Studies of the Composition and Structure of Plasma Lipoproteins. Isolation, Composition, and Immunochemical Characterization of Low Density Lipoprotein Subfractions of Human Plasma*

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ABSTRACT: Immunochemical studies showed that six subfractions isolated by differential ultracentrifugation of plasma low density lipoproteins (LDL subfractions) contain one major (LP-B) and two minor (LP-A and LP-C) lipoproteins. The subfractions from plasma of one group of individuals who were predominantly women had as a minor component only LP-A with little or no LP-C. A second group consisting predominantly of men contained both LP-A and LP-C as minor components in most of the subfractions. Another set of subfractions characterized by the presence of only LP-B (LP-B subfractions) was obtained by immunoprecipitation of LP-A from LDL subfractions isolated from the plasma of the first group of donors. All LP-B subfractions showed a very similar and characteristic phospholipid:protein ratio near unity and displayed also similar values for the neutral lipid:phospholipid-protein ratios. Cholesterol ester content increased and triglyceride content decreased with increasing density up to d 1.040 g/cm^3; the content of unesterified cholesterol varied very little within the entire density range. On the other hand, chemical composition of immunochemically heterogeneous LDL subfractions deviated considerably from the pattern characteristic of LP-B subfractions and varied consistently among individual donors depending most probably on the relative content of LP-A and LP-C families. The phospholipid:protein ratio of LDL subfractions decreased with increasing density. Unesterified cholesterol was distributed randomly throughout the entire density range. Results of the immunochemical studies and amino acid analyses suggested that the protein moiety of LP-B family may consist of two polypeptides.

Detailed distribution analyses (Oncley, 1963; Ewing *et al.*, 1965) have shown that LDL[1] is a heterogeneous system in respect of both particle size and hydrated density. It has been suggested from a limited number of studies with LDL subfractions, that the differences in the hydrated densities reflect, and could be explained by, subtle changes in the chemical composition of a continuous population of lipoprotein molecules. An increasing trend in hydrated densities has been correlated either with an increased content of protein (Oncley *et al.*, 1957; Shore and Shore, 1962; Dearborn and Wetlaufer, 1969) or an increased content of phospholipid (Hayashi as cited in a review by Nichols, 1967). Since no evidence was presented in these studies for chemical and immunological homogeneity of the protein moiety of LDL subfractions, the possibility remains that changes in chemical composition of at least some subfractions resulted from the presence of small amounts of lipoprotein families other than the principal LDL family characterized by the apolipoprotein B. Studies on the immunological properties of LDL have resulted in conflicting findings indicating either an antigenic heterogeneity (Gitlin, 1953; Aladjem and Campbell, 1957; Briner *et al.*, 1959; Lawrence and Shean, 1962) or homogeneity (Korngold and Lipari, 1955; Levine *et al.*, 1955; Scanu *et al.*, 1958; Tracy *et al.*, 1961; Blumberg *et al.*, 1962; Walton and Darke, 1964; Störiko and Fisher, 1965) between or within various LDL subfractions. Although these discrepancies could be partly due to differences in methodology, antibody titers, source of lipoproteins, or presence of other contaminating serum proteins, results of the N-terminal amino acid analyses (Rodbell, 1958; Shore and Shore, 1962; Bobbitt and Levy, 1965) and recent immunochemical and chemical studies (Lee *et al.*, 1966; Alaupovic, 1968) on the distribution of apolipoproteins seem to support the view that lipoproteins in a density range of 1.006–1.063 g/cm^3 may be heterogeneous not only with respect to some physical properties but also with respect to the protein moieties.

The chemical and physical heterogeneity of LDL reflects

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[1] Abbreviations based on the operational classification of lipoproteins: VLDL, very low density lipoproteins (d < 1.006 g/cm^3; S_f = >20); LDL, low density lipoproteins (d 1.006–1.063 g/cm^3; S_f = 0–20); HDL$_3$, high density lipoproteins (d 1.125–1.21 g/cm^3); α_1-LP, α-lipoproteins, lipoproteins with an electrophoretic mobility of α_1-globulins; β-LP, β-lipoproteins, lipoproteins with an electrophoretic mobility of β-globulins. Abbreviations based on the chemical classification of lipoproteins are: LP-A, lipoproteins characterized by the presence of apolipoprotein A; LP-B, lipoproteins characterized by the presence of apolipoprotein B; LP-C, lipoproteins characterized by the presence of apolipoprotein C; LP-X, lipoproteins occurring in obstructive jaundice and characterized by the presence of a protein moiety consisting of 40% albumin and 60% apolipoprotein C; ApoA, apolipoprotein A, protein moiety of LP-A; ApoB, apolipoprotein B, protein moiety of LP-B; ApoC, apolipoprotein C, protein moiety of LP-C and LP-X. At the present time the apolipoproteins could be characterized by terminal amino acids and antigenic determinants as follows: ApoA, aspartic acid as N-terminal and threonine and glutamine as C-terminal amino acids, and at least two antigenic determinants; ApoB, glutamic acid as N-terminal and serine as C-terminal amino acid, and a single antigenic determinant as LDL; ApoC, threonine and serine as N-terminal and valine and alanine as C-terminal amino acids, and at least three antigenic determinants.

a unique system of macromolecules highly adaptable to and influenced by continuous changes in the concentration of transportable lipids. To gain further insight into the nature of protein and lipid components in such a flexible macromolecular continuum we studied the chemical, immunochemical, and physical properties of a number of LDL subfractions rather than those of the entire class. To test the assumption that varying contents of apolipoproteins influence the chemical composition of low density lipoproteins, we prepared two types of LDL subfractions. One group of six subfractions (LDL subfractions) was isolated by differential ultracentrifugation. The other group of six subfractions (LP-B subfractions) was also isolated by differential ultracentrifugation and in addition submitted to a purification procedure by immunoprecipitation.

