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A Method for the Comparison of HDL Containing Apoprotein E and HDL Cholesterol

Steven A. Knizner
Old Dominion University

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A METHOD FOR THE COMPARISON OF HDL CONTAINING APOPROTEIN E AND HDL CHOLESTEROL

by

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B.S. May 1981, James Madison University

A Thesis Submitted to the Faculty of
Old Dominion University in Partial
Fulfillment of the Requirements
for the Degree of

MASTER OF SCIENCE

CHEMISTRY

OLD DOMINION UNIVERSITY
May 1984

Approved by:

James H. Yuan (Director)

John D. Van Norman

Patricia Pleban

ABSTRACT

A METHOD FOR THE COMPARISON OF
HDL CONTAINING APOPROTEIN E
AND HDL CHOLESTEROL

Steven A. Knizner
Old Dominion University, 1984
Director: Dr. James H. Yuan

Human serum high density lipoproteins (HDL) were isolated by preparative salt density gradient ultracentrifugation and analyzed further by heparin-sepharose affinity chromatography. Separation of the major classes of serum lipoproteins was achieved after a single ultracentrifugation on a salt density gradient at 272,000 x g for 36 hours in a swinging bucket rotor. After isolation, the total HDL was subdivided into HDL containing apoprotein E and HDL without this apoprotein by heparin-sepharose affinity chromatography. Relative amounts of these subclasses were then calculated.

HDL-cholesterol was measured after precipitation of LDL and VLDL by phosphotungstic acid and magnesium. The statistical relationship between HDL containing apoprotein E and HDL-cholesterol was then examined.

DEDICATION

The author would like to dedicate this work to his mother,

Lore M. Knizner

for her love and help in solving life's many problems.

The author also dedicates this work to his father,

Anthony A. Knizner

though death cheated him of his opportunity to watch his sons become men, he will always be loved, and never forgotten.

This work is also dedicated to the rest of my terrific family; my sister Barbara, and her beau Larry Potoski; my brother Mike, his wife Sandy and their children, Jeffrey and Lindsay; my brother Marty and his wife Paula, and of course my dear friend James Harris

ACKNOWLEDGMENTS

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Thanks are also given to my fellow graduate students, Ed, Sau, Jean, Jia-Hwei, Ying-Lin, Chin Chan, Dave, Robbo, Mark, Sid, Ki, Steve and Mrs. Mei.

Of course thanks must be given to Mr. John Hill, were it not for him, none of our instruments would ever function, and our softball season would have been a failure. Thanks are also extended to Dr. Charles Bell and Dr. Frank E. Scully, for their patience in teaching me NMR Spectroscopy. Dr. Ken Brown is also sincerely thanked for his advice and help in performing infra-red spectroscopic analyses.

Last, but certainly not least, I thank my friends Gordon Melrose, Wendell Lawrence, Laurie and Kurt Neitzke, and Bill Peterschmidt for making life in Norfolk a little more bearable. Roy, Demon and Tasha are also remembered for the fun times we had together.

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INTRODUCTION

Lipids, due to their inherent hydrophobic nature, are transported in the serum in association with various proteins. Polar lipids, such as free fatty acids, are predominantly bound to small proteins, such as albumin. Non-polar lipids, such as cholesterol esters and triglycerides, are transported in large macromolecular lipid-protein complexes, referred to as lipoproteins. The individual proteins of these complexes are termed apoproteins. The non-polar lipids form the core of the spherical lipoprotein particle, shielded from their aqueous environment by a mixed monolayer of polar lipids and apoproteins. The association between apoproteins and lipids is through hydrophobic forces (1).

When separated by ultracentrifugation, which exploits differences in bouyant densities, lipoproteins fall into four discrete classes. These classes are chylomicrons ($d < 0.95$ g/mL), very low density lipoproteins ($d = 0.95$-1.006 g/mL), low density lipoproteins ($d = 1.006$-1.063 g/mL), and high density lipoproteins ($d = 1.063$-1.210 g/mL). It is recognized, however, that none of these four lipoprotein classes are homogeneous molecular species. Rather, each class consists of a spectrum of particles of differing composition, size and density (2). Densities of the lipoproteins vary due to differences in the lipid to protein ratio, which may range from 99:1 in chylomicrons to 1:1 in high density lipoproteins (HDL) (3).

