Studies on the Isolation and Partial Characterization of Apolipoprotein D and Lipoprotein D of Human Plasma†

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ABSTRACT: This report describes further studies on the characterization of apolipoprotein D (ApoD), a recently recognized human plasma apolipoprotein, and presents results on the isolation and distribution of its lipoprotein form, lipoprotein D (LP-D). ApoD, isolated by a procedure combining hydroxylapatite and Sephadex G-100 column chromatography, migrated on 7% polyacrylamide gel as a single band with a mobility intermediate between those of A-II and C-II polypeptides. On double diffusion and immunoelectrophoresis, ApoD reacted only with antiserum to ApoD. It was characterized by the presence of all common amino acids including half-cystine. The amino terminal acid was blocked. Carbohydrate analysis demonstrated that ApoD is a glycoprotein with glucose, mannose, galactose, glucosamine, and sialic acid accounting for 18% of the dry weight of ApoD. The estimated molecular weight of ApoD is 22 100. ApoD occurs in the serum as a lipoprotein which was isolated from high density lipoproteins$_3$ by two different chromatographic procedures. In the first procedure, high density lipoproteins$_3$ were treated with neuraminidase and chromatographed on concanavalin A. The retained fraction containing LP-D was purified by hydroxylapatite column chromatography. Alternatively, LP-D was isolated by a procedure combining chromatography of high density lipoproteins$_3$ or whole serum on an immunosorber containing antibodies to ApoD, and hydroxylapatite column chromatography. LP-D displayed a single, symmetrical boundary in the analytical ultracentrifuge and a single band on 7% polyacrylamide gel electrophoresis. When injected into rabbits it produced antisera that reacted only with ApoD. On immunoelectrophoresis LP-D had a mobility different from that of lipoprotein A (LP-A). A direct immunological comparison of LP-D and LP-A showed a reaction of nonidentity. LP-D consists of 65–75% protein and 25–35% lipid. The lipid moiety contains cholesterol, cholesterol ester, triglyceride, and phospholipid. The phospholipid composition is characterized by a relative high content of lysolecithin and sphingomyelin and a relatively low content of lecithin. We have concluded from these studies that ApoD is a unique apolipoprotein that exists in the form of a distinct lipoprotein family with a macromolecular distribution extending from very low density lipoproteins into very high density lipoproteins, but with a maximum concentration in high density lipoproteins$_3$ and a minimum concentration in high density lipoproteins$_2$.

Recent investigations of the human plasma lipoprotein system have led to the isolation and characterization of several antigenically distinct polypeptides (Shore and Shore, 1968, 1969; Brown et al., 1969, 1970). According to the nomenclature based on the lipoprotein family concept (Alaupovic, 1972) these polypeptides have been designated as A-I, A-II, C-I, C-II, C-III, and ApoB and the corresponding lipoprotein families as lipoprotein A (LP-A), lipoprotein C (LP-C), and lipoprotein B (LP-B).

We have recently isolated and partially characterized a human plasma apolipoprotein (McConathy and Alaupovic, 1973) which corresponded antigenically to a determinant referred to as "thin-line" polypeptide (Alaupovic et al., 1972; Kook et al., 1970; Kostner, 1974a; Lee and Alaupovic, 1970). This apolipoprotein was named apolipoprotein D (ApoD). In this paper we describe further studies on the characterization of ApoD, and report the isolation and some physical-chemical properties of its corresponding lipoprotein form. Results of these studies support our previous suggestion that apolipoprotein D is the protein moiety of a distinct lipoprotein family designated as lipoprotein D (LP-D).

Experimental Section

Lipoproteins were isolated from a minimum of 500 ml of fresh human plasma obtained by plasmapheresis from normolipemic subjects after an overnight fast or outdated human plasma.

Isolation of Lipoprotein Density Classes. VLDL, LDL, HDL, HDL$_2$, HDL$_3$, and VHDL[1] were isolated by sequential ultracentrifugation as previously described (Alaupovic et al., 1966, 1972). The isolated density classes were recentrifuged under identical isolation conditions until free of albumin as demonstrated by double diffusion analysis.

