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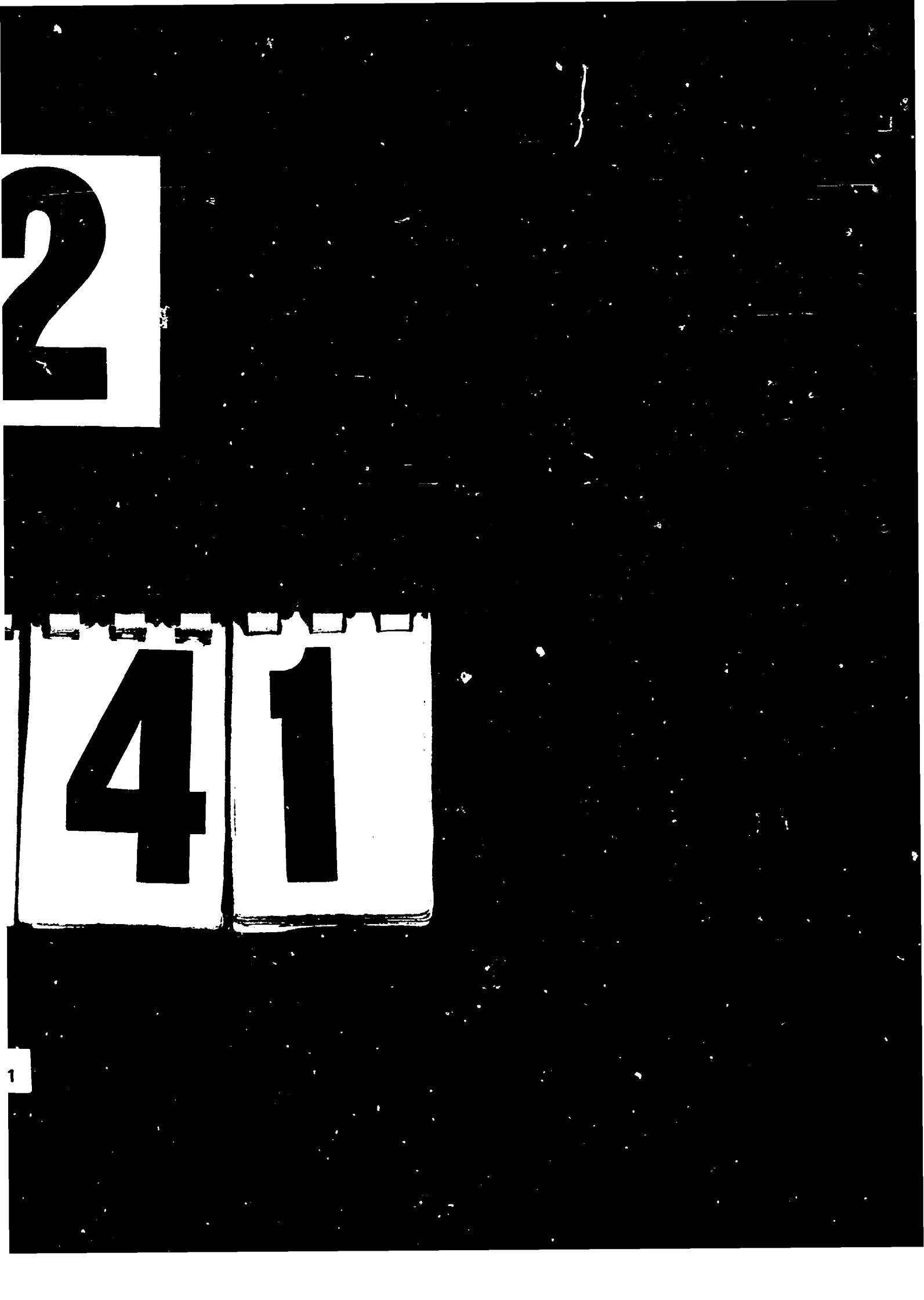
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STUDIES ON THE ISOLATION AND PARTIAL CHARACTERIZATION OF

APOLIPOPROTEIN C FROM HUMAN PLASMA AND CHYLE

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STUDIES ON THE ISOLATION AND PARTIAL CHARACTERIZATION OF APOLIPOPROTEIN C FROM HUMAN PLASMA AND CHYLE

CHAPTER I

INTRODUCTION

Human plasma lipoproteins have been under investigation for several decades. These lipid-protein complexes have been separated by several methods into two major groups which have been designated α_1-lipoprotein and β_1-lipoprotein (the nomenclature of lipoproteins is presented in the Appendix) based on their electrophoretic mobility in the free-boundary electrophoretic system (1). The protein moieties of these two lipoproteins were designated apolipoprotein A (ApoA) and apolipoprotein B (ApoB) (2), respectively.

The purpose of this study was to further characterize a third apolipoprotein, apolipoprotein C (ApoC), which was originally isolated from the very low-density lipoproteins (VLDL) of hypertriglyceridemic subjects (3). In order to facilitate this study, several methods were developed to isolate both partially delipidized lipoprotein C and apolipoprotein C from plasma and chyle. It was established that ApoC is a complex apolipoprotein composed of several polypeptides. The ApoC polypeptides were separated and partially characterized. Distribution studies showed that ApoC occurred in all the major ultracentrifugally defined

lipoprotein classes. From these studies the role of apolipoprotein C in the structure and metabolism of lipoproteins will be discussed with particular emphasis on the very low-density lipoproteins.

CHAPTER II

LITERATURE REVIEW

The concept of lipoproteins has its roots in many early observations such as those of Hoppe-Seyler (4) who found that plasma lipids were incompletely extracted by organic solvents. These findings were amplified by the work of Nerking (5) who observed that lipid phosphorous and cholesterol ester were co-precipitated with the serum globulin fraction. He also found that some of the lipids were extractable with diethyl ether only after proteolytic degradation of the globulin fraction. From these observations Nerking postulated the existence of a lipid-protein complex. Extension of these observations by several investigators (6, 7, 8) demonstrated that these lipid-protein complexes were present primarily in the euglobulin fraction.

The concept of lipoproteins was put on a firm experimental basis in 1929 by Macheboeuf's (9) isolation from horse serum of a lipid-protein fraction of constant composition. Reproducible amounts of cholesterol ester, lipid-phosphorous, and protein were characteristic of this material. It was demonstrated that the protein portion was not albumin or any of the known globulins and therefore represented a specific lipid-binding protein (10).

During the next two decades it was established that all plasma

lipids, excluding unesterified fatty acids, were associated with specific lipoproteins. On free-boundary electrophoresis the majority of lipids were associated with proteins that had either α_1- or β_1-globulin mobility (1). The lipoproteins with α_1- and β_1-mobility have been designated α_1-lipoproteins (α_1-LP) and β_1-lipoproteins (β_1-LP), respectively.

Fractionation of human plasma in ethanol-water mixtures at low temperatures led to the first large scale isolation of two types of lipoproteins (11, 12). Gurd et al. (13) showed that these two lipoprotein fractions differed in their lipid composition, molecular size and shape, and solubility properties. These two lipoproteins exhibited electrophoretic mobilities corresponding to those of α_1-LP and β_1-LP.

Plasma lipoproteins are characterized by low hydrated densities which are due to the relatively low densities of the lipid portions of the lipoprotein molecules (14, 15). The anomalous schileren patterns observed by McFarlane (16) could be explained by the presence of lipoproteins. In fact, by adjusting the density of the suspending media, Pedersen (17) isolated ultracentrifugally a lipoprotein that had the electrophoretic mobility of β_1-LP. On the basis of these findings, Gofman et al. (18) introduced an important ultracentrifugal technique which was soon refined into a method for the quantitative isolation and determination of plasma lipoproteins. This technique involved the flotation of lipoproteins followed by analytical ultracentrifugation at a solvent density of 1.063 g/ml at 26°C. The floating lipoprotein fraction was characterized by a β_1-globulin mobility and a flotation coefficient (S_f) greater than zero (19, 20).

Refinements of the ultracentrifugal method have led to the

separation of lipoproteins into the following major density classes (21-23): (1) chylomicrons (S_f > 400), (2) very low-density lipoproteins (VLDL, d < 1.006 g/ml, S_f 20-400), (3) low-density lipoproteins (LDL, d 1.006-1.063 g/ml, S_f 0-20), (4) high-density lipoproteins (HDL, d 1.063-1.21 g/ml), and (5) very high-density lipoproteins (VHDL, d > 1.21 g/ml). Oncley and coworkers (24) and Ewing et al. (25) have suggested, on the basis of studies on macromolecular distributions, that at least the first four major classes represent polydisperse systems, heterogeneous with respect to both particle size and hydrated density. The above separation and classification system has made ultracentrifugation the most widely used method of isolating lipoproteins for both analytical and preparative purposes. Although chylomicrons are considered particles of dietary origin, they have been detected not only in individuals following an overnight fast but also in some persons maintained on a fat-free diet (26). Unfortunately, there has been no method for differential separation of endogeneous and exogeneous particles; for that reason the flotation coefficient boundary S_f > 400 has remained rather arbitrary.

In general the VLDL, LDL, and HDL corresponded electrophoretically to α_2-LP, β_1-LP, and α_1-LP, respectively, on various supporting media at alkaline pH (26-28). The mobility of chylomicrons varied depending on the media utilized (27, 29, 30).

Another method that was applicable on a preparative scale was the precipitation of lipoproteins with various polyanionic agents. The most commonly used polyanions have been the sulfated polysaccharides in the presence of a divalent cation. Some of the sulfated polysaccharides that have been utilized are heparin (31), sulfated amylopectin (31), dextran

sulfate (32), mepesulfate (33), and others (34). Serum has been the usual starting material because fibrinogen is also precipitated if plasma is utilized (31, 34). The lipoprotein complexes with heparin, dextran sulfate, or mepesulfate can be dissociated by addition of a solution of high ionic strength, increasing the pH to greater than 9, addition of chelating agents, or by selectively precipitating out the sulfated polysaccharides as a Ba^{++}, Sr^{++} or protamine salt complex (34). The addition of polysaccharides resulted in a specific precipitation of β-LP with a simultaneous flocculation of the rather poorly defined chylomicrons (32-34). Interaction of sulfated polysaccharides with β-LP was primarily electrostatic (35-37). It seems that the free amino groups (36) or nitrogenous bases of the phospholipids (37) represented the functional group on β-LP mainly responsible for this interaction. Another polymer, polyvinylpyrrolidone, has been utilized to differentiate and selectively flocculate chylomicrons (38, 39).

Several methods have been developed to precipitate α_1-LP. A sequential addition of sucrose, heparin, magnesium, and a low molecular weight dextran sulfate resulted first in the precipitation of β_1-LP followed by the precipitation of α_1-LP (40). A more recent procedure utilized phosphotungstate for the precipitation of β_1-LP and α_1-LP in a stepwise fashion (41).

Several chromatographic procedures have been utilized for purification or separation of lipoproteins from various density classes. Hydroxyapatite has been used either to isolate β_1-LP from serum in a relatively pure form (42) or to separate the β-LP from α-LP (43-45). Although cross-linked dextrans, particularly Sephadex G-200, fractionated

serum lipoproteins into β-LP and α-LP (46), there was no separation of the larger molecules such as chylomicrons and VLDL from β-LP. Recently, with the availability of commercial agarose preparations, several authors have utilized 2-6% agarose gels for fractionating plasma lipoproteins (47, 48). Due to the larger pore size, 2-6% agarose gels have been shown to be satisfactory for fractionating lipoproteins on the basis of size with the 2% agarose gel being more satisfactory for the separation of the chylomicrons and VLDL (49).

Although very useful for analytical and clinical studies, several other separation techniques have not been utilized on a preparative scale because of limitations in the sample size and other technical difficulties, especially when compared with preparative ultracentrifugation or the precipitation methods. Zone electrophoresis has been utilized with a wide assortment of media to identify lipoproteins, but elution is rather tedious, and sample size is small for preparative purposes. Paper electrophoresis employing buffer containing albumin (50) has become a major tool for differentiating various types of hyperlipidemias (51). With the development of zonal rotors it has been possible to fractionate VLDL and LDL, but not HDL, from whole serum in a density gradient; unfortunately, the lipoprotein fractions are greatly diluted (52).

Discontinuous electrophoresis on polyacrylamide gels as described by Davis (53) has become a potentially powerful method for both diagnostic and clinical screening of various lipid transport disorders (54, 55). Also, analytical electrofocusing of serum after prior staining of lipoproteins with Sudan Black has been shown to separate lipoproteins into several components (56).

Chemistry of Lipoproteins

The chemistry of lipoproteins has been based principally on the study of substances isolated by ultracentrifugation techniques. The lipid composition of various plasma lipoprotein density classes is shown in Table 1. As the density of the lipoprotein class increases (chylomicrons to VHDL), the neutral lipid content decreases and the protein content increases. It has been reported that various density classes contain, in addition to triglycerides, esterified and unesterified cholesterol and phospholipids, also minor components such as glycolipids, gangliosides and hydrocarbons (57-60). The phosphatide composition of the various density classes is summarized in Table 2. Carbohydrate analyses of the delipidated LDL and HDL have indicated the presence of 4-5% and 1.5-3% carbohydrate in the respective apolipoproteins (62-65). Principal reported sugars are hexoses, N-acetylneuraminic acid (NANA), glucosamine, and traces of fucose (62-65).

The structure of various lipoprotein density classes has been studied by several different approaches. Extraction of lipoproteins under appropriate conditions led to the removal of all the lipids except for small amounts of firmly bound long chain fatty acids (66). From this and similar studies, it has been concluded that the forces binding the lipid to the protein are non-covalent in nature.

Electronmicrographs have shown that chylomicrons, VLDL, LDL and HDL are spherical in nature (67-69). Recent results with negative staining of HDL have indicated a subunit structure of 4-5 subunits with some indication of aggregation (70). Similarly, it has been concluded from the results of electron microscopy that LDL consists of 20 subunits (71).

TABLE 1

PERCENT COMPOSITION OF THE MAJOR PLASMA LIPOPROTEIN DENSITY CLASSES

Density Class	Protein	Phospholipid	Cholesterol		Triglyceride	NEFA[a]
			Free	Ester		
Chylomicrons[b]	2.0	7.0	2.0	5.0	84.0	-
VLDL[b]	8.0	18.0	7.0	12.0	50.0	2.0
LDL[b]	21.0	22.0	8.0	37.0	11.0	1.0
HDL[b]	50.0	22.0	3.0	14.0	8.0	3.0
$VHDL_1$[c]	62.4	28.0	0.3	3.2	4.6	0.6
$VHDL_2$[c]	98.45	0.83	0.002	0.05	0.05	0.6

[a]Non-esterified fatty acids

[b]Ref. 14

[c]Ref. 23

TABLE 2

PERCENT COMPOSITION OF PHOSPHOLIPIDS OF SERUM LIPOPROTEIN DENSITY CLASSES

	Chylo-microns[a]	VLDL[b] S_f 20-400	LDL[b] S_f 0-20	HDL_2[b]	HDL_3[b]	> 1.21[b]
Sphingomyelin	18.7 (12-24)	14.8 (14.0-17.0)	25.9 (21.3-29.1)	14.5 (10.8-16.7)	9.2 (5.2-11.9)	5.7 (3.9- 8.9)
Phosphatidylcholine	72 (66-79)	59.7 (48.7-78.5)	63.7 (34.6-73.7)	73.8 (66.1-82.5)	77.1 (70.9-84.2)	47.4 (37.4-54.1)
Lysophatidylcholine	4 (3-5)	5.0 (2.7- 9.0)	2.7 (1.8- 3.8)	2.0 (1.0- 3.6)	5.4 (3.8- 7.6)	34.5 (15.4-44.5)
Phosphatidylinositol	-	3.6 (2.5- 5.4)	1.6 (1.1- 2.8)	2.4 (1.5- 3.0)	2.4 (1.6- 3.1)	1.9 (1.1- 3.7)
Phosphatidylserine	-	1.5 (1.3- 1.7)	0.8 (0.2- 1.4)	0.9 (0.3- 1.2)	0.6 (0.5- 0.8)	0.5 (0.3- 0.8)
Phosphatidylethanolamine	6	4.6 (3.0- 6.4)	2.2 (1.1- 3.9)	3.3 (1.9- 4.0)	2.5 (1.8- 3.0)	4.2 (2.6- 5.5)
Polyglycerophosphatides	-	7.6 (5.0- 9.6)	2.0 (1.2- 3.3)	2.2 (1.5- 2.9)	2.0 (1.5- 3.0)	3.4 (2.4- 5.8)

[a]Averaged values, Ref. 61. [b]Ref. 59.

The S_f > 0 lipoproteins are thought to be micellar in nature (72, 73) in that they contain a neutral lipid micellar core composed of triglycerides and cholesterol ester surrounded by an outer layer or "membrane" composed of phospholipid, protein, and unesterified cholesterol (3). The presence of a portion of the protein on the surface of the LDL and VLDL has been confirmed by N-terminal analysis (74) and chemical modification of free amino groups of lysine (> 90%) (75, 76) of the intact lipoprotein. Phospholipase A and C treatment of S_f > 0 has confirmed the presence of phospholipids at the surface of these lipoproteins (77, 78).

From infrared and circular dichroic spectral studies it has been concluded that LDL contains a significant amount of anti-parallel chain β-structure in addition to random structure and probably some α-helix structure (79), while HDL is relatively richer in α-helix (79, 80). It has been inferred from nuclear magnetic resonances studies that both LDL and HDL exist at least in part as micellar structures (81). The molecular weights and diameters of the various lipoprotein density classes are shown in Table 3.

Studies on the immunochemical properties of the lipoproteins have shown that β-LP and α-LP are antigenically different (83-85). However, there has been a great deal of controversy in the literature concerning the number of antigenic components present in the various density classes. Gitlin (86) reported that the β-lipoprotein fraction was composed of a number of lipoproteins differing markedly in their immunochemical characteristics. Utilizing agar diffusion as described by Oudin (87), Aladjem et al. (83) observed that lipoproteins with S_f = 6 and S_f = 13 gave up to eight precipitin lines with antibodies to LDL after

TABLE 3

MOLECULAR WEIGHTS AND DIAMETERS OF VARIOUS LIPOPROTEIN DENSITY CLASSES[a]

Lipoprotein properties	Chylomicrons	VLDL	LDL	HDL_2	HDL_3	VHDL
Diameter (mμ)	120-1,100	21-76	19	10.3	7.9	7.4
Molecular weight x 10^{-6}	500-430,000	3-128	2.2	0.38	.18	.15

[a]Ref. 82.

five days of diffusion. They did not observe this phenomenon in the reaction of HDL_2 and HDL_3 with the corresponding antisera. It was concluded from these experiments that the lipoproteins were heterogeneous by this criterion and non-identical to the other serum proteins.

Brinner et al. (88) observed, using a hemagglutination technique, that the S_f 3-9 lipoprotein fraction contained one or more antigenic components in addition to the one common to the S_f 10-400 range. The finding of two antigenic determinants for S_f 0-20 (β-LP) has been recently reported (89, 90). However, other investigators have reported the antigenic homogeneity of LDL. Levine and coworkers (85), utilizing techniques similar to those applied by the above investigators, found evidence for antigenic homogeneity by complement fixation, single diffusion, and double diffusion in agar. Similarly, Walton and Darke (91) concluded from double diffusion in agar and a tanned sheep-cell agglutination technique that there was complete antigenic homogeneity throughout the density range ($S_f > 0$) investigated. By immunoelectrophoresis and double-diffusion, Scanu et al. (84) drew the conclusion that the LDL or β-LP fraction was homogeneous. These conflicting views on the antigenic homogeneity or heterogeneity of LDL could be explained either by the differences in methodology, antibody titer, and isolation procedures for lipoproteins or by the presence of contaminating serum proteins such as enzymes (92). Although most investigators considered the presence of a single antigenic component (84, 91), recent results again suggested the probable occurrence of two non-identical antigenic components (90, 93).

In cases of obstructive jaundice an abnormal lipoprotein was reported in the LDL (94, 95). This abnormal lipoprotein (LP-X) was sep-

arated from LP-A and LP-B present in the LDL and shown to be immunochemically different from both LP-A and LP-B (96).

Two different polymorphic groups were identified in β-lipoproteins, the Ag- and Lp-groups. The Ag-groups represented polymorphisms that were detectable by precipitation with isoimmune sera (97), and the Lp-groups were demonstrable by use of precipitating heteroimmune sera from rabbits (98). Recently, the β-LP carrying the LP-(a^+) component was isolated from HDL_2 by two different procedures (60, 44). It was also reported that the protein moiety of β-LP (ApoB) was present in the HDL infranate obtained after centrifugation (99), but this finding has not been confirmed.

Immunochemical studies on the protein moiety of β-LP (ApoB) were hampered by the fact that ApoB was insoluble in aqueous buffers (2, 85, 100). However, either by various chemical modifications (76, 80, 101) or by the use of sodium dodecyl (102) or decyl sulfate (103), a soluble ApoB was obtained. With succinylated ApoB, partial identity or no reaction was observed with anti-serum prepared against β-LP (76, 101). All other chemical derivatives of both the lipoprotein and apolipoprotein showed no identity with β-LP; however, ApoB solubilized with sodium decyl sulfate gave an identity reaction with β-LP when tested with antibodies to ApoB (80, 103).

The immunochemical components of α-LP have also been studied, with some controversy over the number of antigenic components present. Both HDL_3 and the apolipoprotein exhibited two characteristic precipitin lines on the immunodiffusion pattern and two to three precipitin arcs upon immunoelectrophoresis with antibodies to either HDL_3 or to the cor-

responding apolipoprotein (23, 104). The antigenic forms of HDL were interpreted as either various forms of partially delipidized lipoproteins or molecular aggregates formed upon storage (26, 106). The principal apolipoprotein of HDL, apolipoprotein A, was detected also in the chylomicrons, VLDL, and LDL (3, 90, 107, 108).

Work on the immunochemical components of chylomicrons was hampered by the small amount of protein present and also by the large diameter of chylomicrons which might have prevented migration into the support media used for immunochemical studies (48). Both apolipoproteins A and B were identified immunochemically in the chylomicrons of both serum and chyle (107, 108). The presence of apolipoprotein A was supported by chemical evidence such as N-terminal amino acid and peptide fingerprint analyses of the protein moieties of chylomicrons and high density lipoproteins (109, 110).

The VLDL with α_2 mobility (28, 111) was reported to contain only β-LP immunochemically (84, 91). However, the disruption of the VLDL structure with diethyl ether revealed the presence of a second antigenic component, apolipoprotein A (111); dissociation of the heparin precipitate of VLDL also revealed two components which were identified immunochemically as α-LP and β-LP (112). Gustafson et al. (3) reported that VLDL of hypertriglyceridemic subjects contained an additional antigenic component besides those corresponding to α-LP and β-LP.

Until recently, it was accepted by most investigators that HDL had one lipoprotein family, the protein moiety of which was designated apolipoprotein A. This was also the case for LDL, the protein moiety of which was designated apolipoprotein B. The protein moiety of VLDL was

thought to be identical with apolipoprotein B with some disagreement over the presence of apolipoprotein A. The presence of other immunochemical components in the various density classes besides those mentioned above was thought to represent contamination by other density classes or other serum proteins (104).

Surprisingly, the immunochemical heterogeneity of the various densities was not correlated with the terminal amino acid analyses. Table 4 shows the reported terminal amino acids for the various lipoprotein density classes. Terminal amino acid analyses of VLDL indicated glutamic acid, serine, threonine, and aspartic acid as the N-terminal and serine and alanine as C-terminal amino acids. The N-terminal analysis of chylomicrons indicated the same terminal amino acids as VLDL, but glutamic acid was not identified. The LDL and HDL were also heterogeneous with respect to both the N- and carboxyl terminal amino acids. Glutamic acid and aspartic acid were considered the N-terminal amino acids for apolipoprotein B and apolipoprotein A, respectively. Serine has been reported as the carboxyl terminal amino acid for ApoB and threonine for ApoA.

Apolipoproteins

Apolipoproteins of the Very Low-Density Lipoproteins

Results of N-terminal analyses and the immunochemical findings suggested that VLDL and chylomicrons were composed of as many as four proteins, two of which were positively identified as ApoA and ApoB. Similar data from studies with LDL and HDL suggested that the principal apolipoproteins of these density classes were ApoB and ApoA, respectively.

TABLE 4

TERMINAL AMINO ACIDS OF LIPOPROTEIN DENSITY CLASSES

	Amino acids			
	Aspartic acid	Glutamic acid	Serine	Threonine
N-terminal[a,b,c]				
Chylomicrons[a]	+ +	-	+ +	+
VLDL[a,c]	+[a]	+ +	+ +	+ +
LDL[a,b,c]	Trace	+ + + +	+	+
HDL[a,b,c]	+ + + +	+	+	+
	Threonine	**Serine**	**Alanine**	**Glycine**
C-terminal[c]				
S_f 20-60	-	+	+	-
S_f 6-8	+	+ + +	+	+
HDL_2	+ + +	+	+	+
HDL_3	+ +	+	+	+

[a]Ref. 109.

[b]Ref. 74.

[c]Ref. 113.

Due to its apparent protein heterogeneity, the VLDL represented the most likely candidate as a source for the isolation and identification of unknown apolipoproteins.

Gustafson et al. (114) developed a method for the fractionation of VLDL into five subfractions by sequential angle-head ultracentrifugation but concluded from the N-terminal analyses and immunological findings that none of the subfractions consisted of a distinct apolipoprotein. The alternative approach consisted of delipidizing the VLDL and fractionating it into its various apolipoproteins. In order to improve the recovery of soluble proteins, a partial delipidization procedure (115, 116) was developed which consisted of lyophilizing the VLDL in the presence of insoluble potato starch followed by extraction of the neutral lipids with n-heptane at -10°C. The recovery of phospholipid-protein residues soluble in aqueous buffers is presented in Table 5. The phospholipid-protein residue of LDL was sparingly soluble and that of HDL completely soluble; the corresponding residues of chylomicrons and VLDL (S_f > 400 and S_f 20-400) exhibited intermediate solubility.

Fractionation of the VLDL phospholipid-protein residues (117, 3) on Pevikon block electrophoresis resulted in separation of two zones. The slower moving component was shown to be immunologically identical to the antigenic component of β-LP by several different criteria, whereas the second zone consisted of two partially delipidized lipoproteins which were further fractionated by preparative ultracentrifugation at d = 1.105 g/ml for 44 hours at 105,000 g. In this manner the two partially delipidized lipoproteins were separated with the infranate containing protein moiety which was identical to the apolipoprotein of α-LP (Table 6). The

TABLE 5

PARTIAL DELIPIDIZATION STUDIES ON LIPOPROTEIN DENSITY CLASSES[a]

Lipoprotein Density Class	Phospholipid/Protein Ratio Lipoprotein	Residue	Recovery of Soluble Protein %
Chylomicrons S_f > 400	3.5	1.3	93
VLDL S_f 20-400	1.8	1.2	60
LDL	0.8	0.7	10
HDL	0.9	0.8	100

[a]Ref. 116.

TABLE 6

COMPARISON OF PARTIALLY DELIPIDIZED LP-A, LP-B AND LP-C[a]

Lipoprotein Fraction	Electrophoretic Fraction	$S^{o}_{20,w}$	Hydrated Density	Precipitin Reaction with			N-terminal	Peptide
				A-α-LP	A-β-LP	AWS		
HDL (LP-A)	N.D.[b]	4.3	1.17	+	-	+	Asp	
LDL (LP-B)	N.D.[b]	14.5	1.18	-	+	+	Glu	
VLDL (LP-A)	II	4.5	1.17	+	-	+	Asp	Identical to α-LP
VLDL (LP-B)	I	14.0	1.13	-	+	+	Glu	Identical to β-LP
VLDL (LP-C)	II	6.9	1.09	-	-	+	Ser and Thr	Different from α- and β-LP

[a]Ref. 3.

[b]Not determined.

phospholipid-protein residue in the supernate was immunologically different from both α-LP or β-LP, and the protein moiety of this unknown lipoprotein was designated apolipoprotein C (ApoC). The three phospholipid-protein residues isolated from the hyperlipemic VLDL were characterized by their immunochemical behavior, N-terminal amino acid analysis, hydrated densities, peptide patterns of tryptic and peptic hydrolysates, sedimentation and diffusion coefficients, and quantitative composition of the individual phospholipids (3). These results (Table 6) clearly indicated that the VLDL of hyperlipemic subjects was composed of three phospholipid-protein residues, two of which had apolipoproteins A and B as their protein moieties; the third protein moiety, designated as apolipoprotein C, consisted either of two non-identical peptides or represented actually two distinct apolipoproteins (3).

The molecular weight of the phospholipid-apolipoprotein C residue was found to be 834,000 by sedimentation-diffusion data (15). Preliminary results on the percent distribution of three phospholipid-protein residues in VLDL (A, B and C) indicated some variation in the quantities of A and C in two types of hypertriglyceridemias; the relative contents of ApoA and ApoC varied from 4% to 33% and 9% to 48%, respectively (3).

Although the finding of a third apolipoprotein (ApoC) was accepted with some skepticism, recent studies confirmed its occurrence in normal and hypertriglyceridemic plasma (118-122). Brown et al. (118) found that VLDL partially delipidized with n-heptane (116) gave three precipitin lines with antibodies to VLDL, two of which were identical to those obtained with specific antisera to LDL and HDL; the third line was a unique reaction obtained only with antibodies to VLDL. The phospho-

lipid-protein residues obtained by partial delipidization of VLDL were fractionated by Pevikon block electrophoresis into two zones. In contrast to original finding by Gustafson et al. (117), fractions from both zones gave a unique reaction with antibodies to VLDL. The second zone was centrifuged as described by Gustafson et al. (3), but no separation of the 4S and 7S residues was achieved. Since another attempt to separate the ApoA- from ApoC-phospholipid residues by Sepharose 4B column chromatography failed, Brown et al. (118) concluded that the phospholipid-protein residues obtained by partial delipidization of VLDL resulted in an aggregate of phospholipid and protein which prevented their further purification.

From the above results it was concluded that the fractionation of VLDL apolipoproteins might be achieved by column chromatography of totally delipidized VLDL (118). The VLDL, totally delipidized with ethanol-ether, was partially soluble in Tris buffer (pH 8.2). Application of the soluble portion (30% of the apo-VLDL) to a Sephadex G-100 column (115 x 1.2 cm) yielded two peaks. The first peak reacted only with antibodies to HDL and displayed on polyacrylamide gel electrophoresis mobility similar to that of ApoA; the second peak (S2) reacted only with antibodies to VLDL and on immunoelectrophoresis exhibited two precipitin arcs with α_2 and γ mobilities. Eighty percent of the soluble apo-VLDL was found in the second peak. This peak (S2) was then fractionated on DEAE-cellulose with a two-stage linear gradient into four peaks designated D1, D2, D3, and D4. The D1, D3, and D4 peaks were homogeneous on disc gel electrophoresis whereas D2 was not. Purification of D2 was achieved by utilizing essentially the same procedure as already described (119). The

totally delipidized VLDL (apo-VLDL) was dissolved (> 90%) in Tris buffer containing 0.1 M sodium decyl sulfate and passed over a Sephadex G-150 column (1.2 x 110 cm) which resolved the material into three peaks (S1-S3). The S3 peak was then fractionated on a DEAE-cellulose column into 4 peaks, each of which was homogeneous by polyacrylamide gel electrophoresis.

Each peak was characterized by amino acid analysis, N- and C-terminal amino acids, disc gel electrophoresis, sedimentation equilibrium experiments, sialic acid content, and by immunological properties (118, 120). These results are summarized in Table 7. D1 and D2 did not release any amino acids when treated with carboxypeptidase A, but hydrazinolysis revealed valine and glutamic acid, respectively. The amino acid compositions and immunochemical identity of D3 and D4 indicated that these two polypeptides were identical. However, the greater mobility of D4 on the disc gel electrophoresis was explained by the fact that D4 had twice as much sialic acid (1.2 moles/mole protein) as D3 (0.6 moles/mole protein). Treatment of D3 or D4 with neuraminidase for 24 hours resulted in the appearance on polyacrylamide gel of a single band with a mobility slower than that of either D3 or D4. From the sialic acid studies it was concluded that D3 and D4 are polymorphic polypeptides differing only in one mole of sialic acid (120).

Brown et al. (118-120) estimated that the D1-D4 polypeptides comprised 35-50% of the protein moiety of VLDL. They confirmed also the presence of ApoA and ApoB in the VLDL and reported the presence of D1-D4 in normal lipemic subjects (119). In commenting upon the relationship of D1-D4 polypeptides and apolipoprotein C, these authors suggested that

TABLE 7

CHARACTERISTICS OF POLYPEPTIDES ISOLATED FROM VLDL[a]

Polypeptide designation	N-terminal	C-terminal	Missing amino acid	Molecular weight	Immuno-electrophoretic mobility
Apo-LP-Val (D1)	Threonine	Valine	Histidine, tyrosine, cysteine, cystine	7,000	γ
Apo-LP-Glu (D2)	Threonine	Glutamic acid	Histidine, cystine, cysteine	10,000	N.D.[b]
Apo-LP-Ala (D3)	Serine	Alanine	Isoleucine, cystine, cysteine	10,000	α_2
Apo-LP-Ala (D4)	Serine	Alanine	Isoleucine, cystine, cysteine	10,000	α_2

[a]Ref. 120.

