Isolation and Partial Characterization of a New Acidic Apolipoprotein (Apolipoprotein F) from High Density Lipoproteins of Human Plasma†

Sven-Olof Olofsson,‡ Walter J. McConathy, and Petar Alaupovic*

ABSTRACT: This report describes the isolation and partial characterization of a minor polypeptide from human plasma high density lipoproteins. To isolate this polypeptide, a combination of gel permeation and carboxymethylcellulose column chromatography was utilized. This procedure resulted in the isolation of a homogeneous polypeptide designated carboxymethylcellulose:unretained polypeptide (CM:U). On 7% polyacrylamide gel electrophoresis, it migrated as a single band into a position similar to the recently described ApoD (McConathy, W. J., & Alaupovic, P. (1973) *FEBS Lett. 37*, 178). Amino acid analyses demonstrated the presence of all common amino acids except tryptophan. The molecular weight was estimated to be 26 000–32 000. Isoelectric focusing gave a relatively low isoelectric point (p*I* = 3.7). These results suggest that the CM:U may represent the most acidic polypeptide of the human plasma lipoprotein system. Monospecific antiserum to CM:U only reacted with CM:U and not with any of the known apolipoproteins or their constitutive polypeptides. The apolipoprotein nature of this polypeptide was indicated by the uptake of Oil Red O by the precipitin arcs formed when anti-CM:U was reacted against LDL and HDL. Nonidentity reactions between the known lipoprotein families LP-A, LP-B, LP-C, LP-D, and LP-E and the lipoprotein form of CM:U are strong indications that CM:U is the protein moiety of a distinct lipoprotein family. This conclusion was supported by the results of qualitative electroimmunoassay. Whole serum and HDL gave two rockets with a mixture of antisera to CM:U, A-I, and A-II. One rocket corresponded to LP-A and the other to the lipoprotein form of CM:U. These results suggest that the CM:U represents a new minor apolipoprotein designated according to the ABC nomenclature, apolipoprotein F (ApoF), and its corresponding lipoprotein family, lipoprotein F (LP-F).

The protein complement of the lipoprotein system has been shown to consist of a number of nonidentical polypeptides (Shore & Shore, 1968, 1969; Brown et al., 1969, 1970; McConathy & Alaupovic, 1973; Shelburne & Quarfordt, 1974). According to the nomenclature based on the concept of lipoprotein families (Alaupovic, 1972), these polypeptides are referred to as A-I, A-II, ApoB, C-I, C-II, C-III, ApoD, and ApoE and their corresponding lipoprotein families as LP-A, LP-B, LP-C, LP-D, and LP-E.

In addition to these partially or completely characterized apolipoproteins or their constitutive polypeptide chains, immunochemical studies in our laboratory have indicated the presence of antigenic components in the human lipoprotein system not associated with any of the known constituents of this system. Basic polyacrylamide electropherograms have also indicated the presence of uncharacterized protein bands. This report describes the isolation and partial characterization of one of these minor polypeptide components of the human lipoprotein system.

Materials and Methods

Lipoproteins were isolated from fresh human plasma obtained by plasmapheresis from normolipidemic subjects after an overnight fast or from 2 L of pooled outdated plasma.

Isolation of HDL.[1] For preparation of HDL, 2 L of pooled outdated plasma were made 0.01% with respect to sodium azide (Eastman Kodak Co., Rochester, N.Y.) and thiomerosal (Sigma, St. Louis, Mo.). The plasma was adjusted to a solution density of 1.23 g/mL with solid KBr (J. T. Baker Chemical Co., Phillipsburg, N.J.) and centrifuged in a Ti 50.2 rotor (Beckman, Palo Alto, Calif.) at 45 000 rpm for 22 h. The supernate was recovered by a tube slicing technique, readjusted to density 1.23 g/mL with solid KBr and again subjected to ultracentrifugation under the same conditions. The supernate, containing the serum lipoproteins, was recovered by tube slicing, dialyzed against redistilled water for 20 min, and readjusted to a solution density of 1.073 g/mL with KBr. Ultracentrifugation was carried out under the same conditions as described above. The HDL containing infranate was recovered by tube slicing and subjected to two or three washes at 35 000 rpm for 40 h at density 1.23 g/mL. The HDL were free from albumin as demonstrated by double-diffusion analysis.

