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Development of a Procedure for Analysis of High Density Lipoprotein Subclasses

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DEVELOPMENT OF A PROCEDURE FOR ANALYSIS OF HIGH DENSITY LIPOPROTEIN SUBCLASSES

by

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ABSTRACT

DEVELOPMENT OF A PROCEDURE FOR ANALYSIS OF HIGH DENSITY LIPOPROTEIN SUBCLASSES

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Human serum high density lipoprotein subclasses, HDL_2 and HDL_3, were isolated by preparative salt density gradient ultracentrifugation and further analyzed by electrophoresis on a 4 to 15% concentration gradient polyacrylamide gel.

The separation of the major classes of serum lipoproteins was achieved after a single ultracentrifugation for 272,000 g at 15°C in a swinging bucket rotor. High resolution concentration gradient gel electrophoresis was found to be particularly suitable for the separation of lipoproteins. The isolation of HDL by precipitation methods, heparin-manganese and dextran-magnesium have also been evaluated in this study, and were found not to completely separate HDL from apo B containing lipoproteins.

The combined procedure of salt gradient ultracentrifugation for isolation of serum HDL and concentration gradient polyacrylamide gel electrophoresis analysis of HDL_2 and HDL_3 was further evaluated with a series of normal and lipemic sera. The results suggested that the new procedures can be adopted by the clinical laboratory for the evaluation of serum HDL subclasses in patients with various cardiovascular diseases.

DEDICATION

The author would like to dedicate this work to her mother

Mrs. Yi-Ping S. Chin

for her love, devotion, and continued support, and to loving memory of her father for his dedication towards work and discipline, which were a great inspiration throughout her graduate studies.

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Chapter I

INTRODUCTION

In the human body, lipids are transported exclusively in association with specific protein-apolipoprotein. Certain polar lipids, free fatty acid and lysolecithin, are bound predominantly to relatively small proteins such as albumin or to specific binding proteins. Nonpolar lipids are usually transported in much larger macromolecular complexes known as the plasma lipoproteins. These nonpoplar lipids (triglycerides, cholesteryl esters and retinyl esters) comprise the core of the spherical plasma lipoprotein, and are shielded from the aqueous environment by a mixed monolayer of polar lipids (phospholipids and cholesterol) and a group of specific proteins.[1,2]

Application of the analytical ultracentrifugation to lipoprotein separations began in 1935 with McFarlane's early studies of normal and pathological sera.[3] One of the principal differences among the lipoproteins is their lipid-to-protein ratio. Roughly, the ratio may vary from 99:1 in chylomicrons to 1:99 in the albumin-fatty acid complex. Thus, ultracentrifugation may be used for separating lipoproteins into different classes according to their densities.

Lately, the various classes of human serum lipoproteins have been separated from one another according to their size (by gel filtration chromatography),[4] density (by ultracentrifugation),[5,6]

net surface charge (by electrophoresis in various media)[7,8,9] or other surface properties (by precipitation techniques).[10,11]

When separated by ultracentrifugation, lipoproteins fall into four relatively discrete classes: chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL). LDL ($d = 1.006 - 1.063$ g/ml) are also called β-lipoproteins because of their β-globulins mobility in paper electrophoresis. The VLDL ($d < 1.006$ g/ml) are also named pre-β-lipoproteins since they move slightly further from the origin than the LDL fraction. Lipoproteins that have densities between 1.063 and 1.210 g/ml are called high density lipoproteins (HDL), or α-lipoproteins, since their mobilities are similar to those of α-globulins in electrophoresis (Table 1).

The lipoprotein classes have distinctly different roles in lipid transport. Normally, chylomicrons are absent from blood plasma obtained in the postabsorptive state (i.e. 10 to 15 hours after the last meal), so that most plasma triglycerides are found in VLDL. About two-thirds of the plasma cholesteryl esters are found in LDL, one-fourth in HDL and the remainder in VLDL (Table 1). The composition of lipoprotein classes separated by ultracentrifugation is not absolutely fixed. However, these compositional changes are small relative to changes in number of individual lipoprotein particles in each class in disease state.[12]

There are two major types of triglyceride-rich particles in the circulation: chylomicrons, which carry exogenous triglyceride from the intestine; and VLDL, which carry endogenously synthesized triglyceride from liver. Both of these particles interact with

Table 1

Properties of Lipoproteins

	Chylomicrons	VLDL	LDL	HDL
Density (g/ml)	0.95	0.95-1.006	1.006-1.063	1.063-1.210
Electrophoresis Mobility	origin	pre-β	β	α
Diameter (Å)	800-5000	300-800	170-210	70-150
Approximate Composition (% of dry mass)				
Triglycerides	80-95	45-60	14	4
Cholesteryl esters	2-4	10-15	36	15
Cholesterol	1-2	7-10	8	5
Phospholipids	3-6	15-20	21	29
Proteins	1-2	5-10	21	47

lipoprotein lipase (LPL) in extrahepatic capillary beds. As the triglyceride component of the core of these lipoproteins is hydrolyzed and removed, the surface area of the particle diminishes. Some of the free cholesterol and phospholipid at the surface interact with lecithin:cholesterol acyltransferase (LCAT) in the presence of HDL to form cholesteryl esters, which enter the core of the remanant particle. Plasma LDL functions as a storage depot for cholesterol. Low density lipoproteins are catabolized in extrahepatic tissue. Free cholesterol is continually released from cells that degrade LDL by the receptor mechanism or the scavenger pathway.[13] This excreted cholesterol leaves the tissues, in association with HDL, and part of it is subsequently esterified through the action of plasma LCAT. The resultant cholesteryl esters in HDL can be transferred to VLDL and chylomicrons remnants and ultimately to LDL.

The protein portions of lipoproteins are called apolipoproteins that have been found to be synthesized in the parenchymal cells of the liver while some are synthesized in the intestinal mucosa. At the present time, a total of eight apoproteins have been characterized (Table 2). Some of the functions of these apoproteins were suggested to involve in lipid transport as well as to play important role in lipoprotein metabolism. Several of the apoproteins have been shown to interact with enzymes and cell surface receptors to control lipid metabolism. The role of apo-B and LDL in cholesterol synthesis has been extensively studied by Brown et al.[14] The role of apo C-II in the activation of LPL for hydrolysis of chylomicron and VLDL triglycerides is also well established.[15] Apo A-I and C-I are known to activate LCAT, the enzyme believed to be primarily responsible

Table 2

The Human Plasma Apolipoproteins

Apolipoprotein	Density Class	Molecular Wt.
A-I	HDL	28,000
A-II	HDL	17,000
B	VLDL,LDL	250,000
C-I	VLDL	6,500
C-II	VLDL,LDL	10,000
C-III	chylomicrons, VLDL	9,300
E	VLDL,LDL	33,000
D	HDL_3	20,000

for the synthesis of cholesteryl esters.[16] Apo A-I and apo A-II are the major protein constituents of human HDL and are known to bind phospholipid.[17]

The relationship between plasma lipids and atherosclerosis has been exhaustively studied for many decades, and a first integrated approach to mechanism and disorders of fat transport in lipoproteins was reviewed by Fredrickson et al.[18]

The structure, definition and interrelationship of plasma lipoproteins, and the regulation of lipoprotein concentration, have become increasingly important topics because it is evident that abnormal lipoprotein concentrations, hypercholesterolemia or hyper-betalipoproteinemia, accelerate the development of atherosclerosis. The mechanism involves the proposed role of HDL in the regulating of intracellular concentration of cholesterol in which HDL facilitates the removal of cholesterol from peripheral cells and the transport of cholesterol from peripheral cells to the liver for ultimate removal from body. Low levels of plasma HDL would be anticipated to decrease the effective removal rate of cholesterol from peripheral cells.[19]

The recognition by epidemiologists and clinicians that HDL is a negative risk factor related to premature cardiovascular disease has created a heavy demand for serum HDL concentrations in laboratory analytical services.[20,21]