[b]Not determined.

apolipoprotein C probably consisted of a mixture of D1-D4 in combination with large amounts of phospholipids. However, Gustafson et al. (3) considered only the protein moiety as apolipoprotein C which upon total delipidization of its corresponding phospholipid-protein residue gave only serine and threonine as the N-terminal amino acids. The four polypeptides isolated by Brown et al. (118) were designated in the following manner: apolipoprotein-Val (apo-LP-Val, D1), apolipoprotein-Glu (apo-LP-Glu, D2), and apolipoprotein-Ala (apo-LP-Ala, D3 and D4). Unfortunately, these authors have not presented any experimental evidence for the existence of lipoproteins with the designated polypeptides as the sole protein moieties.

Recently a polypeptide with alanine as the carboxyl terminal acid was isolated from both VLDL and HDL_2 (121). Scanu also reported the isolation of a group of polypeptides from HDL_2 (122) which had certain characteristics similar to those isolated by Brown et al. (118).

Results of recent studies indicated that some VLDL polypeptides might play a role as the activators of lipoprotein lipase, an enzyme involved in the hydrolysis of plasma triglycerides (123). It was reported that low concentrations of the human polypeptide R-Glu in the presence of phospholipids activated lipoprotein lipase isolated from either cows' milk (124) or rat adipose tissue (125). Another polypeptide R-Ala was also found to be active to a lesser extent (124-125). The R-Val (apo-LP-Val) and the major polypeptides of HDL (R-Gln and R-Thr) were inactive with or without phospholipids (124-125).

ApoA or the HDL Peptides

Shore and Shore (126) reported the first successful separation

of two different polypeptide components from any density class or lipoprotein. Fractionation of totally delipidized HDL_3 was achieved by DEAE-cellulose column chromatography in the presence of 8 M urea. Three peaks were obtained, the first and second being homogeneous by disc gel electrophoresis, and the third being a mixture of the first two. By carboxypeptidase A and/or B studies it was concluded that the carboxyl terminal amino acids were glutamine and threonine for the first and second peaks, respectively. The results of carboxypeptidase studies were confirmed by hydrazinolysis of apo-HDL_3. Analysis of the hydrazinolysis products revealed threonine (1 mole/50 x 10^3 g of protein) by amino acid analysis while glutamic acid-γ-hydrazide was demonstrated by chromatography of the di-DNP compound. The two isolated polypeptides had dissimilar mobilities on disc gel electrophoresis with the polypeptide designated R-Glutamine (R-Gln) moving faster than the polypeptide designated R-Threonine (R-Thr).

Subsequent work by the Shores (127) resulted in further characterization of the two non-identical polypeptides of HDL. Determination of the molecular weight of R-Gln by sedimentation equilibrium experiments in 8 M urea or 6 M guanidine-HCl gave values of 14,300 and 14,900. The molecular weight of R-Thr measured by sedimentation equilibrium was 31,400; it was concluded from the release of threonine by carboxypeptidase and the minimum molecular weight calculated from the amino acid composition that this value represented a dimer form of R-Thr. The differences in the amino acid composition further confirmed the non-identity of these two polypeptides. Amino acid analysis of R-Glutamine indicated the absence of histidine, arginine, cysteine, and tryptophan whereas the

amino acid composition of R-Threonine revealed only the absence of cystine, cysteine, and isoleucine. Shore and Shore concluded from this study that the two isolated polypeptides were non-identical and that the HDL_3 lipoprotein molecules probably contain a total of six polypeptide units.

The Shores (121) expanded their work by fractionating totally delipidized HDL_2 and lipoproteins of S_f 4-8 and S_f 20-100 on DEAE-cellulose columns. Carboxypeptidase and hydrazinolysis of these totally delipidized density classes revealed only glutamine, threonine, and alanine as the carboxy terminal amino acids. In examining the HDL_2, the Shores reported the presence of three polypeptides having threonine as the C-terminal amino acid. One of these was identical to the R-Thr isolated from apo-HDL_3 (126). This polypeptide was designated R_1-Thr, and the other two were named R_2-Thr and R_3-Thr. The major difference between R_1-Thr, R_2-Thr, and R_3-Thr was the absence of isoleucine from R_1-Thr. R-Ala peptides found in lipoproteins with S_f 20-100 and HDL_2 differed in the amount of isoleucine present. Another peptide with tryptophan missing was found in HDL_2 and S_f 20-100. The Shores concluded (122) that R-Ala and a peptide with tryptophan deleted were common to HDL_3, HDL_2, and S_f 20-100. From these data they suggested that there are multiple forms of R-Thr and R-Ala peptides which might have some importance in lipid binding.

Several other investigators confirmed the Shores' results by fractionating HDL into several polypeptide fractions. Rudman et al. (128) separated HDL delipidized with acetone-ethanol (1:1, v/v) into three peaks on Sephadex G-200 column chromatography equilibrated with 1 N acetic acid. Examination of these fractions by peptide fingerprints, amino

acid composition, and immunodiffusion suggested the presence of at least two non-identical polypeptides. The amino acid composition of these fractions did not agree with the data compiled by the Shores (126) for R-Thr and R-Gln. Modification of the delipidization procedure by Rudman and coworkers (129) led to the introduction of column chromatography on Sephadex LH-20 equilibrated with 2-butanol:acetic acid:H_2O (4:1:5) to achieve total delipidization of HDL. The apolipoprotein eluted from the LH-20 column was resolved by column chromatography on Sephadex G-200, as described previously (128), into two major peaks and one minor peak (129). The major peaks corresponded to R-Thr (peak II) and R-Gln (peak III) by amino acid composition and mobility on disc gel electrophoresis. The minor peak (peak I), which was eluted before the major peaks, appeared to be an aggregate of the second peak (R-Thr). Peak I was greatly increased if the second peak was lyophilized and rechromatographed. This observation was also supported by the similarity in the amino acid composition between the first and second peak. Only peak III reacted with antibodies to HDL and showed an identity reaction with ApoA. Rudman et al. (129) concluded that α-protein (ApoA) consisted of two distinct polypeptides which were very similar to those isolated by the Shores (126).

Fractionation of the totally delipidized HDL_2 into several components by Sephadex gel filtration in the presence of 8 M urea has been reported (122). The apo-HDL_2 was resolved into five peaks (I-V), two of which (III and IV) were similar on the basis of the amino acid composition and electrophoretic mobility on polyacrylamide gel to those isolated by the Shores (126) and Rudman et al. (129). The first peak (I) was thought to be an aggregate of III and IV. Peak II was omitted from dis-

cussion because of its variability in occurrence. Peaks III and IV were thought to correspond to R-Thr and R-Gln. Fraction V comprised approximately 5% of the total recovered material and was heterogeneous on disc gel electrophoresis. All peaks obtained from column chromatography were heterogeneous by the criterion of analytical electrofocusing. The apparent molecular weights determined from a calibrated Sephadex G-200 column were $52\text{-}55 \cdot 10^3$ (I), $25.8\text{-}28.0 \cdot 10^3$ (III), $16.4\text{-}17.6 \cdot 10^3$ (IV), and $11.2\text{-}11.8 \cdot 10^3$ (V). These molecular weights were corroborated by sedimentation equilibrium experiments and by gel electrophoresis in sodium dodecyl sulfate. Double diffusion studies showed that Fraction V reacted with antibodies to apo-HDL, but not with antibodies to either Fraction III or Fraction IV. Reactions of partial identity between Fractions III and IV were obtained with both anti-III and anti-IV. Thus, one could conclude that Fractions III and IV had a common antigenic component and that Fraction V was immunochemically free of both Fractions III and IV. Scanu et al. concluded from this study that each fraction (III-V) was a distinct class of polypeptides, each of which was made up of subcomponents. However, neither Scanu and coworkers (122) nor Rudman et al. (129) investigated the terminal amino acids of the isolated polypeptides.

Investigation of the HDL by Alaupovic et al. (130) was approached in a different manner. The HDL was not totally delipidized but was first fractionated in its intact form into three lipoprotein families: lipoprotein A, LP-A; lipoprotein B, LP-B; and lipoprotein C, LP-C (2). This was done to minimize complications arising from contamination of ApoA by the other apolipoproteins or their polypeptides. The LP-B was precipitated by antibodies to LP-B, and the LP-A was separated from LP-C

by precipitation of LP-C with 33% polyethylene glycol (PEG) or by elution from a hydroxyapatite column with 0.25 M phosphate buffer. The material obtained from the supernate of the PEG precipitate or the elutant of the hydroxyapatite reacted only with antibodies to LP-A. Resolution of totally delipidized LP-A into two major components was achieved by gel filtration on Sephadex G-75. At this point, neither component was immunochemically homogeneous. Each polypeptide fraction was further purified by column chromatography on DEAE-cellulose in the presence of 8 M urea, followed by gel filtration on G-75. The two polypeptides purified in this fashion were tested immunochemically with antibodies to LP-A and/or antibodies to R-Thr; they gave on direct comparison a non-identity reaction. The mobility on polyacrylamide gel and the amino acid composition of each isolated polypeptide were comparable to those reported previously for R-Thr and R-Gln (126). Terminal amino acids of R-Thr and R-Gln were investigated, and the N-terminal for R-Thr was found to be aspartic acid. No N-terminal for R-Gln was identified. By kinetic experiments with carboxypeptidase A, both R-Thr and R-Gln had glutamine as the carboxy terminal amino acid. It was proposed that the "R-Thr" peptide be designated as "R-Gln-I" and "R-Gln" be designated as "R-Gln-II." The minimum molecular weight by sedimentation equilibrium was 14,000 for "R-Gln-II" and 24,000 for "R-Gln-I." The latter value was confirmed by results of tryptic hydrolysis.

Polypeptide Composition of ApoB

At the present time the polypeptide composition of ApoB remains unclear. Several investigators have suggested that ApoB may contain two or more non-identical polypeptides, but there exists no unequivocal proof.

Two antigenic components have been reported to be present in LP-B (89, 90). The differences in amino acid composition of two subfractions of immunochemically pure LP-B suggested the presence of two unequally distributed ApoB polypeptides (90). Two fractions isolated from DEAE-cellulose chromatography had different amino acid compositions and comprised only 30% of the starting material (121). Modification of LP-B with subsequent total delipidization yielded a soluble maleylated apolipoprotein B preparation which was resolved by gel permeation on Sephadex G-150 or G-200 (93, 131) into two fractions with different amino acid analyses and immunochemical behavior. By the use of positively charged disulfide exchange reagents and monitoring the behavior of modified ApoB on electrophoresis, it was suggested that ApoB contained at least two non-identical polypeptides (132).

Classification Systems

Until recently the two classification systems for plasma lipoproteins were based either on their hydrated densities or electrical charge as the two most characteristic physical-chemical properties. The differential flotation of lipoproteins by ultracentrifugation resulted in the recognition of five density classes based on the observed maximal and minimal concentrations of lipoproteins along a density gradient between approximately 0.92 and 1.21 g/ml (21-23). As already mentioned, all the density classes have been shown to be heterogeneous by terminal amino acids (74, 109, 113), immunochemical studies (3, 90, 60, 107, 108), and, most recently, by the isolation of non-identical polypeptides (126, 118, 121, 128). These density classes have also been shown to be polydisperse systems with respect to particle size and hydrated density

(24, 25).

The other classification system, based on electrical charge, recognizes three major bands or groups of lipoproteins designated as α_1-, α_2- and β-LP. However, since lipoproteins with these electrophoretic mobilities correspond (26-28) roughly to the HDL, VLDL, and LDL, respectively, they also represent mixtures rather than distinct lipoprotein species.

To account for the reported protein heterogeneity of the various density classes, a classification system based on apolipoproteins as the only distinct chemical components of lipoproteins has been proposed (2) for the differentiation of lipoprotein families. Lipoprotein families were defined as "polydisperse systems of lipid-protein complexes characterized by the presence of a single, distinct apolipoprotein or its constitutive polypeptides" (130). The chemical concept currently recognizes the existence of three distinct lipoprotein families: lipoprotein family LP-A, characterized by the presence of apolipoprotein A; lipoprotein family LP-B, by apolipoprotein B; and lipoprotein family LP-C, by apolipoprotein C. Some of the characteristics of the lipoprotein families are listed in Table 8 which summarizes the available information such as number of peptides, terminal amino acids, and antigenic components associated with each family. It should be pointed out that this division of lipoproteins into only three lipoprotein families can be readily expanded if some of the already characterized polypeptides are found to be the protein moiety of a distinct lipoprotein or if new polypeptides are isolated and shown to be the apolipoprotein of a new lipoprotein family.

Although the operational nomenclature of plasma lipoproteins

TABLE 8

THE EXPANDED CHEMICAL NOMENCLATURE OF HUMAN PLASMA LIPOPROTEINS

Lipoprotein Family	Apo-lipoprotein	Constitutive Polypeptides	N-terminal Amino Acid	C-terminal Amino Acid	Amino Acid Deletion	Antigenic Component(s)	Literature Designation
LP-A	ApoA	A-I	Aspartic acid	Glutamine	Ile, 1/2 Cys	Single	R-Thr
		A-II	Blocked	Glutamine	His, Arg, Try	Single	R-Gln
LP-B	ApoB	B	Glutamic acid	(Serine)	None	One-two	
LP-C	ApoC	C-I	Threonine	(Valine)	His, Tyr, 1/2 Cys	Single	R-Val
		C-II	Threonine	(Glutamic acid)	His, 1/2 Cys	Single	R-Glu
		C-III-1[a]	Serine	Alanine	Ile, 1/2 Cys	Single	R-Ala
		C-III-2[a]	Serine	Alanine	Ile, 1/2 Cys		

[a]C-III-1 and C-III-2 represent the polymorphic forms of C-III peptide. The Roman numerals refer to the parent polypeptide, while the Arabic numerals designate the polymorphic forms.

based on ultracentrifugal and/or electrophoretic isolation is fully justified when properly applied to specific preparations, the chemical nomenclature based on apolipoproteins as the unique and specific criterion for differentiation of lipoproteins seems to reflect better and describe more adequately the complexity of lipoprotein chemistry and metabolism under normal and pathological conditions. The chemical nomenclature used in this dissertation is presented in its expanded form in Table 8.

CHAPTER III

MATERIALS AND METHODS

Materials

Human Plasma and Serum

Venous blood was obtained from normal, healthy male and female donors after an overnight fast. The plasma was collected by plasmapheresis in a 600 ml plastic bag containing 2.2% sodium citrate (Fenwal Lab., Morton Grove, Ill.). Plasma triglycerides (TG), phospholipids (PL), and cholesterol (C) were analyzed in each case to determine whether or not the lipid values were within the normal levels (TG < 150 mg%, PL < 250 mg%, and C < 250 mg%). The plasma samples with normal lipid values were designated as "normal fasting plasma."

Large quantities of plasma (5-10 liters) were obtained from the plasmapheresis laboratory of the Oklahoma Medical Research Foundation. These plasma samples were designated as "random plasma" because the dietary condition of the presumably normal, healthy donors was unknown.

Blood samples were also obtained from two patients with "mixed type" hypertriglyceridemia (Type V). A small quantity of venous blood was drawn after an overnight fast and was immediately followed by the ingestion of a heavy fat load (milk shake). Five hours after the oral fat load, 500 ml of blood were drawn. The blood was allowed to stand for

several hours, and the clot was removed by low speed centrifugation. The former serum sample was labeled "fasting hypertriglyceridemic serum" and the latter "postprandial hypertriglyceridemic plasma." One large sample (5,000 ml) was obtained from nine hypertriglyceridemic (Type IV and Type V) subjects by plasmapheresis.

Jaundiced subjects with various kinds of liver disease were checked for the presence of LP-X by the immunochemical test described by Seidel et al. (96). If the serum tested was positive for LP-X, then a larger volume of plasma was collected by plasmapheresis and designated "obstructive jaundiced plasma."

Human Chyle

Chyle samples were obtained through the cooperation and courtesy of W. H. Falor, M.D., Akron, Ohio. This material was obtained by cannulation of the left thoracic duct of individuals with the diagnosis of carcinoma of the lung or some other thoracic ailment. Chyle was collected continuously for several days at 12-hour intervals beginning two hours after the patients had ingested 150-200 g of heavy cream; the subsequent diet contained 75% calories from fat which consisted mainly of half-and-half cream. In some cases the diet for the last few collections was dextrose-water. Any fibrin strands formed during shipment via air express were removed by filtration through cheesecloth. The fluid remaining in the clots was recovered by applying pressure to the fibrin strands. Most chyle samples were pooled and used for subsequent preparative procedures; however, some individual samples were studied separately, i.e., the more lactescent sample and the sample obtained after the dextrose-water feeding.

Miscellaneous Material

Human autopsy material was obtained through the courtesy of Y. Stein, M.D., The Hebrew University, Hadassah Medical School, Jerusalem, Israel. The samples including serum, liver tissue, and intestinal mucosal scrapings were obtained within 2 to 4 hours after death. Liver and mucosal scrapings were homogenized, and the cellular debris was removed by low-speed centrifugation.

Fasting (0 hr) and postprandial plasma samples (6 and 24 hrs) from hypertriglyceridemic patients with Type III or Type IV disease were obtained through the cooperation of Wm. Hazzard, M.D., Seattle, Washington. In some cases VLDL subfractions consisting of $S_f > 400$ and S_f 20-400 isolated at each time interval were sent either in the intact form or as β-, α_2- and α_1-fractions separated by starch electrophoresis.

Chemicals

Ammonium carbonate, magnesium chloride, guanidine monohydrochloride, mercaptoethanol, disodium salt of EDTA, sodium hydroxide, hydrochloric acid, glacial acetic acid, potassium phosphate (mono and di-basic), phosphoric acid, 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris), ß-alanine, glycine, urea, iodine, petroleum ether, and n-heptane were purchased from J. T. Baker Chemical Co., Phillipsburg, N. J.; sodium chloride, sodium carbonate, sodium citrate, sulfuric acid, ethylenediamine, barbituric acid, barbital, trichloroacetic acid, chloroform, and peroxide-free diethyl ether, from Mallinckrodt Chemical Works, St. louis, Mo.; potassium bromide, from Fisher Scientific Co., Fair Lawn, N. J.; polyethylene glycol, from Matheson, Coleman, and Bell, Norwood, Ohio; sodium azide, Bromophenol Blue, Amido Black 10B, sodium dodecyl sulfate, and mepesulfate,

from K&K Laboratories, Inc., Hollywood, Calif.; heparin sodium, from Nutritional Biochemical Corp., Cleveland, Ohio; phosphotungstic acid, from B&A, Morristown, N. J.; Coomassie Brilliant Blue R-250, from Co-Lab, Chicago, Ill.; Oil-Red-O and Pyronin B, from Allied Chemical Corp., New York, N. Y.; acrylamide, N,N'-methylenebisacrylamide (Bis), riboflavin, N,N,N'N^{1}-tetramethylethylenediamine (TEMED), and ammonium persulfate, from Canalco, Rockville, Md.; absolute and 95% ethanol, from U.S. Industrial Chemicals Co., New York, N. Y.; special Agar-Noble and Freund's complete adjuvant, from Difco Lab., Detroit, Mich.; agarose from Bio-Rad Lab., Richmond, Calif.; anhydrous hydrazine, from Pierce Chemical Co., Rockford, Ill.; 1-dimethylaminonaphthalene-5-sulfonyl chloride (DNS-Cl), from Calbiochem, Los Angeles, Calif.; DNS-amino acid standards, from Mann Research Lab, New York, N. Y.; carboxypeptidase A, carboxypeptidase B, and trypsin, from Worthington Biochemical Corp., Freehold, N. J.; human albumin, from Certified Blood Donor Service, Woodbury, N. Y.; lysozyme, cytochrome c, ribonuclease, and ovalbumin, from Sigma Chemical Co., St. Louis, Mo.; Blue Dextran, from Pharmacia Fine Chemicals, Inc., Pitscataway, N. J.; and Nojax cellulose casing, Size 18, from Union Carbide, Chicago, Ill.

Methods

Isolation of Lipoproteins

Ultracentrifugal methods. For analytical purposes 7 ml of plasma or serum were overlayered with 4 ml of 0.15 M NaCl in 0.1% EDTA and centrifuged in the Type T150 rotor of the Model L preparative ultracentrifuge (Beckman Instruments, Inc., Palo Alto, Calif.) for 22 hours at 105,000 x g at 4°C. After completion of the ultracentrifugal run, the

top 2 ml were removed by a tube-slicing technique to yield the VLDl , S_f > 20 (3). The LDL (1.006-1.063 g/ml), HDL_2 (1.063-1.125 g/ml), and HDL_3 (1.125-1.21 g/ml) were isolated sequentially by adjusting the solution density of the infranate with KBr to 1.063, 1.125, and 1.21 g/ml and centrifuging for 22 hours at 105,000 x g (3). A scheme for the fractionation of lipoproteins is shown in Fig. 1. Occasionally the LDL was subfractionated into LDL_1 (1.006-1.019 g/ml) and LDL_2 (1.019-1.063 g/ml). In some cases the HDL_2 and HDL_3 were isolated as one density class, HDL (1.063-1.21 g/ml).

The VLDL was fractionated into five subfractions or combinations of these subfractions as described by Gustafson et al. (114). These subfractions are defined as A (S_f > 5,000), B (S_f 400-5,000), C (S_f 100-400), D (S_f 50-100), and E (S_f 20-50). The centrifugally isolated lipoprotein density classes were "purified" by repeated flotations in the preparative ultracentrifuge under their original isolation conditions. Purity of various lipoprotein fractions was determined by the absence of albumin as demonstrated by double diffusion tests with antibodies to albumin.

To isolate larger amounts of VLDL in a single run, the plasma was spun at its original density (1.023-1.027 g/ml) in either the Type Ti60 or H42 rotor of the Model L2-50 or L2-65-B ultracentrifuge (Beckman Instruments, Inc., Palo Alto, Calif.) for 22 hours at 105,000 x g. The lipoprotein layer was removed by a tube-slicing technique and washed two times by recentrifugation under identical conditions. The isolated lipoproteins were used for preparative purposes and were designated VLDL.

In some instances, when large volumes of chyle had to be immediately processed, the chylomicrons (S_f > 400) were isolated by centri-

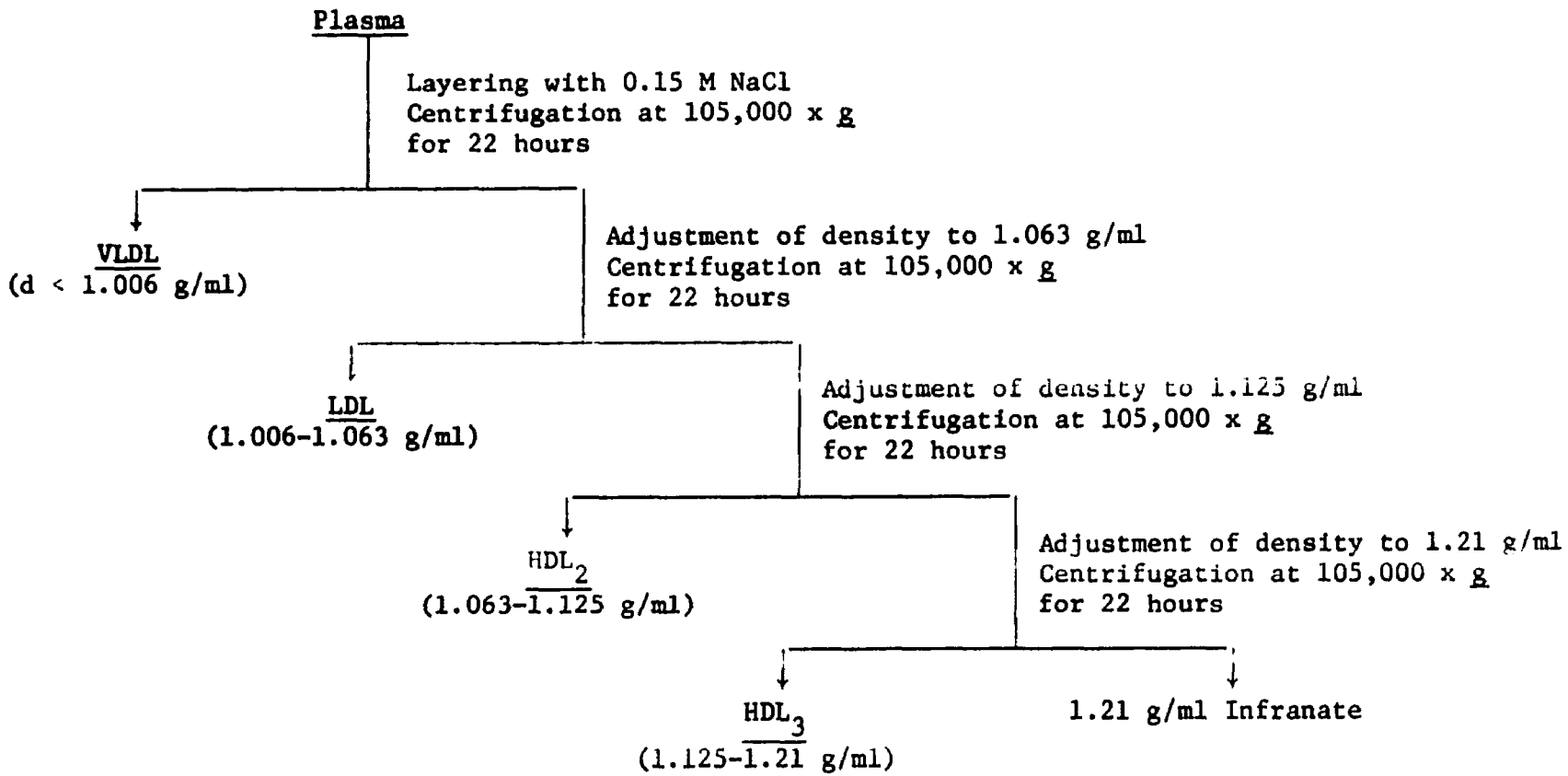


Figure 1 Ultracentrifugal isolation of lipoproteins.

fuging chyle in the GSA rotor of the Servall centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.) at 1 x 10^6 g-min. The required amount of ultracentrifugation was calculated from an experimentally determined nomogram (114). The centrifugation was done at the chyle density with no overlayering of a saline solution. The isolated chylomicrons were then repeatedly washed in a preparative ultracentrifuge until free of albumin.

Precipitation methods. Most of the precipitation procedures were carried out with either plasma or isolated density classes. Cohn's Method 10 (133) was utilized for both plasma and VLDL to obtain Cohn fractions I-III, IV-V, and VI. The sulfated polysaccharides, heparin (40) and mepesulfate (33), were utilized to precipitate lipoproteins with an approximate $S_f > 0$. When plasma was used as the starting material, it was necessary to wash the dissolved lipoprotein precipitate at least once by ultracentrifugation at d 1.073 g/ml for 22 hours at 105,000 g min to remove contaminating serum proteins.

Polyethylene glycol precipitation, as described by Kostner and Alaupovic (134), was applied to intact and partially delipidized VLDL. To a 1 ml sample, 0.1 g of NaBr and 0.6 ml of 33% polyethylene glycol were added. The precipitate separated from the soluble portion by low-speed centrifugation was dissolved in 0.1 M $(NH_4)_2CO_3$. The dissolved precipitate and the soluble portion were tested by double diffusion.

Phosphotungstate precipitation (41) of lipoproteins was found to be a useful procedure for the preparative isolation of large quantities of plasma lipoproteins. Fifty milliliters of a 4% solution of phosphotungstate (pH 7.6) were added to 500 ml of plasma, followed by the addition of 12.5 ml of 2 M $MgCl_2$. An immediate turbidity developed; the

precipitate (ppt. I) was removed by low-speed centrifugation, and the supernates were used for further precipitation. Four hundred and fifty milliliters of the 4% phosphotungstate solution were added to the combined supernates, and the mixture was allowed to stand until a fine precipitate developed. The supernate was aspirated and utilized for the next step. The precipitate was discarded. Addition of 87.5 ml of 2 M $MgCl_2$ to the supernate resulted, after an overnight storage at 4°, in a precipitate (ppt. II) which was recovered by low-speed centrifugation. Both ppt. I and ppt. II were dissolved in 1 M Na_2CO_3 by prolonged stirring. Precipitate I was centrifuged sequentially (Fig. 2) at densities 1.073 g/ml, 1.030 g/ml, and 1.073 g/ml for 22 hours at 105,000 g to isolate the two fractions designated VLDL ($d < 1.030$ g/ml) and LDL (1.030-1.073 g/ml). The density of precipitate II was adjusted to 1.23 g/ml by the addition of KBr, and the mixture was centrifuged in the Ti60 rotor of Spinco Model L2-65-B ultracentrifuge for 22 hours at 165,000 g. The top layer (HDL, $d < 1.23$ g/ml) was collected and washed two times by recentrifugation under identical conditions. Infranates from the original centrifugations and washes were discarded. Whereas precipitate I or its fractions (VLDL and LDL) were free of immunochemically detectable albumin after two ultracentrifugations, HDL still contained traces of albumin. Up to 10 liters of plasma could be handled satisfactorily by this fractionation procedure (Fig. 2). The lipoproteins were dialyzed exhaustively against distilled water, totally delipidized, and used as a source of ApoC, ApoA, and ApoB.

Lipoprotein X (LP-X) was isolated as described previously (96).

Column chromatographic techniques. Sepharose 4B or 6B (Pharmacia

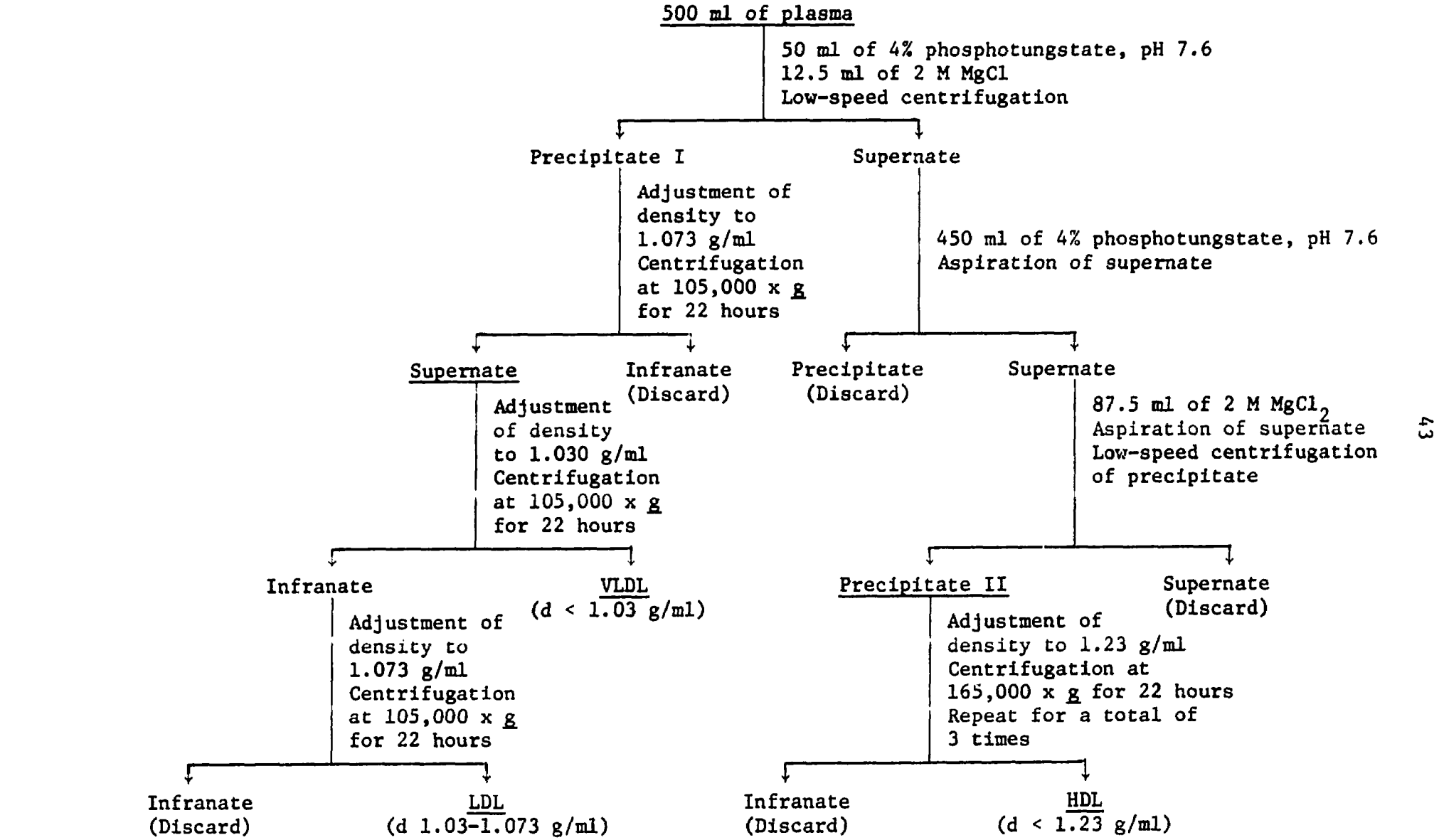


Figure 2. Isolation of lipoproteins by phosphotungstate precipitation.

Fine Chemicals, Piscataway, N. J.) was equilibrated with the buffer, fines removed, deaerated, and poured into a column 2.5 x 100 cm (Glenco Scientific, Inc., Houston, Texas). Flow rate was held constant during each run with all chromatographic procedures being conducted at room temperature. Lipoprotein density classes or partially delipidized lipoproteins were applied to the column in a volume of 3 to 5 ml. Four to five ml fractions were collected and monitored continuously with a Uviscan III-A (Buchler Inst., Inc., Fort Lee, N. J.) at 254 mμ; alternatively, each fraction was monitored at 280 mμ in a spectrophotometer (Gilford Instrument Lab Inc., Oberlin, Ohio).