Column Chromatographic Procedures. Gel chromatography on Sephadex G-100 and hydroxylapatite was carried out according to previously described procedures (McConathy and Alaupovic, 1973).

Affinity chromatography on concanavalin A-Sepharose 4B (Pharmacia Fine Chemicals, Inc., Piscathaway, N.J.)

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[1] Abbreviations used are: VLDL, very low density lipoproteins (d < 1.006 g/ml); LDL, low density lipoproteins (d = 1.006–1.063 g/ml); LDL$_2$, subclass of low density lipoproteins (d = 1.019–1.063 g/ml); HDL, high density lipoproteins (d = 1.063–1.21 g/ml); HDL$_2$, subclass of high density lipoproteins (d = 1.063–1.125 g/ml); HDL$_3$, subclass of high density lipoproteins (d = 1.125–1.21 g/ml); VHDL, very high density lipoproteins (d > 1.21 g/ml).

was carried out with the same buffer system as previously described (McConathy and Alaupovic, 1974a). As a control, HDL_3 was chromatographed on the concanavalin A-Sepharose 4B column prior to neuraminidase treatment. An excess amount of neuraminidase (*Clostridium perfringens*, Worthington Biochem. Corp., Freehold, N.J.) was added to 800 mg of the unretained HDL_3 in a tenfold excess over the calculated molar content of neuraminic acid in HDL_3, and the reaction mixture was incubated for 24 h at room temperature, pH 6.5. Neuraminidase-treated HDL_3 was rechromatographed on the concanavalin A column, and the retained fraction was eluted with 0.2 M methyl α-D-glucopyranoside. The retained fraction was chromatographed on a hydroxylapatite-cellulose column and eluted stepwise with 0.001, 0.01, and 0.05 M KH_2PO_4 (pH 8.0).

Immunosorbers. Rabbit antiserum to apolipoprotein D (McConathy and Alaupovic, 1973) was utilized for isolation of the IgG antibody-containing fraction. A saturated ammonium sulfate solution was added to the antiserum to give 33% saturation. The precipitated IgG fraction was dialyzed against 0.1 M K_2HPO_4 (pH 6.5) and coupled to CNBr-activated Sepharose 4B at pH 6.5 as described by Cuatrecasas (1970). A ratio of 15 mg of protein/ml activated Sepharose was utilized, and a coupling efficiency of 80% was estimated by absorption at 280 nm. To block any unreacted groups, the immunosorber was incubated for 2 h with 0.1 M ethanolamine (pH 8.0) and then washed extensively with the equilibration buffer (0.15 M NaCl, 0.05 M Tris-HCl, and 0.01% NaN_3 (pH 7.5)). An immunosorber with antibodies to A-II polypeptide (anti-A-II-Sepharose 4B) was prepared in the same fashion. To avoid prolonged interaction of the dissociating buffer (3 M NaSCN) with the antibodies and protein eluted from the immunosorber, the chromatography was performed on a column (60 × 2.5 cm) constructed in a fashion similar to that described by Crook et al. (1972). The column was first packed to a height of 15 cm with medium grade Sephadex G-25 followed by the Sepharose-coupled antibodies (40 cm) and another layer of Sephadex G-25 (4 cm). To remove any previously uneluted material, the immunosorber was washed successively with 50 ml of 3 M NaSCN and the equilibration buffer. Application of the sample was followed by extensive washing with the equilibration buffer until the absorbance returned to the baseline. Material bound to the anti-D-Sepharose column was eluted successively with 50 ml of 3 M NaSCN and the equilibration buffer. Approximately 75% of the total bound material was eluted with the equilibration buffer as demonstrated by $FeCl_3$ titration, while the remainder was eluted with NaSCN. Both fractions were first made 0.001 M with respect to phosphate by chromatography on a Sephadex G-25 column (60 × 2.5 cm) equilibrated with 0.001 M KH_2PO_4 (pH 8.0) and were then applied onto the hydroxylapatite-cellulose column. Fractions eluted with 0.001 M KH_2PO_4 (pH 8.0) were rechromatographed under identical conditions and usually showed a single band on basic polyacrylamide gel electrophoresis. Those that did not were rechromatographed until a single band was obtained. After purification, the fractions eluted with 0.001 M KH_2PO_4 were desalted by gel filtration on Sephadex G-25 equilibrated with 0.1 M $(NH_4)_2CO_3$.