This first report sets forth the procedures for the isolation and preparation of both types of LDL subfractions and describes results of a comparative study on their immunochemical properties and chemical composition.

Experimental Section

Blood samples from healthy young men and women who had fasted overnight were collected into plastic bags (Abbott) containing sodium–heparin in saline with sodium citrate as buffer. The blood was allowed to cool for 1–2 hr at 5°, and the plasma was recovered by low-speed centrifugation. Only plasma samples characterized by normal levels of lipids (Furman *et al.*, 1961) were utilized immediately for the isolation of LDL subfractions.

Isolation and Fractionation of LDL. The LDL subfractions were isolated according to a modified procedure of Lindgren *et al.* (1951). Centrifugations were carried out in the No. 50 rotor of the Spinco Model L or L-2 ultracentrifuges at 5°. Eight volumes of fresh plasma was layered under three volumes of NaCl solution containing 0.1% EDTA (d 1.006 g/cm^3) in Beckman polyallomer tubes and centrifuged at 105,000g for 22 hr. The top layer consisting of VLDL (d < 1.006 g/cm^3) was removed by a tube-slicing technique and the bottom portion was utilized for the subsequent isolation of LDL subfractions. A sample of the bottom portion was dialyzed against a NaCl solution of known density (d 1.006 g/cm^3) at 5° until equilibrium was reached. The density difference at equilibrium between the plasma sample and dialysate was considered as the density contributed to the solution by plasma proteins and lipoproteins. These density differences, Δd, ranging between 0.007 and 0.013 g per cm^3 were taken into consideration each time during the actual adjustment of lipoprotein solutions to true solvent densities. Solvent densities of the bottom fractions were increased successively to 1.009, 1.019, 1.030, 1.040, 1.053, and 1.063 g per cm^3 by adding solid NaBr; if, for example, the value for Δd were 0.010 g/cm^3 for all subfractions, the corresponding solution densities would be 1.019, 1.029, 1.040, 1.050, 1.063 and 1.073 g per cm^3. Each subfraction separated by flotation at 105,000g for 22 hr at 5° was removed from the top layer (upper 1 cm) by a tube-slicing technique. The six subfractions were designated according to the *solvent density* ranges in the following order of increased densities: LDL-I (1.006–1.009 g/cm^3), LDL-II (1.009–1.019 g/cm^3), LDL-III (1.019–1.030 g/cm^3), LDL-IV (1.030–1.040 g/cm^3), LDL-V (1.040–1.053 g/cm^3), and LDL-VI (1.053–1.063 g/cm^3).

A β-LP preparation (fraction T) was also obtained from a VLDL-free plasma sample by precipitation with heparin according to the procedure of Burstein and Samaille (1960).

Purification of LDL Subfractions. All subfractions were washed once or twice by recentrifugation at their corresponding upper densities at 105,000g for 22 hr to remove the adsorbed contaminating proteins. The washing procedure was monitored by immunodiffusion tests which finally demonstrated that the subfractions were free of albumin and γ-globulin. These subfractions represented the *LDL subfractions*. LP-A was removed by immunoprecipitation with rabbit antisera to human LP-A (α-LP). (To avoid contamination with rabbit lipoproteins the antisera, adjusted to d 1.25 g/cm^3, were centrifuged at 165,000g for 44 hr, and the floating lipoproteins were removed.) LP-A was precipitated by adding to each subfraction equivalent amounts of the lipoprotein-free antisera determined by a serial microdilution method. The reaction mixtures were allowed to stand for 24 hr at 5° and the immunoprecipitates were sedimented by low-speed centrifugation. The density of LP-A-free supernatants tested immunochemically with rabbit antibodies to LP-A was adjusted to the upper solvent density of each corresponding subfraction and centrifuged at 165,000g for 44 hr to remove the rabbit serum proteins. The purified lipoprotein subfractions designated now as *LP-B subfractions* were removed from the top layer; the LP-B subfractions showed no reaction with goat antibodies to rabbit whole serum, total globulins, and γ-globulin.

Determination of the Flotation Coefficient. The analytical ultracentrifugation of LDL subfractions was carried out at various rotor speeds in a Spinco Model E ultracentrifuge equipped with a schlieren optical system, RTIC unit, and an electronic speed control unit. The LDL subfractions were centrifuged in double-sector cells at solvent density 1.063 g/cm^3 at 26°. The flotation coefficients, $S_i°$, were calculated as outlined by Schachman (1957).

Electrophoresis was performed in 1% agarose gels prepared in the presence of 0.33% bovine albumin. Lipoprotein samples were applied as mixtures with equal volumes of agarose gel and the electrophoresis was carried out at 7 V/cm for 45 min employing Veronal buffer (pH 8.6), ionic strength 0.1.

Cellulose acetate electrophoresis was carried out in Beckman Microzone Cell, Model R-101, at constant voltage (250 V) utilizing Veronal buffer (pH 8.6), ionic strength 0.075, for 45 min at room temperature. The cellulose acetate strips were stained with ponceau red (Allied Chemicals, New York, N. Y.).