Sephadex G-200, G-100, G-75, and G-25 (Pharmacia) were either equilibrated with the appropriate buffer for several days or placed in a boiling water bath to swell the gel and then equilibrated with buffer. Fines were decanted and the gels were poured into the columns. G-200 columns (2.5 x 100 cm) were used primarily for the separation of lipoproteins; Sephadex G-100 and G-75 columns (2.5 x 100 cm) were used for fractionating the apolipoproteins, and Sephadex G-25 columns (2.5 x 60 cm) for desalting.

Diethylaminoethyl (DEAE) cellulose (Bio-Rad Laboratories, Richmond, Calif., 0.40 mEq/g dry weight) was utilized for the ion exchange chromatography of both lipoproteins and apolipoproteins. One-hundred grams of DEAE-cellulose were washed successively with 0.5 N NaOH, distilled water, 0.5 N HCl, and distilled water. The pH value of the washed DEAE-cellulose was adjusted to that desired for the operation of the column. After washing with the appropriate buffer, the DEAE-cellulose was poured into the column (1.5 x 30 cm) and further equilibrated with the

buffer. If 8 M urea was utilized in the buffer system, the most satisfactory equilibration with the urea-buffer system was achieved when the urea solution was added to the already packed column. The pH of the effluent was checked before the sample was added, and the flow rate was usually 30-40 ml/hr. Gradient formation was either linear or concave depending on the manipulation of the gradient vessels (135).

Hydroxyapatite (Bio-Rad) was washed several times with 0.01 M phosphate buffer (pH 6.8) and then poured into a 1.5 x 30 cm column (Glenco). The sample was applied to the hydroxyapatite column and eluted with stepwise increments of phosphate buffer: 0.01 M, 0.25 M, 0.40 M, 0.65 M, and 0.1 M sodium citrate.

All fractions eluted from the columns were monitored by adsorption at 280 mμ or 254 mμ, immunodiffusion, and polyacrylamide disc gel electrophoresis.

Delipidization of Lipoproteins

Partial delipidization. Partial delipidization of lipoproteins was carried out according to the procedure by Gustafson (116) as modified by Ledford et al. (136). The lipoprotein samples were dialyzed exhaustively against distilled water and lyophilized without starch in a 50 ml glass stoppered centrifuge tube. The dried lipoproteins were extracted with n-heptane at least five times or until the organic phase was colorless. The residue was dried at room temperature and then dissolved in 0.1 M borate buffer (pH 8.6). The insoluble material was removed by low-speed centrifugation.

Total delipidization. Total delipidization of lipoproteins was carried out essentially as described by Scanu et al. (137). Exhaustive

dialysis of the sample against distilled water preceded lyophilization. The delipidization procedure was begun by treatment of the lyophilized material with absolute ethanol-diethyl ether (3:1, v/v) on a rotator (15 rpm for 30 min) with a total of 5-8 extractions, followed by low-speed centrifugation to remove the solvent. In the final step the residue was extracted overnight with ethanol-diethyl ether (2:1, v/v) followed by 3-5 extractions with diethyl ether. All extractions were carried out at 6°C. The residue was dried at room temperature and usually contained less than 1% phospholipid.

Isolation of Partially Delipidized Lipoprotein C

Isolation by immunoprecipitation. The soluble portion of the partially delipidized "postprandial hypertriglyceridemic" VLDL represented the principal source for the isolation of partially delipidized LP-C (pdLP-C).

Immunoprecipitation of pdLP-A and pdLP-B was accomplished by the use of delipoproteinated antisera to HDL_3 and LDL, respectively. To avoid contamination with rabbit lipoproteins, the antisera were adjusted to d 1.25 g/ml and centrifuged for 22 hours at 120,000 x g. The floating lipoproteins were removed by a tube-slicing technique and discarded. Antibodies in the infranate were dialyzed against several changes of saline. Optimal ratios for immunoprecipitation of LP-B and LP-A were determined by a serial micro dilution method as described under immunological methods. The appropriate amounts of delipoproteinated antisera were added successively to the pdVLDL dissolved in 0.1 M borate buffer, pH 8.6, and the reaction mixtures were allowed to stand for two hours at 37°C (Fig. 3). The immunoprecipitates were removed by low-speed centri-

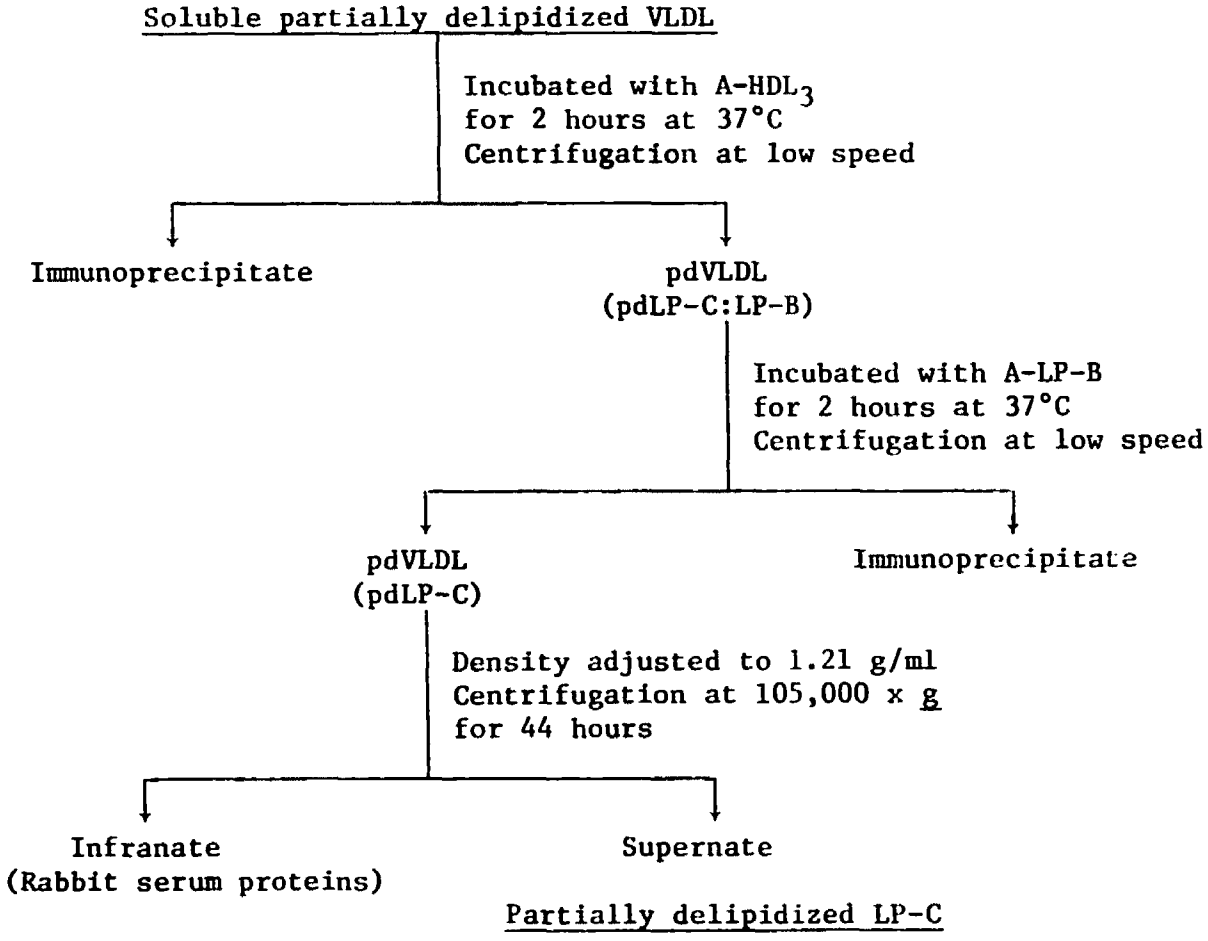


Figure 3. Isolation of partially delipidized LP-C by immunoprecipitation.

fugation, and the supernates were checked for the possible presence of pdLP-B and pdLP-A by double diffusion. If these antigenic components were still present, the precipitation procedure was repeated until there was no reaction with antibodies to LDL or HDL_3. The density of the supernate was adjusted to 1.21 g/ml, and the solution was centrifuged for 22 hours at 105,000 x g. The top fraction contained the pdLP-C and no rabbit plasma proteins, as demonstrated by immunodiffusion with antibodies to rabbit whole plasma.

Isolation by ultracentrifugation. Due to the presence of antibodies to LP-C in the anti-HDL_3 serum, another method was developed to isolate the pdLP-C. This method was based on the hydrated density of pdLP-C ($\bar{d}$ = 1.09 g/ml) determined by Gustafson et al. (3). The density of the soluble pdVLDL was adjusted to 1.04 g/ml, and the solution was centrifuged for 22 hours at 105,000 x g (Fig. 4). The supernate was discarded, and the density of the infranate was increased to 1.12 g/ml. The centrifugation of the infranate for 22 hours at 105,000 x g yielded a floating lipoprotein fraction with a density range of 1.04-1.12 g/ml. This fraction, containing the pdLP-C, was washed several times by recentrifugation under the original conditions until Fraction C was immunochemically free of pdLP-A and pdLP-B. The density of the combined washings and original infranate stemming from centrifugation at 1.12 g/ml was adjusted to 1.21 g/ml, and the mixture was centrifuged for 22 hours at 105,000 x g. The supernate (Fraction ABC) contained an additional amount of pdLP-C.

Isolation and Fractionation of ApoC

Although the ultracentrifugal procedure represents the method of

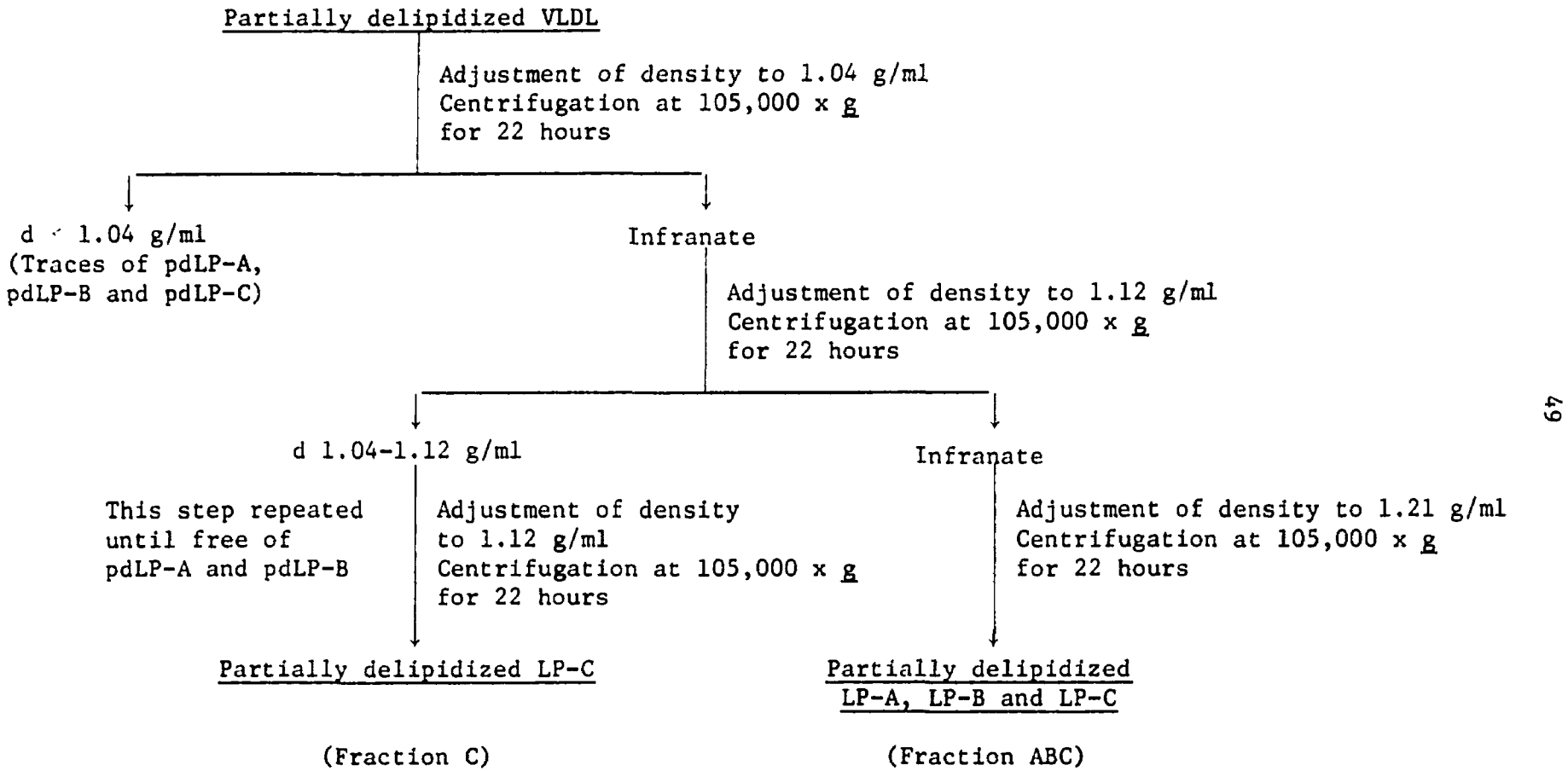


Figure 4. Ultracentrifugal isolation of partially delipidized lipoprotein C.

choice for the isolation of pdLP-C, it is too time consuming and complicated for the preparative isolation of ApoC. To facilitate the isolation of ApoC, either the ultracentrifugally isolated VLDL or the phosphotungstate precipitate was totally delipidized according to the procedure described previously. Precipitate I (d 1.030 g/ml) was the major source of ApoC from the phosphotungstate procedure.

Totally delipidized lipoprotein preparations were only partially soluble in 0.1 M $(NH_4)_2CO_3$ due to the insolubility of ApoB in aqueous buffers. The soluble protein was separated from the aggregated material by low-speed centrifugation. After three repeated treatments of insoluble residue with 0.1 M $(NH_4)_2CO_3$, the soluble portions were combined and lyophilized (solute 1). The remaining aggregated protein was treated with 6 M guanidine·HCl in 0.1 M mercaptoethanol, as described by Lee and Alaupovic (138), with overnight stirring, followed by removal of any insoluble material by low-speed centrifugation. The soluble portion was diluted with distilled water to an approximately 3 M solution with respect to the guanidine·HCl and then dialyzed exhaustively against distilled water to precipitate ApoB and to keep ApoC and ApoA in solution. The insoluble material, representing mainly ApoB, was treated two times in the same fashion; and the three soluble portions were combined (solute 2). Since the remaining insoluble residue was soluble in 50% acetic acid, the extraction procedure involving dialysis of the acetic acid solution against distilled water with ensuing precipitation of ApoB was repeated three times. The soluble portions (solute 3) were combined with solutes 1 and 2, dialyzed against distilled water, and lyophilized. This lyophilized preparation was used for the subsequent isolation of ApoC.

The ApoC was isolated by gel permeation of the soluble apolipoprotein preparation on a Sephadex G-75 or G-100 column (1 x 36 in) equilibrated with 0.1 M $(NH_4)_2CO_3$, pH 9.0. Fractions corresponding to the elution peaks were combined and lyophilized; the ApoC peak was utilized for further fractionation.

Further fractionation of ApoC was attempted by several different procedures. One was the use of preparative electrofocusing as described in the LKB instruction manual. The 440 ml column (LKB, Brona, Sweden) was used for a large amount of ApoC (200 mg) and the 110 column (LKB) for smaller quantities, such as subfractions of the 440 ml column. Selection of the proper ampholine range for preparative focusing was determined from the results of analytical electrofocusing. All solutions were prepared as directed except that they were made 7 M with respect to urea. Electrofocusing was begun at an initial voltage of 400 volts for approximately 12 hours and then increased to 700 volts with the column being cooled with tap water (16-18°C). Focusing was terminated when the amperage became constant (48-72 hr). The column was drained in 2 ml fractions at 2 ml/min and monitored at 280 mμ, with the pH and disc gel pattern determined for every other fraction.

For preparative purposes the 440 ml column was utilized with a 2% ampholine solution. Elution of the 440 ml column was monitored by absorption at 280 mμ and disc gel electrophoresis. From this information fractions were pooled and refocused on the 110 ml column and monitored as described previously.

Another procedure utilized to fractionate the ApoC peak was the use of DEAE-cellulose in the presence of 8 M urea. The gradient-forming

device (Glenco) was set to form a concave gradient by stoppering vessel I which was attached to the column. Vessel I contained 350 ml of .005 M phosphate buffer (pH 7.2) in 8 M urea, and vessel II contained 500 ml of the same buffer with 0.10 M NaCl used to form the gradient. After vessel II was empty, 250 ml of phosphate-urea buffer in 2 M NaCl were added; the elution procedure was concluded by the addition of glacial acetic acid. The elutant was analyzed continuously at 254 mμ and the peaks were examined by disc gel electrophoresis. Elution peaks were dialyzed exhaustively against distilled water and lyophilized.

Preparative discontinuous polyacrylamide gel electrophoresis was performed using the Buchler Fractophorator (Buchler Instruments, Fort Lee, N. J.). The gels were identical to those used on the analytical scale with both the acidic and basic buffer systems. Separation on 5, 6 and 7% separating gels in 8 M urea was examined and it was decided that the 7% gel was the most satisfactory. The separating gel (4 ml) was prepared in 8 M urea, whereas stacking and sample gels were prepared in 4 M urea. Although a Tris-glycine buffer, pH 8.3, was utilized initially, it was found later that the Tris-borate buffer, pH 9.0, was just as satisfactory. Pre-electrophoresis for 30 minutes at 12 milliamperes was followed by application of the sample in a volume of 1-3 ml which was overlayered with buffer containing the tracking dye Bromophenol Blue. Electrophoresis was carried out at a constant amperage (10 ma) and at room temperature. Four milliliter fractions were collected every 2.5 minutes, and the electrophoresis was usually completed in four hours. The fractions were monitored at 280 mμ, and every second tube of the peak and/or peaks was examined by 7% disc gel electrophoresis with an accom-

panying VLDL sample as a standard. Fractions eluted from the gel were combined on the basis of the analytical polyacrylamide patterns. Fractions containing pure polypeptides were dialyzed exhaustively against distilled water and utilized for subsequent analyses.

Electrophoretic Techniques

Polyacrylamide gel electrophoresis. Analytical discontinuous polyacrylamide gel electrophoresis was carried out essentially as described by Davis (53) with equipment from Canalco, Rockville, Maryland. Separating gels of 3% and 7% by weight were used in the basic buffer system, while only 7% gels were used in the acidic buffer system (139). Tris-glycine (pH 8.3) and Tris-β-alanine (pH 4.5) were utilized for the two systems. The electrophoretic separation on 7% gels was usually carried out with 8 M urea in the separating gel and 4 M urea in both the stacking and separating gels. Electrophoresis was carried out at 4.5 ma/tube with the tracking dye (basic, Bromophenol Blue; acidic, Pyronin B) migrating a constant distance of approximately 4 cm into the separating gel during each run. The gels were originally fixed with a solution of 1% Amido Black 10B in 7% acetic acid followed by electrophoretic destaining. This procedure was found to be tedious and somewhat questionable as to whether or not some bands were removed by electrophoretic destaining. In the course of this study, it was found that placing the gels directly into a solution of 0.12% Coomassie Brilliant Blue in 10% trichloroacetic acid (0.25/10, v/v) was very satisfactory for both fixing and staining the separated polypeptides. After approximately 48 hours, the background became perfectly clear with only the stained, fixed protein bands visible. The gels were then transferred to a water solution to

prevent destaining of the fixed band.

Polyacrylamide gel electrophoresis combined with immunodiffusion. Samples were separated by the basic 7% polyacrylamide gel electrophoresis system, and the gels were sliced longitudinally into halves. Sliced gels were placed flat side down on microscopic slides, and approximately 4 ml of a 1% agarose solution in Veronal buffer, pH 8.6, at 50°C were poured around each gel. The agarose-acrylamide gel was allowed to set for one hour. Longitudinal troughs were then cut on each side of the acrylamide gel and the appropriate antisera were added.

The horizontal regions, corresponding to bands visualized in separately stained gels, were sliced from the 7% gels and examined by immunodiffusion and immunoelectrophoresis. The slices from the acrylamide gel were treated in the same manner as described for the longitudinally sliced gels.

Scanning of some of the separating gels was carried out using the linear transport module (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) at 550 mμ at a scan speed of 1 cm/min with recording speed of 4 cm/min in order to achieve maximum resolution of the bands in the gel. The areas under the peaks were determined by triangulation ($H \times W_{1/2\ H}$) or by use of a planimeter (Keuffer & Esser Co., New York).

Analytical electrofocusing (56) was carried out in the Canalco disc gel electrophoresis equipment employing a constant voltage power supply. Ampholine 3-10 (LKB, Bromma, Sweden) was found to be the most useful carrier ampholyte for screening purposes, although ampholines with narrower pH ranges were also utilized when necessary. Samples were mixed with ampholine, acrylamide, bisacrylamide, TEMED, and ammonium persulfate

and allowed to polymerize in a I.D. tube (10 x 0.4 cm) to form a 1% ampholine solution in a 5% polyacrylamide gel. Electrofocusing was carried out for 16 hours at 200 volts and 6-8° with 1% phosphoric acid at the cathode and 1% ethanolamine at the anode. The most satisfactory staining procedure was found to be 0.12% Coomassie Brilliant Blue in 10% TCA (.25:10, v/v) as described for the disc gels.

Agarose or agar electrophoresis was carried out as described for immunoelectrophoresis except that the sample (20-30 λ) mixed with equal volumes of agar or agarose (all done at approximately 50°C) was placed into a slot (1 x 0.1 cm) which was covered with agarose to give a leveled surface. After completion of electrophoresis, the slides were fixed in 10% TCA for one hour and washed in distilled water for approximately two hours with two changes of water. Washed slides were then covered with lintless paper and allowed to dry overnight with subsequent staining with Amido Black 10B or Oil Red O.

Paper electrophoresis was carried out as described by Lees and Hatch (50) with a Durrum cell and Veronal buffer, pH 8.6, containing 1% human serum albumin. The dried paper strips were stained with Oil Red O in an 80°C oven.

Molecular Weight Determination by Polyacrylamide Gel Electrophoresis

Determination of the molecular weight of polypeptides by 5% polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was carried out according to the procedure described by Shapiro *et al*. (140). Human albumin, ovalbumin, trypsin, and lysozyme were used as standards. The gels were fixed and stained with the Coomassie Bril-

liant Blue-TCA staining procedure.

Analytical Ultracentrifugation

The analytical ultracentrifugation of lipoproteins was carried out in a Spinco Model E analytical ultracentrifuge equipped with schlieren and interference optical systems, a RITC unit, and an electronic speed control unit. Photographs were taken automatically by the photographic apparatus of the ultracentrifuge. Plate measurements were made with a Nippon Kogaku KK Shadowgraph Model 6.

Samples for sedimentation rate were equilibrated with 0.15 M NaCl or some other appropriate buffer. Sedimentation experiments were carried out in single-sector cells at 56,100 rpm at 25°C, and the coefficients were calculated according to Svedberg and Katsurai (141). The observed S values were corrected to $s_{20,w}$, the sedimentation coefficient in water at 20°C according to Svedberg and Pedersen (142), and expressed in seconds or Svedberg units (1S = 10^{-13} cgs. units).

Amino Acid Analysis

Samples of apolipoproteins or polypeptides were dried over P_2O_5 under vacuum and then hydrolyzed in constant boiling HCl in an evacuated sealed tube at 110 $\pm$ 1° for 24 and/or 72 hours. The hydrolysates were evaporated to dryness, redissolved in distilled water, and evaporated again to dryness. Solubilization of the residue in 3 ml of 0.2 N sodium citrate buffer (pH 2.2) was followed by filtration through a fine frit sintered glass filter. This solution was analyzed on a Beckman Model 120C amino acid analyzer according to the accelerated procedure of Benson and Patterson (143) on spherical resins (Beckman Custom Spherical Resins,

Type PA-28 for the acidic and neutral amino acids, and PA-35 for the basic amino acids). The analyzer was calibrated with a mixture of known amino acids. Half-cystine was determined as cysteic acid after oxidation of the apolipoproteins or polypeptides with performic acid. The values for cysteic acid were corrected for the 94% recovery found by Moore (144). Tryptophan was determined by the procedure of Gaitonde and Dovey (145).

Analysis of C- and N-terminal Amino Acids

Carboxyl terminal analysis. Carboxypeptidase A (CPA) and/or carboxypeptidase B (CPB) were used for the enzymatic determination of the carboxyl terminal amino acids as described by Ambler (146). The protein sample was dissolved in 0.1 M N-ethylmorpholine (pH 8.5), and the CPA and/or CPB were added in the usual ratio of 1 part enzyme to 80 parts protein. In some cases the protein sample was heated in a boiling water bath before addition of the enzyme, or the reaction was run at a lower pH value (pH 6.5). Incubation of the enzyme-protein mixture was usually carried out at 37°C for varying time periods. A sample was withdrawn initially to serve as the blank, and the subsequent samples were taken at suitable time intervals after addition of the enzyme. The protein was precipitated with 6 N HCl, and the remaining supernate was evaporated to dryness. The residue was dissolved in 0.2 N sodium citrate, pH 2.2, and analyzed on the Beckman 120C amino acid analyzer. Values obtained were corrected by subtraction of the blank levels of amino acids and contributions from the autodigestion of CPA and/or CPB.

The carboxyl terminal amino acids were also determined with anhydrous hydrazine by the method of Braun and Schroeder (147).

Amino terminal analysis. Dansylation of the protein and/or

peptide was carried out as described by Gray (148). After dansylation the protein was precipitated with 10% trichloroacetic acid (TCA) and centrifuged at low speed to remove the excess reagents and by-products (149). The dansylated protein was then washed two times with 0.1 N HCl. Hydrolysis of the dansylated protein with constant boiling 6 N HCl was carried out in an evacuated sealed tube for 18 hours at 110°C. Following hydrolysis the ampule was opened and dried *in vacuo* in the presence of NaOH pellets. The sample and standards were then spotted on a thin-layer plate coated with Silica Gel G and the dansyl amino acids were separated as described by Morse and Horecker (150).

Carbohydrate Analysis

Sialic acid was determined by the thiobarbituric acid method (151). Samples were hydrolyzed with 0.1 N HCl for 1 hour at 80°. Neutral sugars were analyzed by gas-liquid chromatography as alditol acetates according to the procedure described by Kim *et al*. (152). Neutral sugars were released from the polypeptide preparations by hydrolysis with 1 N HCl for 4 hours at 100°. Galactosamine and glucosamine were determined on the amino acid analyzer by comparison with the elution time of standards following hydrolysis of the sample with constant boiling 6 N HCl for 24 hours at 110°C.

Lipid and Protein Analyses

Lipid and protein analysis. Whole serum and various lipoprotein preparations dialyzed against 0.15 M NaCl (pH 7.0) with 0.01% EDTA were utilized for lipid analysis. Free and total cholesterol were determined by the method of Schoenhiemer and Sperry (153). Cholesterol ester was

calculated as cholesterol oleate. Lipid phosphorus was analyzed by the method of Fiske and Subbarow (154), after wet digestion by the procedure of Youngburg and Youngburg (155), or by the method of Gerlach and Deuticke (156). Lipid phosphorus was converted to phospholipid by multiplication with the factor 25. Triglyceride was measured either by the fluorometric method of Kessler and Lederer as applied to the auto-analyzer (157) or by the procedure of Van Handel and Zilversmit (158).

The analysis of individual phosphatides was performed on the organic extracts of totally delipidized lipoprotein fractions. The extracts were evaporated to dryness in vacuo at room temperature, and the residues were dissolved in chloroform. Phosphatides were separated by one (59) or two (3) dimensional thin-layer chromatography and visualized either with iodine or molybdenum blue (159). Quantitation of the phosphatides was achieved by scraping the individual spots off the plate and determining the phosphorus (156). The neutral lipids from the organic extracts were separated on silica gel G with petroleum ether:diethyl ether (90:10) and the spots were visualized either with iodine or by charring with sulfuric acid.

Protein was determined by the method of Lowry et al. (160), with human serum albumin as the standard.

Immunological Methods

Immunodiffusion and immunoelectrophoresis. Double diffusion (161) and immunoelectrophoresis (162) were carried out on glass slides (25 x 75 mm) coated with 1% agar or agarose employing veronal buffer, pH 8.6, ionic strength 0.1. Immunoelectrophoresis was carried out at 6.5 volts/cm for 60 minutes. A drop of 10% dextran was sometimes added

to the center well of an immunodiffusion pattern to enhance the development of precipitin lines. The precipitin patterns were allowed to develop for 48-72 hours at ambient temperature, although they appeared to be satisfactory already after only 24 hours. Plates were washed in distilled water for 48 hours and then dried under a strip of lintless paper. A 1% Amido Black 10B or a 0.06% Coomassie Brilliant Blue in water:ethanol (55:45, w/v) solution was utilized for protein staining with the background stain removed with 10% acetic acid or 95% ethanol, respectively. Lipid staining was accomplished with Oil Red O.

The optimal ratio of antibody to antigen for the absorption of either antibody or antigen was determined with the Micro-titer set (Cooke Engineering Co., Alexandria, Va.). Both antigen and antibody were diluted serially in 0.1 M borate buffer, pH 8.6, and then the appropriate component was added. Plates were incubated overnight at 37°C, and the optimal ratio was established by selecting the well displaying the maximal precipitation. Using this ratio, the undesirable antigen or antibody was removed by addition of the appropriate antibody or antigen, incubation of the reaction mixture for two hours at 37°C, and low-speed centrifugation of the immunoprecipitate. If necessary, the entire procedure was repeated to remove the last traces of either antigen or antibody.

Preparation of antibodies. Antigens for injection were obtained from various sources. LP-A and its two peptides were furnished by Dr. G. Kostner; LP-X by Dr. D. Seidel and Dr. H. Magnani; density classes and various ApoC preparations were prepared by the methods described. Prior to administration, lipoprotein, apolipoprotein, and polypeptide preparations were dialyzed against 0.9% NaCl in 0.1% EDTA. Equal volumes

of antigen (protein concentration 0.5-10 mg/ml) and Freund's complete adjuvant were homogenized and then injected intraperitoneally into white New Zealand rabbits, Angora goats, or Black Kekule sheep. One or two additional injections *via* the same route, at intervals of ten days, were usually sufficient to elicit antibody titers adequate for our studies. Subsequent booster injections were used only when the titer of the antisera became low as demonstrated either by immunodiffusion or immunoelectrophoresis against whole serum or corresponding lipoprotein preparations.

The drawing of blood from the immunized animals commenced two to three weeks following the initial immunization. Blood samples (35-40 ml) were collected from the rabbits by cardiac puncture at weekly intervals. An evacuated flask was utilized to draw blood from the jugular vein of the goats and sheep in a volume of 300 to 500 ml at intervals of 2-3 weeks. The collected blood was allowed to stand at both room temperature and 4°C to allow the clot to form and retract. The clot was removed by low-speed centrifugation, and the antisera were examined by immunodiffusion and/or immunoelectrophoresis against various antigenic lipoprotein preparations. When necessary, the antisera were treated with appropriate antigens to remove undesirable antibodies. The antisera, with sodium azide (1 mg/ml of antiserum) added as a preservative, were stored at -20°C.

Commercial antisera. Antisera prepared commercially were purchased from Behringwerke AG (Marburg-Lahn, Germany). Antisera to the following human serum components were utilized in this study: albumin, α_1-lipoprotein, β_1-lipoprotein, α_2-lipoprotein, whole serum, γ_1A-globulin, γ-globulin, α_1-acid glycoprotein, α_2-macroglobulin, transferrin,

fibrinogen, γ_1-macroglobulin, β_2-glycoprotein, cerruloplasmin, IgA, IgM, IgG, C-reactive protein, and Bence-Jones κ and λ chain. Also, anti-rabbit whole serum and γ-globulin were purchased from Behringwerke.

CHAPTER IV

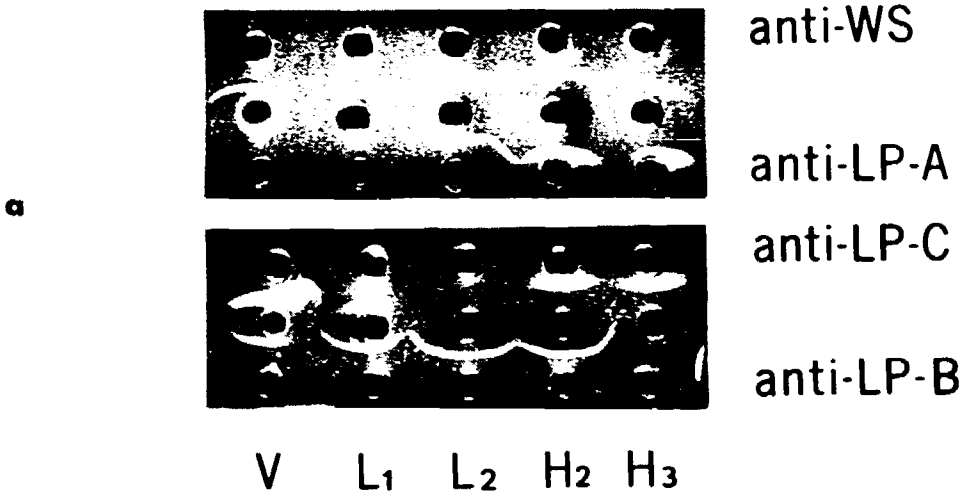
RESULTS

Antigenic Characterization of Density Lipoprotein Classes

Normal Fasting Plasma

Normal fasting plasma was fractionated into the major density classes, each of which was examined for the presence of various lipoprotein families by immunological and electrophoretic techniques. Double diffusion studies (Fig. 5a) demonstrated the presence of the following lipoprotein families: A-LP-A gave precipitin lines with LDL_2, HDL_2, HDL_3, and the 1.21 infranate; A-LP-C reacted positively with all density classes except the 1.21 infranate; A-LP-B gave precipitin lines with VLDL, LDL_1, LDL_2, HDL_2, and occasionally with HDL_3. Another precipitin line between HDL_3 and A-HDL_3, closer to the antibody well, was always present in this high-density lipoprotein fraction (Fig. 5b). This antigenic component has been designated by Lee and Alaupovic (90) as the "thin-line" polypeptide. A double diffusion study of HDL_2 (Fig. 5b) revealed reactions of non-identity between A-LP-A, A-LP-B, and A-LP-C.