The core of chylomicrons and very low density lipoproteins (VLDL) consists primarily of triglycerides, while cholesterol esters predominate in the core of low density lipoproteins (LDL) and HDL (Table 1). Normally chylomicrons are absent in the post-absorptive state, so most of the

Table 1

Distribution of lipids in serum lipoproteins

TG = triglycerides, CE = cholesterol esters,
C = cholesterol, PL = phospholipids (1).

Lipids (% dry mass)

Class	TG	CE	C	PL
Chylomicrons	80-95	2-4	1-2	3-6
VLDL	45-60	10-15	7-10	15-20
LDL	14	36	8	21
HDL	4	15	5	29

triglycerides are present in VLDL (4).

Five immunochemically different subclasses of apoproteins exist, which may be further subdivided by differences in electrophoretic mobilities. HDL contain predominantly apoproteins AI, AII: apoproteins C and E are also present to a much lesser extent. LDL contains almost exclusively apoprotein B. VLDL has mainly apoproteins B, CI, CII, CIII, and E (Table 2). As may be expected, the different apoproteins have different functions. Apoprotein AI is a cofactor of the enzyme lecithin-cholesterol acyl transferase (5). Apoprotein CII is an activator of the enzyme lipoprotein lipase (LPL) (6). Apoproteins B and E are involved in recognition of lipoproteins by cell surface receptors (3).

The different classes of lipoproteins perform various functions in lipid metabolism. The primary function of chylomicrons is to transport exogenous triglycerides from the small intestines to peripheral tissues. VLDL transport endogenously synthesized triglycerides from the liver to extrahepatic tissues. Both of these lipoproteins interact with lipoprotein lipase, situated on the surface of capillary endothelia, catalyzing the hydrolysis of triglycerides to form monoglycerides and free fatty acids (7). There is a close relationship between the catabolism of VLDL and the formation of LDL. The liver does not secrete LDL directly into the circulatory system. Instead, virtually all of the LDL is derived from the metabolism of VLDL (8).

LDL are metabolized by either of two pathways, referred to as the LDL receptor pathway or the scavenger pathway. The LDL receptor pathway is more properly known as the apoprotein B - apoprotein E receptor pathway (9). When they need cholesterol, certain cells are capable of

Table 2

Apoprotein composition of major serum lipoprotein classes (1).

Class	Apoproteins
Chylomicrons	B, CI, CII, CIII
VLDL	B, CI, CII, CIII, E
LDL	B
HDL	AI, AII, E

synthesizing cell surface receptors specific for either of these apoproteins. Uptake of LDL via this high affinity receptor is associated with a decrease in endogenous cholesterol biosynthesis, and an increase in cholesterol esterification, followed by a subsequent decrease in the number of cell surface receptors (10).

The scavenger pathway is receptor independent and represents a non-specific catabolism of LDL. LDL are ingested by cells via bulk fluid endocytosis, in a manner analogous to the clearance of albumin and other serum proteins (1).

HDL appear to be synthesized in the liver and released into circulation as a bilayer disc of phospholipids, cholesterol, apoproteins AI, E, and C. This nascent HDL disc is acted upon by lecithin-cholesterol acyl transferase, which esterifies most of the cholesterol present. As cholesterol esters are formed, they migrate to the center of the particle, due to their hydrophobic nature. This results in the spherical shape of the mature HDL (11).

Information concerning the removal sites of HDL is inconclusive. In several studies using HDL labeled with radioactive iodine, it appears that HDL is predominantly removed by hepatocytes via lysosomal degradation (12, 13). However, others have found that the liver is not the exclusive site of HDL removal, the intestines (14), and kidney (15) have also been shown to contribute to HDL uptake.