Lately, two methods have often been described for the isolation of HDL from serum, i.e. ultracentrifugation and precipitation.[22,23] In the method of ultracentrifugation, serum sample was first adjusted to a density of 1.063 g/ml with KBr and then was overlayered with

a KBr solution (d = 1.063 g/ml) in a cellulose nitrate tube (13 x 63.5 mm) for the Beckman 40.3 rotor. The centrifugation was performed at 105,000 x g for 24 hours at 10°C. The top layer which contained LDL and VLDL was removed, and the bottom layer was further adjusted to a density of 1.21 g/ml with KBr. The fraction of HDL can then be separated into its subclass, HDL_2 and HDL_3 (d = 1.063 - 1.21 g/ml) through a second ultracentrifugation under similar conditions. In the method of precipitation, HDL was isolated by specific precipitation of non-HDL apo-B containing lipoproteins (LDL and VLDL) with polyanions and divalent cations (Fig. 1). The precipitating agents such as heparin and Mn^{2+}, dextran and Mg^{2+} and phosphotungstate and Mg^{2+} were added to serum samples and mixed well. The samples were incubated at room temperature for 15 minutes giving sufficient time for the reagents to complex with apo-B containing lipoproteins and to precipitate them from the solution. The precipitates were removed by centrifugation at 1,500 x g for 30 minutes at 4°C.

The fact that HDL is heretogeneous in terms of density was first appreciated by researchers from Donner Laboratory in the early 1950's. At that time, they separated HDL into three components: HDL_1, HDL_2 and HDL_3.[24] HDL_1 and HDL_2 were later shown to be identical by radio-immunoassay and are now designated as HDL_{2a} and HDL_{2b}. In comparison, HDL_2 was shown to have abundance of lipid, lower density and larger particle weight[25] and a considerably higher apo A-I to apo A-II ratio than that of HDL_3.[26] There is considerable evidence that the HDL subclasses have different metabolic significance.[27] In general, when HDL is increased, the HDL_2 is increased disproportionally. HDL was

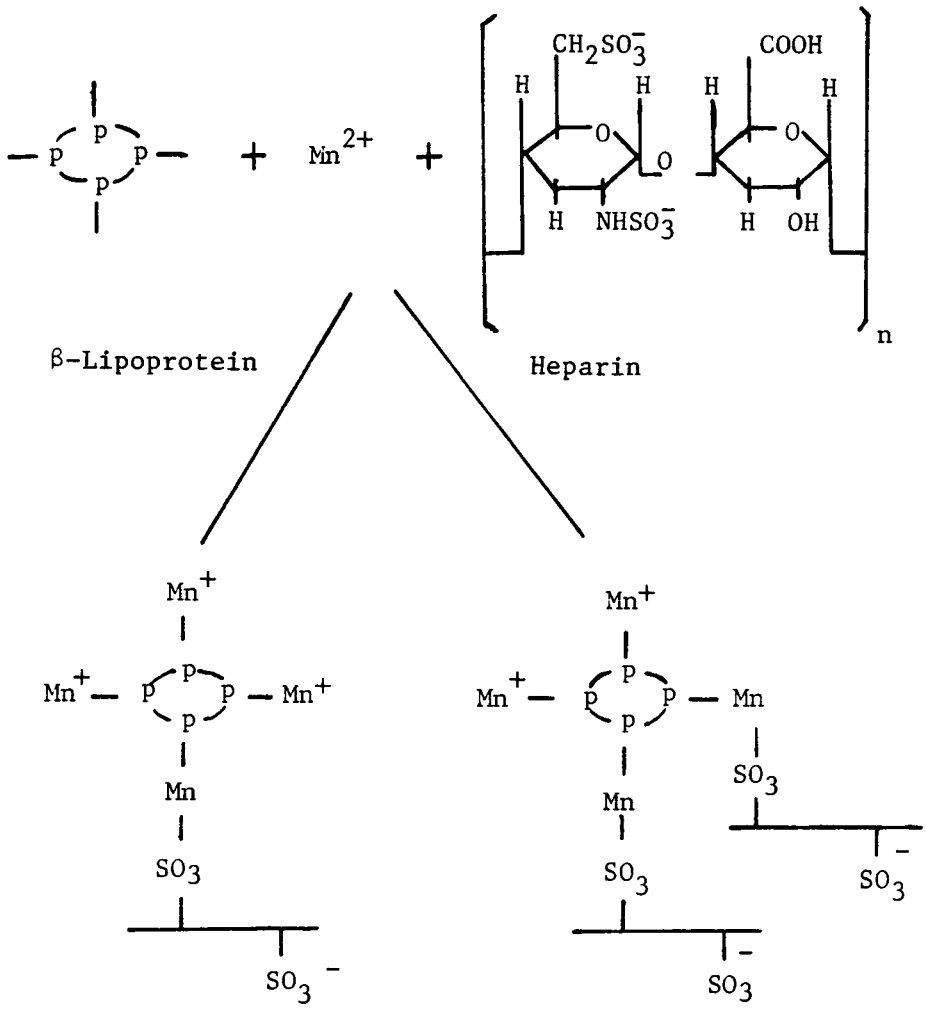

Fig. 1. A hypothetical and schematic diagram of the mechanism of formation of complexes between β-lipoprotein and heparin.

appreciably higher in females than males, whereas HDL_3 was very similar in males and females. HDL is reduced in patients with coronary heart disease and the major reduction was shown to be in HDL_2.[28,29] Measurements of HDL subclasses may be especially important to clinicians and epidemiologists in that determination of HDL_2 may provide a more sensitive discrimination of changes in metabolism leading to increased risk from atherosclerosis.

Chapter II

STATEMENT OF THE PROBLEM

High density lipoproteins have been implicated as a negative risk factor in cardiovascular diseases.[20] Reduction of HDL was found in patients with heart diseases and the major reduction was found to be in the HDL_2 subclass.[29] There is a need for an accurate method for quantitative determination of HDL subclasses in the clinical laboratory. The isolation of HDL by the precipiation technique is much simpler than the ultracentrifugal technique. However, whether the apo-B containing lipoproteins are completely precipitated and whether the technique is applicable for HDL subclass analysis needs to be determined. Comparison of the HDL isolated by single-step ultracentrifugation method and that of precipitation method[30] will be made. It is proposed to investigate the feasibility of using high resolution gradient gel electrophoresis as the method for analysis of HDL subclasses. It is believed that once the method is perfected, it will provide a useful diagnostic tool for clinicians to correlate the variation of HDL subclasses in normal and lipemic samples.

Chapter III

EXPERIMENTAL TECHNIQUES

A. Materials

The following materials were purchased from Sigma Chemical Company: acrylamide, N, N'-methylene-bis-acrylamide, bovine serum albumin, coomassie brilliant blue R-250, sodium dodecyl sulfate (SDS), Trizma base, Sudan Black B and heparin (grade 1, sodium salt). Dextran sulfate and magnesium sulfate were obtained from DOW Chemical Company. Bromophenol, blue (BPB) and N,N,N', N'-tetramethylethylene-diamine (TEMED) were obtained from Eastman Kodak Chemical Company. Stable Biuret Reagent was the product of Hycel Inc. All other chemicals were reagent grade quality and purchased commercially.

B. Equipment

A Beckman Model L5-65 preparative ultracentrifuge with either SW-27 or SW-60 rotor was used for all ultracentrifugal studies. An ISCO Model 185 density gradient fractionator connected with an ISCO Model UA-5 UV monitor and a Gilson Model FC-100 microfraction collector were used for fractionation of density gradient tubes. All UV-visible spectral measurements were carried out on a Beckman Model 25 spectrophotometer equipped with a constant temperature cuvette holder.

Concentration gradient slab gels were cast using a gradient mixer fabricated in the Old Dominion University Science Shop. The electrophoresis chamber was designed and fabricated and is shown in Figure 2. An E-C Apparatus Corporation Model E-C 400 power supply was used for the electrophoretical separations. Concentration Gradient Electrophoresis (CGE) gels and SDS disc gels were destained using BioRad's gel electrophoresis diffusion destainers.