Immunological Methods. Immunoelectrophoretic and double diffusion analyses in 1% agarose gels, and the preparation and characterization of antisera to human A-I, A-II, C-I, C-II, C-III, LP-B, albumin, α-lipoprotein (α-LP), and HDL_3, respectively, were carried out as previously described (Alaupovic et al., 1972). Antiserum to human ApoD was prepared as recently reported (McConathy and Alaupovic, 1973).

Electrophoresis. Basic (Davis, 1964) and acidic (Reisfeld et al., 1962) polyacrylamide gel electrophoreses (PAGE) were carried out in 7% acrylamide gels in the presence of 8 M urea as previously described (McConathy et al., 1973). The molecular weight determinations by sodium dodecyl sulfate-PAGE were performed according to Weber and Osborn (1969). Lipoprotein electrophoresis was performed on cellulose acetate strips (Helena Laboratories, Beaumont, Texas).

Analytical Ultracentrifugation. Determination of the sedimentation coefficient of a LP-D preparation (4 mg/ml) dissolved in a mixture of 0.15 M NaCl and 0.05 M Tris-HCl (pH 7.5) was carried out as previously described (Lee and Alaupovic, 1974). The determination was carried out at 56 000 rpm, 25 °C, and d = 1.0087 g/ml.

Amino Acid Analyses. Protein samples were hydrolyzed with constant-boiling HCl in evacuated, sealed tubes at 110 °C for 24 and 72 h. The amino acid analyses and the performic acid oxidation were done as previously described (Lee and Alaupovic, 1970). Tryptophan was determined according to the *p*-toluenesulfonic acid hydrolysis procedure of Liu and Chang (1971).

Terminal Amino Acids. C-terminal amino acids of ApoD were analyzed by digestion with carboxypeptidase A and/or B according to the procedure of Ambler (1967). The possible occurrence of glutamine in the carboxypeptidase digests was determined by use of the lithium citrate buffer as described by Benson et al. (1967). N-terminal amino acids were determined by dinitrophenylation (Fraenkel-Conrat et al., 1955), a modified (Seidel et al., 1970) dansylation procedure of Gray (1967), and the automated Edman degradation method (Niall, 1973).

Carbohydrate Analyses. Neutral and amino sugars were determined by a modification (Wang et al., 1974) of the gas–liquid chromatographic procedure of Griggs et al. (1971). Sialic acid was determined by the same procedure after mild acid hydrolysis (0.01 N HCl for 90 min at 30 °C) of ApoD.

Protein and Lipid Analyses. Protein was determined by the method of Lowry et al., (1951). Lipid analyses of lipoprotein preparations were performed according to the procedures described previously (Alaupovic et al., 1966). For these analyses, lipoproteins were totally delipidized by chloroform–methanol (2:1, v/v).

Results

Characterization of Apolipoprotein D. Isolation of ApoD by a previously described procedure (McConathy and Alaupovic, 1973) combining hydroxylapatite and Sephadex G-100 column chromatography yielded a product that displayed a single broad band on both acidic and basic PAGE. The elution volume of ApoD from the Sephadex G-100 column was between those of A-I and A-II. On basic PAGE apolipoprotein D had a mobility intermediate between the bands corresponding to A-II and C-II polypeptides. On acidic PAGE ApoD displayed a single band in the separating gel near the juncture of the separating and stacking gels. Double diffusion analysis demonstrated that ApoD reacted only with antiserum to ApoD but not with antisera to A-I, A-II, C-I, C-II, C-III, LP-B, or albumin. ApoD gave a single precipitin arc on agarose immunoelectrophor-

Table I: Amino Acid Composition of Apolipoprotein D.[a]

