A Lipoprotein Characterizing Obstructive Jaundice

II. ISOLATION AND PARTIAL CHARACTERIZATION OF THE PROTEIN MOIETIES OF LOW DENSITY LIPOPROTEINS

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ABSTRACT The plasma low density lipoproteins (LDL) in biliary obstruction are characterized almost exclusively by the presence of the immunochemically distinct lipoprotein families, lipoprotein B (LP-B) and lipoprotein X (LP-X). It is suggested that LP-X, with its uniquely high content of unesterified cholesterol and phospholipid, is primarily responsible for the unusual lipid composition of LDL and the abnormal plasma lipid composition in obstructive jaundice.

To show their protein moieties, we isolated LP-X and LP-B from the LDL in plasma obtained from patients with obstructive jaundice. A separation procedure was employed which combines ultracentrifugation, heparin precipitation, and ethanol fractionation. Whereas LP-B was characterized by the presence of apolipoprotein B (ApoB), intact LP-X contained a protein moiety of unique composition consisting of a mixture of albumin (approximately 40%) and the specific apolipoprotein, ApoX (60%). These two protein moieties were separated by preparative ultracentrifugation at d 1.21 g/ml of a solution of partially delipidized LP-X. LP-X thus comprises an albumin-lipoprotein complex in which the masked antigenic site of albumin can be revealed by partial or total delipidization.

Apolipoprotein X, the characteristic nonalbumin protein moiety of intact or partially delipidized LP-X, was immunochemically different from ApoA, ApoB, albumin, γ-globulins, and other serum proteins. The results of analytical ultracentrifugation and the immunochemical and electrophoretic properties of ApoX indicated it to be a complex protein consisting possibly of several nonidentical polypeptides. ApoX was characterized by its amino acid composition, and by serine and threonine as the major N-terminal and alanine as the major C-terminal amino acids. It has been suggested that ApoX is similar to, if not identical with, apolipoprotein C.

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INTRODUCTION

The plasma lipoprotein pattern in subjects with biliary obstruction is characterized after ultracentrifugation by an increased concentration of LDL[1] and a decreased

[1] *Abbreviations based on the operational classification of lipoproteins:* VLDL, very low density lipoproteins, lipoproteins of $d < 1.006$ g/ml ($S_f > 20$); LDL, low density lipoproteins, lipoproteins of d 1.006–1.063 g/ml (S_f 0–20); HDL, high density lipoproteins, lipoproteins of d 1.063–1.210 g/ml; 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: 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 the obstructive jaundice and characterized by the presence of apolipoprotein X; LP-Xpd, partially delipidized LP-X; ApoA, apolipoprotein A, protein moiety of LP-A; ApoB, apolipoprotein B, protein moiety of LP-B; ApoC, apolipoprotein C, protein moiety of LP-C; ApoX, apolipoprotein X, the non-albumin protein moiety of LP-X.

Tentatively, the apolipoproteins may be characterized by terminal amino acids and antigenic determinants as follows:

Apolipoprotein	N-terminal amino acid	C-terminal amino acid	Antigenic determinants
ApoA	Aspartic Acid	Threonine Glutamine	At least 2
ApoB	Glutamic Acid	Serine	Single (as LDL)
ApoC	Threonine Serine	Alanine Valine Glutamic acid	At least 3

concentration of HDL (1–4). However, it has been demonstrated (5) that the characteristic elevation of plasma unesterified cholesterol and phospholipid concentrations is due to the presence of abnormal lipoproteins in Cohn fractions IV–VI which normally contain HDL or α-LP. Russ, Raymunt, and Barr (6) partially resolved these paradoxical findings by demonstrating that the lipoproteins present in Cohn fractions IV–VI had low hydrated densities (1.035–1.049 g/ml) but failed to react with antibodies to normal serum LDL. Switzer (7) established that serum LDL from patients with extrahepatic biliary obstruction contains, in addition to the usual amounts of LP-B, a high content of an abnormal lipoprotein which does not react with antibodies to normal LDL and is characterized by a low content of protein and a very high content of phospholipid and unesterified cholesterol. The immunochemical properties and the amino acid composition of this abnormal lipoprotein differ from those of LP-A and LP-B. In contrast to these studies, several investigators (8–10) demonstrated immunochemically the presence of LP-A in the LDL fraction, and Fredrickson, Levy, and Lees (11) suggested that the increased concentration of LDL in patients with biliary obstruction is caused by a shift of LP-A from the HDL into the LDL fraction. Recently, Burstein and Caroli (12) isolated a lipoprotein with the electrophoretic mobility of β-globulin but which was nonreactive with antibodies to normal LDL. Since, when delipidized, the lipoprotein moved on paper electrophoresis in the α_1-globulin area, they concluded that the abnormal lipoprotein in patients with obstructive jaundice is a "particular" α-LP characterized by a low protein and high lipid content. However, this conclusion, based solely on electrophoretic mobility, has not yet been supported by immunochemical and chemical identification of ApoA.

To study the individual lipoprotein families in LDL fractions from patients with obstructive jaundice, a procedure was developed in our laboratory (13) which permitted the separation of three immunochemically distinct lipoproteins, LP-A, LP-B, and LP-X. Quantitative determination showed that the combined LP-X and LP-B accounted for 98%, and the LP-A for only 2%, of the total protein content of LDL fraction.

The purpose of this paper is to present additional evidence that the abnormal lipoprotein, LP-X, with its high content of phospholipid and unesterified cholesterol is primarily responsible for the characteristic lipid and lipoprotein pattern of the plasma and of the LDL fraction in subjects with obstructive jaundice. This evidence is based on (*a*) differences in the chemical composition, immunochemical properties, and quantitative distribution of LP-A, LP-B, and LP-X, and (*b*) certain physical, chemical, and immunochemical characteristics of partially and totally delipidized LP-X. A preliminary report describing studies on the identification of the protein moiety of LP-X has appeared (14).

MATERIALS AND METHODS

Blood samples were obtained from four patients with extrahepatic biliary obstruction and from two patients with intrahepatic biliary obstruction. The diagnoses were established by the standard laboratory tests, liver biopsy, and surgical procedures. Blood drawn after an overnight fast was collected in plastic bags containing 2.2% Na citrate (Fenwal Laboratory, Morton Grove, Ill., and the plasma was recovered by low-speed centrifugation at room temperature.