Recently, a vast amount of epidemiological data has established that the level of HDL is inversely correlated with the occurrence of atherosclerosis (16-20). Several mechanisms by which HDL serves to slow the atherogenic process have been postulated. Miller and Miller (16) proposed that HDL facilitates the uptake of cholesterol from peripheral

tissues and its transport to the liver for subsequent catabolism and excretion. This represents a reverse transport process for cholesterol. Carew and coworkers (21) observed that HDL and LDL compete for cell surface receptor binding sites of arterial smooth muscle cells. However, HDL is internalized and degraded to a much lesser extent than LDL. Therefore, HDL inhibits the uptake and subsequent metabolism of LDL and suppresses the net increase in cell sterol content induced by LDL (21). It has been previously shown that smooth muscle cells of the artery play a key role in the atherogenic process (22).

Based upon density, HDL can be further subclassified into two groups designated HDL_2 (d = 1.063-1.125 g/mL), and HDL_3 (d = 1.125-1.210 g/mL). The significance of measuring HDL subclasses has been noted by Anderson and coworkers, who have shown that HDL_3 levels are fairly constant, and that HDL_2 levels account for most of the differences in total HDL concentration (23). Total HDL is reduced in patients with coronary heart disease, and the major reduction is in HDL_2 (24, 25). Anderson goes on to suggest that HDL_2 may be the critical factor in accounting for the inverse correlation between HDL concentration and atherosclerosis (23).

Mahley and coworkers (24) have observed that only HDL_2 contains apoprotein E, while HDL_3 lacks this key apoprotein. The metabolic role of apoprotein E and apoprotein B, as previously mentioned, appears to be related to the transport of cholesterol between lipoproteins and cells. Both apoprotein E and apoprotein B interact with the same high affinity receptor on the cell surface of fibroblasts and smooth muscle cells (25). Of the total HDL only 5-17% contain apoprotein E (24). However, even if apoprotein E content represents less then 10% of the individual HDL

apoprotein, these lipoproteins exhibit an even greater receptor binding activity than LDL (26). The potency of the HDL fraction containing apoprotein E with respect to receptor binding results from the 10 to 100-fold enhanced binding activity of apoprotein E as compared to apoprotein B containing LDL (27).

Hepatic parenchymal cells also take up apoprotein E HDL in a similar receptor mediated process. HDL containing apoprotein E is rapidly removed from the serum and may represent one of the reverse cholesterol transport vehicles (25).

Isolation of HDL by ultracentrifugation is expensive, time consuming, and requires highly skilled technicians, making it impractical for routine hospital laboratory analysis. Methods involving the preciptation of apoprotein B containing lipoproteins, and subsequent quantitation of HDL cholesterol remaining in the supernatant solution have been more widely used clinically. Precipitation techniques generally employed make use of polyanions and divalent cations. Examples include; heparin and Mn^{+2}, dextran sulfate and Mg^{+2}, polyethylene glycol, and phosphotungstate and Mg^{+2} (28). Of all these methods, precipitation with phosphotungstate and Mg^{+2}, as originally described by Burstein (29), is one of the most widely used. This method is inexpensive, simple, fast, and solutions of phosphotungstate are stable over extended periods of time (30). The heparin and Mn^{+2} method will not be used, since the heparin may precipitate HDL containing apoprotein E, and since Mn^{+2} interferes with enzymatic cholesterol assays (31).

STATEMENT OF PROBLEM

High density lipoproteins have been shown to be a negative risk factor for atherosclerosis (16-20). The HDL_2 subclass seems to be most important in this protective function (23). Previous workers (24) have demonstrated that only HDL_2 contains apoprotein E. Apoprotein E is involved in the transport of cholesterol between lipoproteins and cells. Also, apoprotein E has a 10 to 100 fold greater binding affinity than apoprotein B for LDL receptors (26). Therefore, apoprotein E appears to be involved in preventing atherosclerosis, by reducing the increase in cell sterol content associated with the ingestion of LDL.