Immunoelectrophoretic studies of the various density classes revealed that VLDL and LDL_1 gave similar precipitin lines of α_2 mobilities with antibodies to LP-B, LP-C, and WS. The LDL_2 showed a similar reactiv-



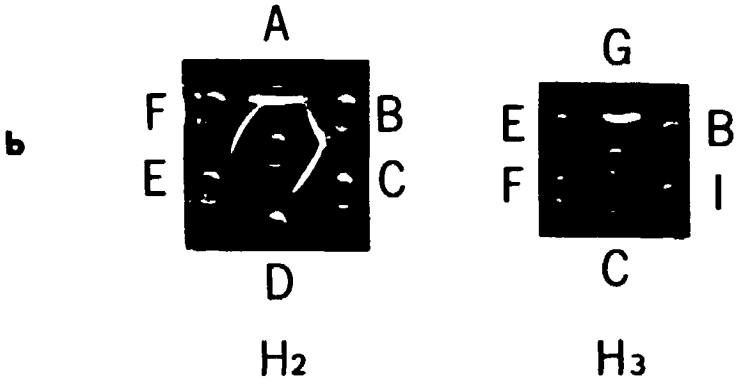


Figure 5. Immunodiffusion patterns of lipoprotein density classes from a normal female donor (pattern a) and a normal male donor (pattern b). Lipoproteins were placed in the central wells. V = VLDL, L_1 = LDL_1, L_2 = LDL_2, H_2 = HDL_2, H_3 = HDL_3. Outer wells contain antibodies to whole serum (anti-WS); LP-A (A); LP-C (B); LP-B (C); IgG (D); albumin (E); whole serum (F); HDL_3 (G); and VLDL (I).

ity, but the mobility was slower, and the precipitin arc with A-LP-C was much weaker. The HDL_2 fractions reacted with A-WS, A-LP-C, A-LP-B, and A-LP-A. Whereas the precipitin lines of LP-B and LP-C had similar mobilities, the LP-A precipitin line was somewhat elongated, streaking through the α_1 and α_2 regions. The HDL_3 gave precipitin arcs with A-WS, A-LP-C, and A-LP-A. The major LP-A precipitin arc migrated faster than that due to LP-C. Relative mobilities of density classes on paper and agarose electrophoresis were similar to those observed on immunoelectrophoresis.

Examination of the basic polyacrylamide gel patterns of various density classes revealed characteristic patterns (Fig. 6). The VLDL isolated from fasting plasma typically yielded three fast-moving bands when electrophoresed in the presence of 8 M urea but only two bands (C-III-1 and C-III-2) in the absence of urea. The mobility of all bands was greater in the absence than in the presence of urea. A slower moving band was also observed occasionally in the separating gel. The loading and stacking gels, as well as the junctions between the three gels, were always stained with the protein stain. Similarly, the loading and stacking gels were always stained for lipid. However, the two fast-moving bands were stained for lipid in only 50% of the VLDL samples examined.

The electrophoretic pattern of LDL was very similar to that of VLDL. Another faint band was occasionally noted midway between the fast-moving bands and the heavily stained region at the juncture of the separating and stacking gels. Loading and stacking gels were always stained for both protein and lipid. However, the fast-moving bands were stained only for protein.

The HDL_2 and HDL_3 patterns were similar except that the HDL_3

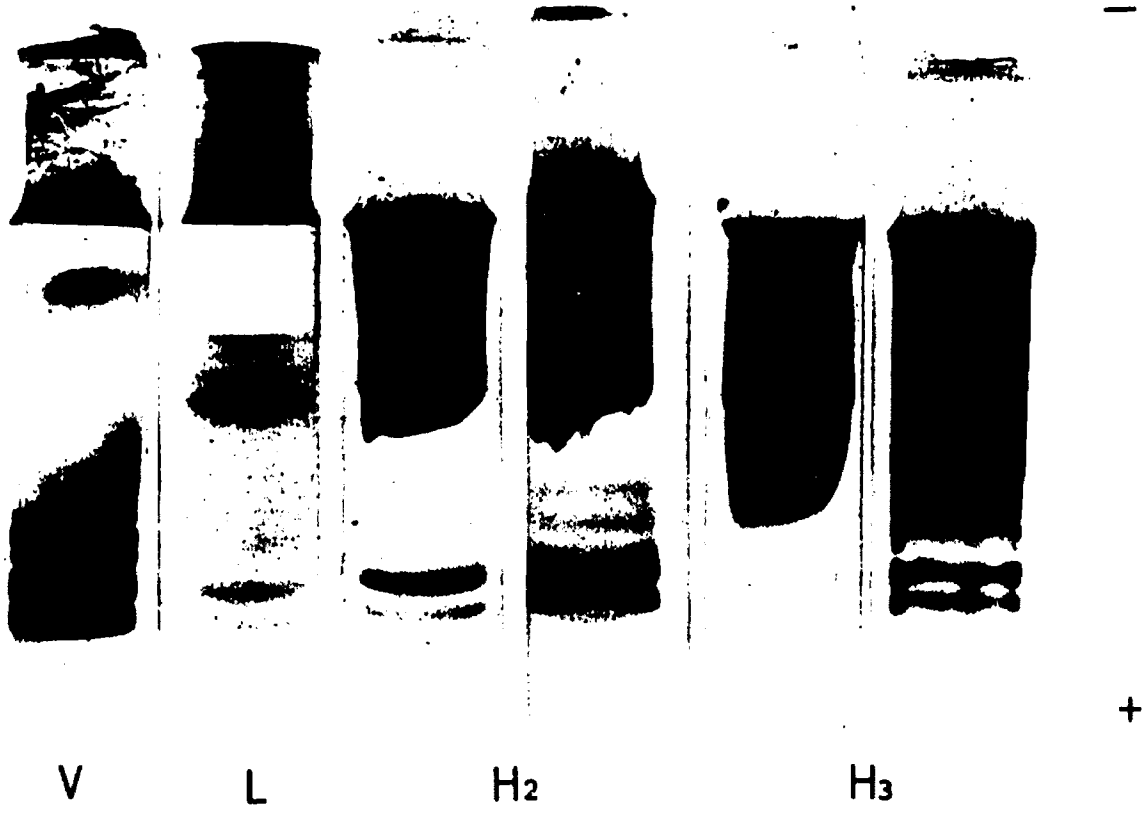


Figure 6. Basic polyacrylamide gel electrophoresis (PAGE) of lipoprotein density classes. PAGE was run on 7% gels containing 8 M urea and stained with Amido Black. V = VLDL, L = LDL, H_2 = HDL_2, and H_3 = HDL_3.

sample usually showed no protein stained band at the junction of the separating and stacking gels. There were several protein stained regions between the juncture of the stacking gel and the fast-moving bands. Only the principal bands were stained intensively with Oil-Red-O; there was no lipid staining of the fast bands. A better resolution of the heavily stained bands was achieved with low concentrations of HDL_2 and HDL_3, but the fast-moving bands were very faint or absent.

Immunochemical investigation of the patterns obtained on 7% polyacrylamide by imbedding the gels in agarose revealed certain characteristic precipitin patterns. The LDL, HDL_2, and HDL_3 exhibited three regions of reactivity in the separating gels: the fast-moving bands reacted with A-LP-C, the junction of the separating and stacking gels with A-LP-B, and the intermediate region with A-LP-A. In some instances a reaction was observed with A-LP-C in the region immediately adjacent to the juncture of the separating gel. If LDL_1 or LDL was examined in the same system, reactions with A-LP-B, A-LP-C, and A-LP-A were usually observed in the loading and stacking gels.

Three percent polyacrylamide patterns of these three density classes were less complex when stained with protein or lipid stain, but there was no clear-cut separation of lipoprotein families. The faster moving band(s) reacted with both A-LP-A and A-LP-C while the slower moving zone reacted with A-LP-B and A-LP-C. Occasionally a reaction with A-LP-A was observed with the slower moving zone. The intermediate region reacted with both A-LP-C and A-LP-A while the stacking and loading gels had the same pattern of reactivity as the 7% gels.

As already mentioned, VLDL reacted with A-LP-B and A-LP-C and

very infrequently with A-LP-A. Since the LP-A antigenic component(s) was possibly buried in the VLDL molecule, the partially delipidized VLDL produced very faint precipitin lines with A-LP-A. It was concluded from these immunochemical studies that there is very little, if any, LP-A in the normal fasting VLDL. When VLDL was examined by double diffusion against A-LP-B and A-LP-C, a line of complete fusion was observed between these two antigen-antibody complexes (Fig. 7a). All VLDL samples gave at least one precipitin line with A-C-I, but some gave two distinct lines. Most VLDL preparations reacted positively also with A-C-III.

Immunoelectrophoretic analysis of various VLDL preparations on agarose gels revealed at least two lipoprotein bands with different mobilities. The faster moving band reacted with antibodies to C-I, C-II, C-III, and LP-B, whereas the slower moving band gave precipitin arcs only with antibodies to C-II and C-III (Fig. 7b). The presence of a slow-moving band seems to suggest a partial dissociation from the VLDL complex of C-II and C-III polypeptides or polypeptide-lipid units, but not C-I. Reaction of VLDL with antibodies to LP-B and C-I resulted in a complete fusion of precipitin arcs.

Presence of urea had a striking effect on the separability and mobility of various VLDL bands in 7% polyacrylamide gel electrophoresis at pH 8.8. In the absence of urea from gels, only two C-III bands were clearly distinguished; however, when 4 M urea was incorporated into the loading gel, a third band corresponding to the C-II polypeptide became much more prominent. A 20% decrease in the mobility of all the fast-moving LP-C bands was noted when 8 M urea was incorporated into the separating gel. The LP-C bands present in LDL and HDL also showed this re-

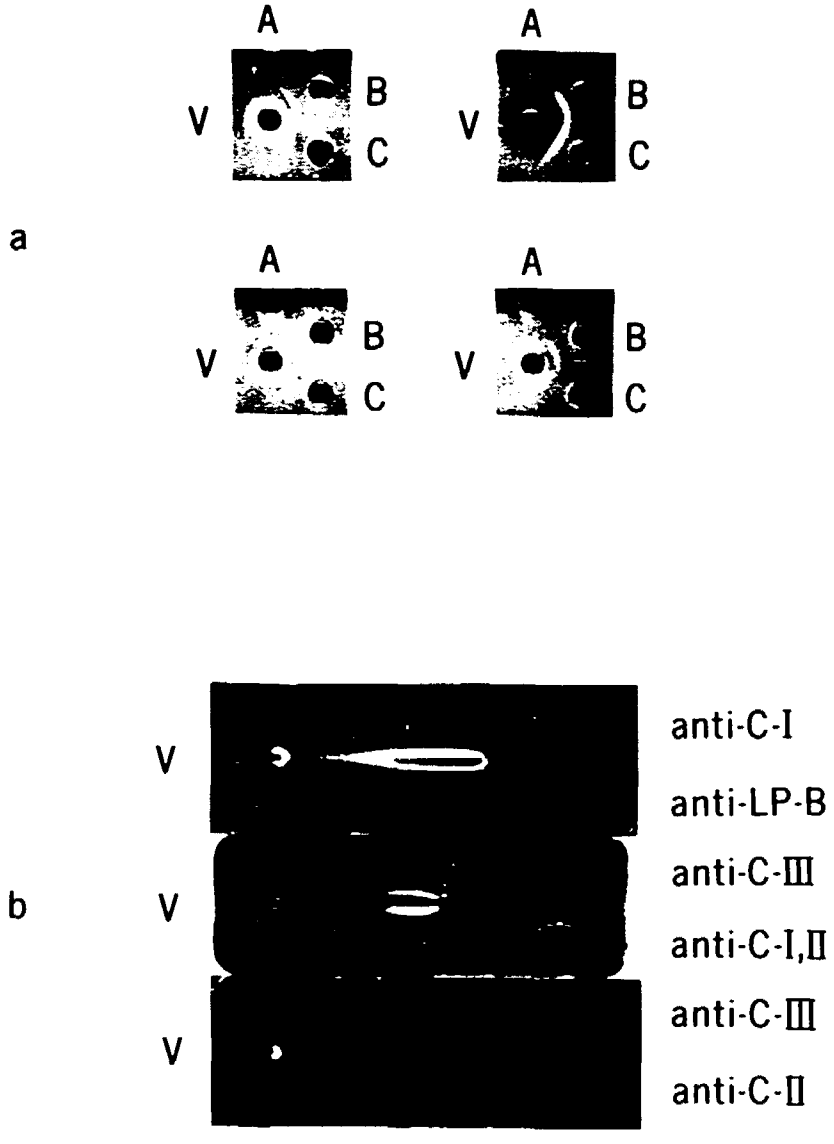


Figure 7. Immunodiffusion of VLDL from four normal subjects (pattern a) and immunoelectrophoresis in 1% agarose gel of three different normal VLDL samples (pattern b). Anti-sera were placed in the outer wells. V = VLDL, A = anti-LP-A, B = anti-LP-C, and C = anti-LP-B.

duction in mobility. In the acidic (pH 4.3) polyacrylamide system, the sharpest resolution of C-I was obtained when urea was present in all gels. Urea had no apparent effect on the mobility of this faint fast-moving band.

It was demonstrated by immunological examination of the VLDL bands separated by 3 or 7% polyacrylamide gel electrophoresis, pH 8.8, that they reacted positively with A-LP-C, A-LP-B, A-WS, and in a few cases, with A-LP-A. Reactions with A-LP-C, and A-LP-B were observed at the junction of the separating and stacking gels in both systesm (Fig. 8). In the 3% system the VLDL bands formed in the separating gel ("beta" and "post-beta" bands) reacted both with the antibodies to LP-B and LP-C (Fig. 8a). In the 7% system the fast-moving bands in the separating gel gave precipitin lines only with antibodies to LP-C (Fig. 8b).

Results of these studies showed that intact lipoproteins are separated by 7% polyacrylamide gel electrophoresis into three main zones. The fast-moving bands in the separating gel are those of LP-C; the intermediate region consists of LP-A bands; and the band at the juncture of the separating and stacking gels is LP-B. Lipoproteins retained in the loading and stacking gels represent a mixture of LP-A, LP-B, and LP-C species of the sizes and molecular weights characteristic primarily of VLDL or LDL_1.

Chyle

Chyle samples obtained following a fat meal or dextrose-water were fractionated into VLDL, LDL and HDL. Testing by immunodiffusion demonstrated that LP-A was present in all density classes except the VLDL isolated during the glucose-water regimen; the LP-C was present in all

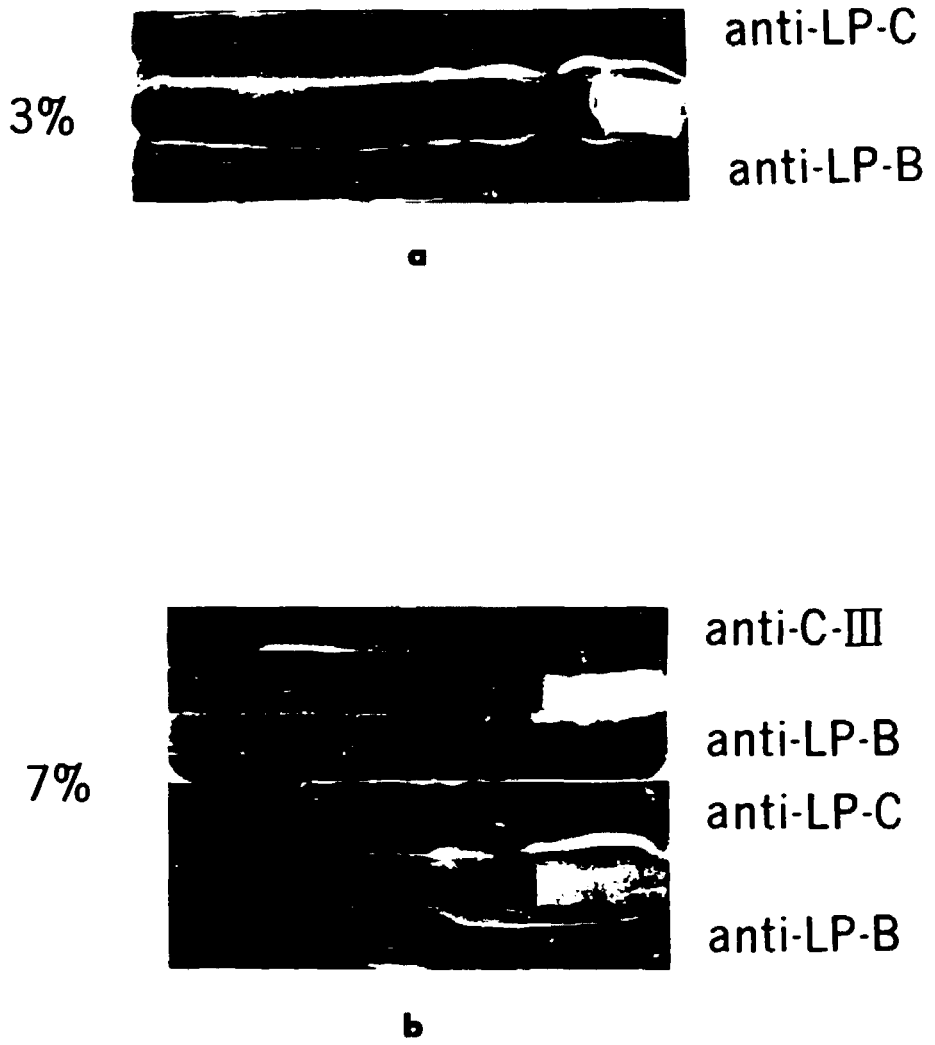


Figure 8. Combined basic polyacrylamide gel electrophoresis and immunodiffusion of VLDL on a 3% gel (pattern a) and 7% gel (pattern b). Acrylamide gels were embedded in 1% agarose.

density classes examined. The LP-B was absent immunochemically from the postprandial HDL in 2 of 3 different chyle samples examined.

The subfractionation of chyle into VLDL subclasses was performed only with a sample obtained following a high fat diet. The VLDL subfraction with S_f > 5,000 gave an indistinct halo rather than distinguishable precipitin lines with A-LP-A, A-LP-B, and A-LP-C. However the S_f 400-5,000, S_f 20-400, LDL, and HDL all reacted with A-LP-A and A-LP-C. It seems that all of these density classes also contained LP-B.

Paper electrophoretic studies revealed that the postprandial chyle VLDL had a heavily lipid-stained region at the origin, while both VLDL and LDL displayed a lipid-stained band extending from the origin through the pre-beta region. All three density classes of the postprandial chyle had an α_1-band while the HDL had no β_1-band. The glucose-water VLDL displayed lipid staining from the origin to the pre-beta region while LDL and HDL both had bands in the β_1 and α_1 region.

Agarose electrophoresis of VLDL demonstrated that the mobility of the postprandial sample extended from the origin through the pre-beta region while the glucose-water sample was characterized principally by the pre-beta band. The postprandial LDL resolved into three bands in the α_2-region. The sample from postprandial and glucose-water state revealed only a single α_1 band.

Disc gel electrophoresis of three VLDL subfractions, LDL, and HDL isolated from postprandial chyle showed a similarity in their resolution but a variation in the staining intensity of individual bands (Fig. 9). Whereas VLDL subfractions with S_f 20-400 and 400-5,000 had several prominent bands in the LP-A region, the subfraction with S_f >

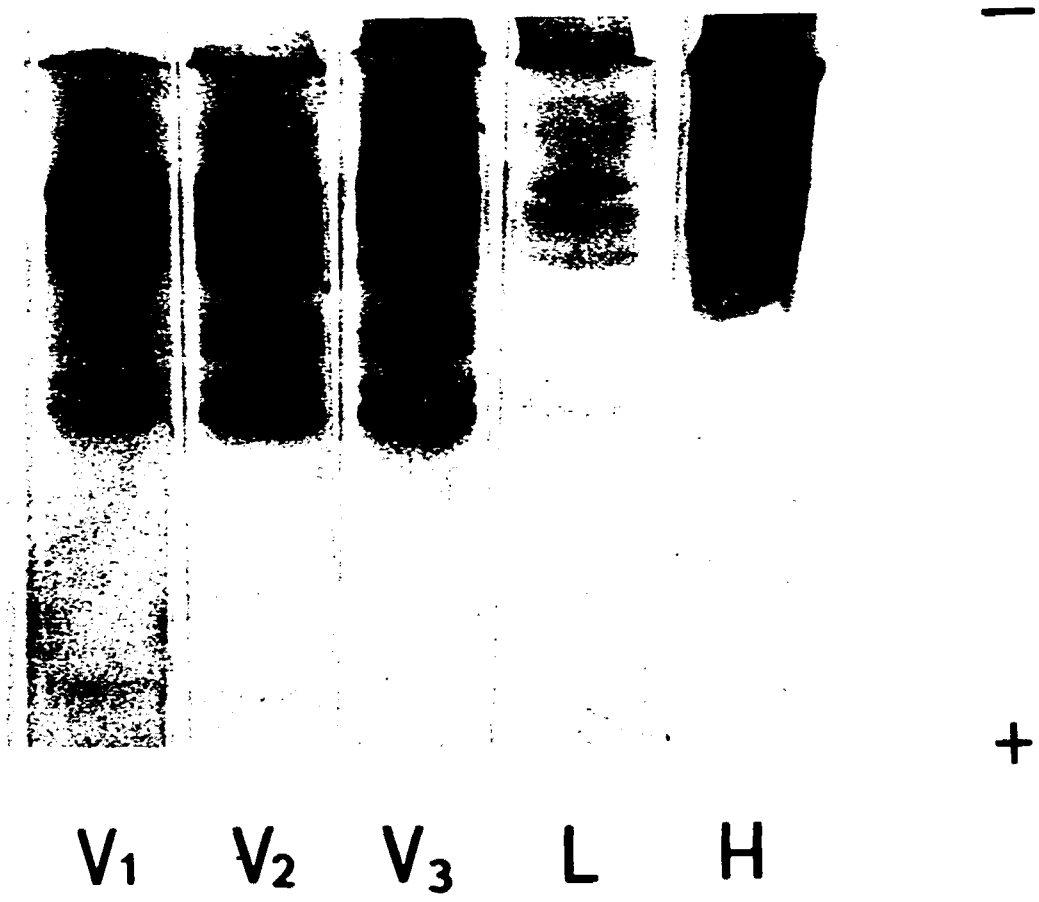


Figure 9. Basic polyacrylamide gel electrophoresis of postprandial chyle lipoproteins. PAGE was run on 7% gels containing 8 M urea and stained with Coomassie Brilliant Blue. V_1 = S_f 400-5,000, V_3 = S_f 20-400, L = LDL, and H = HDL.

5,000 was characterized by one major and several minor bands in the same region. Similar faint bands in the LP-A zone were also seen in the LDL fraction. Although the HDL exhibited the highest staining density in the LP-A region, the bands were poorly resolved. Staining of the juncture of the separating-stacking gel, noted in the VLDL subfractions and LDL, was indicative of the presence of LP-B. The fast-moving LP-C bands were present in all density classes (Fig. 9).

Totally delipidized VLDL subfractions with S_f 400-5,000 gave positive immunoprecipitin lines with antibodies to A-I and A-II polypeptides (Fig. 10a). The A-I polypeptide separated from the totally delipidized chyle subfraction with S_f 400-5,000 by Sephadex G-100 gel filtration and DEAE-cellulose chromatography showed immunochemical identity with the A-I polypeptide isolated from totally delipidized plasma HDL (Fig. 10b).

To obtain some information about the relative distribution of various LP-A and LP-C bands in three VLDL subfractions isolated from a postprandial chyle sample, the 7% polyacrylamide gels were stained with Coomassie Blue, and the bands in the separating gels were measured by densitometric scanning. The results presented in Table 9 do not include the LP-B band occurring at the juncture of stacking and separating gels and the LP-A, LP-B,and LP-C species present in the loading and stacking gels. Each lipoprotein band was characterized by its mobility relative to that of the principal fast-moving LP-C band (C-III-2) and by comparison of these mobilities with those of corresponding totally delipidized species. The scans revealed that the LP-A region accounted for approximately 85% (83-88%) and the LP-C region for about 15% (12-18%) of the

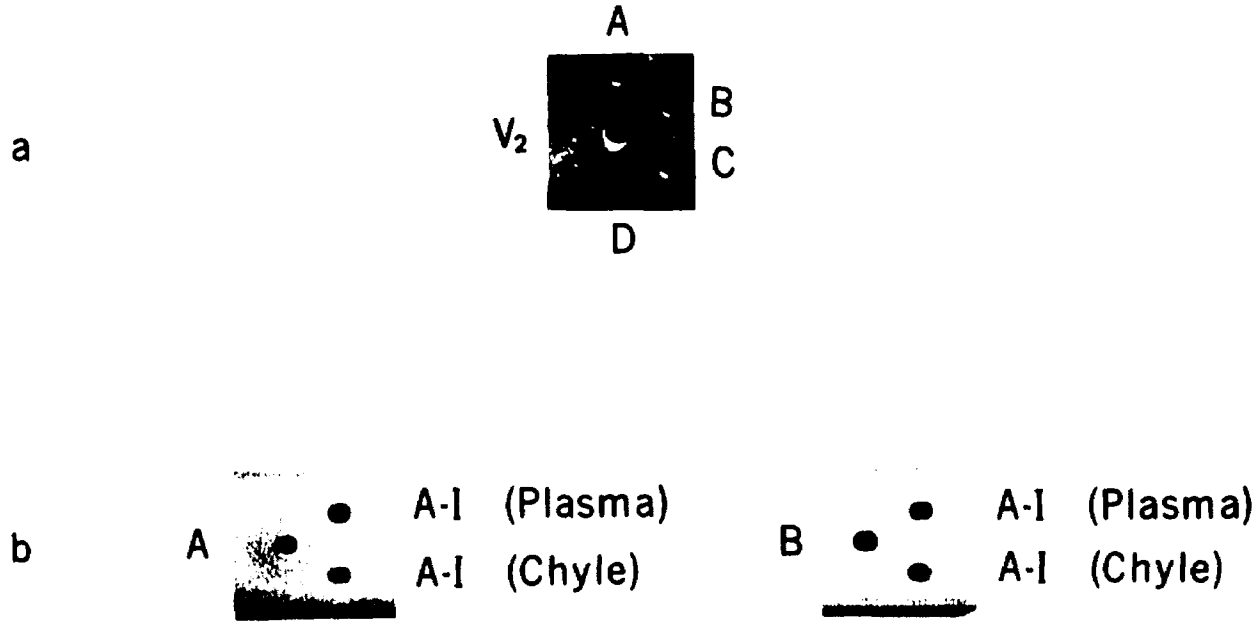


Figure 10. Immunodiffusion (pattern a) of totally delipidized postprandial chyle subfraction S_f 400-5,000 (V_2). Immunodiffusion of A-I polypeptide isolated from chyle VLDL subfraction (S_f 400-5,000) and plasma HDL. A = anti-A-I, B = anti-A-II, C = anti-C-III, and D = anti-C-I (pattern a); A = anti-A-I and B = anti-LP-A (pattern b).

TABLE 9

RELATIVE CONTENT OF LP-A AND LP-C POLYPEPTIDES IN SUBFRACTIONS OF CHYLE VLDL

Band	Polypeptide Designation	Chyle VLDL Subfractions[a]			R_f[b]
		S_f > 5,000	S_f 400-5,000	S_f 20-400	
		%	%	%	
A	C-III-2	5.46	7.19	8.33	1.00
B	C-III-1	3.85	3.13	5.08	0.926
C	C-II	2.84	4.76	3.41	0.819
D	A-peptide	3.44	5.97	5.76	0.764
E	A-peptide	11.29	12.87	6.09	0.577
F	A-peptide	30.58	31.23	19.36	0.465
G	A-peptide	29.38	23.41	22.68	0.375
H	A-peptide	13.16	11.43	29.28	0.228
A-C	LP-C	12.15	15.08	17.82	
D-H	LP-A	87.85	84.92	83.18	

[a]Determined by densitometric scanning of the separating gel, pH 8.8 in 8 M urea and expressed as percent of the sum of individually determined bands.

[b]Average mobility of each band for three subfractions. The mobility of C-III-2 was taken as unity.

stained lipoprotein bands; the LP-C bands increased with a decrease in the S_f-values of VLDL subfractions. The average ratio of the C-III-2 band to the C-III-1 band (R = 1.79) in the three VLDL fractions was similar to the ratio found with the C-III polypeptides from some preparations of ApoX.

To study the effect of diet on the relative distribution of LP-A and LP-C bands in 7% polyacrylamide gels, the VLDL, LDL, and HDL were isolated from chyle samples collected from a patient during a prolonged fast, after a high-fat meal, and on a diet consisting of only dextrose and water. Results of densitometric evaluation of the LP-A and LP-C bands of major lipoprotein density classes are presented in Table 10. Diet had very little effect on the relative distribution of LP-A and LP-C bands in HDL. On the other hand, dietary fat had an appreciable effect on the distribution of LP-A and LP-C in VLDL; with an increased fat-load, there was an increase in the relative content of LP-A and a decrease in the content of LP-C. The densities of bands in the stacking and loading gels and of the LP-B band were not estimated.

A sample obtained by cannulation of the right thoracic duct was compared to a sample drawn at the same time from the left thoracic duct. The chyle from the right thoracic duct showed on immunoelectrophoresis positive reactions with A-LP-A, A-LP-B, and A-WS but not with A-LP-C (Fig. 11). On double diffusion the only difference noted between the two chyle samples was that the chyle from the right thoracic duct did not react with A-LP-A. The ultracentrifugation of the chyle from the right thoracic duct at d 1.006 g/ml demonstrated the absence of a VLDL class. However, the 1.006 infranate reacted with both A-LP-B and A-LP-A.

TABLE 10

THE EFFECT OF DIET ON THE RELATIVE CONTENT OF LP-C AND LP-A IN THE MAJOR DENSITY LIPOPROTEIN CLASSES OF CHYLE

Diet	Density Class	Lipoprotein[a]	
		LP-A	LP-C
		%	%
75% Fat	VLDL	72.99	27.00
	LDL	81.12	18.18
	HDL	81.57	18.43
Fasting	VLDL	33.81	66.19
	LDL	77.88	22.12
	HDL	86.69	13.31
Dextrose-Water	VLDL	32.53	67.47
	LDL	-	-
	HDL	82.31	17.68

[a]Determined by densitometric scanning of the basic separating gel in 8 M urea.



Figure 11. Immunoelectrophoresis in 1% agarose gel of right thoracic duct chyle. A = right thoracic duct chyle.

It was concluded from these studies that the chyle isolated from the right thoracic duct had very little, if any, VLDL and LP-C.

Plasma from Hypertriglyceridemic Patients

The qualitative apolipoprotein composition of VLDL, LDL, and HDL isolated from plasma samples of patients with type I, III, IV, and V hypertriglyceridemia was determined by examining the intact lipoproteins with immunological methods and 7% polyacrylamide gel electrophoresis. Results of these studies (Table 11) showed that neither dietary regimen nor the type of lipid transport derrangement had any effect on the qualitative distribution of LP-B and LP-C; these two lipoprotein families were detected in all density classes of hypertriglyceridemic patients. Similarly, the LP-A family was detected in HDL of all hypertriglyceridemic patients. However, the LP-A was positively identified only in VLDL isolated from Types I and V but not in VLDL from Types III and IV. The LP-A could not be detected in intact LDL. In hypercholesterolemic (Type II) patients the LP-B and LP-C were positively identified in VLDL and LDL whereas HDL was not examined.

The analytical polyacrylamide patterns demonstrated that C-I, C-II, and C-III polypeptides were present in the VLDL of all Type I, III, IV, and V hypertriglyceridemic patients.

