Electrophoresis was carried out using a constant current (10 ma) at room temperature.

Four milliliter fractions were collected every 2.5 minutes after the tracking dye reached the end of the separating gel. The electrophoresis was usually completed in four hours. The fractions were monitored for protein concentration at 280 nm and by analytical polyacrylamide gel electrophoresis. Fractions eluted from the gel were combined on the basis of the analytical polyacrylamide gel patterns. Those fractions which showed only one band on polyacrylamide gels were dialyzed exhaustively against distilled water and used for subsequent analyses.

Analytical Techniques

Immunodiffusion and immunoelectrophoresis. Double diffusion (133) and immunoelectrophoresis (134) were carried out on glass slides (25 x 75 mm) coated with 1 per cent agarose employing veronal buffer, pH 8.6, ionic strength 0.10. Immunoelectrophoresis was carried out at 6.5 volts/cm for 60 minutes. The precipitin patterns were allowed to develop for 48-72 hours at room temperature. The patterns were usually

fully developed after 24 hours. Plates were washed for 48 hours in several changes of distilled water to remove the buffer and unprecipitated proteins, then dried under a strip of lintless paper. A 1 per cent solution of Amido Black 10B in 3 per cent acetic acid was used to stain the precipitin lines for the presence of protein. Excess stain was removed by rinsing in distilled water. The precipitin lines were sometimes stained with a solution of 0.06 per cent Coomassie Brilliant Blue in water:ethanol (55:45, w/v), but the Amido Black stain was preferred. Lipid staining of the precipitin lines was accomplished by using an acidic ethanol solution of Oil Red O.

The optimal ratio of antibody to antigen for the absorption of antibody or antigen was determined by use of the Micro-titer set (Cooke Engineering Co., Alexandria, Virginia). Both antigen and antisera were diluted serially in 0.1 M borate buffer, pH 8.6, and the appropriate antigen or antisera were added. The plates were incubated overnight at 37°C and the optimum ratio was established by selecting the well which showed maximum precipitation. Using this ratio, the undesired antigen or antibody was removed by adding the appropriate component. After incubation for 2 hours at 37°C, the immunoprecipitate was removed by low speed centrifugation. If necessary, the procedure was repeated to remove the last traces of antigen or antibody.

Preparation of antibodies. The lipoprotein density classes which were used as antigens were dialyzed against 0.9 per cent NaCl containing 0.1 per cent EDTA. Apolipoproteins, which were lyophilized, were dissolved in the same salt solution. Equal volumes of antigen (protein concentration 0.5-10 mg/ml) and Freund's complete adjuvant were homogen-

ized, then injected intraperitoneally into white New Zealand rabbits. One or two additional injections via the same route, at intervals of 10 days, were usually sufficient to elicit antibody titers adequate for our studies. Subsequent booster injections were used only when the titer of the antisera became low as demonstrated by immunodiffusion or immunoelectrophoresis against the antigen which was initially injected.

Blood was first drawn from rabbits two to three weeks after the initial immunization. Blood samples (30-40 ml) were collected from the rabbits by cardiac puncture at weekly intervals. The collected blood was allowed to stand at room temperature for several hours, then at 4°C to allow the clot to form and retract. The clot was removed by low-speed centrifugation, and the antisera were examined by immunodiffusion and immunoelectrophoresis with various antigens and lipoprotein preparations. When necessary, the antisera were treated with appropriate antigens to remove contaminating antibodies. The antisera were stored at -20°C with sodium azide (1 mg/ml of antisera) as a preservative.

Agarose electrophoresis. Agarose gel electrophoresis was carried out as described for immunoelectrophoresis except that the sample (20-30 µl) was mixed with an equal volume of 2 per cent agarose (cooled to 50°C) and placed into a slot (1.0 x 0.1 cm) cut in the gel. The sample and agarose were added until the slot was completely filled. After completion of the electrophoresis, the slides were fixed in 10 per cent TCA for an hour, then washed in distilled water for two hours with several changes of water. Washed slides were covered with a lintless paper strip and allowed to dry overnight. After drying, the slides were stained with Amido Black 10B and Oil Red O as described previously.

Polyacrylamide gel electrophoresis. Analytical discontinuous polyacrylamide gel electrophoresis (PAGE) was done essentially as described by Davis (135) with equipment from Canalco, Rockville, Maryland. Separating gels of 7 per cent acrylamide were used employing a Tris-glycine buffer (pH 8.3). The electrophoretic separation of polypeptides was usually carried out with 8 M urea in the separating gel and 4 M urea in the stacking and sample gels. A constant current of 4.0 ma/tube was used. The 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 by placing them in a solution of 0.12 per cent Coomassie Brilliant Blue in 10 per cent trichloroacetic acid (0.25:10.0, v/v). After 48 hours, the protein bands were visualized against a clear background. The gels were then transferred to tubes containing distilled water to prevent destaining of the bands.

Some of the gels were scanned using the linear transport module for the Gilford Model 240 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). Gels were scanned using visible light at 550 nm and a scan rate of 1 cm/min. A chart recording speed of 1 in./min was chosen to obtain maximum resolution of the bands. The areas under the peaks were determined by use of a planimeter (Keuffer and Esser Co., New York).

Polyacrylamide gel electrophoresis combined with immunodiffusion. Samples were first separated by electrophoresis on 7 per cent polyacrylamide gel using the Tris-glycine buffer, then the gels were sliced longitudinally into halves. The sliced gels were placed, with the flat side down, on microscope slides (25 x 75 mm). About 4 ml of 1 per cent

agarose solution in Veronal buffer (pH 8.6) (50°C) were poured around each gel. The agarose was allowed to set for one hour. Longitudinal troughs parallel to the acrylamide gel were then cut on each side of the gel and the desired antisera were added.

Sometimes horizontal regions of the polyacrylamide gel corresponding to bands visualized in separately stained gels were sliced from the gels and examined by immunodiffusion. The horizontal slices were treated in the same manner as described for the longitudinally sliced gels.

Molecular weight determination by SDS-polyacrylamide gel electrophoresis. Determination of the molecular weights of polypeptides by 10 per cent polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed according to the procedure of Shapiro et al. (136) as described by Weber and Osborn (137). Human serum albumin, ovalbumin, myoglobin, lysozyme and cytochrome c were used as reference proteins. Samples were dissolved in 1 per cent SDS-phosphate buffer, pH 7.0, and incubated at 37°C overnight. The electrophoresis was run at 6 ma/tube until the tracking dye (Bromophenol Blue) had migrated approximately 7.0 cm into the gel. The gels were removed from the tubes and the portion of the gel in front of the leading edge of the dye front was cut off using a razor blade. In this manner, the length of the gel could be used to calculate R_f values of the protein bands.

The gels were stained in a solution prepared by dissolving 1.25 g of Coomassie Brilliant Blue in a mixture of 454 ml of 50 per cent methanol and 46 ml of glacial acetic acid. The insoluble material was removed by filtration through Whatman No. 1 filter paper. The gels were

stained at room temperature from 2 to 10 hours. Usually 2 hours were sufficient for maximum staining of the gels.

Destaining was accomplished by placing the gels in a solution containing 75 ml of acetic acid, 50 ml of methanol, and 875 ml of water. An alternate method of destaining was to electrophoretically destain the gels using the Canalco quick gel destainer (Canalco, Rockville, Maryland). Ten per cent acetic acid was used as the destaining solution. After destaining, the gels were placed in distilled water overnight. The relative mobility of the protein bands was measured from the leading edge of the band. The molecular weights were read directly from a plot of log MW vs relative mobility of the reference proteins.

Analytical electrofocusing. Analytical isoelectric focusing in polyacrylamide gels (138) was done by using the Canalco disc gel electrophoresis equipment and a constant voltage power supply (Model E-C 454, E-C Apparatus Corp., Philadelphia, Pennsylvania). Ampholine 3-10 (LKB, Bromma, Sweden) was found to be the most useful carrier ampholine for screening purposes. Other ampholines with narrower pH ranges were used when appropriate. Samples were mixed with ampholine, acrylamide, bisacrylamide, TEMED, and ammonium persulfate, and allowed to polymerize in a tube (10 x 0.4 cm) to form a 1 per cent ampholine solution in a 5 per cent polyacrylamide gel. Electrofocusing was performed at 200 volts for 16 hours at 6-8°C. The electrode solutions were: 1 per cent phosphoric acid at the cathode and 1 per cent ethanolamine at the anode. The gels were removed from the tubes and stained in 0.12 per cent Coomassie Brilliant Blue in 10 per cent TCA (0.25:10, v/v) as described for analytical polyacrylamide gels.

Amino acid analysis. Samples of apolipoproteins or polypeptides were dried over P_2O_5 under vacuum and then hydrolyzed in constant boiling HCl in an evacuated tube at 110 ± 1°C for 24 hours and/or 72 hours. The hydrolyzates were evaporated to dryness, redissolved in distilled water, and evaporated again to dryness. The residue was solubilized in 3 ml of 0.2 N sodium citrate buffer (pH 2.2) and filtered through a fine frit sintered glass filter. The filtered hydrolysate was analyzed in a Beckman Model 120 C amino acid analyzer (Beckman Instruments, Inc., Palo Alto, California) according to the accelerated procedure of Benson and Patterson (139) on spherical resins (Beckman Custom Spherical Resins, Type PA-28 for the acidic and neutral amino acids and PA-35 for the basic amino acids).

The amino acid analyzer was calibrated with a mixture of known amino acids. Amino-guanidine propionic acid (AGPA) and norleucine were used as internal standards. Half cystine and methionine were determined as cysteic acid and methionine sulfoxide after oxidation of the apolipoproteins or polypeptides with performic acid (140). Values for cysteic acid were corrected for the 94 per cent recovery found by Moore (140). Typtophan was determined on the amino acid analyzer after hydrolysis with p-toluenesulfonic acid (141) or spectrophotometrically by the method of Gaitonde and Dovey (142). Amino acid analysis of stained bands from polyacrylamide gels was performed by the procedure of Houston (143). It was found that the recovery of amino acids from hydrolysis of stained bands could be increased if the gels were first oxidized with performic acid.

Carboxyl terminal analysis. Carboxypeptidase A (CPA) and/or

carboxypeptidase B (CPB) were used for the enzymatic determination of the carboxyl terminal amino acids as described by Ambler (144). The protein sample was dissolved in 0.1 M N-ethylmorpholine (pH 8.5) and the CPA and/or CPB were added in the usual ratio of 1 part enzyme to 80 parts protein. Other ratios were examined, but the ratio of 1:80 was found to be optimal. If the protein was not soluble in N-ethylmorpholine alone, 0.1 per cent SDS was added. At this concentration, SDS did not inhibit the release of the C-terminal amino acids by the carboxypeptidases. Incubation of the enzyme-protein mixture was carried out at 37 C for varying time periods. A sample was withdrawn initially to serve as the zero time blank. Subsequent samples were taken at suitable time intervals after the addition of the enzyme. The protein was precipitated with 6 N HCl or 10 per cent TCA, and the remaining supernate was evaporated to dryness. The residue was dissolved in 0.2 N sodium citrate buffer (pH 2.2) and analyzed in the Beckman 120 C amino acid analyzer using the same procedure as used for amino acid composition of apolipoproteins or polypeptides. Values obtained were corrected by subtraction of the zero time values and contribution from the autodigestion of CPA and/or CPB.

The carboxyl terminal amino acids were also determined with anhydrous hydrazine by the procedure of Braun and Schroeder (145). The protein sample (0.2-1.0 μM) was added to a hydrolysis ampule containing 50 mg of dry Amberlite CG-50 (100-200 mesh) (Mallinckrodt Chemical Works, St. Louis, Missouri). The Amberlite functioned as a proton donor during the reaction with hydrazine. Two milliliters of anhydrous hydrazine were added to the hydrolysis tube, then the tube was sealed under vacuum. The reaction mixture was incubated in a constant temperature oven (Fisher

Isotemp, Fisher Scientific Co., Fair Lawn, New Jersey) for 48-96 hours at 80°C. After incubation, the reaction mixture was lyophilized to get rid of excess reagent. When the sample was dry, it was suspended in 3 ml of glass distilled deionized water and centrifuged to separate the free amino acid(s) and hydrazides from the Amberlite CG-50. The supernate was recovered, acidified with HCl and chromatographed on a column (1 x 15 cm) of Amberlite CG-50 which was equilibrated with 0.01 M pyridine-acetate buffer, pH 5.9. The hydrazides and the basic amino acids were retained on the column, whereas the neutral and acidic amino acids were eluted in the void volume. After chromatography, the neutral and acidic amino acids were evaporated to dryness under vacuum. The residue was dissolved in the sodium citrate buffer (pH 2.2), and analyzed on the Beckman Model 120 C amino acid analyzer. An alternate method to separate the neutral and acidic amino acids from the hydrazides was to precipitate the hydrazides with benzaldehyde. The basic amino acids (lys, his, arg) can be recovered by chromatography on phosphocellulose.

Amino terminal analysis. The N-terminal amino acids were determined by dansylation and dinitrophenylation of the proteins. Dansylation of the proteins and/or polypeptides was done according to the procedure of Gray (146). After dansylation, the proteins were precipitated with 10 per cent trichloroacetic acid (TCA) and centrifuged at low speed to remove excess reagents and by-products (147). The precipitated dansyl protein was washed twice with 0.1 N HCl. The dansylated protein was hydrolyzed with constant boiling HCl in an evacuated sealed ampule for 18 hours at 110°C. Following hydrolysis, the ampule was opened and the mixture was dried *in vacuo* in the presence of NaOH pellets. The sample and

standards were applied on a thin-layer plate coated with Silica Gel-G, and the dansyl amino acids were separated as described by Morse and Horecker (148).

Dinitrophenylation of proteins and polypeptides was performed essentially as described by Fraenkel-Conrat et al. (149). A protein sample (5-10 mg) was dissolved in Tris buffer (pH 8.6), 8 M urea, or 6 M guanidine-HCl and the solution was adjusted to 1 per cent with sodium bicarbonate. The addition of bicarbonate was not necessary when Tris buffer was used. An equal volume of 10 per cent 1-fluoro-2-4-dinitrobenzene (FDNB) in 95 per cent ethanol was added to the protein solution and the mixture was stirred for 2 hours in the dark at room temperature. The reaction mixture was acidified with 1 N HCl and extracted with peroxide-free anhydrous diethyl ether until no yellow color appeared in the ether phase. The ether extraction removed excess FDNB and other by-products.

During the ether extraction, the DNP-protein usually precipitated and was recovered by low-speed centrifugation. The DNP-protein was washed several times with diethyl ether and lyophilized to remove dinitrophenol. After the protein was dry, it was transferred to a 10 ml hydrolysis ampule (Wheaton Glass Company, Millville, New Jersey) and hydrolyzed with 5 ml of constant boiling HCl for 16 hours at 110°C. After hydrolysis, the reaction mixture was diluted to a concentration of 1 N HCl and extracted with diethyl ether. An acid concentration greater than 1 N inhibits the extraction of DNP-amino acids with ether.

The amino terminal DNP-amino acid(s) should be in the ether phase with the exception of DNP-arg which is water soluble. The ether phase was evaporated to dryness and chromatographed on paper (150) or on

thin-layers of polyamide (151). In order to further identify the DNP amino acids, the yellow spots were eluted from the paper chromatogram with methanol and hydrolyzed with a saturated aqueous solution of barium hydroxide for 1 hour at 110°C. The free amino acids were qualitatively identified by paper chromatography (150) or quantitatively by analysis on the amino acid analyzer.

Carbohydrate analysis. Sialic acid was determined by the thiobarbituric acid method of Warren (152). Samples were hydrolyzed with 0.1 N HCl for 1 hour at 80°C. Neutral sugars were analyzed by gas liquid chromatography in a Packard-Becker, Model 420, gas chromatograph equipped with dual hydrogen flame ionization detectors as alditol acetates according to the procedure of Kim et al. (153). The neutral sugars were hydrolized with 1 N HCl for 4 hours at 100°C. Amino sugars were hydrolyzed with 4 N HCl for 6 hours at 100°C. Glucosamine was also determined on the amino acid analyzer by comparison with the elution time of standards after hydrolysis of the sample with constant boiling HCl for 24 hours at 110°C.

Lipid and protein analysis. Whole serum and various lipoprotein preparations dialyzed against 0.15 M NaCl (pH 7.0) were used for automated lipid analysis. Free and total cholesterol were determined by the method of Schoenheimer and Sperry (154). Cholesterol ester was calculated as cholesterol oleate. Lipid phosphorus was analyzed by the method of Fiske and Subbarow (155) after wet digestion by the procedure of Youngburg and Youngburg (156). Lipid phosphorus was converted to phospholipid by multiplication by the factor 25. Triglyceride was measured by the fluorometric method of Kessler and Lederer as applied to the auto-analyzer

(157).