Immunological Methods. The immunochemical characteristics of LDL and LP-B subfractions were studied by double diffusion and immunoelectrophoresis as described previously (Alaupovic *et al.*, 1966).

Rabbit sera containing antibodies to human whole serum, α_1-LP, β-LP, albumin, γ-globulin, and to fibrinogen (Behringwerke Reagents) were purchased from Hoechst Pharmaceuticals, Inc., Cincinnati, Ohio. Goat sera containing antibodies to rabbit whole serum, total globulins, and γ-globulin were obtained from Mann Research Laboratories, New York, N. Y. A rabbit antiserum to an abnormal low density lipoprotein, LP-X, isolated from patients with obstructive jaundice was prepared as described previously (Seidel *et al.*, 1969). Since the nonalbumin protein moiety obtained by partially delipidized LP-X is immunochemically identical

TABLE I: Characterization of Rabbit Antisera.

Antisera	Reactivity[a]					
	LP-A	LP-B	LP-C[b]	Albumin	γ-Globulin	Fibrinogen
β-LP (Behringwerke)	−	++	−	−	−	−
LDL (d 1.019–1.053 g/cm^3)	−	++	−	−	−	−
α_1-LP (Behringwerke)	++	−	−	−	−	−
HDL_3 (d 1.150–1.21 g/cm^3)	++	−	●	−	−	−
LP-X	−	−	+++	+	−	−
Albumin (Behringwerke)	−	−	−	+	−	−
Fibrinogen (Behringwerke)	−	−	−	−	−	+
γ-Globulin (Behringwerke)	−	−	−	−	+	−
Whole serum (Behringwerke)	+±	++	−	+	+	+

[a] Each + sign represents a distinct immunoprecipitin line obtained in 1% agar or agarose double-diffusion experiments. [b] LP-C in its partially delipidized form was obtained according to the procedure described by Alaupovic *et al.* (1969).

with apolipoprotein C (Alaupovic *et al.*, 1969), the antibodies obtained by immunizing rabbits with partially delipidized LP-X are identical with antibodies obtained by immunizing rabbits with partially delipidized LP-C isolated from human plasma or chyle VLDL. Rabbit antisera to LDL (d 1.019–1.053 g/cm^3) and HDL_3 (d 1.150–1.21 g/cm^3) were prepared according to a standardized procedure described previously (Alaupovic *et al.*, 1968). The characterization of all rabbit antisera used for immunochemical study is shown in Table I. The LP-B immunoprecipitin lines represent in order of increasing diffusion rates from antigen well the antigenic determinants B_1 and B_2. The thin LP-A immunoprecipitin line close to the antigen well represents the antigenic determinant A_1 and the heavy immunoprecipitin line the antigenic determinant A_2. In some LDL subfractions prepared from non-

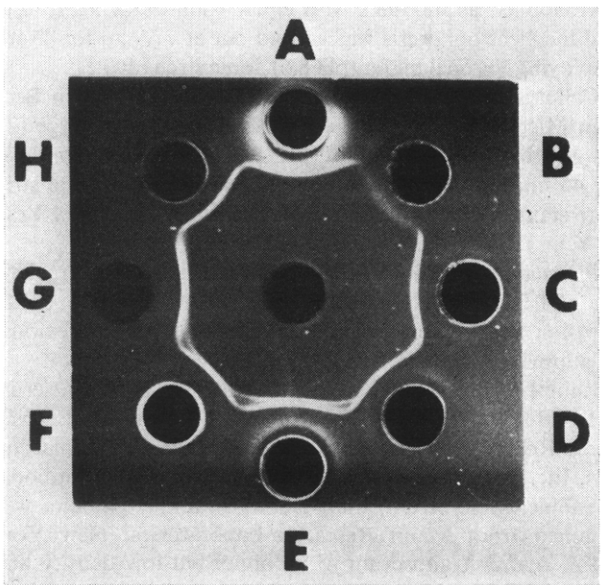

FIGURE 1: Immunodiffusion pattern of LDL subfractions in 1% agar gel. Center well contains antibodies to human β-LI. Outer wells contain VLDL (A), LDL-I through LDL-VI (B–G) and LDL-III (H).

fasting plasma LP-A showed three distinct immunoprecipitin lines indicating the possible presence of a third antigenic determinant. Some batches of Behringwerke anti-α_1-LP sera gave two immunoprecipitin lines with HDL_3, which showed reaction of identity with A_1 and A_2, respectively, but other batches, of lower titer, contained antibodies only to the A_2 determinant. The antibodies to LP-X gave a single precipitin line with albumin, and three characteristic immunoprecipitin lines (C_1, C_2, and C_3 counting from antigen to antibody well) with partially delipidized LP-C isolated from human chyle or plasma VLDL (Alaupovic *et al.*, 1968; McConathy and Alaupovic, 1969).

Total Delipidization of LP-B and LDL Subfractions. The LP-B and LDL subfractions were totally delipidized by extraction with ethanol–diethyl ether (3:1, v/v) followed by washing with diethyl ether at 5°. The final diethyl ether extract tested by thin-layer chromatography contained no neutral lipids and phospholipids.