C. Methods

1. Serum Samples

Fasting blood was drawn from the antecubital vein into vacutainer tubes without anticoagulant. Serum was obtained by centrifugation at 1,200 x g for 30 minutes. Sera was stored at 4°C and processed within 3-4 days. Patients were considered normal when their multiphasic profile testing results were normal (usually includes cholesterol and triglycerides). Both normal and lipemic serum samples were donated by Bayside, Maryview, Portsmouth Naval and Norfolk Community Hospitals and represent specimens from their normal daily workload. Clinical diagnostic data was not available for their patients.

2. Isolation of Serum High Density Lipoproteins (HDL)

a. By Density Gradient Ultracentrifugation

The ultracentrifugal method reported by Chapman et al.[31] was modified for the isolation of the HDL from serum. The serum sample was first adjusted to a density of 1.210 g/ml by the addition of solid potassium bromide (KBr). A discontinuous density

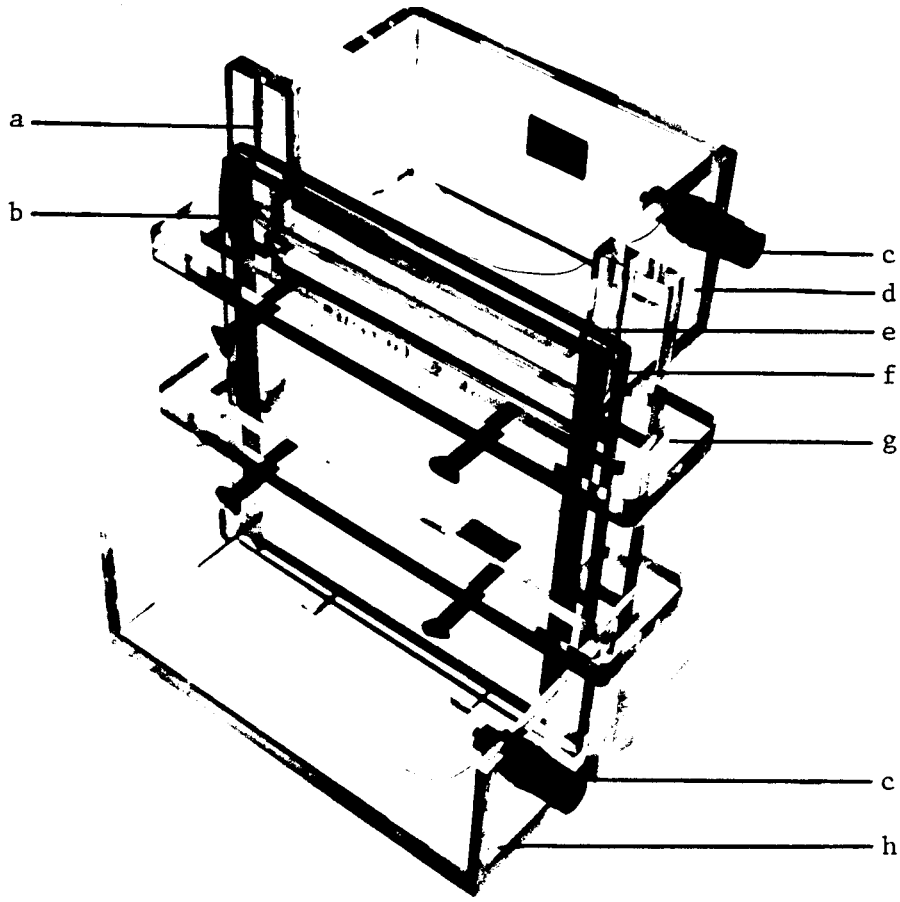

Fig. 2. Slab gel electrophoresis chamber: (a) grove for faceplate gasket; (b) spacer; (c) platinum wire; (d) upper buffer chamber; (e) notched plate; (f) front plate; (g) clamp; (h) lower buffer chamber.

gradient was constructed at ambient temperature in a 4-ml polyallomer centrifuge tube (7/16" diameter x 2-3/8" length) of the Beckman SW-60 Ti swinging bucket rotor (capacity 6 tubes). A 0.64 ml of NaCl-KBr solution (d = 1.240 g.ml) was pipetted into the bottom of the tube, and the following solutions were then layered onto the latter: 0.96 ml of serum sample at d 1.210 g/ml, 0.64 ml of NaCl-KBr solution of d 1.063 g/ml, 0.80 ml of d 1.019 g/ml and 0.96 ml of NaCl solution of d 1.006 g/ml (Table 3). In the control tube, the serum sample was replaced by a NaCl-KBr solution of d 1.210 g/ml. Immediately after layering, the gradients were centrifuged at 45,000 rpm (272,000 x g, ave.) for 36 hours at 15°C in a Beckman L5-65 preparative ultracentrifuge; no braking was used at the end of the run.

After centrifugation, tubes were fractionated on an ISCO Model 185 density gradient fractionator connected with an ISCO UA-5 UV-monitor and a Gilson Model FC-100 microfraction collection. The fractionations were monitored at 280 mm, and 0.5 ml fractions were collected. The fractions containing HDL (1.063 - 1.200 g/ml) were pooled and immediately dialyzed against a solution containing NaN_3 (0.5 g/l), NaCl (11.3 g/l) and EDTA (0.1 g/l), pH 8.6 at 4°C.

b. By Precipitation

Two precipitation methods were used for evaluation. In the heparin-Mn^{2+} precipitation method, 0.2 ml of combined heparin-Mn^{2+} reagent (14.3 mg of heparin and 210 mg of $MnCl_2 \cdot 4H_2O$ in 1 ml of H_2O) was added sequentially to 2.0 ml of serum sample with thorough mixing by a vortex mixer. The final concentrations of heparin and Mn^{2+} in the serum were 1.43 mg/ml and

Table 3

The Composition of the Salt Solution of Different Density at Room Temperature

Density (g/ml)	NaCl (g/100 ml)	KBr (g/100ml)
1.240	1.1	33.4
1.210	1.1	28.9
1.063	1.1	7.4
1.019	1.1	1.18
1.006	1.1	0

0.046M, respectively. After the reagent addition, sample was incubated for 15 minutes at 23°C before centrifugation at 1,500 x g for 30 minutes at 4°C. The supernatant was then transferred to tube, sealed and stored at 4°C until electrophoresis. In the dextransulfate-Mg^{2+} precipitation method, the procedure is essentially similar to that of heparin-Mn^{2+} precipitation method except the combined reagent in which 0.1 ml of dextran sulfate reagent (20 mg/ml) and 0.2 ml of 1.1 M magnesium sulfate were added instead.

3. Protein Determination

Protein determination was performed on each sample so that the same relative amount of protein could be applied to each gel. The determination was performed by incubating sample with 3 ml of Hycel Biuret Reagent for 30 minutes at 37°C in a heating block. Absorbance was measured at 540 nm and compared with a protein standard, bovine serum albumin.[32]

4. Concentration Gradient Polyacrylamide Electrophoresis (CGE)

a. Reagents

CGE Acrylamide: 14.55% acrylamide, 0.45% N,N' methylene-bis-acrylamide (T = 15%, C = 3%)

Gel Buffer: 0.09 M Tris-HCl, 0.08 M borate 0.01% EDTA, 0.05% NaN_3, pH = 8.35

b. Preparation of Concentration Gradient Gels

Glass plates were assembled with spacers and sealing gaskets as shown in Figure 2 (p. 13). A 135 x 150 x 30 mm resolving gel was poured with a linear 4-15% acrylamide gradient. After pouring, the gels were gently overlayered with the sample wellmaker. Polymerization was completed in one to two hours. The sealing and

the sample wellmaker were removed. The upper and lower gel surfaces were gently flushed with gel buffer. The sample was loaded into sample well with a micropipet. Nine samples can be analyzed in one gel slab.

c. Sample Preparation

For each serum, HDL and HDL subclasses sample, one drop of 0.05% bromophenol blue was added as a tracking dye and one drop of glycerol was mixed with the sample. For electrophoresis, 20 µl of serum sample and 200 µl of HDL or HDL subclasses were applied to the sample well of the gel with micropipets.

d. Electrophoresis

Degassed gel buffer was placed in the upper and lower chambers with a filter paper as a bridge between the upper chamber and sample well. The gels were run at 150 V constant voltage per slab gel at room temperature until the tracking due reached the bottom edge of the gel (20 hours).