Although the dietary fat had little effect on the qualitative composition of apolipoproteins in major density classes, an increased fat intake seems to influence considerably the absolute levels as well as the percent distribution of lipoprotein families or their protein moieties in at least VLDL and LDL. Preliminary results of such a study with a Type III hypertriglyceridemia patient showed not only a marked

TABLE 11

DISTRIBUTION OF APOLIPOPROTEINS IN THE MAJOR LIPOPROTEIN DENSITY CLASSES ISOLATED FROM PATIENTS WITH VARIOUS TYPES OF HYPERTRIGLYCERIDEMIA

Hypertriglyceridemia	Dietary regimen	Density Class	Apolipoproteins ApoA	ApoB	ApoC
Type I	Fasting	VLDL	+	+	+
	40% fat diet	LDL	-	+	+
		HDL	+	+	+
Type III	Fasting	VLDL	-	+	+
	Non-fat diet	LDL	-	+	+
		HDL	+	+	+
	Fasting	VLDL	-	+	+
	40% fat diet	LDL	-	+	+
		HDL	+	+	+
Type IV	Fasting	VLDL	-	+	+
	40% fat diet	LDL	-	+	+
		HDL	+	+	+
	Fasting	VLDL	-	+	+
	75% fat diet	LDL	-	+	+
		HDL	+	+	+
Type V	Fasting	VLDL	+	+	+
	40% fat diet	LDL	-	+	+
		HDL	+	+	+
	Six hours	VLDL	+	+	+
	after a	LDL	-	+	+
	high-fat diet	HDL	+	+	+

increase in the absolute amounts but also a redistribution of LP-C and LP-B families in VLDL and LDL. However, the number of patients studied is still too small to permit a more thorough evaluation of the significance of these results.

Tissue homogenate studies of intestinal mucosa and liver obtained from recently deceased (3-4 hours) human cadavers were examined by immunodiffusion. Reactions with A-LP-C were obtained in three of four intestinal mucosal samples examined; and, in two samples, two precipitin arcs were present, one of which apparently fused with a precipitin line present in a whole serum that contained LP-X. A-LP-A precipitin arcs were observed only when the A-LP-A was diffused against whole serum, while faint precipitin lines were observed when A-LP-B was diffused against both the liver and intestinal homogenates of one subject.

Antisera

Random plasma, fasting plasma, and postprandial plasma were utilized as the sources of antigens which were isolated either as intact lipoproteins or as apolipoproteins. The antisera were characterized by immunodiffusion and immunoelectrophoresis against whole serum, density classes, or purified polypeptides. The reactions of all antisera with whole plasma were comparable on agar and agarose gels except that the mobilities of antigens were reduced on agar gels. The characteristics of antisera obtained against major density classes are presented in Table 12. Antisera to fasting (VLDL) and postprandial VLDL (PP-VLDL) differed only in that the latter contained antibodies to LP-A. Some HDL_3 antisera contained antibodies not only to the two ApoA polypeptides but also to a polypeptide referred to as the "thin-line" polypeptide (90).

TABLE 12

CHARACTERIZATION OF ANTISERA TO ULTRACENTRIFUGALLY ISOLATED LIPOPROTEIN DENSITY CLASSES

Designation of antisera	Host animal	Total protein injected	Specificity			Comment
			LP-A	LP-B	LP-C	
A-VLDL	Rabbit	3-8 mg	-	+	+	Fasting normal VLDL was the source of antigen (pool of five subjects).
A-PP-VLDL	Rabbit	5-15 mg	+	+	+	Postprandial hypertriglyceridemic VLDL (Type V) was the source of antigen.
A-LDL	Rabbit	5-16 mg	-	+	-	One of seven A-LDL's reacted with LP-X on agar immunoelectrophoresis.
A-HDL_3	Rabbit	10-20 mg	+	+	+	Three of six A-HDL_3's also contained antibodies directed against the "thin-line" polypeptide.

Table 13 shows the source and reactivity of antisera directed against purified lipoprotein families and the polypeptides of ApoA and ApoC.

Examination of immunoelectrophoretic patterns of plasma samples from normal subjects and patients with obstructive jaundice revealed some characteristic precipitin arcs when they were tested with various antisera. Whereas antibodies to C-I gave a similar anodal precipitin arc with both normal and jaundiced plasma, the typical cathodal arc (LP-X) was formed only with the jaundiced plasma (Fig. 12a). Reaction between the antibodies to LP-B and normal or jaundiced plasma samples resulted in the formation of identical precipitin arcs which extended from the well into the β_1-region (Fig. 12b). Both plasma samples also gave essentially the same pattern with A-LP-A; it consisted of two converging precipitin arcs with a spur between them (Fig. 12c).

The cathodal mobility of LP-X was very useful as a test for the possible presence of antibodies to LP-C in various antisera. When tested against jaundiced plasma, most antisera to VLDL, several antisera to HDL, all A-LP-X, and one antiserum to LDL displayed the typical cathodal migration indicating the presence of antibodies to LP-C.

Column Chromatography and Precipitation Methods

In an attempt to separate lipoprotein families, especially LP-C, the major density classes were chromatographed on several different media.

Ion exchange chromatography of lipoprotein density classes on DEAE-cellulose, with or without 8 M urea, was only partially successful. There was no resolution of either the VLDL or LDL. However, the HDL_3 was separated into four peaks with a NaCl linear gradient (0.01 M - 0.5 M, pH 7.2 40 cm column). An antiserum prepared by immunizing rabbits

TABLE 13

CHARACTERIZATION OF ANTISERA TO LIPOPROTEINS AND POLYPEPTIDES

Designation of antisera	Host animal	Total protein injected	Method of preparation of antigen
A-LP-A	Goat	40 mg	Phosphotungstate precipitation (ppt. II), ultracentrifugation (1.12-1.21 g/ml) and hydroxyapatite
A-A-I	Sheep	23 mg	Column chromatography of ApoA
A-A-II	Sheep	20 mg	Column chromatography of ApoA
A-LP-B	Rabbit	5-16 mg	Ultracentrifugally isolated LDL
A-LP-C	Rabbit	5.0 mg	Partial delipidization of LP-X followed by ultracentrifugation
A-C-I	Rabbit	10 mg	DEAE-cellulose chromatography
$A\text{-}C\text{-}I_{Ab}$	Rabbit	10 mg	Partial delipidization of LP-X followed by ultracentrifugation
A-C-II	Rabbit	2.0 mg	DEAE-cellulose chromatography followed by polyacrylamide gel electrophoresis (PAGE)
A-C-I, II	Sheep	10 mg	Preparation of LP-X
A-C-III	Rabbit	5.0 mg	DEAE-cellulose chromatography followed by PAGE

TABLE 13--Continued

Specificity						Comment
A-I	A-II	B	C-I	C-II	C-III	
+	+					Absorption of A-albumin and A-LP-B.
+						
	+					Titer to unknown component which may be due to a polymorphic form of A-II.
		+				
			+	+	+	
			+			Absorption of A-albumin
			+			Absorption with HDL_3. Titer to an unknown component present.
				+		
			+	+		Absorption with 1.25 g/ml infranate and LDL.
					+	One of the two A-C-III may contain an additional antibody directed against C-III-3.

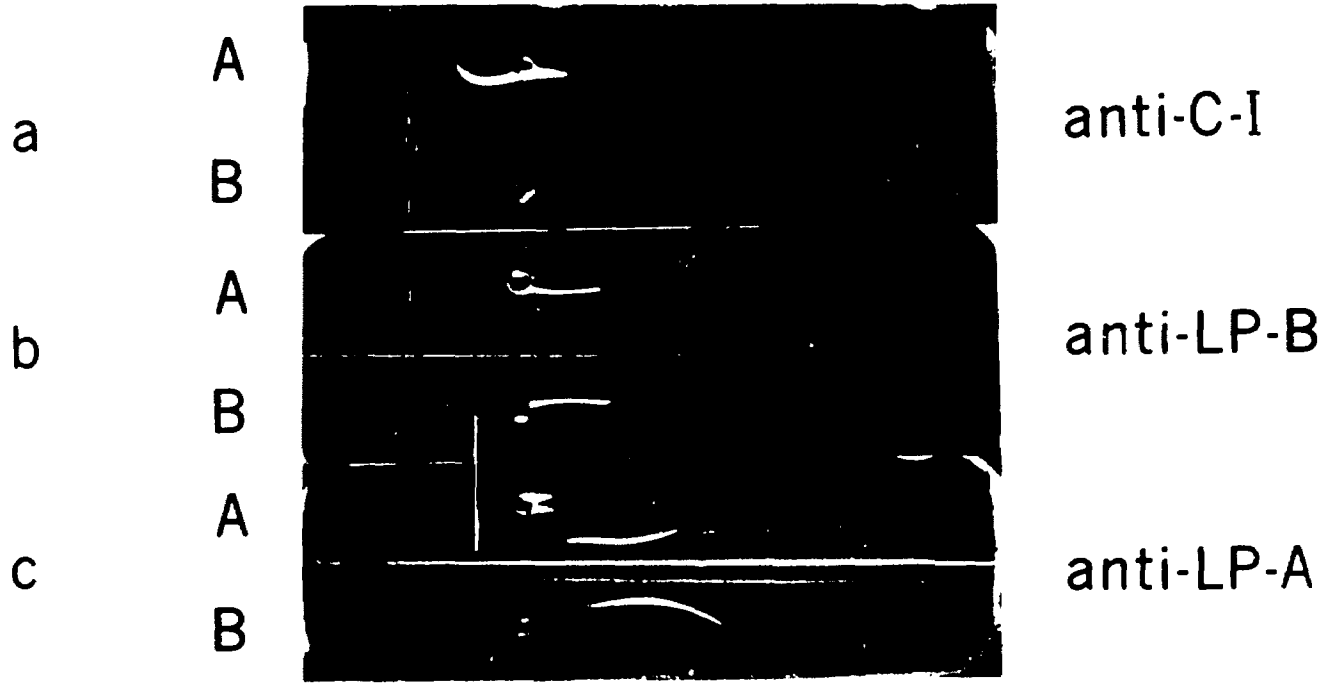


Figure 12. Immunoelectrophoresis in 1% agar gel of whole plasma. A = obstructive jaundiced plasma and B = normal fasting plasma.

with the second peak reacted only with A-I and A-II polypeptides. The parent HDL_3 also produced antibodies to LP-B and, possibly, LP-C.

Column chromatography on Sephadex G-200, Sepharose 4B, and Sepharose 6B was utilized in an attempt to separate the lipoprotein families present in the various density classes. Sephadex G-200 was found to be unsatisfactory for separating LP-A, LP-B, LP-C, and the density classes because the larger lipoproteins (those in VLDL and LDL) were eluted in the void volume. In fact, none of the molecular seive media could be successfully applied to the resolution of lipoprotein families in the VLDL, LDL, or HDL as judged by immunodiffusion and disc gel electrophoresis. However, the VLDL, LDL, and HDL were resolved by Sepharose 6B or 4B into three peaks. The VLDL eluted before the major Blue Dextran peak exhibited some trailing (Fig. 13) while the LDL was resolved into a major peak eluted immediately after Blue Dextran and into a minor late peak which was not identified immunologically. The HDL (1.063-1.21 g/ml) was eluted as two discernible peaks, the first of which consisted of a mixture of LP-B and LP-C and the second, principally, of LP-A. Each of the three major peaks corresponding to VLDL, LDL, and HDL were identified, in order of elution, as LP-B and LP-C, LP-B and LP-C, and LP-A and LP-C, respectively.

Attempts to resolve VLDL by hydroxylapatite column chromatography into LP-C and LP-B families were not successful. On the other hand, some separation of LP-C from LP-A was achieved when HDL_3 was used as the starting material with LP-C eluted primarily with 0.25 M and 0.40 M phosphate buffer (pH 6.8).

Heparin precipitation, phosphotungstate, ethanol-water, and poly-

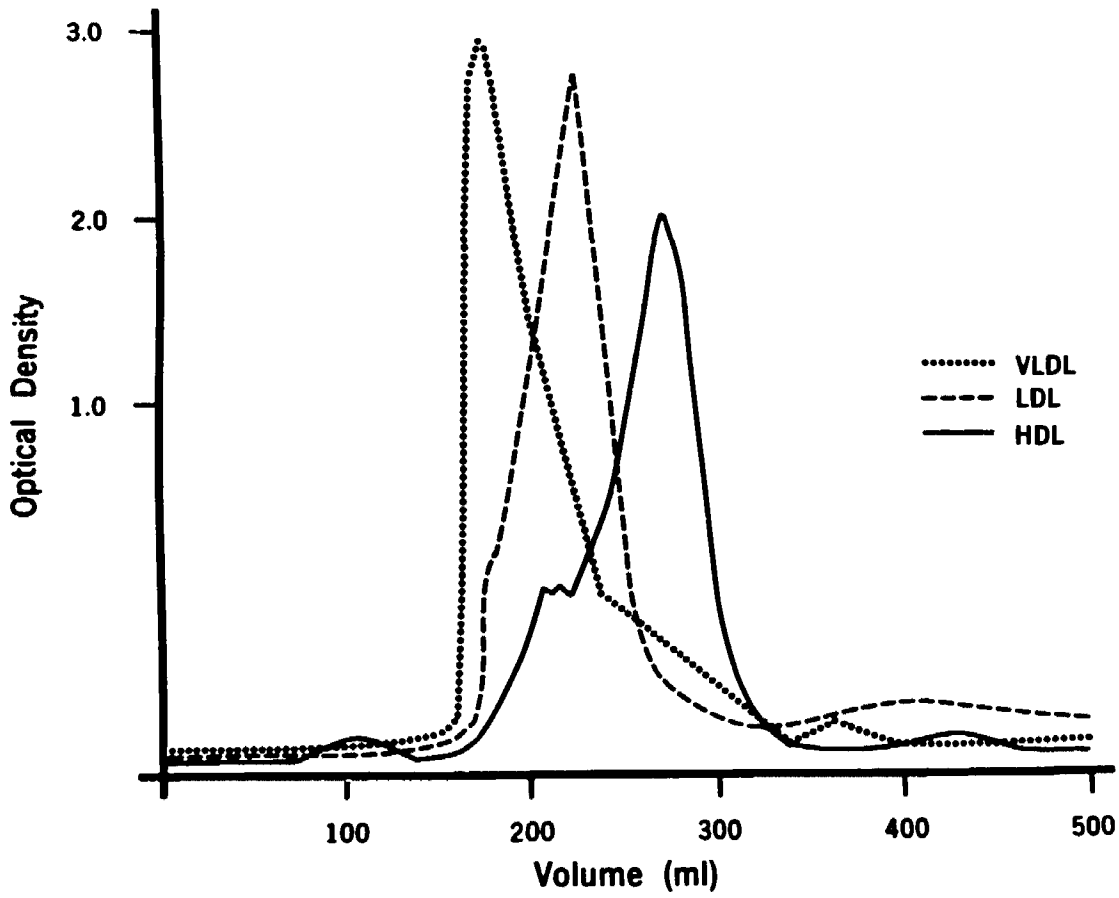


Figure 13. Elution pattern of VLDL, LDL and HDL from Sepharose 6B. Lipoproteins were eluted with 0.1 M borate buffer (pH 8.6).

ethylene glycol precipitation of VLDL yielded no separation of LP-B from LP-C as determined by immunodiffusion.

Isolation of Partially Delipidized LP-C

The scheme for isolation of pdLP-C by immunoprecipitation was outlined in Figure 3. The partially delipidized lipoprotein C from chyle and serum obtained after the immunoprecipitation procedure and removal of the rabbit serum proteins was tested by immunodiffusion, immunoelectrophoresis, and basic disc gel electrophoresis. The pdLP-C gave a negative reaction on immunodiffusion with A-LP-A, A-LP-B, or any other of the commercially available antisera listed under the immunochemical procedures. The pdLP-C reacted positively with both A-LP-C and A-VLDL with fusion of the two precipitin lines (Fig. 14a). On immunoelectrophoresis the pdLP-C usually gave a single precipitin arc with A-LP-C and/or A-VLDL (Fig. 14b); however, in some cases pdLP-C gave two precipitin arcs with A-LP-C (Fig. 14b). On disc gel electrophoresis the pdLP-C consisted of four fast bands (Fig. 15a). Unfortunately, the A-HDL_3 utilized for immunoprecipitation was found to contain a weak titer for LP-C, and therefore this approach for the isolation of partially or totally delipidized LP-C was abandoned.

For the above reason the ultracentrifugal isolation of partially delipidized C was developed (Fig. 4). Both postprandial VLDL from hyperlipemic patients and postprandial chyle lipoproteins ($S_f > 100$) were utilized as the starting material. The two fractions isolated by the ultracentrifugal procedure (Fig. 4) were designated as Fraction C (d 1.04-1.12 g/ml) and Fraction ABC (d 1.12-1.21 g/ml). Fractions C and ABC reacted with antibodies to LP-C and VLDL while only Fraction ABC reacted

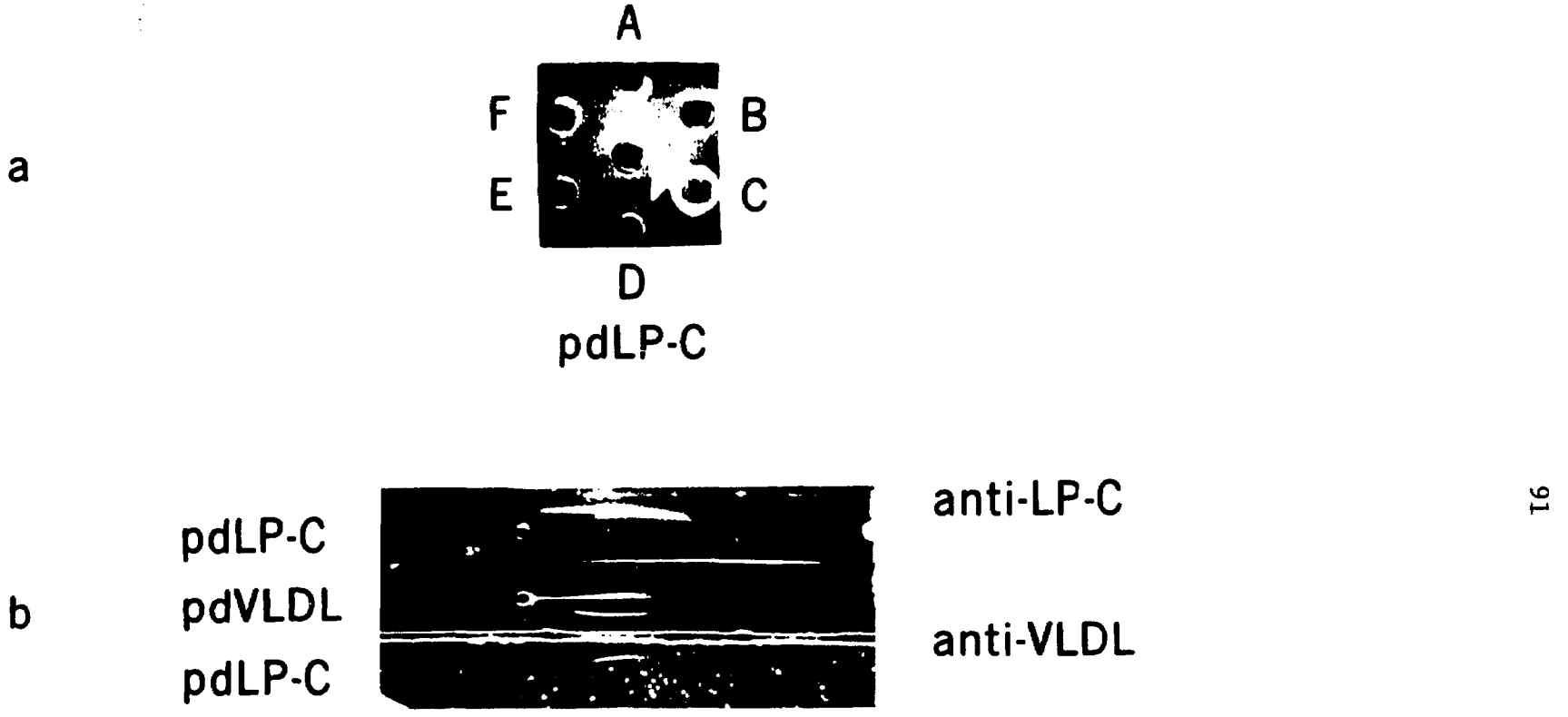


Figure 14. Immunodiffusion and immunoelectrophoresis of pdLP-C isolated by immunoprecipitation. pdLP-C was placed in central well (pattern a). Outer wells contain antibodies to LP-A (A); VLDL (B), LP-C (C); LP-B (D); whole serum (E); and albumin (F).

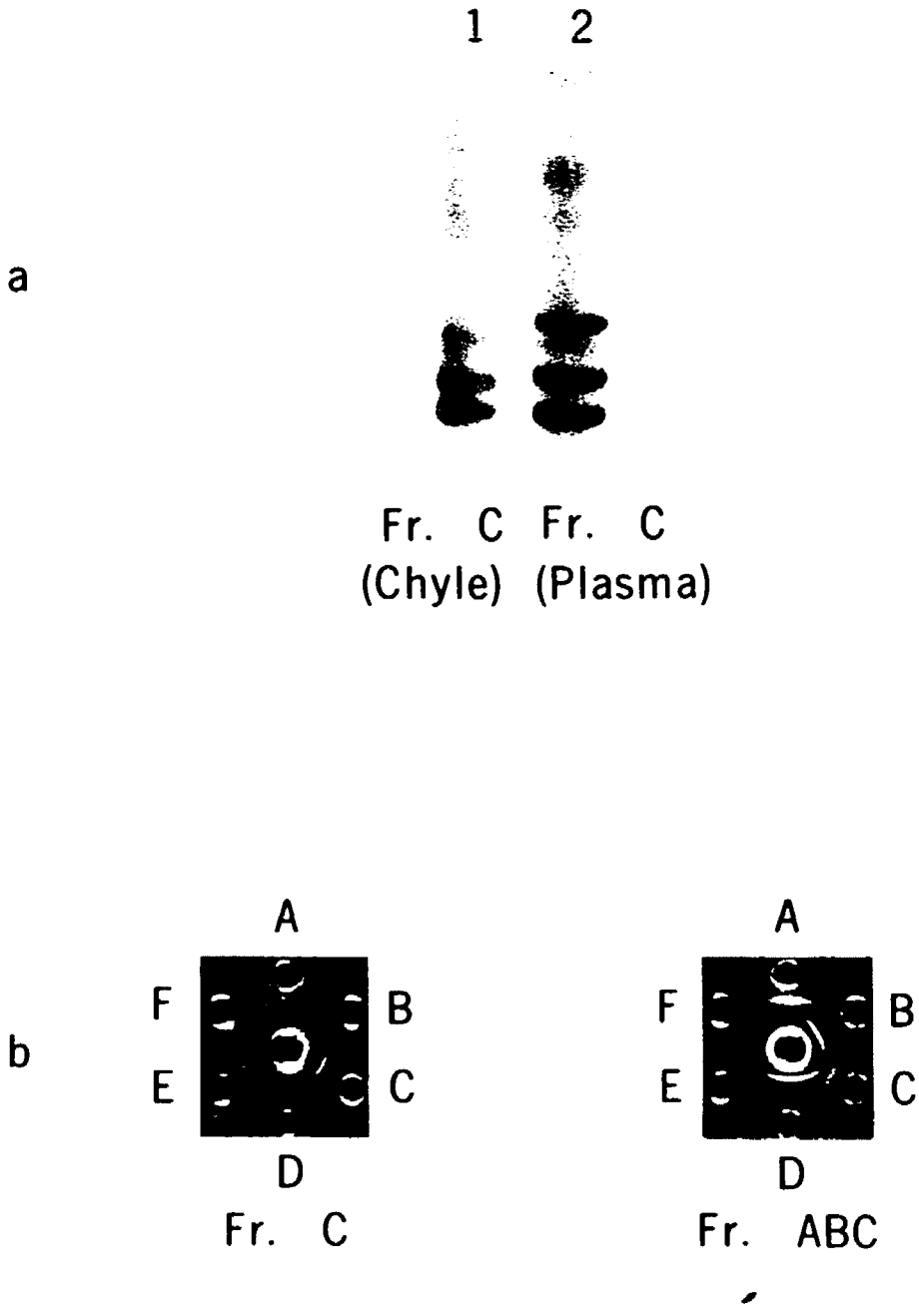


Figure 15. Basic polyacrylamide gel electrophoresis and immunodiffusion of pdLP-C obtained by ultracentrifugation. The 7% acrylamide gels were stained with Coomassie Brilliant Blue (pattern a). Fr. C (d = 1.04-1.12 g/ml) and Fr. ABC (d = 1.12-1.21 g/ml) were placed in the central wells (pattern b). Outer wells contain antibodies to LP-A (A); VLDL (B); LP-C (C); LP-B (D); whole serum (E); and albumin (F).

with A-LP-A and A-LP-B (Fig. 15b). The basic polyacrylamide pattern of partially delipidized lipoprotein C isolated from both chyle and serum (Fig. 15a) again had three to four fast-moving bands.

The ultracentrifugal pattern of pdLP-C preparations isolated from both chyle and serum revealed single symmetrical sedimenting peaks with $s_{w,20}$ of 7.56 and $s^{o}_{w,20}$ of 8.5, respectively (Fig. 16).

The pdLP-C from chyle and serum migrated on paper electrophoresis into the α_1-region (Fig. 17). On agarose electrophoresis the pdLP-C band had a β-α_2 mobility and stained with both protein and lipid stains.

The pdLP-C obtained by both immunoprecipitation and the ultracentrifugal procedure was totally delipidized, and the ApoC was used for further studies. The phospholipid to protein ratio of pdLP-C was approximately two. Kinetic studies with carboxypeptidase A revealed that alanine was the principle C-terminal amino acid released from both serum and chyle ApoC (Table 14). Serine and threonine were the N-terminal amino acids for both chyle and serum ApoC.

Thin-layer chromatography of lipid extracts of pdLP-C demonstrated the presence of cholesterol, cholesterol esters, triglycerides, and phospholipids. The average phosphatide composition of the serum and chyle pdLP-C (Table 15) had some dissimilarities in that the chyle pdLP-C had a lower relative content of sphingomyelin and a higher percent of phosphatidylethanolamine than the serum.

Isolation, Fractionation, and Characterization of ApoC

Due to the tedious and time consuming procedures involved in preparing partially delipidized LP-C, it was decided to utilize totally

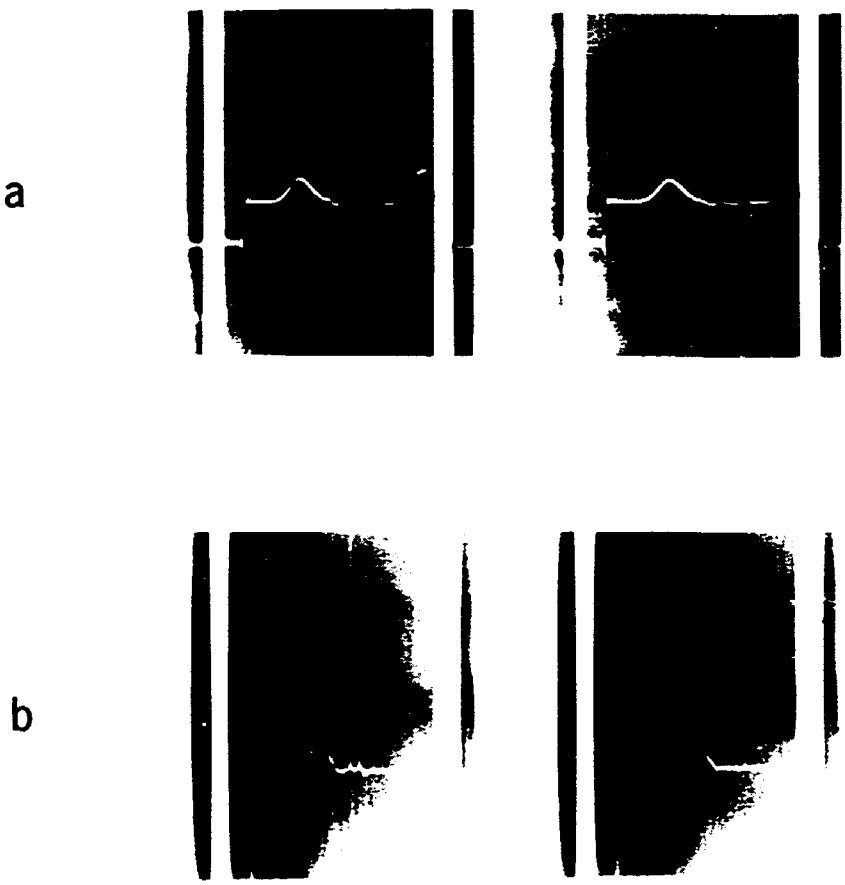


Figure 16. Sedimentation pattern of plasma and chyle pdLP-C. Plasma pdLP-C (pattern a) was run at 44,770 rpm with photographs taken at 36 and 40 minutes. Chyle pdLP-C (pattern b) was run at 52,640 rpm with photographs taken at 24 and 32 minutes.

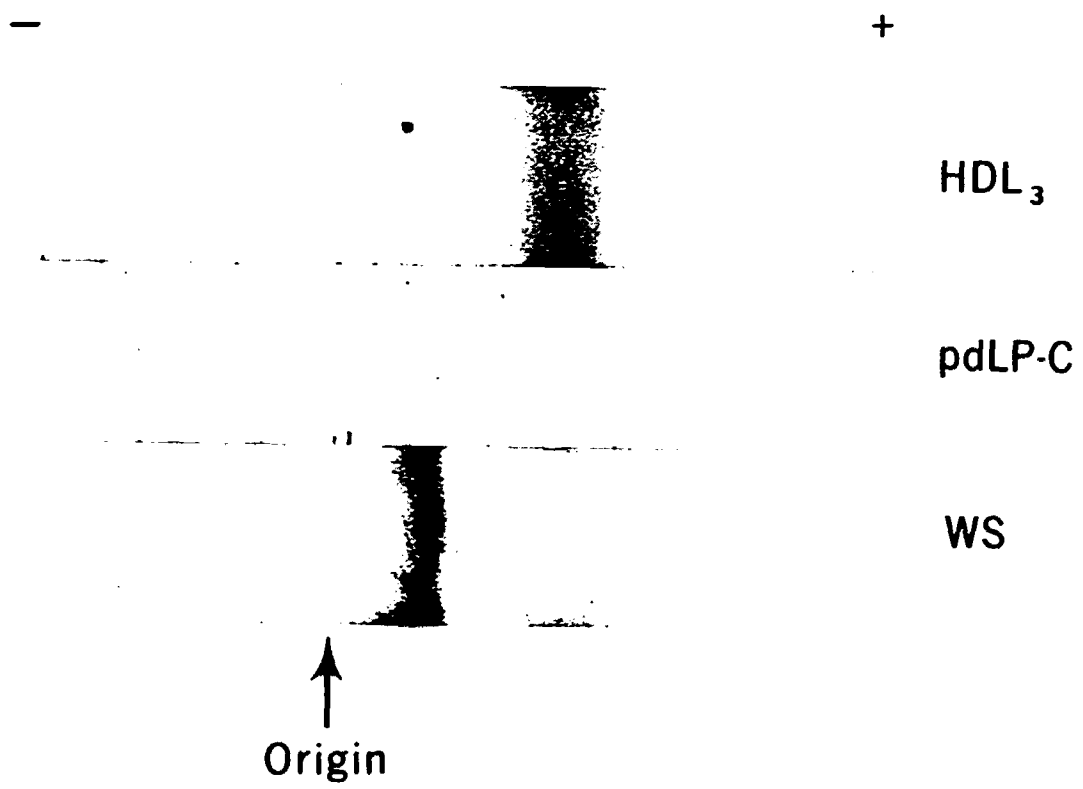


Figure 17. Paper electrophoresis of pdLP-C. Papers were stained with Oil Red O. WS = normal fasting serum.

TABLE 14

CARBOXYL-TERMINAL ANALYSIS OF ApoC[a]

Amino Acids	Time					
	1'	10'	30'	2 hrs	5 hrs	24 hrs
	μmoles/mg x 10^2					
Alanine	5.70	6.76	6.65	7.40	7.41	8.11
Valine	2.30	3.37	3.27	3.25	3.61	3.70
Glycine	0.50	1.02	0.83	0.74	0.51	0.92
Serine	0.18	0.85	0.73	0.88	0.83	0.94

[a]Time-study was performed with carboxypeptidase A.

TABLE 15

PHOSPHATIDE COMPOSITION OF PARTIALLY DELIPIDIZED LP-C FROM PLASMA AND CHYLE

Source	Lysophatidyl-choline	Phosphatidyl-inositol	Phosphatidyl-choline	Sphingo-myelin	Phosphatidyl-ethanolamine	Reference
	%	%	%	%	%	
Chyle (6)[a]	6.6 (1.7 -20.8)	1.10 (0-2.01)	73.9 (70.0-79.7)	5.40 (2.0- 8.2)	12.36 (2.6-15.8)	
Plasma (6)[a]	10.74 (2.94-26.8)	1.81 (0-5.33)	63.43 (40.0-79.8)	15.75 (9.3-26.18)	6.64 (2.9-20.1)	
pdLP-C	10.8	-	57.0	26.9	5.3	(3)
pdLP-X	2.6 (2.6)	-	75.0 (71.4-78.5)	22.6 (20.8-24.4)	1.2 (0.7- 1.6)	(163)

[a]Number of determinations.

delipidized plasma or chyle VLDL as a source for the isolation of ApoC. The introduction of the phosphotungstate procedure (41) as a preparative method for the isolation of large quantities of lipoproteins was found to be very satisfactory. Fractionation (Fig. 2) of the precipitate I resulted in the separation of VLDL (d < 1.030 g/ml) and LDL (1.03-1.07 g/ml), and the centrifugation of precipitate II yielded HDL (p < 1.23 g/ml). Immunodiffusion and disc gel patterns revealed that LP-C was present in all three fractions, LP-B in VLDL and LDL, and LP-A only in the HDL (Fig. 18). Unfortunately, albumin was not removed from the HDL by repeated washes in the ultracentrifuge.

All three fractions (VLDL, LDL, and HDL) obtained by phosphotungstate precipitation as well as VLDL isolated by ultracentrifugation and pdLP-C prepared by immunoprecipitation procedure were utilized, after total delipidization, as a source of ApoC.