Isolation and separation of lipoproteins. The separation scheme including the partial and total delipidization of LP-X is outlined in Fig. 1. The VLDL were obtained by layering plasma samples under equal volumes of NaCl solution (d 1.006 g/ml) and by centrifuging in the Type Ti 50 rotor of the Spinco Model L-2 ultracentrifuge (Beckman Instruments, Inc., Fullerton, Calif.) for 22 hr at 105,000 *g* and 4°C. The top fraction ($d < 1.006$ g/ml) containing VLDL was removed by a tube-slicing technique, resuspended in 3 vol of 1.006 g/ml NaCl solution, and recentrifuged under identical conditions. This washing procedure was repeated three times to remove traces of albumin. The LP-B, LP-X, and HDL were isolated from the bottom fraction ($d > 1.006$ g/ml) by a previously described procedure which combines heparin precipitation, ethanol fractionation, and ultracentrifugation (13). The isolated LP-B and LP-X fractions were resuspended in NaCl solution (1.063 g/ml) and the HDL in the NaCl-KBr solution (1.210 g/ml) and recentrifuged at 105,000 *g* for 44 hr. This washing procedure was repeated several times until lipoprotein fractions were free of albumin, as demonstrated by immunodiffusion tests.

Plasma samples were also fractionated into VLDL, LDL, and HDL by standard sequential preparative ultracentrifugation (4). The partially delipidized LP-C were isolated as described previously (14).

Lipoprotein fractions, dialyzed exhaustively against distilled water containing 0.05% EDTA, pH 7.0, were characterized by protein and lipid analyses and by determination of electrophoretic and immunochemical properties.

Partial and total delipidization of lipoproteins. Partial delipidization of purified LP-X fractions was accomplished according to a slight modification of the procedure by Gustafson (15). The lipoprotein fractions (5–20 mg of protein) were lyophilized in the absence of starch or any other additive, and the dry samples were extracted four times by vigorous shaking with 40 ml of *n*-heptane, spectranalyzed grade (Fisher Scientific Co., Pittsburgh, Pa.), for 1 hr at 4°. After each treatment with solvent, the partially delipidized lipoprotein was sedimented by low-speed centrifugation for 20 min at 4°C, the *n*-heptane extract was decanted, and fresh solvent was added. The final sediment was dried under nitrogen and dissolved in 0.15 M NaCl. The solution density was adjusted to 1.21 g/ml by adding KBr, and the mixture was centrifuged at 105,000 *g* for 44 hr. The top fraction (upper 1 cm) containing the partially delipidized lipoprotein fraction was removed by a tube-slicing technique.

Total delipidization of LP-A, LP-B, and LP-Xpd was performed according to a modification of the procedure of Scanu, Lewis, and Bumpus (16). Each lipoprotein fraction was delipidized by five successive extractions with ethanol-diethyl ether (3:1, v/v) followed by five extractions with diethyl ether at 0°C. After the final solvent treatment the essentially lipid-free (phosphorus content less than 0.01%) protein residues were removed by low-speed centrifugation

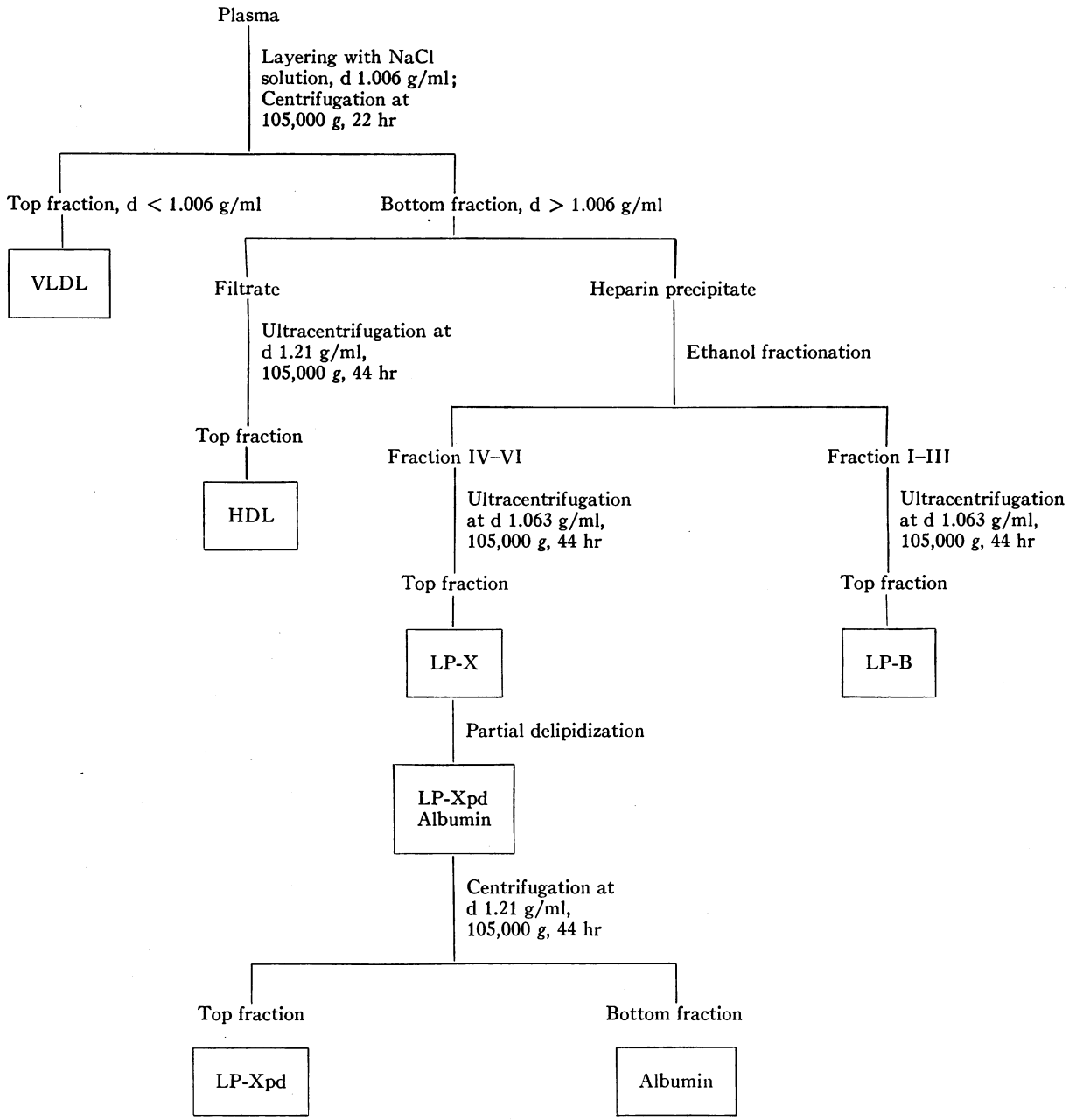

FIGURE 1 Procedure for the isolation of plasma lipoprotein fractions from patients with obstructive jaundice.

and were dried under nitrogen for several hours to remove the last traces of solvents.