	M/10^5 g[b]		M/10^5 g[b]
Lysine	61.3 ± 0.97	Alanine	46.2 ± 3.39
Histidine	10.2 ± 0.93	Half-cystine	19.2 ± 2.49
Arginine	19.1 ± 2.09	Valine	55.2 ± 2.19
Aspartic acid	100.5 ± 2.58	Methionine	9.6 ± 2.93
Threonine	50.4 ± 3.25	Isoleucine	48.2 ± 2.01
Serine	34.1 ± 3.08	Leucine	64.6 ± 2.01
Glutamic acid	94.4 ± 2.02	Tyrosine	26.4 ± 1.21
Proline	52.2 ± 4.37	Phenylalanine	26.0 ± 3.17
Glycine	32.3 ± 2.12	Tryptophan	17.8 ± 0.58

[a] Analysis of three different ApoD samples. [b] Standard deviation.

Table II: Carbohydrate Content of Apolipoprotein D.[a]

	% Weight
D-Glucose	1.89 (1.62–2.16)
D-Galactose	4.42 (3.98–4.86)
D-Mannose	2.96 (2.22–3.24)
D-Glucosamine	4.49 (4.07–4.92)
Neuraminic acid	4.82 (4.50–5.14)

[a] Analysis of two ApoD samples in duplicate.

esis with a mobility similar to the corresponding antigenic determinant present in whole plasma. Partially purified ApoD preparations isolated from the water soluble portion of totally delipidized VLDL and LDL contained a protein which was immunologically and electrophoretically (Figure 1) identical with ApoD isolated from HDL_3. There was, however, no demonstrable ApoD in HDL_2. In all subsequent studies, ApoD was isolated from HDL_3.

Amino acid analyses demonstrated the presence of all common amino acids including half-cystine (Table I). The amino acid composition of ApoD differs from any of the previously isolated polypeptides of the lipoprotein density spectrum (Shore and Shore, 1969). To exclude the possibility that half-cystine stemmed from any contaminating A-II, an ApoD preparation was chromatographed on an anti-A-II-Sepharose 4B immunosorber. The analysis of the ApoD preparation treated in this fashion had essentially the same amino acid composition as untreated ApoD including half-cystine.

Carboxyl terminal amino acid analyses with carboxypeptidases A and B as well as by hydrazinolysis did not yield unequivocal results. Presence of glutamine in the C-terminal portion of ApoD was excluded by analyzing the 30-min carboxypeptidase A and B digest with the lithium citrate buffer system.

Dansylation, dinitrophenylation, and the automated Edman degradation procedures yielded no detectable N-terminal amino acid. It was concluded from these studies that the N-terminal amino acid of ApoD is blocked.

Carbohydrate analyses indicated that ApoD is a glycoprotein containing glucose, mannose, galactose, glucosamine, and sialic acids as the sugar components (Table II). Glucosamine was also identified by the amino acid analysis. These sugars accounted for approximately 18% of the dry weight of ApoD.

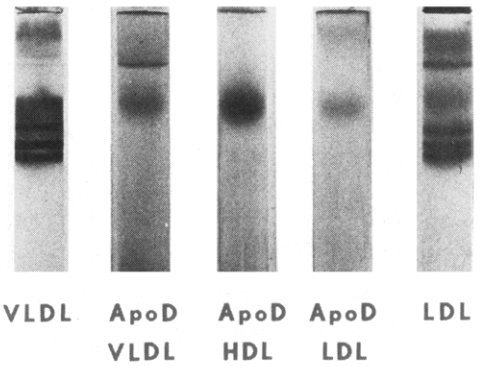

FIGURE 1: Polyacrylamide gel patterns of ApoD isolated from totally delipidized VLDL, LDL, and HDL. PAGE were run on 7% gels in presence of 8 M urea and stained with Coomassie Brilliant Blue. 30–50 μg of protein was applied to each gel.

Molecular weight analyses of ApoD by the Weber–Osborn procedure in the presence and absence of a reducing agent gave values between 19 000 and 26 000. Only a single band was seen on the sodium dodecyl sulfate PAGE gels. Since the molecular weight did not change in the presence or absence of a reducing reagent, this result indicates that ApoD is a single chain polypeptide. Calculation of the minimum molecular weight from the amino acid composition based on 2 mol of methionine per mol of protein gave a value of 18 130. The estimated molecular weight of ApoD, corrected for the 18% carbohydrate content, was 22 100.