5. Sodium Dodecyl Sulfate (SDS) Disk Gels Electrophoresis

a. Reagents

Gel buffer: 0.78% $NaH_2PO_4H_2O$, 3.86% $Na_2HPO_4 \cdot 7H_2O$
0.2% of SDS

Stock acrylamide solution: 22.2% acrylamide and 0.6% methylene-bis-acrylamide (T = 22.8%, C = 2.6%)

Stacking gel solution: 4 ml gel buffer, 1.4 ml stock acrylamide solution, 2.2 ml H_2O, 6 µl TEMED and 0.4 ml 10% ammonium persulfate (APS)

b. Preparation of Gels

The glass gel tubes were 10 cm long with inner diameter of 6 mm. For a typical 10 gel-run, 15 ml of gel buffer were degassed

and mixed with 13.5 ml of stock acrylamide solution. After further deareation, 1.5 ml of freshly prepared APS and 0.045 ml of TEMED were added and mixed. The gel solution was then poured into each of the gel tubes and was overlayered with a few drops of water. The polymerization was usually complete with 30 minutes. The overlayered water was then removed and the stacking gel was then layered on the separation gels.[33]

c. Preparation of Sample

The HDL_2, HDL_3 and HDL, isolated either by ultracentrifugation or precipitation methods, were delipidated by two 12 hour extractions with freshly prepared ether-ethanol (3:1 v/v) at 4°C and further washed twice with diethyl ether and dried under N_2 gas. The apoprotein residues were then dissolved completely in a Tris-HCl buffer.[33] For each gel, 3 µl of tracking dye (0.05% Bromophenol blue) and one drop of glycerol were added to an appropriate amount of apoprotein solution (50-100 µl) and mixed well before applying to the gel.

d. Electrophoresis

The two compartments of the electrophoresis apparatus were filled with gel buffer and diluted 1:1 with water. Electrophoresis was performed at a constant current of 8 ma per gel until the tracking dye reached the bottom edge of the gel (about 8-9 hours).

6. Staining Methods

a. Reagents

Protein staining solution: It was prepared by dissolving 1.23 g of Coomassie brilliant blue in a mixture of 454 ml of

methanol and 46 ml of glacial acetic acid and the insoluble material was removed by filtration through a glass fritted funnel.

Destaining solution: 25% ethanol and 8% acetic acid.

Gel preserving solution: 5% acetic acid.

Lipid staining solution: The saturated solution of Sudan Black B in ethanol 100 ml, glycerol 40 ml, water 60 ml and glacial acetic acid 10 ml, mixed in that order.

Lipid destaining solution: This solution was prepared in the same manner as staining solution with the omission of Sudan Black B.

b. Protein Staining

The gels were removed from the plates and the upper right corner of each gel was marked with India ink. The gels were stained at room temperature for 2-3 hours in the protein staining solution and were destained by diffusion destaining overnight in the Bio-Rad diffusion chamber, and stored either in preserving solution or in zip-lock sandwich bags.

c. Lipid Staining

The basic staining procedure of Pratt et al.[34] was followed. The gels were removed from the plates, and were stained at room temperature for six hours, then destained in the destaining solution.

Chapter IV

RESULTS

A. Gradient Ultracentrifugal Isolation of HDL

The ultracentrifugations were carried out for various periods of time (30, 36 and 48 hours) at 45,000 rpm in a Beckman SW Ti-60 rotor. As the time period of ultracentrifugation increased, the peaks of the pattern moved to the left as indicated in Figure 3 and the VLDL and LDL peaks merged into one. In the 36 hours ultracentrifugation, the HDL_2 and HDL_3 peaks were better resolved as shown in Figure 3. The final condition of ultracentrifugation was thus established after the examination of the density profile of the control which was spun for 36 hours parallel with the samples. The density profile is plotted as a function of fraction number, and the results plotted in Figure 4 demonstrate a linear gradient. The typical lipoprotein profiles of normal and lipemic serum samples in a 36 hour centrifugal separation are shown in Figure 5.

In normal serum lipoprotein profiles, five absorption peaks were consistently present, and three of these typically fell within the ranges of characteristic density of LDL (1.025 -1.40 g/ml), HDL_2 (1.069 -1.11 g/ml, fractions 4 and 5 in Figure 5) and HDL_3 (1.11 -1.16 g/ml, fractions 6 and 7 in Figure 5).[35,36] A fifth broad peak presented at the bottom of the gradient corresponded to yellow layer which included albumin, bilirubin and very high density

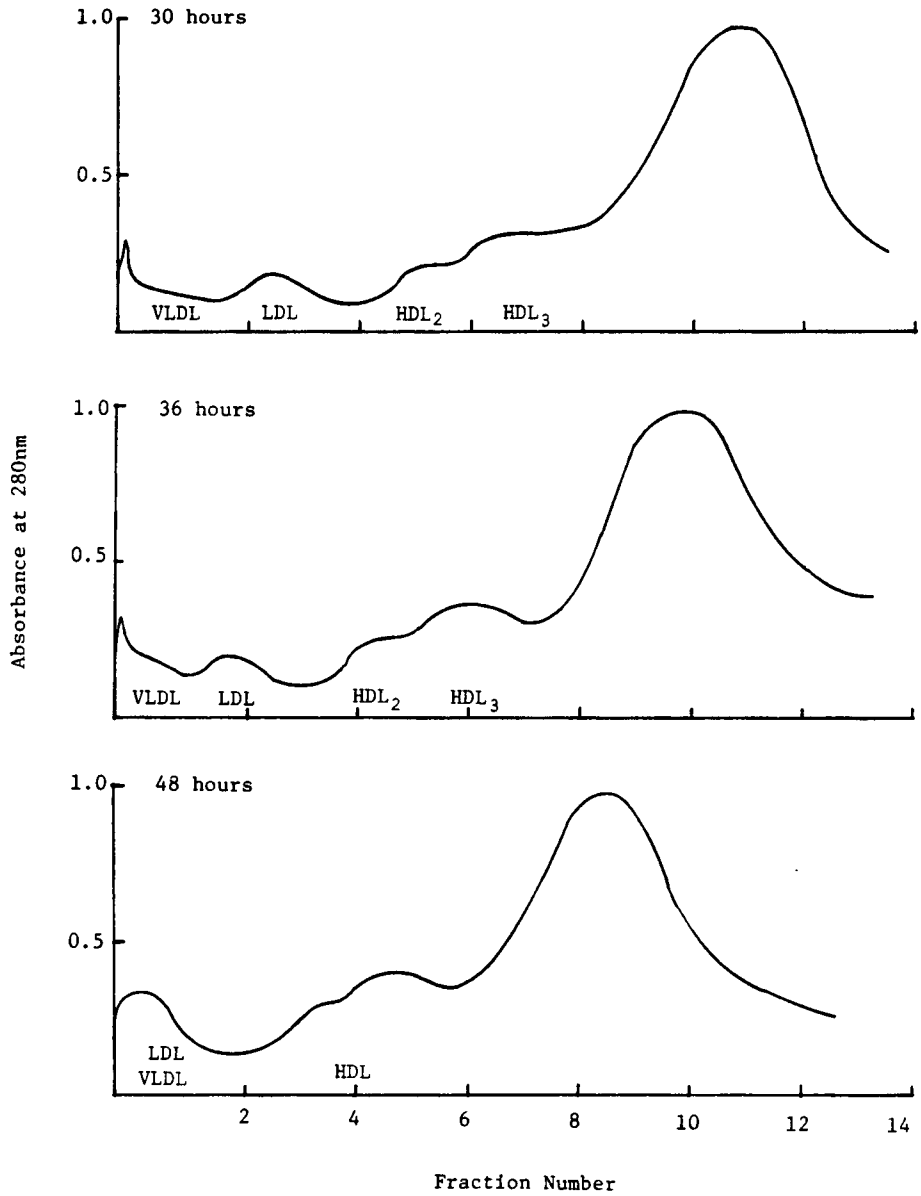

Fig. 3. Effect of time on the rate-zonal flotation of serum lipoprotein in the SW Ti-60 rotor.