Immunochemical methods. Samples of whole plasma, intact and partially delipidized lipoprotein fractions, and apolipoproteins were studied by double diffusion (17) and immunoelectrophoresis (18) in 1% agar (Special Agar-Noble, Difco Laboratories, Detroit, Mich.) or 1% agarose (Seakem Agarose, Bausch & Lomb, Incorporated, Rochester, N. Y.) employing barbital buffer, pH 8.7, ionic strength 0.05. Plates, allowed to develop for 24–30 hr, were washed several times with 0.15 M NaCl and distilled water and were dried at room temperature. They were stained for protein and lipid with Amido Black 10B and Oil Red 0, respectively.

Rabbit antihuman sera α_1-LP, β-LP, albumin, IgA, IgG, IgM, and whole human serum (Behringwerke A.G., Marburg an der Lahn, Germany) were used. The antisera to α_1-LP gave, in most instances, two precipitin lines and the antisera to β-LP gave a single precipitin line with whole serum from normal subjects. The antisera to α_1-LP and

β-LP showed no reaction with human serum albumin, and the antisera to albumin did not react with HDL and LDL.

The purified rabbit antisera to LP-X were prepared and characterized as described previously (13). The antisera prepared by immunizing rabbits with LP-Xpd (protein concentration 10 mg/ml) formed a single precipitin line with LP-X, but showed no reaction with human albumin. The rabbit antiserum to human VLDL was prepared as described previously (19).

Electrophoresis. Starch gel electrophoresis was performed according to Smithies (20) utilizing a discontinuous buffer system, pH 8.2 (21). The electrophoresis on 1% agar and 1% agarose gels was carried out as described under Immunochemical Methods.

The polyacrylamide gel disc electrophoresis was performed in Canalco Model 6 unit according to the procedure by Davis (22). Electrophoresis was carried out at 5 ma per tube until the tracking dye had migrated approximately 4.2 cm into the separating gel. The acrylamide monomer concentration was 7.5% and a continuous buffer system of Tris-glycine, pH 8.8, was used.

Analytical ultracentrifugation. The ultracentrifugal analysis of LP-Xpd and ApoX was carired out in a Spinco Model E ultracentrifuge equipped with a phase plate schlieren diaphragm and an automatic temperature control unit. Plate measurements were made with a Nikon microcomparator (Nikon Co., Tokyo, Japan). Samples of LP-Xpd and ApoX dissolved in 0.15 M NaCl solutions were run in single sector cells at 52,640 rpm at 25°C.

Analysis of C- and N-terminal amino acids. A water suspension of DFP-treated carboxypeptidase A (COADFP) and a frozen solution of carboxypeptidase B (COBDFP) were obtained from Worthington Biochemical Corporation, Freehold, N. J. The Apox, dissolved in 0.2 M N-ethylmorpholine acetate, pH 8.5, was digested by carboxypeptidase A or carboxypeptidase B at 37°C according to the procedure outlined by Ambler (23). The ratio of enzyme to substrate was 1:80 (w/w). Aliquots of the reaction mixture were taken at various intervals from 0 to 24 hr, and the reaction was stopped by adding 0.1 N HCl. The precipitated protein was removed by low-speed centrifugation, and the supernatant portion was dried in a vacuum desiccator. The residue was dissolved in 0.2 N sodium citrate, pH 2.2, and analyzed on the amino acid analyzer. The blank levels of amino acids and the contribution from autodigestion were determined in separate samples of ApoX and enzyme preparation, respectively. The C-terminal amino acids were determined also by hydrazinolysis according to the procedure by Braun and Schroeder (24).

The qualitative N-terminal amino acid analysis of approximately 1 mg of proteins was carried out using dansyl chloride as described by Gray (25). The dansylated product was separated from excess reagents and hydrolyzed for 18 hr, and the *N*-dansyl amino acids were extracted according to the procedure by Gros and Labouesse (26). The dansylated amino acids were separated on polyamide layers as described by Woods and Wang (27).

Amino acid analysis. Samples of ApoA, ApoB, ApoX, and albumin (1–2 mg) were hydrolyzed in constant boiling HCl (1 ml for each milligram of protein) in evacuated, sealed tubes in an oven maintained at 110°C for 24 and 72 hr. The hydrolysate was extracted with chloroform to remove any trace of fatty acids. The aqueous layer was separated, and the HCl was evaporated *in vacuo* in a rotary evaporator. The residue was then dissolved in 1 ml of 0.2 N sodium citrate buffer, filtered through a fine frit sintered glass filter, and analyzed on a Beckman Model 120C amino acid analyzer according to an accelerated automatic procedure (28) 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). Half-cystine and methionine were determined as cysteic acid and methionine sulfoxide after the oxidation of apolipoprotein samples and albumin with performic acid according to the procedure by Hirs (29). The values for cysteic acid were corrected for the 94% recovery found by Moore (30).

Lipid, protein, and carbohydrate analyses. Esterified and unesterified cholesterol, triglycerides, phospholipids, and total protein were determined as described previously (13). Free fatty acids were determined according to the method of Dole (31). Quantitative analysis of individual phospholipids was performed by two-dimensional thin layer chromatography as described previously (32). The anthrone-positive carbohydrates were estimated according to the method of Koehler (33).

RESULTS

Characterization of LDL obtained from patients with obstructive jaundice. The LDL obtained from patients with obstructive jaundice by the standard sequential preparative ultracentrifugation (4) contained, as usual (13), LP-A, immunochemically distinct from LP-B and LP-X (Fig. 2 *a*). In contrast, the lipoproteins isolated by heparin precipitation of VLDL-free plasma samples (Fig. 1) contained only LP-B and LP-X (Fig. 2 *b*). Traces of albumin were removed by one or two successive ultracentrifugations of LDL or heparin precipitates at solution density 1.063 g/ml (Fig. 2). It has been already established (13) that LP-A accounted for only 2% of the total protein content of LDL fraction.