Studies on the Lipoprotein Nature of Apolipoprotein D. The ApoD-containing lipoprotein was isolated by two different procedures. The first was based on the assumption that in the carbohydrate moiety the terminal sialic acid is followed either by glucose or mannose. The HDL_3 preparation was treated with neuraminidase and chromatographed on a concanavalin A-Sepharose 4B column. A single immunoprecipitin line formed between the retained fraction and antibodies to HDL_3 gave an identity reaction with ApoD. The unretained fraction, however, exhibited two immunoprecipitin lines with anti-HDL_3. The line closer to the antigen well displayed an identity reaction with ApoD, while the line closer to the antibody well was identified as LP-A. The presence of LP-D in the unretained fraction was due most probably to incomplete removal of sialic acid. The retained fraction was further purified by chromatography on the hydroxylapatite-cellulose column, and the presumably desialylated LP-D fraction was eluted with 0.01 M phosphate buffer. It gave a single precipitin line with antibodies to HDL_3. When injected into a rabbit it produced an antiserum that reacted only with ApoD, but not with A-I, A-II, C-I, C-II, C-III, LP-B, "arginine-rich" polypeptide, or albumin. Basic PAGE analysis demonstrated that the LP-D band had a decreased electrophoretic mobility in comparison with that of untreated LP-D (Figure 2). A similar decrease in electrophoretic mobility, though less pronounced, has been observed when C-III-2 polypeptide was treated with neuraminidase (Brown et al., 1970). Total delipidization of LP-D followed by the thin-layer chromatography of the lipid extract demonstrated the presence of triglycerides, cholesterol, cholesterol esters, and phospholipids.

An alternative procedure for the isolation of LP-D was based on the utilization of a monospecific antiserum to ApoD. Density classes or whole plasma were chromatographed on the anti-ApoD-Sepharose 4B column. The unretained fraction of HDL_3 reacted with antibodies to A-I,

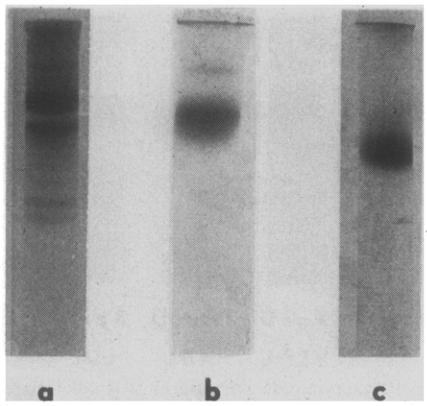

FIGURE 2: Basic polyacrylamide gel electrophoretic pattern of a neuraminidase treated LP-D. 7% gels were run in 8 M urea and stained with Coomassie Brilliant Blue. (a) ApoHDL_3; (b) neuraminidase treated LP-D; and (c) LP-D. 30–50 μg of protein applied to each gel.

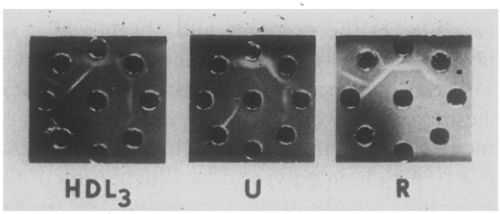

FIGURE 3: Double diffusion patterns of fractions isolated by affinity chromatography of HDL_3 on anti-ApoD-Sepharose 4B. Central wells contain: HDL_3 = starting material; U = unretained fraction; R = retained fraction. Outer wells from the top, clockwise, contain antisera to human A-II, A-I, C-III, C-II, C-I, LP-B, albumin, and ApoD.