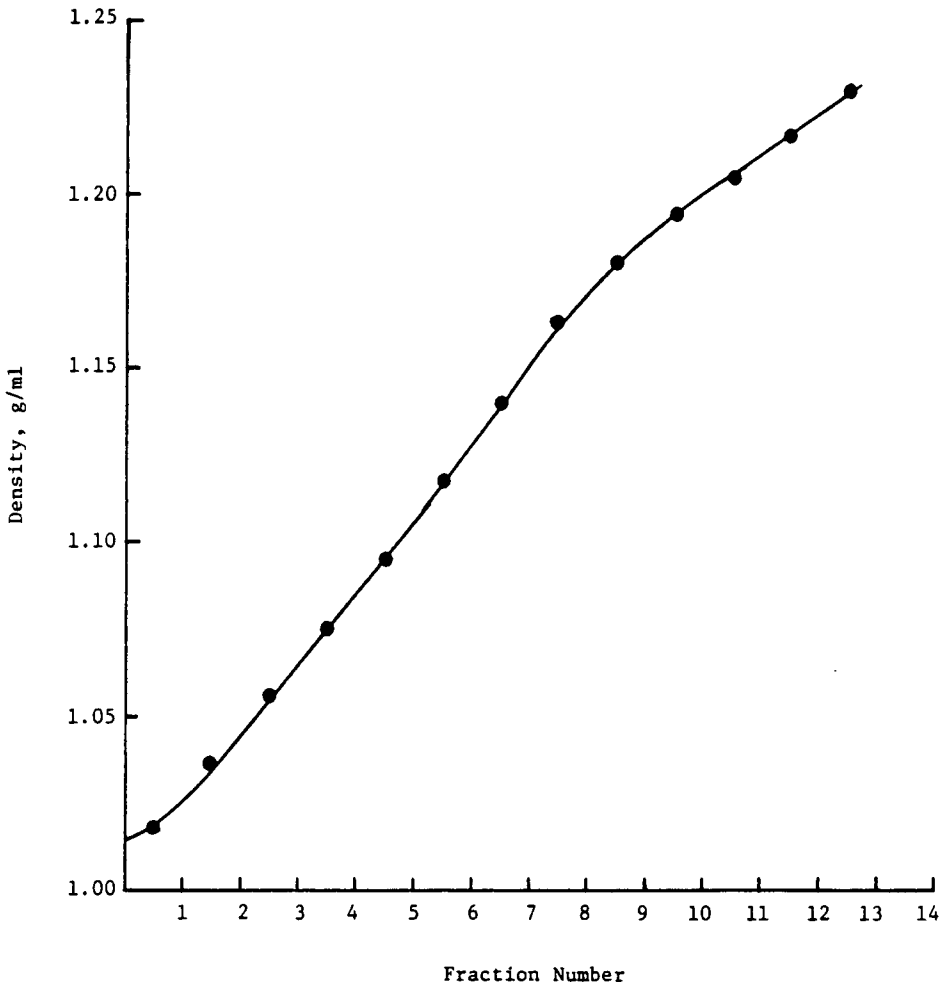

Fig. 4. Salt gradient after 36-hour ultracentrifugation in SW Ti-60 rotor.

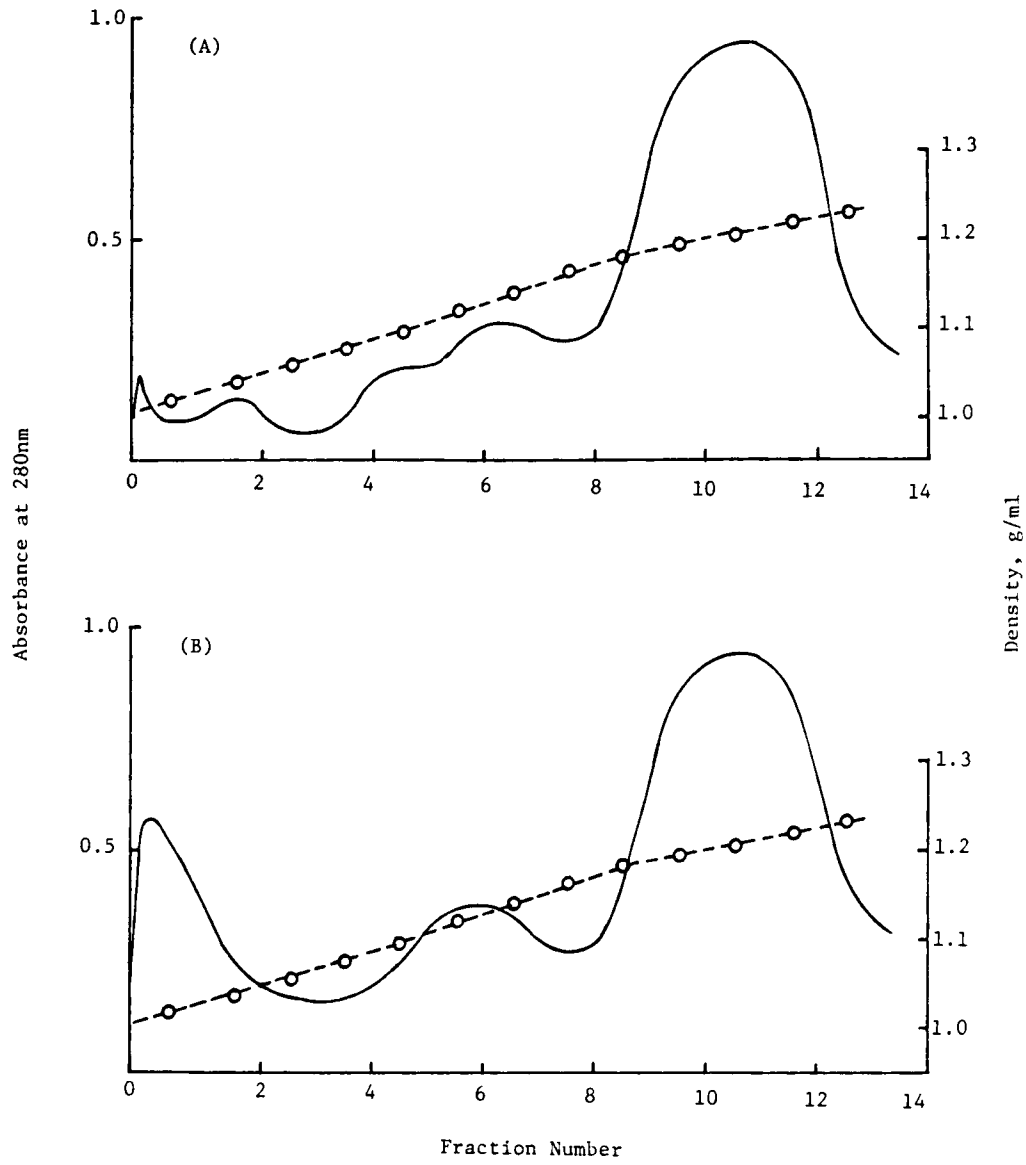

Fig. 5. Lipoprotein profile pattern in the density gradient after 36-hour ultracentrifugal separation of a normal serum (A) and lipemic serum (B). The dotted line (--0--0--0) indicates the density gradient along the tube. The solid line (————) indicates the lipoprotein profile.

lipoprotein (VHDL).[31] In most of the lipemic serum lipoprotein profiles, only three broad peaks existed. When compared to the normal serum lipoprotein profiles, VLDL and LDL were overlapped together in lipemic serum samples (Fig. 5B, fractions 1 and 2). Also, the HDL_2 and HDL_3 could not be resolved in the broad peak (Fig. 5B, fractions 4-7).