Lipid analysis was also performed on the ethanol:diethyl ether extracts of lipoprotein preparations. After evaporation of the solvent, the residue was dissolved in chloroform and quantitatively transferred to volumetric flasks. Cholesterol, cholesterol esters, diglycerides and triglycerides were separated on pre-coated thin-layer plates (Silica Gel G, Schwarz/Mann, Orangeburg, New York) using a solvent system containing 113 ml hexane, 35 ml diethyl ether, and 3 ml glacial acetic acid. The lipid spots visualized by exposing the plates to iodine vapor were scraped into tubes for quantitative analysis.

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, California). The Hycel cholesterol determination was more sensitive at lower concentrations of cholesterol, but the color was unstable. The Oxford cholesterol determination was more sensitive at higher concentrations of cholesterol and had a stable color. Cholesterol esters were calculated as cholesterol oleate.

Diglycerides and triglycerides were determined by the method of Van Handel and Zilversmit (158) using triolein as the reference. Diglycerides were calculated as diolein. Fatty acids of the triglycerides and cholesterol esters were transesterified by the method of Mason and Waller (159) and chromatographed as methyl esters.

Individual phosphatides from ethanol:diethyl ether extracts were separated on thin-layer plates coated with silica gel G (Schwarz/Mann, Orangeburg, New York) 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 using iodine vapor were scraped into tubes for phosphorus determination. Phosphorus was quantitated by the method of Gerlach and Deuticke (160) in the presence of silica gel.

Protein content of lipoprotein solutions was determined by the method of Lowry et al. (161) using human serum albumin as the standard. The amount of protein in totally delipidized lipoproteins was quantitated gravimetrically by drying to a constant weight in pre-weighed tubes under vacuum in the presence of P_2O_5.

Analytical ultracentrifugation. The analytical ultracentrifugation of lipoproteins was carried out in a Spinco Model E analytical ultracentrifuge equipped with schlieren and interference optical systems, a RITC unit, and an electronic speed control unit. Photographs were taken automatically by the photographic apparatus of the ultracentrifuge. The plate measurements were made on a Nippon Kogaku KK Shadograph, Model 6.

Samples for the determination of sedimentation rate were dialyzed against 0.15 M NaCl (pH 7.0) overnight. Sedimentation experiments were done in single-sector cells at 56,000 rpm at 25°C and the coefficients were measured according to Svedberg and Katsurai (162). The observed s values were corrected to $s_{20,w}$ (the sedimentation coefficient in water at 20°C) according to Svedberg and Pederson (163), and expressed in seconds or Svedberg units (1S = 10^{-13} cgs. units).

CHAPTER IV

RESULTS

Characterization of Lipoprotein Density Classes

Isolation

Serum lipoproteins from fasting male turkeys were separated by preparative ultracentrifugation into three density classes: VLDL ($d < 1.006$ g/ml), LDL (1.006-1.063 g/ml), and HDL (1.063-1.21 g/ml). Sometimes, the HDL were further subfractionated into HDL_2 (1.063-1.12 g/ml) and HDL_3 (1.12-1.21 g/ml). The VLDL appeared slightly opalescent, whereas LDL and HDL appeared bright yellow. The yellow color increased in intensity with increasing density (VLDL to HDL). All fractions were recentrifuged at the appropriate density until no albumin was detected by immunodiffusion.

Chemical Characterization

Quantitation of protein and lipoproteins was done gravimetrically after drying to a constant weight. Cholesterol, cholesterol esters, glycerides, and phospholipids were determined colorimetrically as outlined in the Methods section. Cholesterol esters were calculated as cholesterol oleate. The HDL represent the major lipoprotein density class in turkey serum (Table 11). VLDL were the least abundant lipoprotein density class.

TABLE 11[a]

CONCENTRATION OF SERUM APOLIPOPROTEINS AND INDIVIDUAL LIPIDS OF SERUM DENSITY CLASSES

Density Class	Protein	Phospholipid	Cholesterol		Triglyceride	Diglyceride	Lipoprotein
			Free	Ester			
			mg/100 ml				
VLDL (n = 4)	2.20± 0.02	1.89±0.25	0.32±0.13	2.32±0.37	1.52±0.26	1.46±.34	9.71
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±.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±.52	225.25

[a]Results presented in this table and all following tables and figures were obtained using sera from male turkeys.

Most of the phospholipid was found in the HDL. Both LDL and HDL had about the same absolute amount of total cholesterol, but the ratio of cholesterol to cholesterol ester was different.

The per cent composition of the major serum lipoprotein density classes is shown in Table 12. These values were computed from Table 11 by expressing the individual components of each density class as the per cent of the total amount of material in that density class. This calculation allows a comparison of components within each density class. As an example, in the LDL density class the molecules contain on the average 26 per cent protein, 26 per cent phospholipid, 5 per cent free cholesterol, 38 per cent cholesterol ester, 2 per cent triglyceride and 3 per cent diglyceride. The HDL contained about 40 per cent lipid which consisted mainly of phospholipid. VLDL contained more glycerides than the other density classes. Diglycerides represent about half of the total glycerides in VLDL.

Table 13 shows the distribution of lipid and protein across the density spectrum. For example, of the total apolipoprotein, 78 per cent was found in HDL, 20 per cent in LDL, and only 2 per cent in VLDL. The majority of the lipid as well as protein was found in the HDL. Although HDL were responsible for most of the lipid found in the lipoproteins, over 50 per cent of the cholesterol esters and 30 per cent of the free cholesterol was found in the LDL.

Phosphatidylcholine (lecithin) was the major phospholipid found in all lipoprotein density classes (Table 14). VLDL and LDL had a very similar distribution of phosphatides which differed from those found in HDL. HDL had more lecithin and less sphingomyelin and lysolecithin than

TABLE 12

PER CENT COMPOSITION OF THE MAJOR SERUM LIPOPROTEIN DENSITY CLASSES

Density Class	Protein	Phospholipid	Cholesterol		Triglyceride	Diglyceride
			Free	Ester		
			%			
VLDL (n = 4)	22.66	19.46	3.30	23.89	15.65	15.04
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 13

DISTRIBUTION OF SERUM LIPOPROTEINS AND LIPIDS OF THE DENSITY CLASSES

Density Class	Lipoprotein	Protein	Phospholipid	Cholesterol		Triglyceride	Diglyceride
				Free	Ester		
				%			
VLDL (n = 4)	2.76	1.47	2.44	3.21	5.10	20.03	22.50
LDL (n = 4)	19.68	10.70	20.90	31.90	51.85	16.07	29.89
HDL (n = 6)	77.56	87.83	76.66	64.89	43.04	63.90	47.61

TABLE 14

PER CENT COMPOSITION OF PHOSPHOLIPIDS OF SERUM LIPOPROTEIN DENSITY CLASSES

Density Class	Phosphatidylethanolamine	Phosphatidylcholine	Sphingomyelin	Lysophosphatidylcholine
		%		
VLDL (n = 4)	8.80 (6.37–12.47)	58.87 (48.93–73.63)	21.59 (15.60–27.00)	13.37 (11.90–15.45)
LDL (n = 4)	6.06 (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)

VLDL and LDL.

The composition of fatty acids of triglycerides and cholesterol esters is presented in Table 15. Palmitic and oleic acids were the major fatty acids found in both triglycerides and cholesterol esters. Linoleic acid was not found in triglycerides, but was found in cholesterol esters. More palmitoleic acid was found in cholesterol esters than in triglycerides in all density classes.

Antigenic Characterization

Serum lipoproteins have traditionally been characterized by their mobility in an electric field. The isolated serum lipoproteins were subjected to electrophoresis in 1 per cent agarose (Figure 3). VLDL had a mobility slightly faster than LDL. The LDL migrated in the position of β-globulins and HDL migrated in the position of α-globulins. In the terminology based on electrophoretic mobility, the LDL contain β-lipoproteins and the HDL contain α-lipoproteins. From the agarose electrophoresis, it could be seen that the LDL contained small amounts of α-lipoproteins and HDL contained detectable amounts of β-lipoproteins.

Because of the heterogeneity of density classes, the lipoproteins which had β-mobility on agarose were called LP-B and the lipoproteins which had α-mobility were called LP-A. Using this nomenclature, it was more logical to say that LDL contained LP-A and LP-B rather than to say the LDL contained trace amounts of HDL.

To explore the antigenic differences of serum lipoprotein density classes, the density fractions were injected into rabbits to induce the formation of antibodies (Table 16). Antiserum to VLDL contained antibodies to only LP-B. The LDL and HDL antisera contained antibodies

TABLE 15

RELATIVE FATTY ACID COMPOSITION OF TRIGLYCERIDES AND CHOLESTEROL ESTERS OF THE SERUM LIPOPROTEIN DENSITY CLASSES

Density Class		Fatty Acid				
		16	16:1	18	18:1	18:2
				%		
VLDL	TG	39.51	5.21	8.16	47.12	0
	CE	33.07	7.99	8.58	48.32	2.04
LDL	TG	25.56	3.24	13.75	57.45	0
	CE	35.28	8.10	8.10	43.38	5.15
HDL	TG	34.19	4.30	11.63	49.88	0
	CE	30.86	8.07	7.07	45.20	8.79

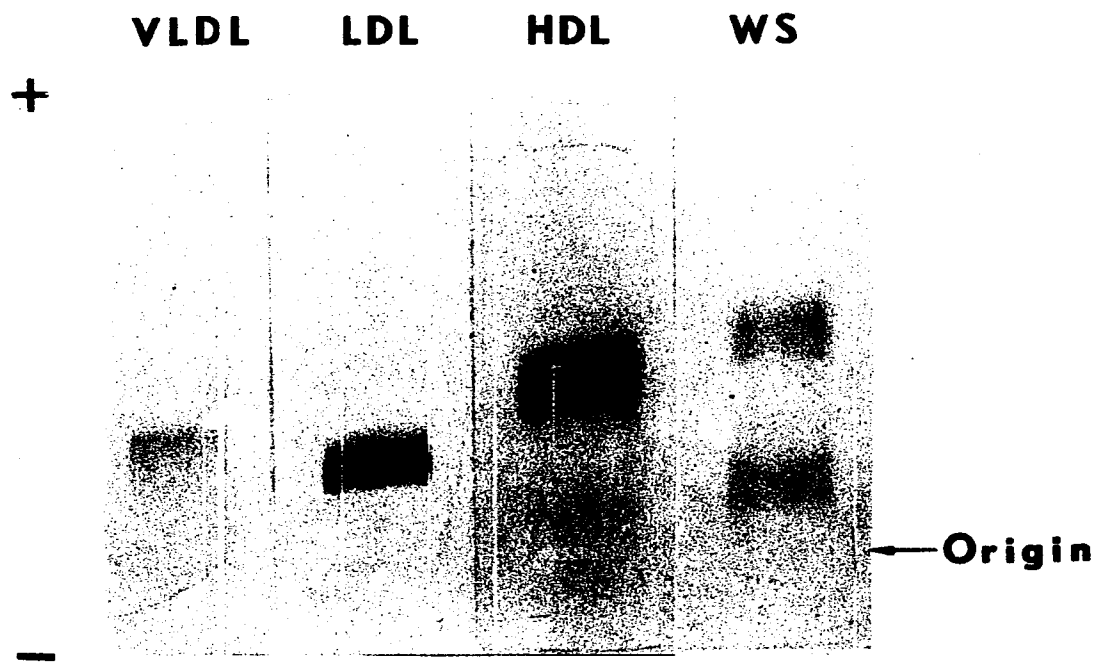

Figure 3. Agarose gel electrophoresis of serum lipoprotein density classes. Pattern was stained with Oil Red O.

TABLE 16

CHARACTERIZATION OF ANTISERA TO ULTRACENTRIFUGALLY ISOLATED LIPOPROTEIN DENSITY CLASSES

Designation of Antisera	Host Animal	Total Protein Injected	Specificity			Comment
			LP-A	LP-B	Albumin	
A-VLDL	Rabbit	3-8 mg	-	++	-	
A-LDL	Rabbit	5-10 mg	+	++	-	Antibodies to LP-A were removed by adding HDL_3
A-HDL	Rabbit	10-20 mg	++	+	-	Antibodies to LP-B were removed by adding VLDL
$A\text{-}HDL_3$	Rabbit	10-20 mg	++	-	-	

to both LP-B and LP-A. Antiserum to HDL_3 contained antibodies to only LP-A. The antibodies to LP-B which were found in antiserum to HDL could be removed by adding VLDL to the antiserum. Likewise, the antibodies to LP-A which were found in antiserum to LDL could be removed by treating the antiserum with HDL_3.

Heteroimmune antisera further indicated the antigenic heterogeneity of the density classes. With monospecific antiserum to LP-A and LP-B, the density classes were characterized by immunodiffusion (Figure 4) as to whether they contained these two lipoprotein families. VLDL, LDL, HDL_2, and WS gave a single line of identity when reacted against anti-LP-B. A single broad precipitin line of identity was seen when anti-LP-A reacted with LDL, HDL_2, HDL_3, WS, and the 1.21 g/ml infranate. Sometimes a weak reaction of anti-LP-A with VLDL could be seen. LDL and HDL_2 showed two precipitin lines when reacted against anti-WS; one which was identical to LP-B and the other identical to LP-A. From these studies, it was shown that only HDL_3 was antigenically homogeneous; it contained only LP-A. VLDL contained only trace amounts of LP-A. LDL and HDL_2 contained both LP-A and LP-B. None of the lipoprotein density fractions reacted with antiserum to albumin.

The results of immunoelectrophoresis of density classes were quite similar to those obtained by immunodiffusion. It was clear by immunoelectrophoresis that LDL and HDL_2 contained LP-A as well as LP-B (Figure 5 c, d). For the first time, heterogeneity of LP-A was observed in HDL_2 and HDL_3 (Figure 5 d, e). A precipitin line was seen which migrated slightly slower than the major precipitin line. This slower line coalesced with the major line suggesting an identity reaction.

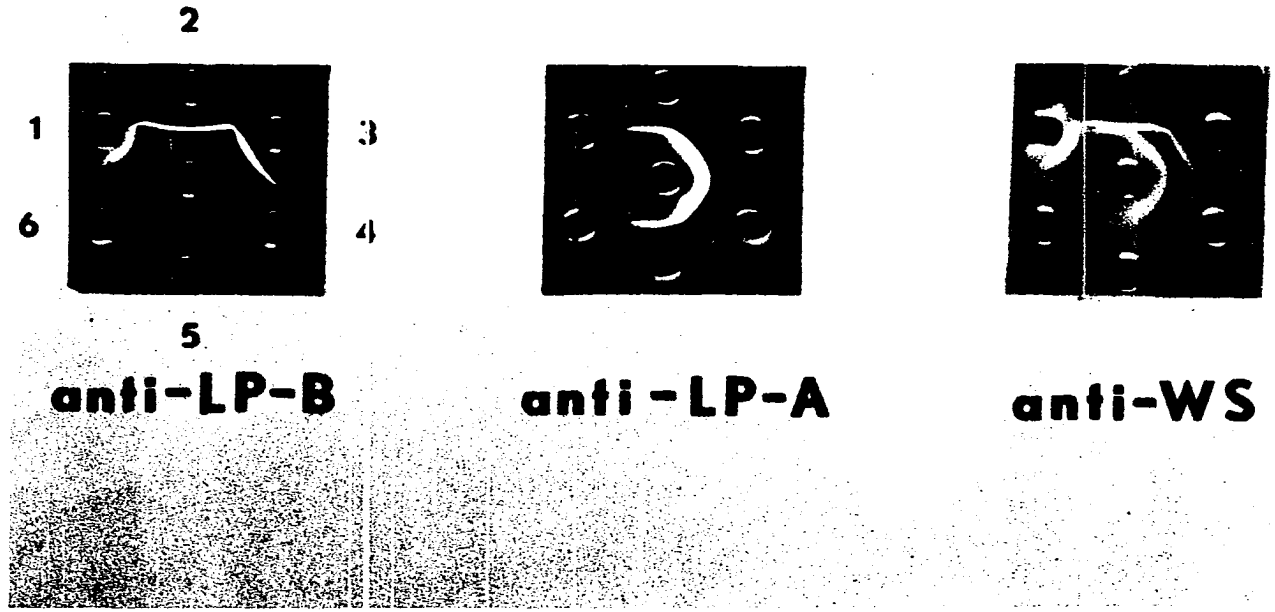

Figure 4. Immunodiffusion of serum lipoprotein density classes. Antisera to LP-B, LP-A, and WS were placed in the center wells. The outer wells contain (1) VLDL, (2) LDL, (3) HDL_2, (4) HDL_3, (5) 1.21 g/ml infranate.