Characterization of LP-X. The LP-B and LP-X fractions utilized for chemical and immunochemical studies of their protein moieties were separated by Cohn fractionation of the heparin precipitate (Fig. 1). The LP-X present in Cohn fractions IV-VI gave no reaction with antibodies to normal whole serum, LP-B, or LP-A. It displayed a characteristic migration pattern in agar-gel

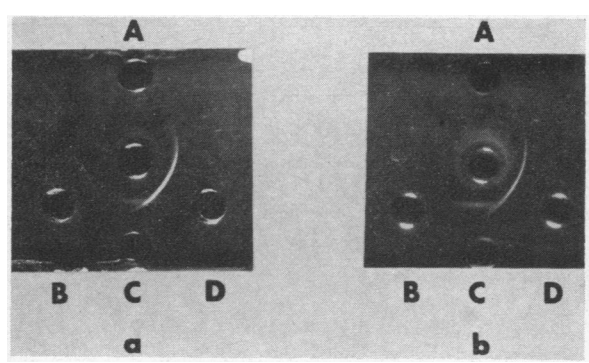

FIGURE 2 Immunodiffusion patterns of LDL. Central wells contain (*a*) LDL isolated by ultracentrifugation, and (*b*) lipoproteins isolated by heparin precipitation. Outer wells contain antibodies to albumin (*A*), LP-A (*B*), LP-X (*C*), and LP-B (*D*).

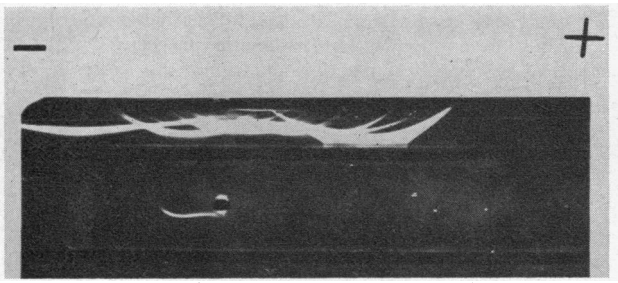

FIGURE 3 Immunoelectrophoresis patterns in 1% agar-gel of control serum, upper well, and LP-X, lower well. The upper trough contains antibodies to normal whole serum, the lower trough anti-LP-X serum.

electrophoresis (Fig. 3) and reacted only with antibodies to LP-X. In contrast to agar gel electrophoresis, LP-X moved towards the anode on agarose gel and starch gel. On starch gel electrophoresis LP-X migrated with a mobility slightly less than that of LP-B (Fig. 4).

The chemical composition of LP-X was characterized by a high content of phospholipid (66.5%), a low content of protein (5.8%), and the presence of cholesterol almost exclusively in unesterified form (93% of total cholesterol) (13). On the other hand, the phospholipid composition of LP-X (Table I) was characterized, like that of LDL and of HDL, by lecithin and sphingomyelin as principal components. The high lecithin: sphingomyelin ratio of LP-X resembled that of HDL rather than LDL.

Characterization of partially delipidized LP-X. The extraction of LP-X by *n*-heptane resulted not only in complete removal of neutral lipids, but also in substantial removal of phospholipids; in contrast to the high phospholipid: protein ratio of 11.5 for LP-X (13), the ratio for various preparations of LP-Xpd was approximately

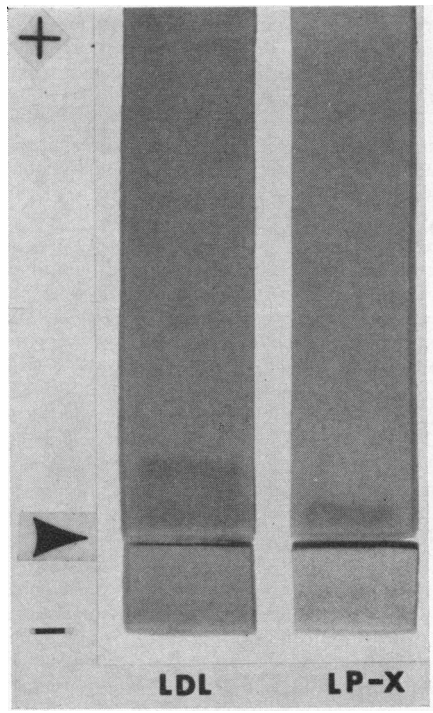

FIGURE 4 Electrophoresis pattern of LDL from a patient with obstructive jaundice and LP-X in starch gel. Amido Black stain.

2. The preferential extraction of lecithin, lysolecithin, and cephalin was reflected in a decreased lecithin: sphingomyelin ratio of LP-Xpd (Table I).

Whereas LP-X showed a single, slowly migrating band in agar (Fig. 3), agarose, and starch gel (Fig. 4) electrophoresis, and a single immunoprecipitin arc with anti-LP-X, the partially delipidized LP-X fraction gave

TABLE I

*Per Cent Phospholipid Compositon of Intact and Partially Delipidized LP-X**

Lipoprotein fraction	Lysolecithin	Sphingomyelin	Lecithin	Cephalin	Lecithin: sphingomyelin
	%	%	%	%	
LP-X	4.1 (3.9–4.3)	14.2 (13.3–15.2)	77.5 (72.1–81.0)	2.5 (1.5–3.4)	5.4
HDL_2‡	2.0	14.5	73.8	3.3	5.1
LDL‡	2.7	25.9	63.7	2.2	2.5
Partially delipidized LP-X	2.6 (2.6)	22.6 (20.8–24.4)	75.0 (71.4–78.5)	1.2 (0.7–1.6)	3.4

* Expressed as per cent of the sum of individually determined phosphorus values. Figures for intact and partially delipidized LP-X represent mean values of samples from three subjects.

‡ Values for normal serum HDL and LDL are taken from a study by Skipski, Barclay, Barcley, Fetzer, Good, and Archibald (34).

