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THE PRIMARY STRUCTURE OF HUMAN HIGH DENSITY

APOLIPOPROTEIN A-II (ApoLP-Gln-II)

APPROVED BY

Robert Delaney

Jordan J. H. Tang

Leon Unger

Martin J. Griffin

Joseph J. Ferretti

DISSERTATION COMMITTEE

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THE PRIMARY STRUCTURE OF HUMAN HIGH DENSITY APOLIPOPROTEIN A-II (ApoLP-Gln-II)

CHAPTER I

INTRODUCTION

Plasma lipoprotein was first generally recognized early in the 1920's as a soluble lipid-protein complex present in the plasma. The successful isolation of a lipoprotein from horse plasma with constant composition by Macheboeuf in 1929 (1) marked the beginning of an extensive research and understanding of plasma lipoproteins. During the next two decades, the accumulated knowledge obtained from the study of lipoproteins led to the recognition of the significance of abnormal concentrations of plasma lipids in the genesis and development of atherosclerosis. This period, focused on lipid study, resulted in a very detailed characterization of the lipid components of lipoproteins. However, it was not until the late fifties that the protein moieties of lipoproteins were recognized as important for the structural stability and specificity of plasma lipoproteins. Additional information revealed both the necessity for the presence of certain protein moieties for lipid transport and that many human

genetic disorders are due to the alteration or absence of these protein moieties (2). Recent studies are mostly concentrated on the structure of lipoproteins, mainly the primary structure of the protein moieties, and the interactions between lipids and proteins as well as between different protein moieties. Undoubtedly, new knowledge in this field will assist in understanding the lipid transport mechanisms and the mutations which affect these processes.

Classifications and Nomenclature of Plasma Lipoproteins

The major systems of classification of plasma lipoproteins were based on sets of operational terms which have been derived from the various methods of isolation and study. Plasma lipoproteins were classified into α-lipoproteins, β-lipoproteins, and pre-β-lipoproteins because their electrophoretic mobilities corresponded to those of α and β globulin fractions (3).

When ultracentrifugal techniques were used for analysis and preparative isolation of plasma lipoproteins, a second classification and nomenclature was established (4). Based on density ranges, plasma lipoproteins were usually classified into four major groups; chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL), and high density lipoproteins (HDL). The protein moieties of all densities were divided into three groups designated as Apolipoprotein A (ApoA), Apolipoprotein B (ApoB), and Apolipoprotein C (ApoC). The density spectrum, electrophoretic mobility,

molecular weight, and protein components of lipoproteins based on these two systems are summarized in Table 1. The classifications according to electrophoretic mobility and ultracentrifugation represent a heterogenous system with respect to protein moieties; possibly additional polypeptides will be identified in the future. To overcome these inadequate systems, a new classification based on apoproteins as the only distinct chemical components for the differentiation of lipoproteins was proposed recently (5). Three lipoprotein families were recognized, each of which is characterized by the presence of a single apoliprotein: lipoprotein family A (Lp-A) is characterized by the presence of ApoA, lipoprotein family B (Lp-B) by ApoB, and lipoprotein family C by ApoC. Each family provides protein homogeneity, but may have various hydrated densities and occur simultaneously in several density classes. However, in each density class, there is only one dominant apolipoprotein, ApoA in HDL, ApoB in LDL, and ApoC in VLDL. Fig. 1 presents the correlations among these three classification systems.

Structure and Function of Plasma Lipoproteins

According to Cook et al. (8), two types of lipoproteins have been postulated. They are micellar lipoproteins and so called pseudomolecular lipoproteins. The micellar type contain less than 30 per cent protein and includes those at the low density end of the spectrum: chylomicrons, VLDL, and LDL. The general structural features of micellar lipoproteins

TABLE I

CLASSIFICATIONS OF HUMAN PLASMA LIPOPROTEINS BASED ON ELECTROPHORETIC MOBILITY AND HYDRATED DENSITY RANGE (6)

Density Class (a)	Density Range (g/ml)	Electrophoretic mobility on paper	Flotation Characteristics sf (b)	Approximate Molecular Weight (in millions)	Apolipoproteins (c)
Chylomicrons	< 0.95	origin	> 400	10^3-10^4	C,B,A
VLDL	0.95-1.006	pre-β	20-400	5-100	C,B,A
LDL	1.006-1.063	β	0-20	2-3	B,C,A
HDL	1.063-1.21	α	-	0.25	A,C,B

(a) VLDL = very low density lipoproteins; LDL = low density lipoproteins; and HDL = high density lipoproteins. (b) sf designates the negative sedimentation coefficient in Svedbergs in density 1.063 g/ml NaCl solution at 26° C. (c) In order of relative abundance.

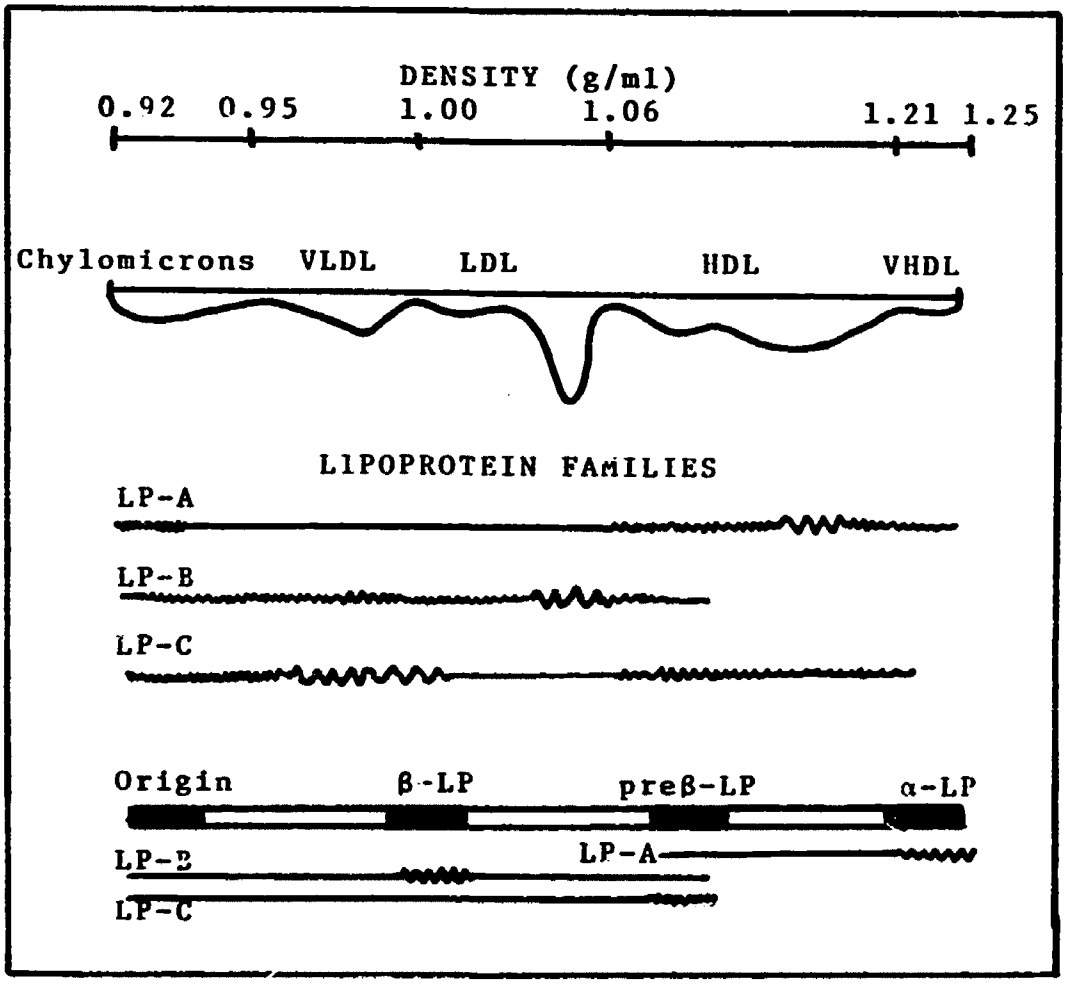


Figure 1--Correlation of the Classification Systems of Human Plasma Lipoproteins (7). The amplitudes of the zigzag lines are approximately proportional to the concentrations of lipoprotein families along the density gradient.

were suggested to consist of a hydrophobic core of triglycerides and cholesterol esters surrounded by a hydrophilic coat of protein, phospholipid, and free cholesterol. Calculations indicated that the amount of protein, phospholipid, and free cholesterol would just be sufficient to form a surface coat with the molar ratio of phospholipid to free cholesterol approximately 1:1. By electron microscopy all micellar lipoproteins appeared as spherical particles which had diameters ranging from 1200-11,000 Å, 300-700 Å, 170-260 Å for chylomicrons, VLDL, and LDL, respectively (13). Recently it was reported that this coat material was able to be separated from the core by the freeze-thawing of chylomicrons (9,10). A possible model suggested for micellar lipoprotein is a spherical particle with mosaic composed of extended protein interacting with 1:1 phospholipid-cholesterol complexes. The polar side of this surface coat would be exposed to the surrounding aqueous medium, while the apolar side would interact with the hydrophobic core of triglyceride and cholesterol ester.

The pseudomolecular lipoproteins contain more than 30 per cent protein and include the high density end of the spectrum, mainly HDL. Electron microscopy showed HDL had diameters ranging from 74-98 Å, frequently with an electron dense region in the center (13). This suggested a structure composed of subunits aggregating to form an intact macromolecule. Thus HDL is thought to consist of protein-lipid

subunits that are associated in some quaternary structure. The number of apparent subunits in such quaternary structure varies from 3 to 5. In each subunit, the lipid is perhaps located in the interstices of the folded peptide chain. Recent studies (11) indicated that native HDL by circular dichroism criteria contained about 70% α helical, 5 to 15% β, and 15 to 20% disordered structure. Results showed that both phospholipid and cholesterol esters are required to complete the reorganization of the secondary and tertiary structure of HDL.

The major function of plasma lipoprotein is to transport the biologically important lipid compounds which include triglycerides, phospholipids, cholesterol and cholesteryl esters. The kind and amount of lipid transported by lipoproteins is presented in Table 2. The importance of the lipoproteins may be reflected, in part, by the physiological functions of these lipid compounds. Triglycerides are a source of fatty acids which in turn are a most important energy source for living cells. Phospholipids are the basic structural units for plasma membranes and membranes of cytoplasmic organelles, such as mitochondria and microsomes. Free cholesterol is also a component of many cell membranes and a major source of bile acids and steroid hormones. Thus the overall major function of plasma lipoprotein is to be involved in energy metabolism, sterol metabolism, and stabilization of highly apolar lipids in plasma. The composition and normal concentration in plasma of the four major lipoprotein groups

TABLE 2

MAGNITUDE OF MAJOR LIPID TRANSPORT TASKS IN PLASMA (2)

Lipid	Lipoprotein or carrier	Grams/day (a)
Free fatty acids	Albumin	50-150
Triglycerides		
Exogenous	Chylomicrons	70-150
Endogenous	VLDL	25-50
Cholesterol	Chylomicrons VLDL,LDL,HDL	1

(a) Estimates for an adult.

is summarized in Table 3.

The high content of triglycerides in chylomicrons and VLDL clearly indicates that they could be the major vehicle for triglyceride transport. Chylomicrons which are formed in the intestine in response to fat ingestion serve to transport primarily exogenous (dietary) triglycerides to liver, adipose tissue, and other tissue sites where they may be stored or utilized. VLDL which is synthesized in the liver in response to carbohydrates or fatty acid mobilized from adipose tissue, serve to transport primarily endogenous triglycerides. The complete picture of the removal of the triglycerides of chylomicrons and VLDL from plasma by the tissues is not yet clear. However, there is abundant evidence to suggest that upon entrance into the circulation, chylomicrons and VLDL may pick up ApoA from HDL (6). ApoA, once bound, may serve to facilitate the action of lipoprotein lipase. Lipoprotein lipase catalyzes the removal of triglycerides from chylomicrons and VLDL into smaller lipoprotein products that have ultracentrifuge flotation properties similar to LDL and HDL. These degraded products are thought to be a source of normal plasma LDL and, possibly, HDL. Studies show that the chemical properties of the LDL products are comparable to those of the normally occuring LDL (13). However, the HDL products have a slightly different lipid content from that of normal HDL. These HDL are suggested to be normalized during their circulation in plasma to that of regular HDL by the action of

TABLE 3

COMPOSITION AND NORMAL CONCENTRATION IN PLASMA OF THE FOUR MAJOR LIPOPROTEIN GROUPS (12)

	Chylomicrons	VLDL	LDL	HDL
Apoprotein	2.0	5-12	22	50
Phospholipid	4.3	13-20	20	24
Cholesterol alcohol	2.1	3-5	10	2
Cholesterol ester	3.9	10-13	36	20
Triglyceride	87	50-60	12	4
Approx.conc. mg/100 ml				
Male	12 ± 13	129 ± 122	439 ± 99	300 ± 83
Female	2 ± 3	5.9 ± 63	389 ± 79	457 ± 115

All composition values are weight per cent.

lecithin:cholesterol acyltransferase which catalyzes transesterification of an acyl group of lecithin to cholesterol.

Besides triglycerides transport, the HDL and particularly the LDL could be considered major transport vehicles for cholesteryl esters. In addition the synthesis and appropriate availability of Apo LDL or LDL appear to be crucial requirements for the formation and secretion of chylomicrons and VLDL by the intestine and liver. Inhibition of synthesis or secretion of LDL leads to triglyceride accumulation and inhibition of VLDL formation and secretion (13,14). In vitro studies indicated one significant function for HDL in addition to lipid transport. HDL or ApoA-phospholipid complexes can activate one or both of the enzymes, lipoprotein lipase and lecithin:cholesterol acyltransferase. Patients with HDL deficiencies often have elevated levels of triglycerides and extensive accumulation of cholesteryl esters. It has been shown that lipoprotein lipase in vitro is dependent on the presence of HDL or ApoA-phospholipid complexes and the absence or alteration of ApoA may prelude the normal clearing of chylomicrons. It has also been reported that the preferred lipoprotein substrate for lecithin:cholesterol acyltransferase is HDL and that the HDL surface is the probable locus for the transesterification reaction (13). However, the actual interaction mechanism between these two enzymes and HDL is still not well understood.

Structure of Apolipoproteins

Apolipoprotein C (ApoC) is the major protein component present in VLDL and constitutes over 50% of the protein content in VLDL. In addition ApoC is 20-25% of the protein in LDL_1, (1.006-1.019) but a more minor protein component (about 10%) in LDL_2 and HDL. ApoC was first reported by Gustafson et al. in 1964 (15). They obtained three phospholipid-protein fractions from partially delipidized VLDL. Two of the protein moieties of these three phospholipid-protein fractions were identical to ApoA and ApoB. The third one not only contained a protein moiety which had different immunochemical properties, but N-terminal amino acid and peptide patterns different from those of the other Apoproteins. This protein moiety was designated as ApoC. ApoC was also found in chyle, VLDL, and in all lipoprotein density classes isolated from plasma of normal, fasting man. The presence of ApoC in lipoproteins was confirmed by several other investigators. Brown, et al. (16) reported that phospholipid-protein complex isolated from VLDL reacted not only with antibody to HDL and LDL, but gave at least one specific immunoprecipitin line with antibody to VLDL. They further fractionated the soluble portion of totaly delipidized VLDL on a Sephadex G-100 column into two major fractions. First fraction contained all the proteins reacting with Anti-ApoA whereas the second fraction contained a mixture of polypeptides reacting only with anti-VLDL. The second fraction was purified further by DEAE-

cellulose chromatography giving rise to four fractions designated as D_1, D_2, D_3, and D_4. D_1 had N-terminal threonine, C-terminal valine and contained no tyrosine, cystine, nor cysteine. D_3 and D_4 were eluted from DEAE-cellulose in two closely contiguous peaks. Both of them had N-terminal serine, C-terminal alanine, and the same amino acid compositions containing no isoleucine, cystine and cysteine. However, they gave bands of slightly different mobility on polyacrylamide gel electrophoresis. No explanation was given in this report for the apparent difference in charge or size of these two polypeptides. D_2 was found to be impure. In a subsequent paper published by the same authors (17) they described an improved series of procedures which enabled them to establish D_2 as a separate protein. By using detergent to solubilize the Apoproteins of VLDL, they obtained three fractions from a Sephadex column, two of which were identified as ApoA and ApoB. The third fraction, ApoC, was purified on a DEAE-cellulose column under different conditions. They were able to obtain pure D_2 which had no histidine or half-cysteine but had N-terminal threonine and C-terminal glutamic acid. The complete characterization of these four polypeptides was also made by the same group (18). The different chromotographic behavior of D_3 and D_4 was due to the difference in carbohydrate composition. D_3 had twice as much sialic acid as D_4. Thus they were considered as one apoprotein. The molecular weight of D_1 (ApoLP-Val), D_2 (ApoLP-Gln), and $D_3 + D_4$ (ApoLP-Ala)

was determined to be 7000, 10,000, 10,000 respectively. Apo-Lp-Val was established to have 62 amino acid residues, Apo-Lp-Glu 95, and Apo-Lp-Ala 81. The circular dichroic and optical rotatory dispersion study indicated that Apo-Lp-Val had significant content of α-helical structure whereas Apo-Lp-Ala contained mainly disordered structure. Two other research groups soon confirmed these results. By using isoelectric focusing in a narrow pH gradient, Scanu et al.(19) were able to separate ApoC into four fractions (A, B, C, D) that were identified as distinct from ApoA. and ApoB. Three of these four fractions (A, B, D) had identical amino acid compositions and C-terminal amino acids, but different pI. Fraction A contain 2 moles of sialic acid per mole of protein, fraction B 1 mole per mole of protein, and fraction D no sialic acid. It appeared that fraction A was essentially identical to D_4, fraction B identical to D_3, and fraction C identical to D_2 on the basis of amino acid composition, N-terminal amino acid, C-terminal amino acid, sialic acid content, and position in polyacrylamide gel electrophoresis. However, fraction D which had identical amino acid composition to fraction A and B but no sialic acid was not reported by Brown et al. On the other hand fraction D_1(ApoLP-Val) reported by Brown et al. was not found by Scanu. The discrepancy might be that Brown solubilized all apoproteins of VLDL and fractionated them while Scanu only took the Tris-soluble apoproteins for fractionation. However, a revision has been made by Brown et al.(20) that serine rather

than valine is the COOH-terminal amino acid of D_1 (ApoLp-Val). The fractionation of ApoC isolated from VLDL was also studied by Alaupovic et al.(21). ApoC was obtained by immunoprecipitation and further separated into four fractions by DEAE-cellulose chromatography and by preparative polyacrylamide gel electrophoresis. First fraction designated as ApoC-I seems to correspond to D_1 or ApoLp-Ser. Second fraction designated as ApoC-II is correspond to D_2 or ApoLp-Gln. The third and fourth fractions are designated as ApoC-III correspond to D_3 + D_4 or ApoLp-Ala. Recently Shore et al. (22) have published studies of VLDL. They used a different delipidation technique to solubilize the protein moieties which subsequently were fractionated on DEAE-cellulose column. A total of six different fractions were obtained; all of them were different from ApoA and ApoB. Two major fractions had essentially the same amino acid composition and same C-terminal amino acid. They were very similar to that of Apo C-III or ApoLp-Ala. However, the remaining four fractions can not be positively identified as corresponding to the other two polypeptides reported by different investigators. It was suggested that the source of plasma, different techniques of delipidation, and different ways of fractionating the proteins might explain these differences. Nevertheless, it seems generally agreed by different investigators that ApoC isolated by different ways of fractionation contains at least three well characterized polypeptides. ApoC-I (ApoLp-Ser), ApoC-II (ApoLp-Gln), and

ApoC-III(ApoLp-Ala). Although Alaupovic reported that there are three forms of ApoC-I, two forms of ApoC-II, and four forms of ApoC III (23), they probably represented polymorphic forms of the three major polypeptides. In fact one of these three major polypeptides, ApoC III (ApoLp-Ala), has been sequenced very recently by Brewer et al. (24). It is a single polypeptide chain of 79 amino acid residues containing 1 or 2 residues of sialic acid, and 1 residue each of galactose and galactosamine. The single carbohydrate chain is attached to threonine, residue 74. This is the first human plasma apoprotein to be sequenced.