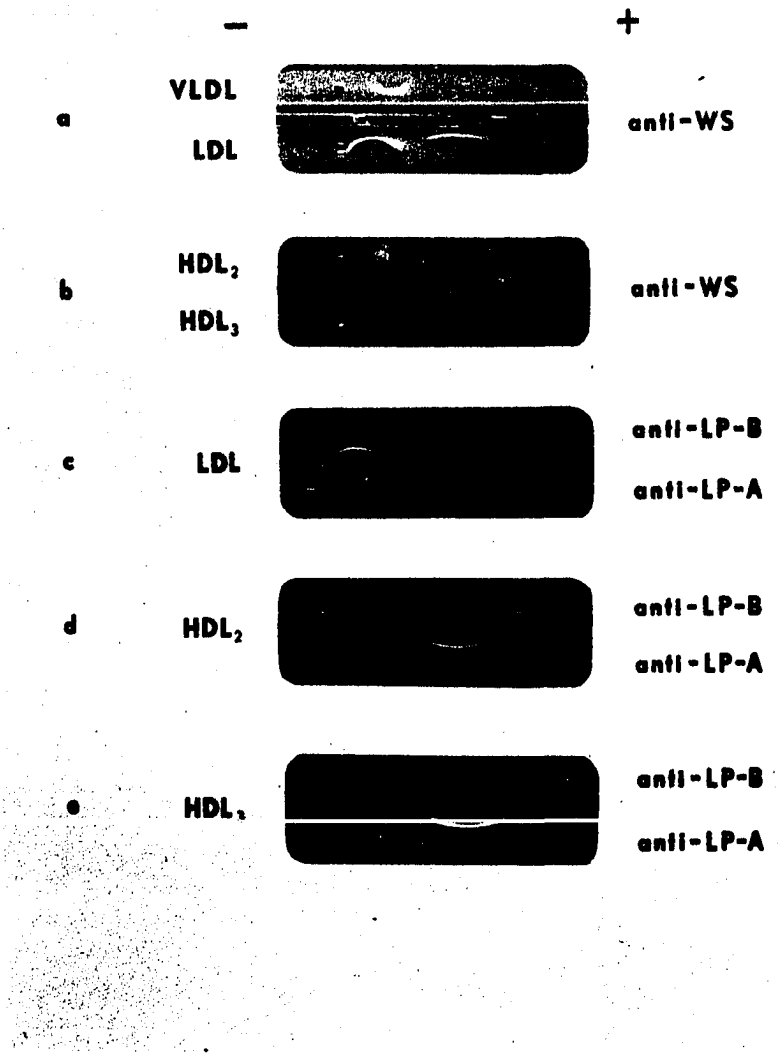

Figure 5. Immunoelectrophoresis of serum lipoprotein density classes. Antigens were placed in the wells and the appropriate antisera in the troughs parallel to the well.

The density classes were also characterized by their pattern on polyacrylamide gel electrophoresis (Figure 6). When stained with Coomassie Brilliant Blue, several bands were seen in all density classes. VLDL and LDL looked similar except LDL showed heavier staining bands in the separating gel. The patterns for HDL_2 and HDL_3 were nearly identical showing three major bands. A fast moving band was seen in all density classes. Some material which stained for lipid as well as protein remained in the stacking gels.

In order to determine where lipoprotein families migrated on PAGE, the gels were embedded in 1 per cent agarose and examined by immunodiffusion (Figure 7). Although some LP-B remained in the stacking gel, most of the LP-B was seen at the junction between the stacking and separating gels. When HDL was run on PAGE and embedded in agarose, the LP-A reaction was seen in the region of the gel corresponding to the major protein staining bands shown in Figure 6.

Heterogeneity of the density classes was also observed by studies in the analytical ultracentrifuge. LDL showed the formation of a slower floating peak after 32 minutes (Figure 8). HDL also showed heterogeneity, but not as pronounced as seen in LDL (Figure 9). VLDL had a flotation coefficient of 33.2 S at a density of 1.063 g/ml. LDL had a flotation coefficient of 9.5S at a density of 1.063 g/ml and HDL had a flotation coefficient of 4.65S at a density of 1.21 g/ml.

In order to further investigate the antigenic components of the density classes, they were totally delipidated by extraction with organic solvents. After delipidization, VLDL and LDL were mostly insoluble in aqueous buffers, but were partially soluble in 8 M urea-0.01 M phosphate

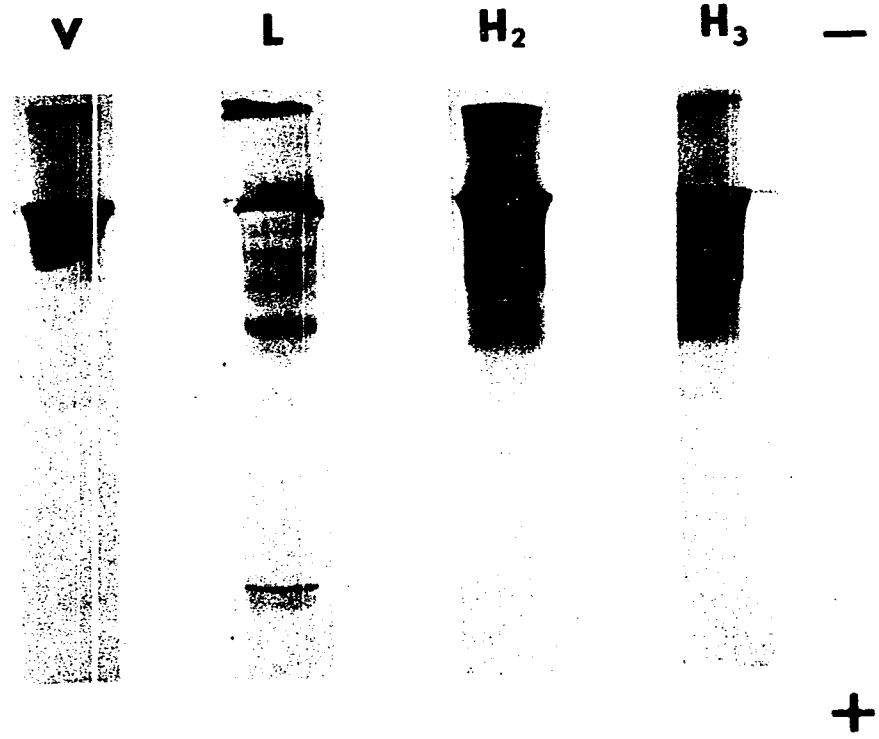

Figure 6. PAGE of serum lipoprotein density classes. Gels contained 7 per cent acrylamide and 8 M urea. Gels were stained with Coomassie Brilliant Blue.

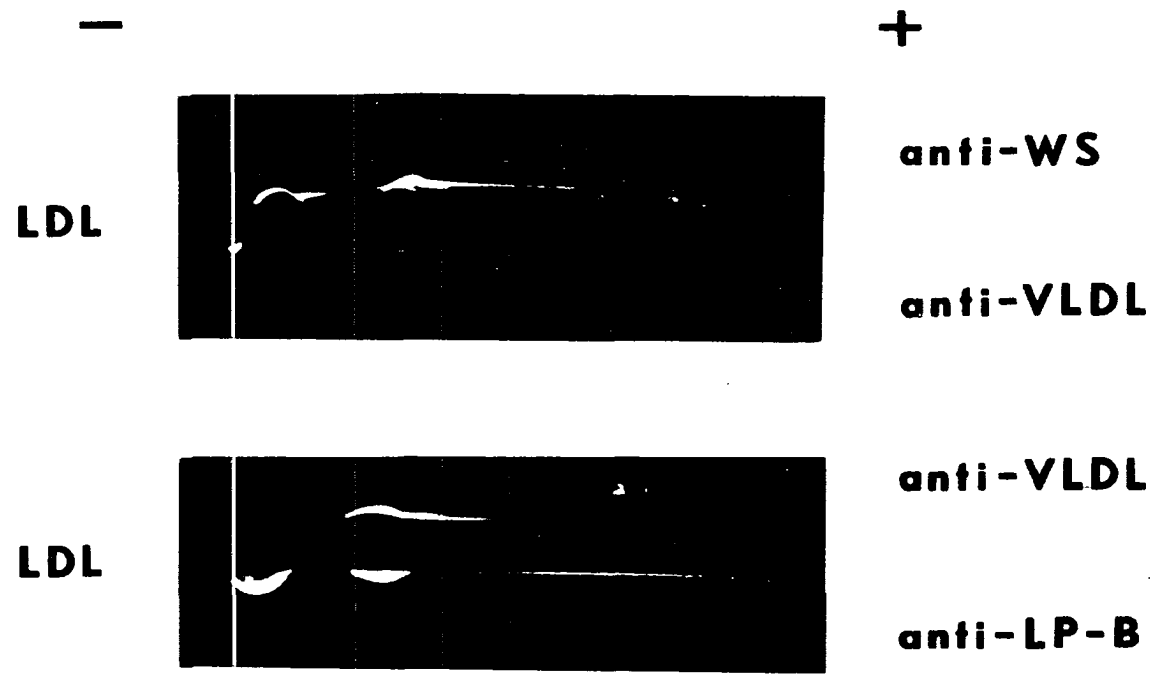

Figure 7. Combined PAGE and immunodiffusion of LDL on 7 per cent acrylamide gel embedded in 1 per cent agarose.

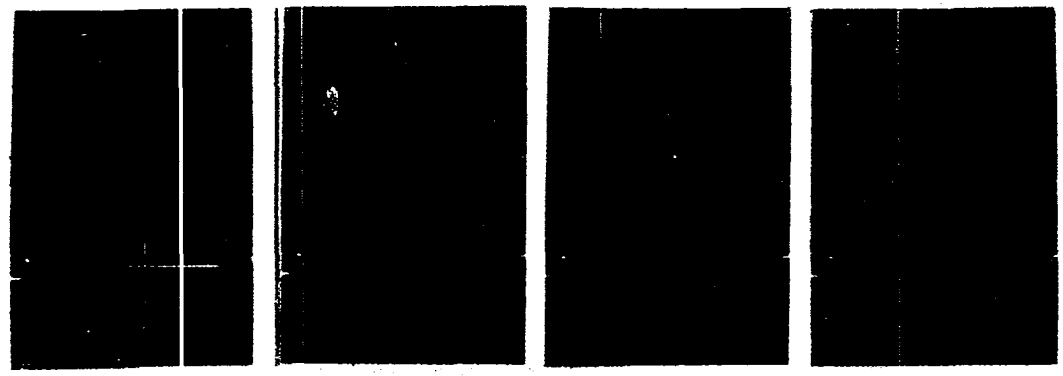

Figure 8. Ultracentrifugal flotation pattern for LDL. Experimental conditions: rotor speed, 52,000 rpm; temperature, 25°C; solvent density, 1.063 g/ml. Pictures were taken from left to right at 0, 8, 16, and 32 minutes after full speed was reached.

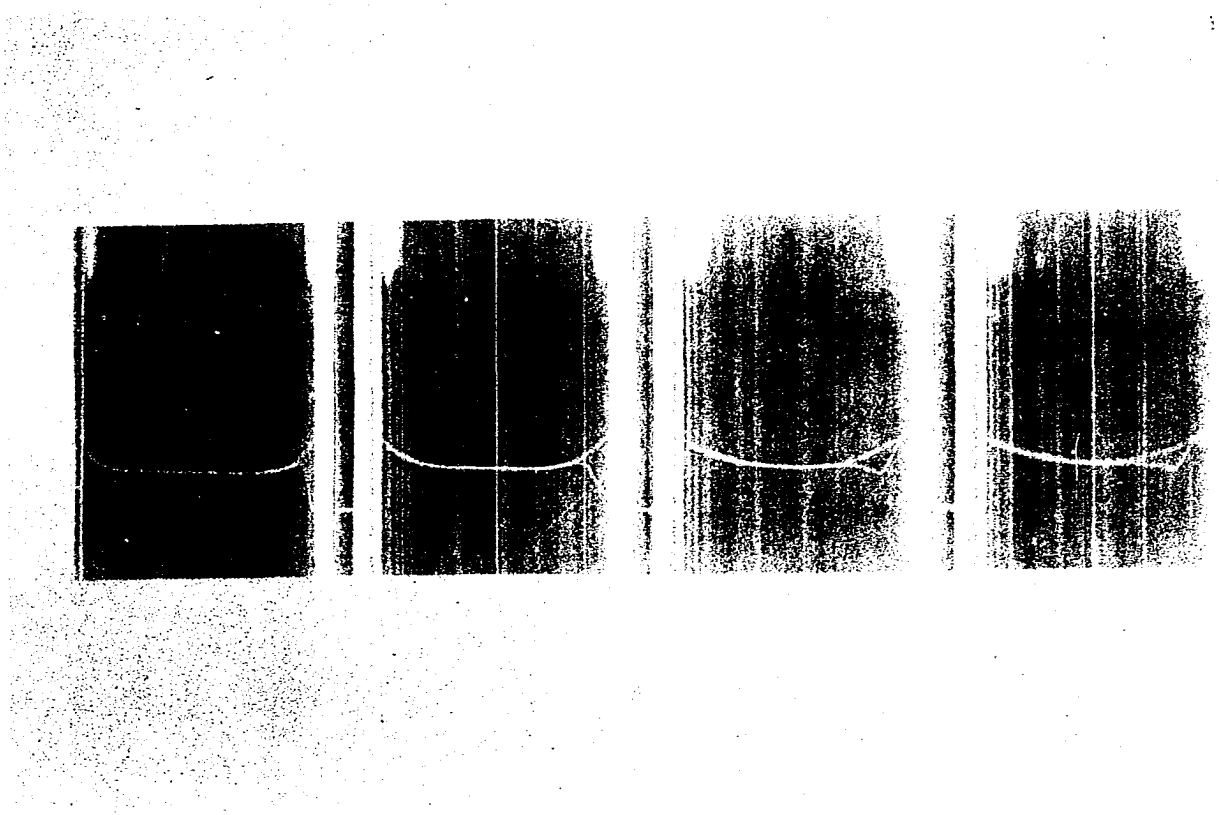

Figure 9. Ultracentrifugal flotation pattern for HDL. Experimental conditions: rotor speed, 52,000 rpm; temperature, 25°C; solvent density, 1.21 g/ml. Pictures were taken from left to right at 0, 8, 16, and 32 minutes after full speed was reached.

buffer, pH 8.0. Apolipoproteins of VLDL and LDL which were soluble in 8 M urea no longer reacted with antisera to LP-B, but did react with antisera to LP-A. ApoHDL$_2$ was partially insoluble in 0.01 M Tris buffer, pH 8.6, presumably due to the insolubility of apoB. ApoHDL$_3$ was soluble in aqueous buffers and reacted strongly with antisera to LP-A.

On immunoelectrophoresis, apolipoproteins of density classes which were solubilized in 8 M urea, did not react with antisera to LP-B (Figure 10 a, b). Totally delipidized VLDL showed several precipitin lines with antibodies to LP-A. The heterogeneity of apoA was more prominent than that of LP-A (Figure 10 c, d). A precipitin line closer to the antiserum trough was different from the faster migrating precipitin line. No new antigenic determinants were observed after total delipidization of the density classes, but it was established that VLDL contained apoA.

The amino acid compositions of apoVLDL and apoLDL were very similar (Table 17), but were different from the composition of apoHDL. Aspartic acid, glutamic acid, alanine and leucine were the major amino acids found in apoVLDL and apoLDL. HDL contained more glutamic acid and leucine than LDL or VLDL. HDL showed only trace amounts of half cystine and glucosamine. The ratio of phenylalanine to tyrosine was greater than unity in VLDL and LDL, but less than unity in HDL. A similar relation existed between threonine and serine. No cysteic acid was detected in HDL$_3$ (Table 18). The presence of cysteic acid in HDL and HDL$_2$ could be due to the presence of LP-B.

Alanine and leucine were identified as the carboxyl terminal amino acids of apoHDL$_3$ by enzymatic assay with carboxypeptidases as well

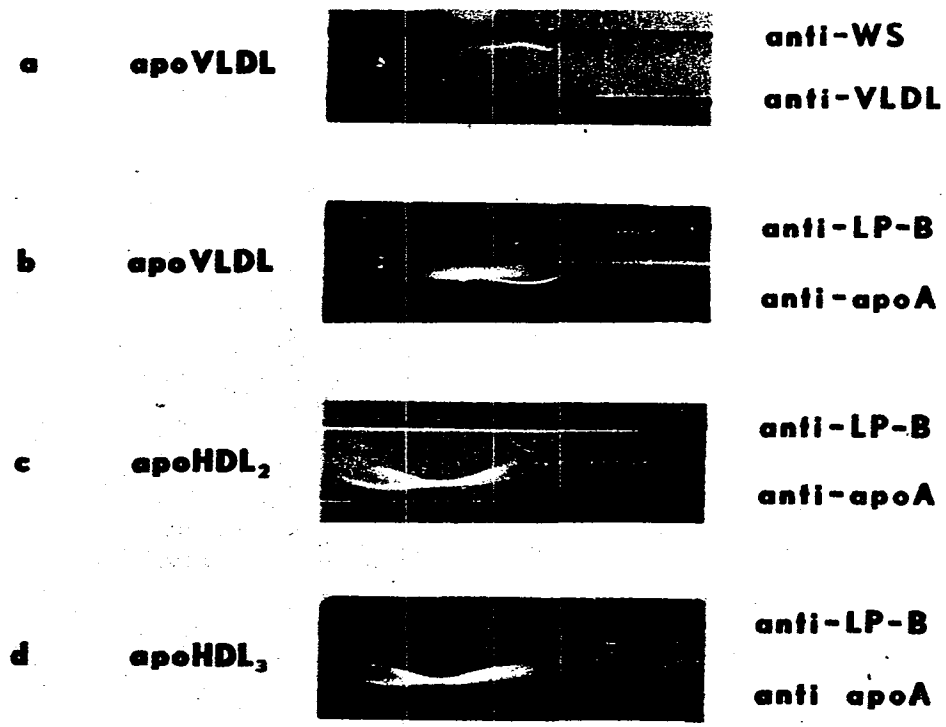

Figure 10. Immunoelectrophoresis of serum apolipoproteins in 1 per cent agarose.