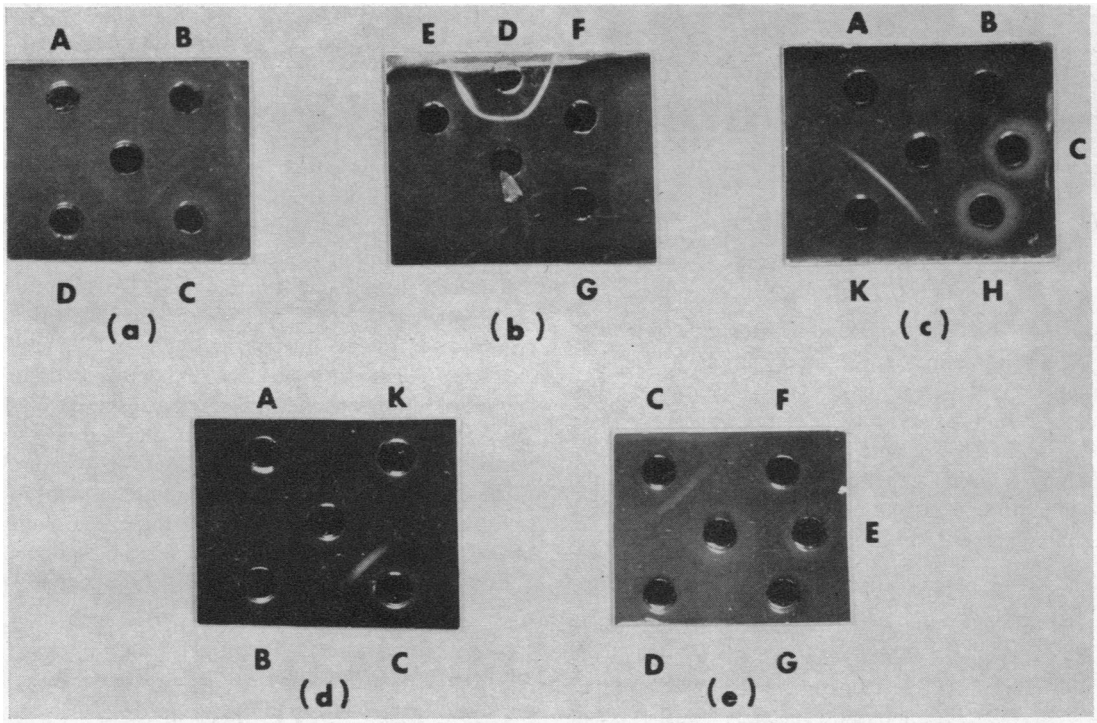

FIGURE 5 Immunodiffusion patterns of partially delipidized LP-X. Central wells contain (*a*) partially delipidized LP-X before ultracentrifugation at d 1.21 g/ml, (*b*) and (*c*) bottom fraction (albumin) of partially delipidized LP-X centrifuged at 1.21 g/ml, and (*d*) and (*e*) top fraction of partially delipidized LP-X centrifuged at d 1.21 g/ml. Outer wells contain antibodies to LP-A (*A*), LP-B (*B*), LP-X (*C*), albumin (*D*), IgG (*E*), IgA (*F*), IgM (*G*), VLDL (*H*), and whole serum (*K*).

a positive immunoprecipitin reaction not only with antibodies to LP-X, but also with antibodies to albumin (Fig. 5, pattern *a*). To separate albumin from the immunochemically characteristic protein of LP-Xpd, the density of the partially delipidized lipoprotein solution was adjusted to 1.21 g/ml and the mixture was centrifuged at 105,000 *g* for 44 hr (Fig. 1). The bottom fraction contained immunochemically identified albumin (Fig. 5, pattern *b*). The nonalbumin component of LP-Xpd present in the top fraction showed a broad single immunoprecipitin line with antibodies to LP-X (Fig. 5, patterns *d* and *e*); occasionally, this broad line resolved into two separate lines. However, this fraction showed no reaction with antibodies to LP-A, LP-B, albumin, IgG, IgA, IgM, and whole serum. Except for the occasional presence of trace amounts of LP-Xpd, the albumin-containing fraction was free of immunochemically detectable IgA, IgG, IgM, partially delipidized LP-A and LP-B, and other serum proteins (Fig. 5, patterns *b* and *c*). The albumin fraction was characterized also by the terminal amino acid analysis and by amino acid composition. Results of these studies showed that aspartic acid was the only detectable N-terminal (35) and leucine the only C-terminal amino acid (36). Except for a higher content of glutamic acid and a lower content of valine and lysine, the amino acid composition of albumin was similar to that reported by Schultze, Heimburger, and Frank (37). Chloroform extraction of the albumin fraction revealed the presence of fatty acids (15–20 moles/mole of albumin) and unidentified phospholipids (0.1–0.2 mg/mg of albumin). The dissociation of LP-X caused by partial delipidization was reflected also in an electrophoretic mobility of LP-Xpd in agar gels different from that of LP-X; the LP-X migrated towards the cathode (Fig. 3) while both the albumin and nonalbumin components of the LP-Xpd migrated towards the anode (14). The nonalbumin component of LP-Xpd showed a single, broad sedimenting boundary ($s_{20,w}$ = 4.0S); further flattening of the boundary upon prolonged ultracentrifugation indicated the possible presence of a multicomponent system of macromolecules of similar hydrodynamic properties.

After partial delipidization of LP-X the protein content of separated albumin and nonalbumin protein was expressed as per cent of the sum of these two values. Basically, the same result was obtained when the individually determined values were expressed as percentage of the protein value of LP-X. Analyses of ten separate

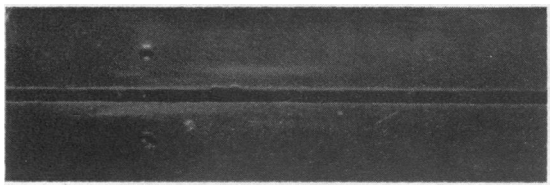

FIGURE 6 Immunoelectrophoresis pattern in 1% agar gel of ApoX, upper well, and ApoC, lower well. The trough contains antibodies to LP-X.

LP-X samples showed that albumin accounted for 40% (38–42%) and the nonalbumin moiety for 60% (58–62) of the total protein content of LP-X.

Characterization of the nonalbumin protein moiety (ApoX) of LP-X. Total delipidization of the albumin-free LP-Xpd fraction resulted in the isolation of the water-soluble protein moiety, ApoX, which showed three distinct immunoprecipitin arcs in agar-gel electrophoresis (Fig. 6, upper pattern); whereas one component migrated toward the anode and the other toward the cathode, the third, a relatively weak immunoprecipitin arc, was located in the middle close to the antigen well. In 7% polyacrylamide electrophoresis the ApoX preparations showed a characteristic pattern consisting of three fast moving bands (Fig. 7, pattern *a*) and a weak band close to the stacking gel (because poorly stained by Amido Black 10B, this fourth band was identified by a positive immunoprecipitin reaction with antibodies to LP-X). The results of the polyacrylamide gel electrophoresis (Fig. 7) and immunoelectrophoresis (Fig. 6) indicate that the nonalbumin protein moiety of LP-X consists of at least three antigenic components or nonidentical polypeptides. The presence of several polypeptides of similar hydrodynamic properties was reflected also in the schlieren pattern of ApoX showing a single but broad and rapidly flattening peak.