A-II, and C-III, but not with antibodies to ApoD (Figure 3). On basic PAGE (Figure 4b), the unretained fraction was characterized by the typical slowly migrating broad A-I and A-II bands and fast moving C-II and C-III bands. The retained fraction reacted strongly with anti-ApoD, and only weakly with antibodies to A-I and A-II. On basic PAGE, the retained fraction displayed three bands with mobilities corresponding to those of A-I, A-II, and ApoD (Figure 4c). Retention of a portion of LP-A with LP-D on the immunosorber indicated either some association of LP-D and LP-A or a nonspecific absorption of LP-A. The LP-A with its A-I and A-II polypeptides was separated from LP-D by hydroxylapatite-cellulose column chromatography (Figure 4d). The fraction eluted with 0.001 M phosphate buffer reacted with antibodies to ApoD but not with any of the other available antisera (Figure 5). On basic PAGE, this LP-D preparation migrated as a single band with a mobility similar to that of ApoD (Figure 1). The LP-D preparations isolated by the same procedure from LDL_2, HDL, VHDL, and whole plasma gave a positive immunoprecipitin reaction only with antibodies to ApoD, and displayed on basic PAGE a single band with a mobility similar to that of ApoD. On immunoelectrophoresis, these LP-D preparations had similar mobilities. On cellulose acetate electrophoresis, LP-D isolated from HDL_3 gave an Oil Red O positive band in the α_1 region. The LP-D preparation from HDL_3 sedimented in the analytical ultracentrifuge as a single symmetrical boundary with $S_{obsd} = 4.75$.

Immunoelectrophoretic analysis of HDL_3 with anti-D and a mixture of antibodies to A-I and A-II demonstrated dissimilar mobilities for LP-D and LP-A (Figure 6). The single precipitin line formed between HDL_3 and a mixture of anti-A-I and anti-A-II supports previous evidence that

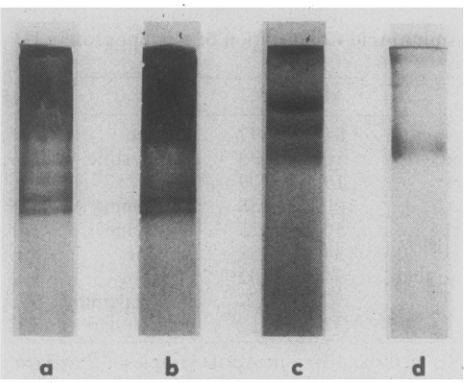

FIGURE 4: Basic polyacrylamide pattern of LP-D isolated by a combination of an immunosorbent and hydroxylapatite column chromatography. 7% gels were run in 8 M urea and stained with Coomassie Brilliant Blue. (a) HDL_3, starting material; (b) unretained fraction eluted from immunosorber; (c) retained fraction; (d) LP-D isolated from the retained fraction, pattern c, by hydroxylapatite column chromatography. 30–50 μg of protein applied to each gel.

A-I and A-II are the constitutive polypeptide chains of LP-A (Albers and Aladjem, 1971; Kostner and Alaupovic, 1972). Direct comparison of LP-D and LP-A on double diffusion against anti-HDL_3 showed a reaction of nonidentity (Figure 7). These results indicate that LP-D and LP-A are not part of the same lipoprotein molecule but occur as separate physical–chemical entities.

Lipid analyses of three LP-D preparations from HDL_3 demonstrated the presence of all common lipid classes (Table III). However, the major constituent of LP-D was protein which accounted for 65–75% of the total weight. The phospholipid composition of four different LP-D preparations revealed relatively high contents of lysolecithin (26.4%, range 24.1–29.3%) and sphingomyelin (33.3%, range 30.0–35.5%) and a relatively low content of lecithin (40.1%, range 35.2–43.0%).

Discussion

In continuation of our previous studies (McConathy and Alaupovic, 1973) we have provided additional evidence that ApoD differs immunologically and chemically from any other human apolipoprotein or its constitutive polypeptides, i.e., A-I, A-II, ApoB, C-I, C-II, C-III, and "arginine-rich" polypeptide. ApoD is characterized by the presence of all common amino acids including half-cystine. Results based on three different methods indicate that the N-terminal amino acid of ApoD is blocked. Studies on the C-terminal amino acid were inconclusive. The uniqueness of ApoD among serum apolipoproteins lies, however, in its high content of sugar constituents which account for approximately 18% of its dry weight. In contrast, other apolipoproteins or polypeptides contain less than 10% carbohydrate (Ayrault-Jarrier et al., 1961; Marshall and Kummerow, 1962; Shulman et al., 1974). The molecular weight of ApoD estimated from the amino acid and carbohydrate analyses was 22 100 in agreement with results reported by Kostner (1974a). This molecular weight was supported by the results of the dodecyl sulfate PAGE experiments. Slightly variable molecular weights obtained by the dodecyl sulfate PAGE method may be due to the anomalous behavior of glycoproteins in this system (Segrest et al., 1971).