Apolipoprotein B

Apolipoprotein B (ApoB) constitutes 75-85% of the protein content in LDL, 85-95% in LDL_2, 40% in VLDL, 42% in HDL_2, but is absent from HDL_3. Because it is sparingly soluble, ApoB has been the most difficult of the apoproteins to characterize. After total delipidation with ether-ethanol ApoB yields a gel-like material that is insoluble in neutral salt systems. This tendency of ApoB to form water-insoluble aggregates represented a major problem in efforts to estimate its various physical-chemical properties. However, efforts were made recently for the preparation of soluble ApoB by different procedures. Among them are the use of the anionic detergents. Sodium dodecyl and decyl sulfate, succinylation with or without detergent, concentrated solutions of urea and detergent, reduction and alkylation, and maleylation prior to delipidation (25). Although delipidation and chemical

modification of LDL altered its properties to some extent, the soluble ApoB retains prominent immunological and optical characteristics of the intact lipoprotein. In general ApoB which was chemically unmodified and dissolved in 0.02 N NaOH showed a molecular weight of 70,000 by ultracentrifugation. By gel filtration chromatography it exhibited a molecular weight of 80,000 at high concentrations of urea and sodium dodecyl sulfate (26). Recently sedimentation equilibrium study demonstrated (27) that in the absence of urea ApoB had an average molecular weight of 80,000-100,000. However, in the presence of urea the minimum molecular weight of ApoB was 27,500. These findings provided the basis for subunit structure and possible heterogenity of ApoB. An early study (28) showed that ApoB had N-terminal glutamic acid and C-terminal serine. It also contained 2.7% hexose, 0.3% fucose, 1.2% glucosamine and 1.4% sialic acid.

The heterogeneity of ApoB has been investigated recently by several groups. Shore et al. obtained two fractions from a LDL fraction (sf 4-8) by DEAE-cellulose column chromatography in urea. The amino acid composition of these two fractions were distinctly different from the polypeptides of ApoA and ApoC (22). The immunochemical studies of the LP-B by Lee (29) suggested that LP-B may contain two polypeptides or antigenic determinants separable by diffusion in agar or agarose gels. The amino acid composition difference between the LP-B subfractions might also suggest the possible presence

of two unequally distributed ApoB proteins in Lp-B. Kane et al. (30) prepared a lipid-free and soluble maleylated ApoB using the technique of interfacial extraction in the presence of guanidine hydrochloride. The maleylated ApoB then was separated into two fractions by gel filtration. The second fraction corresponded to a random coil protein having a molecular weight of 26,000 and representing up to 25% of the total soluble ApoB. The large particle size of fraction I suggested that it still represented some form of oligomeric protein. Neither of these 2 fractions were found to have similar amino acid compositions to those of ApoA or ApoC. So it was concluded that at least two species of protein subunit was present in the ApoB and Fraction II likely represented a unique previously unrecognized protein constituent of ApoB. However the amino acid composition of these two fractions differed from those isolated by Shore and Shore. The comparison was not made and the reason for the difference is not yet clear. Neither Shore nor Kane have characterized their isolated two fractions other than by amino acid composition.

In summary, the recent development of new techniques to solubilize ApoB has made the further study of ApoB practical. From immunochemical, ion exchange and gel filtration studies, it seems that ApoB contains at least two polypeptides. However, results from various investigators disagree with each other, thus no conclusion can be drawn at the present time as to

their identity. More information is needed to clarify the discrepancy.

Apolipoprotein A

Apolipoprotein A (ApoA) comprises about 90% of the protein content in HDL_3, 50% in HDL_2, 10% in VLDL and a few per cent or trace amounts in LDL. ApoA was first commonly thought to consist of identical subunits with a molecular weight between 23,000 to 31,500 (31,32) and the following sugar composition: glucosamine 1.4%, fucose 0.5%, sialic acid 0.4% and a mixture of glucose and mannose 0.7% (31). Ultracentrifugation studies gave a single, symmetrical sedimenting peak in the presence of dissociating agents and terminal amino acid analysis showed only N-terminal aspartic acid and C-terminal threonine (28). Furthermore ApoA moved as a single band in zone electrophoresis, on paper, agar gel, or cellulose acetate (31,32,33). However, some later preliminary reports suggested possible heterogeneity in ApoA 28,34,35). In 1968 Shore et al. (36) observed that two non-identical polypeptides were revealed by carboxypeptidase A and B digestions. Polyacrylamide gel electrophoresis and actual isolation of these two polypeptides by DEAE-cellulose chromatography in urea further supported this finding. They were designated on the basis of C-terminal amino acids as R-Gln and R-Thr. In polyacrylamide gel R-Gln moves faster than R-Thr. The amino acid composition of R-Gln was characterized by the absence of histidine, arginine and tryptophan. The

total number of residues was 133 and the molecular weight based on amino acid composition was 14,900 which was confirmed by sedimentation equilibrium experiments. The second polypeptide R-Thr was characterized by the absence of isoleucine, cystine or cysteine. The total number of residues was 133 and the molecular weight from amino acid composition was 15,500. However, sedimentation equilibrium experiments showed a molecular weight of 31,400 which was thought to be the dimer form. The C-terminal sequences were suggested to be -Thr-Gln for R-Gln and -Lys-Tyr-Lys-Asn-Leu-Thr for R-Thr based on the actions of carboxypeptidase A and B. The occurance of these two major polypeptides in HDL was confirmed by two groups of investigators. Scanu et al.(37) reported that ApoHDL was separated by gel filtration chromatography in 8 M urea into two major fractions, fraction III and fraction IV. Fraction III accounted for about 70% of the total ApoHDL protein and had a molecular weight of approximately 27,000 in urea by ultracentrifugation, gel filtration, and disc gel electrophoresis. The M.W. for fraction IV was 17,000 by the same criteria. The amino acid compositions and electrophoretic mobilities indicated that fraction III was identical to R-Thr and fraction IV to R-Gln, although there was a molecular weight discrepancy between fraction III and R-Thr. Rudman et al.(33) resolved the protein moiety of HDL into three fractions by gel filtration on Sephadex G-200. The three fractions were analyzed by amino acid composition, electrophoresis, immunodiffusion, finger printing of the tryptic

digestion, paper chromatography and rechromatography on Sephadex G-200 in the absence and presence of SDS. These results indicated that ApoA contains two subunits of molecualr weight 20,000-25,000. Further study (38) made by the same investigators enabled them to prepare these two different polypeptides in pure forms. HDL was totaly delipidized by gel filtration on Sephadex LH-20 in organic solvent. The delipidated protein moieties were resolved into two major fractions by Sephadex G-200 chromotography in 1 N acetic acid. Again these two fractions were identified to be identical to R-Thr or fraction III and R-Gln or fraction IV by amino acid composition and electrophoretic mobility. The weight ratio between R-Thr and R-Gln was demonstrated to be 3:1 in ApoA.

It seems clearly established that ApoA contains two immunochemically different polypeptides. However, some corrections were made recently concerning the molecualr weight and terminal amino acids of these two polypeptides. Kostner et al. (40) obtained three fractions corresponding to R-Thr, R-Gln, and ApoC by a combination of gel filtration and DEAE-cellulsoe chromatography. The terminal amino acid analysis showed the fraction corresponding to R-Thr had glutamine instead of the threonine reported previously by Shore as C-terminal amino acid and had aspartic acid as its N-terminal amino acid. The fraction corresponding to R-Gln had glutamine as C-terminal amino acid. Its N-terminal was judged to be blocked rather than containing the previously reported aspartic acid. A very recent study showed the N-terminal of R-Gln is

pyrrolidone-carboxylic acid (39). Thus these two polypeptides were redesignated as ApoA-I (ApoLp-Gln-I) and ApoA-II (ApoLp-Gln-II)(40). Further report made by Scanu (41) indicated that upon reduction and carboxylmethylation, ApoA-II (ApoLp-Gln-II, fraction IV) can be reduced to half its size while ApoA-I was not affected, suggesting that ApoA-II is composed of two polypeptide chains connected by a disulfide bridge. These two polypeptides were soon proven to be identical (39). It was concluded that ApoA-I has N-terminal aspartic acid, C-terminal glutamine, molecular weight 25,000-27,000 whereas ApoA-II has N-terminal pyrrolidonecarboxylic acid, C-terminal glutamine, molecular weight 8,500 in the reduced monomer form and 17,000 in the oxidized dimer form. The primary structure of ApoA-I is under extensive study at present and part of its sequence has been published recently (42).

The purpose of the work presented here is to study the primary structure of ApoA-II (Apo-Gln-II) which is one of the two major polypeptides in HDL, in order that this knowledge can be used subsequently for further studies of lipid-protein binding in HDL. The study of this ApoA-II together with the other polypeptides in all density classes may provide the basic information to interpret for the study of protein-lipid as well as protein-protein interactions of lipoproteins. Eventually this might lead to a full understanding of plasma lipoprotein structures. This information might help to give some clue to the understanding of plasma hypo- or hyperlipo-

proteinemias which are thought to accelerate the development of atherosclerosis.

ApoA-II was purified by gel filtration from HDL which was prepared from a pool of human plasmas by salt precipitation. The purified ApoA-II was subjected to trypsin digestion and these tryptic peptides were purified and their amino acid sequence determined. CNBr cleavage was also performed and further digestion of the CNBr fragments was intended to produce overlap peptides by which to align the tryptic peptides and complete the entire sequence of ApoA-II. This was not needed because at that time Brewer et al.(39) published the complete sequence of ApoA-II from one individual donor. However, the detailed procedures of their sequence work have not yet been reported. The results obtained from this laboratory and those from Brewer lead to the conclusion that extensive polymorphism is not likely present in ApoA-II.

CHAPTER II

MATERIALS AND METHODS

Materials

Source of Human Plasma

All the human plasma used in this experiment consisted of a pool from healthy donors (90% male and 10% female). The plasma was at least one month in storage (4°C) before use.

Sodium Phosphotungstate

Phosphotungstic acid (reagent grade) was purchased from either Merck or Baker Chemical Company. Sodium phosphotungstate was prepared by titrating phosphotungstic acid with 1 N NaOH to pH 7.6 and diluting to 4% (w/v).

Cellulose Casing

Cellulose casing (18/100) was used for dialysis throughout the experiment. It was obtained from Union Carbide (Chicago, Ill.). A roll of cellulose casing was cut into suitable lengths and boiled with Na_2CO_3 for 30 minutes to remove heavy metals. The tubings were then rinsed with distilled water and boiled in distilled water for another 30 minutes. After rinsing they were stored in distilled water

at 4° ready for use.

Ethyl Ether

Analytical grade anhydrous ethyl ether was purchased from Mallinckrodt (St. Louis, Mo.). All the ether was precooled to 4° before use.

Ethyl Alcohol

Reagent grade ethyl alcohol (absolute) was purchased from U.S. Industrial Chemical Co. (New York, N.Y.). It was also precooled to 4° before use.

Nitrogen

Nitrogen (dry) used to evaporate the ether-ethanol in this experiment was purchased from National Cylinder Gas Co. (Chicago, Ill.).

Polyacrylamide Gel Electrophoresis Solutions

For apolipoprotein. All the ingredients for polyacrylamide gel electrophoresis were purchased from Canalco (Rockville, Md). The solutions were prepared according to the instructions except catalyst (ammonium persulfate) was prepared twofold concentrated. The 8 M urea gel was prepared as following: separating gel contained 3.75 ml C, 3.75 ml A, 2.1 ml G, and 7.2 g urea; stacking gel contained 1 ml B, 1 ml D, 1 ml E, 2 ml sucrose, 3 ml H_2O, and 2.88 g urea; sample gel contained 1 ml B, 1 ml D, 1 ml E, 2 ml sucrose, and 2 g urea.

For lipoprotein. All the solutions were prepared according to the directions described by Frings (47).

Dansyl Chloride

Dansyl Chloride (5-dimethylamino-1-naphthalene sulfanyl chloride) B grade, was purchased from Calbiochem (Los Angeles, Calif.). It was dissolved in acetone at a concentration of 5 mg/ml for amino acid dansylation reactions.

Carboxypeptidase A and B

Carboxypeptidase A, dialyzed and recrystallized, diisopropylfluorophosphate treated, was obtained from Sigma Chemical Company (St. Louis, Mo.). Hog pancreas carboxypeptidase B, diisopropylfluorophosphate treated was obtained as a frozen solution from the same source.

Proteolytic Enzymes

Trypsin (B grade, essentially free of chymotrypsin treated with PTCK) and thermolysin (crystalline, B grade) were purchased from Calbiochem (Los Angeles, Calif.). 2,4,6-Trinitrobenzenesulfonic acid (TNBS) was purchased from Pierce Chemical Company (Rockford, Ill.).

Pyridine

Pyridine (analytical grade) was obtained from Baker Chemical Company (Phillipsburg, N.J.). It was redistilled after refluxing with ninhydrin to free it from reactive amines.

Phenylisothiocyanate

Phenylisothiocyanate (PITC) was obtained from Baker Chemical Company. PITC (5%,v/v) was prepared in redistilled pyridine.

Dithiothreitol

Dithiothreitol (Cleland's reagent, A grade) was obtained from Calbiochem Company (Los Angeles, Calif.).

Ethylenimine

Ethylenimine was obtained from K & K Chemical Company. It was stored with KOH to keep it from absorbing moisture.

Thioglycolic Acid

Thioglycolic acid was obtained from Fisher Chemical Company.

Cyanogen Bromide

Cyanogen bromide was obtained from Pierce Chemical Company (Rockford, Ill.)

The other common chemicals used were reagent or analytical grade and were purchased from various suppliers without further purification or special treatment.

Methods

All operations, reactions, centrifugations were performed at room temperature except the following.

All dialyses and delipidation of lipoproteins were performed in the cold room at 4°. Reactions above room

temperature were carried out in a heating block (Cole-Parmer, Chicago, Ill.) which maintained a desired temperature within ± 1 degree. All the drying steps for amino acid sequence determination and N-terminal analyses were carried out in a desiccator which was evacuated with an oil pump to accelerate the process. The desiccator was placed in a heating mantle (Curtin, Tulsa, Okla.) and the temperature was maintained at 70°.

Fractionation of Human Plasma

About 20 liters of plasma were fractionated for use in this study. The plasma was fractionated by a sodium phosphotungstate (NaPhT) and magnesium chloride ($MgCl_2$) precipitation described by Burstein *et al* (43). Plasma was centrifuged at 10,000 rev/min for 30 min in a SS2 rotor (Sorvall) to remove the chylomicrons. To each 500 ml of clear plasma was added 50 ml of 4% NaPhT pH 7.6 and 12.5 ml of 2 M $MgCl_2$. Precipitation was complete in several minutes and the precipitate was removed by centrifugation at 6,000 g for 10 min. Precipitate I contained mainly LDL and VLDL. To the supernatant I a second portion (450 ml) of NaPhT (4%) was added. Precipitation was complete in an hour and the mixture was centrifuged at 600 g for 10 min. This white precipitate (II) contained mostly Γ-globulin. To the supernatant II a second portion, 87.5 ml of $MgCl_2$ (2 M) was added and the precipitation was complete in three to four hours but sometimes was left overnight. The mixture was centrifuged at 20,000 g for 30 min. The final

supernatant was discarded. The precipitate III which contained HDL was washed once by suspension in 250 ml of 1% NaCl, 0.4% NaPhT, and 0.1 M $MgCl_2$. The washed HDL was recovered by centrifugation at 6000 g for 10 min and suspended in 40 ml of 1% NaCl. Sodium carbonate (10%) was added dropwise until all the HDL precipitate was in solution. Approximately 10 ml sodium carbonate was needed.