TABLE 17

AMINO ACID COMPOSITION OF LIPOPROTEIN DENSITY CLASSES[a]

	VLDL	LDL	HDL	Albumin
Lysine	5.89	6.55	7.90	6.62
Histidine	1.13	.89	.55	1.83
Arginine	3.84	3.07	5.51	2.57
Aspartic acid	10.38	11.97	9.77	11.56
Threonine	5.70	6.95	6.09	4.41
Serine	7.57	9.48	3.68	8.50
Glutamic acid	13.40	13.45	22.97	17.35
Proline	5.08	5.55	6.47	6.29
Glycine	6.30	5.83	2.76	6.20
Alanine	10.40	7.73	8.40	10.35
Half cystine[b]	1.00	1.00	.46	1.00
Valine	3.89	4.14	4.50	4.29
Methionine	.67	.63	1.09	2.11
Isoleucine	4.59	4.49	1.92	3.87
Leucine	9.27	10.92	13.96	6.66
Tyrosine	4.94	2.41	2.30	2.72
Phenylalanine	5.26	4.18	1.67	4.03
Glucosamine	1.04	.72	.35	Trace

[a]Moles/100 moles in sample.

[b]Determined as cysteic acid after performic acid oxidation.

TABLE 18

AMINO ACID COMPOSITION OF HDL SUBFRACTIONS

	HDL_2	HDL_3	HDL
Lysine	60.67	66.37	66.76
Histidine	2.80	2.57	2.56
Arginine	52.74	46.99	49.40
Aspartic acid	42.06	42.18	37.98
Threonine	27.44	26.62	24.83
Serine	17.07	15.27	14.16
Glutamic acid	100	100	100
Proline	24.31	26.00	31.32
Glycine	11.85	13.58	9.86
Alanine	43.24	44.71	40.94
Half cystine[a]	3.60	0	2.05
Valine	21.52	23.55	19.36
Methionine	5.60	5.45	6.40
Isoleucine	8.90	10.21	8.95
Leucine	58.94	66.23	66.43
Tyrosine	11.59	13.08	11.87
Phenylalanine	8.93	8.60	7.90

[a]Determined as cysteic acid after performic acid oxidation.

as by hydrazinolysis (Table 19). Results of dansylation and dinitrophenylation of apoHDL$_3$ indicated aspartic acid and isoleucine as the N-terminal amino acids. The disclosure of two amino and two carboxyl terminal amino acids suggested that LP-A contained two polypeptides. From end group analysis, it was shown that VLDL, LDL, and HDL$_2$ also contained LP-A. By indirect evidence, tyrosine and lysine may be the terminal amino acids of LP-B.

Characterization of Albumin

Albumin was isolated from the 1.21 g/ml infranate by precipitation with 10 per cent TCA and extraction with ethanol. Although this precipitate consisted mostly of albumin, it still contained trace amounts of LP-A. In the absence of urea, albumin migrated faster than apoA when electrophoresed on polyacrylamide gel. This preparation of albumin was further purified by preparative polyacrylamide gel electrophoresis in the absence of urea.

Purified albumin exhibited one major band and two minor bands on 7 per cent analytical PAGE (Figure 11) in 8 M urea. By SDS-polyacrylamide gel electrophoresis, albumin had a molecular weight of 64,000 daltons. Albumin gave a single precipitin line with anti-WS or anti-albumin (Figure 11). The anti-albumin was prepared by injecting a purified albumin preparation into white New Zealand rabbits.

Aspartic acid was found as the only amino-terminal for albumin. Alanine was the first amino acid released by digestion with carboxypeptidase A and B (Table 20). After fifteen minutes, the release of glycine was almost twice as high as that of alanine, suggesting two glycine residues in sequence. Valine was also released, but at a very low concen-

TABLE 19

CARBOXYL AND AMINO TERMINAL AMINO ACIDS OF THE LIPOPROTEIN DENSITY CLASSES

Density Class	C-terminal	N-terminal
VLDL	Ala, Leu, Tyr	Asp, Ileu, Lys
LDL	Ala, Leu, Tyr	Asp, Ileu, Lys
HDL_2	Ala, Leu, Tyr	Asp, Ileu, Lys
HDL_3	Ala, Leu	Asp, Ileu

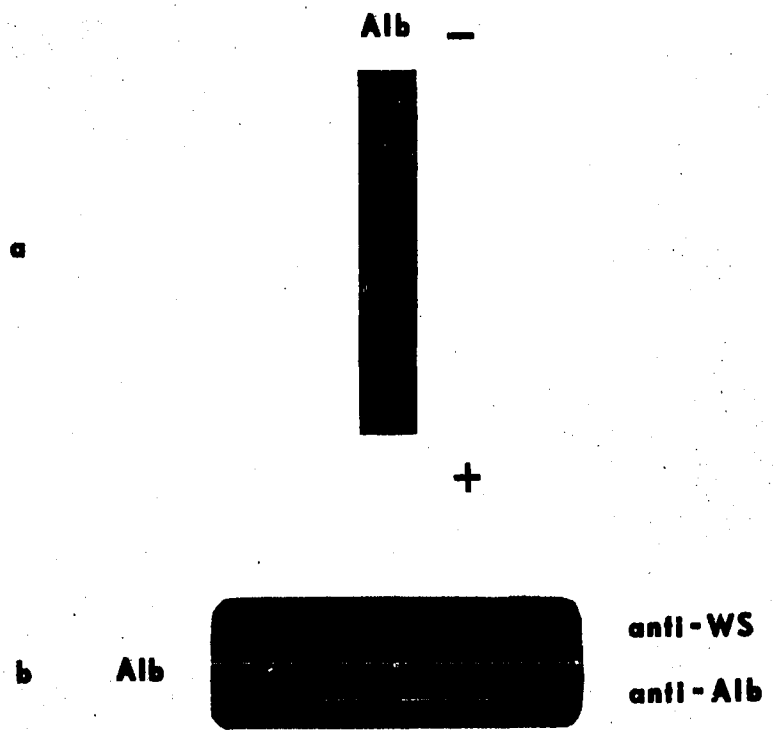

Figure 11. PAGE and immunoelectrophoresis of serum albumin.

TABLE 20

RELEASE OF THE CARBOXYL TERMINAL AMINO ACID OF ALBUMIN BY CARBOXYPEPTIDASE A AND B[a]

	5 min.	15 min.	30 min.	60 min.
Alanine	.71	.84	.97	1.08
Leucine	.49	.49	.58	.78
Glycine	.46	1.28	1.29	1.30
Valine	.25	.28	.34	.41

[a]Values expressed as micro moles amino acid released per mg protein X 10^{-2}.

tration. Alanine was confirmed as the C-terminal by hydrazinolysis for 48 and 72 hours. Digestion with the carboxypeptidases suggested the following C-terminal sequence: R-val-leu-gly-gly-ala. Albumin was characterized by aspartic acid as the N-terminal and alanine as the C-terminal amino acids.

Characterization of Lipoprotein Families

Isolation and Immunochemical Characterization

The unique binding characteristics of Concanavalin-A-Sepharose were used to separate LP-A and LP-B families. Whereas LP-B was retained by binding to Con-A, LP-A was eluted at the void volume of the column. The retained LP-B was then eluted with α-D-methyl-glucoside buffer. Chromatography of density classes on Con-A-Sepharose always resulted in the separation of two fractions; one which was not retained by the column and the other which could be obtained when the elution buffer was added. The ratio of peak 1 and peak 2 was different for the individual density classes.

The chromatography pattern of VLDL consisted of a small peak which was not retained and a large peak which was retained by the column (Figure 12). Peak 1 did not contain any detectable protein either by immunology or by chemical determination, but did contain lipid. It did not react with anti-LP-B or anti-LP-A. Peak 2 reacted with anti-LP-B, but not with anti-LP-A (Figure 13).

The chromatographic pattern of LDL was characterized by more of the first peak than VLDL (Figure 14). Peak 1 reacted with anti-LP-A, but not with anti-LP-B (Figure 15). Peak 2 gave a single precipitin line

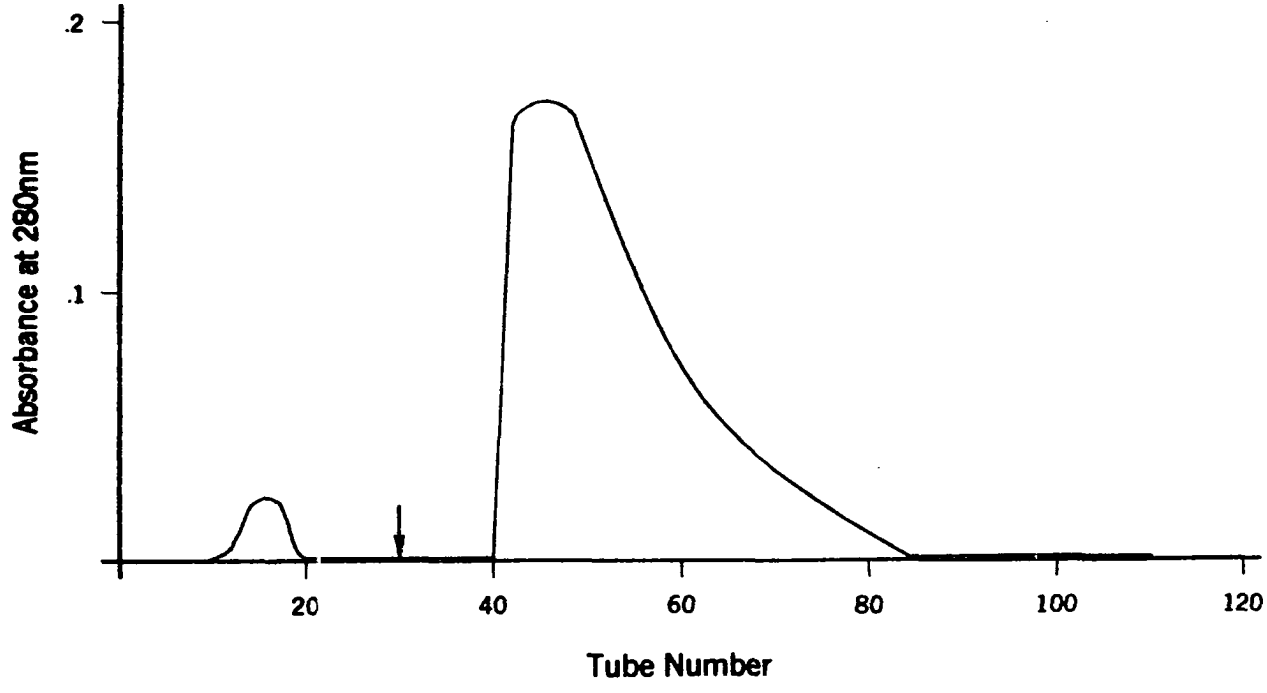

Figure 12. Elution profile of VLDL on Con-A-Sepharose. The arrow indicates the application of the elution buffer.

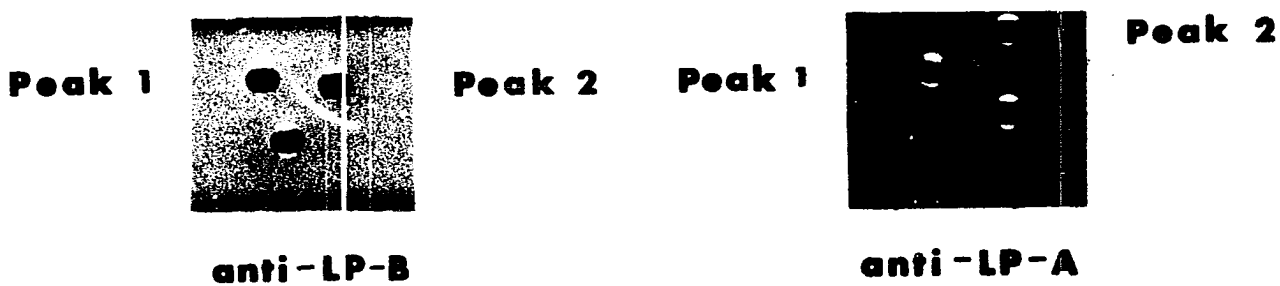

Figure 13. Immunodiffusion of lipoprotein families separated from VLDL.

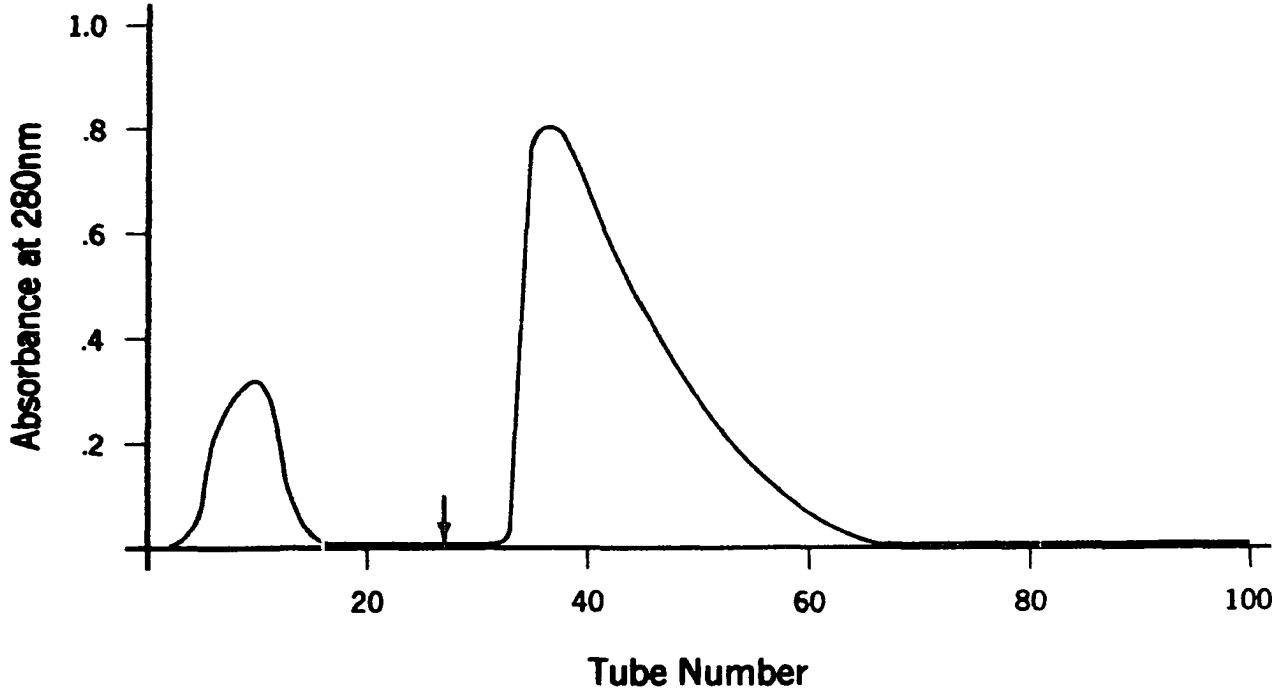

Figure 14. Elution profile of LDL on Con-A-Sepharose. The arrow indicates the application of the elution buffer.

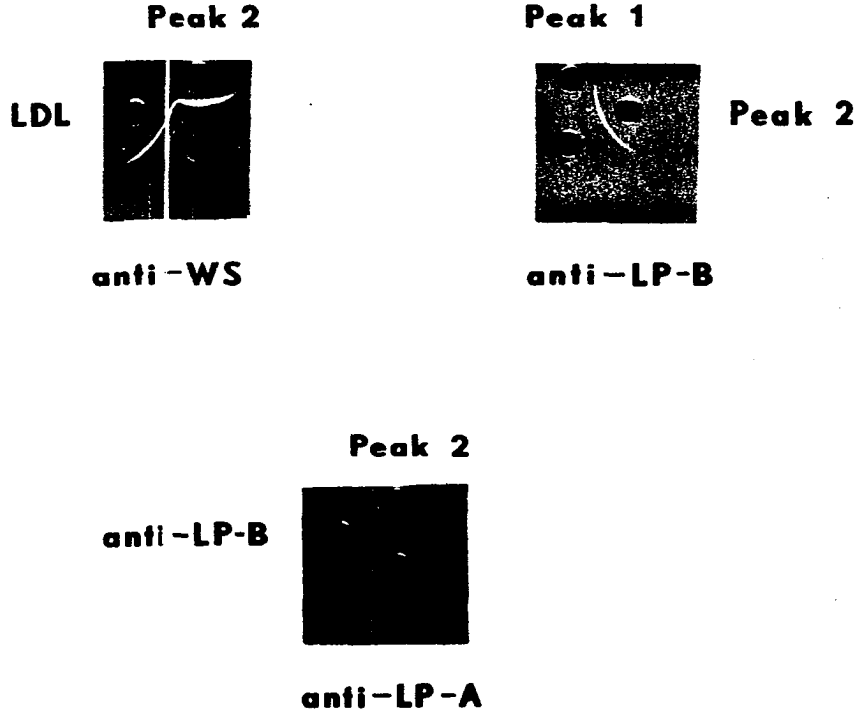

Figure 15. Immunodiffusion of lipoprotein families separated from LDL.

with anti-LP-B, but showed no reaction with anti-LP-A. Peak 1 (LP-A) and peak 2 (LP-B) gave symmetrical peaks in the analytical ultracentrifuge (Figures 16, 17).