Amino Acid Analysis. Triplicate aliquots of totally delipidized LP-B and LDL subfractions (3–4 mg) were hydrolyzed with constant-boiling HCl in evacuated, sealed tubes at 110 ± 1° for 24 and 72 hr. The hydrolysates were evaporated to dryness in a rotary evaporator, redissolved in distilled water, and evaporated again to dryness. The residues were then dissolved in 3 ml of 0.2 N sodium citrate buffer (pH 2.2), filtered through a fine frit sintered glass filter, and analyzed on a Beckman Model 120C amino acid analyzer according to an accelerated automatic procedure (Benson and Patterson, 1965) on spherical resins (Beckman Custom Spherical Resins Type PA-28 for the acidic and neutral amino acids and Type PA-35 for the basic amino acids). The analyzer was calibrated with a mixture of known amino acids and, in studies of the performic acid oxidized protein samples, with a mixture of oxidized calibration mixture and cysteic acid. The analyzer was calibrated separately with a glucosamine sample. Half-cystine and methionine were determined as cysteic acid and methionine sulfoxide after oxidizing protein samples with performic acid. The values for cysteic acid were corrected for the 94% recovery found by Moore (1963). Tryptophan was determined spectrophotometrically by the method of Spies and Chambers (1949).

FIGURE 2: Immunoelectrophoresis pattern of LP-B-V subfraction in 1% agarose gel. The trough contains antibodies to β-LP.

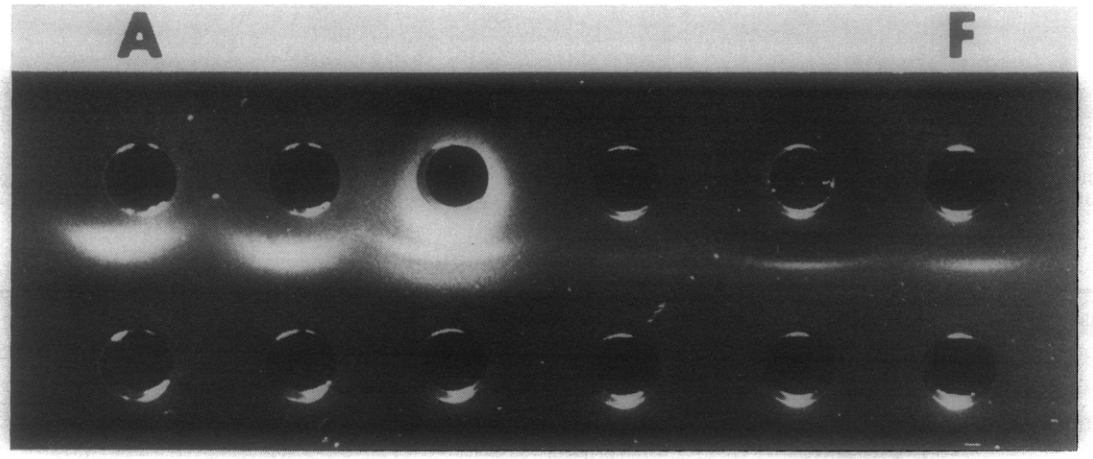

FIGURE 3: Immunodiffusion pattern of LDL subfractions in 1% agarose gel. Top wells contain LDL-I through LDL-VI (A–F); all bottom wells contain antibodies to HDL_3.

Protein and lipid analyses were performed according to the methods described previously (Alaupovic *et al.*, 1966).

Results

The ultracentrifugal pattern of the combined subfractions LDL-I and LDL-II showed a single boundary with S_f° = 18.8. Similarly, the combined subfractions LDL-III through LDL-V displayed a single peak with S_f° = 7.0.

Immunochemical Characterization of LDL and LP-B Subfractions. Immunochemical studies of LDL subfractions showed that individual donors with normal plasma lipid levels could be divided into two groups. Both groups contained LP-B as the dominant and typical lipoprotein family; however, one group of donors (group I) was characterized by the presence of LP-A and very little or no LP-C and the other (group II) by the occurrence of both LP-A and LP-C in most LDL subfractions.

The LP-B subfractions were prepared only from the corresponding LDL subfractions of group I donors by immunoprecipitation of LP-A. All LP-B and LDL subfractions showed single, identical immunoprecipitin lines (B_1) with antibodies to β-LP. Some subfractions gave a second immunoprecipitin line (B_2), clearly developed and resolved after 40 hr (Figure 1). These LP-B subfractions showed also in agarose immunoelectrophoresis two crossing precipitin arcs of the same mobility (Figure 2) indicating a possible nonidentity between antigenic determinants B_1 and B_2. The affinity of both immunoprecipitin lines of LP-B for lipid and protein stains indicated clearly their lipoprotein nature.

Although there was some variation in the distribution of antigenic determinants of LP-A and LP-C in the VLDL and LDL subfractions isolated from individual donors of

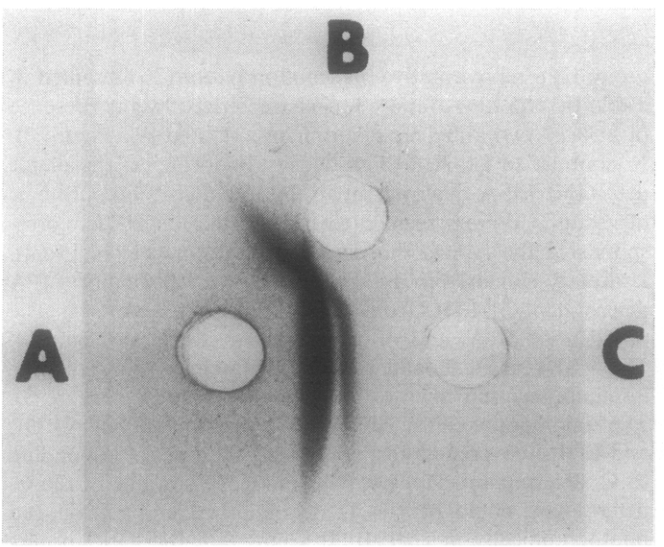

FIGURE 4: Immunodiffusion pattern of LDL-VI and HDL_3 in 1% agarose gel. Antibodies to HDL_3 (A), LDL-VI (B), and HDL_3 (C).