Further Purification of HDL

Suspended HDL was dialyzed at 4° against H_2O with frequent changes for a minimum of 48 hours to remove NaPhT. After dialysis the density of HDL was adjusted to 1.21 by potassium bromide. The density was checked by measuring the volume and weight of the solution. After adjusting the density, the HDL solution was subjected to ultracentrifugation in a Beckman Model L Ultracentrifuge (Beckman Instruments, Inc., Palo Alto, Calif.) using a no. 40 rotor at 36,000 rpm for 60 to 70 hours. About 1 ml of the yellowish HDL top layers were removed from each centrifuge tube and combined. If not immediately used, HDL solution was dialyzed against 0.1 M NaCl for 48 hours and then stored frozen until use.

Immunochemical Analysis

Double diffusion was performed on a glass slide. 1% agar was prepared in 0.15 M NaCl and poured evenly over a glass slide. The gel was hardened; the wells were cut and agar removed from them by gentle suction. Antiserum was

placed in the center well and samples were placed in outside wells. The precipitin line was developed in a moist chamber in 1-2 days.

Single diffusion was performed in 6 x 50 mm culture tubes. 1% agar solution was made in 0.15 M NaCl which also contained an appropriate amount of antiserum. The agar solution was then quickly pipeted into a series of tubes. The agar was allowed to harden and then different dilutions of samples containing known or unknown concentrations of albumin were placed on top of the gels. The tubes were covered with parafilm and allowed to stand at 37° for about 2 days. The distance of migration of the precipitin line of each unknown was measured and compared to that of known concentrations of albumin solutions run in parallel. A plot of logarithm of the concentration versus distance of migration was used as a standard curve.

Immunoelectrophoresis was carried out with a Gelman apparatus. Glass slides were covered first with 0.1% adhesive agar and then with 1% agar solution in Tris barbital-sodium barbital pH 8.8, 0.007 ionic strength, buffer solution. HDL samples were placed in the holes and electrophoresed for 60 min at 350v. Agar in the trough was removed and antiserum was placed in the trough. Then the agar plate was kept in a moist chamber to develop precipitin lines. They were inspected at 24 and 48 hours and the results were recorded.

Protein Concentration Determination

Protein concentrations of all HDL preparations were

determined by the biuret reaction (44), using bovine serum albumin as a standard.

Total Delipidation of HDL

The total delipidation of HDL was performed according to the procedures described by Shore et al. (45). The purified HDL was dialyzed against 0.1 M NaCl at 4°C for 48 hr and adjusted to a concentration of about 4 mg/ml lipoprotein. The HDL solution was extracted first with an equal volume of ether-ethanol (3:2, v/v) overnight. The aqueous soluble fraction was then extracted repeatedly five to six times with aliquots of ether-ethanol (3:1, v/v) until the volume of the aqueous phase was about the same as the initial volume. The time for each repeated extraction was about three to four hours. After extraction ether and ethanol were removed by blowing nitrogen over the solution until no odor of ether or ethanol could be detected. The protein solution was then dialyzed against H_2O for 48 hr at 4°C.

Analysis of Phospholipid

The phospholipid content of the delipidated HDL was analyzed according to a modification of the prodecure by Rouser et al (46). Appropriate amounts of ApoHDL were evaporated to dryness and 0.25 ml of perchloric acid ($HClO_4$) was added. It was then pyrolized at 180° to dryness. To the pyrolized sample was added 0.2 ml of $HClO_4$ and warmed in a boiling water bath for several seconds. Then, with one

immediately following the other, 0.1 ml of H_2O, 0.4 ml of ammonium molybdate (1.25% in H_2O), and 0.4 ml of ascorbic acid (5% in H_2O) were added to the sample and heated in a boiling water bath for 5 min to develop the color. The optical density of the color was read at 797.5 mμ and quantitated with pure lecithin used as a standard.

Isolation of ApoA-II

The delipidated ApoHDL was concentrated by flash evaporation and its concentration was adjusted to approximately 15 mg/ml (based on biuret determination) in 1 N acetic acid. A column of 3.3 x 190 cm was poured with Sephadex G-100 in 1N acetic acid. ApoHDL was chromatographed on this column and 5 ml was collected per tube. Six fractions were obtained from this chromatography. Fraction 4 and fraction 5 were rechromatographed separately twice on a smaller column of Sephadex G-100 (2.1 x 190 cm) in 1 N acetic acid with 3 ml collected per tube for each rechromatography. Optical density at 280 mμ was recorded for all the chromatographies.

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was performed with a Canalco Model 6 apparatus and 4 x 66 mm tubes were used. All the gels were prepared in 8 M urea at pH 8.9. The polyacrylamide concentration in the separating gel was 7%, in the stacking gel was 1%, and in the sample gel was 1.54%. Protein samples containing 0.2 to 0.5 mg were mixed with sample gel

and polymerized on top of the stacking gel. A Tris-glycine buffer at pH 9.5 was used as running buffer in the lower and upper chambers. A stock solution of 0.05% bromphenol blue was added to the upper chamber buffer (0.5%, v/v) as a tracking dye. Electrophoresis was carried out at 5 mA per tube until the tracking dye reached the bottom of the gel. The gels were stained with aniline black in 7% acetic acid or with coomassie blue (0.2 ml of 0.12% coomassie blue in H_2O + 8 ml of 10% trichloroacetic acid). When aniline black was used, destaining was done in 7% acetic acid either by electrophoresis or just by washing.

Lipoprotein polyacrylamide gel electrophoresis was also carried out with a Canalco Model 6 apparatus according to the procedure of Frings et al (47). The concentration of polyacrylamide was 3.75% in the separating gel, 2.50% in the concentrating gel, and 2.22% in the sample gel. Lipoprotein sample was polymerized with sample gel which contained lipid staining solution, Sudan Black B. During the polymerization process, lipoprotein was prestained with Sudan Black B in the sample gel. The electrophoresis was run at 5 mA per gel tube for 35 min using tris-glycine (pH 8.3) running buffer.

Amino Acid Analysis

Appropriate amounts (0.01-0.03 μmole) of protein or peptides were dried in an 18 x 150 mm pyrex test tube and 1 ml of constant boiling hydrochloric acid was added. The tube was stretched, evacuated, and sealed with an oxygen torch flame.

The sample was hydrolyzed in a heating block maintained at 110° C for 24 hours. The hydrochloric acid was evaporated by flash evaporation and the hydrolysate was redissolved in 2.5 ml of pH 2.2, 0.2 N citrate buffer. The amino acid was analyzed on a Biocal Biochrom Amino Acid Analyzer equipped with an automated sample injector using the 2 column system (48).

N-Terminal Amino Acid Analysis

The qualitative N-terminal amino acid analysis of protein or peptide was determined using a dansyl chloride procedure described by Gray (49). 5-10 nmoles of sample were dried in a small 6 x 60 mm culture tube over P_2O_5 and NaOH in a desiccator. It was then dissolved in 20 µl of 0.1 M sodium bicarbonate and the solution was dried again *in vacuo*. The sample was redissolved in 15-20 µl of deionized H_2O and the pH of the solution was checked by pipeting a tiny drop onto pH indicator paper. If the pH was below 8, more sodium bicarbonate was added. Then an equal volume of dansyl chloride (1.5 mg/ml in acetone) was added and the reaction mixture was incubated in a heating block at 37°C for 2 to 3 hours. After dansylation, the mixture was dried *in vacuo* and 50 µl of constant boiling HCl was added. The tube was sealed and hydrolysis was allowed to proceed 14 to 16 hours at 105°. After hydrolysis HCl was removed *in vacuo* and hydrolysate was dissolved in 5 µl of 50% pyridine.

The identification of the dansyl amino acid was accomplished by polyamide thin layer chromatography described

by Woods and Wang (50). The dansyl hydrolysate was spotted on both sides of a polyamide sheet and about 0.02 mμmole of a standard mixture of dansyl amino acids was placed on one side of the sheet on the top of the sample spot. The chromatography was developed in two dimensions. The first solvent was water 88% formic acid (200:3, v/v) and the second solvent was benzene-glacial acetic acid (9:1, v/v). The N-terminal dansyl amino acid was visualized by a long wave UV lamp and identified by comparison with standard dansyl amino acids. When aspartic acid or glutamic acid, and serine or threonine occur as the N-terminal amino acid, a third solvent of n-heptane-n-butanol-glacial acetic acid (3:3:1, v/v/v) was used to resolve these pairs for more positive identification.

C-Terminal Amino Acid Analysis

The C-terminal amino acids of proteins or peptides were determined by treatment with carboxypeptidase A and B. Carboxypeptidase A (CPA) was prepared by the procedure of Ambler (51): 1.25 mg CPA (normally 0.025 ml) was diluted with 1 ml of H_2O in a small centrifuge tube. The CPA solution was mixed well and H_2O was removed by centrifugation. CPA crystals were resuspended in 0.1 ml of 0.1 N $NaHCO_3$ in an ice bath and 0.1 N NaOH was added dropwise with thorough mixing until the CPA was dissolved. Then an equal volume of 0.1 N HCl was added to neutralize the NaOH. The volume was made up to 1.25 ml 1 mg CPA/ml with 0.2 M, pH 8.5, N-ethylmorpholine acetate buffer. Protein or peptide samples containing 0.02-0.05 μmole

were dried in a desiccator and redissolved in 0.1 ml of 0.2 M, pH 8.5 N-ethylmorpholine acetate buffer. 10 µl to 20 µl of freshly prepared CPA and/or CPB (1 mg/ml) was added to the sample solution and incubated at 37° for various periods of time. The reaction time was stopped by adding several drops of glacial acetic acid to the 0.1 ml reaction volume. The mixture was dried and dissolved in pH 2.2, 0.2 N citrate buffer for amino acid analysis.

Sialic Acid Analysis

Sialic acid content of ApoA-II was determined as N-acetylneuraminic acid by the method of Warren (52). ApoA-II was hydrolyzed in 0.1 N H_2SO_4 at 80°C for 1 hour to liberate N-acetylneuraminic acid. The sulfate was removed by barium precipitation and the acetylneuraminic acid was separated by ion exchange column chromatography using coupled columns with two resins (Dowex 50W-X8 [H^+],20 to 50 mesh and Dowex I-X8 [Formate]50-100 mesh, from Sigma)(53). Color was developed and optical density was recorded at 549 mµ. N-acetylneuraminic acid was used as the standard.

Performic Acid Oxidation

The performic acid oxidation of ApoA-II was carried out according to the procedure described by Hirs (54). Performic acid was prepared by mixing 5 volumes of 30% hydrogen peroxide and 95 volumes of 97% formic acid and allowed to stand in a closed container at room temperature

for 120 minutes. The freshly generated performic acid was cooled in an ice bath for 30 min. ApoA-II (58 mg) was dissolved in 2.5 ml of 97% performic acid and also cooled in an ice bath for 30 minutes. Then 5 ml of this cold performic acid was added to the protein solution and reaction was allowed to proceed for 150 minutes in the ice bath. To end the reaction, the protein solution was diluted to 200 ml with cold water and lyophilized. An aliquot of the oxidized protein was acid hydrolyzed for a cysteic acid determination on the amino acid analyzer to check the extent of the oxidation.

Trypsin Digest

Performic acid oxidized ApoA-II was dissolved in H_2O to a concentration of 1% and the pH was adjusted to 8.0 with 0.1 N NaOH and 0.01 N NaOH using a pH meter. An aliquot of trypsin solution (1% protein in 10^{-3} N HCl) calculated to give a ratio of 1 to 100 of trypsin to protein (w/w) was added to the protein solution. The reaction mixture was incubated at 37°C in a heating block for 6 hours. After three hours incubation a second aliquot of trypsin solution was added. The pH of the solution was kept at 8.0 throughout the reaction course by titrating it with 0.01 N NaOH using a pH meter. After digestion, the pH of the solution was adjusted to 4.0 and allowed to stand overnight at 4°. Some precipitate was formed and removed by centrifugation.

Isolation of Tryptic Peptides by Ion Exchange Chromatography

The clear supernatant from the trypsin digest was flash evaporated almost to dryness and redissolved in 2.43 M acetic acid. The tryptic peptides were isolated by cation exchange chromatography with a peptide monitoring system developed by Delaney (55). A column (0.9 x 60 cm) was poured with Aminex Q 15S (BioRad) and maintained at 50°. The buffer gradient was developed by two buffer chambers, one of which contained 500 ml of 0.05 M pyridine/acetic acid, pH 3.0, and the other chamber contained 500 ml of 2 M pyridine/acetic acid, pH 5.0. The peptide mixture was applied to the column and the fraction collector timer, the chromatography pump, and the recorder chart were started simultaneously. The flow rate of the gradient buffer was 60 ml/hr. Most of the effluent from the column went to the fraction collector and a small portion was diverted for peptide analysis. This small portion of peptide sample was mixed with approximately 3 N NaOH and maintained in heated coils for 60-80 min at 97°C for hydrolysis. Then this mixture was pumped through a second set of coils together with 0.5 M boric acid containing 0.15% trinitrobenzenesulfonic acid at room temperature for about 40 min to develop color. The concentration of NaOH was carefully calculated so that the pH of the whole buffer system, sodium hydroxide, boric acid, and acetic acid would be 8.9-9.4. The solution was passed through a flow cell and the optical density

at 430 mμ was recorded. Using the peaks on the recorder chart as reference, pools from the fraction collector were made and concentrated by flash evaporation. They were dissolved in a known amount of water or dilute acetic acid and stored frozen.

Further Purification of Tryptic Peptides by Paper Electrophoresis

The impure tryptic peptides obtained from cation ion exchange chromatography were further purified by high voltage paper electrophoresis. Paper electrophoresis was also used for molecualr weight and ionic charge determination as well. The electrophoresis were run in electrophoresis tanks at pH 2.1, 3.5 and/or 6.0 for various periods of time. The voltage used for all electrophoresis was 52 volts per cm of paper. Whatman No. 3 or 1 paper (46 x 57 cm, W. and R. Balston LTD., England) was used depending upon the amount of peptides. After electrophoresis a strip of paper was cut and dipped with cadmium acetate-ninhydrin to locate the peptide spots. Peptides were eluted off the paper either by 0.1 N NH_4OH or by 0.1 N acetic acid and stored frozen.

Thermolysin Digest

Thermolysin digestion of peptides was carried out in 0.2 M N-ethylmorpholine acetate buffer at pH 8.5. Both peptide sample and enzyme were dissolved in this buffer. The ratio of thermolysin to peptide was 1 to 50 (w/w). In

addition, 0.1 M $CaCl_2$ was added to give a final concentration of .002 M in the mixture. The reaction was incubated at 37° for various periods of time and stopped by adding acetic acid to pH 4 or lower. The peptides were then separated and purified by high voltage paper electrophoresis.

Amino Acid Sequence Determination

The amino acid sequence of the purified tryptic peptides was determined by the procedure according to Gray et al. (56). The coupling mixture was prepared freshly for each cycle and consisted of 30 µl of phenylisothiocyanate, 600 µl of pyridine, and 400 µl of water. Approximately 10 nmoles of peptide were used for each cycle. Peptide sample was placed in a series of 6 x 50 mm tubes depending upon the number of residues of the peptide and dried over P_2O_5 and NaOH in a desiccator at 70°. To each of the sample tubes was added 50 µl of the coupling mixture and the tubes were allowed to stand in a heating block at 50° for 45 min. Then the samples were dried. A white or lightly brown deposit was left in the bottom of the tube. In succeeding cycles if the deposit appeared oily, it was redissolved in 30 µl of ethanol and the drying repeated. The cleavage reaction was carried out by adding 50 µl of anhydrous trifluoroacetic acid to each sample tube. They were left uncovered in the heated desiccator. After 10-15 minutes the vacuum was applied to the desiccator and samples were dried. An Edman cycle was completed at this stage. The tube containing the N-terminal residue to be

analyzed was set aside and another Edman cycle was performed on the samples in the remaining tubes. After each cycle, the tube containing the newly exposed residue was set aside until the last Edman cycle was carried out. The by-products of all the cycles were removed at the same time by ethylacetate extraction. Ethylacetate was satruated with H_2O and then the two phases (ethylacetate and water) were separated.

The N-terminal amino acid from each cycle was identified by one of two methods. The first method was dansylation coupled with polyamide thin layer chromatography as described earlier. The second method was subtractive analysis. After each cycle the peptide sample was hydrolyzed in constant boiling HCl for 24 hr and amino acids were analyzed on the amino acid analyzer. The amino acid with reduced concentration was the N-terminal amino acid removed by that Edman cycle from that peptide.

Reduction and Aminoethylation of ApoA-II

The disulfide chain of ApoA-II was reduced by dithiothreitol and then blocked by ehtylenimine in guanidine hydrochloride. ApoA-II was dissolved in 6.7 M guanidine hydrochloride which was prepared in 0.55 M tris-HCl, pH 8.4, containing 5×10^{-3} M EDTA to give 1% protein solution. The protein solution was flushed several times in an evacuated desiccator with dry nitrogen. Dithiothreitol was then added to give an SH concentration of 0.1 M. The reaction mixture was allowed to stand at room temperature for 24 hours. An appropriate

amount of ethylenimine (80 fold excess over half-cystine) was added to the mixture. It was added at three separate times and each addition was 10 minutes apart. After a total reaction time of 35 minutes, mercaptoethanol was added to destroy the excess ethylenimine. Then the mixtrue was placed on a Sephadex G-25 column, (4.5 x 30.5 cm) equilibrated with 2 N acetic acid, to remove the reagents and by-products.