The HDL isolated by this procedure were compositionally indistinguishable from normal fasting HDL isolated by standard sequential ultracentrifugation (Alaupovic et al., 1966, 1972).

Delipidization. HDL were dialyzed against five changes of redistilled water and lyophilized in eight 50-mL centrifuge tubes. Lyophilized HDL (1.2–1.6 g of protein) were mixed with 1 volume of chloroform and shaken by inverting the tube several times and 2 volumes of methanol was added. The tubes

† From the Laboratory of Lipid and Lipoprotein Studies, Oklahoma Medical Research Foundation and Department of Biochemistry and Molecular Biology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73104. *Received July 14, 1977.* This study was supported in part by Grants HL-6221 and HL-7005 by the U.S. Public Health Service and by resources of the Oklahoma Medical Research Foundation.

‡ Present address: University of Göteborg, Department of Medicine I, Sahlgren's Hospital, Göteborg, Sweden.

[1] Abbreviations used: VLDL, very low density lipoproteins ($d < 1.006$ g/mL); LDL, low density lipoproteins ($d = 1.006$–1.063 g/mL); HDL, high density lipoproteins ($d = 1.063$–1.21 g/mL); CM:U, the carboxymethylcellulose unretained polypeptide.

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were shaken three times while stored at −10 °C for 30 min. Following low speed centrifugation at 6 °C the solvent was aspirated and the precipitated protein was treated again in the same fashion. The residue was extracted four additional times with chloroform:methanol (2:1, v/v) followed by two extractions with diethyl ether. Delipidized HDL (apoHDL) were solubilized in 2 M acetic acid and the diethyl ether was evaporated under a gentle stream of nitrogen. Ninety-nine percent of apoHDL was soluble after the addition of UltraPure urea crystals to approximately 2 M with respect to urea.

The degree of delipidization of HDL was assessed in two ways. Organic phosphorus was determined as described by Alaupovic et al. (1966). Fatty acid content of two different apoHDL samples (40 mg) was determined after hydrolysis of apoHDL in 6 N HCl for 24 h at 110 °C and methylation of the fatty acids (Mason & Waller, 1964). Analyses of the methylated fatty acids were performed in duplicate by gas–liquid chromatography (Packard Becker 420, Packard, Inc., Downes Grove, Ill.) utilizing N_2 as carrier gas (flow rate, 20 mL/min) and 15% DEGS on 80–180 mesh Chromasorb W AW. Injection block temperature was 220 °C and the oven, 185 °C. The two procedures indicated the presence of less than 0.02% organic phosphorus and less than 0.2% fatty acids. These analyses demonstrated a relatively complete delipidization of HDL.

Gel Chromatography. ApoHDL (1.2–1.6 g) in a volume of 50–60 mL was applied to a Sephadex G-100 (Pharmacia Fine Chemicals, Piscataway, N.J.) column (5.0 × 150 cm, Glenco Scientific, Inc., Houston, Texas) equilibrated with 2 M acetic acid (flow rate, 30 mL/h). Thirteen-milliliter fractions were collected and monitored at 280 nm with a spectrophotometer (Gilford Inst., Oberlin, Ohio). Fractions were combined on the basis of elution pattern and lyophilized.

Chromatography on Sephadex LH-20. Apolipoprotein fractions were redissolved in 2-butanol:glacial acetic acid:water (4:1:5, v/v/v) and chromatographed on a Sephadex LH-20 (Pharmacia Fine Chemicals, Piscataway, N.J.) column (2.5 × 90 cm) equilibrated with the same solvent (Rudman et al., 1970); the flow rate was 30 mL/h. Aborbancy at 296 nm was measured and the protein fractions were pooled and evaporated to dryness in a rotatory evaporator.