When HDL_2 were chromatographed on Con-A, peak 1 and peak 2 were present in almost equal amounts. HDL_2 seemed to be an intermediate density class containing equal amounts of LP-A and LP-B.

Most of the HDL were not retained when chromatographed on Con-A, but a small peak was retained (Figure 18). Peak 1 showed an identity reaction with HDL in reaction with anti-LP-A; peak 2 reacted with anti-LP-B (Figure 19). Peak 1 and peak 2 showed a nonidentity reaction when reacted with anti-WS. Non-identity of peak 1 and peak 2 also means that LP-A is not identical to LP-B.

In order to obtain monospecific antisera to lipoprotein families, peak 1 (LP-A) and peak 2 (LP-B) were injected into rabbits (Table 21). The availability of these antisera offered an invaluable means for differentiating and identifying lipoprotein families in any density class. LP-B, purified by Con-A chromatography, reacted only with anti-LP-B and LP-A reacted only with anti-LP-A (Figure 20).

Chemical Characterization

Quantitation of lipoprotein families separated by chromatography on Con-A was carried out gravimetrically after exhaustive dialysis and drying to a constant weight. Protein, cholesterol, cholesterol esters, glycerides, and phospholipids were quantitated colorimetrically by the same methods used for the density classes.

The distribution of lipoprotein families across the density spectrum is shown in Table 22. Of the total apoA in the different den-

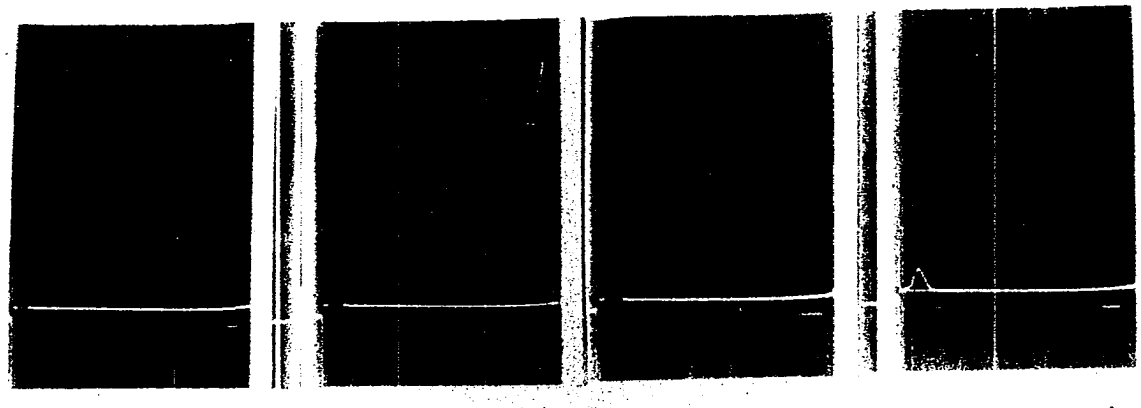

Figure 16. Ultracentrifugal sedimentation pattern of LP-B from LDL. Experimental conditions: rotor speed, 52,000 rpm; temperature, 25°C; solvent density, 1.006 g/ml. Pictures were taken from right to left at 0, 4, 8, and 12 minutes.

Figure 17. Ultracentrifugal sedimentation pattern of LP-A from LDL. Experimental conditions: rotor speed, 52,000 rpm; temperature, 25°C; solvent density, 1.006 g/ml. Pictures were taken from right to left at 0, 4, 8, and 12 minutes.

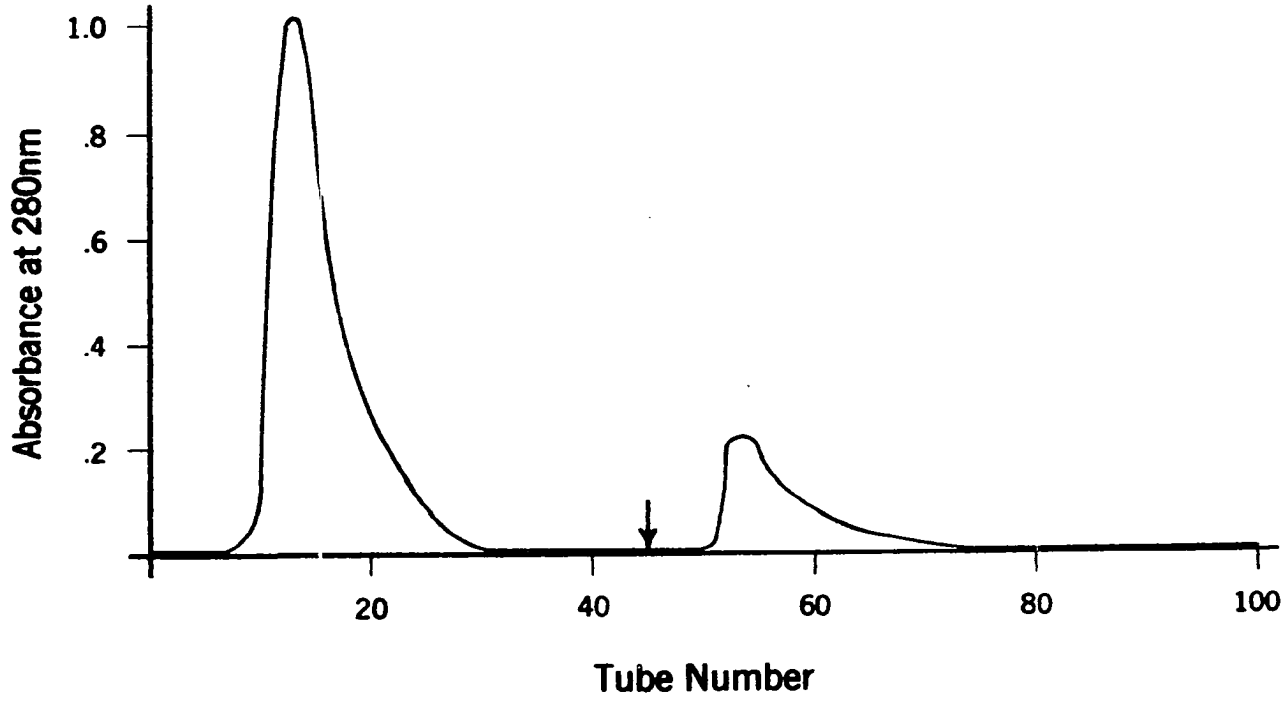

Figure 18. Elution profile of HDL on Con-A-Sepharose. The arrow indicates the application of the elution buffer.

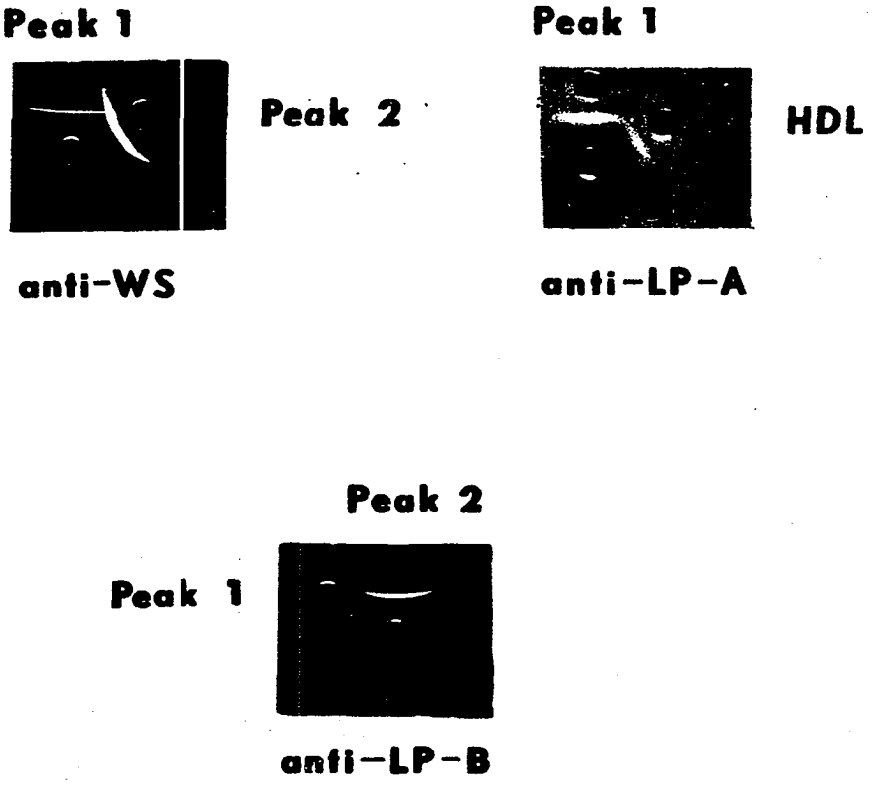

Figure 19. Immunodiffusion of lipoprotein families separated from HDL.

TABLE 21

CHARACTERIZATION OF ANTISERA TO LIPOPROTEIN FAMILIES AND POLYPEPTIDES

Designation of Antisera	Host Animal	Total Protein Injected	Source of Antigen	Specificity			
				A-I	A-II	B	Albumin
A-LP-A	Rabbit	10-20 mg	Ultracentrifugally isolated HDL_3 or from chromatography on Con-A	+	+	-	-
A-apoA	Rabbit	10-30 mg	Totally delipidized HDL_3	+	+	-	-
A-A-II	Rabbit	10-20 mg	Chromatography on Biogel A-5m in the presence of guanidine-HCl	-	+	-	-
A-LP-B	Rabbit	5-10 mg	From chromatography on Con-A	-	-	+	-
A-Alb	Rabbit	10-20 mg	Preparative polyacrylamide gel electrophoresis	-	-	-	+

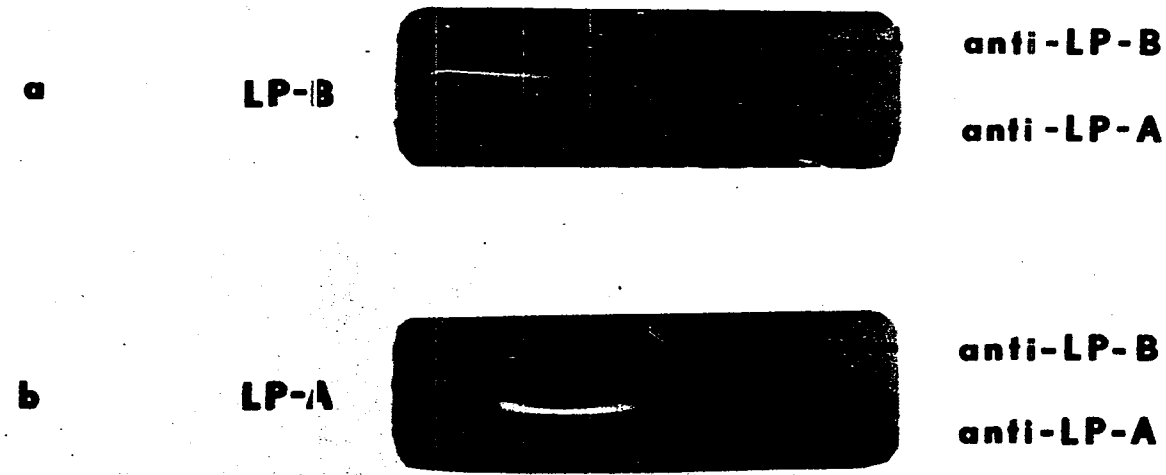

Figure 20. Immunoelectrophoresis of lipoprotein families.

TABLE 22

DISTRIBUTION OF LIPOPROTEIN FAMILIES

Density Class	apoA	apoB
	%	
VLDL	Trace	21.07
LDL	0.75	71.51
HDL_2	22.31	16.41
HDL_3	76.94	0

sity classes, 77 per cent was found in HDL_3, 22 per cent in HDL_2, less than 1 per cent in LDL and only trace amounts in VLDL. Nearly 72 per cent of the apoB was found in LDL, 21 per cent in VLDL and 16 per cent in the HDL_2. No apoB was detected in HDL_3.

The per cent composition of lipoprotein families was different from that of the corresponding density classes (Table 23). The increased protein and phospholipid contents were probably due to selective losses of neutral lipids during chromatography. The phospholipid/protein ratio seems to remain constant for the lipoprotein families separated from each density class. For example, the phospholipid/protein ratio of LP-A and LP-B in LDL and HDL_2 was close to unity. The phospholipid/protein ratio of LP-A in HDL_3 was less than unity.

The phospholipid composition of lipoprotein families LP-A and LP-B was similar to that of the lipoprotein density classes (Table 24). Lecithin and sphingomyelin were the major phospholipids of both lipoprotein families. LP-A from HDL_2 contained relatively more lecithin than LP-B from that density class.

Characterization of Apolipoproteins

Characterization of ApoB

The LP-B purified by chromatography on Con-A was totally delipidated by extraction with ethanol-diethyl ether. ApoB prepared in this manner was insoluble in 0.01 M Tris buffer, pH 8.6, but was partially soluble in 0.01 M Tris-8 M urea buffer, pH 8.6. ApoB could also be solubilized in buffer containing 0.1 per cent SDS. When apoB was chromatographed on Sephadex G-100, equilibrated with Tris-urea buffer, a single

TABLE 23

PER CENT COMPOSITION OF LIPOPROTEIN FAMILIES

Density Class	Lipoprotein Family	Protein	Phospholipid	Cholesterol Free	Cholesterol Ester	Glycerides
LDL	LP-A	32.91	28.94	8.66	28.39	1.02
	LP-B	37.78	34.13	10.63	14.65	2.81
HDL_2	LP-A	47.26	39.73	5.22	6.64	1.15
	LP-B	42.22	32.12	9.53	13.21	2.92
HDL_3	LP-A	54.47	29.55	1.05	14.46	.48

TABLE 24

PER CENT COMPOSITION OF PHOSPHOLIPIDS OF LIPOPROTEIN FAMILIES

Density Class	Lipoprotein Family	Phosphatidyl-ethanolamine	Phosphatidyl-choline	Sphingo-myelin	Lysophosphatidyl-choline
VLDL	LP-B	7.43	38.86	35.43	18.29
LDL	LP-A	7.84	39.71	36.28	16.18
	LP-B	9.34	43.39	27.24	20.04
HDL_2	LP-A	5.42	55.16	20.77	18.66
	LP-B	8.86	35.44	37.98	17.72
HDL_3	LP-A	8.19	76.59	7.92	7.30

symmetrical peak eluted slightly after the void volume of the column. The peak obtained from chromatography on G-100 did not react with anti-apoA. It was shown previously that apoB does not react with anti-LP-B.

The most consistent protein band seen when apoB was run on SDS-PAGE had a molecular weight of 100,000 daltons. This observation also correlated with the elution volume of apoB when chromatographed on G-100 in the presence of 8 M urea. Due to the insolubility of apoB the observed band on SDS-PAGE may represent an aggregation of polypeptide subunits.

Since LP-B was retained by Con-A, it was concluded that apoB contained carbohydrates. Carbohydrate analysis showed that apoB contained mannose, galactose, glucose, and glucosamine (Table 25). When moles/10^5g of protein were converted to the number of residues/10^5g of protein, there were 2 residues of mannose, 1 of galactose, and 1 of glucosamine. The amount of glucosamine was also determined on the amino acid analyzer, and the results were very close to those found by gas chromatographic analysis.

The amino acid composition of apoB from VLDL, LDL, and HDL_2 (Table 26) was very similar. The values were expressed relative to glutamic acid for comparison. ApoB contained relatively large amounts of aspartic acid, glutamic acid, and leucine. Cysteic acid was also found in apoB. The similarity of amino acid compositions further supported the immunochemical identity of the LP-B from the three density classes.