Reduction of Methionine Sulfoxide

The reduction of methionine sulfoxide to methionine was carried out by thioglycolic acid in guanidine hydrochloride, a modified procedure of Jori (57). The aminoethylated ApoA-II was flash evaporated almost to dryness and a 1% solution was prepared in 6 M guanidine hydrochloride. Thioglycolic acid and dithioerythritol were added to the protein solution to give 2% thioglycolic acid (v/v) and 0.06 M dithioerythritol solution. The pH of the solution was titrated to pH 2-3 with acetic acid and the sample was incubated at 37° for 24 hours. After reduction, the mixture was placed on a Sephadex G-25 column (4.5 x 30.5 cm) equilibrated with 2 N acetic acid to remove by-products. The degree of reduction was checked by measuring the disappearance of methionine in a small amount of reduced ApoA-II reacted with CNBr.

Cyanogen Bromide Cleavage

Cyanogen bromide cleavage of ApoA-II was accomplished in 70% formic acid at room temperature for 24 hours. The

aminoethylated methionine reduced ApoA-II solution was concentrated and 97% formic acid was added to give a 2% protein solution in 70% formic acid. Cyanogen bromide was weighed out in an amount that was a threefold excess of the protein weight. It was dissolved rapidly in 70% formic acid and the reaction mixture was covered with parafilm and allowed to stand at room temperature for 24 hours. After reaction, an aliquot was taken for amino acid analysis to check the extent of methionine disappearance. The remaining mixture was passed through a Sephadex G-25 column (4.5 x 30.5 cm) equilibrated in 2 N acetic acid to remove the reagents.

The CNBr fragments were separated by gel filtration in 8 M urea. Urea solution (10 M) was prepared in H_2O and passed through a column (4.5 x 45 cm) containing a mixed bed ion exchange resin (AG501-X8(D), 20-50 mesh, BioRad) to generate deionized urea. The solution was then diluted to 8 M urea containing also 10^{-4} M EDTA, 0.2% thiodiglycol, and 0.1 N acetic acid.

Sephadex G-50 was poured into a column, 2.1 x 190 cm, and equilibrated thoroughly with the deionized acid urea solution. Cyanogen bromide fragments of ApoA-II were separated on this column and four major fractions were obtained from the separation. Each fraction was passed through a Sephadex G-25 column (4.5 x 30.5 cm) in 2 N acetic acid to remove salts. They were then concentrated and stored frozen in H_2O or dilute acetic acid.

CHAPTER III

RESULTS

Purity of HDL

HDL was isolated from human plasma by sodium phosphotungstate and magnesium chloride precipitation and was further purified by ultracentrifugation to remove a small amount of albumin contaminant. The purity of HDL was then checked by polyacrylamide gel electrophoresis, double and single immunodiffusion analysis, and immunoelectrophoresis.

HDL was prestained during the polymerization process with a lipid staining solution of Sudan Black B which was present in the sample gel. The electrophoretic result showed that there was only one band on the gel. The position of the band corresponded to that of HDL described by Frings (47). This indicated that the HDL preparation was one electrophoretic component and had no contamination with LDL and VLDL.

Double immunodiffusion analysis showed that HDL gave a single precipitin line with anti-ApoA-I and with anti-ApoA-II, but no detectable precipitin line with antihuman albumin (this analysis was the courtesy of Dr. P. Alaupovic).

Single immunodiffusion was used to quantitate albumin contamination in HDL preparations. Rabbit antihuman albumin

was used. A standard curve was constructed by plotting the distance of migration of the precipitin band versus the logarithm of the concentration of standard human serum albumin. Comparison of the albumin in the HDL preparation with the standard curve indicated that only trace amounts of albumin (1-2%) were present in the HDL preparations.

In immunoelectrophoretis, rabbit antihuman serum was used. After electrophoresis and development, HDL formed only one precipitin line with antihuman serum. No albumin or other lipoprotein precipitin lines were found.

Delipidation of HDL

Total delipidation of HDL was carried out with ether-ethanol extraction. After the first extraction, a saline-soluble (approximately 50% of the total protein) and saline-insoluble fractions were obtained. These two fractions were treated separately throughout the remaining extractions. Preliminary polyacrylamide gel electrophoresis indicated that the saline-soluble fraction was rich in ApoA-II and ApoC whereas the saline-insoluble fraction was rich in ApoB and ApoA I. Therefore, the saline-soluble fraction was analyzed for residual phospholipid content not removed by the delipidation. The phospholipid analysis showed that there was 2-3% phospholipid remaining in the apoprotein preparation when pure lecithin was used as standard.

Isolation of ApoA-II

ApoA-II was isolated from the saline-soluble fraction

of Apo HDL by gel filtration on Sephadex G-100 in 1 N acetic acid. The chromatographic pattern of the separation is shown in Figure 2. Based on polyacrylamide gel electrophoresis, six fractions (designated by arrows in Figure 2) were obtained. The distribution of HDL apoproteins in these six fractions was as follows: fraction 1 and fraction 2 contained mostly an unidentified yellowish substance and ApoA-I; fraction 3 contained mostly one of the ApoC polypeptides and some ApoA-I and ApoA-II; fraction 4 and fraction 5 contained ApoA-II as the major protein and ApoC polypeptides as minor components; fraction 6 contained mostly ApoC polypeptides. All fraction 4 pools from different preparations were combined and re-chromatographed twice on Sephadex G-100 in 1 N acetic acid. Fraction 5 was similarly repurified. The results are shown in Figures 3 and 4 for fraction 4 and Figures 5 and 6 for fraction 5. The arrows in these Figures indicate the limits of the pools after each rechromatography. The pooling limits were governed by analytical polyacrylamide gel electrophoresis. Then fractions 4 and 5 were lyophilized and stored frozen.

Characterization of ApoA-II

The purity of the isolated protein was investigated by polyacrylamide gel electrophoresis, amino acid composition, N-terminal amino acid analysis, and its sialic acid content.

The polyacrylamide gel electrophoresis for each chromatography of fraction 4 and fraction 5 are shown in

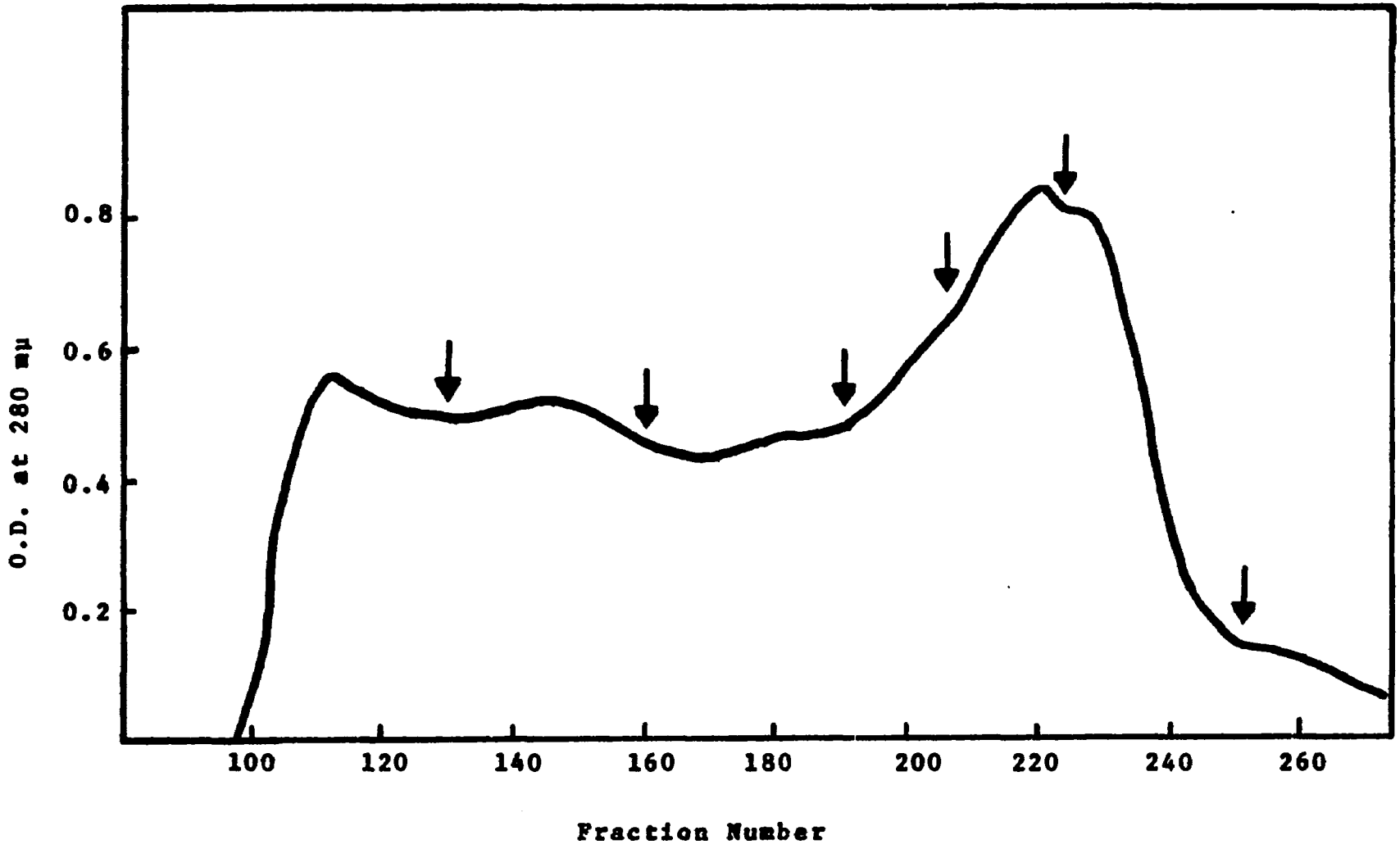


Figure 2--Chromatographic Patterns of ApoHDL on Sephadex G-100.

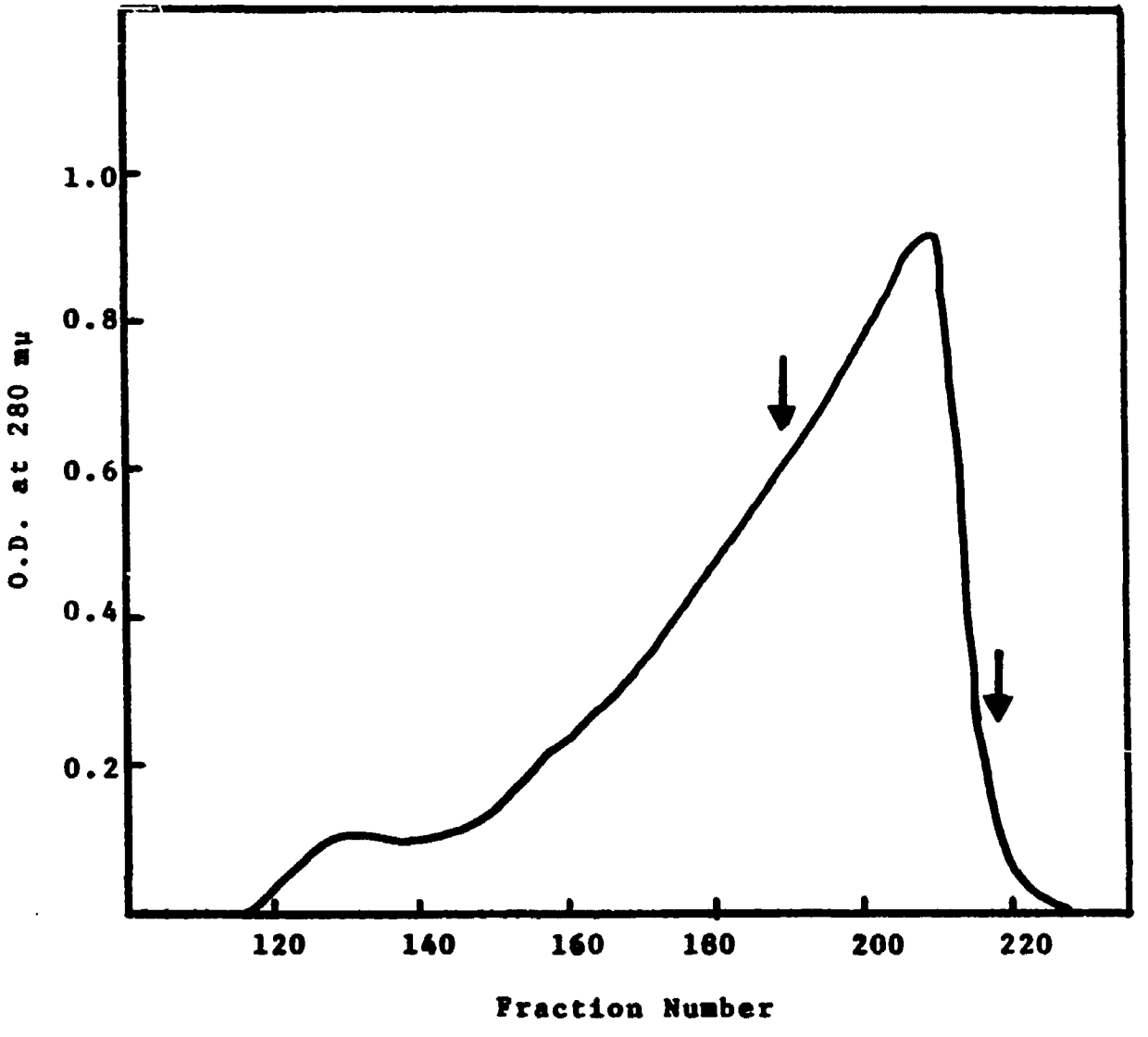


Figure 3--First Rechromatographic Patterns of Fraction 4.

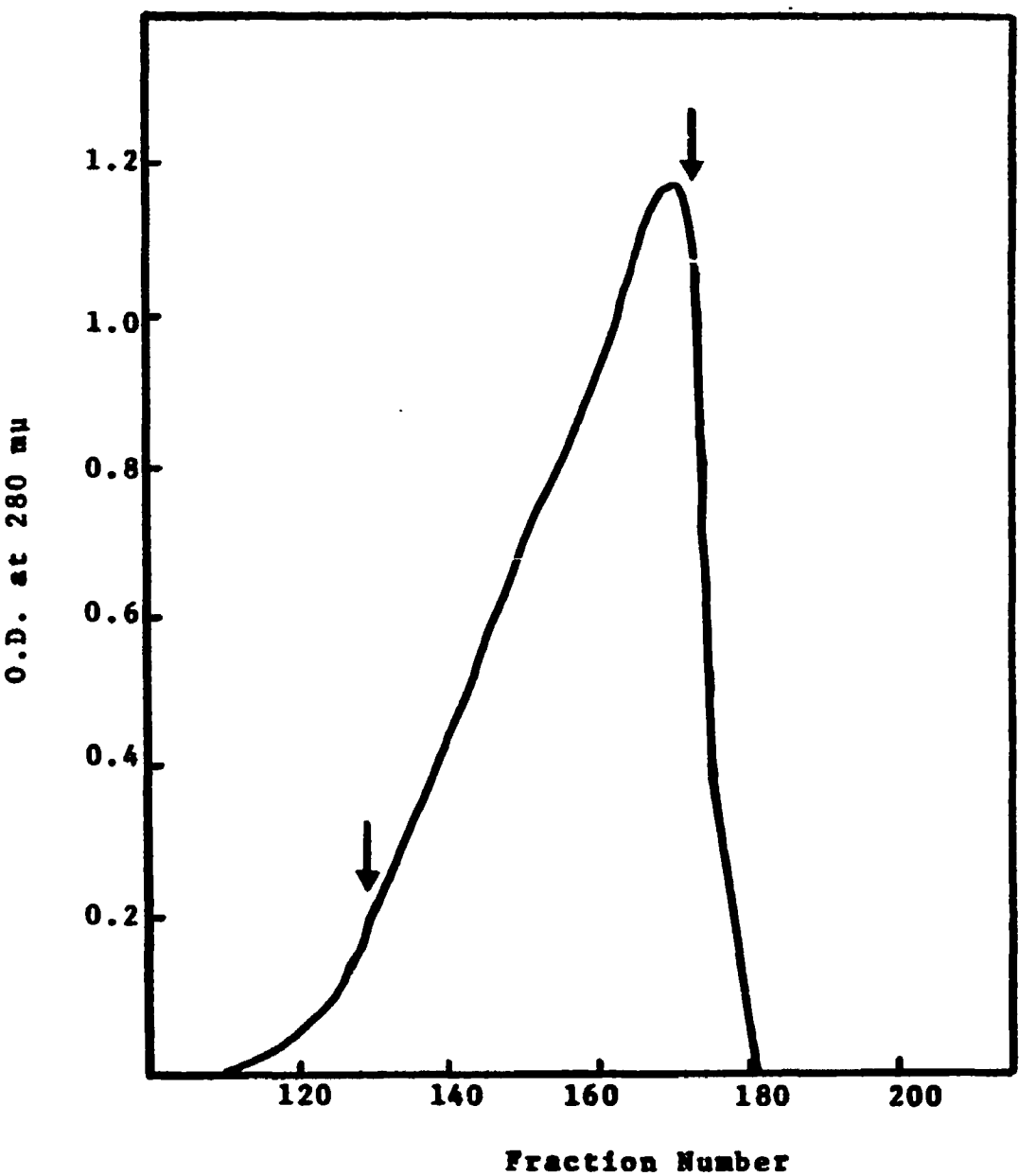


Figure 4--Second Rechromatographic Patterns of Fraction 4.