Ion-Exchange Chromatography. Carboxymethylcellulose (Cellex-CM, Bio-Rad Lab., Richmond, Calif.) was extensively washed with 0.5 N NaOH, water, 0.5 N HCl, water, 0.5 N NaOH and again with water. The slurry of carboxymethylcellulose was adjusted to pH 3.5 with 1.0 N hydrochloric acid and poured into a column (k15/35, Pharmacia Fine Chemicals, Piscataway, N.J.). Prior to use the ion-exchange bed was equilibrated with the eluting buffer 0.001 M KH_2PO_4, pH 3.5, in 8 M urea. The urea solution was deionized on a mixed bed ion-exchange resin prior to addition of the KH_2PO_4 and adjustment of pH. All column chromatographic procedures were run at room temperature.

Electrophoresis. Basic (Davis, 1964) and acidic (Reisfeld et al., 1962) polyacrylamide gel electrophoreses were carried out on 7% acrylamide gels containing 8 M urea. Gels were stained with Coomassie Brilliant Blue (Chrambach et al., 1967).

Molecular Weight Determination. Two procedures were utilized for estimation of molecular weight: sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Weber & Osborn, 1969) in the presence of mercaptoethanol and gel chromatography on a Sephadex G-100 column (2.5 × 100 cm), equilibrated with 0.001 M KH_2PO_4, pH 3.5, containing 8 M urea and 0.1 M mercaptoethanol. Distribution coefficients (K_d) were calculated for standards and samples (Gelotte, 1960).

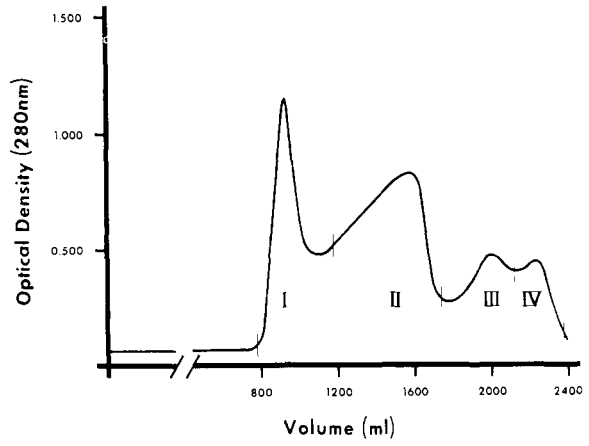

FIGURE 1: Elution profile of apoHDL applied to a Sephadex G-100 column (5.0 × 150 cm) equilibrated in 2 M acetic acid.

Isoelectric Focusing. Analytic isoelectric focusing in 7% polyacrylamide gels was performed with an ampholine range of pH 4–6 in the presence of 8 M urea (Wrigley, 1968). Gels were stained with Coomassie Brilliant Blue (Chrambach et al., 1967).

Preparative isoelectric focusing of apoHDL at 8 °C was carried out in the presence of deionized 8 M urea on a LKB ampholine electrofocusing column (Type 8101, LKB, Bromma, Sweden) utilizing a pH gradient of 3.5 to 5. The column was eluted in 2-mL fractions and each fraction was analyzed by double diffusion, the pH, basic polyacrylamide gel electrophoresis, and absorbancy at 280 nm.

Amino Acid Analysis. Hydrolysis was performed at 110 °C for 24 h in 6 N HCl in evacuated sealed tubes. Analyses were performed as previously described (Lee & Alaupovic, 1970) except a single column system with the Bechman AA 15 resin was utilized. Half-cystine was determined as cysteic acid (Moore, 1963). Tryptophan was determined following hydrolysis in *p*-toluenesulfonic acid as described by Liu & Chang (1971).

Immunological Methods. Double diffusion analyses in 1% agar and immunoelectrophoresis in 1% agarose were performed as previously described (Alaupovic et al., 1972). Preparation of A-I, A-II, ApoB, C-I, C-II, C-III, ApoD, ApoE, and LP-B and their corresponding antisera have been previously described (Alaupovic et al., 1972; McConathy & Alaupovic, 1973, 1976; Curry et al., 1976). The antiserum to Lp(a) was provided by K. Berg, M.D., University of Oslo, Norway. Qualitative electroimmunoassay was done as described by Curry et al. (1977).