There is a need for an accurate method to measure the proportion of HDL containing apoprotein E. In addition, since precipitation techniques for the quantification of HDL-cholesterol are becoming increasingly common in the clinical laboratory, the relationship between HDL-cholesterol and HDL containing apoprotein E merits close examination.

EXPERIMENTAL TECHNIQUES

A. Materials

Sigma Chemical Company supplied the following materials: trizma base, cyanogen bromide, acrylamide, N,N'-methylene-bis-acrylamide, heparin (grade 1, sodium salt), sodium dodecyl sulfate (SDS), Coomassie Brilliant Blue R-250, potassium bromide (grade 1), sodium chloride (Sigma grade), and bovine serum albumin. Sepharose 6-B was purchased from Pharmacia Fine Chemicals. Bromophenol blue and N,N,N',N'-tetramethylethylenediamine (TEMED) were obtained from Eastman Kodak Chemical Company. Phosphotungstic acid (Fisher certified), manganese chloride (Fisher certified ACS) and magnesium chloride (Fisher certified ACS) were purchased from Fisher Scientific. All other chemicals used were reagent quality and purchased commercially.

B. Equipment

Ultracentrifugations were performed in a Beckman L5-65 Preparative Ultracentrifuge equipped with either a SW-60Ti or SW-27 rotor. Fractionation of density gradients was accomplished through use of an ISCO Model 185 Density Gradient Fractionator, connected to an ISCO Model UA-5 Absorbance Monitor and a Gilson Model FC-100 Microfraction Collector. All refractive indices were measured using a Bausch and Lomb Abbe-3L Refractometer.

Concentration gradient slab gels were cast using a locally fabricated gradient mixer. The electrophoresis chamber was also locally manufactured. An E-C Apparatus Corporation Model 400 Power Supply was used for all electrophoretic separations. A Cary 219 UV-Visible Spectrophotometer equipped with a Gel Scanner and interfaced with an Apple II Computer was used for densiometric scans of polyacrylamide gels. The gel scanning

program used was developed by the manufacturer.

BioRad Econo-Columns (inner diameter 1 cm, length 20 cm) connected to an ISCO UA-5 Absorbance Monitor and a Gilson Model FC-100 Micro-fraction Collector, were used in all heparin-sepharose affinity chromatography separations. When concentration of samples were required, it was accomplished through use of an Amicon Model 12 Ultrafiltration Cell with a Diaflo PM-10 Ultrafiltration Membrane (molecular weight exclusion 10,000 daltons).

Dr. Hsiu of DePaul Hospital, Norfolk, Va., kindly permitted the use of a Technicon SMAC for the quantitation of HDL-cholesterol.

C. Methods

1. Serum Samples

Serum samples were obtained from the daily inpatient workload of Maryview Hospital Laboratory. All samples were fasting specimens, and were free of chylomicrons and turbidity, as visually confirmed. No diagnostic information was available for the samples. By virtue of this fact, and the origin of the serum samples, the results presented clearly cannot be thought of as representing a "normal", healthy population.

Serum specimens were stored at $4^{o}C$, and were processed within three days. It has been previously shown (32) that storage of serum at low temperatures is acceptable before HDL-cholesterol determination. Bachorik et. al. (33) recommend that serum be kept at $2\text{-}4^{o}C$ in the dark, and that lipoprotein analyses be performed as soon as possible.

2. Isolation of HDL by Density Gradient Ultracentrifugation

A density gradient ultracentrifugation procedure, first developed by Chapman et. al. (34) and modified by Chin (35), was used to obtain HDL from serum. A discontinuous density gradient was first constructed.