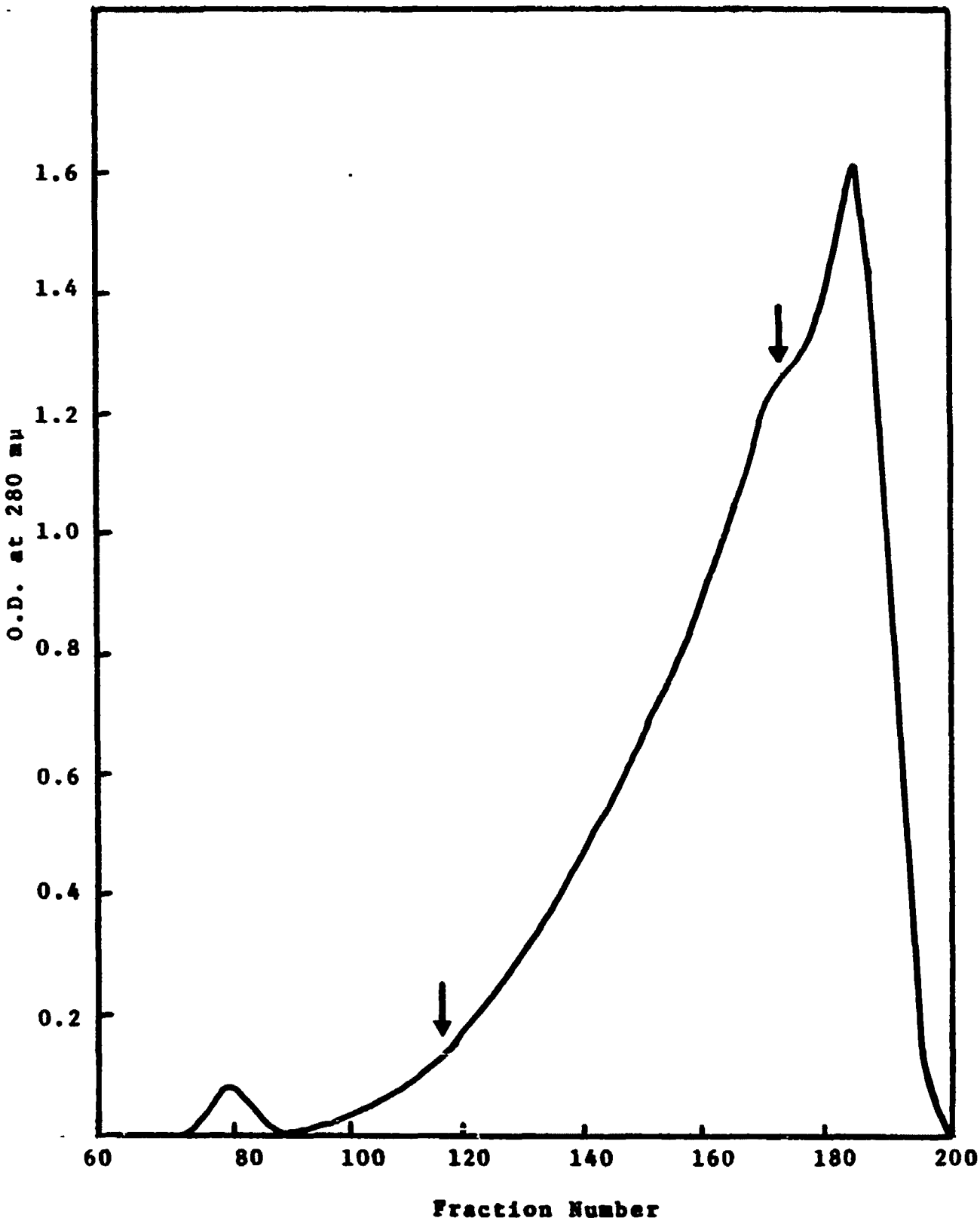


Figure 5--First Rechromatographic Pattern of Fraction 5.

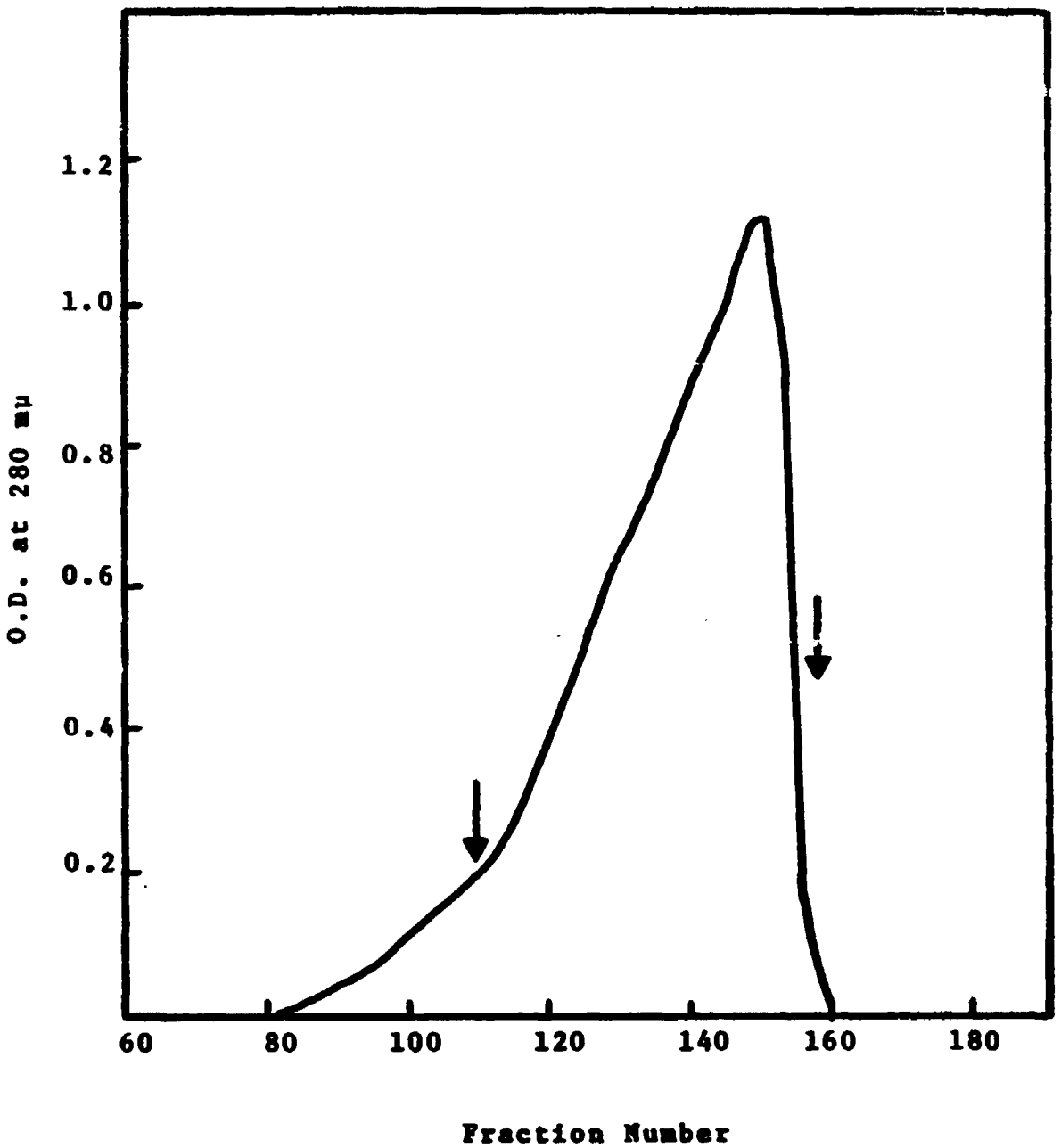


Figure 6--Second Rechromatographic Patterns of Fraction 5.

Figure 7. Both fractions showed one dominant homogeneous band with a small amount of contaminants, which was estimated to be $\leq$ 10%. These two dominant bands in fraction 4 and 5 migrated to the same extent in 8 M urea polyacrylamide gel. To further demonstrate that these two bands were the same protein, a polyacrylamide gel electrophoresis of a mixture of the third rechromatography of two fractions was carried out. The result (Figure 7) showed that fraction 4 and fraction 5 migrated as one band. In addition, the amino acid compositions of these two fractions were determined spearately and the result indicated they were identical within experimental error. Based on these observations, fraction 4 and fraction 5 were judged indeed to consist of one predominant protein and therefore were combined for further primary structure study.

The amino acid composition of the isolated protein is shown in Table 4. For the purpose of comparison, the amino acid compositions of ApoA-II published by other investigators (37,38,59) are also presented. The composition of the isolated protein closely resembled the published results. It was even more evident by examining the low amount of histidine and arginine. Therefore, based on the comparison of their position in polyacrylamide gel and amino acid composition with published results, the isolated protein was considered as ApoA-II. It contains one disulfide bond and the minimum total residues per molecule was calculated to be 77.

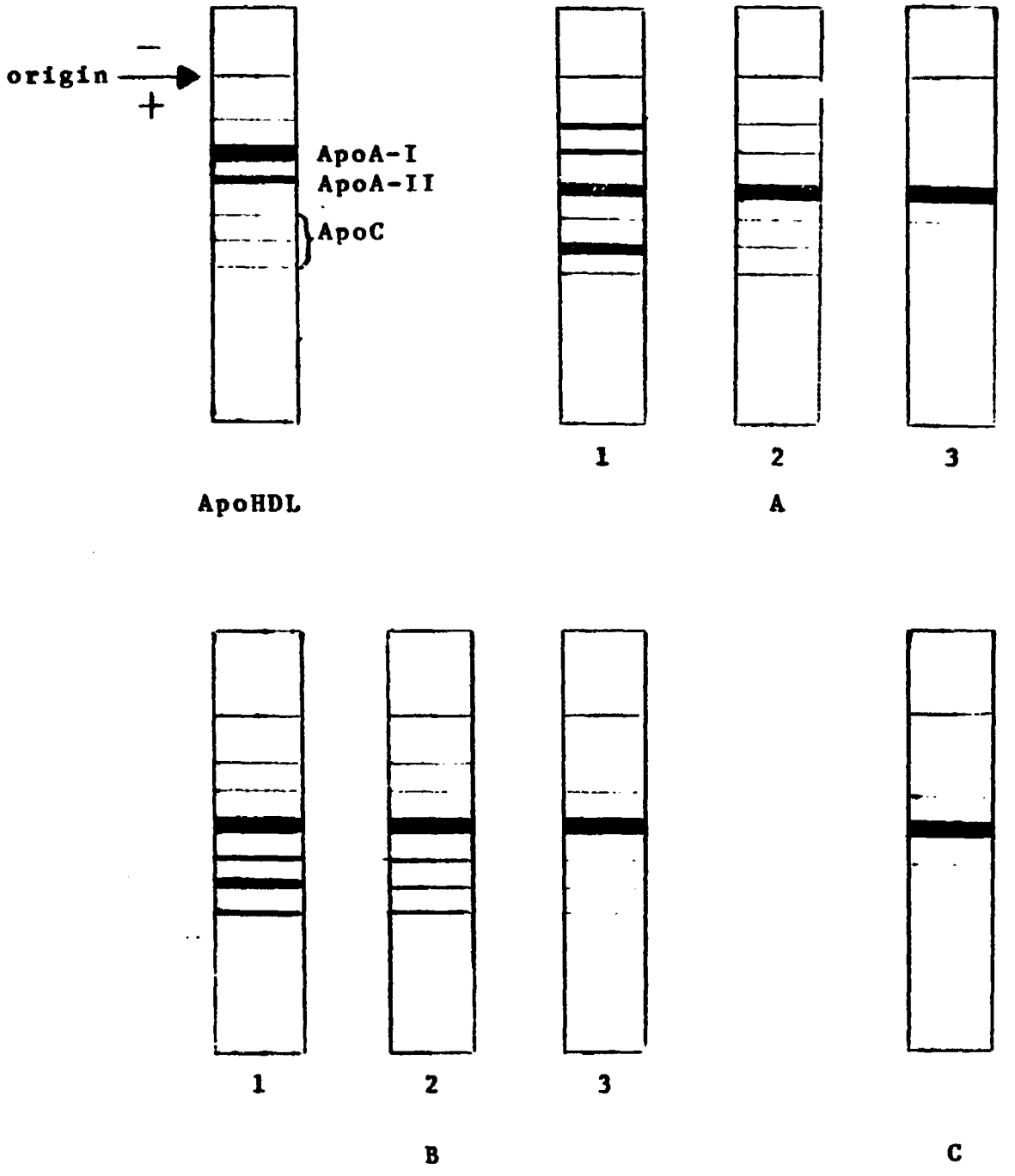


Figure 7--Polyacrylamide Gel Electrophoresis of Fraction 4 and Fraction 5. A,B - Fraction 4 and Fraction 5 from Sephadex G-100 column. 1,2,3 - First, Second and Third Chromatography. C - Mixture of A_3 and B_3.

TABLE 4

AMINO ACID COMPOSITION OF ApoA-II

	Isolated ApoA-II	(a) R-Gln (ApoA-II)	(b) Fraction III (ApoA-II)	(c) Fraction IV (ApoA-II)
Aspartic acid	4.2	3.1	4.2	3.9
Threonine	5.3	6.3	5.5	6.2
Serine	5.4	6.3	5.6	6.0
Glutamic acid	16.5	15.2	15.6	15.8
Proline	4.7	4.0	4.1	3.0
Glycine	3.4	3.3	3.6	2.9
Alanine	5.3	5.2	5.5	5.0
Valine	5.2	5.9	5.8	5.4
1/2 Cystine	0.92(d)	1.2	1.0	2.0
Methionine	1.07	1.2	1.1	1.2
Isoleucine	0.71	1.2	1.5	1.5
Leucine	9.0	8.0	7.7	7.7
Tyrosine	3.3	3.5	3.3	3.0
Phenylalanine	3.5	3.8	3.9	3.1
Tryptophan		0	0	
Lysine	7.6	8.9	10.0	10.0
Histidine	0.42	0	0.29	0
Arginine	1.4	0	0.77	0

Values represent residues per 77 residues. (a) from reference 58; (b) from reference 38; (c) from reference 37; (d) determined as cysteic acid after performic acid oxidation.

The N-terminal amino acid analysis of ApoA-II by dansylation showed no major NH_2-terminal dansyl amino acids on the polyamide sheet. This led to the assumption that the N-terminal amino acid of ApoA-II was blocked. However, a faint dansyl glutamic acid did appear on the polyamide sheet. Thus pyrrolidone carboxylic acid was considered as the likely N-terminal amino acid of ApoA-II.

The sialic acid content of ApoA-II was determined as N-acetylneuraminic acid. The analysis showed less than 0.03 mole of sialic acid per mole of ApoA-II.

In summary, the isolated protein was highly purified ApoA-II. From polyacrylamide gel electrophoresis and amino acid composition data this ApoA-II preparation contained small amounts of ApoA-I and ApoC as contaminants. However, it was decided that the primary structure could be defined on this protein with this small amount of contamination providing we restricted our attention to the sequence of peptides produced in greater than 10% yield.

From Table 4 it can be seen that the protein is most likely to contain about 77 residues. Therefore, the study of complete amino acid sequence seems feasible. The planned steps to approach the primary structure of ApoA-II may be outlined as follows: 1. preparation, purification, and characterization of the tryptic peptides; 2. sequence of tryptic peptides (Edman degradation and/or carboxypeptidase); 3. production of overlap peptides by hydrolysis at points

other than lysines by: (a) fragmentation with cyanogen bromide at methionine residues; (b) hydrolysis with chymotrypsin and thermolysin; 4. ordering of the tryptic peptides.