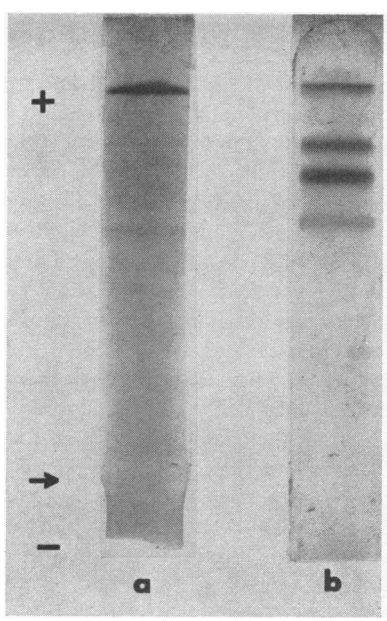

FIGURE 7 Electrophoretic patterns in 7% polyacrylamide gel of ApoX, pattern *a*, and ApoC, pattern *b*. A weak band close to the stacking gel in both patterns stained poorly with Amido Black 10B. It was identified by immunochemical reaction with antibodies to LP-X. The arrow indicates the boundary between the stacking and separating gels.

To test for the presence of fatty acids, intact and acid-hydrolyzed ApoX was extracted exhaustively with chloroform; the titrimetric analysis of both chloroform extracts indicated the absence of fatty acids. The ApoX contained no detectable anthrone-positive carbohydrates. Analyses of neuraminic acid and glucosamine were not performed; however, amino acid analysis of ApoX failed to reveal the presence of glucosamine.

Results of the analyses of the amino acid composition of apolipoproteins are shown in Table II. Apolipoproteins B and X were prepared by total delipidization of LP-B

TABLE II

*Amino Acid Composition of the Apolipoproteins from Patients with Obstructive Jaundice**

Amino acids§	Moles of amino acids per 100,000 g of protein‡			
	ApoB	ApoA	ApoX	Albumin from LP-X
Lysine	54.6	42.6	75.6	65.6
Histidine	17.9	8.8	4.6	19.0
Arginine	22.1	25.0	32.4	30.1
Aspartic acid	82.3	55.8	68.1	62.8
Threonine	47.9	39.7	48.5	31.1
Serine	60.5	58.8	88.0	27.2
Glutamic acid	96.0	157.3	135.8	168.9
Proline	30.2	36.8	21.9	32.3
Glycine	38.8	66.2	27.6	21.6
Alanine	50.2	82.3	61.2	75.1
Half-cystine	5.2	4.8	0	31.1
Valine	43.0	44.1	35.0	42.4
Methionine	11.4	14.7	15.1	6.6
Isoleucine	42.5	8.8	26.8	12.0
Leucine	94.8	102.9	76.0	79.8
Tyrosine	20.7	23.5	14.9	21.6
Phenylalanine	37.7	22.1	36.0	34.8

* ApoB and ApoX were isolated from corresponding lipoproteins present in the LDL fraction, and ApoA was isolated from the HDL fraction.

‡ The values represent average of analyses on two separate apolipoprotein preparations.

§ Values for serine, threonine, and tyrosine were obtained by linear extrapolation of average recoveries from 24 and 72 hr of hydrolysis. Values for valine and isoleucine represent average recoveries obtained after 72 hr hydrolysis. Values for half-cystine were calculated from the cysteic acid content after treatment of apolipoproteins with performic acid.

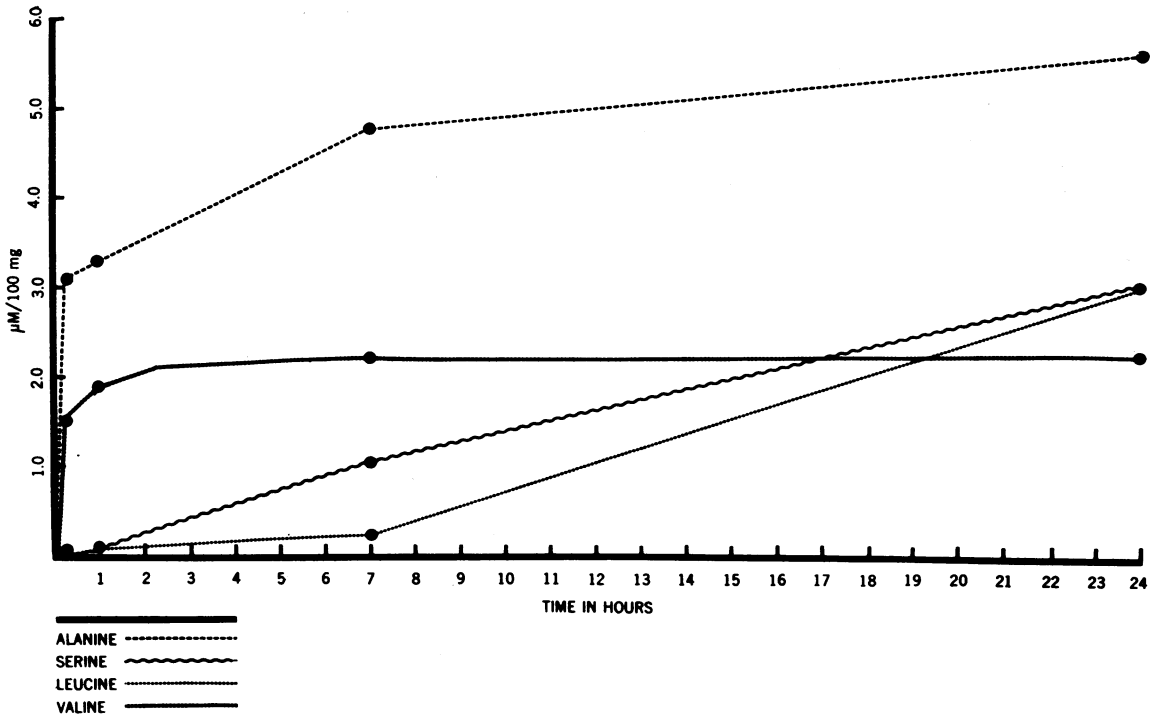

FIGURE 8 Release of principal amino acids from ApoX with carboxypeptidase A at 37°C.