Antiserum to CM:U was prepared by dissolving CM:U (0.3 mg) in 1 mL of sterile 0.9% sodium chloride. The CM:U solution was mixed with equal amounts of Freund's complete adjuvant and injected intraperitoneally into white New Zealand rabbits. Three or four additional injections were necessary to obtain precipitating antibodies. Blood was drawn by cardiac puncture.

Results

The polypeptide designated carboxymethylcellulose unretained polypeptide (CM:U) was isolated from apoHDL. ApoHDL was fractionated into four fractions by gel filtration on Sephadex G-100 (Figure 1). Several experiments indicated that the first fraction (fraction I) was the most suitable for the isolation of CM:U. However, CM:U was also present in the second fraction (fraction II) which contained A-I as the main constituent. The major constituents of third and fourth frac-

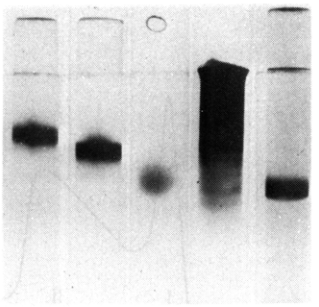

FIGURE 2: Basic polyacrylamide gel electrophoresis of CM:U. a = A-I; b = A-II, c = ApoD; d = fraction I; e = CM:U.

tions were A-II and ApoC polypeptides, respectively.

Fraction I was lyophilized, dissolved in 2-butanol:glacial acetic acid:water (4:1:5, v/v/v) (Rudman et al., 1970), and chromatographed once on Sephadex LH-20 in order to remove any residual lipid. The protein eluate (100–150 mL; 100–200 mg of protein) from Sephadex LH-20 was evaporated to dryness, dissolved in 0.001 M KH_2PO_4, pH 3.5, containing 8 M urea, and chromatographed on carboxymethylcellulose (flow rate, 25 mL/h) using the solubilizing buffer to elute the unretained fraction. The unretained fraction was eluted in a volume of 60–80 mL. Chromatography of this fraction was repeated until it was free from A-I, A-II, and ApoE as demonstrated by double-diffusion analyses. Three chromatographies over carboxymethylcellulose were usually sufficient to yield a homogeneous preparation of CM:U. This isolated polypeptide gave no reaction with antisera to LP-B; ApoB; C-I, C-II, C-III; or LP-D.

The CM:U was desalted on a Sephadex G-25 column equilibrated with 2 M acetic acid. This material was lyophilized and used for all subsequent analyses. The yield of CM:U from fraction I was approximately 2 mg.

Polyacrylamide Gel Electrophoresis. On basic polyacrylamide gel electrophoresis CM:U (Figure 2e) moved as a single protein band in the region of ApoD (Figure 2c). Reduction of CM:U with mercaptoethanol or carboxymethylation of CM:U had no effect on the mobility of CM:U. It did not migrate into the separating gel on acidic polyacrylamide gel electrophoresis.

Molecular Weight Estimation. The CM:U gave a single protein band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis with an R_f value of 0.47 ± 0.01 ($\bar{x}$ ± SD, n = 8) corresponding to a molecular weight of 28 000 ± 1200.

On gel chromatography, the CM:U was eluted with a K_d of 0.14 (mean of five experiments), corresponding to a molecular weight of 26 000.

Estimation of the minimum molecular weight from the amino acid composition (Table I) assuming 1 mol of histidine per mol of protein gave a calculated molecular weight of 32 000. These different procedures are indicative of a molecular weight of 26 000–32 000 for CM:U.