Studies on the immunochemical identification and preparative isolation of ApoD from major density classes indicated that ApoD is distributed across the entire density

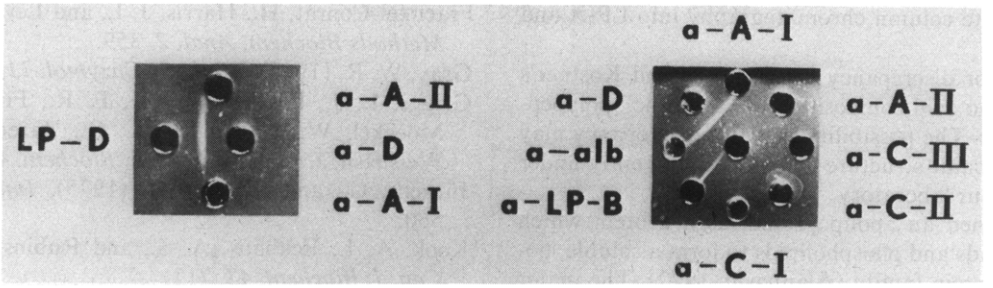

FIGURE 5: Double diffusion pattern of LP-D isolated from HDL_3 by a combination of immunosorber and hydroxylapatite chromatography.

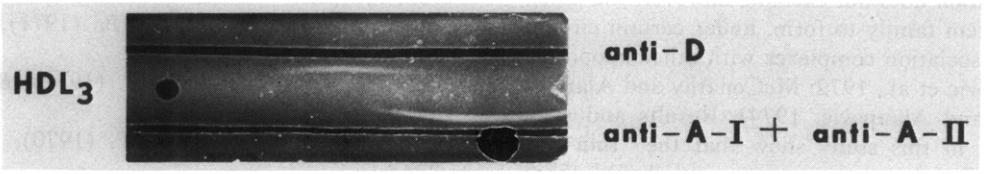

FIGURE 6: Nonidentical mobilities of LP-D and LP-A present in HDL_3.

Table III: Lipid Composition of LP-D.[a]

Triglycerides	Cholesterol Ester	Free Cholesterol	Phospholipids
11.15	19.12	19.02	50.71
12.21	33.69	17.06	37.04
Trace	30.20	18.16	51.61

[a] Three different preparations of LP-D.

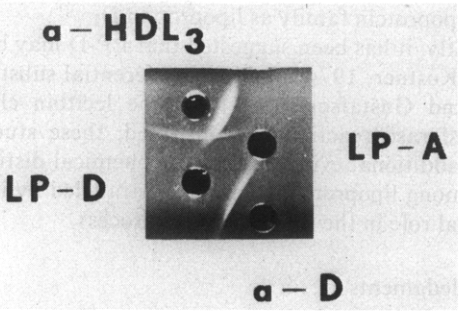

FIGURE 7: Nonidentity of LP-D and LP-A isolated from HDL_3. a = anti.

spectrum with the maximum concentration in HDL_3 and the minimum concentration in the HDL_2 segment. These results agree with previous reports (Alaupovic et al., 1966; Lee and Alaupovic, 1970; Alaupovic et al., 1972).

ApoD occurs in the serum as a lipoprotein which was isolated from HDL_3 by two different chromatographic procedures. Concanavalin A-Sepharose chromatography of neuraminidase-treated HDL_3 followed by hydroxylapatite chromatography yielded a lipoprotein that gave a reaction of identity with ApoD and upon immunization a monospecific antiserum to ApoD. Alternatively, LP-D was isolated from whole serum, LDL, HDL, or VHDL by a procedure utilizing an immunosorber to ApoD and hydroxylapatite column chromatography. We have concluded from these studies that ApoD exists mainly in the form of a distinct, unassociated lipoprotein family.