and the nonalbumin containing LP-Xpd. Because of a very low content of LP-A in the low density segment of the lipoprotein spectrum, analysis of the amino acid composition of ApoA was performed on a preparation isolated from the corresponding lipoprotein fraction of HDL. The differences between apolipoproteins were reflected primarily in the different content of such amino acids as lysine, aspartic acid, glutamic acid, alanine, isoleucine, and leucine. The values for ApoB agreed well with those reported for ApoB isolated from normal serum (38–40). On the other hand, there were some differences in the content of basic amino acids, alanine, and methionine between the ApoA preparation of this study and those preparations reported recently in the literature (40–43). It has been suggested (43) that the discrepancy in the results of amino acid composition of ApoA preparations from various laboratories may be due to a varying degree of lipoprotein heterogeneity in the HDL spectrum of individual plasma donors. The apolipoprotein heterogeneity of serum and chyle HDL has been established by our studies (44–45) indicating the presence of ApoB, and by Shore and Shore (46–47) who have demonstrated that ApoA consists of two nonidentical polypeptides. Although a characteristic amino acid composition of ApoA from patients with obstructive jaundice cannot be excluded by these preliminary experiments, the immunochemical behavior of ApoA was identical with that of an ApoA preparation from a normal subject. The main characteristic of the amino acid composition of ApoX is the absence of cystine and cysteine and a relatively low content of histidine; no cysteic acid was detected after performic acid oxidation of ApoX. The difference between the amino acid composition of ApoX and the "obstructive lipoprotein" (OLP) isolated by Switzer (7) was, in all probability, due to the presence of albumin in the totally delipidized OLP preparation.

The qualitative N-terminal amino acid analysis of ApoX showed the presence of dansylated serine and threonine as the major terminal amino acids; aspartic and glutamic acids were present in trace amounts. The results of a time study of the carboxypeptidase A digestion of ApoX are presented in Fig. 8. The release of major C-terminal amino acids alanine and valine in an approximate molar ratio of 2:1 seemed to level off after 7 hr of digestion. Other amino acids liberated after 24 hr, not shown in Fig. 8, include, in decreasing molar amounts, threonine, aspartic acid, glutamic acid, tyrosine, phenylalanine, glycine, and isoleucine. There were no additional amino acids released by the action of carboxypeptidase B. In a separate experiment, the action of carboxypeptidase A on ApoX for 1 min at 25°C resulted in the release of alanine in a molar ratio of 10:1 over valine. After hydrazinolysis of ApoX several amino acids could be detected by chromatography; alanine, glutamic acid, and valine were detected in decreasing or-

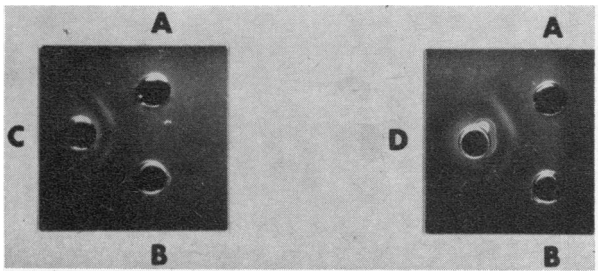

FIGURE 9 Immunodiffusion of ApoC (*A*) and ApoX (*B*). The wells on the left side of each pattern contain antibodies to LP-X (*C*) and antibodies to VLDL (*D*).

der as the major amino acids, while serine, aspartic acid, and glycine were present in trace amounts. Although additional quantitative studies will be required, results of these analyses indicate that alanine is the major and valine and glutamic acid the minor C-terminal amino acids of ApoX.

The evidence for an immunochemical similarity, if not identity, between the partially delipidized nonalbumin protein moiety of LP-X and a partially or totally delipidized LP-C, isolated from VLDL of patients with "mixed type" hypertriglyceridemia, has already been presented in a preliminary communication (14). Recent studies on the characterization of ApoC provided additional evidence for a close relationship between this apolipoprotein and ApoX. Similarly to ApoX, the ApoC gave three distinct immunoprecipitin lines with antibodies to LP-X in 1% agar electrophoresis (Fig. 6, lower pattern) and displayed three fast moving bands in 7% polyacrylamide electrophoresis (Fig. 7, pattern *b*). A fourth, poorly stainable band close to the stacking gel was identified by immunochemical reaction with antibodies to LP-X. The ApoX and ApoC gave a complete fusion of immunoprecipitin lines with antibodies to LP-X and VLDL (Fig. 9).

DISCUSSION

The low density lipoprotein pattern in biliary obstruction is characterized almost exclusively by the presence of the immunochemically distinct lipoprotein families LP-B and LP-X (7, 12, 13). Since LP-B in obstructive jaundice is of an almost normal chemical composition, it has been suggested (7, 13) that LP-X, with its uniquely high content of unesterified cholesterol and phospholipid, is primarily responsible for the unusual lipid composition of LDL and the characteristic plasma lipid concentrations in obstructive jaundice.

The results of the present study indicate that intact LP-X contains a protein moiety of unique composition consisting of a combination of approximately 40% albumin and 60% of the apolipoprotein, ApoX. Although this finding represents the first description of a lipoprotein containing an apolipoprotein (ApoX), albumin, and lipids, there are several reports in the literature about the association of plasma globulins with β-LP. The well known tendency of albumin to adsorb onto the surfaces of various lipoproteins during preparative ultracentrifugation (13, 39, 45, 48–51) or to coprecipitate with β-LP upon addition of the dextran sulfate (52) or heparin and $MnCl_2$ (53) results in a weak association which disrupts spontaneously during electrophoresis or ultracentrifugation. The antigenic site of albumin in these associations is readily detectable by immunochemical procedures. In contrast, the lipid-rich union of albumin and LP-X masks the antigenic site of albumin and results in a binding so strong that *n*-heptane extraction of intact LP-X albumin complex is required for its cleavage. Similar complexes of plasma β-LP with IgG (54), IgA (55, 56), and IgM (57) globulins of varying strength have been isolated from patients with hyperlipemia and/or multiple myeloma: for example, a β-LP-IgA complex (55), resistant to dissociation by electrophoresis or ultracentrifugation, could be split only after repeated precipitations with $(NH_4)_2SO_4$ and $ZnSO_4$. Omission of the partial delipidization procedure, necessary for unmasking the antigenic site of albumin prior to immunochemical testing, may explain why other investigators (7, 12) failed to identify albumin as an integral part of LP-X complex. The finding of a single, fast moving electrophoretic band of totally delipidized LP-X (12) may be due to very similar paper electrophoretic mobilities of ApoX and albumin.