Trypsin Digest of ApoA-II

The disulfide bridge which holds the two identical polypeptide chains of ApoA-II together was cleaved by performic acid oxidation. The oxidation was checked to be complete by the recovery of cysteic acid. Approximately 58 mg (7.25 μmoles) of oxidized ApoA-II were subjected to trypsin digestion. The soluble tryptic peptide mixture from this trypsin digest was separated on a cation exchange column. The result is shown in Figure 8. A total of 27 peaks were obtained and the amino acid composition of each peak was analyzed. On the basis of yield (over 20%), 10 tryptic peptides (T-1 through T-10b) were considered as major peptides. All others were considered minor peptides except T-10a. Although it was recovered only in 2% yield, it was also considered as a major peptide and will be discussed in detail in the next chapter. Two kinds of minor peptides were found. One type resulted from incomplete hydrolysis by trypsin or from hydrolysis at bonds other than lysine (chymotrypsin-like hydrolysis). Peptides T-1+2 (incomplete hydrolysis of T-1 plus T-2), T-2a and T-2b (chymotryptic hydrolysis derived from peptide T-2), and T-8a and T-8b (chymotryptic hydrolysis derived from peptide T-8) were this type of minor peptides. They were found useful in determining the overlaps of some

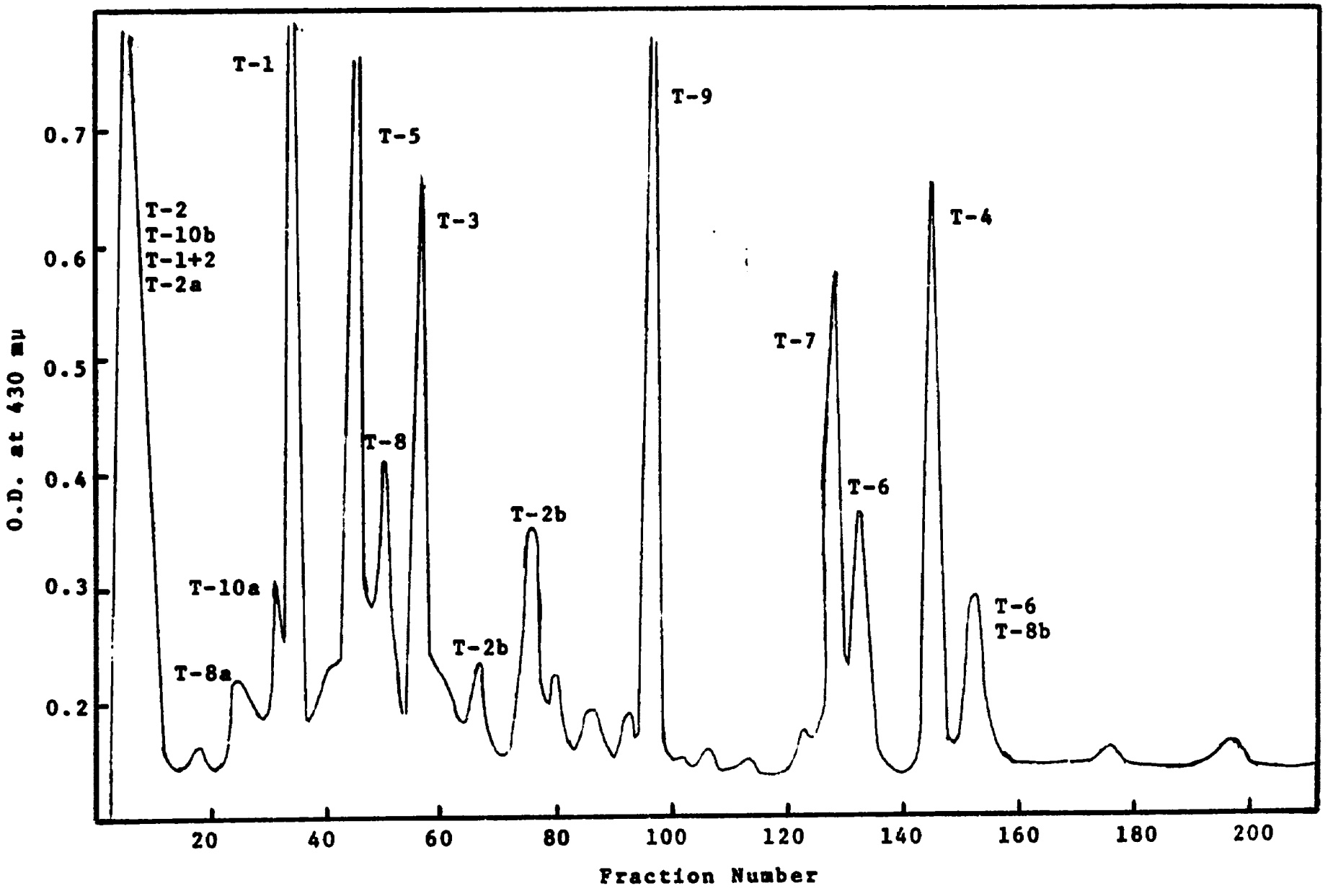


Figure 8--Chromatographic Patterns of Tryptic Peptides of ApoA-II on Cation Exchange Column.

major tryptic peptides. The second type of minor peptides resulted from trypsin digestion of contaminant proteins and they were not studied further.

The purity of all major tryptic peptides and minor peptides was checked by paper electrophoresis at pH 2.1 and pH 6.0 and by integral mole ratios of their constituent amino acids. Peptides T-3, T-4, T-5, T-7, and T-9 gave only one major dominant spot on electrophoresis at both pH's. Based on thier analysis by amino acid composition and paper electrophoresis, these peptides were judged to be pure and ready for amino acid sequence determination. The other major peptides, (T-1, T-2, T-6, T-8, T-10a and T-10b) and some minor peptides (T-2a, T-2b, T-8a and T-8b) were resolved from their mixtures by paper electrophoresis at different pH's and for various periods of time. The purification conditions are presented in Table 5. The characterizations of all purified major tryptic peptides by paper electrophoresis and their amino acid compositions are shown in Table 6 and Table 7 respectively.

Three peaks are of special interest because of the number of components they contained. The breakthrough peak from the ion exchange chromatography (Figure 8) of the trypsin digest was a most complicated one. By purification on paper electrophoresis, this peak was resolved into 4 peptides; two major peptides, T-2 and T-10b; two minor peptides, T-1+2 and T-2a. The yield of peptide T-2 itself was only 0.62 μmole

TABLE 5

PURIFICATION OF TRYPTIC PEPTIDES BY PAPER ELECTROPHORESIS

Peptide	pH	Time (min.)	No. of Ninhydrin Positive Spots			Elution Solvent
			Major	Minor	Ignored	
T-1	2.1	60	T-1		5	0.1 N CH_3CO_2H
T-2	6.0	90	T-2			0.1 N NH_4OH
			T-10b			0.1 N NH_4OH
				T-1+2		0.1 N NH_4OH
				T-2a		0.1 N NH_4OH
T-6[a]	2.1	35	T-6		3	0.1 N CH_3CO_2H
T-6[a]	2.1	30	T-6	T-8b		0.1 N CH_3CO_2H
T-8	3.5	90	T-8		1	0.1 N NH_4OH
T-10a	6.0	100	T-10a		4	0.1 N NH_4OH
T-2b[a]	2.1	55		T-2b	1	0.1 N CH_3CO_2H
T-2b[a]	2.1	45		T-2b	2	0.1 N CH_3CO_2H
T-8b	6.0	110		T-8b	3	0.1 N NH_4OH

All purifications were carried out with Whatman 3 MM paper at 53 volts/cm.

[a]The same peptide came off the ion exchange column in two peaks differing only in the oxidation state of the tyrosine residue.

TABLE 6

CHARACTERIZATION OF TRYPTIC
PEPTIDES BY PAPER ELECTROPHORESIS

Peptide		T-1	T-2	T-3	T-4
R Value	Rser (pH 2.0)	0.83	0.39	1.05	1.69
	Rasp (pH 6.0)	--	0.36	0.34	0.62
Color of Ninhydrin Spot		Red	Red	Red	Red
Molecular Weight	pH 2.0	400	2400	800	280
	pH 6.0	-	2100	660	280
N-terminal Amino Acid		Blocked	Glu	Asp	Val
Yield	µmole	3.2	2.7 (a)	5.0	4.7
	percentage	44.4%	37.5%	69.5%	65.3%

Rser = relative migration distance to that of serine
Rasp = relative migration distance to that of aspartic acid
(a) = corrected yield

TABLE 6--Continued

T-5	T-6	T-7	T-8	T-9	T-10a	T-10b
0.88	1.08	1.79	0.94	-	0.49	0.45
0.23	--	0.65	--	-	0.25	0.22
Yellow /Red	Yellow /Red	Yellow /Red	Red	Red	Red	Red
1180	740	250	1000	-	1350	1700
1120	-	255	--	-	1050	1250
Ser	Ser	Ser	Glu	Freely	Als	Phe
5.4	2.3	4.2	2.6(a)	4.8	0.14	1.6
75.0%	32.0%	58.3%	36.1%	66.8%	2%	22.2%

TABLE 7

AMINO ACID COMPOSITION OF TRYPTIC PEPTIDES OF ApoA-II

	T-1	T-2	T-3	T-4	T-5	T-6
Aspartic acid		1.05	1.02			
Threonine		2.06				
Serine		1.80			1.01	0.99
Glutamic acid	1.02	3.92	1.04		3.06	1.05
Proline		1.19			1.01	
Glycine		1.11				
Alanine	0.98				1.92	
Valine		2.78		0.98		
Methionine			0.83			
Isoleucine						
Leucine		1.23	1.00		0.98	
Tyrosine		1.87				0.96
Phenylalanine		1.24				1.03
Lysine	1.00	1.18	1.10	1.02	1.02	0.98
Cysteic acid		1.09				
SCMC[a]						
Residues	3	20	5	2	9	5

[a] S-carboxymethylcysteine

TABLE 7--Continued

T-7	T-8	T-9	T-10a	T-10b	Sum	ApoA-II (b) Composition
			1.06		3	3
	0.93		1.00	1.77	6	6
0.85			1.00		6	6
	2.10		1.18	2.84	16	16
	1.04			1.16	4	4
			1.23	1.06	3	3
			0.94	0.97	5	5
			1.00	1.05	6	6
					1	1
	0.91				1	1
	2.05		1.76	1.16	8	8
			0.91		4	4
			1.00	0.96	4	4
1.15	0.96	1.00			9	9
					1	
						1
2	8	1	11	11	77	77

(b) from reference 32

(8.6%). Evidence is presented later to support the assumption that T-1+2, T-2a and T-2b are derived from T-2. When the yields of the three major peptides T-1+2, T-2a and T-2b were considered, they gave a final corrected total yield of 2.7 μmoles or 37.5%. Therefore it was concluded that T-2 was indeed a major tryptic peptide.

Peptide T-6 was eluted from the ion exchange column in two distinct peaks (Figure 8). These two peaks were demonstrated later to be the same peptide, T-6, except that the first T-6 peak contained an intact tyrosine residue whereas the second T-6 peak contained an oxidized tyrosine residue. The total yield of these two T-6 peptides was 2.3 μmoles (32%) and the oxidized T-6 accounted for one third of this total.

Peptide T-8 also had minor peptides T-8a and T-8b. T-8a came off the ion exchange column as a separate peak and T-8b came off coincident with the oxidized T-6 peptide. The yield for intact T-8 alone was 1.3 μmoles (18%). However, with the two minor peptides, the total final yield for T-8 was 2.6 μmoles (36%).

Minor peptide T-2b also contained intact and oxidized tyrosine, but they were eluted from the column very close to each other.

Amino Acid Sequence of Tryptic Peptides

Amino acid sequence determination of the tryptic peptides was carried out by using Gray's dansyl-Edman procedure

with the aid of carboxypeptidase A and B digestions. Large peptides were further digested with different proteolytic enzymes to yield smaller peptides for sequence determination. For the purpose of clarity the peptides will be considered in the order in which they occur in the intact protein.

Sequence of peptide T-1. Upon acid hydrolysis T-1 gave a composition of Glu, 1.02; Ala, 0.98; Lys 1.00. Dansylation failed to yield any major N-terminal amino acid but a small amount of dansyl glutamic acid was seen. By paper electrophoresis, T-1 showed one positive charge at pH 2.1 and no charge at pH 6.0. A combined carboxypeptidase A and B digestion gave the sequence of Ala-Lys and no glutamic acid or glutamine was found. All these results indicated that the N-terminal amino acid of T-1 was probably blocked. It also strongly suggested that T-1 was derived from the N-terminal of ApoA-II which was known to have blocked N-terminus. Thus the complete amino acid sequence of this N-terminal T-1 peptide was PCA-Ala-Lys.

Sequence of peptide T-2: T-2 was a 20 residue peptide. Glutamic acid was found to be the N-terminal and lysine to be the C-terminal amino acid of T-2. In order to determine the sequence of this long peptide, it was necessary to reduce T-2 to smaller peptides. About 0.4 μmole of T-4 was digested with thermolysin at 37° for 2 hours and the mixture was separated by paper electrophoresis at pH 2.1. Four peptides were obtained and they were designated as T-2a_1, T-2a_2, T-2b_1

and T-2b$_2$. The enzyme digestion was close to completion and the recoveries of these four peptides were very high. The amino acid composition of these four peptides is shown in Table 8. The amino acid sequence of T-2a$_2$ and T-2b$_2$ were done by dansyl-Edman procedure alone and the sequence of T-2a$_1$ and T-2b$_1$ was done by dansyl-Edman, Edman-subtractive, and carboxypeptidase A. The sequence for T-2a$_1$ was Glu-Pro-Cys-Val-Glu-Ser; for T-2a$_2$ was Leu-Val-Ser-Gln-Thr; for T-2b$_1$ was Phe-Gln-Thr; for T-2b$_2$ was Val-Thr-Asp-Tyr-Gly-Lys. The identifications of glutamic acid or glutamine, and aspartic acid or asparagine were determined by ionic charge during paper electrophoresis at pH 6.0. Peptide T-2a$_1$ was the only peptide having glutamic acid as N-terminal amino acid, therefore T-2a$_1$ was assigned as the N-terminal peptide of T-2. Similarly T-2b$_2$ contained the only lysine residue as its C-terminal amino acid, thus it was assigned as the C-terminal peptide of T-2. To assign the order of the middle two peptides (T-2a$_2$ and T-2b$_1$), minor peptides (T-2a and T-2b) from the trypsin digest of ApoA-II were utilized. T-2a had the amino acid composition of T-2a$_1$ plus T-2a$_2$ and contained N-terminal glutamic acid while T-2b had the amino acid composition of T-2b$_1$ plus T-2b$_2$ and contained N-terminal phenylalanine. Hence, it would appear that the order of the four thermolysin peptides was T-2a$_1$-T-2a$_2$-T-2b$_1$-T-2b$_2$, although actual overlap peptides were not obtained. In addition, the minor peptide T-1+2 had the amino acid composition of T-1

TABLE 8

AMINO ACID COMPOSITION
OF THERMOLYSIN PEPTIDES OF T-2

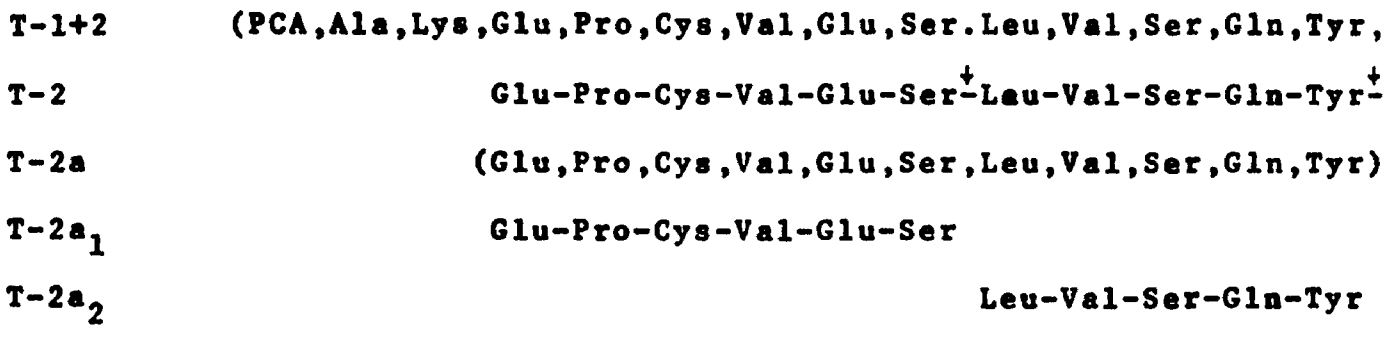
	$T\text{-}2a_1$	$T\text{-}2a_2$	$T\text{-}2b_1$	$T\text{-}2b_2$
Aspartic Acid				1.06
Threonine			1.03	1.00
Serine	0.91	0.89		
Glutamic Acid	2.00	1.14	0.95	
Proline	1.23			
Glycine				1.03
Valine	1.06	1.02		1.00
Leucine		1.07		
Tyrosine		0.85		0.94
Phenylalanine			1.03	
Lysine				0.97
Cysteic Acid	0.79			
Rser pH 2.0	—	0.56	0.67	0.91
Yield µmole	0.37	0.36	0.32	0.32
Yield percentage	92.5%	90%	80%	80%
Residues	6	5	3	6
N-terminal	Val	Leu	Glu	Phe

and T-2, the first N-terminal 23 residues of ApoA-II could be ordered as peptide T-1 followed by peptide T-2. The sequence of these 23 residues is summarized in Figure 9.

Sequence of peptide T-3. T-3 was a 5 residue peptide and the only methionine-containing peptide. Sequening of this peptide was carried out by the dansyl-Edman procedure. The methionine residue had been oxidized to methionine sulfone by performic acid and its dansyl derivative of the sulfone migrated to the serine-threonine area on the polyamide sheet. The amino acid sequence of T-3 was Asp-Leu-Met-Glu-Lys. Both acidic amino acids were identified as aspartic and glutamic acid by paper electrophoresis at pH 6.0.

Sequence of peptide T-4. Amino acid composition and electrophoretic mobility at different pH's showed that T-4 was a dipeptide. The actual amino acid sequence was determined as Val-Lys for T-4.

Sequence of peptide T-5. T-5 was obtained in the highest yield (5.4 μmole or 75%) of all tryptic peptides. It was a 9 residue peptide containing serine as the N-terminal and lysine as the C-terminal amino acid. The dansyl-Edman procedure gave the order of the first 4 residues, Ser-Pro-Glu-Leu-(Glu_2, Ala_2, Lys_1). Degradation of T-5 to smaller peptides was attempted. Approximately 0.6 μmole of peptide T-5 was incubated with thermolysin at 37° for 6 hours. The mixture was separated by paper electrophoresis at pH 6.0 for 50 minutes. Three peptides were obtained and they were



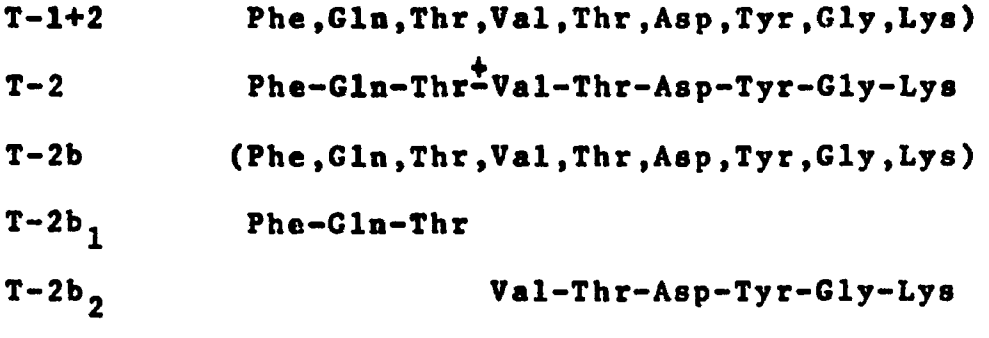


Figure 9--The Amino Acid Sequence of Peptide T-2. Thermolysin hydrolysis sites are shown by arrows.

designated at T-5a, T-5b, and T-5c. The amino acid composition, yield, and N-terminal amino acid of these three peptides are presented in Table 9. Since the only serine and proline of T-5 were contained in T-5a, it was the N-terminal peptide of T-5. T-5c was the C-terminal peptide because it contained the lysine. Hence T-5b must be the middle peptide. The acid residue in T-5a was identified as glutamic acid by ionic charge at pH 6.0. Thus, by the dansyl-Edman procedure the amino acid sequence was determined to be Leu-Gln-Ala-Glu for T-5b and Ala-Lys for T-5c. The glutamine and glutamic acid in T-5b were determined by paper electrophoresis at pH 6.0 after one and two Edman cycles. Thus, the complete sequence of T-5 was Ser-Pro-Glu-Leu-Gln-Ala-Glu-Ala-Lys.