In a recent study on the isolation of "thin-line" polypeptide, Kostner (1974a) has suggested that this protein moiety should be considered another constitutive polypeptide of LP-A rather than a separate apolipoprotein. Consequently, he has proposed that "thin-line" polypeptide be designated as A-III. The main argument for considering "thin-line" polypeptide as an integral component of LP-A particles was based on immunosorption experiments which indicated that "thin-line" polypeptide could be completely coprecipitated from whole serum by monospecific antibodies to A-I polypeptide. In contrast, our conclusion leading to the designation of "thin-line" polypeptide as a distinct apolipoprotein capable of forming its own lipoprotein family was based on the following experimental results: (1) in double diffusion experiments LP-D and LP-A form not a single but two separate precipitin lines against a mixture of their corresponding antibodies or an antiserum to HDL_3; the LP-A line encompasses both of the constitutive A-I and A-II polypeptides, whereas the LP-D line only contains ApoD; (2) when whole serum is tested in double diffusion against a mixture of antibodies to ApoD, A-I, and A-II polypeptides, LP-D gives a nonidentity reaction with LP-A; (3) when whole serum is tested in immunoelectrophoresis against antibodies to LP-A and LP-D (McConathy and Alaupovic, 1974b), the immunoprecipitin arc of LP-D has a slower mobility than that of LP-A; (4) ApoD occurs in LP-X (Magnani et al., 1973) and in LP(a) (Jürgens and Kostner, 1975) in the absence of A-I and A-II polypeptides; (5) in the LP-A deficiency disease (Gustafson et al., unpublished results) LP-D occurs in LDL in the absence of A-I; (6) LP-D can be found in normal LP-A-free VLDL; and (7) LP-D can be isolated from whole serum by an immunosorber to ApoD. One of the explanations for the coprecipitation of LP-D by an antiserum to A-I may be the possible occurrence of a small amount of undetected antibodies to ApoD sufficient to precipitate an equally small amount of LP-D present in the whole serum. It should be noted that Kostner and Alaupovic (1971) utilized similar methodology, i.e., immunoprecipitation of HDL_3 by antibodies to either A-I or A-II polypeptides presented evidence that "thin-line" polypeptide is not associated with LP-A. On the other hand, it is possible that the coprecipitation may have been brought about either by a nonspecific interaction or the presence of a certain amount of an association complex of LP-A and LP-D. Present results show, however, that the weak association complex of these two lipoproteins can easily be dissociated

by hydroxylapatite column chromatography into LP-A and LP-D.

The reasons for discrepancy between our and Kostner's data on the amino acid composition of "thin-line" polypeptide are not clear. The possibility that this discrepancy may be due to the subunit structure of ApoD is currently under investigation in our laboratory.

We have defined an apolipoprotein as a protein which binds neutral lipids and phospholipids to form a soluble, polydisperse lipoprotein family (Alaupovic, 1972). The major characteristic of a lipoprotein family is the presence of a single, distinct apolipoprotein or its constitutive polypeptides. This definition does not exclude, however, the potential of a lipoprotein family to form, under certain circumstances, weak association complexes with other lipoprotein families (Alaupovic et al., 1972; McConathy and Alaupovic, 1974a; Lee and Alaupovic, 1974). Results and arguments presented in this study show that the "thin-line" polypeptide satisfies these requirements and should, therefore, be considered as a unique apolipoprotein designated according to the ABC nomenclature as apolipoprotein D and its lipoprotein family as lipoprotein D.

Recently, it has been suggested that LP-D may be the activator (Kostner, 1974b) and/or preferential substrate (Olofsson and Gustafson, 1974) for the lecithin–cholesterol acyltransferase reaction. If confirmed, these studies may provide additional evidence that the chemical distinction of LP-D among lipoprotein families is paralleled by its special functional role in the lipid transport process.

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