It seems that the characteristic lipid composition of intact LP-X is primarily due to an extraordinary lipid-binding capacity of ApoX: partially delipidized LP-X retained twice as much phospholipid (phospholipid: protein ratio 2) as the LP-A or LP-B extracted by *n*-heptane under identical conditions (58). However, despite this high binding capacity of ApoX, it is possible that albumin plays an important role in maintaining the structural integrity of a lipoprotein particle characterized by a phospholipid: protein ratio of 11–12. In comparison, the next highest value known for the phospholipid: protein ratio of a lipoprotein is the relatively low value of 4.7, determined for a $S_f > 5000$ chylomicron fraction (58). Since albumin derived from LP-X contained free fatty acids and an unidentified phospholipid, presumably lysolecithin, the LP-X and albumin may be interwoven into a structural entity through both lipid-lipid and protein-protein interactions. The relatively high degree of structural orderliness for a macromolecule consisting of noncovalently linked constituents is illustrated indirectly, but significantly, by excellent agreement among several laboratories regarding the per cent composition of protein and lipid components and by a constant ratio of albumin and ApoX of isolated LP-X preparations. The

intriguing questions about the molecular arrangement in the albumin-LP-X complex, the site of its formation and the reasons for its apparently exclusive occurrence in patients with obstructive jaundice (13) remain to be answered by further investigations. We have proposed as a working hypothesis that the formation and plasma accumulation of LP-X may be the result of an impaired lipoprotein catabolism caused by inhibitory action of increased concentrations of bile salts. It has been assumed in this hypothesis that ApoX and the corresponding lipoprotein (the latter not necessarily of the same composition as LP-X in obstructive jaundice) represent normal constituents of the lipid transport system.

The ApoX, with its characteristic electrophoretic and immunochemical behavior, lipid-binding capacity, and its terminal amino acids and amino acid composition, represents an apolipoprotein different from ApoA, ApoB, and albumin. A broad, fast-spreading schlieren boundary, the presence of multiple bands in polyacrylamide electrophoresis, the detection of at least three antigenic determinants, and the results of terminal amino acid analyses suggest strongly that ApoX may possess a complex quaternary structure composed of several nonidentical polypeptides. Alternatively, it may represent a complex consisting of albumin and three separate lipoproteins.

Before presenting evidence for the possible identity of ApoX with ApoC, it is necessary to discuss briefly the present state of our knowledge regarding the chemical and immunochemical properties of ApoC. In 1964 Gustafson, Alaupovic, and Furman (59) separated from partially delipidized VLDL three phospholipid-protein residues; protein moieties of two of the three phospholipid-protein residues were identical with the apolipoproteins A and B. The third phospholipid-protein residue contained a protein moiety characterized by a high capacity for lipid binding, immunochemical properties, and peptide patterns different from other plasma apolipoproteins, and by serine and threonine as N-terminal amino acids. This newly recognized protein moiety was designated as apolipoprotein C. The presence of ApoC has been demonstrated immunochemically also in chyle VLDL (19) and in all density lipoprotein classes isolated from plasma of normal, fasting men (60). The distribution studies have shown (32, 61) that ApoC appears to be present in highest amounts in VLDL of subjects with mixed type hypertriglyceridemia. Although the demonstration of ApoC as a separate apolipoprotein had been accepted initially with certain skepticism (48, 62, 63), the results of recent studies from two laboratories have fully confirmed its occurrence in human plasma lipoproteins. Brown, Levy, and Fredrickson (64) confirmed that phospholipid-protein residues isolated from human plasma VLDL reacted not only with antibodies to HDL and LDL, but gave also at least one specific immunoprecipitin line with antibodies to VLDL. Moreover, these authors were able to fractionate the soluble portion of totally delipidized VLDL on Sephadex G-100 column into two major fractions. One fraction contained primarily ApoA, whereas the second fraction consisted of a mixture of peptides differing in the electrophoretic mobility and N-terminal amino acids. Further fractionation of this mixture on DEAE-cellulose column resulted in the isolation of four fractions (D_1–D_4), two of which were characterized by N-terminal serine and C-terminal alanine (D_3 and D_4) and one by N-terminal threonine and C-terminal valine (D_1). Very recently, the same authors characterized fraction D_2 by N-terminal threonine and C-terminal glutamic acid (65). Shore and Shore (66) isolated from VLDL and HDL_2 a polypeptide characterized by alanine as C-terminal amino acid. Results of recent studies in this laboratory have shown that ApoC isolated from plasma VLDL (14, 67) and characterized by serine and threonine as N-terminal amino acids, contained alanine as the major C-terminal amino acid. The ApoC was resolved in 7% polyacrylamide gel electrophoresis into three very characteristic fast moving bands and a weak band close to the stacking gel; however, some preparations showed additional weak bands. Immunochemical characterization of LP-C has shown (68) that the protein moiety consists of at least three antigenic determinants. On the basis of all these experiments, we suggest that ApoC is a distinct human plasma apolipoprotein, the quaternary structure of which is composed of nonidentical polypeptides characterized by a high affinity for lipid binding. Although additional studies are essential for establishing the number and properties of constitutive ApoC polypeptides, it seems clear that ApoC contains three well characterized polypeptides (N-terminal serine and C-terminal alanine; N-terminal threonine and C-terminal valine; and N-terminal threonine and C-terminal glutamic acid) and, possibly, two additional polypeptides; it is easily detectable by its characteristic behavior in 7% polyacrylamide gel electrophoresis and, in its totally delipidized form, by three distinguishable antigenic determinants.

The possible identity of ApoX with ApoC is based on following evidence: (*a*) similarity of the electrophoretic patterns in different supporting media such as agar, agarose, and polyacrylamide gels, (*b*) similarity of phospholipid: protein ratios of LP-X and LP-C delipidized under identical conditions, (*c*) identical N-terminal (threonine and serine) and C-terminal (alanine, valine, and glutamic acid) amino acids, and (*d*) complete fusion of ApoX and ApoC precipitin lines in reaction with antibodies to VLDL (LP-C) or LP-X. Although only a complete chemical characterization and analysis of quaternary structure or polypeptide composition of both ApoX and ApoC will provide the final proof, we

propose a chemical identity of these two apolipoproteins as the most plausible working hypothesis.

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