Sequence of peptide T-6. Approximately one third of the tyrosine residues were oxidized in peptide T-6 by performic acid and hence T-6 was eluted from the ion exchange column as two separate peaks. Both peaks were purified and their amino acid sequences were determined separatley. On the amino acid analyzer, oxidized tyrosine appeared on the short column after the phenylalanine peak. On the polyamide sheet, its dansyl derivative migrated about halfway between the origin and O-dansyltyrosine. The amino acid sequence of these two peaks proved to be identical as Ser-Tyr-Phe-Glu-Lys. The acid group was identified as glutamic acid by peptide mobility at pH 6.0.

TABLE 9

AMINO ACID COMPOSITION

OF THERMOLYSIN PEPTIDES OF T-5

	T-5a	T-5b	T-5c
Serine	0.95		
Glutamic acid	1.06	1.96	
Proline	0.99		
Alanine		1.00	0.99
Leucine		1.04	
Lysine		1.01	
Rasp pH 6.0	0.52	0.38	0.66
Yield μmole	0.51	0.47	0.53
%	87%	78%	88%
Residues	3	4	2
N-terminal	Ser	Leu	Ala

Sequence of peptide T-7. Both amino acid composition and paper electrophoresis at different pH's showed that T-7 was a dipeptide. The amino acid sequence was determined to be Ser-Lys for T-7.
$\rightarrow \rightarrow$

Sequence of peptide T-8. T-8 contained 8 residues and was the only isoleucine-containing peptide. It had the composition of Glx_2,Leu_2,Thr_1, Pro_1,Ile_1,Lys_1. The sequence of the first 5 residues was carried out by dansyl-Edman procedure, Glx-Glx-Leu-Thr-Pro(Leu,Ile,Lys). The sequence
$\rightarrow \rightarrow \rightarrow \rightarrow \rightarrow$
was confirmed by quantitative subtractive sequence determination. After the first Edman cycle, the remaining peptide lsot one negative charg, therefore the first two residues were Glu-Gln. Incubation of peptide T-8 with a mixture of carboxypeptidase A and B revealed the C-terminal sequence to be -Leu-Ile-Lys. Therefore the complete amino acid sequence
$\leftarrow \leftarrow \leftarrow$
of T-8 was Glu-Gln-Leu-Thr-Pro-Leu-Ile-Lys.
$\rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \leftarrow \leftarrow \leftarrow$

Sequence of peptide T-9. T-9 contained only one lysine residue. This lysine residue was confirmed by amino acid composition (with and without acid hydrolysis) and by paper electrophoresis at both pH 2.1 and pH 6.0.

Sequence of peptide T-10a. Only 0.14 μmoles of T-10a were recovered from trypsin digest, so special caution was taken for the sequence determination of this peptide. It had the composition of Ala_1,Gly_1,Thr_1,Glx_1,Leu_2,Val_1,Asx_1,Phe_1, Ser_1,Tyr_1. Dansyl-Edman procedure proceeded 8 steps and the amino acid sequence was Ala-Gly-Thr-Glx-Leu-Val-Asx-Phe-Leu-
$\rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow$

(Ser,Tyr). In a low temperature carboxypeptidase A digestion, tyrosine was revealed to be the C-terminal amino acid of peptide T-10a. The experimental conditions used for this digestion were--a peptide enzyme mixture (100/1, wt/wt) was incubated at 7° for 5 min. The mixture was dried and dansylated. In addition to the dominant bis-dansyl tyrosine spot on the polyamide sheet, a trace amount of dansyl serine was also found. Thus the sequence of the last two residues was demonstrated to be Ser-Tyr.
← ←

At pH 6.0 peptide T-10a showed only one negative charge on paper electrophoresis. Therefore, either Glx or Asx should be in acid form. During a separate carboxypeptidase A digestion experiment in which more enzyme was used, dansyl asparagine was found to be one of the amino acids released by carboxypeptidase A. It was evident that Glx must be a glutamic acid. The complete sequence of T-10a was clearly established to be Ala-Gly-Thr-Glu-Leu-Val-Asn-Phe-Leu-Ser-Tyr.
→ → → → → → → → → ← ←

Sequence of peptide T-10b. T-10b was a peptide containing 11 residues and had the composition $Thr_2Glx_3Pro_1Gly_1Ala_1Leu_1Phe_1$. Dansylation gave phenylalanine as the N-terminal amino acid and carboxypeptidase A revealed the last two residues as -Thr-Gln. Since it was reported (43) that ApoA-II
← ←
had -Thr-Gln as its C-terminal sequence, peptide T-10b was assigned to be the C-terminal peptide of ApoA-II. The dansyl-Edman procedure was unequivocal in identifying the order to the 7th residue and the sequence was Phe-Val-Glx-Leu-Gly-Thr-
→ → → → → →

Glx-(Pro,Ala)-Thr-Gln. In order to complete the sequence, further degradation of T-10b was necessary. Thermolysin was again used to produce smaller peptides. The peptide-enzyme mixture was incubated at 37° for 6 hours. The peptides in the mixture were separated by paper electrophoresis at pH 2.1. Three degraded peptides were obtained and they were designated as T-10b$_1$, T-10b$_2$, and T-10b$_3$. The characterization of these three peptides is presented in Table 10. By amino acid composition the order of the three peptides was easily assigned as T-10b$_1$-T-10b$_2$-T-10b$_3$. The sequence of T-10b$_2$ was determined to be Leu-Gly-Thr-Gln-Pro and T-10b$_3$ to be Ala-Thr-Gln. The acid or amide Glx in all three peptides was determined by ionic charge at pH 6.0. Thus, the complete amino acid sequence of T-10b was Phe-Val-Glu-Leu-Gly-Thr-Gln-Pro-Ala-Thr-Gln.

The amino acid sequence of all tryptic peptides of ApoA-II was compelte at this stage and they are presented in Figure 10. In order to align these tryptic peptides, cyanogen bromide cleavage of ApoA-II to produce overlap peptides was undertaken.

Cyanogen Bromide Fragmentation of ApoA-II

There is only one methionine residue in ApoA-II; a preliminary cyanogen bromide cleavage experiment showed that the yield was less than 10%. This indicated that methionine was probably in methionine sulfoxide state. Since methionine sulfoxide does not react with cyanogen bromide, it was

TABLE 10

AMINO ACID COMPOSITION OF

THERMOLYSIN PEPTIDES OF T-10b

	$T\text{-}10b_1$	$T\text{-}10b_2$	$T\text{-}10b_3$
Threonine		0.85	1.03
Glutamic acid	1.14	1.10	1.01
Proline		0.95	
Glycine		1.04	
Alanine			0.96
Valine	1.04		
Leucine		1.03	
Phenylalanine	0.81		
Rser pH 2.0	0.70	0.63	0.77
Yield μmole	0.22	0.26	0.27
%	37%	43%	45%
Residues	3	5	3
N-terminal	Phe	Leu	Ala

T-1 PCA-Ala-Lys

T-2 Glu-Pro-Cys-Val-Glu-Ser↓-Leu-Val-Ser-Gln-Tyr↓-Phe-Gln-Thr↓-Val-Thr-Asp-Tyr-Gly-Lys

T-3 Asp-Leu-Met-Glu-Lys

T-4 Val-Lys

T-5 Ser-Pro-Glu↓-Leu-Gln-Ala-Glu↓-Ala-Lys

T-6 Ser-Tyr-Phe-Glu-Lys

T-7 Ser-Lys

T-8 Glu-Gln-Leu-Thr-Pro-Leu-Ile-Lys

T-9 Lys

T-10a Ala-Gly-Thr-Glu-Leu-Val-Asn-Phe-Leu-Ser-Tyr

T-10b Phe-Val-Glu↓-Leu-Gly-Thr-Gln-Pro↓-Ala-Thr-Gln

Figure 10--Amino Acid Sequence of Tryptic Peptides of ApoA-II. Thermolysin hydrolysis sites are shown by arrows.

necessary to reduce methionine sulfoxide to methionine. The reduction was carried out by thioglycolic acid in the presence of guanidine hydrochloride and dithioerythritol. Approximately 45% of the methionine sulfoxide was reduced to methionine as shown by amino acid analysis, the remainder was still in the sulfoxide state. Despite this, cyanogen bromide cleavage was attempted with the reduced ApoA-II.

Cyanogen bromide reaction was carried out in 70% formic acid and the resulting fragments were separated on Sephadex G-50 in 8 M urea and 0.1 N acetic acid. The chromatographic pattern of the separation is shown in Figure 11. Four peaks were obtained and their amino acid compositions are presented in Table 11. By amino acid composition it was difficult to judge what the first three peaks were. It seemed that the first peak contained intact ApoA-II and some amount of impurity proteins because of the high arginine content; the second peak was a mixture of intact ApoA-II and C-terminal fragment of ApoA-II; the third peak contained primarily the C-terminal fragment and a small amount of intact ApoA-II plus some material (whole or methionine cleavage products) from the impurity proteins. Preliminary SDS-polyacrylamide gel electrophoresis indicated that these suggestions might be the case. Peak 1 showed heterogenous 4-5 bands and one of which seemed to be the major one. Peak 2 showed two major bands which did not have a large difference in quality. Peak 3 had one major band and 2-3 minor bands. However, it

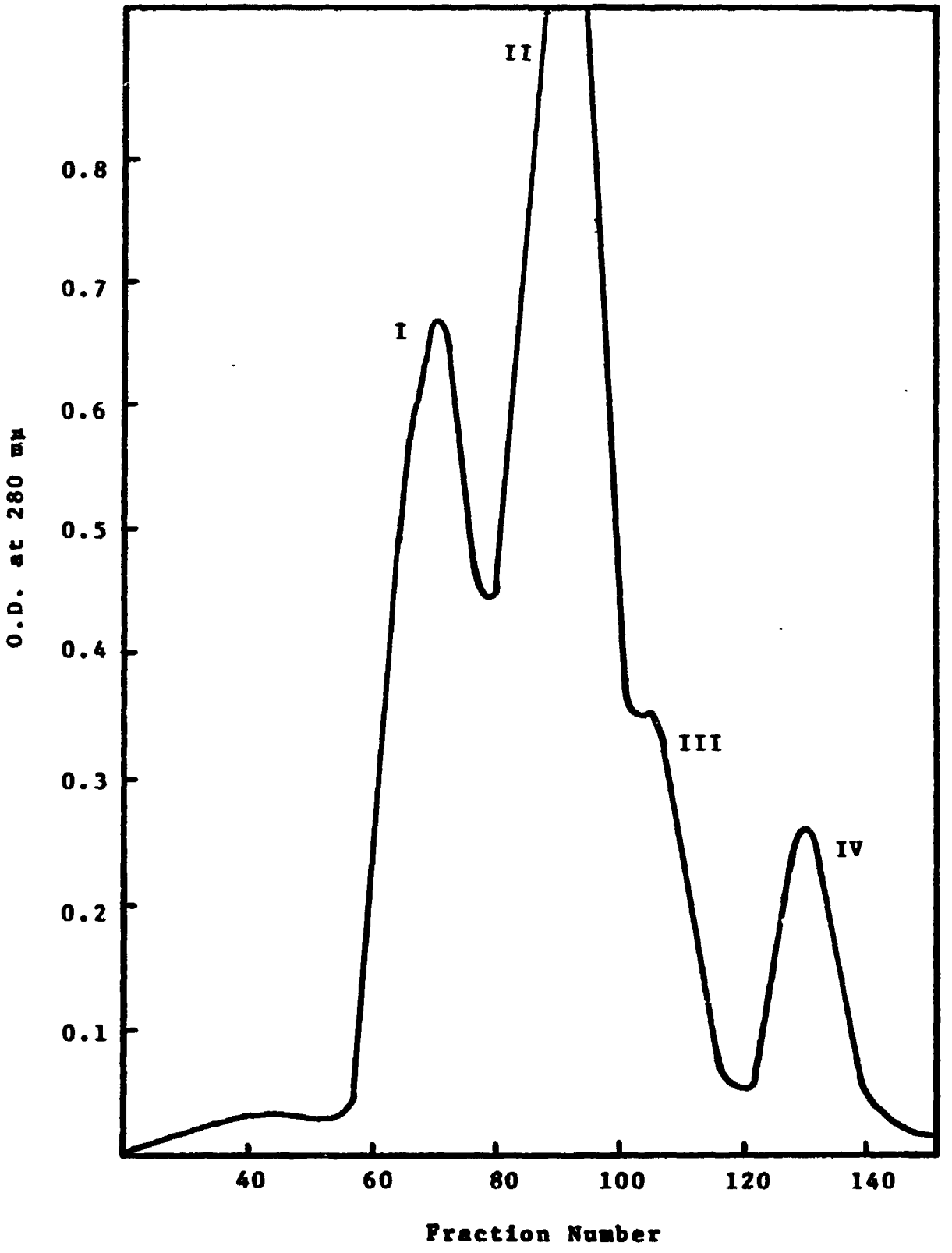


Figure 11--Chromatographic Patterns of CNBr Fragments of ApoA-II on Sephadex G-50.

TABLE 11

AMINO ACID COMPOSITION OF CNBr
FRAGMENTS AND N-TERMINAL 23 RESIDUES OF ApoA-II

	Peak I	Peak II	Peak III	Peak IV	N-terminal 23 Residues of ApoA-II
	(a)	(a)	(a)	(d)	
Aspartic Acid	8.4	5.5	3.8	1.7	1
Theronine	5.3	7.3	6.9	1.8	2
Serine	6.7	7.1	7.0	1.9	2
Proline	4.4	4.7	5.8	1.1	1
Glutamic Acid	21.0	22.0	24.7	5.3	5
Glycine	5.2	4.3	4.8	1.1	1
Alanine	4.6	7.0	8.4	0.9	1
Valine	7.2	8.4	4.4	2.8	3
Aminoethylcysteine (b)	0.39	1.2		1.0	
Methionine	1.2	1.2	0.43	1.0(c)	
Isoleucine	0.67	1.3	1.5		
Leucine	13.2	10.9	11.0	2.1	1
Tyrosine	3.2	4.5	3.2	1.7	2
Phenylalanine	3.7	3.7	4.6	4.8	1
Lysine	10.0	10.0	11.7	2.0	2
Histidine	0.33	0.35			
Arginine	4.7	0.8	1.5		
Cysteic Acid					1

(a) values expressed as residues per 100 residues (b) estimated values (c) estimated as homoserine and homoserine lactone (d) residues per 26 residues.

was evident that peak 4 was the pure N-terminal fragment of ApoA-II. It showed one single band on SDS-polyacrylamide gel electrophoresis, contained homosetine and homoserinelactone, and its N-terminal amino acid was shown to be blocked. The only difference revealed by close examination of the compositions (Table 11) between the N-terminal fragment and the first N-terminal 23 residues from the tryptic peptides, was that the N-terminal fragment from cyanogen bromide cleavage had three more amino acids: aspartic acid, leucine, and methionine. Tryptic peptide T-3 was the only methionine-containing peptide and had the sequence, Asp-Leu-Met-Glu-Lys. Therefore, it was evident taht peptides T-1-T-2 were followed by T-3 and the sequence could be extended to residue 28. Based on the published results (42), the tryptic peptides could be aligned (Figure 12).

PCA - Ala - Lys ± Glu - Pro - Cys - Val - Glu - Ser - Leu -
1 2 3 4 5 6 7 8 9 10

Val - Ser - Gln - Tyr - Phe - Gln - Thr - Val - Thr - Asp -
11 12 13 14 15 16 17 18 19 20

Tyr - Gly - Lys ± Asp - Leu - Met - Glu - Lys ± Val - Lys ±
21 22 23 24 25 26 27 28 29 30

Ser - Pro - Glu - Leu - Gln - Ala - Glu - Ala - Lys ± Ser -
31 32 33 34 35 36 37 38 39 40

Tyr - Phe - Glu - Lys ± Ser - Lys ± Glu - Gln - Leu - Thr -
41 42 43 44 45 46 47 48 49 50

Pro - Leu - Ile - Lys ± Lys ± Ala - Gly - Thr - Glu - Leu -
51 52 53 54 55 56 57 58 59 60

Val - Asn - Phe - Leu - Ser - Tyr ± Phe - Val - Glu - Leu
61 62 63 64 65 66 67 68 69 70

Gly - Thr - Gln - Pro - Ala - Thr - Gln
71 72 73 74 75 76 77

Figure 12--Primary Structure of ApoA-II. Trypsin digestion sites are shown by arrows.

CHAPTER IV

DISCUSSION

Human high density lipoprotein was isolated for this study from a mixed pool of plasma. Salt precipitation (sodium phosphotungstate and magnesium chloride) instead of conventional ultracrntrifugal flotation was used to fractionate the plasma. Isolated HDL was established to be pure by electrophroetical and immunological means and the procedure was demonstrated to be successful in producing HDL of high purity. The advantages of this method were that large qunatities could be processed rapidly. In a compartivie study of the two methods, Alaupovic et al (21) used the same procedure, as well as the ultracentrigugation method, to isolate HDL, LDL, and VLDL. They also reported that the precipitation was highly specific for each class of lipoprotein.