B. Electrophoresis Gell Procedure

Several gradient and non-gradient acrylamide gels were evaluated. The resolution of the 7.5% and 12% non-gradient gels are not as good as the resolution of the gradient gels. After 20 hours electrophoresis of the 4-30% gel, most of the samples were separated in the upper one-third of the gel. Because the pores of the lower part of 4-30% gel are too small, the lipoproteins could not be separated further. In addition, the 30% acrylamide gel was brittle for accurate work. The resolution of 2-15% CGE gels were evaluated and the pattern was similar to that of 4-15% CGE gels. The top of the 2% acrylamide gel was too soft for accute work. The optimum gradient concentration of acrylamide for separating gels was, therefore, 4-15%.

Because laboratory temperature significantly affects slab gel polymerization, the acrylamide solutions were kept on ice to prevent polymerization prior to the preparation of the slab gel.

C. Electrophoresis of HDL Separated by Ultracentrifugation Method vs. Heparin-Mn^{2+} and Dextran sulfate-Mg^{2+} Precipitation Methods

The pattern obtained from the electrophoresis of the HDL which was isolated by ultracentrifugation and precipitation methods is shown in Figure 6. The results were the same when comparing the CGE

Fig. 6. Four to 15% polyacrylamide gel electrophoresis of HDL_2: (1) protein staining, (2) lipid staining.

of HDL with both lipid and protein stainings. The pattern from lipid staining was weak as shown in Figure 6. So, all the CGG in this study were stained with the protein stain.

The HDL was distributed broadly in the lower part of the concentration gradient gel (CGG) as in Figure 6. The HDL from lipemic serum was compared with HDL_2 and HDL_3 from a normal serum in CGG, both HDL's were isolated by ultracentrifugation. The position on CGG of HDL from lipemic serum was relative to the position of HDL_3 from the normal serum. This phenomenon can be explained only by the sole presence of HDL_3 in HDL from lipemic serum. The HDL isolated by heparin-Mn^{2+} and dextran-Mg^{2+} precipitation methods migrated over a very broad region in the CGG, and showed a banding pattern almost the same as total serum (Fig. 7).

D. Concentration Gradient Electrophoresis (CGE) from Normal and Lipemic Serum Samples

Several distinct patterns of serum protein distribution can be observed in Figure 8. The clear bands were observed in the area of the gel when HDL_2 was shown to migrate. These two bands were also identified by Scanu and Kruski[36] as belonging to the α-lipoprotein fraction. When comparing normal and lipemic CGE patterns only one weak band was observed in the lipemic serum analyses, while two bands were always observed in the analyses of normal serum.

E. SDS Polyacrylamide Gel Electrophoresis (PAGE) of the Protein of HDL

The two major high density proteins, apo A-I and apo A-II, differed in their relative mobility in SDS PAGE. Apo A-I had a broader and more intense band than apo A-II in disc gel with the HDL

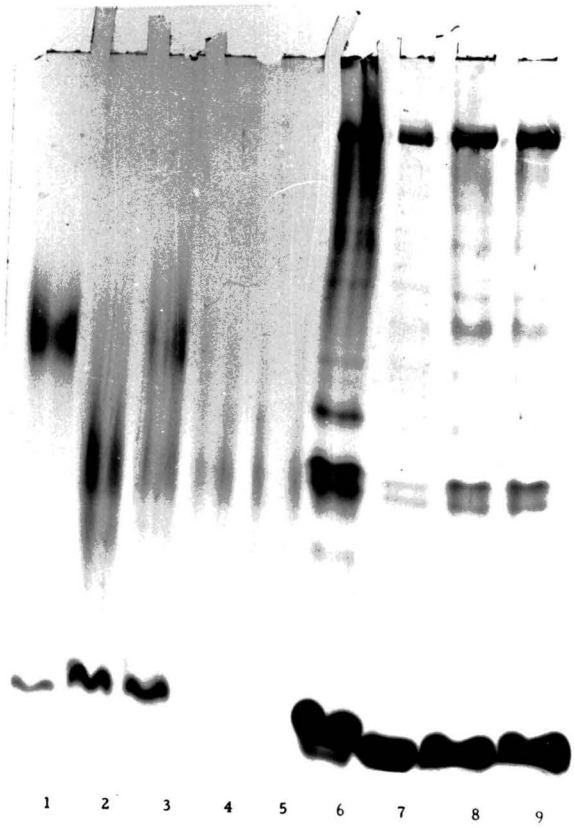

Fig. 7. Four to 15% polyacrylamide gel electrophoresis of: (1) HDL_2, (2) HDL_3, (3) HDL_2+HDL_3, (4) patient HDL, (5) patient HDL, (6) serum, (7-9) HDL were isolated by precipitation methods.

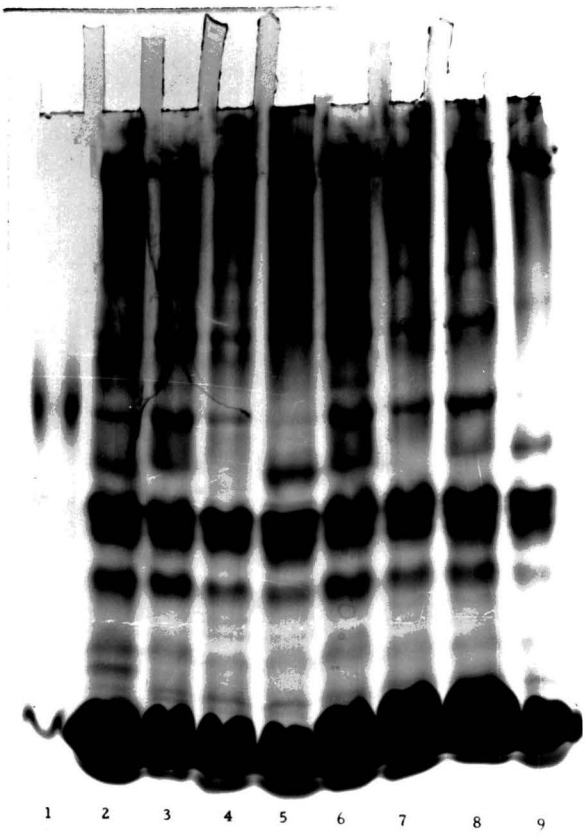

Fig. 8. Four to 15% gradient gel electrophoresis of: (1) HDL_2, (2-8) lipemic serum samples, and (9) control serum.

isolated by heparin-Mn^{2+} precipitation (Fig. 9B). This was also consistent with the approximate apo A-I:apo A-II ratio of 3:1 as previously reported.[37,38] Several other minor bands existed in the gel were reported as apo B.[39] The HDL was isolated by different methods from the same serum sample. The apo-HDL_3 isolated from ultracentrifugation showed only two bands in PAGE (Fig. 9A).