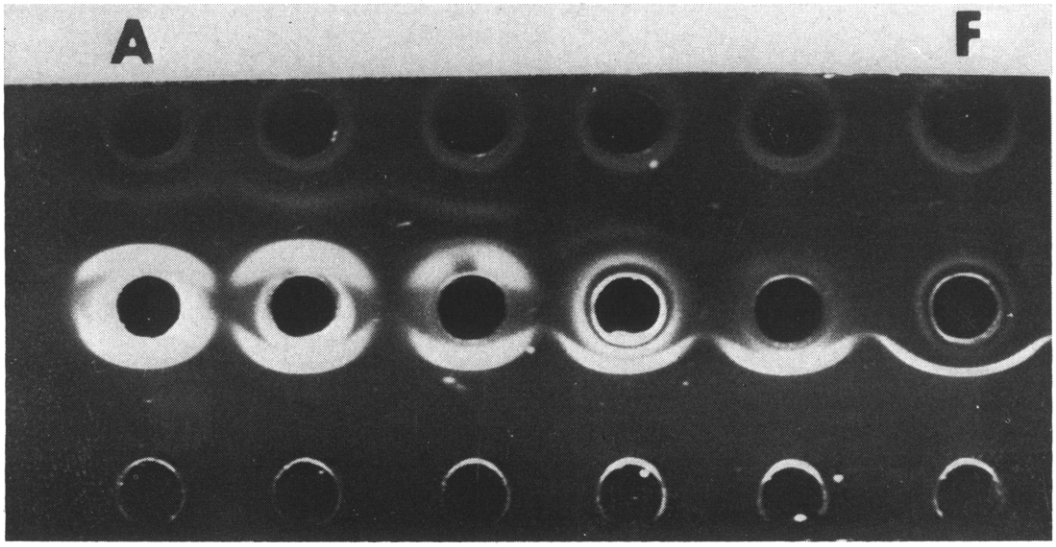

FIGURE 5: Immunodiffusion pattern of LDL subfractions in 1% agar gel. Top wells A–F contain antibodies to LP-X; center wells A–F contain VLDL and LDL-I–LDL-V; all bottom wells contain antibodies to β-LP.

TABLE II: Distribution of Antigenic Determinants in LDL Subfractions.[a]

Subfraction	Density Range (g/cm³)	Antigenic Determinants[b]						
		A_1	A_2	B_1	B_2	C_1	C_2	C_3
VLDL	<1.006	+	−	+		+	±	+
LDL-I	1.006–1.009	+	−	+	+	+	±	+
LDL-II	1.009–1.019	+	−	+	+	+	±	±
LDL-III	1.019–1.030	+	±	+	+	−	±	±
LDL-IV	1.030–1.040	+	−	+	+	−	±	±
LDL-V	1.040–1.053	+	±	+	±	−	±	±
LDL-VI	1.053–1.063	+	−	+	+	−	±	●

[a] Each + sign represents a distinct immunoprecipitin line obtained in 1% agar or agarose double-diffusion experiments. [b] Results in this table represent the distribution of antigenic determinants in LDL subfractions isolated from a male subject who had normal plasma lipid levels.

group II, a representative distribution pattern is presented in Table II. The LP-A family was characterized by the presence of at least two antigenic determinants A_1 and A_2 (Figure 3). In contrast to LP-A in high density lipoproteins, the major detectable LP-A determinant in the low density lipoproteins of group II donors seemed to be A_1 which in HDL_3 corresponds to the typical thin immunoprecipitin line (Figure 4). However, similarly to HDL_3, the A_2 was the major LP-A determinant in LDL from female donors of group I. The nonidentity of A_1 and A_2 is illustrated clearly in Figure 4. The VLDL, LDL-I, and LDL-II subfractions showed three immunoprecipitin lines with antibodies to LP-X (Figure 5). The immunoprecipitin line of C_1 determinant in VLDL and LDL-I covered almost completely the line corresponding to C_2 determinant. However, the characteristic line of the C_2 determinant could be clearly distinguished and reproduced photographically in LDL-II. It seems that the LP-C occurs in the lowest concentration between *d* 1.040 and 1.053 g per cm³. In immunoelectrophoretic pattern of VLDL and LDL subfractions the LP-C appeared to have the same mobility as LP-B. All precipitin lines stained for lipid and protein.

Washed LDL subfractions were immunochemically free of albumin, γ-globulin, fibrinogen, and any other plasma proteins.

Electrophoretic Behavior of LDL Subfractions. The agarose gel electrophoresis of subfractions from both groups of donors showed that VLDL, LDL-I, and LDL-II had higher mobility than LDL-III through LDL-VI.