After ultracentrifugation a linear density gradient was formed, which allowed for the separation of HDL from other lipoproteins. The density of serum was first adjusted to d = 1.210 g/mL by addition of solid potassium bromide (KBr). The discontinuous density gradient was then formed, at ambient temperature, in 4 mL polyallomer centrifuge tubes (1 cm diameter x 6 cm length). Into the bottom of the centrifuge tube, 0.64 mL of a d = 1.240 NaCl-KBr solution was pipeted. Then the following solutions were gently layered over each other: 0.96 mL of serum at d = 1.210 g/mL, 0.64 mL of d = 1.063 g/mL NaCl-KBr solution, 0.80 mL of d = 1.019 g/mL NaCl-KBr solution, and 0.96 mL of d = 1.006 g/mL NaCl solution. A control tube was used to check the density gradient profile with a NaCl-KBr solution of d = 1.210 g/mL replacing the serum specimen. Refractive indices of all solutions were measured prior to ultracentrifugation and following fractionation. Table 3 gives the densities, amounts, and refractive indices of solutions used to construct the discontinuous density gradient.

Following construction of the discontinuous density gradient, the tubes were centrifuged at 272,000 x g average for 36 hours at 15^{o}C in a Beckman L5-65 Preparative Ultracentrifuge. No braking was used at the end of the centrifugation.

The centrifuge tube contents were fractionated by use of an ISCO Model 185 Density Gradient Fractionator connected to an ISCO UA-5 Absorbance Monitor, monitoring absorbance at 280 nm, and a Gilson Model FC-100 Microfraction Collector. Fractions of 0.5 mL were collected. The refractive indices of all fractions obtained from the control tube were measured on a Bausch and Lomb Refractometer.

The fractions containing HDL (d = 1.063-1.210 g/mL) were then

Table 3

Description of solutions used to construct discontinuous density gradient.

Density (g/mL)	Amount (mL)	Refractive Index (25°C)
1.006	0.96	1.3371
1.019	0.80	1.3394
1.063	0.64	1.3464
1.210	0.96	1.3697
1.240	0.64	1.3746

dialyzed against 0.20 mol/L NaCl, 1.0 mmol/L NaN_3, 0.3 mmol/L EDTA solution, pH 8.9, at 4^oC for 16 hours, with two changes of dialysis buffer.

3. Concentration Gradient Polyacrylamide Electrophoresis

a. Reagents

Acrylamide Solutions: 144.0 g/L acrylamide, 6.0 g/L N,N'-methylene-bis-acrylamide (T = 15%, C = 4%).

Gel Buffer: 0.09 mol/L trizma base, 0.08 mol/L boric acid, 0.8 mmol/L sodium azide, 0.3 mmol/L EDTA, pH 8.35.

b. Preparation of Concentration Gradient Slabs

Concentration gradient slab gels were cast using glass plates with spacers and sealing gaskets (135 mm x 150 mm x 10 mm). A linear 4% to 15% acrylamide gradient was cast into the gel mold using a gradient mixer. Because ambient laboratory temperature greatly affects the rate of polymerization, all acrylamide solutions were kept on ice prior to pouring the gel. After pouring the gel, sample wells were formed by use of a polystyrene comb. Polymerization was completed in one to two hours. The sealing gasket and well maker were removed, and the upper and lower gel surfaces were rinsed with gel buffer prior to electrophoresis.

c. Sample Preparation

One drop of glycerol and 10 uL of 0.05% bromophenol blue tracking dye were added to each serum and HDL sample. For electrophoresis, 6 uL of whole serum and 100 uL of HDL sample obtained by ultracentrifugation were applied to the sample wells.

d. Electrophoresis

Degassed gel buffer was placed in the upper and lower

chambers of the electrophoresis apparatus. Filter paper served as a bridge between the upper buffer chamber and the top of the gel. The electrophoresis was performed at 10 mA constant current, until the tracking dye reached the lower edge of the gel, approximately 18 hours.

4. Sodium Dodecyl Sulfate (SDS) Disc Gel Electrophoresis

a. Reagents

Stock Acrylamide Solution: 222.0 g/L acrylamide, 6.0 g/L N,N'-methylene-bis-acrylamide (T = 22.8%, C = 2.6%).

Gel Buffer: 7.8 g/L $NaH_2PO_4 \cdot H_2O$, 38.6 g/L $Na_2HPO_4 \cdot 7H_2O$, 2.0 g/L SDS.