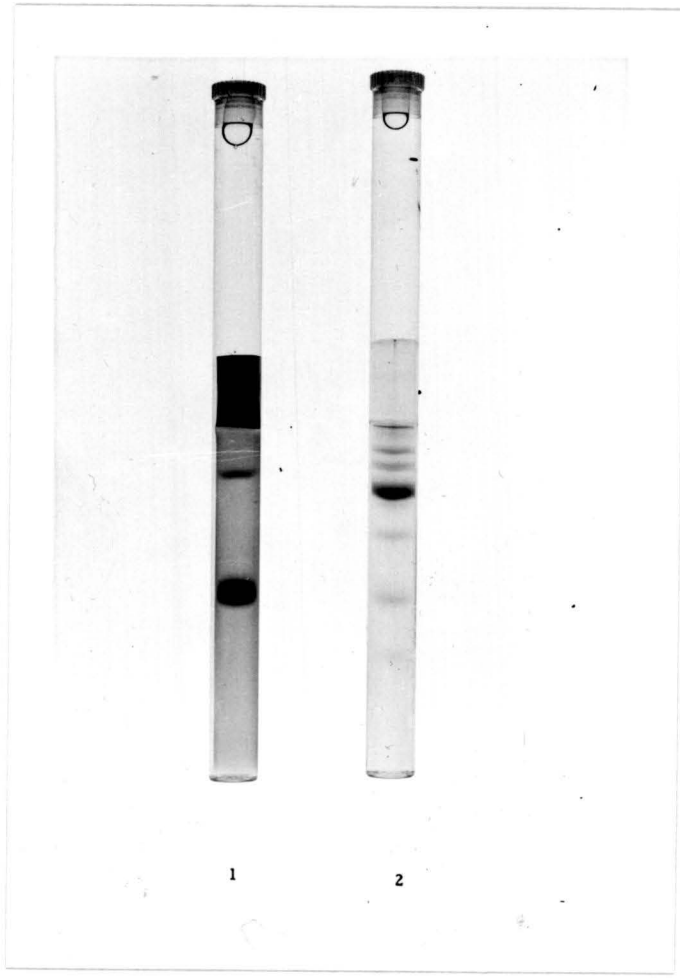

Fig. 9. SDS polyacrylamide gel electrophoresis of the apoproteins of HDL. (1) HDL were isolated by ultracentrifugation. (2) HDL were isolated by heparin-Mn^{2+} precipitation method.

Chapter V

DISCUSSION

Due to the implication of HDL as a negative risk factor in cardiovascular diseases, a method to determine HDL with accuracy and relative simplicity is needed. The isolation of HDL from serum by precipitation techniques is much simpler than the two ultracentrifugations. The accuracy of heparin-Mn^{2+} precipitation procedure has been examined by Alber et al.[23] with quantitation of the principal apoproteins of the isolated HDL by radioimmunodiffusion, and was considered by them to be a reasonably specific method. In the concentration gradient gel electrophoretic studies of HDL isolated by the precipitation technique, many bands in addition to HDL_2 and HDL_3 were observed. When comparing patterns for heparin-Mn^{2+} precipitated specimens to those obtained with unprepared serum, similar but weaker lipoprotein-banding for the precipitated specimens was found. In addition, when the isolated HDL was subjected to delipidation and then to SDS-polyacrylamide gel electrophoresis, several extra bands belonging to apo B class apoproteins were observed along with the expected apolipoproteins A-I and A-II. The observed apo B protein bands are similar to those reported by Rudel.[39] These results indicate that precipitation techniques do not separate HDL from apo B containing lipoproteins such as LDL and VLDL completely. These results also agree well with those

reported by Farrell[40] in which they utilized the conventional electrophoresis procedure.

In studying the time required for separation of serum lipoproteins by density gradient ultracentrifugation, the experiments were performed by a single centrifugation at 272,000 x g at 15°C at various durations of centrifugation. The profiles of these studies showed that with longer centrifugation times, time-dependent mixing of the HDL subclass occurred. These studies show that the 36-hour centrifugation is the time for the best separation of HDL_2 and HDL_3. Numerous experimental conditions were reported for ultracentrifugal separation of serum lipoproteins. Alber et al.[41] used 1.090 to 1.130 g/ml CsCl gradient ultracentrifugation in a Beckman SW 41 rotor at 40,000 rpm (272,000 x g) at 10°C for 72 hours. Nichols[28] used a background NaBr/NaCl density gradient of 1.063 to 1.210 g/ml and centrifuged twice in a Beckman 40.3 rotor at 114,000 x g for 24 hours. The technique described in the Manual of Laboratory Operations, N.I.H.,[22] as mentioned earlier, utilized centrifugation at 105,000 x g for 24 hours at 10°C.

Generally, HDL was delipidated by two 12-hour extractions either with ethanol-ether (3:2 v/v) at -20°C or with ethanol-ether (1:3 v/v) at 4°C. The latter method is simpler and easier to operate and is adapted in this study for the delipidation of HDL. An apolipoproteins A-I and A-II ratio of 1.6:1 for HDL_3 fraction was reported previously by Scanu et al.,[42] and this is distinctly different from the 1:1.2 ratio for the HDL_3 isolated by ultracentrifugation in this study. The decrease in ratio as obtained in this study may possibly be due to the degradation of HDL during preparative centrifugation.

It has been reported[43] that the different methodologies in separating HDL may have accounted for the differences in results.

The polyacrylamide gradient gel electrophoresis allows better resolution of serum lipoproteins than the electrophoresis in a constant concentration of polyacrylamide. This is due to a progressively smaller pore size along the gel that increases resistance of the gel network.[44] High molecular weight lipoproteins such as VLDL and LDL move more slowly as the pore size diminishes, and thus remain close to the point of application. Whereas low molecular weight lipoproteins travel further through the gel and are well separated.

The turbidity of polyacrylamide gels depends on both the quantity of monomers (T = acrylamide + cross-linking monomer) and the amount of cross agent as a percentage of the total monomers (C). When T is 30%, C must be lowered to 4% in order to prevent the opalescence of the gel based on the equation proposed by Margolis and Wrigley.[44]

In the lipoprotein profiles obtained from ultracentrifugation of lipemic serum samples, HDL_2 and HDL_3 were found to migrate broadly in the gel. Two hundred µl and 15 µl of HDL isolated from ultracentrifugation and precipitation methods, respectively, were applied to the gel. In order to avoid the band broading observed, it would be best to use a more concentrated HDL sample from ultracentrifugation so that a smaller volume of HDL can be used for concentration gradient gel electrophoresis. It is well documented that HDL classes are the smallest molecules among lipoproteins and the diffused band observed with the HDL subclasses in concentration

gradient gel electrophoresis can be explained by the ellipsoidal shape of these molecules.[45] Furthermore, the heterogeneity in size (70-150 Å) is also well documented even though they are generally classified as α-lipoproteins in paper electrophoresis.[46]

When serum samples were applied directly to the 4 to 15% gradient gel slab and were subjected to electrophoresis, several protein bands were found to exist in 4 to 7% range. These protein bands most likely belong to the LDL class since a similar report was made by Bautovich et al.[47] in which they found proteins of the LDL class extended from the 5 to 7% region of the gradient gel.

In the HDL_2 region of the gel, two protein bands which were always observed with normal serum samples, were found to have a great degree of variation among lipemic samples. However, the nature of these protein bands and the causes of the variation are unclear. It is felt that further effort should be made to investigate these bands because the result may provide important information about the types of abnormality in these lipemic samples.

The technique developed in this study was applied to the investigation of several lipemic samples. Twelve lipemic samples studied were shown to have a reduction or total absence of HDL_2. Two other lipemic specimens were found to have a reduction in HDL_3. All the lipemic sera were obtained from patients with various cardiovascular diseases and with a total triglycerides 100 mg/dl or more above normal range (10-190 mg/dl). The observation of the variation of HDL_2 in these patients is consistant with the report by Anderson et al.[28] in which they indicate that HDL_2 concentration various in these patients while the HDL_3 concentrations remained constant.

However, the reduction of HDL_3 observed in the two patients deviates from this hypothesis. This may indicate the need for further quantitative investigation of the HDL subclasses for verification of the validity of the hypothesis of Anderson and co-workers.

With the lipoprotein profile developed from the modified single ultracentrifugation and the concentration gradient gel electrophoresis, the quantitation of the HDL subclasses, HDL_2 and HDL_3, can thus be easily made. Although the basic technique has been laid out, additional work needs to be done to further improve the usefulness of this procedure. As with any analytical technique, sampling is a critical factor affecting the ultimate results of the experiment. Care should be taken in the future quantitative analysis of HDL subclasses. Care should also be taken in specimen handling and storage. It has been reported[48] that the serum samples for lipoprotein analysis should be stored at $4^{o}C$ and should be analyzed within 3 to 4 days. Storage of serum samples at $4^{o}C$ for 7 days had resulted in a net decrease of HDL_2 in the same samples.