Cellulose acetate electrophoresis showed that each LP-B subfraction displayed a single band near origin. The VLDL, LDL-I, and LDL-II subfractions of group II donors displayed a second weak band with a mobility slightly higher than that of LP-B.

Chemical Composition of Subfractions. Results of the chemical analyses of LP-B subfractions characterized immunochemically by the absence of LP-A and by the presence of little or no LP-C are presented in Table III. Duplicate or triplicate aliquots of several separate LP-B subfractions isolated from a number of individual subjects were analyzed

for protein and lipid content and the results were expressed as per cent of sum of individually determined mean lipid and protein values. Standard deviations were calculated for all subfractions except LP-B-VI. No attempt was made to determine separately minute amounts of hydrocarbons, or mono- and diglycerides. The free fatty acid content of LP-B subfractions was less than 2.0%. Cholesterol ester content increased and triglyceride content decreased with increasing density. Typical changes in the relative content of these two lipid components were more pronounced in subfractions of lower densities (LP-B I–IV) than in those of higher densities (LP-B IV–VI). The content of unesterified cholesterol varied very little within the entire density range. All LP-B subfractions showed a very similar and characteristic phospholipid: protein ratio near unity. All subfractions also displayed similar values for the neutral lipid:phospholipid-protein ratios.

In contrast to these results, chemical composition of immunochemically heterogeneous LDL subfractions deviated considerably from the pattern characteristic of LP-B subfractions and varied consistently among individual donors (Table IV). Although the cholesterol ester content tended to increase and triglyceride content to decrease with increasing density, there was no continuity in the change of these two lipid components so characteristic of LP-B subfractions. Unesterified cholesterol was distributed randomly throughout the entire density range. In contrast to LP-B subfractions, the protein content of LDL subfractions increased continually with increasing density. Correspondingly, the phospholipid: protein ratio decreased with increasing density of lipoprotein subfractions.

Amino Acid Composition of LDL and LP-B Subfractions. The amino acid composition of totally delipidized LP-B subfractions IV and V are shown in Table V. For comparison, the results of amino acid analyses of totally delipidized LDL preparation (fraction A; d 1.009–1.050 g/cm^3) and a preparation (fraction T) obtained by heparin–$MnCl_2$ precipitation of a VLDL-free plasma sample are also included in the same table. The amino acid composition of LDL and LP-B subfractions was similar but not identical. The statistical evaluation of any possible combination of two subfractions showed that the contents of at least three to six of the amino acids were significantly different. Since the determination of half-cystine, methionine, and tryptophan was done with duplicate rather than triplicate aliquots, the standard deviation for these amino acids was not calculated. A significant difference in the amino acid composition between fractions A and T was observed only for valine, isoleucine, and tyrosine. Both fractions A and T contained immunochemically detectable LP-C. However, there was also a significant difference between LP-B subfractions IV and V in the content of alanine ($p < 0.001$), histidine, serine and isoleucine ($0.001 < p < 0.01$). Comparison of any other two fractions revealed a further increase in the number and extent of differing amino acids.

Glucosamine content of ApoB isolated from five LDL and LP-B subfractions ranged between 1.5 and 3.2%.

TABLE III: Per Cent Composition of LP-B Subfractions.

Subfraction	Density Range (g/cm^3)	Cholesterol Ester[b] (%)	Unesterified Cholesterol (%)	Triglyceride (%)	Phospholipid (%)	Protein (%)	Phospholipid:Protein	Neutral Lipid: Phospholipid-Protein
LP-B-I (5)[a]	1.006–1.009	19.46 ± 1.79	4.95 ± 0.91	27.94 ± 2.03	25.03 ± 2.95	22.42 ± 0.94	1.12	1.10
LP-B-II (3)	1.009–1.019	24.99 ± 2.14	6.83 ± 2.09	17.54 ± 0.35	27.02 ± 2.94	23.59 ± 1.63	1.15	0.98
LP-B-III (3)	1.019–1.030	34.97 ± 1.97	7.55 ± 1.77	8.75 ± 0.14	23.13 ± 0.82	24.00 ± 0.11	0.96	1.09
LP-B-IV (6)	1.030–1.040	41.30 ± 1.48	6.70 ± 0.42	6.35 ± 1.71	21.60 ± 1.86	22.60 ± 1.56	0.96	1.23
LP-B-V (6)	1.040–1.053	40.64 ± 3.49	7.41 ± 0.73	5.60 ± 2.32	21.60 ± 2.09	24.35 ± 2.40	0.89	1.16
LP-B-VI (2)	1.053–1.063	42.12	6.74	4.45	22.19	23.43	0.94	1.17

[a] Figures in parentheses represent the number of preparations from different donors of group I. [b] Values for cholesterol esters were calculated as cholesteryl linoleate (mol wt 649.1).