The totally delipidized material from VLDL and LDL preparations dissolved in various solvents (see the section on Methods) and that from HDL soluble in aqueous buffers were chromatographed on Sephadex G-75 or G-100 columns equilibrated with either 0.1M $(NH_4)_2CO_3$ or 2M CH_3COOH. A typical elution pattern of a soluble apoVLDL preparation chromatographed on a Sephadex G-100 column and the polyacrylamide gel patterns of each major eluate indicated clearly the protein heterogeneity of this density class (Fig. 19a). Each fraction was characterized by polyacrylamide gel electrophoresis (Fig. 19b), immunodiffusion, and immunoelectrophoresis (Fig. 20a and b). Fraction 1 consisted of ApoA, ApoC, and albumin and was thought to be an aggregated protein complex while fraction 2 contained A-I, albumin, and ApoC. Analysis of fraction 3 indicated that it was

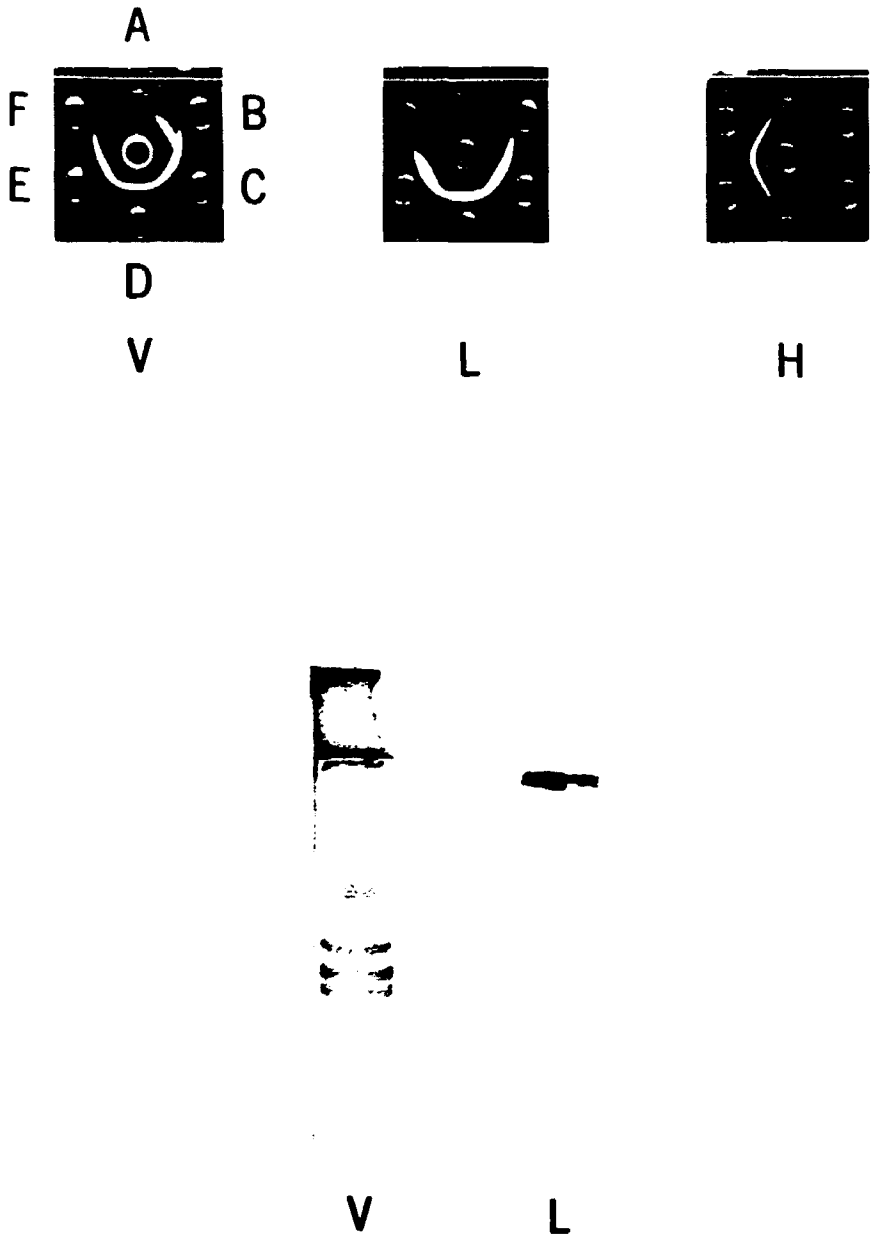


Figure 18. Immunodiffusion and basic polyacrylamide gel electrophoresis of lipoproteins isolated by phosphotungstate precipitation and ultracentrifugation. PAGE was run on 7% gels with 8 M urea and stained with Coomassie Brilliant Blue. V = VLDL (d < 1.030 g/ml), L = LDL (d 1.030-1.073 g/ml), and H = HDL (d < 1.23 g/ml) were placed in central wells. Outer wells contain antibodies to LP-A (A); LP-C (B); VLDL (C); LP-B (D); whole serum (E); and albumin (F).

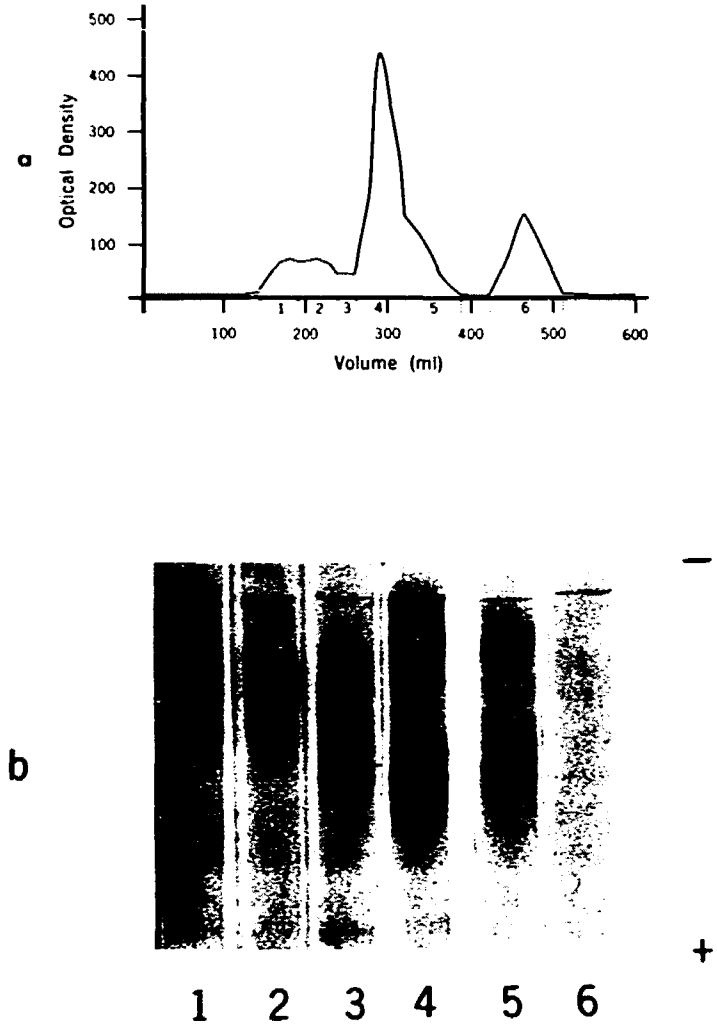


Figure 19. Elution profile of soluble apo-VLDL from Sephadex G-100 (pattern a) and basic PAGE of the fractions eluted from the Sephadex G-100 run (pattern b). Numbers 1 through 6 correspond to Sephadex G-100 fractions. The PAGE was run in 7% gels in 8 M urea and stained with Coomassie Brilliant Blue. Elution buffer for chromatography was 0.1 M $(NH_4)_2CO_3$ (pH 9.0).

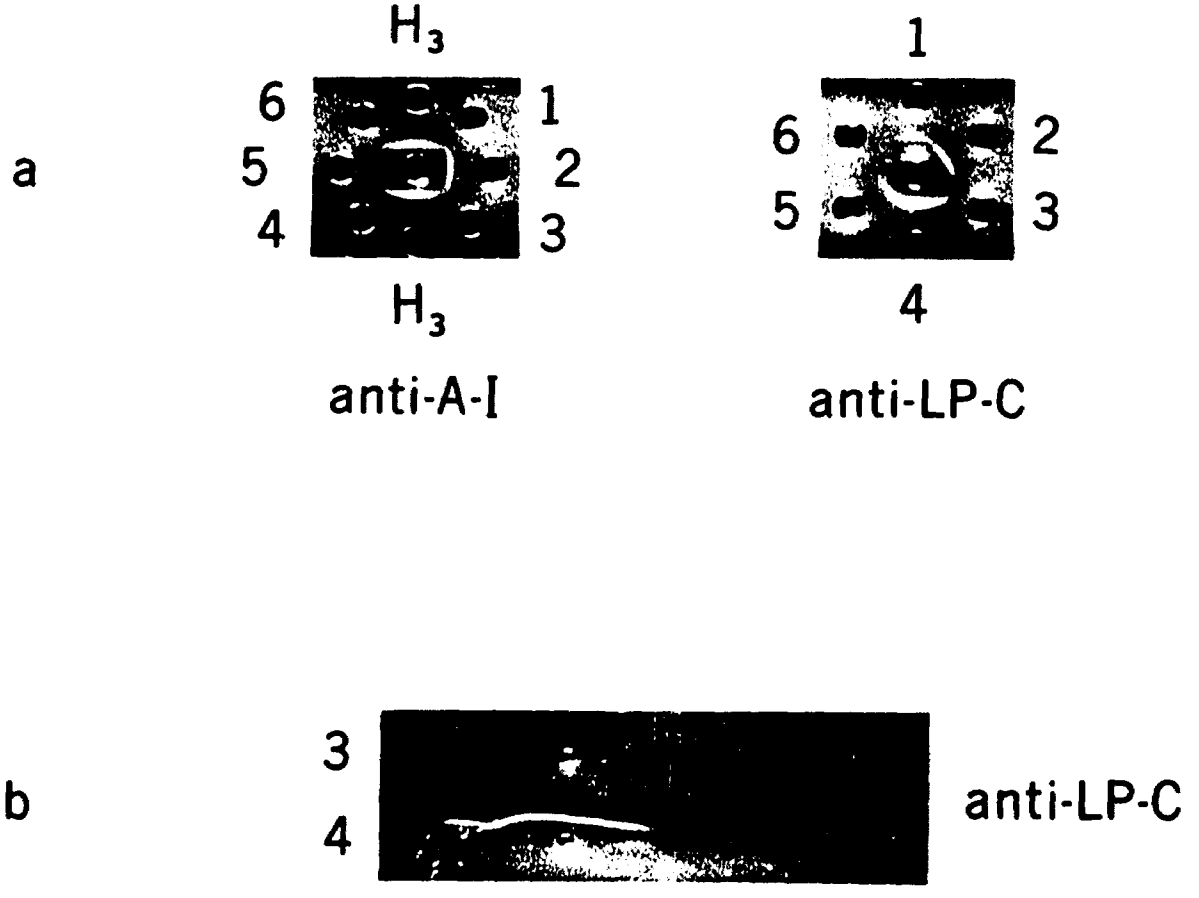


Figure 20. Immunodiffusion and immunoelectrophoresis of fractions (1-6) eluted from the Sephadex G-100 column (Figure 19a). Antisera were placed in the central wells. Immunoelectrophoresis was run in 1% agar. H_3 = HDL_3.

an intermediate fraction. Fractions 4 and 5 consisted of the ApoC peptides and were utilized for further fractionation of ApoC. The last fraction (fraction 6) gave no precipitin lines with any antisera, no bands on disc gel and had less than 5% amino acids. Its further study was not pursued. Although the immunoelectrophoretic precipitin arcs of ApoC were formed in fractions 1, 2, 4, and 5, the highest concentration of ApoC was found in fraction 4 (Fig. 20b). The number of fractions and their immunological and disc gel electrophoretic characteristics were similar for all soluble apoVLDL's examined with the exception that the first fraction (fraction 1) was usually more prominent and had discernible ApoC bands on basic disc gel. Extraction of this fraction with ethanol-ether, followed by rechromatography, usually resulted in recovery of an additional quantity of ApoC.

Column chromatography of ApoHDL on Sephadex G-75 gave a similar elution pattern except that the fractions 1 and 2 were greatly increased due to the presence of A-I and A-II peptides, respectively. The ApoC peak was eluted in a volume similar to that of ApoC from VLDL, but it was necessary to rechromatograph this fraction (fraction 4) to free it from some contaminating A-II peptide.

Soluble portions of totally delipidized chyle VLDL ($S_f > 100$) and apolipoprotein X chromatographed on Sephadex columns gave elution patterns similar to those obtained with soluble apoVLDL. Fusion of immunoprecipitin lines demonstrated a marked immunological similarity, if not identity, of ApoC preparations isolated from chyle, apoHDL, ApoX, and apoVLDL (Fig. 21a). Polyacrylamide patterns of these ApoC preparations were similar with respect to the fast-moving bands (Fig. 21b). In some

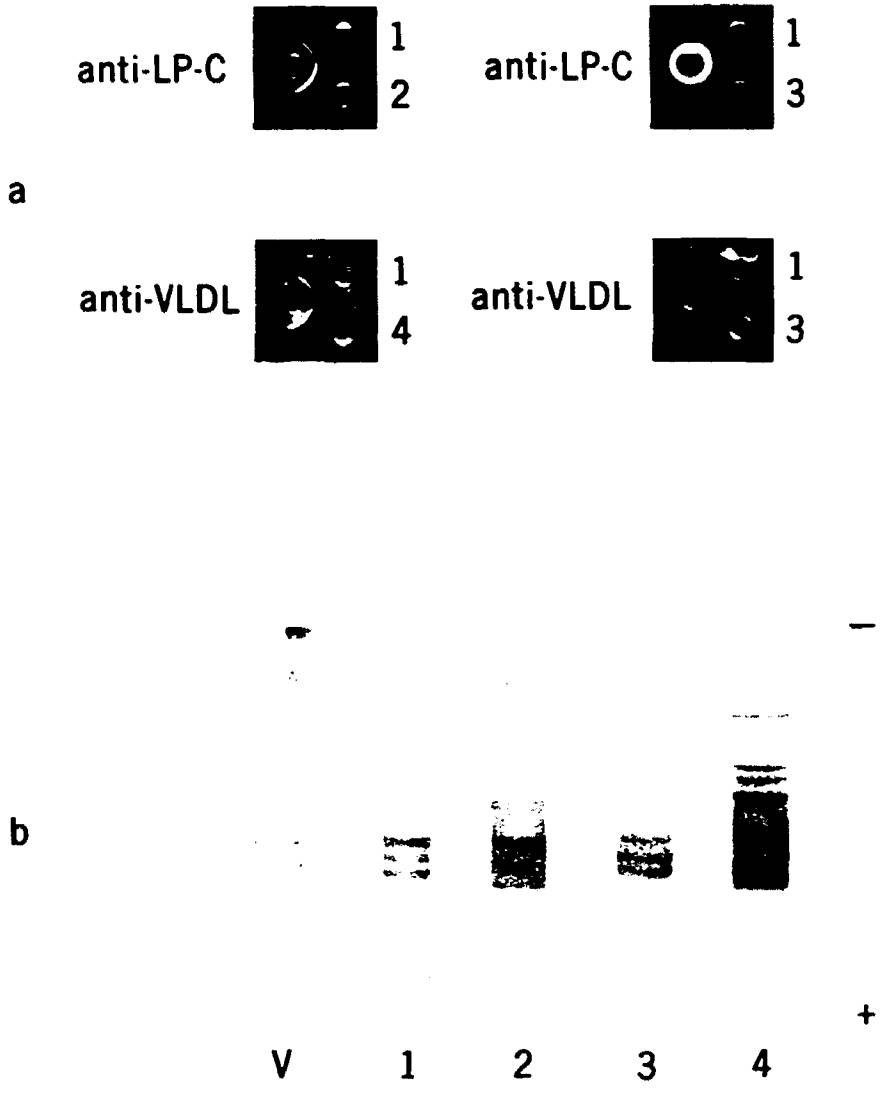


Figure 21. Immunodiffusion and basic PAGE of ApoC from various sources. The 7% gels were run in 8 M urea and stained with Coomassie Brilliant Blue. 1 = ApoC from LP-X, 2 = ApoC from VLDL, 3 = ApoC from HDL, 4 = ApoC from chyle, and V = VLDL.

ApoC preparations, such as those from HDL or LP-X (Fig. 21b), the slow-moving bands could not be visualized by protein staining; however, other ApoC preparations from the same sources contained the slow migrating bands. The ApoC peak from chyle had several bands in the ApoA region which were not identified.

The residual protein components remaining after removal of the soluble portions of totally delipidized lipoprotein preparations were found to be soluble in 50% acetic acid. Chromatography of protein residue on Sephadex G-75 or G-100 equilibrated with 50% acetic acid yielded an asymmetrical elution peak which came off the column immediately after the void volume as determined with Blue Dextran. The finding of a small ApoC fraction in only one out of four fractionation experiments indicated that ApoC had been removed almost quantitatively during the solubilization of delipidized lipoprotein preparations.

Isolation of lipoproteins from ten liters of random plasma by the phosphotungstate precipitation method yielded approximately 10 grams of total protein. The ApoC amounted to 600 mg, or 5-10% of the total lipoprotein protein. It was also estimated that lipoproteins with $d < 1.03$ g/ml contained approximately 10% ApoC and HDL contained 5-6% ApoC. The highest amount of ApoC (35% of the total protein) was isolated from VLDL of a type III patient.

The basic disc gel pattern of ApoC clearly indicated that the protein moiety consisted of several polypeptides (Fig. 22a). Two groups of polypeptides are distinguishable: the fast-moving bands (C-II and C-III) and the slow-moving, less distinct bands (C-I). To characterize the ApoC bands by their migration rates, the mobility of C-III-2 was

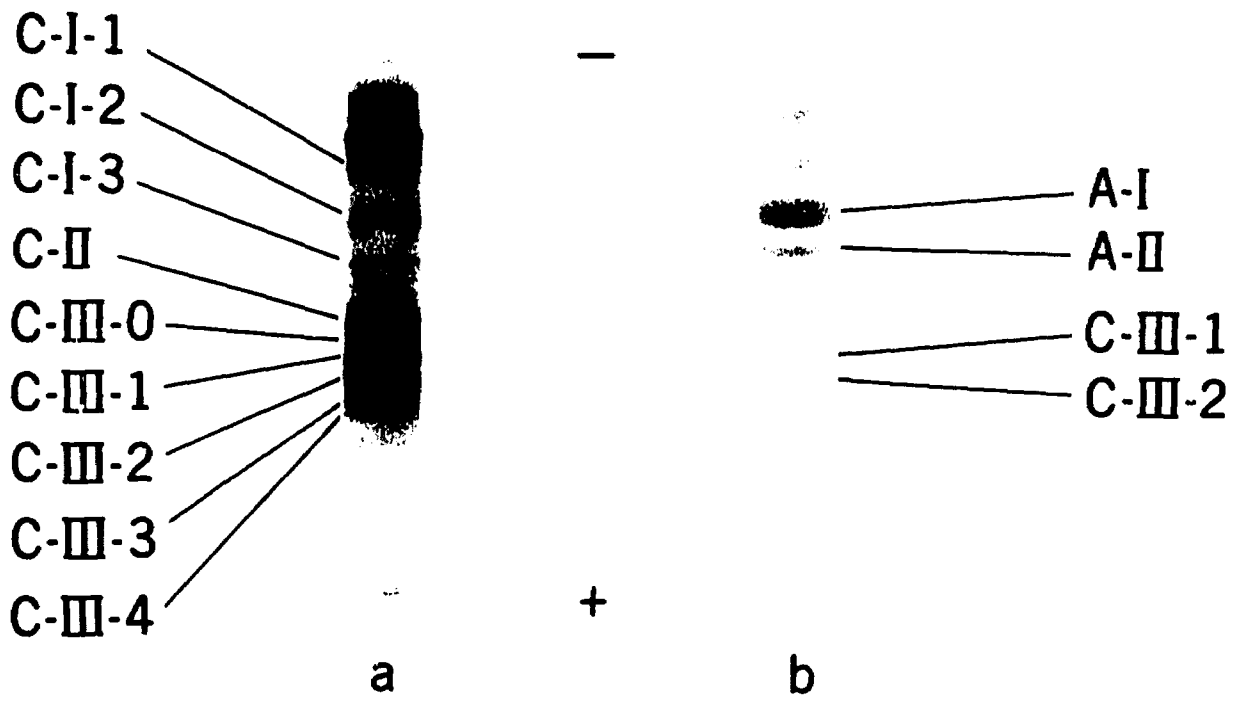


Figure 22. Identification of ApoC and ApoA polypeptides on basic PAGE. The 7% acrylamide gels in 8 M urea were stained with Coomassie Brilliant Blue. a = ApoC from VLDL and b = ApoHDL.

taken as unity, and the Rf-values of the other fast-moving bands were calculated (Table 16). The Rf-values for C-I polypeptides were not calculated because the bands seen on basic disc gel were not sharply resolved. Since the ApoHDL was resolved into two distinct regions (Fig. 22b), one of which was characterized by the slow-moving bands (A-I and A-II) and the other by the fast-moving ApoC polypeptides (C-II and C-III), the Rf-values of A-I and A-II were also calculated on the basis of the mobility of C-III-2 taken as unity (Table 16).

A semi-quantitative determination of the principal ApoC polypeptides was made by scanning their band patterns on acidic and basic polyacrylamide gels (Table 17) and by determining the absorption at 260 mμ of the principal peaks eluted from DEAE-cellulose columns (Table 18). The high percentage of C-I from the polyacrylamide scans probably reflected the high affinity of Coomassie Brilliant Blue for basic polypeptides. Variability in the composition of the ApoC polypeptides seen in Table 17 may reflect either selective losses during isolation of ApoC, non-linear dye uptake by the various polypeptides, or variability in the composition of ApoC. This variability has been demonstrated by gel scans of ApoC from LP-X, plasma VLDL, and plasma HDL_3 which indicated that the ratio (R) of C-III-2 to C-III-1 was less than one for ApoC from VLDL (R = 0.856) and HDL_3 (R = 0.798) but greater than one for ApoC from LP-X (R= 1.134). The ratio of C-III-2 to C-III-1 in VLDL subfractions from chyle was similar to that of LP-X.

Results of immunological and disc gel electrophoretic characterization of various ApoC preparations demonstrated a marked polypeptide heterogeneity of this apolipoprotein. To isolate individual polypeptides,

TABLE 16

RELATIVE MOBILITIES OF POLYPEPTIDES OF ApoC AND ApoA ON BASIC POLYACRYLAMIDE GEL ELECTROPHORESIS IN 8 M UREA

Polypeptide	$R_{f_{ab}}$
C-III-4	1.129
C-III-3	1.060
C-III-2	1.000
C-III-1	0.918
C-III-0	0.829
C-II	0.800
A-II	0.547
A-I	0.426

TABLE 17

RELATIVE CONTENT OF ApoC POLYPEPTIDES[a]

Polypeptide	Acidic System	Basic System
	%	%
C-I (1-3)	40.28	39.90
C-II	17.18	18.91
C-III-1	24.18	16.50
C-III-2	18.36	13.95
C-III-3		12.02

[a]Determined by densitometric scanning of ApoC bands on acidic and basic acrylamide gels in 8 M urea. Expressed as percent of the sum of individually determined bands.

TABLE 18

RELATIVE CONTENT OF ApoC POLYPEPTIDES[a]

Polypeptide	VLDL	HDL
	%	%
C-I (1-3)	21.40	11.30
C-II	5.32	15.52
C-III-1	44.20	35.24
C-III-2	27.20	27.06
C-III-3	1.91	11.78

[a]Determined by absorption (260 mμ) of peaks eluted from DEAE-cellulose chromatographic run. Expressed as percent of the sum of individually determined peaks.

the ApoC was submitted to several fractionation procedures including DEAE-cellulose column chromatography, isoelectric focusing, and preparative polyacrylamide gel electrophoresis.

The DEAE-cellulose exchange chromatography was carried out with a concave gradient in the presence of 8 M urea. A typical elution pattern (Fig. 23) obtained with ApoC from VLDL usually revealed three major and several minor fractions. An additional fraction eluted with 2 M acetic acid gave no immunochemical reaction or disc gel pattern and was not studied further. Similar elution patterns were also obtained with ApoC isolated from chyle or apoHDL. Each fraction was tested by disc gel electrophoresis and in some cases by immunoelectrophoresis. Although the polyacrylamide gel electrophoresis showed that most fractions were still heterogeneous (Fig. 24), chromatography on DEAE-cellulose resulted at least in a partial separation of ApoC polypeptides.

Since analytical isoelectric focusing revealed separate bands in each major fraction from the DEAE-cellulose column, the separation of the more acidic ApoC polypeptides was attempted on the smaller preparative Ampholine column (110 ml). From a single run on Ampholine (pH 5-7) in the presence of 8 M urea, the isoelectric points of several of the ApoC polypeptides were determined (Table 21). Unfortunately, an adequate separation was not obtained when a preparative run on the larger column (440 ml) was made with Ampholine (pH 4-6) even after refocusing of selected fractions on the smaller column. For this reason, isoelectric focusing was abandoned as a possible preparative method for the isolation of the various ApoC polypeptides.

The separation of ApoC polypeptides by preparative polyacryl-

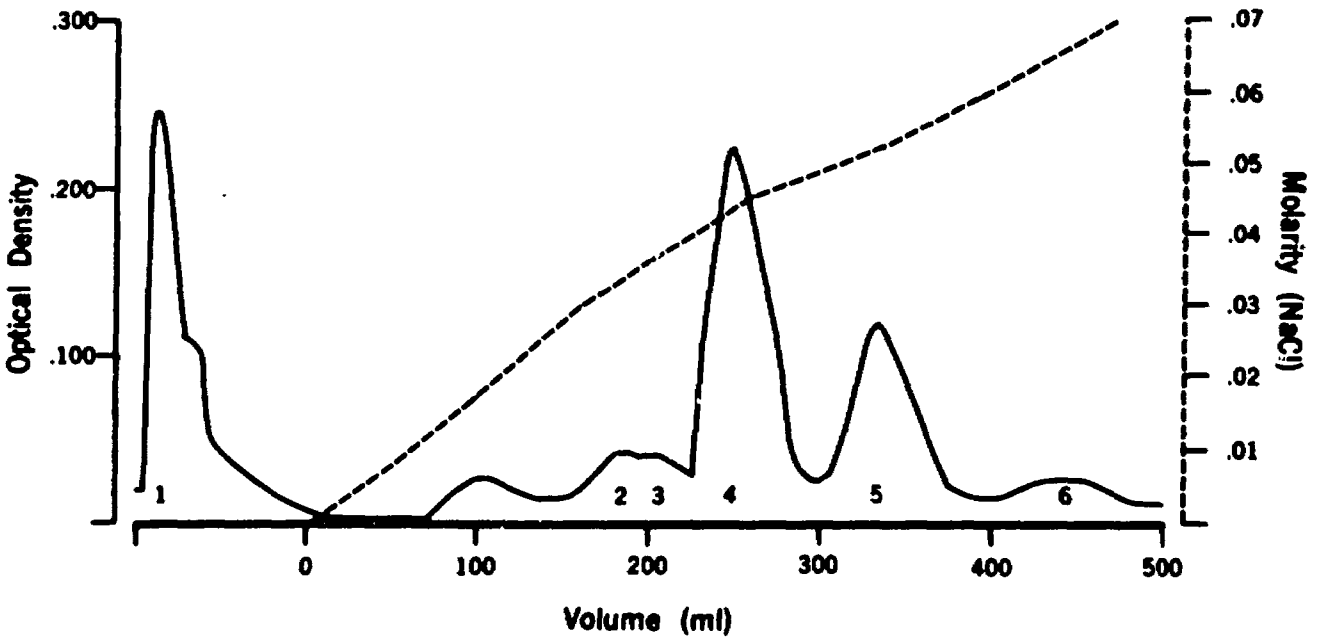


Figure 23. Elution pattern of ApoC from a DEAE-cellulose column. Chromatography was carried out in phosphate - 8 M urea buffer (pH 7.2) with the NaCl gradient indicated by the dashed line.

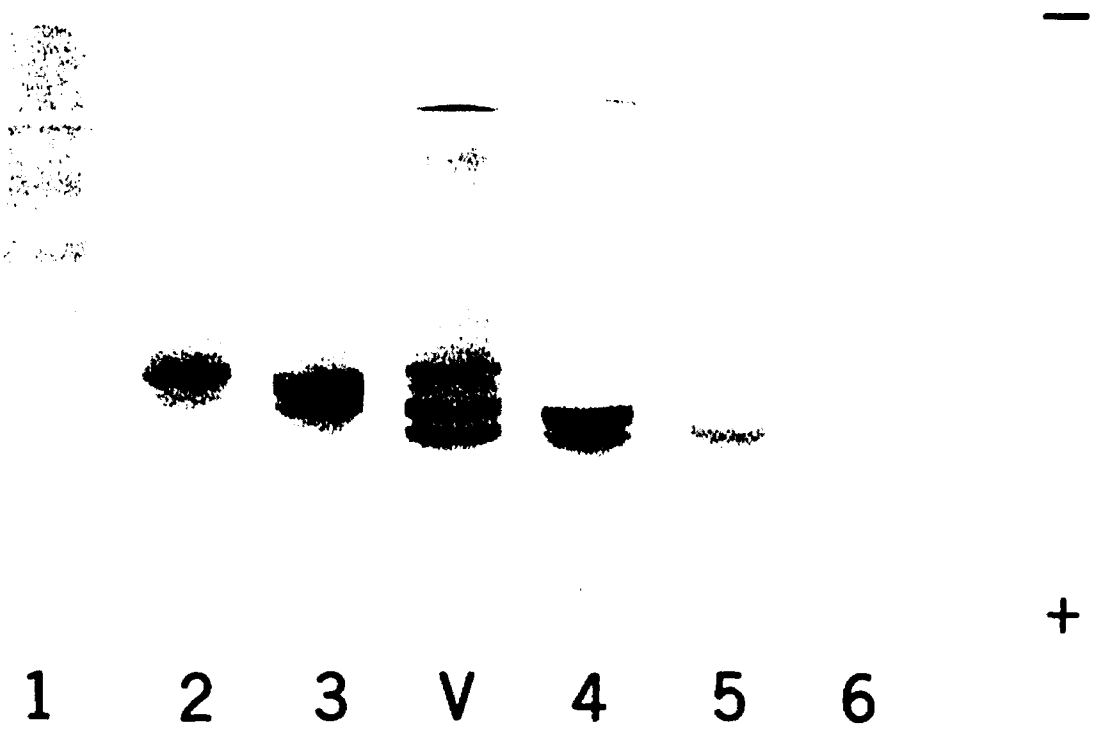


Figure 24. Basic PAGE patterns of fractions separated by DEAE-cellulose column chromatography of ApoC. The 7% acrylamide gels in 8 M urea were stained with Coomassie Brilliant Blue. Numbers 1 through 6 correspond to fractions from DEAE-cellulose chromatogram (Figure 23). V = VLDL.

amide electrophoresis was examined utilizing both the basic and acidic systems. A typical elution pattern (Fig. 25a) obtained by monitoring the absorbance at 280 mμ usually consisted of one peak with some indication of shoulders. The examination of this peak by disc gel electrophoresis revealed regions of polypeptide homogeneity (Fig. 25b). The separation on the basic system was found to be satisfactory, while that obtained on the acidic was not reproducible. Separations were most satisfactory when only two components were to be separated. Preparative polyacrylamide gel electrophoresis with a basic buffer system resolved successfully the fast-moving ApoC bands into homogeneous polypeptides. Results of these studies have shown that the C-III polypeptides consist of at least five, and the C-II polypeptide of possibly two polymorphic forms.

Immunochemical Properties of the Various Purified Polypeptides of ApoC

The immunoelectrophoretic pattern of the fastest moving bands comprising the C-III group of polypeptides was compared with their pattern on basic disc gel electrophoresis (Fig. 26). It was found that the mobilities of C-III-0 to C-III-3 on disc gel correlated well with the corresponding mobilities on agar gels (Fig. 27); in both supporting media the anodal migration increased progressively from C-III-0 to C-III-3.

The C-II polypeptide moved on polyacrylamide gel at a slightly slower rate than the C-III-0 polypeptide (Figs. 26, 28) and had an electrophoretic mobility of β_2-globulin on agar (Fig. 27) and β_1-globulin on agarose immunoelectrophoresis. Another band, designated C-II-0, moving slightly slower (Fig. 28) than the C-II polypeptide, was isolated in quantities insufficient for further characterization.