By hydrazinolysis, tyrosine was found as the C-terminal of apoB. Lysine was found as the N-terminal amino acid. No definite C-terminal was found by digestion of apoB with a mixture of carboxypeptidases A and

TABLE 25

CARBOHYDRATE COMPOSITION OF ApoB

Sugars	Moles/10^5g	Residue
Mannose	12	2
Galactose	5	1
Glucose	3	< 1
Glucosamine	6	1

TABLE 26

AMINO ACID COMPOSITION ApoB FROM THREE DIFFERENT DENSITY CLASSES[a]

	apoB (VLDL)	apoB (LDL)	apoB (HDL_2)
Lysine	59.60	52.22	55.01
Histidine	7.12	9.76	3.88
Arginine	47.65	43.42	36.61
Aspartic acid	81.20	84.70	76.12
Threonine	51.40	52.04	53.28
Serine	61.61	64.90	60.36
Glutamic acid	100.00	100.00	100.00
Proline	36.66	36.56	33.32
Glycine	48.66	48.48	48.66
Alanine	56.01	54.11	64.43
Half cystine[b]	7.59	7.35	6.74
Valine	42.95	41.75	42.36
Methionine	9.04	9.12	5.45
Isoleucine	40.04	28.50	34.74
Leucine	79.04	75.18	77.00
Tyrosine	23.19	22.03	22.13
Phenylalanine	36.40	29.50	31.81
Glucosamine	5.65	6.64	5.53

[a]All values relative to glutamic acid equal to 100.

[b]Determined as cysteic acid after performic acid oxidation.

B.

Characterization of ApoA

LP-A family prepared by chromatography on Con-A-Sepharose or by ultracentrifugation was totally delipidated either by chromatography on Sephadex LH-20 in butanol-acetic acid-water, or by extraction with ethanol-diethyl ether. Since only a small quantity of material could be processed by a single chromatography on LH-20, the extraction of LP-A with organic solvents was chosen as the method of delipidization.

The amino acid compositions of apoA from LDL, HDL_2, and HDL_3 were almost identical (Table 27). The variation in the amino acid compositions were small and could have been due to experimental error. The results were compared relative to glutamic acid. ApoA was characterized by the absence of half cystine and glucosamine and by very small amounts of methionine and histidine. No carbohydrate could be detected in apoA. The lack of carbohydrate accounted for the observation that LP-A was not retained by Con-A-Sepharose columns.

ApoA was characterized by aspartic acid and isoleucine as the N-terminal amino acids and leucine and alanine as the C-terminal amino acids. Because of the chemical and immunochemical evidence suggesting at least two polypeptides of apoA, it was decided to attempt to separate the apoA polypeptides.

Chromatography of apoA on DEAE-cellulose was performed using a 0.01 M Tris-8 M urea buffer, pH 8.6. ApoA was solubilized in the eluting buffer. The apoA was not totally soluble in this buffer at a concentration greater than 10 mg/ml. A gradient of 0.01 to 0.1 M NaCl was used to elute the protein from the column. Immunodiffusion as well as analyt-

TABLE 27

AMINO ACID COMPOSITION OF ApoA FROM THREE DIFFERENT DENSITY CLASSES[a]

	apoA (LDL)	apoA (HDL_2)	apoA (HDL_3)
Lysine	60.41	61.27	69.90
Histidine	3.30	3.05	2.07
Arginine	51.15	57.46	47.19
Aspartic acid	43.06	43.46	42.69
Threonine	28.06	28.04	26.90
Serine	19.10	17.86	14.77
Glutamic acid	100.00	100.00	100.00
Proline	27.18	24.48	26.23
Glycine	19.79	12.82	14.40
Alanine	51.91	43.41	45.59
Half cystine[b]	0	0	0
Valine	20.54	22.60	25.30
Methionine	4.00	4.89	4.34
Isoleucine	12.18	8.86	10.84
Leucine	57.59	59.75	64.43
Tyrosine	12.83	11.40	13.32
Phenylalanine	8.22	9.62	8.36

[a]All values relative to glutamic acid equal to 100.

[b]Determined as cysteic acid after performic acid oxidation.

ical PAGE was used to monitor the column fractions.

When a gradient concentration of 0.1 M NaCl was reached, only trace amounts of protein had been eluted. When 1.0 M NaCl was added to the column, more protein was eluted, but not to any significant extent. Most of the original sample could be eluted with 1.0 N NaOH. The conclusion from chromatography of apoA on DEAE-cellulose was that apoA binds so strongly to the cellulose that separation of the polypeptides could not be achieved by this method.

Chromatography of apoA on columns of hydroxylapatite resulted in some separation of the apoA polypeptides, but the majority of the protein was retained on the column and could not be eluted with any solvent which was used. The results achieved with DEAE-Sephadex and Amberlite CG-50 were similar to those obtained with the DEAE-cellulose.

Since separation on columns based on absorption or charge distribution was not successful, gel permeation chromatography in denaturing solvents was investigated. ApoA separated into three peaks when chromatographed on Sephadex G-75, or G-100, equilibrated with 2 M acetic acid. All three peaks were immunochemically identical. It seemed as though the apoA polypeptides aggregated in acetic acid.

When apoA was applied to a column of Sephadex G-100, equilibrated with 0.01 M Tris-8 M urea buffer, pH 8.6, three peaks similar to those found in the previous experiments with G-100 in acetic acid were seen (Figure 21). Each of the three peaks showed two precipitin lines against anti-LP-A or anti-apoA (Figure 22b). The line closer to the antigen well was designated A-I and the line closer to the antiserum well was designated A-II. Peaks 1 and 2 showed a single protein staining band when

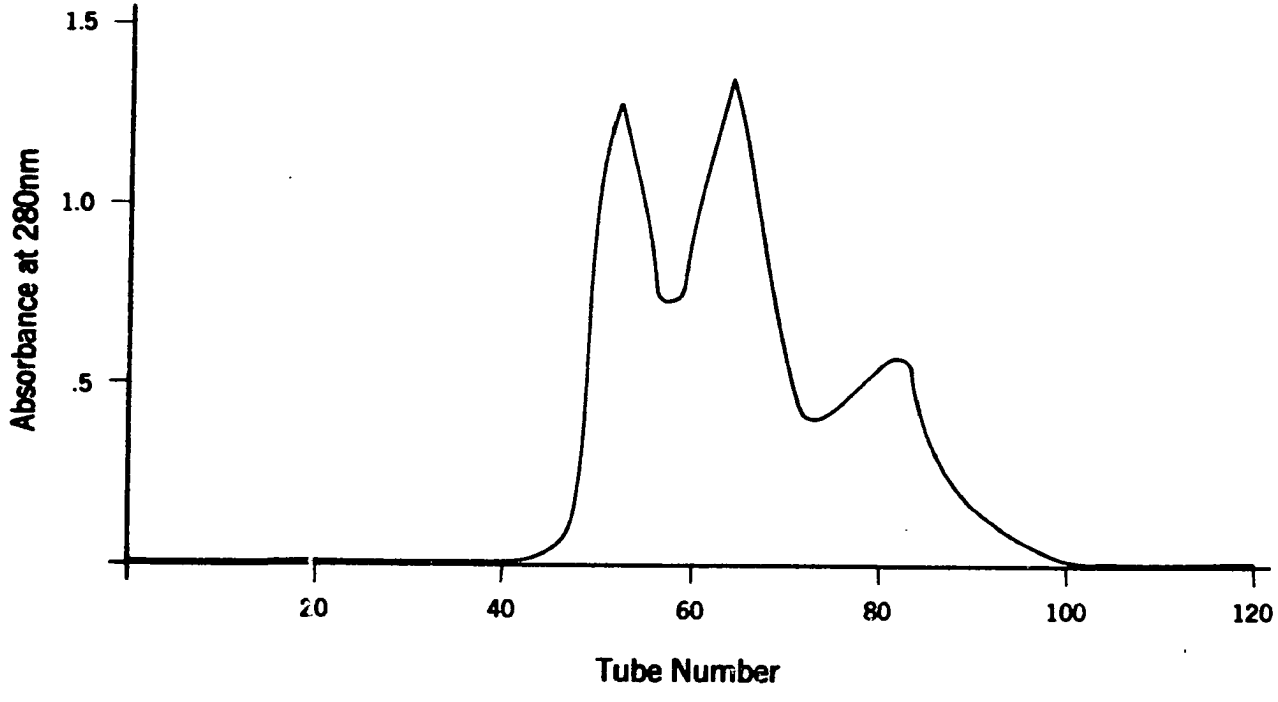

Figure 21. Elution profile of apoHDL$_3$ from Sephadex G-100 in 8 M urea-Tris buffer. Three ml fractions were collected. Thirty mg of apoHDL$_3$ were applied to the column.

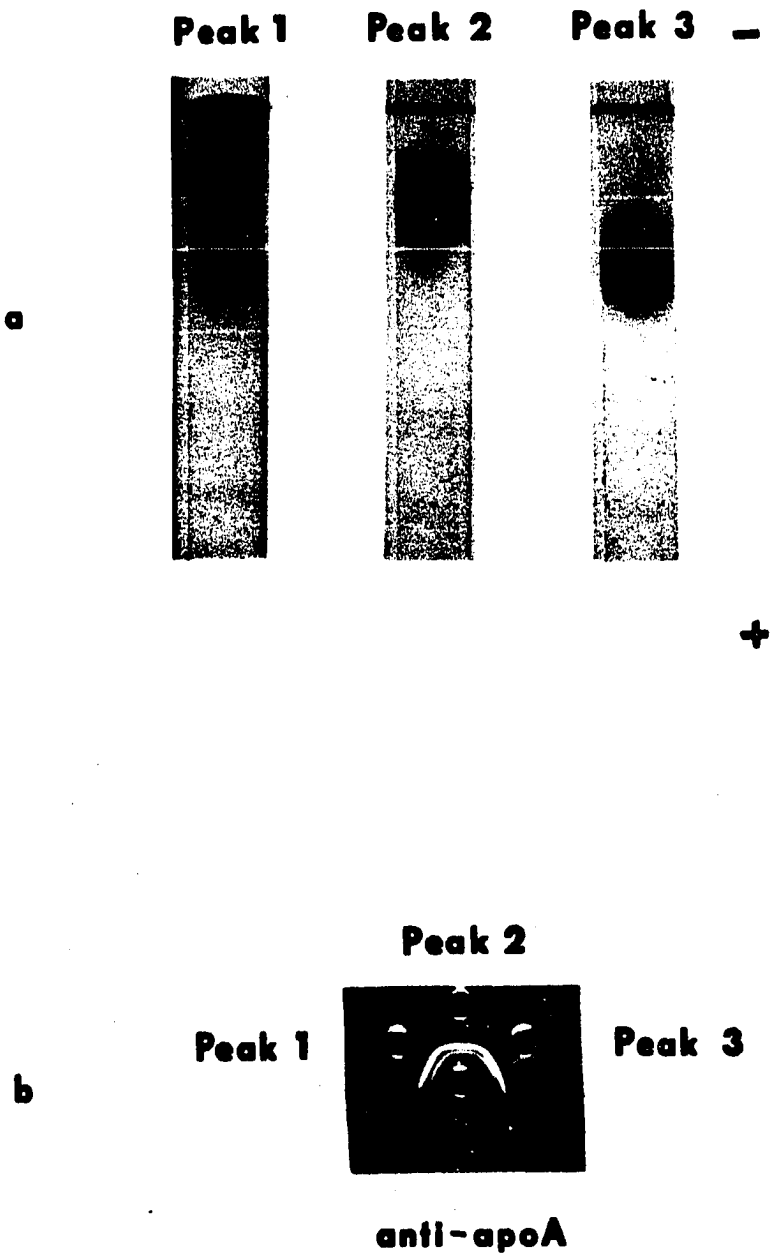

Figure 22. PAGE and immunodiffusion of peaks from chromatography of apoHDL$_3$ on Sephadex G-100 in 8 M urea-Tris buffer. Acrylamide gels were stained with Coomassie Brilliant Blue.

analyzed by PAGE (Figure 22a). Peak 3 showed several bands which migrated slightly faster than the bands in peak 1 and peak 2.

In order to identify these protein staining bands, they were sliced horizontally and embedded in 1 per cent agarose and examined by immunodiffusion (Figure 23). The major band from peak 1 and peak 2 gave two precipitin lines against anti-apoA. The major precipitin line in these two peaks was closer to the antigen which would correspond to the A-I precipitin line. In peak 3, the major precipitin line was the one closer to the antiserum trough which corresponded to the A-II precipitin line.

The amino acid compositions of the three peaks obtained from chromatography of apoA in Sephadex G-100 in the presence of 8 M urea were very similar (Table 28). Peak 3 showed increased amounts of aspartic acid, threonine, glycine, and alanine.

Although some separation of the apoA polypeptides was achieved by gel filtration in the presence of urea, it was necessary to investigate another denaturing agent. Chromatography of apoA on Bio-gel A-5m, equilibrated with 6 M guanidine-HCl, pH 6.5, resolved the apoA into five reproducible peaks (Figure 24).

By immunodiffusion, peaks 1-4 contained both A-I and A-II. Only peak 5 was immunochemically homogeneous, containing only A-II. When these peaks were examined by analytical PAGE, peaks 1 and 2 showed two slowly migrating bands (Figure 25). Peak 3 showed one major band. Peak 4 showed several faster migrating bands and a band with the same mobility as the major band of peak 3.

Although exhibiting a single precipitin line on immunodiffusion,

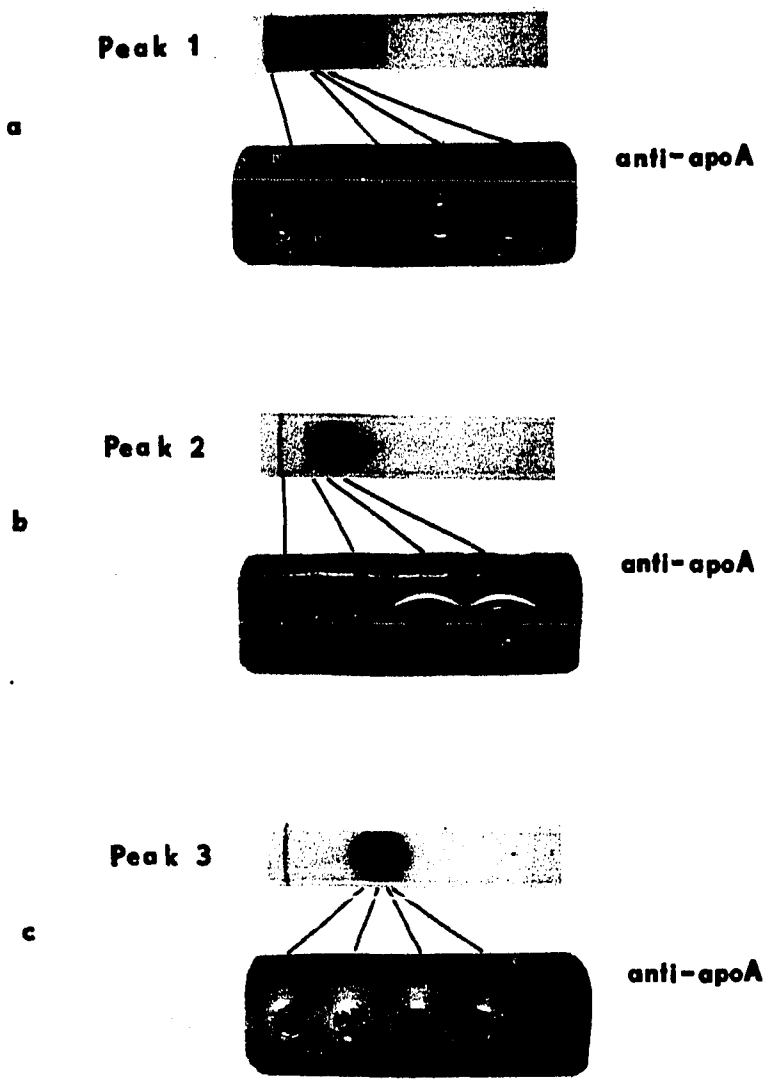

Figure 23. Immunodiffusion of bands sliced from PAGE of peaks from chromatography of apoHDL$_3$ on Sephadex G-100.

TABLE 28

AMINO ACID COMPOSITION OF PEAKS FROM CHROMATOGRAPHY OF ApoHDL$_3$ ON SEPHADEX G-100[a]

	P1	P2	P3
Lysine	51.40	56.56	66.54
Histidine	3.53	3.63	4.34
Arginine	43.75	51.07	56.36
Aspartic acid	41.92	43.61	56.49
Threonine	23.87	20.08	33.58
Serine	14.76	13.36	19.36
Glutamic acid	100.00	100.00	100.00
Proline	20.76	20.25	27.54
Glycine	7.46	7.42	17.41
Alanine	25.92	28.58	55.22
Half cystine[b]	0	0	0
Valine	17.82	18.78	18.10
Methionine	5.69	5.37	5.83
Isoleucine	9.50	8.88	6.50
Leucine	53.80	53.03	58.97
Tyrosine	15.58	15.49	14.38
Phenylalanine	12.12	10.08	7.90

[a]Values relative to glutamic acid equal to 100.