Isoelectric Focusing. Analytical isoelectric focusing on polyacrylamide gels in 8 M urea utilizing a pH gradient between 4 and 6 revealed a single band in the lower pH range corresponding to a p$I \leq 4$ (Figure 3). This band was not present in blanks; however, to ascertain that it corresponded to the CM:U, the polyacrylamide gel was sliced longitudinally and embedded in 1% agar. The embedded gel reacted with monospecific antiserum to the CM:U (a-CM:U) and the observed precipitin arc (Figure 3) was in the same region as the stained band. Data from analytical isoelectric focusing experiments correlated with the preparative focusing results indicating a pI = 3.7 for CM:U. The CM:U isolated by preparative isoelectric focusing of apoHDL was identified by basic polyacrylamide gel electrophoresis and double diffusion analyses with anti-CM:U. Besides CM:U, no other protein staining bands were detectable on basic polyacrylamide gel electrophoresis gels of fraction with pH <3.7.

TABLE I: Amino Acid Composition of the CM:U.

	mol/10^3 mol of amino acids[a]
Lys	44.5 ± 3.8
His	3.2 ± 1.6
Arg	23.1 ± 3.3
Asp	95.2 ± 7.2
Thr	59.6 ± 2.1
Ser	81.4 ± 3.0
Glu	136.0 ± 7.2
Pro	24.7 ± 6.9
Gly	118.1 ± 13.0
Ala	122.0 ± 13.2
Val	73.5 ± 5.1
½-cystine[b]	14.1 ± 2.2
Met	16.8 ± 3.0
Ile	38.1 ± 2.7
Leu	93.8 ± 5.0
Tyr	42.6 ± 6.3
Phe	5.7 ± 0.6
Trp	0

[a] $\bar{x}$ ± SD, n = 5 different preparations. [b] Determined as cysteic acid.

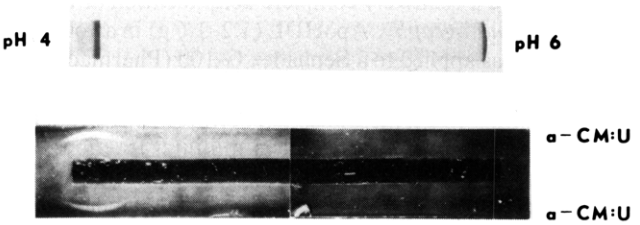

FIGURE 3: Analytical isoelectric focusing of the CM:U. Protein stained gel (upper pattern) and the embedded gel in 1% agar (lower pattern). Embedded gel was reacted against antiserum to the CM:U (a-CM:U). Ampholine gradient between pH 4 and 6 was used.

Amino Acid Composition. Amino acid analyses indicated the presence of all common amino acids except tryptophan. The amino acid composition of CM:U differed from those of previously reported polypeptide chains (Shore & Shore, 1969, 1973; Shelbourne & Quarfordt, 1974; Kostner, 1974; McConathy & Alaupovic, 1976; Lim et al., 1976; Kostner, 1976).

Immunochemistry. Antiserum raised to CM:U reacted with CM:U but gave no reaction with the known apolipoproteins or albumin. Immunoelectrophoresis of freshly drawn whole serum with anti-CM:U only gave a single precipitin arc in the region of α_1-globulins. By double diffusion analyses, anti-CM:U gave a single precipitin line with all fresh whole serum and HDL samples tested. These data indicated the monospecific nature of this antiserum and the occurrence of CM:U in all examined normal sera ($n > 20$).

The CM:U did not react with antisera to A-I, A-II, C-I, C-II, C-III, ApoD, LP-B, and ApoE, but reacted with monospecific antiserum to the CM:U. A reaction of complete identity between CM:U, fraction I, HDL, and whole serum was observed when tested against the monospecific antiserum to CM:U.

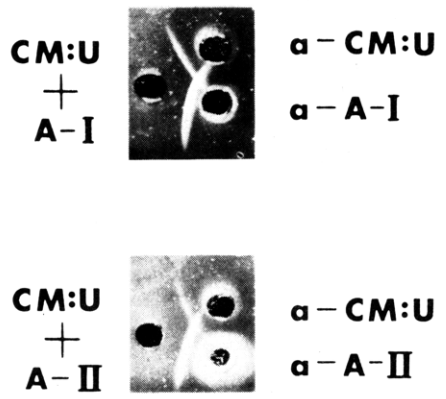

FIGURE 4: The nonidentity reaction of CM:U with A-I and A-II by double diffusion in 1% agar. a = anti.