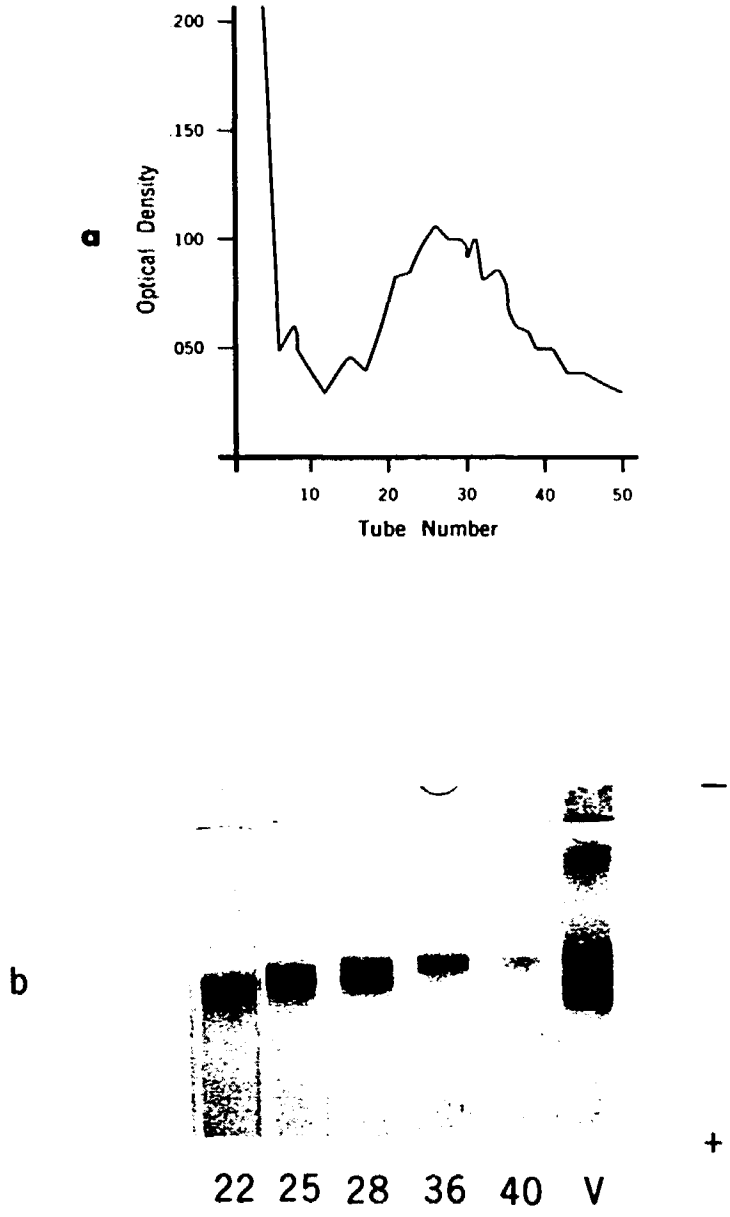


Figure 25. Preparative basic PAGE elution profile (pattern a) of a ApoC fraction eluted from a DEAE-cellulose column. Elution profile (pattern a) and the analytical PAGE of corresponding fractions (pattern b). Both systems were run with 7% acrylamide in 8 M urea. Numbers (pattern b) correspond to tube numbers of preparative elution profile. V = VLDL.

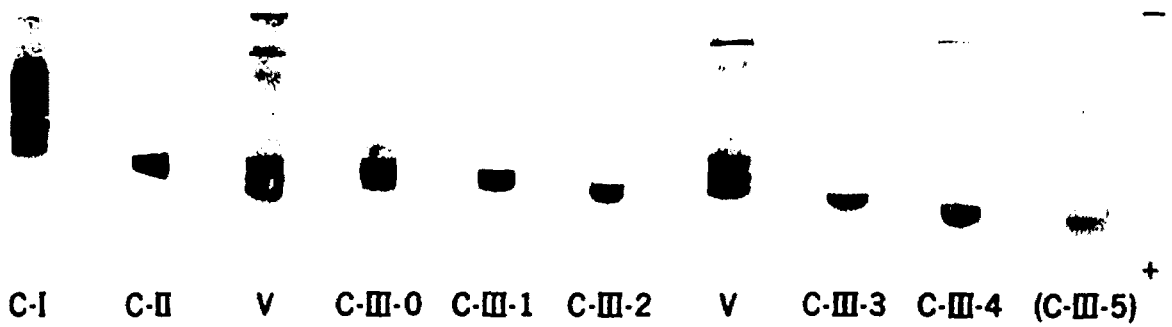


Figure 26. Basic PAGE of purified ApoC polypeptides. PAGE was run in 7% acrylamide in 8 M urea and stained with Coomassie Brilliant Blue. V = VLDL.

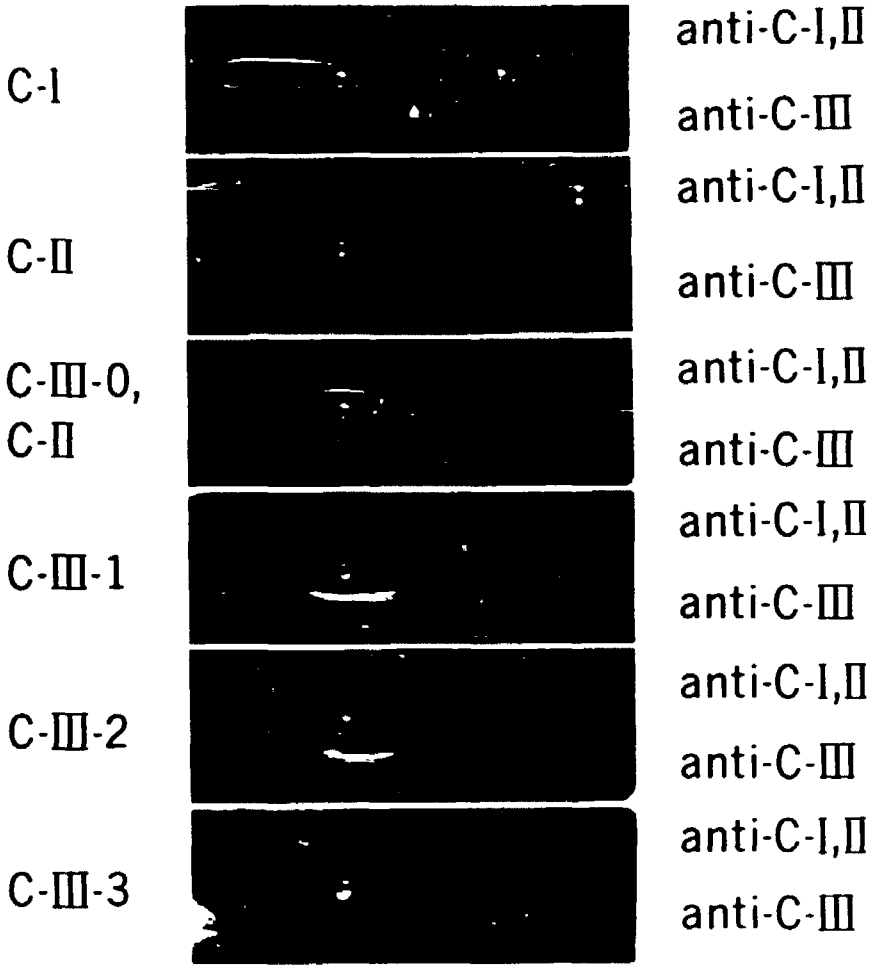


Figure 27. Immunoelectrophoresis in 1% agar gel of ApoC polypeptides.

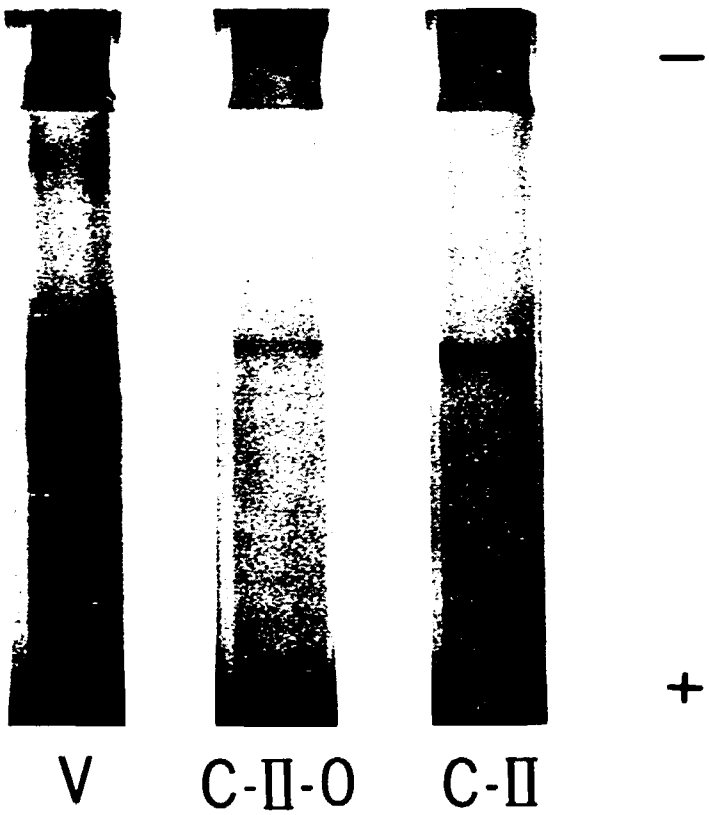


Figure 28. Basic PAGE of C-II polypeptides. PAGE was run in 7% acrylamide in 8 M urea and stained with Coomassie Brilliant Blue. V = VLDL.

The group of C-I polypeptides present in the first fraction eluted from the DEAE-cellulose column (Fig. 23) was resolved by disc gel electrophoresis into three slow-moving bands (Figs. 24, 26). Examination of C-I by the acidic disc gel electrophoresis revealed three major fast-moving bands (Fig. 29) accompanied in some ApoC preparations from chyle, plasma VLDL, and HDL by two minor slow-moving bands. In a fortuitious experiment on acidic preparative disc gel electrophoresis, two of the major components were separated (Fig. 30), which indicated that at least two of these forms were stable. Another experiment on analytical disc gel utilizing the acidic system demonstrated that the three major bands isolated from VLDL were immunochemically identical as indicated by the lines of fusion with various antisera (Fig. 31). On agarose embedding, the three bands had mobilities inversely related to those observed on the acidic polyacrylamide (Fig. 31).

With the availability of more specific antisera, it was found that the purified ApoC polypeptides reacted only with antisera which had specificity for ApoC or its various polypeptides. By direct comparison C-III-0, C-III-1, and C-III-2 gave a complete fusion of immunoprecipitin lines (Fig. 32a). On immunoelectrophoresis, reaction between ApoC and A-C-III resulted in a single precipitin arc while a reaction between the combination of C-III-1, C-III-2 and C-III-3, and A-C-III gave two precipitin arcs (Fig. 32b). These results indicated that C-III-3 might possibly have another or a different antigenic component. Direct comparisons of C-I and C-II, and of C-I and C-III showed non-identity of their immunoprecipitin lines (Fig. 33). Partial or non-identity of C-III and C-II was not demonstrated.

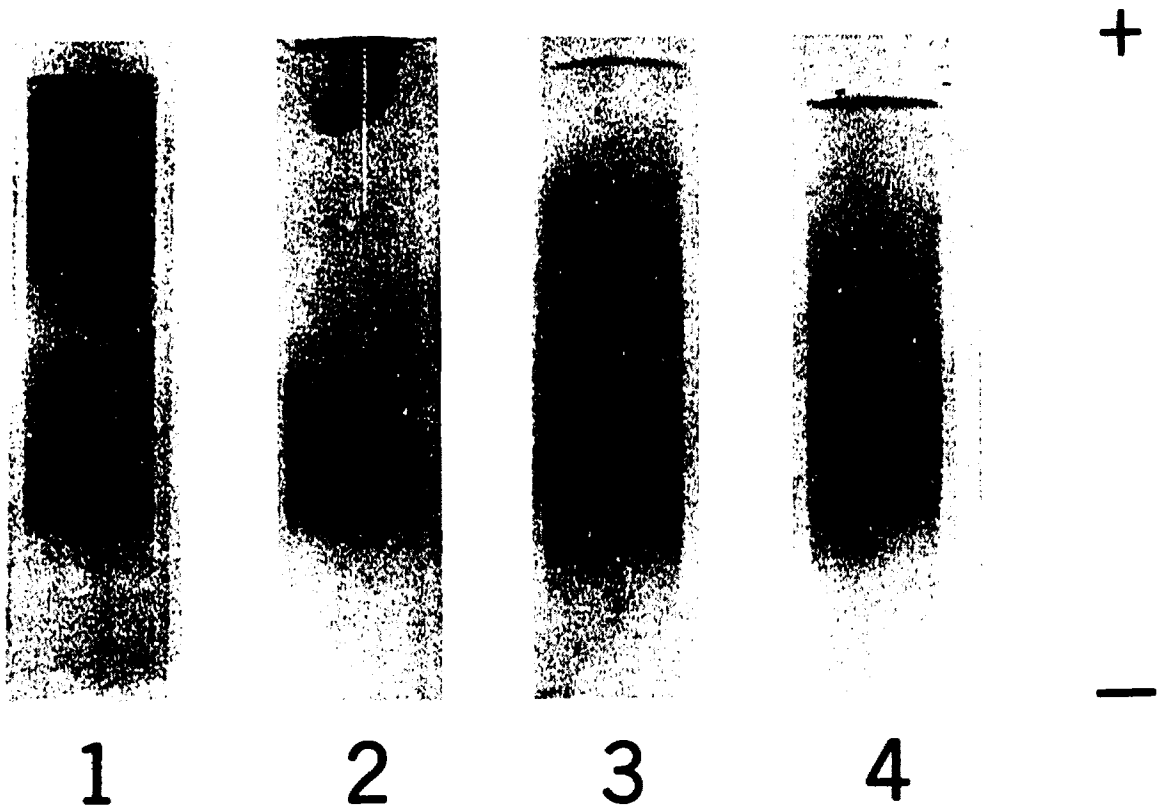


Figure 29. Acidic PAGE of C-I polypeptides from various sources. PAGE was run in 7% acrylamide in 8 M urea and stained with Coomassie Brilliant Blue. 1 = ApoC from VLDL, 2 = C-I from VLDL, 3 = C-I from HDL, and 4 = C-I from chyle.

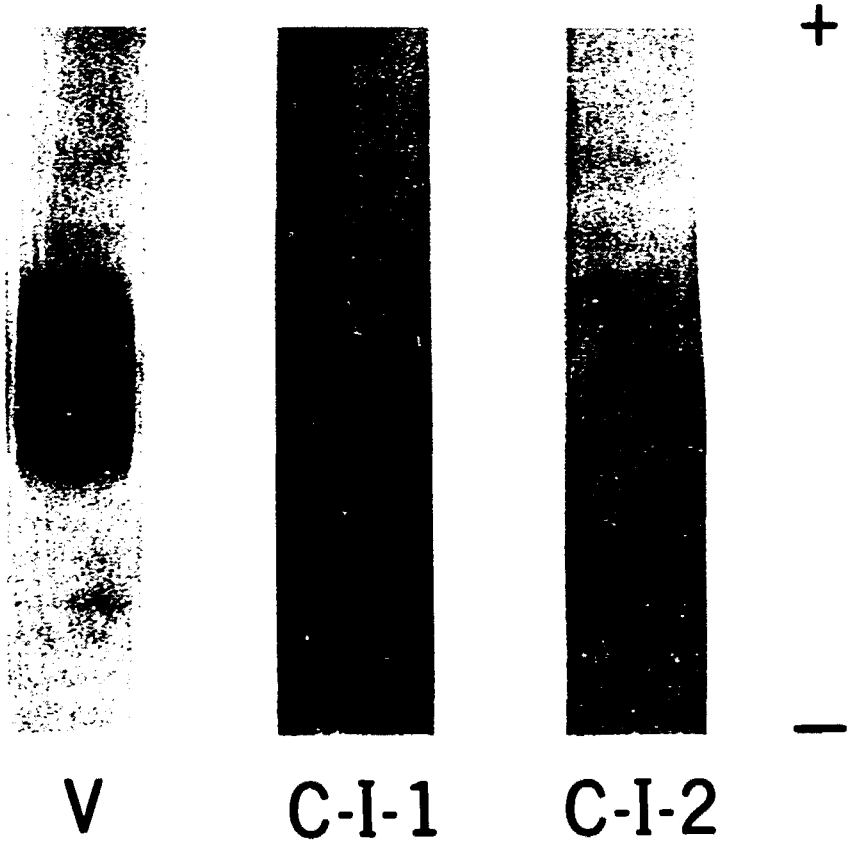


Figure 30. Acidic PAGE of C-I polypeptides. PAGE was run in 7% acrylamide in 8 M urea and stained with Coomassie Brilliant Blue. V = C-I from VLDL.

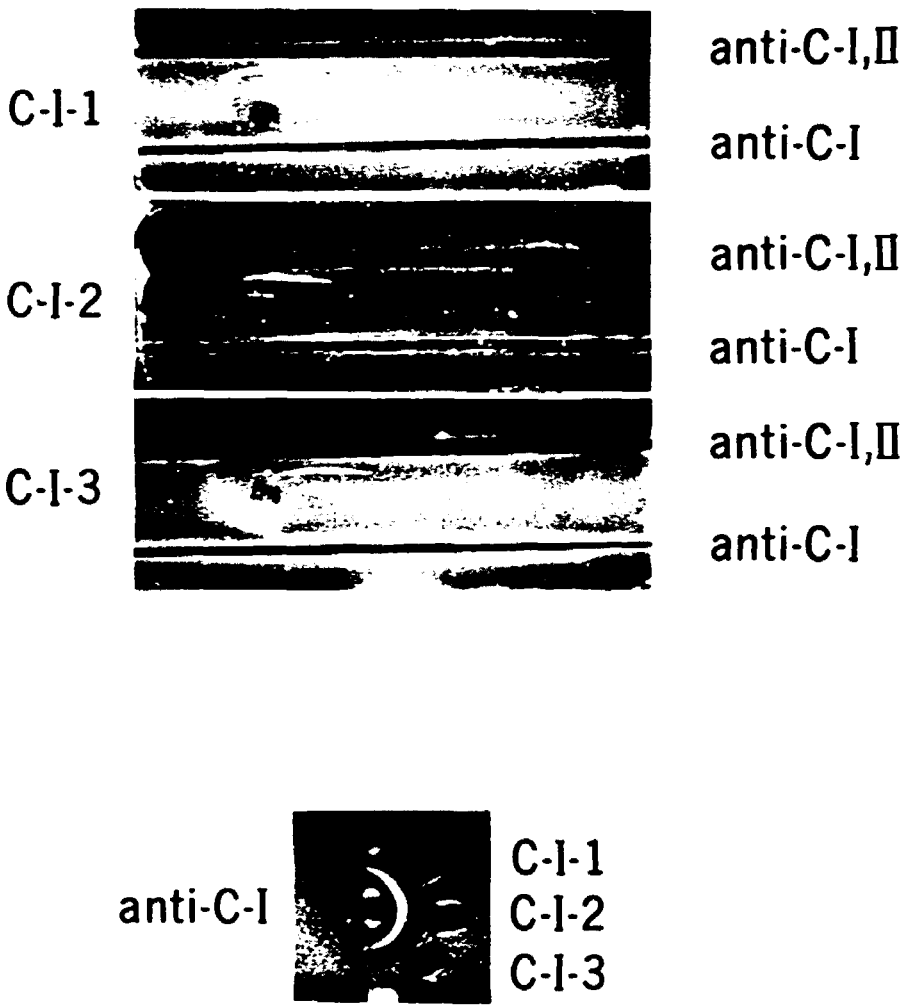


Figure 31. Immunoelectrophoresis and immunodiffusion of C-I-1, C-I-2 and C-I-3 polypeptides separated by 7% acidic PAGE. The C-I bands from acrylamide gel were embedded in 1% agarose gels.

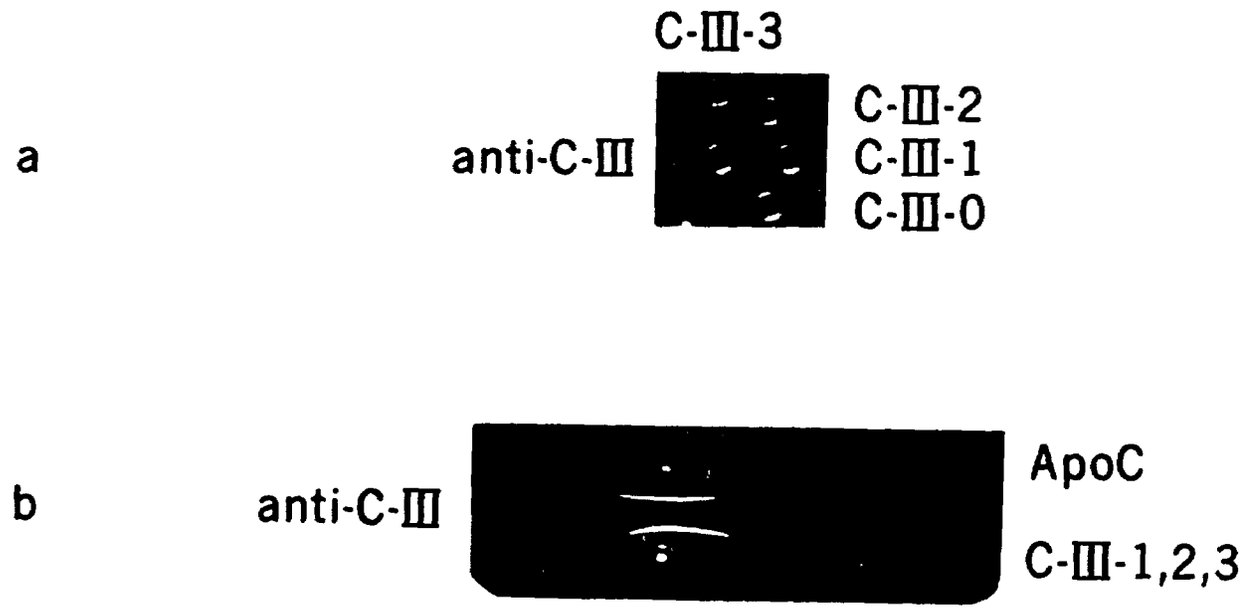


Figure 32. Immunodiffusion and immunoelectrophoresis of C-III polypeptides. Anti-C-III added to central well (pattern a). Purified C-III-1, C-III-2, and C-III-3 polypeptides were added to same well (pattern b).

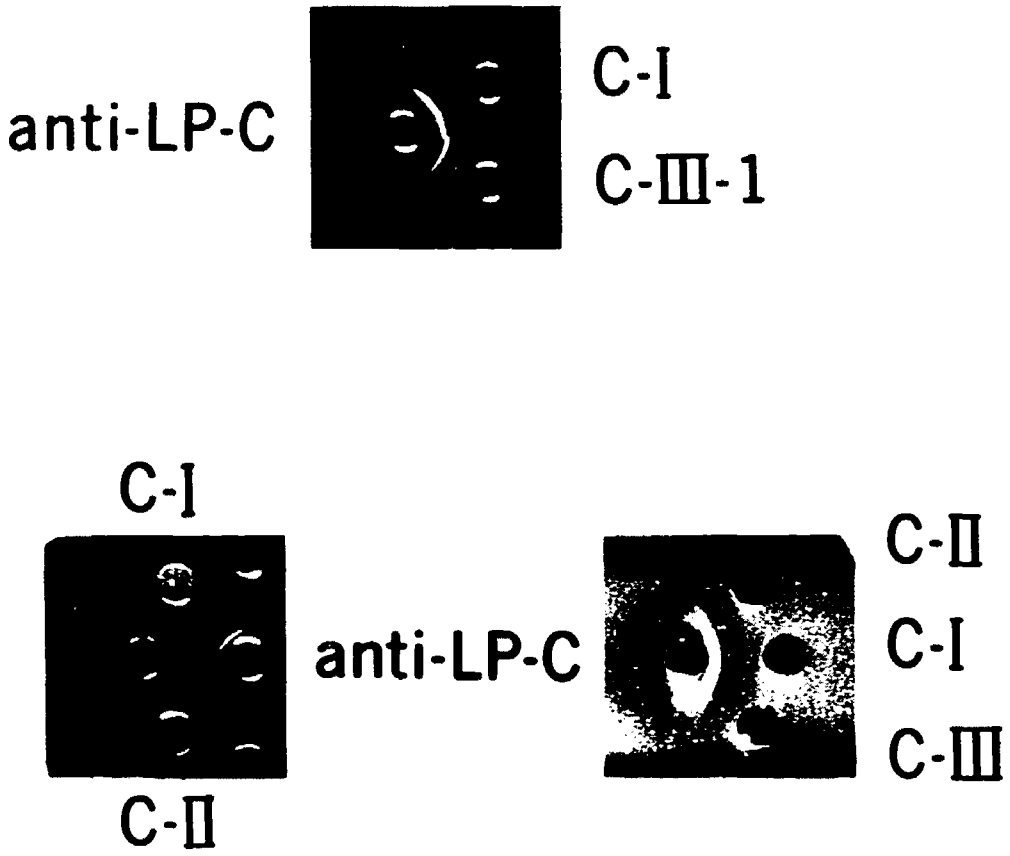


Figure 33. Comparison of C-I, C-II and C-III polypeptides by double diffusion. Anti-LP-C was added to central well.

Partial Characterization of the Various Polypeptides of Apolipoprotein C

Amino Acid Composition

Amino acid analyses of C-I, C-II, and C-III demonstrated that the polypeptides of ApoC isolated from VLDL have different amino acid compositions (Table 19). A characteristic feature of the amino acid composition of C-I was the absence of histidine, tyrosine, cystine, and cysteine. The amino acid composition of the C-II polypeptide showed that all amino acids were present. All of the C-III polypeptides isolated from VLDL had essentially no isoleucine with the exception of C-III-0. The ApoC polypeptides, with the exception of C-II, were characterized by the absence of cystine and cysteine as determined by performic acid oxidation.

Terminal Amino Acids

Qualitative examination of ApoC polypeptides by dansylation demonstrated that serine was the N-terminal amino acid for C-III-1 through C-III-3 and threonine for C-I and C-II. Trace amounts of serine and aspartic acid were present in one C-I preparation, while minor amounts of serine and glutamic acid were found in two C-II fractions.

Serine and threonine were also shown to be the N-terminal amino acids for ApoC isolated from LP-X.

Alanine was identified by kinetic studies with carboxypeptidase A as the carboxyl-terminal amino acid for all C-III polypeptides (0-4) isolated from VLDL. Valine was the only other amino acid released from these polypeptides. The C-III-1 and C-III-2 isolated from chyle and plasma HDL also had alanine as the C-terminal amino acid. There was no

TABLE 19

AMINO ACID COMPOSITION OF ApoC POLYPEPTIDES ISOLATED FROM PLASMA VLDL
($M/10^5$ g)

Amino Acid	Polypeptide						
	C-I	C-II	C-III-0	C-III-1	C-III-2	C-III-3	C-III-4
Lysine	88.46	51.85	61.42	55.48	52.81	52.80	44.07
Histidine	-	3.32	7.26	10.86	8.03	4.67	13.88
Arginine	34.40	14.84	20.89	15.30	14.51	17.28	20.37
Aspartic Acid	65.74	62.23	62.60	71.06	71.39	86.20	74.68
Threonine	36.40	47.79	54.43	48.12	49.92	54.77	48.16
Serine	89.17	100.56	109.22	99.52	98.50	116.47	101.64
Glutamic Acid	129.88	121.23	118.11	114.08	113.96	126.58	120.13
Proline	17.84	42.85	22.72	26.78	29.10	37.70	29.18
Glycine	27.74	85.07	N.D.[a]	31.38	32.45	51.84	77.10
Alanine	52.53	82.42	85.65	100.00	101.08	98.00	104.99
Half Cystine[b]	-	5.99	-	-	-	-	-
Valine	27.96	42.22	43.18	59.17	61.22	52.88	63.23
Methionine	7.85	10.30	10.23	15.46	15.92	7.92	14.32
Isoleucine	34.46	9.70	5.72	-	-	-	-
Leucine	82.62	51.94	49.92	49.15	48.40	47.44	48.33
Tyrosine	-	18.89	23.70	18.73	18.63	14.47	17.01
Phenylalanine	39.78	31.31	27.82	38.10	39.71	39.40	38.48
Tryptophan	8.61	N.D.[a]	N.D.[a]	25.65	28.11	N.D.[a]	N.D.[a]

[a]Not determined.

[b]Determined as cysteic acid.

release of carboxyl terminal amino acids from C-I or C-II polypeptides treated with either carboxypeptidase A or B.

A forty-hour hydrazinolysis of ApoC from VLDL resulted in release of the following amino acids ($\mu M/mg \cdot 10^{-2}$): alanine (2.08), glycine (1.54), aspartic acid (1.15), leucine (0.98), and glutamic acid (0.75). That alanine was released in this experiment from the C-III polypeptides was confirmed by the hydrazinolysis of C-III which yielded alanine (3.24 $\mu M/mg \cdot 10^{-2}$) as the carboxyl terminal amino acid. No unequivocal results were obtained when C-I and C-II were examined by hydrazinolysis. The previous report (124) indicating valine as the C-terminal amino acid of C-I could not be confirmed.

Carbohydrate Analyses

There was some variability in the content of sialic acid among various C-III polypeptides. The C-III-0 had none, and C-III-2 contained two moles of sialic acid/mole of peptide; both C-III-1 and C-III-3 had one mole of sialic acid/mole peptide. The analysis of sugars and hexosamines showed that both C-III-1 and C-III-2 had two moles of galactose and one mole of galactosamine per mole of peptide.

The carbohydrate composition of C-I polypeptide was characterized by 6-8% glucose and small quantities of mannose and galactose (Table 20). The presence of glucosamine was observed in some C-I preparations, but it was not calculable; galactosamine was not determined. No sialic acid was found in C-I.

Molecular Weight Determination

The polyacrylamide SDS procedure showed that C-II and C-III

TABLE 20

CARBOHYDRATE COMPOSITION OF ApoC POLYPEPTIDES

Polypeptide	Mannose		Galactose		Glucose		Glucosamine		Galactosamine		Sialic acid	
	%	Mole/ Mole[a]	%	Mole/ Mole[a]	%	Mole/ Mole[a]	%	Mole/ Mole[a]	%	Mole/ Mole[a]	%	Mole/ Mole[a]
C-I	0.71	0.29	0.84	0.34	8.04	3.30	Trace	Trace	N.D.[b]	N.D.	-	-
C-III-0	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	-	-
C-III-I	-	-	4.12	2.29	-	-	-	-	1.33	0.70	2.68	0.88
C-III-2	-	-	3.22	2.03	-	-	-	-	1.99	1.04	4.87	1.645
C-III-3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	-	-	N.D.	N.D.		0.97
C-III-4	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	-	-	Trace	Trace	N.D.	N.D.

[a]Mole of carbohydrate/Mole of peptide.

[b]Not determined.

polypeptides had similar molecular weights. The molecular weight of C-I was lower than those of C-II and C-III. Minimal molecular weights of ApoC polypeptides calculated from the amino acid composition were in agreement with the values found by the polyacrylamide-SDS gel electrophoresis.

Table 21 summarizes the characteristics of the ApoC polypeptides.

Study on Immunochemical Properties and Polypeptide Composition of LP-X

Plasma samples from patients with obstructive jaundice contain the abnormal lipoprotein X (LP-X) which moves on agar electrophoresis towards the cathode and gives a precipitin arc with A-C-I but not with A-C-III (Fig. 34a,b); some plasma samples, however, show in the anodal compartment the characteristic LP-C precipitin arc (Fig. 34b). Since unabsorbed antisera to LP-X react with C-III polypeptide and since pdLP-X displays on polyacrylamide gels bands characteristic of C-III-1 and C-III-2, these results suggest that in LP-X the C-III peptides are immunochemically buried while the C-I antigenic component is exposed.

On agarose immunoelectrophoresis the LP-X moves towards the anode (Fig. 34c). Plasma samples containing LP-X gave three precipitin lines with A-VLDL (Fig. 34d). Since the slower moving component with a mobility similar to that of LP-X showed a non-identity reaction with the second arc typical of LP-B, it was considered, indeed, to be due to LP-X. The similarity in the mobility of faster moving arc with that of C-III suggested that this arc represented the LP-C. These findings were supported by the fact that the A-VLDL reacted with C-I, C-III, and LP-B.

It has been demonstrated by Seidel *et al.* (96) that partially

TABLE 21

SOME PHYSICAL AND CHEMICAL PROPERTIES OF APOLIPOPROTEIN C POLYPEPTIDES

Poly-peptide	Carboxyl terminal	Amino terminal	Amino acid deletion
C-I	-	Threonine	Cystine, cysteine, tyrosine and histidine
C-II	-	Threonine	None
C-III-0	Alanine	N.D.	Cystine
C-III-1	Alanine	Serine	Cystine, cysteine and isoleucine
C-III-2	Alanine	Serine	Cystine, cysteine and isoleucine
C-III-3	Alanine	Serine	Cystine, cysteine and isoleucine
C-III-4	Alanine	N.D.	Cystine, cysteine and isoleucine

[a]Neutral sugars and hexosamines were not determined.