Because ApoA polypeptides have a marked tendency towards aggregation, it was not until recently that the concept of an HDL consisting of two major polypeptides and minor ApoC polypeptides was well documented (33,36,37,38 and 58). A great deal of effort was made to find methods for completely separating the two ApoA polypeptides (ApoA-I and ApoA-II) and isolating each in pure form. This has been very

difficult because the polypeptides have an even greater tendency towards aggregation after their lipid has been removed. Various procedures were tried in the course of this study to separate these two peptides. Among them were Sephadex of different pore sizes in alkaline pH and in acid pH, in SDS, and chromatography on DEAE-cellulose without urea. Isolation and purification of ApoA-II by gel filtration on Sephadex G-100 in 1 N acetic acid was most satisfactory.

The purity and identification of ApoA-II were demonstrated by polyacrylamide gel electrophoresis, amino acid composition, N-terminal amino acid analysis, and sialic acid content. The polyacrylamide gel electrophoresis of ApoA-II showed one dominant homogeneous band in 8 M urea. Its relative migration corresponded to the published pattern for ApoA-II (40). However, the polyacrylamide gel also showed a small amount of other proteins (ApoA-I and ApoC) which were estimated to be approximately 10% of the total. This figure seemed to agree with the yield of impurity protein peptides after trypsin digestion of ApoA-II. The amino acid composition of this ApoA-II preparation closely resembled the compositions reported by other investigators (37,38,58). This ApoA-II preparation contained essentially no histidine, no tryptophan, and a low amount of contaminant arginine. Furthermore N-terminal analysis of ApoA-II by dansylation showed no major reactive N-terminal amino acid, although a very small amount of dansyl glutamic acid was detected. This result agreed with the report by

Kostner et al (40) in which they demonstrated that dansylation and dinitrophenylation methods failed to produce reactive N-terminal amino acid and it was concluded that the N-terminal amino acid of ApoA-II was blocked. We further assumed that the blocked N-terminal was likely to be pyrrolidone carboxylic acid because during the alkaline conditions of the dansylation procedure a very small amount of pyrrolidone carboxylic acid could be hydrolyzed to yield glutamic acid. This assumption was demonstrated to be true recently by other investigators (39). The sialic acid analysis indicated that essentially no sialic acid was present in ApoA-II. This again confirmed a previous report (39) that no carbohydrate was detected in ApoA-II. Based on all the results obtained it was concluded that the isolated protein was indeed ApoA-II. This highly purified ApoA-II was used in this attempt to determine its amino acid sequence.

It was reported (41) that ApoA-II contains two polypeptides which are held together by a single disulfide bond. Performic acid oxidation was performed on the isolated ApoA-II and its subsequent trypsin digestion gave rise to major peptides (yield over 20%). The number of tryptic peptides obtained seemed to eliminate the possibility of two non-identical polypeptides in ApoA-II. The amino acid sequences of all tryptic peptides were determined. Peptide T-1 was assigned to the N-terminal peptide of ApoA-I because of the blocked N-terminal amino acid. Peptide T-10b was assigned to the

C-terminal peptide because it contained no lysine and had the C-terminal sequence of -Thr-Gln. With the information of minor peptides (T-2a, T-2b, and T-1+2) from trypsin digestion and the N-terminal cyanogen bromide cleavage fragment, the first 28 residues from the N-terminal molecule of ApoA-II appeared to be Pca-Ala-Lys-Glu-Pro-Cys-Val-Glu-Ser-Leu-Val-Ser-Gln-Tyr-Phe-Gln-Thr-Val-Thr-Asp-Tyr-Gly-Lys-Asp-Leu-Met-Glu-Lys, although the actual overlap peptide was not sequenced to confirm it.

Preliminary cyanogen bromide cleavage of a small amount of ApoA-II indicated that over 90% of the methionine residue was present as methionine sulfoxide but not as methionine sulfone. Methionine sulfone is stable to acid hydrolysis and would be revealed by amino acid analysis; methionine sulfoxide is converted mostly to methionine during acid hydrolysis but it does not react with cyanogen bromide. The presence of the methionine sulfoxide was not detected nor suspected until the methionine failed to react with the cyanogen bromide. The reason for this oxidation was not clear and presumably the oxidation occurred during the fractionation and isolation of ApoA-II. The possible steps during which the oxidation occurred might be speculated as follows: 1. During sodium phosphotungstate-magnesium chloride precipitation. It would be possible that the phosphotungstate serves as an oxidizing agent. 2. During the ether-ethanol extraction. In the six day period of exposure of ApoA-II to ether-ethanol, peroxide

might be generated to cause the oxidation. 3. During the removal of ether-ethanol from the aqueous phase by drying with nitrogen. Prepurified nitrogen was not used at this step to remove ether-ethanol. It was conceivable that the presence of oxygen in the nitrogen might have caused the oxidation. In addition, it seemed that storage was also an important factor in the oxidation. Methionine in ApoA-II could be oxidized slowly even in the dry state. The longer the period of storage, the more difficulty the methionine sulfoxide could be reduced back to methionine. It appeared to be true by the fact that in an earlier experiment cyanogen bromide cleavage was performed on an ApoA-I-like peptide (60). It was also found that almost all methionine was oxidized to sulfoxide. After reduction with thioglycolic acid in the presence of 6 M guanidine hydrochloride 80-85% of the methionine was recovered. However, some months later only 45% of the methionine was reduced in ApoA-II under the same reducing condition.

A total of 66 residues was obtained from 10 tryptic peptides (yield over 90%). Based on the composition in Table 4 (58), a tryptic peptide which contained 11 residues and had the composition, $Asx_1Thr_1Ser_1Glx_1Gly_1Ala_1Val_1Leu_2Thr_1Phe_1$ was missing. Since there are 9 lysine residues in ApoA-II and 10 tryptic peptides were already obtained, this missing peptide probably resulted from chymotrypsin-like hydrolysis by trypsin. This was not unusual in this particular trypsin digestion because it was found earlier that peptides T-2a,

T-2b and T-8a, T-8b were also chymotrypsin-like hydrolysis products of peptides T-2 and T-8, respectively. Therefore, a close reexamination of the low yield peptides was attempted. Peptide T-10a was found and its amino acid sequence was determined. Thus, 11 tryptic peptides with a total of 77 amino acid residues were obtained. While efforts were being made on cyanogen bromide cleavage fragments to produce overlap peptides, Brewer et al (39) published the entire amino acid sequence of ApoA-II. The sequence of the first 28 residues from the N-terminal molecule and the sequence of the remaining tryptic peptides from this work were compared to Brewer's independently derived sequence. They were shown to be essentially identical and further overlap peptides to align the tryptic peptides were found unnecessary.

Examination of the sequence indicated peptide T-10a occurred from a chymotrypsin-like hydrolysis of 22 C-terminal residues by trypsin. The hydrolysis occurred between residue 66 (tyrosine) and residue 67 (pheylalanine). Therefore, they were designated as T-10a and T-10b indicating that they were from one tryptic peptide. The identical hydrolysis also occurred at residue 14 (tyrosine) and residue 15 (phenylalanine) to give rise to minor peptides T-2a and T-2b. These two chymotryptic sites hydrolyzed by trypsin might be due to the highly hydrophobic nature of the peptides, particularly peptide T-10a and T-10b. Since peptide T-9 (a single lysine residue at position 53) had a much higher yield (66.8%0 than

peptide T-10b (22.2%), theoretically peptide T-10a could also have at least the same yield as T-10b. However, only 2 % of T-10a was recovered. The possible explanations for such low yield are stated as follows. 1. There was evidence (paper electrophoresis) to show that peptide T-10a was eluted from the ion exchange column as a broad peak under peak T-8a, T-10a, and probably T-1 (Figure 8). Only this peptide from under peak T-10a was recovered because this peptide from under the preceeding and following peaks was ignored during repurifications and lost. Thus 2% was the minimun yield for T-10a. In addition, peptide T-10a was a difficulty-soluble peptide and handling losses during the purification process would be high. 2. According to its sequence peptide T-10a at pH 3 and pH 5 should carry a partial negative charge and normally should be eluted off the cation ion exchange column near the breakthrough peak. However it appeared in the chromatogram much later than expected. Possible hydrophobic interactions between the resin and the peptide might have occurred. Thus a significant amount of T-10a was probably never eluted from the column. In general peptides hydrophobic in nature tend to give low yields off polystyrene ion exchange columns. 3. It was possible that additional hydrolysis by trypsin between residue 63 (phenylalanine) and residue 64 (leucine) might have occurred. If this happened, it would make T-10 even more heterogeneous and lower the yield.

Although the entire amino acid sequence of ApoA-II was published, the details fo their procedure have not been reported.

However based on the information reported (39), there are few apparent different features between the two independent studies.

1. Brewer obtained the plasma from one female donor and all the primary structure work was done on this sole plasma donor. HDL was isolated by conventional ultracentrifugal flotation and ApoA-II was purified by DEAE-cellulose chromatography in 6 M urea.

We obtained the plasma from a mixed pool of at least 30 healthy donors' plasmas (90% male and 10% female). HDL was fractionated by sodium phosphotungstate-magnesium chloride precipitation and ApoA-II was purified by chromatography on Sephadex G-100 in 1 N acetic acid.

For the purpose of this study the two procedures are comparable. The amino acid sequence of ApoA-II derived from either preparation was essentially identical. In neither case was apparent polymorphism found. Scanu (59) reported earlier that polymorphism was detected in ApoA-II. Brewer suggested that the difference might be due to heterogeneity in the donors or due to the differences in isolation techniques. Based on our results, those two possibilities are not likely to be the reasons.

2. The amino acid sequence was determined by both manual Edman degradation and automated sequencer by Brewer et al. The dansyl-Edman procedure and carboxypeptidase were the only techniques for sequence determination in this study.

However, both results were compatible and confirmed each other.

3. The only apparent difference between the two sequences was the residue at position 37. Glutamine was assigned to this position by Brewer. However, an abundance of evidence in our study suggested that this residue was glutamic acid. Residue 37 belongs to tryptic peptides T-5 which has the sequence $\overset{31}{\text{Ser}}\text{-}\overset{32}{\text{Pro}}\text{-}\overset{33}{\text{Glx}}\text{-}\overset{34}{\text{Leu}}\text{-}\overset{35}{\text{Glx}}\text{-}\overset{36}{\text{Ala}}\text{-}\overset{37}{\text{Glx}}\text{-}\overset{38}{\text{Ala}}\text{-}\overset{39}{\text{Lys}}$ and had the highest yield (75%) of all ApoA-II tryptic peptides. The intact T-5 showed one net negative charge and had Rasp value of 0.23 at pH 6.0. This indicated that two of the three Glx must be glutamic acids. Thermolysin digestion of T-5 yielded three peptides T-5a (residue 31 to 33), T-5b (residue 34-37), and T-5c (residue 38-39). Glx (residue 33) in T-5a was demonstrated to be glutamic acid by having the Rasp value (Table 6) of 0.52 at pH 6.0. Therefore one of the two Glx in T-5b must be glutamic acid and this is by the fact that T-5b indeed had Rasp value of 0.38 at pH 6.0. To further demonstrate which Glx was glutamic acid, Edman cycles were carried out on T-5b and peptides Glx-Ala-Glx and Ala-Glx were separated by electrophoresis at pH 6.0. Thus glutamic acid was assigned to position 37. The possible explanations for this sole difference can be visualized in two ways; 1. Brewer might have simply misjudged glutamic acid for glutamine. 2. A possible deamidation reaction might have occurred. Since peptide T-5 had the highest yield (75%), it came off ion-exchange column as a very symmetric peak (Figure 9), and it gave one pure spot on paper

electrophoresis at pH 6.0, these results indicate that residue 37 already exhisted as glutamic acid in native ApoA-II and not a mixture of glutamic acid and glutamine. If deamidation of glutamine occurred, it probably occurred during the purification of ApoA-II stage where 1 N acetic acid was used a solvent for the gel chromatography. This would require that this one amide group of glutamine is extremely labile, and, particularly sensitive amide groups have been reported in the literature. One instance reported by Riniker et al (61) showed the amide group of Asn-Gly- sequence in porcine and human ACTH to be a very labile bond.

Nichols et al (62) reported recently that dehydration by rotary evaporation and solubilization at pH 8.6 of HDL resulted in a highly specific and reproducible redistribution of the lipid and protein components of the parent material into four new ultracentrifugal fractions. These four fractions were d<1.006, d 1.006-1.063, d 1.063-1.21, and d 7.1>1.21 g/ml. The redistribution of lipid components indicated that apolar cholesteryl esters and triglycerides were primarily in the d<1.006 and d 1.006-1.063 g/ml fractions whereas phospholipids were predominately in the d 1.006-1.063 and d 1.063-1.21 g/ml fractions. There was little lipid present in d>1.21 g/ml fraction and ApoA-I was almost exclusively in this fraction. The AooC minor proteins in HDL were found primarily associated with apolar lipid fractions. The other HDL major apoprotein, ApoA-II, was predominately in phospholipid rich fractions,

almost exclusively in d 1.063-1.21 g/ml phospholipid fraction. Hirz and Scanu (63) also observed the similar redistribution of lipids and apoproteins when HDL was disrupted mildly by sonication. These results suggested that apoproteins possibly have different affinities for different lipids in native HDL and that ApoA-II is primarily associated wit polar phospholipid. The elucidation of the sequence of ApoA-II wil enable the more detailed study of ApoA-II-phospholipid binding sites on the molecule. One approach to locate the potential binding sites of ApoA-II was proposed by Delaney (64) according to Welscher's method (65). A plot was constructed based on the relative hydrophobicities of amino acid side chains using the ΔH in calories for transfer of the R gourp from benzene to water. Amino acids with nonpolar side chains have high hydrophobicities and amino acids with polar side chains have little or no hydrophobicity. Four charge clusters and two hydrophobic regions were found in the sequence of ApoA-II. The four charge clusters are residue 3-8, residue 20-28, residue 43-47 and residue 54-59 containing the amino group of lysine and carboxylic group of glutamic acid and/or aspartic acid. They are presumably the potential sites for the ionic interactions between the charged groups of phospholipid and ApoA-II. The two hydrophobic regions (residue 48-53, 60-70) might also contribute to stabilize the binding by interaction with the non-polar part of a phospholipid molecule.

In a comparison with the recently determined amino

acid sequence of ApoLP-ala, isolated from very low density lipoprotein, there is no visual sequence homology to ApoA-II.

CHAPTER V

SUMMARY

Human high density lipoprotein (HDL) was isolated from a mixed pool of plasmas (90% male and 10% female) by sodium phosphotungstate-magnesium chloride precipitation and further purified by ultracentrifugation. The purity of HDL was demonstrated by immunological and electrophoretical means.

ApoA-II was isolated from delipidated HDL by gel filtration on Sephadex G-100 in 1 N acetic acid. The purity and identification of ApoA-II were characterized by polyacrylamide gel electrophoresis, amino acid composition, N-terminal amino acid analysis, and sialic acid content.

Performic acid oxidation was carried out to cleave the disulfide bond which holds two identical ApoA-II polypeptides together. The oxidized ApoA-II was subjected to trypsin digestion and 10 unique tryptic peptides were purified, totaling 77 amino acid residues. The sequences of the tryptic peptides were determined by a manual dansyl-Edman procedure.

Over 90% of the single methionine residue in ApoA-II was found to be in the methionine sulfoxide form. After treatment of aminoethylated ApoA-II with thioglycolic acid in

6 M guanidine hydrochloride, 45% of the methionine sulfoxide was reduced to methionine. Cyanogen bromide was performed on the reduced aminoethylated ApoA-II and four peaks were resolved by gel filtration. Peak IV was demonstrated to be the N-terminus of the molecule containing 26 amino acids.

The complete sequence of ApoA-II was established to be PCA-Ala-Lys-Glu-Pro-Cys-Val-Glu-Ser-Leu-Val-Ser-Gln-Tyr-Phe-Gln-Thr-Val-Thr-Asp-Tyr-Gly-Lys-Asp-Leu-Met-Glu-Lys-Val-Lys-Ser-Pro-Glu-Leu-Gln-Ala-Glu*-Ala-Lys-Ser-Tyr-Phe-Glu-Lys-Ser-Lys-Glu-Gln-Leu-Thr-Pro-Leu-Ile-Lys-Lys-Ala-Gly-Thr-Glu-Leu-Val-Asn-Phe-Leu-Ser-Try-Phe-Val-Glu-Leu-Gly-Thr-Gln-Pro-Ala-Thr-Gln.. The alignment of the first three N-terminal tryptic peptides (28 residues) was based on the minor peptides from trypsin digestion and the N-terminal fragment of cyanogen bromide cleavage. The remaining tryptic peptides were ordered according to the sequence published by Brewer et al (Brewer, H.B., Jr., Lux, S.E., Ronan, R., and John, K.M.,1970 Proc. Nat. Acad. Sci. U.S.A. 69:1304.). It was concluded that the amino acid sequence of ApoA-II derived by these independent studies was essentially identical and that no apparent polymorphism was detected in ApoA-II. However, the residue at position 37 was questionable. Glutamine was assigned to residue 37 by Brewer et al. After consideration of all the evidence obtained in this study, glutamic acid has been assigned to that particular position.

A study of the primary structure elucidated for ApoA-II

reveals four potential phospholipid-binding sites containing charge clusters near residues 3-8, 20-28, 43-47, and 54-59. An extensive hydrophobic region (48-53, 60-70) is also a possible interaction site for the less polar lipid components. No sequence homology between this protein and ApoLP-ala (a VLDL polypeptide) was visually apparent.

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