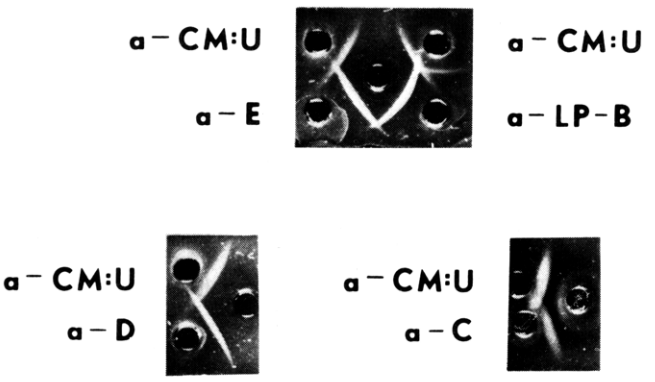

FIGURE 5: The nonidentity reaction of the lipoprotein form of CM:U and LP-B, LP-C, LP-D, and LP-E. Central wells contain HDL. a = anti.

FIGURE 6: The nonidentity reaction between LP-A and the lipoprotein form of CM:U in LDL. a = anti.

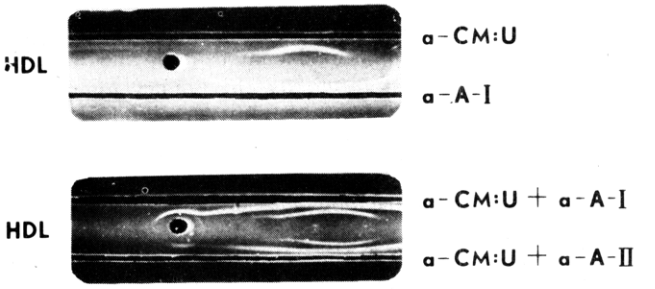

FIGURE 7: The immunoelectrophoretic mobility of the lipoprotein form of CM:U and LP-A in HDL. a = anti.

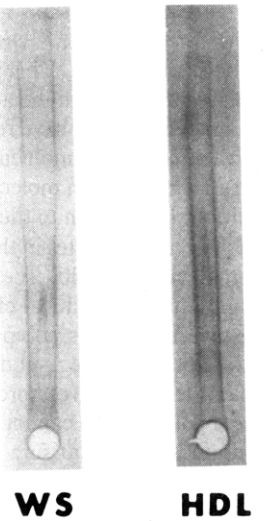

FIGURE 8: Qualitative electroimmunoassay of whole serum and HDL against a mixture of antisera to A-I, A-II, and CM:U. Outer rocket represents LP-A and the inner rocket, the lipoprotein form of CM:U. WS = whole serum.

The CM:U showed nonidentity reactions both with anti-A-I and anti-A-II sera (Figure 4). As shown in Figure 5 the reaction of nonidentity of CM:U and the minor components of HDL, i.e., LP-B, LP-C, LP-D, and LP-E, was also demonstrated. In addition, the nonidentity of Lp(a) and CM:U was demonstrated by the reaction of nonidentity between antisera to Lp(a) and CM:U when tested against Lp(a) positive whole serum. These data are indicative of the homogeneity of CM:U and its corresponding antiserum.

The apolipoprotein nature of CM:U was suggested by the ability of the precipitin line to take up Oil Red O stain, when HDL and LDL were reacted with anti-CM:U. Furthermore, double-diffusion studies utilizing monospecific antiserum to CM:U gave strong reactions with HDL and LDL but only faint or no reaction with VLDL and the portion of plasma of density greater than $d = 1.23$ g/mL.