TABLE 21--Continued

Molecular weight	Carbohydrate moles/mole peptide	Electrophoretic mobility on agar	Isoelectric point (pI)
7,400	3 moles glucose	γ	> 6
10,500	N.D.	β_2	5.46
8,750	No sialic acid[a]	β_2-β_1	5.39
10,650	1 mole sialic acid 2 moles galactose 1 mole galactosamine	β_1-α_2	5.17
11,250	2 moles sialic acid 2 moles galactose 1 mole galactosamine	α_2	4.93
11, 275	1 mole sialic acid[a]	α_2	4.86
10,800	N.D.	α_2	4.79

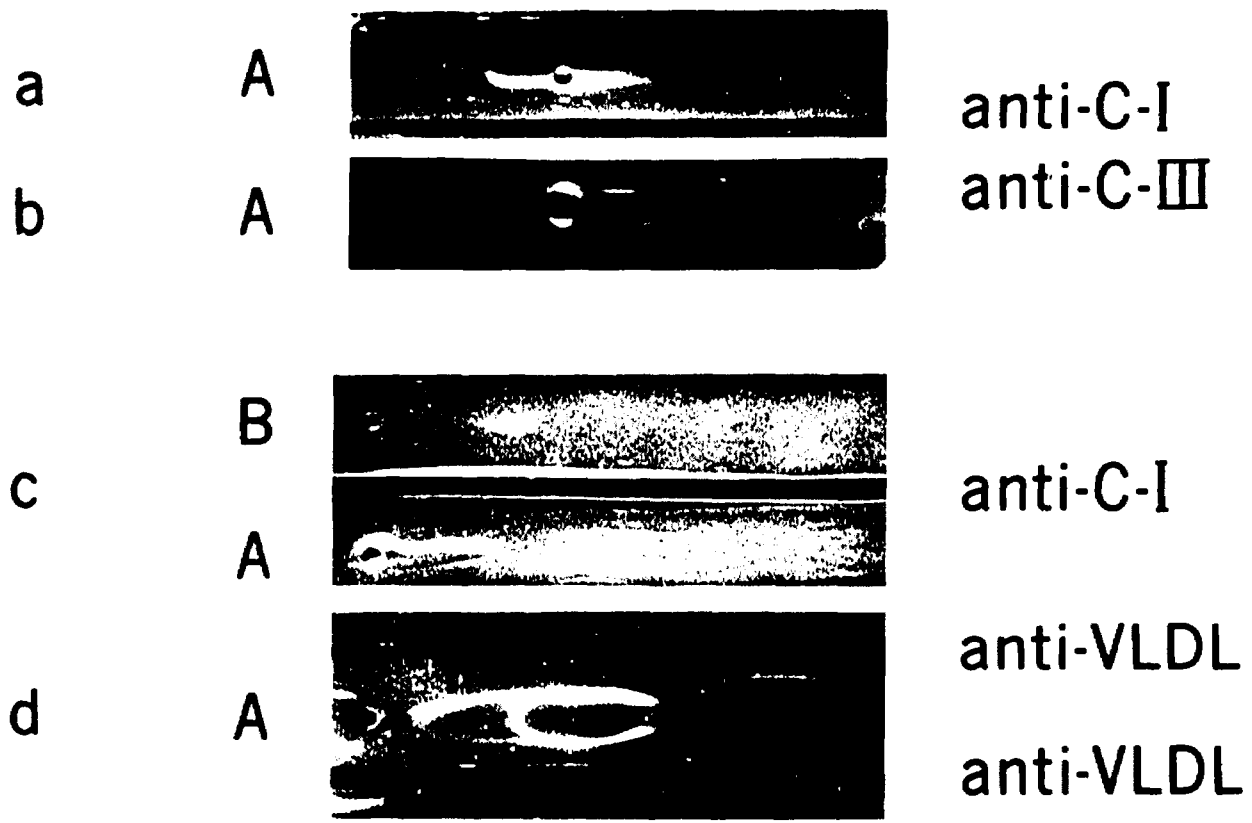


Figure 34. Immunoelectrophoresis of obstructive jaundiced plasma. Pattern a and b were run on 1% agar gels. Pattern c and d were run on 1% agarose gels. A = obstructive jaundiced plasma and B = normal fasting plasma.

delipidized LP-X contained two protein components, i.e., albumin and partially delipidized LP-C. The partially delipidized LP-X gave no reaction with A-LP-A or A-LP-B; however, the precipitin lines obtained with anti-albumin and A-VLDL showed a non-identity reaction. Also, two to three lines were observed with A-LP-C and A-VLDL. When albumin was separated by ultracentrifugation from pdLP-C following partial delipidization, the latter showed a disc gel pattern very similar to that of pdLP-C obtained from hyperlipemic VLDL. On direct comparison by double diffusion, pdLP-C obtained from LP-X showed two lines of identity with pdLP-C from VLDL when it was reacted with A-LP-C and only one line of fusion when it was tested with A-VLDL.

CHAPTER V

DISCUSSION

The studies of the various density lipoprotein classes isolated from postprandial and fasting chyle, fasting normal plasma, random plasma, and hyperlipemic plasma have shown that each density class examined was heterogeneous with respect to its protein moieties. In fact, all density classes examined contained the three lipoproteins LP-A, LP-B, and LP-C in varying concentrations under one metabolic circumstance or another. For example, LP-A was found to be present in postprandial chyle VLDL and essentially absent in normal fasting VLDL of plasma. The LP-B and LP-C were present in all VLDL samples examined except for one VLDL preparation that consisted only of LP-C. The VHDL represented the only exception to this general finding because they contained only LP-A. These results, supported by immunodiffusion, immunoelectrophoresis, and PAGE, are in agreement with previous observations on the protein heterogeneity of the various density classes (3, 83, 90, 96, 108, 109, 113).

Originally, ApoC was found only in the VLDL of hyperglyceridemic subjects (3) and postprandial VLDL chyle (108). Present studies have clearly established that LP-C occurs in all density classes of hypertriglyceridemic, as well as normal plasma and chyle. This is also supported by the recent findings of ApoC polypeptides in the various density classes

(118-122). This accomplishment has been a result of refinements in techniques, such as the introduction of disc gel electrophoresis for the routine identification of the acidic ApoC polypeptides and the availability of specific antisera.

Polyacrylamide gel electrophoresis (PAGE) studies indicated a relatively simple way to examine the lipoprotein composition of the various density classes. These studies have demonstrated that there are three principal regions on the 7% basic polyacrylamide separating gel that correspond to the three currently recognized lipoprotein families: LP-B occurring at the juncture of the stacking and separating gels, LP-A present in the intermediate region between the fast-moving bands, and a slower moving band in the LP-A region. Unfortunately, there are several difficulties associated with this system. Low concentrations of HDL must be utilized to visualize the bands associated with LP-A. At high concentration where the LP-C bands can be most readily visualized, the LP-A bands are blurred and apparently have a tendency to aggregate. A second difficulty is that the C-I polypeptide(s) overlap(s) with the LP-A bands and cannot be positively identified in the basic system. It is, therefore, necessary to run the acidic system in order to verify its presence. The correlation of the LP-A bands seen on PAGE with the known ApoA polypeptides and the characterization of several unknown bands in that region has not been shown in this study. Also, the detection of LP-B, LP-C, and, in some cases, LP-A in the loading and stacking gels has been indicative of a probable association of lipoprotein families that cannot be separated by this technique. The possibility of denatured material in the loading, stacking, and juncture of the stacking and loading gels can-

not be excluded and is always a potential problem in the evaluation of a PAGE gel pattern. In contrast to 7%, the 3% polyacrylamide gel electrophoresis was found to be completely unsatisfactory for identifying the lipoprotein families or the polypeptides associated with these families.

The pattern of the apolipoproteins on basic PAGE is similar to that observed with intact lipoproteins; and, again, if high concentrations are utilized, the separation of ApoA is not sharp. A scheme presenting the localization of various apoHDL and ApoC polypeptides is shown in Fig. 22. Identification of the ApoC polypeptides by analytical polyacrylamide gel electrophoresis has been found to be more reliable than immunochemical techniques. In addition, the analytical polyacrylamide gel electrophoresis, but not the immunological techniques, distinguishes the various polymorphic forms of polypeptides which have similar, if not identical, immunochemical properties.

Combining acrylamide electrophoresis and immunodiffusion to investigate normal fasting VLDL has demonstrated that some of the protein and lipid remains in the loading and stacking gels, while at least a portion of the C-III polypeptides is readily dissociated. Seven percent basic acrylamide patterns have demonstrated that the C-III peptides can always be detected in the separating gel whether urea is present or not; the addition of urea has no apparent effect on the intensity of the C-III staining. In the absence of urea in the loading gel, the bands of C-I and C-II were either not present or present only in small amounts. Since C-II is dissociated and detected in the separating gel when urea is added to the loading gel, it seems that the C-II has a higher affinity for the VLDL molecule, or one of its components, than the C-III polypeptides.

C-I is occasionally seen on basic acrylamide but it can always be detected as a faint band(s) on acidic acrylamide. These studies have indicated that ApoC polypeptides dissociate from VLDL in the following order: C-III>C-II>C-I. This has been further supported by the immunological studies of VLDL which have indicated that the C-I is not dissociated from the VLDL molecule while C-III frequently is. In conclusion, the normal fasting VLDL molecule is a complex consisting of LP-B and LP-C with the C-III-1 and C-III-2 polypeptides being dissociated to some extent.

Paper and agarose electrophoretic results were of questionable value in determining the presence or absence of a particular lipoprotein family. The α_2-mobility of normal fasting VLDL was associated with LP-B and LP-C and not with LP-B and LP-A as previously reported (111, 112). The lipoprotein with α_1-mobility found after dissociation of VLDL is most likely due to pdLP-C (Fig. 17) and not pdLP-A since none, or very little, LP-A was immunochemically detectable in partially delipidized, normal fasting VLDL.

Examination of VLDL from chyle under various dietary conditions revealed the presence of LP-A, LP-B, and LP-C in agreement with previous reports (107, 108, 110). The principal difference between fasting plasma and postprandial chyle VLDL with respect to their apolipoproteins was the presence of ApoA in chyle VLDL. Although LP-B was not positively identified in the chyle $S_f > 400$, its presence has been reported previously (107, 108); the occurrence of LP-B is further supported by the presence of some insoluble material in the totally delipidized lipoprotein fraction with $S_f > 5,000$. In fact, the finding of approximately 80% LP-A by polyacrylamide gel electrophoresis of the postprandial chyle chylomicrons

seems to indicate that ApoA is very important in the transport of exogeneous triglycerides. The decrease in the relative LP-A concentration, with an accompanying increase in the relative LP-C concentration, in the chyle VLDL seen in the transition from a postprandial to a fasting state was suggestive of a selective removal or dissociation of LP-A from the chylomicron molecule yielding the postulated chylomicron remmants (164). In fact, since LP-A was found to be essentially absent from normal fasting VLDL, it was hypothesized that the presence of LP-A in fasting plasma VLDL (S_f > 20) was indicative of a metabolic disorder or impairment associated with removal or dissociation of these molecules of exogeneous origin. This hypothesis was supported by the finding of LP-A in both type I and type V hypertriglyceridemic plasma, with both types being characterized by the presence of chylomicrons (51).

The immunochemical identification of A-I and A-II in the chyle chylomicrons supports the finding that ApoA is composed of these two nonidentical polypeptides (126, 130) and extends the reports of ApoA being associated with chylomicrons (107, 108).

Band G (Table 9) found in chylomicrons had a mobility on disc gel electrophoresis similar to that seen with purified plasma lipoprotein lipase (165). The mobility of band G was similar to that of A-I and albumin, but since neither A-I nor albumin were identified immunochemically in the S_f > 5,000, it is suggested that this band may be due to plasma lipoprotein lipase.

One site of biosynthesis of LP-A, LP-B, and LP-C is the liver, as demonstrated by a combination of radioisotope and immunochemical techniques for LP-A and LP-B (166, 167), while the ApoC polypeptides have

been identified in the Golgi apparatus isolated from rat liver (168). Ockner et al. identified immunochemically the presence of LP-A in rat intestinal lymph; this supported earlier findings (171, 108). The biosynthesis of LP-B has been demonstrated in the intestine (169, 170). The large percent of ApoA found in thoracic chyle chylomicrons indirectly supports its biosynthesis in the intestine, even though the possibility exists that a portion is of plasma origin (172, 173). Identification of ApoC in thoracic lymph and the immunoprecipitin arc seen when mucosal scrapings were examined by double diffusion with A-LP-C are indications of the intestinal origin of ApoC. From these findings, and earlier reports, it appears that all three lipoprotein families are synthesized in both the liver and the intestine.

Recent cooperative work with D. Ganesan et al. (174) on the activation of heparin-released plasma lipoprotein lipase (LPL) from human, dog, and rat revealed that the ApoC polypeptide, C-I, gave the maximal activation of this purified enzyme while C-II activated lipoprotein lipase (LPL) to a lesser extent and C-III had no effect. Since these results were different from those reported previously (124, 125), studies on human and rat adipose tissue and cow and human milk LPL were carried out. In agreement with published reports, it was found that C-II activated the LPL from both adipose tissue and milk while C-III activated human milk LPL but had little effect on human adipose LPL (125). C-I had no effect on the activation of LPL of either adipose tissue or milk origin. This difference in the activation of plasma and tissue lipolytic activities has been interpreted as evidence for two different lipoprotein lipases. These findings also suggest that one of the biological functions of two

ApoC polypeptides may be in the removal of triglycerides from the systemic circulation by activation of lipolytic enzymes. In fact, the suggestion that the underlying cause of hyperchylomicronemia (Type I) is a defective plasma LPL (175) has been supported in this study by finding that the purified heparin-released plasma LPL isolated from Type I was not activated by C-I but that there was some activation by C-II (174). It has been suggested, on the basis of this experiment, that two enzymes may be present in the systemic circulation.

These results seem to indicate that chylomicron catabolism is probably effected primarily by the heparin released LPL. The primary substrate for the adipose tissue lipase may be the very low density lipoproteins or, more specifically, the triglyceride-rich LP-A released from chylomicrons during catabolic reactions occurring either in the systemic circulation or at the surface of endothelial linings. It is possible that, in a synchronized mechanism, both enzymes act simultaneously on chylomicrons as well as on VLDL. Alternatively, plasma LPL might catabolize the chylomicron triglyceride leaving the remnants as the substrate for the adipose tissue LPL. The latter hypothesis is favored.

To facilitate the study of lipoproteins, the production of antisera was undertaken. The heterogeneity of the antisera produced by immunization with various density classes supported the protein heterogeneity detected in this study by disc gel and previously reported (3, 83, 90, 108). Results of this study have shown that it is very difficult to produce a specific antibody to one lipoprotein family by injecting a given density class. In fact, the injection of HDL_3 results in the production of A-LP-A, A-LP-C, and, usually, A-LP-B. A-HDL_3 contained anti-

body to LP-B, even though the original antigen contained no detectable LP-B by double diffusion. This phenomenon may be explained by the fact that the LP-B is easily denatured and is probably adsorbed on the lipoproteins to be injected. Since LP-B is a good antigen, and denatured material is even better (176), antibody to LP-B is commonly obtained. Identification of LP-A in a fasting "normal" VLDL with A-HDL_3 is most likely due to LP-C and not LP-A. It is possible that the earlier reports of LP-A being present in normal fasting VLDL (111, 112) either reflects the identification of LP-C or suggests that the designation of subjects as normal is questionable. Injection of normal fasting VLDL as antigen resulted in antisera with specificity for both LP-C and LP-B while the injection of LDL usually gave antisera specific for LP-B.

Injection of individual polypeptides resulted in the preparation of specific antisera. The immunoelectrophoretic pattern of whole plasma tested with some of these antisera illustrates (Fig. 35) the complexity of lipoproteins found in normal plasma.

Whereas LP-X and LP-B families can be readily identified, the patterns of LP-A and LP-C families are somewhat more complex. Two immunoprecipitin arcs formed with A-LP-A and its two polypeptides indicate the probability of two lipoproteins carrying the LP-A antigenic components, one of which is associated with A-I and A-II and the other associated only with A-I. This interpretation is supported by the finding of only A-I in a lipoprotein isolated from HDL_2, while A-I and A-II were found to be associated together in the HDL_3 (177). Two forms of LP-A have been recently identified (178); one contained only A-I and the other both A-I and A-II.

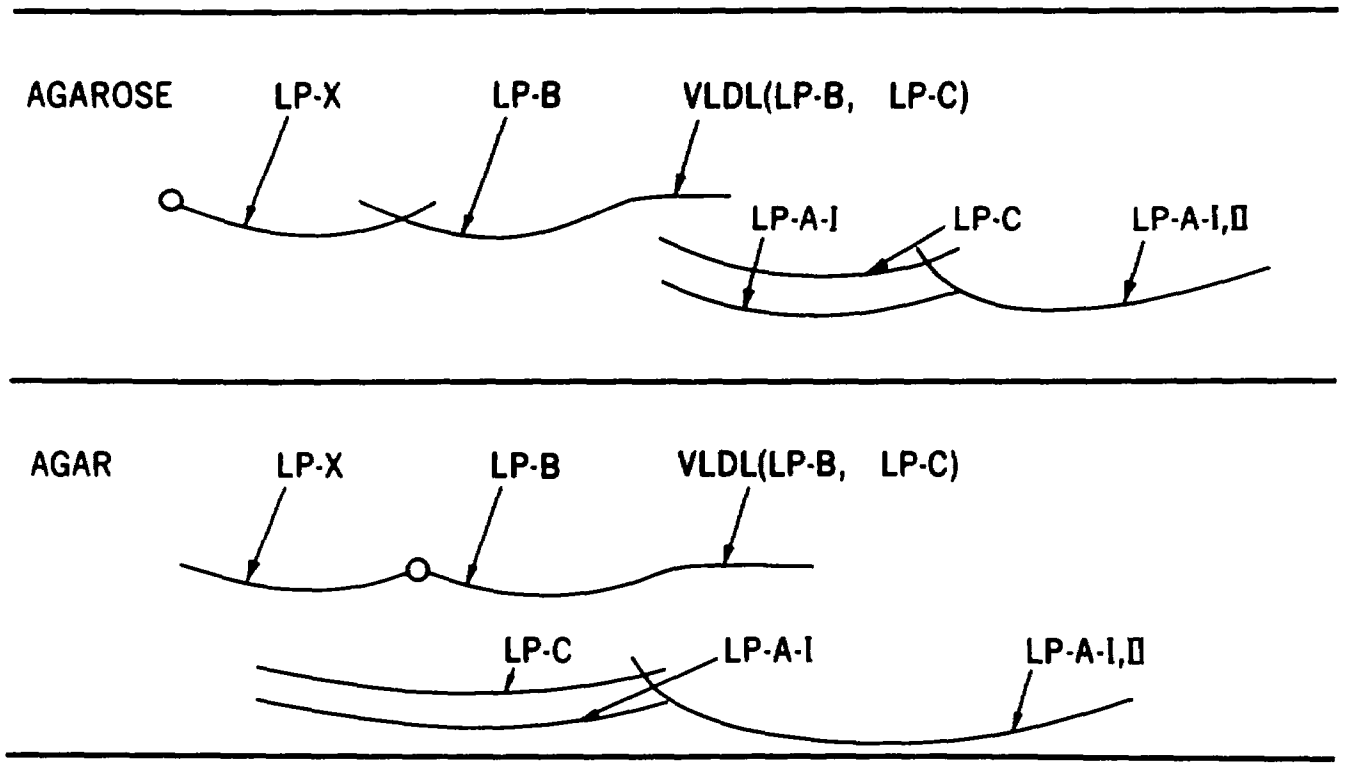


Figure 35. Schematic presentation of immunoelectrophoretic patterns of lipoprotein families in whole plasma.

The antigenic complexity of ApoC was supported by the production of antisera to ApoC and LP-X. A minimum of three antigenic components were identified in association with ApoC: C-I, C-II, and C-III-0, 1, 2. From these studies it was concluded that the polypeptides C-I, C-II, and C-III are antigenically distinct. Further evidence for this conclusion was obtained from results of studies on the amino acid composition, terminal, amino acids, and electrophoretic behavior. Two additional antigenic components may exist, one of which may be associated with C-III-3. The other, detected with A-C-I_{Ab}, may be due to the aggregation of C-I with some other polypeptide or protein, such as albumin, or may represent an unknown haptenic substance such as that reported by Beaumont and Baudet (179). In normal whole plasma two types of LP-C lipoproteins are visualized on immunoelectrophoresis: one is present as an association with LP-B in the VLDL complex, whereas the other is apparently a free LP-C. At the present time it is not possible to conclude whether the unassociated LP-C found in HDL represents an entity consisting of all three ApoC antigenic components or a dissociated LP-C.

The detection of antibodies to albumin in the C-I antiserum was surprising since albumin should have been removed by exclusion on Sephadex G-75 or by DEAE-cellulose chromatography in 8 M urea. From this finding it was concluded that albumin has an affinity for the C-I polypeptide.

Attempts to separate LP-C from either VLDL or from other density classes were unsuccessful utilizing several different approaches, with the exception of hydroxylapatite column chromatography. The latter was not successful, but it was promising. Apparently, the most promising

procedure to isolate LP-C as a lipoprotein is by immunoprecipitation of LP-B and LP-A from HDL or LDL_2.

The results of successful isolation and characterization of pdLP-C were in agreement with those previously reported by Gustafson et al. (3). Serine and threonine were found to be the N-terminal amino acids for both plasma and chyle pdLP-C, and a sedimenting peak with an $s_{w,20}$ of approximately 7S was obtained with pdLP-C from both sources. PdLP-C was also found to be immunochemically distinct from LP-A and LP-B. From these data it has been concluded that this partially delipidized lipoprotein corresponds to the lipoprotein previously designated as LP-C. Separation on disc gel, DEAE-cellulose chromatography, and the immunochemical results have established clearly that the protein moiety of pdLP-C consists of C-I, C-II, and C-III.

Although Brown et al. (118) claimed not to be able to reproduce the ultracentrifugal method, this procedure was reproduced rather consistently in our hands. However, this isolation procedure was abandoned because it had resulted in a low yield of ApoC (10% ApoC from the total protein in VLDL).

Examination of the non-albumin protein portion of LP-X by polyacrylamide gel electrophoresis and immunodiffusion revealed that its polypeptide composition was very similar, if not identical, to that of pdLP-C from VLDL, as reported previously (180). The terminal amino acids were essentially the same as determined by dansylation, hydrazinolysis, and carboxypeptidase analysis (163). The fact that the antisera produced with LP-X reacted with C-I, C-II, and C-III was also a strong indication that the principal polypeptides of ApoC from VLDL and dealbuminated ApoX

were the same. The possible difference between the ApoC preparations from LP-X and LP-C was in the ratio of C-III-1 to C-III-2 polypeptides; this ratio was greater than one for ApoC from VLDL whereas it was less than one for ApoC from LP-X. The ratio of C-III polypeptides of LP-X is similar to that of ApoC isolated from thoracic duct chyle. The significance of the C-III-1 to C-III-2 ratio is not known, but it may reflect the differences in origin or metabolism of these polypeptides. It is interesting to note that LP-C of individuals with the genetic disorder abetaproteinemia consists of C-I, C-II, and C-III-2, but not C-III-1 (181). These findings may indicate either a possible difference in tissue origin of C-III-1 and C-III-2 or a possible block in the interconversion of C-III-2 to C-III-1 in abetaproteinemic subjects.

In contrast to the 50% recovery of ApoC reported by Brown et al. (118), the yield of ApoC never exceeded 35% in the present study. The former finding is in variance not only with results of Gustafson et al. (3) but also with figures from these studies. It has been concluded that the percentage of ApoC is not a constant value but that it depends upon the particular metabolic state of an individual, and then it can be influenced by the dietary regimens and other unknown metabolic factors.

The fractionation of ApoC resulted in the isolation of four major polypeptides. The amino acid composition of these four polypeptides was similar to that reported previously (120) with the exception of the polypeptide designated C-II which was found to contain both histidine and cysteic acid. The detection of threonine as the N-terminal amino acid of C-I and C-II and serine as the N-terminal amino acid of C-III was in full agreement with some results reported by Brown et al. (120). No

valine, however, was released from C-I by hydrazinolysis or carboxypeptidase. Only alanine was identified as carboxyl terminal for the C-III polypeptides.

The molecular weights and the sialic acid content of C-III-1 and C-III-2 were in good agreement with those reported previously (120). Carbohydrate analysis of C-III-1 and C-III-2 revealed 1 mole of galactosamine and 2 moles of galactose for each polypeptide. The only difference noted between these polypeptides was the difference in sialic acid content.

The high percent of glucose and trace amounts of mannose and galactose found in several preparations of C-I might account for some of the heterogeneity of these particular polypeptide preparations.

From the similarities in amino acid deletions, PAGE patterns, immunoelectrophoretic mobility, N-terminal analyses, and molecular weights, it has been concluded that the polypeptides isolated by Brown et al. (118-120) and the C-I, C-II, and C-III-1, 2 polypeptides isolated from ApoC are identical.

Results of the present study, however, extended these findings by identifying several polymorphic forms of C-I and C-III polypeptides. Since the three major bands of C-I were immunologically identical, it has been assumed that certain major characteristics of these three polymorphic forms must be similar. Although two of these forms were isolated, the quantities were not sufficient for a detailed characterization. Since all three forms of C-I were immunochemically identical, but different in electrophoretic mobility, the microheterogeneity could be due to either a difference in the carbohydrate content or traces of albumin. Other

possible reasons for this microheterogeneity (182) include differences in tissue origin, artifacts arising from the isolation procedures, conformers (183), differences due to action of peptidase, amino acid replacements, or amide nitrogen.

The detection of five polymorphic forms of C-III is another example of polypeptide microheterogeneity. Differences between C-III-1 and C-III-2 observed on disc gel electrophoresis have already been shown to be due to the difference in sialic acid content (120) which probably also accounts for the observed mobility of C-III-0. The cause of heterogeneity in C-III-3 and C-III-4 is not known; the C-III-3 polypeptide contained one, rather than three moles of sialic acid.

The C-II polypeptide may also be polymorphic since, in some samples, a minor band was found adjacent to the principal band. The C-II polypeptide was usually recovered in a yield lower than that expected from its estimated content determined by densitometric scanning of polyacrylamide gels. It appears that C-II may be lost during the purification procedure. Additional studies on its chemical and physical characteristics are necessary to resolve the differences between data obtained in this study and those reported by Brown et al. (120).

The column chromatographic elution patterns, electrophoretic behavior, and immunological properties of ApoC isolated from various sources such as plasma and chyle VLDL, plasma HDL, and LP-X indicate that its qualitative polypeptide composition is similar, if not identical. The possible differences in the stoichiometric proportions of polypeptides in these ApoC preparations may be due to the tissue origin, interconversion of polymorphic forms, differential removal rates, or a general

or specific derangement of lipid transport processes.

In contrast to the view that each ApoC polypeptide forms its own lipoprotein (118), results of this study suggest an association of C-I, C-II, and C-III in a lipoprotein designated as LP-C. The evidence for this association is based on the following observations: 1) the partially delipidized LP-C isolated from chyle and plasma VLDL behaved as a chemical entity and contained only C-I, C-II, and C-III; 2) the protein moiety of an abnormal low-density lipoprotein, LP-X, consisted of C-I, C-II, C-III and albumin; after partial delipidization, albumin could be removed from a phospholipid-protein residue which still contained C-I, C-II, and C-III as its constitutive polypeptides; 3) purified post-heparin lipoprotein lipase was found to be associated only with C-I, C-II, and C-III.

It is hoped that the utilization of monospecific antibodies may provide an unequivocal answer to the question whether ApoC-polypeptides are constitutive polypeptides of ApoC or separate apolipoproteins.

CHAPTER VI

SUMMARY

All ultracentrifugally isolated density lipoprotein classes from chyle and from normal and hypertriglyceridemic plasma samples were found to be heterogeneous with respect to their apolipoprotein moieties. The distribution of apolipoprotein C was expanded to include all the ultracentrifugally defined density classes with the possible exception of the 1.21 infranate.

Three regions on 7% polyacrylamide gels have been correlated with the currently recognized lipoprotein families: LP-A, LP-B, and LP-C. Utilization of both acidic and basic polyacrylamide gel electrophoresis in 8 M urea resulted in the identification of C-I, C-II, and C-III polypeptides, both in their intact or totally delipidized form.

Based on immunodiffusion patterns and polyacrylamide gel electrophoresis data, it was concluded that normal, fasting VLDL is a complex consisting of LP-B and LP-C. The C-III-1 and C-III-2 polypeptides were found to be dissociated to some extent from the VLDL by polyacrylamide gel electrophoresis and immunoelectrophoresis.

Examination of thoracic duct chyle confirmed the presence of LP-A and LP-C in chyle chylomicrons and extended these observations by the immunochemical identification of A-I and A-II.

Antisera to the three lipoprotein families and their major polypeptides were prepared. These antisera had specificity for LP-A, LP-B, LP-C, A-I, C-I, C-II, and C-III.

Two procedures were developed to isolate partially delipidized LP-C. Both procedures were based on partial delipidization of hypertriglyceridemic VLDL followed by ultracentrifugation or immunoprecipitation to remove pdLP-A and pdLP-B. Characteristics of the pdLP-C isolated by ultracentrifugation or immunoprecipitation were identical to those of the pdLP-C described by Gustafson et al. (3). Furthermore, pdLP-C contained C-I, C-II, and C-III polypeptides. In addition, C-III-1 and C-III-2 were positively identified in pdLP-C.

Apolipoprotein C and its polypeptides were isolated by a procedure consisting of gel filtration, DEAE-cellulose chromatography, and preparative polyacrylamide gel electrophoresis. Three groups of polypeptides, C-I, C-II, and C-III, were isolated and partially characterized. From these data it was concluded that C-I, C-II, C-III-1, and C-III-2 correspond to the polypeptides described previously by Brown et al. (118-120).

Several polymorphic forms of C-I and C-III were identified and designated as C-I-1, C-I-2, C-I-3, C-III-0, C-III-1, C-III-2, C-III-3, and C-III-4. The polymorphism of C-III-0, C-III-1, and C-III-2 was apparently due to variations in the content of sialic acid, while that of the other polypeptides has not yet been explained since C-I-1, C-I-2, and C-I-3 do not contain sialic acid and C-III-3 contains the same amount of sialic acid as C-III-1.

Lipoprotein C was not isolated in its intact form. However, the isolation of pdLP-C and the presence of only pdLP-C and albumin in pdLP-X indirectly support the existence of a lipoprotein designated as LP-C.

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APPENDIX

NOMENCLATURE AND ABBREVIATIONS OF LIPOPROTEINS

Operational Nomenclature of Lipoproteins

VLDL — Very low-density lipoproteins, lipoproteins of d < 1.006 g/ml and S_f > 20; α_2-LP, pre-β-LP, lipoproteins displaying the electrophoretic mobility of α_2-globulins.

LDL — Low-density lipoproteins, lipoproteins of d 1.006-1.063 g/ml and S_f 0-20; β_1-LP, β-LP, lipoproteins displaying the electrophoretic mobility of β_1-globulins.

LDL_1 — Subfraction of low-density lipoproteins of d 1.006-1.019 g/ml.

LDL_2 — Subfraction of low-density lipoproteins of d 1.019-1.063 g/ml.

HDL — High-density lipoproteins, lipoproteins of d 1.063-1.210 g/ml; α_1-LP, α-LP, lipoproteins displaying the electrophoretic mobility of α_1-globulins.

HDL_2 — Subfraction of high-density lipoproteins of d 1.063-1.125 g/ml.

HDL_3 — Subfraction of high-density lipoproteins of d 1.125-1.210 g/ml.

VHDL — Very high-density lipoproteins, lipoproteins of d > 1.210 g/ml.

$VHDL_1$ — Very high-density lipoproteins, lipoproteins of d 1.210-1.250 g/ml.

$VHDL_2$ — Very high-density lipoproteins, lipoproteins of d > 1.250 g/ml.

Chemical Nomenclature of Lipoproteins

LP-A — Lipoproteins containing apolipoprotein A.

LP-B — Lipoproteins containing apolipoprotein B.

LP-C — Lipoproteins containing apolipoprotein C.

LP-X — Abnormal low-density lipoprotein characterizing obstructive jaundice. Protein moiety consists of apolipoprotein C and albumin.

Abbreviations of Lipoproteins

α-LP	Alpha-lipoproteins
α_1-LP	$Alpha_1$-lipoproteins
α_2-LP	$Alpha_2$-lipoproteins
ApoA	Apolipoprotein A
ApoB	Apolipoprotein B
ApoC	Apolipoprotein C
apoHDL	Totally delipidized high-density lipoproteins
$apoHDL_2$	Totally delipidized subfraction of high-density lipoproteins
apoVLDL	Totally delipidized very low-density lipoproteins
ApoX	Apolipoprotein X
β-LP	Beta-lipoproteins
β_1-LP	$Beta_1$-lipoproteins
pdLP-A	Partially delipidized lipoprotein A
pdLP-B	Partially delipidized lipoprotein B
pdLP-C	Partially delipidized lipoprotein C
pdLP-X	Partially delipidized lipoprotein X
pdVLDL	Partially delipidized very low-density lipoproteins

Abbreviations

A-LP-A, A-C-I, etc.	A - = antisera with specificity for the designated antigenic component(s)
A-WS	Anti human whole serum
C	Cholesterol
CPA	Carboxypeptidase A
CPB	Carboxypeptidase B

d	Isolation density of lipoproteins
$\bar{d}$	Hydrated density of lipoproteins
DEAE	Diethylaminoethyl
di-DNP	Di-dinitrofluorobenzene
EDTA	(Ethylenedinitrilo)tetraacetic acid
g	Gravity units
g-min	Product of gravity units and minutes, "centrifugal force"
LPL	Lipoprotein lipase
N.D.	Not determined
PL	Phospholipids
PAGE	Polyacrylamide gel electrophoresis
rpm	Revolutions per minute
S	Observed sedimentation coefficient
$s_{20,w}$	Observed sedimentation coefficient in water at 20°C
$s^{\circ}_{20,w}$	Sedimentation coefficient at infinite dilution in water at 20°C
SDS	Sodium dodecyl sulfate
S_f	Flotation coefficient in a NaCl solution of density 1.063 g/ml
TEMED	N,N,N',N'-tetramethylethylene diamine
TG	Triglycerides
Tris	2-amino-2(hydroxymethyl)-1,3-propanediol
TCA	Trichloroacetic acid