Discussion

The results of this study indicate that the low density (1.006–1.063 g/cm^3) segment of the normal human plasma

TABLE IV: Per Cent Composition of VLDL and LDL Subfractions.[a]

Fraction	Density Range (g/cm³)	Cholesterol Ester (%)	Unesterified Cholesterol (%)	Triglyceride (%)	Phospholipid (%)	Protein (%)	Phospholipid: Protein	Neutral Lipid: Phospholipid-Protein
VLDL	<1.006	13.5 (9.8–18.5)	5.6 (4.6–6.4)	55.9 (50.8–59.9)	16.4 (14.9–17.9)	8.6 (7.3–9.8)	1.92 (1.68–2.05)	3.03 (2.74–3.53)
LDL-I	1.006–1.009	22.6 (19.8–25.4)	7.9 (6.7–9.0)	31.5 (31.3–31.8)	22.1 (21.1–23.1)	15.9 (15.0–16.8)	1.40 (1.38–1.41)	1.64 (1.51–1.77)
LDL-II	1.009–1.019	29.1 (25.7–32.0)	8.4 (5.9–10.4)	20.2 (17.1–24.9)	23.3 (23.1–23.7)	19.0 (17.8–20.3)	1.23 (1.15–1.32)	1.36 (1.30–1.41)
LDL-III	1.019–1.030	34.2 (33.6–34.9)	10.5 (9.4–11.6)	9.5 (8.5–10.1)	23.6 (21.3–24.8)	22.2 (22.1–22.3)	1.06 (0.96–1.11)	1.19 (1.12–1.31)
LDL-IV	1.030–1.040	39.4 (37.2–41.0)	9.7 (9.5–9.9)	4.9 (4.1–6.2)	22.7 (22.5–23.3)	23.3 (22.4–24.0)	0.98 (0.94–1.00)	1.17 (1.14–1.23)
LDL-V	1.040–1.053	37.8 (36.7–39.7)	9.1 (7.0–10.7)	4.5 (4.5–4.6)	23.5 (22.7–24.1)	25.1 (21.4–27.6)	0.95 (0.86–1.11)	1.06 (0.93–1.21)
LDL-VI	1.053–1.063	38.2 (36.2–40.9)	6.7 (5.6–7.6)	5.5 (4.6–6.1)	22.0 (21.7–22.8)	27.6 (26.0–30.6)	0.81 (0.71–0.88)	1.08 (1.05–1.10)

[a] Results represent the mean values of separate samples obtained from three normal men (donors from group II). The range of values is given in parentheses.

lipoprotein spectrum consists of LP-B as the major and LP-A and LP-C as the minor lipoprotein families. Since the ultracentrifugal analyses showed that all subfractions contained lipoproteins with flotation coefficients characteristic of the low density lipoprotein range (S_f = 0–20), the presence of LP-A and LP-C is not due to the contamination of LDL subfractions with VLDL and/or HDL. The LP-A and LP-C families probably occur in the LDL density range either as complexes with the major LP-B family or as separate entities with hydrated densities characteristic of LDL range. We favor the latter alternative because LP-A not removable by repeated ultracentrifugal washing of LDL subfractions could be precipitated individually by antibodies to LP-A.

The presence of LP-A has also been detected in the LDL range by Aladjem and Campbell (1957) using immunological techniques. More recently, Seidel *et al.* (1969) showed that LDL in patients with obstructive jaundice consists of a mixture of 2% LP-A with 98% of varying amounts of LP-B and the abnormal low density lipoprotein, LP-X.

The present immunochemical identification of LP-A and LP-C clarifies the ambiguous results of the terminal amino acid analyses of various LDL preparations. Thus, the detection of the minor N-terminal aspartic acid (N-terminal amino acid of ApoA; Avigan *et al.*, 1956) and the minor C-terminal threonine (C-terminal amino acid of ApoA; Shore, 1957) in LDL (Avigan *et al.*, 1956; Rodbell, 1958; Shore and Shore, 1962; Bobbitt and Levy, 1965) represents, indeed, the chemical evidence for the presence of LP-A in LDL. Similarly, the detection of the minor N-terminals serine and threonine (N-terminal amino acids of ApoC; Gustafson *et al.*, 1966) and the minor C-terminal alanine (C-terminal amino acid of ApoC; Alaupovic *et al.*, 1969) indicates the presence of LP-C in LDL. We suggest that most of the previous discrepancies regarding the immunochemical properties of LDL fractions may now be resolved by the recognition of apolipoprotein C or its constitutive peptides (Gustafson *et al.*, 1964, 1966; Alaupovic *et al.*, 1968, 1969; Brown *et al.*, 1968, 1969a,b; Shore and Shore, 1969) as the protein moiety of a family of lipoproteins (LP-C) which are present in all density segments of the lipoprotein spectrum.

The majority of donors who were characterized by very little or no detectable LP-C in LDL were females. Conversely, it was the male subjects who had the largest content of LP-C in the LDL fraction. However, in both groups of donors the detectable content of LP-C varied between individual subjects as well as between various subfractions. The detailed quantitative differences in the LP-C content of LDL subfractions between individuals of both sexes could not be determined by the procedures used in the present study.

The difference in the chemical composition between the LDL and LP-B subfractions as well as the variation in the chemical composition of LDL subfractions between individual subjects was confined mainly to the subfractions at the opposite ends of LDL density range. The low value for phospholipid:protein ratio of the highest density LDL subfraction was caused most probably by the presence of an increased amount of LP-A in subfraction bordering with HDL range. On the other hand, the high values of phospholipid:protein ratio of the lower density LDL subfractions were due to the presence of LP-C which, in its delipidized form (Gustafson *et al.*, 1966) and as LP-X (Seidel *et al.*, 1969), is characterized by high phospholipid:protein ratio. These

TABLE V: Amino Acid Composition of the Protein Moieties of Human Plasma LDL and LP-B Subfractions.[a]