In order to investigate whether CM:U forms its own lipoprotein family or should be considered a constitutive polypeptide of an already known lipoprotein family, HDL were tested by double diffusion against anti-CM:U and antisera to ApoE, LP-B, LP-D, and ApoC. The nonidentity between the lipoprotein formed by the CM:U and these various lipoproteins was clearly demonstrated (Figure 5). The large differences in concentrations between LP-A and the lipoprotein formed by CM:U in HDL made the interpretation of the double-diffusion patterns difficult when HDL was tested against anti-CM:U and anti-A-I or anti-A-II. However, the nonidentity reaction between these two lipoprotein families in LDL was evident (Figure 6). To achieve further information about the relationship between LP-A and CM:U within HDL, the reactivity of HDL against monospecific antiserum to CM:U and anti-A-I or A-II was tested by immunoelectrophoresis (Figure 7). The immunoelectrophoretic patterns of HDL with mixtures of antisera to CM:U and either A-I or A-II clearly demonstrated that the lipoprotein corresponding to CM:unretained polypeptide precipitated as a single arc distinct from that of LP-A. These data were verified by the qualitative electroimmunoassay. Whole serum and HDL gave two rockets with a mixture of antisera to the CM:U, A-I, and A-II. The outer rocket corresponded to LP-A, the protein moiety of which consists of A-I and A-II (Curry et al., 1977). The inner rocket represented the lipoprotein form of CM:U (Figure 8).

Discussion

In this report, we have described a reproducible procedure for the isolation of an acidic polypeptide from human plasma high density lipoproteins utilizing a combination of gel- and ion-exchange chromatography. Characterization of the polypeptide as to its behavior on polyacrylamide gel electro-

phoresis, isoelectric point, Oil Red O staining of precipitin lines, molecular weight, amino acid composition, and immunochemical properties has shown that this is a new minor apolipoprotein of the human lipoprotein system.

Basic polyacrylamide gel electrophoresis of CM:U gave a single protein band with a migration similar to that of several polypeptides found in the human lipoprotein system. Thus, the recently described ApoD (McConathy & Alaupovic, 1973, 1976) has a similar migration on basic polyacrylamide gel electrophoresis. However, these two polypeptides differ in molecular weight and amino acid composition. Furthermore, the CM:U did not react with antiserum to ApoD and there was a reaction of nonidentity between the lipoprotein forms of the two polypeptides (Figure 5). Kostner has reported the isolation of a polypeptide with a mobility similar to CM:U which was designated "A-III" (Kostner, 1974). This polypeptide differed from the CM:U with respect to its amino acid composition and molecular weight. Lim et al. (1976) have reported the presence of a polypeptide with an apparent molecular weight of 7000. It differed, however, from CM:U in molecular weight and the amino acid composition. In addition to these polypeptides, the monomeric form of A-II also migrates in this region, but differs from CM:U in its molecular weight, isoelectric point, amino acid composition, and immunochemical characteristics. Since there are possibly several distinct polypeptides that have a similar migration rate between the A-II dimer and C-II band on basic polyacrylamide gel electrophoresis, an unequivocal identification of these polypeptides cannot be achieved without the availability of monospecific antisera to each of these polypeptides. This is particularly important for HDL, since all of the polypeptide chains that are localized on basic polyacrylamide gel electrophoresis between A-II and C-II have been isolated from this density class.

The results of isoelectric focusing indicated a relatively low p*I* as demonstrated by preparative and analytical isoelectric focusing of the CM:U. This characteristic is in agreement with its relatively rapid migration on basic polyacrylamide gel electrophoresis, and the lack of cathodal migration on acidic polyacrylamide gel electrophoresis. Our studies have shown that the CM:U has the lowest isoelectric point of the described apolipoproteins and may represent the most acidic polypeptide chain of the human lipoprotein system.

The relation between the lipoprotein form of the CM:U and the known lipoprotein families was studied. The immunochemical studies indicate that the CM:U forms its own lipoprotein family separate from the known lipoprotein families LP-A, LP-B, LP-C, LP-D, and LP-E. Utilizing the concept of lipoprotein families (Alaupovic, 1972), we suggest that the carboxymethylcellulose unretained polypeptide should tentatively be designated as apolipoprotein F (ApoF) and its corresponding lipoprotein family as lipoprotein F (LP-F). Further work is now in progress to isolate and characterize this lipoprotein family.

Acknowledgments

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