[b]Determined as cysteic acid after performic acid oxidation.

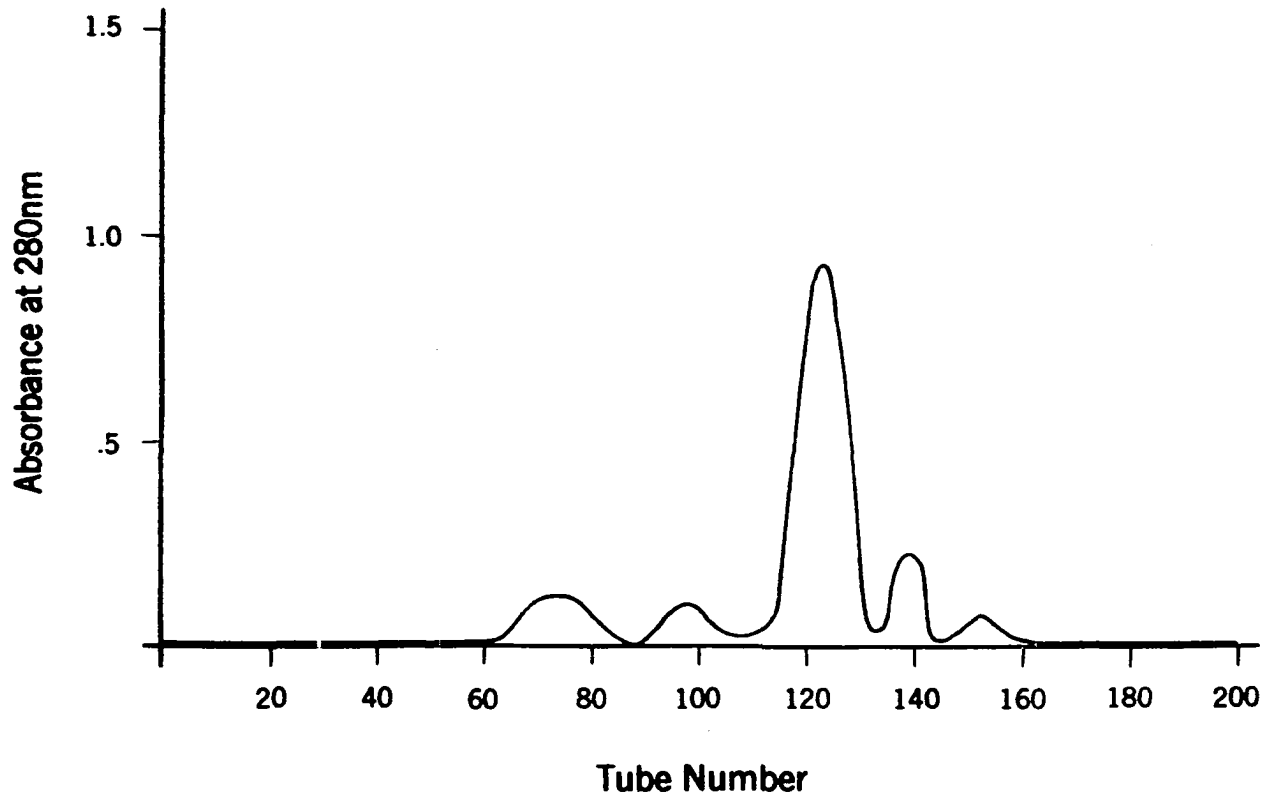

Figure 24. Elution profile of apoHDL$_3$ from Bio-gel A-5m in the presence of guanidine-HCl. Two ml fractions were collected. Approximately 25 mg of apoHDL$_3$ were applied to the column.

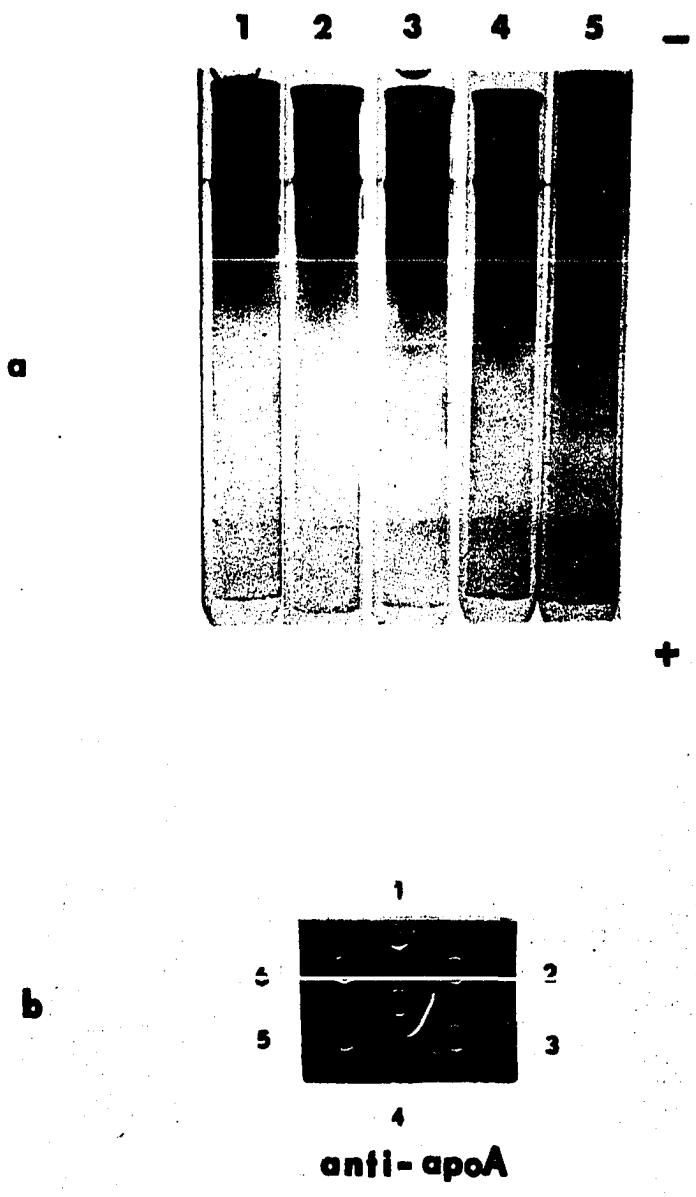

Figure 25. PAGE and immunodiffusion of peaks from chromatography of apoHDL$_3$ on Bio-gel A-5m in guanidine-HCl. Antisera to apoHDL$_3$ is in the center well and peaks 1-5 are in the outer wells.

peak 5 showed three bands on PAGE. In order to determine the immunochemical nature of these three bands, they were sliced horizontally and embedded in agarose for examination by immunodiffusion (Figure 26). All three bands were immunochemically identical. Peak 5 also showed three bands when examined by analytical isoelectric focusing.

The fractions separated by chromatography of apoA on Bio-gel A-5m were examined by SDS-PAGE in order to determine molecular weights of the apoA polypeptides (Figure 27). Peak 1 and peak 2 showed only two bands. The slowest band was seen at the top of the gel, suggesting aggregation. Peak 3 showed one major and two minor bands. The faster band of peak 3 had a molecular weight of 10,000 daltons, while the slower, major band had a molecular weight of 27,000 daltons. Peak 4 had more of the faster migrating band than peak 3. Peak 5 showed only the fast migrating band. The major band in peak 5, when sliced horizontally and embedded in agarose, reacted with anti-apoA to give the characteristic line for A-II. The major band in peak 3 corresponded to A-I.

The amino acid compositions of the five peaks from Bio-gel A-5m are shown in Table 29. Peak 3 consisted mainly of A-I with a small amount of A-II and peak 5 contained only A-II. No half cystine was found in any of the fractions after performic acid oxidation. There were differences in the amino acid compositions between the five peaks. Peak 3 and peak 5, which correspond to A-I and A-II, are compared in Table 30. The A-I polypeptide contained more lysine and arginine than A-II, hence A-I was more basic than A-II.

Peak 5 was injected into rabbits to obtain a monospecific antiserum to A-II. With this antiserum, it was possible to show the non-

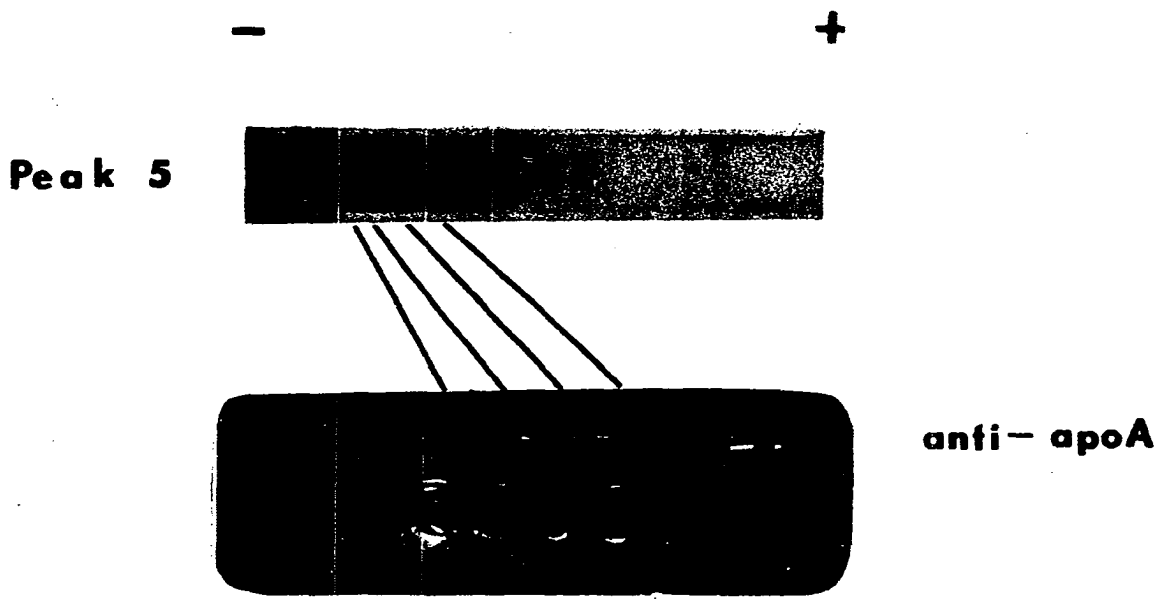

Figure 26. Immunodiffusion of horizontally sliced bands from PAGE of peak 5. Gel slices were embedded in 1 per cent agarose.

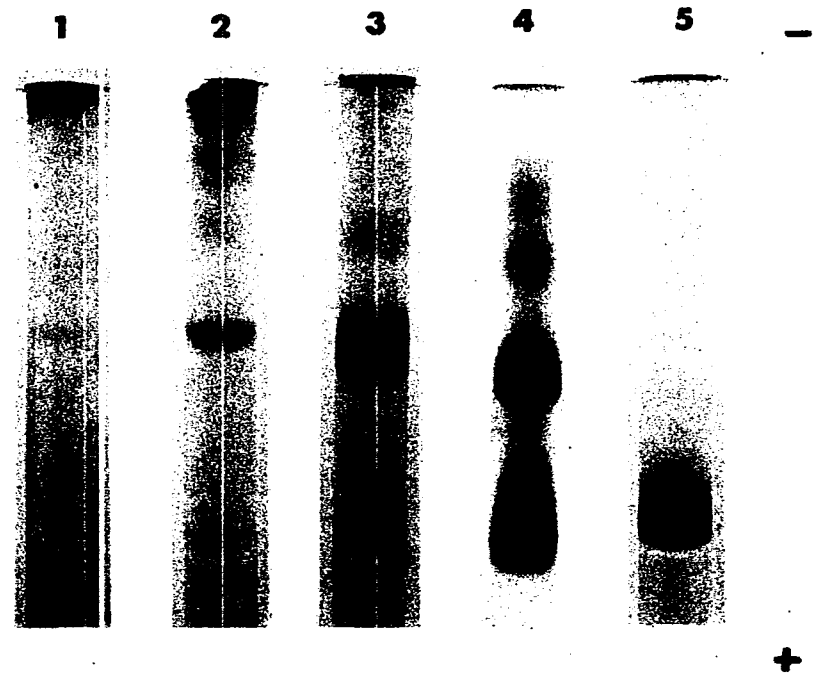

Figure 27. SDS-PAGE of peaks from chromatography of apoHDL$_3$ on Bio-gel A-5m.

TABLE 29

AMINO ACID COMPOSITION OF PEAKS OBTAINED FROM CHROMATOGRAPHY OF ApoHDL$_3$ ON BIO-GEL A-5m[a]

	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5
Lysine	45.37	58.22	60.82	50.42	42.80
Histidine	5.44	3.45	3.60	4.53	3.25
Arginine	32.65	56.35	47.79	53.89	25.51
Aspartic acid	43.09	41.13	46.77	50.23	45.33
Threonine	24.97	27.71	27.66	32.83	35.74
Serine	20.01	16.00	16.41	16.74	21.06
Glutamic acid	100.00	100.00	100.00	100.00	100.00
Proline	22.13	25.35	20.13	25.73	28.72
Glycine	12.65	16.46	12.94	15.60	26.64
Alanine	39.67	42.34	44.44	48.54	44.69
Half cystine[b]	0	0	0	0	0
Valine	22.07	23.52	24.82	26.03	20.15
Methionine	3.69	3.62	4.63	4.53	3.06
Isoleucine	11.15	10.17	11.79	10.03	12.09
Leucine	54.18	58.20	64.72	65.67	59.14
Tyrosine	11.61	13.36	12.70	12.78	10.16
Pheynlalanine	11.73	11.11	9.98	11.40	6.81

[a]Values are relative to glutamic acid equal to 100.

[b]Determined as cysteic acid after performic acid oxidation.

TABLE 30

AMINO ACID COMPOSITION OF THE POLYPEPTIDES OF ApoA[a]

	A-I[b]	A-II
Lysine	60.82	42.80
Histidine	3.60	3.25
Arginine	47.79	25.51
Aspartic acid	46.77	44.33
Threonine	27.66	35.74
Serine	16.41	21.06
Glutamic acid	100.00	100.00
Proline	20.13	28.72
Glycine	12.94	26.64
Alanine	44.44	44.69
Half cystine	0	0
Valine	24.82	20.15
Methionine	4.63	3.06
Isoleucine	11.79	12.09
Leucine	64.72	59.14
Tyrosine	12.70	10.16
Phenylalanine	9.98	6.81

[a]Values relative to glutamic acid equal to 100.

[b]Contains a small amount of immunochemically-detectable A-II polypeptide.

identity of A-I and A-II (Figure 28). So far, all attempts to prepare an immunochemically homogeneous A-I polypeptide have not been successful.

Table 31 shows the results of end group analysis of separated apoA polypeptides. Leucine was found to be the C-terminal amino acid and aspartic acid the N-terminal amino acid of A-I polypeptide. Alanine was found as the C-terminal and isoleucine the N-terminal amino acids of A-II polypeptide. C-terminal was determined by carboxypeptidase digestion and confirmed by hydrazinolysis. The N-terminal was determined by dansylation and dinitrophenylation. The DNP amino acids were hydrolyzed and the free amino acids identified on the amino acid analyzer.

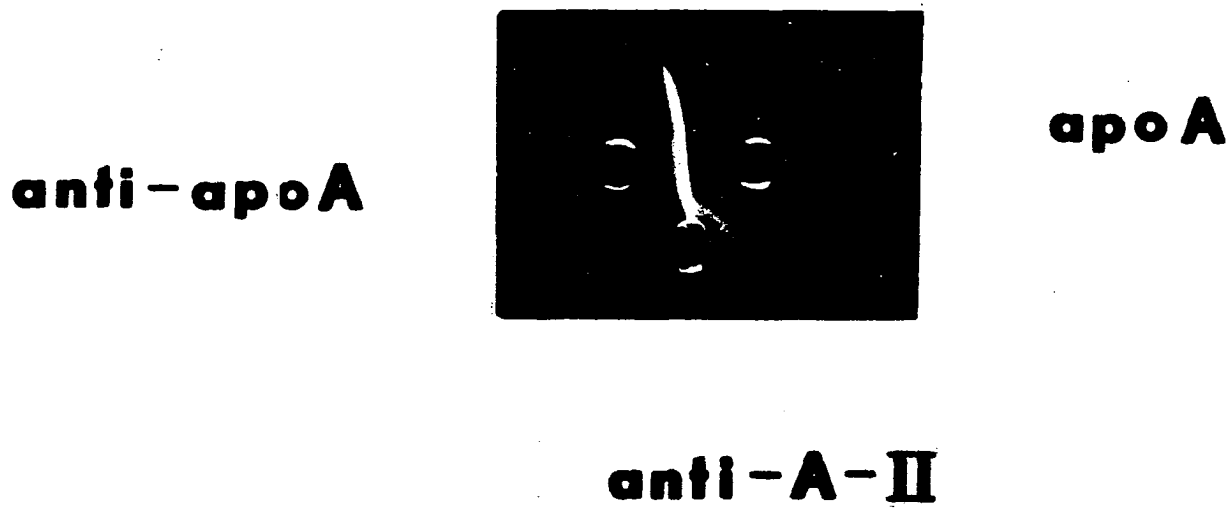

Figure 28. Immunodiffusion of apoA against monospecific antisera to A-II.