Fractions:	T	A	LP-B-IV	LP-B-V
Density Range (g/cm^3):		1.009–1.050	1.030–1.040	1.040–1.053
Amino Acid	$M/10^5$ g ± σ	$M/10^5$ g ± σ	$M/10^5$ g ± σ	$M/10^5$ g ± σ
Lys	49.77 ± 0.85	49.38 ± 1.68	54.99 ± 6.20	57.02 ± 0.69
His	15.33 ± 0.98	17.51 ± 0.89	21.69 ± 1.02	18.16 ± 0.29
Arg	17.90 ± 0.49	21.91 ± 1.80	20.18 ± 3.23	23.82 ± 3.46
Asp	81.39 ± 1.41	86.33 ± 3.36	90.30 ± 1.80	88.07 ± 7.92
Thr	48.82 ± 0.43	54.57 ± 2.13	51.96 ± 0.31	51.55 ± 0.93
Ser	64.63 ± 0.61	67.01 ± 1.88	69.12 ± 1.87	62.10 ± 1.11
Glu	102.29 ± 0.27	102.03 ± 2.66	95.35 ± 2.56	99.78 ± 0.99
Pro	24.18 ± 0.81	29.22 ± 1.54	33.30 ± 2.06	29.29 ± 1.26
Gly	39.31 ± 0.44	36.46 ± 0.91	45.40 ± 0.77	43.15 ± 1.72
Ala	49.03 ± 0.73	52.16 ± 1.25	55.49 ± 0	60.73 ± 1.03
$^1/_2$-Cys	ND[b]	4.47	7.06	4.69
Val	44.68 ± 0	36.67 ± 1.32	35.31 ± 0.53	36.52 ± 4.12
Met	ND[b]	12.07	10.59	11.72
Ile	49.02 ± 0.99	39.84 ± 1.31	35.31 ± 0.53	37.49 ± 0.52
Leu	91.26 ± 2.13	90.92 ± 1.91	85.76 ± 2.31	82.99 ± 0.99
Tyr	28.33 ± 0.71	24.87 ± 0.32	24.72 ± 0.79	23.82 ± 0.31
Phe	40.15 ± 4.41	36.58 ± 0.72	35.31 ± 1.34	33.78 ± 0.30
Trp	ND[b]	5.07	3.03	6.44

[a] Values for serine, threonine, and tyrosine were obtained by linear extrapolation of average recoveries from 24- and 72-hr hydrolysis. Values for valine and isoleucine represent average recoveries obtained after 72-hr hydrolysis. [b] ND, not determined.

results and conclusions are convincingly corroborated by studies on the quantitative determination of terminal amino acids of LDL subfractions which show the largest content of the minor N-terminal aspartic acid in fractions with S_f = 11–20 and 0–3 (Shore and Shore, 1962) and the minor C-terminal alanine in the LDL fractions with S_f = 12–20 and 0–3 (Shore and Shore, 1969).

So far, there have been no literature reports on the chemical composition of more than three density segments of human plasma LDL. However, studies on the chemical composition of three LDL subfractions (Oncley *et al.*, 1957; Shore and Shore, 1962; Dearborn and Wetlaufer, 1969) indicated that protein content increased with increasing density. These latter findings could be explained now by the possible presence of LP-A and LP-C in these LDL subfractions.

Since LDL subfractions IV and V from group II donors occur in the highest concentration and usually contain the smallest immunochemically detectable amount of LP-A and LP-C, their chemical composition should not only be closest to the chemical composition of the entire LDL class but also to LP-B subfractions of the same density range. And indeed, the phospholipid:protein ratios of LDL and LP-B subfractions in the density range 1.030–1.053 g/cm^3 had values close to unity. Several investigators (Bragdon *et al.*, 1956; Heide *et al.*, 1957, 1958; Oncley, 1958; Cornwell and Kruger, 1961; Crámer, 1962) reported a similar value for the phospholipid:protein ratio of LDL isolated between *d* 1.019 and 1.063 g per cm^3.

The immunochemical studies of the LP-B subfractions suggested that this lipoprotein family may consist of two peptides or antigenic determinants separable by diffusion in agar or agarose gels. Since both LP-B species displayed identical electrophoretic mobility, it seems that they differed in their respective molecular weights or diffusion rates rather than net electrical charges. The LP-B with slower diffusion rate (antigenic determinant B_1) was stained more readily with lipid stain than the faster moving LP-B species (antigenic determinant B_2). The latter, lipid-poor lipoprotein seemed not to be a centrifugal degradation product of lipid-rich LP-B_1, because both lipoproteins were detected frequently in fresh native plasma. These findings are very similar to those observed by Lawrence and Shean (1962) who identified two immunoelectrophoretic lines as β-LP of the same electrophoretic mobility but different diffusion rates in agar gels.

Of all the LDL or LP-B fractions studied the amino acid composition of immunochemically heterogeneous fractions A and T resembled most closely that of a β-lipoprotein isolated by dextran sulfate precipitation and subsequent ultracentrifugation (Margolis and Langdon, 1966), except for a 20–25% difference in the content of proline and histidine in case of fraction T and valine and isoleucine in case of fraction A. The amino acid composition of fraction A was also in a good agreement with that of an ultracentrifugally prepared LDL fraction (Rudman *et al.*, 1968). However, the amino acid composition of LP-B subfractions differed not only from those of fractions A and T but also from those of similar provenance reported in the literature. Since both subfractions contained only ApoB as the protein moiety, the difference in the amino acid composition suggested also

the possible presence of two unequally distributed ApoB proteins or peptides in these two subfractions.

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