The fact that HDL is heterogeneous in terms of density is well appreciated. It has been suggested[49] that HDL subclasses play a role in the catabolism of VLDL. In vitro studies of lipolysis of triglyceride-rich lipoproteins, several components such as lipids, cholesterol and apoproteins of the surface monolayer were transferred to HDL_3 resulting in a particle closely resembling HDL_2 in density. The reduction of HDL subclasses in lipemic samples may be due to the abnormality of the HDL subclass surface components necessary for proper transfer of lipids and apoproteins from VLDL, or may be due to a defective external enzyme system which is responsible for

this transfer. It is felt that future effort should be made to investigate the protein constituent of HDL subclasses of these lipemic patients. Quantitative determination of HDL subclasses along with the component apoproteins, especially apoproteins C and E should be made. The quantitative relationship between HDL subclasses and their phospholipids, unesterified cholesterol and cholesterol ester content should also be further studied for a better understanding of HDL metabolism.

REFERENCES

1. Forte, T. and Nicholas, A. V. 1972. Adv. Lipid. Res. 10:1-28.

2. Richard, J. M., Goldstein, J. L., and Brown, M. S. "Lipoprotein and lipid transport" in Metabolic Control and Disease ed. Bondy, P. K. and Rosenberg, L. E. (W. B. Saunders Company Press, 1980), Ch. 7, p. 276.

3. McFarlane, A. S. 1935. Biochem. J. 29:1175.

4. Rudel, L. L., Lee, J. A., Morris, M. D., and Felts, J. M. 1974. Biochem. J. 139:89-95.

5. Havel, R. J., Eder, H. A., and Bragdon, J. H. 1955. J. Clin. Invest. 34:1345-1353.

6. Hinton, R. H., Kawalski, A. J., and Mallinson, A. 1973. Clin Chim Acta 44:267-270.

7. Narayan, K. A., Narayan, S., and Kummerow, F. A. 1965. Nature (London) 205:246-248.

8. Noble, R. P. 1969. J. Lipid. Res. 9:693-700.

9. Fletcher, M. J. and Styliou, H. A. 1970. Clin. Chem. 16:362.

10. Burstein, M., and Scholnick, H. R. 1973. Adv. Lipid. Res. 11:67-108.

11. Albers, J. J., Bachorik, P. S., Wood, P. D., Steiner, P., Dempsey, M., Kuba, K., Warnick, R., and Karlsson, L. 1976. Clin. Chem. 22:1827-1834.

12. Meyers, L. H., Phillips, N. R., and Havel, R. J. 1976. J. Lab. Clin. Med. 88:491-505.

13. Goldstein, J. L., and Brown, M. S. 1976. Curr. Top. Cell Reg. 11:147-181.

14. Goldstein, M., and Brown, M. S. 1977. Ann. Rev. Biochem. 46: 897-930.

15. Havel, R. J., Shore, V. G., Shore, B., and Vier, D. M. 1970. Circ. Res. 27:597-600.

16. Fielding, C. J., Shore, V. G., and Fielding, P. E. 1972. Biochem. Biophys. Res. Commun. 46:1493-98.

17. Soutar, A. K., Garner, C. W., Wordean-Baker, H., Sparrow, J. T., Jackson, R. L., Gotto, A. M., and Smith, L. C. 1975. Biochemistry 14:3057-3064.

18. Fredrickson, D. S., Levy, R. I., and Lees, R. S. 1967. N. Engl. J. Med. 276:34-44, 94-103, 148-156, 273-281.

19. Turner P., Miller, N., Chrystie, I., and Lewis, B. 1979. Lancet 1:645.

20. Miller, G. J., and Miller, N. E. 1975. Lancet 1:16.

21. Rhoads, G. G., Culbrandsen, C. L., and Kagan, A. 1976. New Engl. J. Med. 294:293.

22. Manual of Laboratory Operations, Lipid Research Clinical Program, Lipid and Lipoprotein Analysis. DHEW Publication No. (NIH) 75-628, May 1974.

23. Albers, J. J., Warnick, G. R., and Smith, L. 1978. Clin. Chem. 24:853-856.

24. Gofman, J. W., Delalla, O., Glazier, F., Freeman, N. K., Lindgren, F. T., Nichols, A. V., Strisower, B. and Tamplin, A. R. 1954. Plasma 2:414-484.

25. Patsch, J. R., Stailer, S., Kostner, G., Sandhofer, F., Holasek, A. and Braunsteiner, H. 1974. J. Lipid, Res. 15: 356-366.

26. Kostner, G. M., Patsch, J. R., Saiter, S., Holasek, A., and Braunsteiner, H. 1974. Env. J. Biochem. 45:611-621.

27. Tall, A. R., and Small, D. M. 1978. N. Eng. J. Med. 299: 1232-1236.

28. Anderson, D. W., Nichols, A. V., Pan, S. S., and Lindgren, F. T. 1978. Atherosclerosis 29:161-179.

29. Castelli, W. P., Doyle, J. T., Gordon, T., Hanes, C. G., Hjortland, M. L., Hulley, S. B., Kaga, A., and Zuker, W. J. 1977. Circulation 55:767-772.

30. Warnick, G. R., and Alber, J. J. 1978. J. Lipid Res. 19:65-76.

31. Chapman, M. J., Goldstein, S., Lagrange, D., and Laplaud, M. P. 1981. J. Lipid Res. 22:339-358.

32. Hycel, Inc. For the quantitation determination of total serum protein. Lit. No. 5773, 1976.

33. Weber, K., and Osborn, M. 1969. J. Biol. Chem. 244:4406-4412.

34. Pratt, A. J., and Dangerfield, N. G. 1969. Clin. Chem. Acta. 23:189-201.

35. Scanu, A. M. 1972. Biochem. Biophys. Acta. 265:471-508.

36. Scanu, A. M., and Krusky, A. W. In International Encyclopedia of Pharmacology and Therapeutics, E. Masoro, editor. Plenum Press, New York. 21-28.

37. Melish, J. S., and Waterhouse, C. 1972. J. Lipid. Res. 13: 193-200.

38. Albers, J. J., and Aladjem, F. 1971. Biochemistry 10:3436.

39. Rudel, L. L., Greene, D. G., and Shah, R. 1979. J. Lipid. Res. 20:55-65.

40. Farrell, E. C. 1979. Clin. Chem. 25:1146.

41. Cheung, M. C., and Albers, J. J. 1979. J. Lipid. Res. 20: 200-207.

42. Scanu, A. M., Lagocki, P., and Chung, J. 1980. The New York Academy of Sciences, p. 166.

43. Fainaru, M., and Glangeand, M. L. 1975. Biochem. Biophys. Acta. 386:432-443.

44. Margolis, J., and Wrigley, C. W. 1975. J. Chromatogr. 106: 209.

45. Lindgren, F. T., and Nichols, A. V. 1960. In The Plasma Proteins, Vol. II, F. W. Putman, ed. Academic Press, New York. p. 43.

46. Ritter, M. C., and Scanu, M. 1979. J. Biol. Chem. 254:2517-2525.

47. Bautovich, G. J., Dash, M. J., Hensley, W. J., and Turtle, J. R. 1973. Clin. Chem. 19:415-418.

48. Gidez, L. I., Miller, G. J., Burstein, M., and Eder, H. A. "Analyses of plasma high density lipoprotein subclasses by a precipitation procedure:correlation with preparation and analytical ultracentrifugation" in Report of the High Density Lipoprotein Methodology Workshop. Ed. Lippel, K. National Institute of Health Publication No. 79-1661, August 1979.

49. Patsch, J. R., Gotto, A. M., Olivecrona, T., and Eisenberg, S. 1978. Proc. Natl. Acad. Sci. USA 75:4510-4523.