TABLE 31

CARBOXYL AND AMINO TERMINAL AMINO ACIDS OF THE NON-IDENTICAL POLYPEPTIDES OF ApoA

	C-terminal	N-terminal
ApoA	Leu, Ala	Asp, Ileu
A-I	Leu	Asp
A-II	Ala	Ileu

CHAPTER V

DISCUSSION

Characterization of Lipoprotein Density Classes

Turkey serum lipoprotein density classes were heterogeneous with respect to the distribution and composition of their lipid and protein moieties. The chemical composition was in close agreement with the composition of chicken serum lipoprotein density classes (32, 33, 4, 36). The finding that the HDL were the major lipoprotein density class was also established in chickens, goose, pigeon, and quail (32), in chickens (34, 36) and in several mammals such as horse, sheep, pig, and dog (164). Mills and Taylaur (32) found large amounts of VLDL (S_f 100-400) in turkey serum. In contrast, we found very low concentrations of VLDL in serum from male turkeys. It is not known whether the high concentration of VLDL found by English investigators was due to an unusually rich triglyceride diet or to the fact that serum samples were drawn from laying turkeys. The discrepancy between our data and those of Mills and Taylaur points out the danger of making conclusions about the entire population of a species on the basis of a single serum sample.

Diglycerides were found to compose a fairly large proportion of the VLDL. The diglycerides might play a role in egg formation since they were found as part of avian egg shells and associated membranes (165,

166). Diglycerides in lipoproteins may also represent an intermediate in the evolution of triglycerides since diglycerides are the major glycerides found in lower invertebrates (167-169).

Lecithin was found as the major phospholipid in turkey serum lipoproteins. Christie and Moore (170) found lecithin as the major phospholipid in chicken plasma. Although inositol was detected in small amounts in chicken plasma, it was not detected in our samples. Phosphatides from turkey serum have not been studied previously, so no direct comparison could be made.

No linoleic acid was found in serum triglycerides of the lipoprotein density classes. Christie and Moore (170) found 11 per cent of the fatty acids of serum triglycerides of chicken to be linoleic acid. Similar to our findings, the major fatty acids of chicken lipids were palmitic and oleic acids. It should be emphasized, however, that absence or presence of a fatty acid in animal lipids could be related to the fatty acid composition of lipids in the diet.

Little linoleic acid was found in turkey serum cholesterol esters. Linoleic and palmitic acids were the major fatty acids found in chicken serum cholesterol esters (171). Linoleic acid was the major fatty acid found in pigeon (172) and human (173) serum cholesterol esters. The lack of linoleic acid in cholesterol esters of turkey plasma may be due to a different fatty acid specificity of the turkey cholesterol esterifying system.

The electrophoretic pattern of male, turkey serum lipoprotein density classes resembled that of chicken serum lipoprotein density classes (55) except that turkey serum VLDL had a faster mobility than

chicken serum VLDL. Hillyard et al. (55) used a pool of serum from both laying and non-laying birds. The slower mobility of chicken serum VLDL was probably due to the increased triglyceride content. The electrophoretic pattern of turkey serum was similar to that of other birds (174).

Turkey serum lipoprotein density classes were found to be antigenically heterogeneous, but this was not peculiar to turkeys. Antigenic heterogeneity of human serum lipoprotein density classes has been well documented (67, 76, 175-181). Also, rat serum lipoprotein density classes were shown to be heterogeneous (182-186). Hillyard et al. found that the chicken serum LDL contained small amounts of HDL (55).

In the past, the observation of LP-A in LDL was considered as a contamination of LDL with HDL. Kostner (187) showed that an LP-A isolated from the LDL density class had a hydrated density of a low-density lipoprotein. Hillyard et al. (55) considered the trace amounts of LP-A in the LDL of chicken serum to be a contamination of LDL with HDL. Our data showed that the LP-A in LDL had the same hydrated density as the LP-B in LDL. The LP-A was not just contamination of LDL, but had a hydrated density which was characteristic of LDL.

HDL_3 was the only density class that was antigenically homogeneous. VLDL contained only trace amounts of LP-A which were detected after total delipidization. The use of immunodiffusion and immunoelectrophoresis clearly indicated that VLDL, LDL, and HDL_2 contained both LP-A and LP-B.

The amino acid composition of turkey serum lipoprotein density classes compared very closely with those determined for chicken serum lipoprotein density classes (55). Determination of the amino acid com-

position of the density classes yielded little information about the protein moieties of lipoprotein families since each density class, except HDL_3, represented a mixture of LP-A and LP-B.

Alanine, leucine, and tyrosine were found as the C-terminal amino acids of turkey VLDL, LDL, and HDL_2. Alanine and leucine were the C-terminal amino acids of HDL_3. Since HDL_3 contained only LP-A, the C-terminals for LP-A were alanine and leucine. It has been shown by immunochemistry that VLDL, LDL, and HDL_2 contained LP-A; therefore the alanine and leucine found in these density classes was derived from LP-A. By deduction, tyrosine was the C-terminal of LP-B. By similar reasoning, the N-terminal of LP-A was aspartic and isoleucine and the N-terminal of LP-B was lysine.

Turkey serum albumin was characterized by aspartic acid as the only N-terminal and alanine as the only C-terminal amino acid. Peters et al. (188) found aspartic acid as the N-terminal of chicken, duck and turkey serum albumin. They also found alanine as the C-terminal of chicken and duck serum albumin, but found valine as the C-terminal of turkey serum albumin. The detection of valine as the C-terminal amino acid of turkey serum albumin is in disagreement with our data. Since the techniques used by Peters et al. were not as refined as those available at the present time, they may have mistaken valine for alanine when these two amino acids were run on paper chromatography.

Characterization of Lipoprotein Families

Chromatography of density classes on Con-A-Sepharose resulted in a sharp resolution of lipoprotein families A and B. LP-B, but not LP-A, were retained by binding to the Con-A. Affinity chromatography

allowed the separation of lipoprotein families which could not be resolved by separation techniques based on hydrated density.

Purified LP-B and LP-A, when examined by immunodiffusion with antibodies to whole serum, gave a reaction of non-identity. Immunochemical evidence for the non-identity of protein moieties was further substantiated by the difference in amino acid composition, carbohydrate composition and C- and N-terminal amino acids.

The chemical composition of the separated lipoprotein families was different from that of isolated density classes. The relatively higher protein content of purified lipoprotein families suggested a selective loss of neutral lipid during gel filtration. The per cent content of phospholipid increased slightly. The lipoprotein families isolated from the same density class had similar chemical compositions. As was stated before, the LP-A and LP-B isolated from LDL had similar hydrated densities. In order to have similar hydrated densities, LP-A and LP-B should have similar chemical compositions.

Separation of lipoprotein families allowed a comparison of the distribution of the apolipoproteins across the density spectrum. Most of the LP-A were found in HDL_3 and most of the LP-B in LDL. HDL_2 contained both LP-A and LP-B families. The LP-B present in HDL_2 was not considered as a contamination, but as a lipoprotein species with a hydrated density characteristic of that density range. Lipoprotein families A and B were immunochemically and chemically different from one another.

Characterization of Apolipoproteins

Characterization of ApoB

ApoB represented a minor apolipoprotein of turkey serum lipoprotein system. Because of the small amount of apoB, fewer studies were performed than with apoA. ApoB becomes very unstable when separated from other serum proteins and readily precipitates from aqueous solution. ApoB can be solubilized in strong dissociating agents such as urea and guanidine-HCl. Because of the insolubility of apoB in aqueous buffers, the N-terminal and C-terminal amino acids were very difficult to determine. At the present time, the available evidence indicates lysine to be the N-terminal amino acid, but further studies with larger quantities of protein need to be done. Results of hydrazinolysis indicated tyrosine as the C-terminal amino acid. Hillyard et al. (55) found lysine as the N-terminal and tyrosine as the C-terminal of apoB in the chicken. If turkey apoB is similar to chicken apoB, then the tentatively identified C- and N-terminals are correct.

The LP-B from turkeys did not cross-react with antiserum to human LP-B. Antisera to human LP-B did not react with any of the serum lipoprotein density classes of turkey. Walton and Darke (189) observed that human anti-LP-B did not react with chicken serum. Our results showed that LP-B isolated from turkey serum was chemically and immunochemically different from human LP-B.

Although turkey apoB is chemically different from human apoB, it seems to be more soluble than human apoB. Since both apolipoproteins may have a similar function, further studies on the quaternary structure of turkey apoB may help in resolving the structural and functional char-

acteristics of human apoB.

After separation of the lipoprotein families from LDL, it was clear that apoB was responsible for the presence of half cystine and glucosamine observed in LDL and HDL. The half cystine observed in the HDL from chicken serum (55) was probably due to the presence of apoB. The apoB preparations isolated from VLDL, LDL, and HDL_2 were identical by immunochemical analysis and also showed very similar amino acid compositions.

Characterization of ApoA

ApoA was prepared by total delipidization of LP-A or HDL_3. ApoA from LDL, HDL_2, and HDL_3 showed similar amino acid compositions. Cysteic acid was not found after performic acid oxidation and no carbohydrates were detected. ApoA was characterized by low contents of histidine and methionine.

Aspartic acid and isoleucine were found as the N-terminal amino acids of apoA. Alanine and leucine were found as the C-terminal amino acids. The chemical evidence for the presence of two polypeptides in apoA was supported by the occurrence of two precipitin lines when apoA was reacted against anti-apoA. Hillyard *et al.* (55) found alanine and leucine as the C-terminal amino acids of chicken apoHDL. Aspartic acid was the only N-terminal found in chicken apoHDL.

Since we had evidence for more than one polypeptide in turkey apoA, attempts were made to separate these non-identical polypeptides and characterize them according to their chemical and immunochemical properties. ApoA was bound to ion-exchange columns to such an extent that the separation of polypeptides could not be achieved. Hydroxylapa-

tite columns offered some separation, but most of the material was retained.

When apoA was chromatographed on Sephadex G-100, each of the three eluted fractions gave two precipitin lines with antibodies to apoA. Since gel permeation chromatography separates molecules according to size, it seems that each fraction consisted of a different ratio of A-I and A-II polypeptides.

The combination of PAGE and immunodiffusion proved to be a powerful technique for identifying the protein stained bands on PAGE (Figure 23). By slicing the bands of each of the three fractions from the Sephadex column and embedding them in agarose, it was possible to show that each band gave two precipitin lines when reacted with anti-apoA. From these experiments, it was clear that a protein stained band on PAGE does not necessarily correspond to a single antigen. Gel permeation chromatography in urea suggested that A-II was smaller than A-I, and that both polypeptides were strongly associated with one another.

Isolation of an immunochemically homogeneous preparation of A-II was achieved by chromatography of apoA on Bio-gel A-5m in 6 M guanidine-HCl. Five peaks were obtained when apoA was chromatographed on Bio-gel A-5m. On PAGE, all five peaks showed multiple bands. Immunochemically, peaks 1-4 were shown to contain both A-I and A-II polypeptides. Peak 5, however, contained only the A-II polypeptide. Although peak 5 showed only one precipitin line on immunodiffusion, it produced three bands on PAGE. These three bands, when sliced horizontally and embedded in agarose, showed a line of identity with antibodies to apoA. The above experiment showed how misleading the electrophoretic patterns could be. How-

ever, electrophoretic patterns of all five peaks run in the presence of SDS gave a more correct picture of the antigenic components. Peak 5 showed only one protein stained band which had a molecular weight of 10,000. The formation of three bands on analytical PAGE in the presence of urea and a single band on SDS-PAGE suggested that A-II may be heterogeneous with respect to charge distribution.

Peak 3 contained mainly A-I polypeptide. The molecular weight of A-I was 27,000. Rechromatography of peak 3 did not totally eliminate the presence of A-II. It appears that A-I and A-II are so strongly bound to one another that even powerful dissociating agents could not effect a total dissociation. The results of SDS-PAGE indicate that preparative electrophoresis may be a useful technique to obtain pure A-I. Preparative SDS-PAGE will be done in the near future.

There were some differences in the amino acid composition of enriched A-I and purified A-II. A-I contained relatively more basic and less acidic amino acids than A-II. Neither A-I nor A-II contained half cystine.

With the availability of a monospecific antiserum to A-II, it was possible to show the non-identity reaction of A-I and A-II. The antibodies to A-II precipitated both A-I and A-II from intact HDL, showing that A-I and A-II are associated in the LP-A molecule.

No apolipoprotein similar to apolipoprotein C of human plasma VLDL was detected in turkey serum. Since VLDL are such a small proportion of male turkey serum lipoproteins, the apoC of turkey serum may have gone undetected. In order to get larger amounts of VLDL, experiments have been planned to administer estrogens to male turkeys. As described

earlier, estrogen treatment caused a large increase in serum VLDL. If apoC is present in turkey serum, the estrogen treatment should give adequate amounts of this apolipoprotein for characterization.

CHAPTER VI

SUMMARY

Lipoprotein density classes isolated by preparative ultracentrifugation were found to be heterogeneous with respect to their apolipoprotein moieties. VLDL, LDL, and HDL_2 contained both apoA and apoB. Only HDL_3 was antigenically homogeneous containing only apoA.

HDL were the major lipoprotein density class found in male turkey serum. The HDL were responsible for transport of most of the phospholipids, cholesterol, cholesterol esters, and glycerides. The LDL contained a relatively large proportion of the cholesterol and VLDL contained a large proportion of glycerides.

Chromatography of lipoprotein density classes on Con-A-Sepharose allowed the separation of lipoprotein family A and lipoprotein family B. The lipoprotein family A was characterized by apolipoprotein A, and lipoprotein family B was characterized by apolipoprotein B. LP-B was found mainly in LDL and LP-A was found mainly in HDL.

The apolipoproteins of the lipoprotein families were characterized according to their chemical and immunochemical properties. ApoB was characterized by N-terminal lysine and C-terminal tyrosine. ApoA contained two non-identical polypeptides, A-I and A-II. A-I was characterized by aspartic acid as the N-terminal and leucine as the C-terminal

amino acid. A-II was characterized by isoleucine as the N-terminal and alanine as the C-terminal amino acid.

No apolipoprotein similar to apoC of human serum was detected in the turkey serum lipoprotein system.

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APPENDIX

NOMENCLATURE AND ABBREVIATIONS OF LIPOPROTEINS

Operational Nomenclature

VLDL — Very low-density lipoproteins, lipoproteins of d < 1.006 g/ml and S_f > 20; pre-β-LP, lipoproteins displaying the mobility of α_2-globulins.

LDL — Low-density lipoproteins, lipoproteins of d 1.006-1.063 g/ml and S_f 0-20; β-LP, lipoproteins displaying the electrophoretic mobility of β_1-globulins.

HDL — High-density lipoproteins, lipoproteins of d 1.063-1.21 g/ml; α-LP, lipoproteins displaying the electrophoretic mobility of α_1-globulins.

VHDL — Very high-density lipoproteins; lipoproteins of d > 1.21 g/ml.

HDL_2 — Subfraction of high-density lipoproteins of d 1.063-1.125 g/ml.

HDL_3 — Subfraction of high-density lipoproteins of d 1.125-1.21 g/ml.

Chemical Nomenclature

LP-A — Lipoproteins containing apolipoprotein A.

LP-B — Lipoproteins containing apolipoprotein B.

LP-C — Lipoproteins containing apolipoprotein C.

Abbreviations of Lipoproteins

α-LP — Alpha-lipoproteins.

ApoA — Apolipoprotein A.

ApoB — Apolipoprotein B.

ApoVLDL — Totally delipidized very low-density lipoproteins.

ApoLDL	Totally delipidized low-density lipoproteins.
ApoHDL	Totally delipidized high-density lipoproteins.
$ApoHDL_2$	Totally delipidized subfraction of high-density lipoproteins.
$ApoHDL_3$	Totally delipidized subfraction of high-density lipoproteins.
β-LP	Beta-lipoproteins
LDF	Low-density fraction of egg yolk.
HDF	High-density fraction of egg yolk.

Other Abbreviations

A-LP-A, A-A-I, etc.	A- = antisera with specificity for the designated antigen
Alb.	Albumin
Con-A	Concanavalin-A
CPA	Carboxypeptidase A
CPB	Carboxypeptidase B
C-terminal	Carboxyl-terminal
d	Isolation density of lipoproteins
DEAE	Diethylaminoethyl
DES	Diethylstilbestrol
DNP	2, 4 dinitroflourobenzene
EDTA	(Ethylenedinitrilo) tetra acetic acid
N.D.	Not determined
N-terminal	Amino-terminal
PAGE	Polyacrylamide gel electrophoresis
rpm	Revolutions per minute
S	Observed sedimentation coefficient
S_f	Flotation coefficient

SDS	Sodium dodecyl sulfate
TCA	Trichloroacetic acid