Stacking Gel Solution: 4 mL gel buffer, 1.4 mL stock acrylamide, 10 µL TEMED, 300 uL of 100 g/L ammonium persulfate (APS).

Running Gel Solution: 15 mL gel buffer, 13.5 mL stock acrylamide, 10 µL TEMED, 250 uL of 100 g/L APS.

b. Preparation of SDS Gels

Gels were poured into glass tubes 10 cm long, with an inner diameter of 0.6 cm. All solutions were thoroughly degassed prior to use. The running gel was approximately 7.0 cm long, with a 1.0 cm stacking gel.

c. Preparation of Sample

Prior to electrophoresis, it was necessary to delipidate HDLs. This was accomplished by two 12 hour extractions with freshly prepared ether-ethanol solution (3:1 v/v) at $4^{o}C$. Typically, 1.0 mL of HDL solution isolated from density gradient ultracentrifugation was delipidated using 40 mL of the ether-ethanol solution. Lipids were extracted into the organic phase, while the apoproteins formed percipitates. The precipitated apoproteins were then dried under nitrogen gas. The dried apoprotein residues were then dissolved in 10 mmol/L tris buffer, pH 8.2.

Samples were mixed with an equal amount of 10 g/L SDS and heated to 95°C for five minutes. For each sample 10 µL of 0.05% bromophenol blue and a drop of glycerol were added and mixed well prior to electrophoresis.

d. Electrophoresis

Gel buffer was diluted 1:1 with deionized water and placed in the upper and lower buffer chambers. Electrophoresis was carried out at 6 mA per gel until the tracking dye reached the bottom of the gel (6-8 hours).

5. Staining Procedures

a. Reagents

Protein Staining Solution: 1.25 g Coomassie Brilliant Blue R-250, 454 mL of 50% methanol, and 46 mL of glacial acetic acid.

Destaining Solution: 25% (v/v) ethanol, 8% (v/v) acetic acid.

Gel Preserving Solution: 5% (v/v) acetic acid.

b. Procedure

Slab gels and gel rods were stained at room temperature for two hours in the protein stain. Gels were destained by diffusion using the destaining solution, until the background was colorless.

6. Gel Scanning

Gel scanning was accomplished using a Cary 219 UV-Visible Spectrophotometer equipped with a gel scan accessory and interfaced with an Apple II computer. Absorbance was monitored at 555 nm, a maximum for Coomassie Brilliant Blue R-250.

7. Heparin-Sepharose Affinity Chromatography

a. Reagents

Sepharose-6B, 0.1 mol/L $NaHCO_3$, pH 9.5, 5.0 g CNBr in

5.0 mL CH_3CN, 40 mL 0.1 mol/L $NaHCO_3$ containing 2.32 g NaCl and 0.50 g heparin (grade 1, sodium salt), 1 mol/L $NaHCO_3$ pH 8.0 and, 0.1 mol/L $NaCH_3COO$, pH 5.0.

b. Procedure for Coupling of Heparin to Sepharose-6B

1. A 50 mL packed volume of Sepharose-6B was washed with cold deionized water and 0.1 mol/L $NaHCO_3$ buffer, pH 9.5, and filtered with a coarse sintered glass funnel. The gel was then placed in a 50 mL of 0.1 mol/L $NaHCO_3$ buffer, pH 9.5, and stirred gently in a cold water bath at 10-15°C.

2. Then, 5.0 g of CNBr was dissolved in 5.0 mL of CH_3CN and added to the sepharose solution. The pH of this solution was maintained at 11.0 $\pm$ 0.2 by addition of 0.1 mol/L NaOH as necessary.

3. After twenty minutes, the activated sepharose was filtered within 30 seconds and washed first with 500 mL of 0.1 mol/L $NaHCO_3$ buffer, pH 9.5, then with 200 mL of cold deionized water. All of the washings were completed within 2 minutes. The sepharose was then filtered to a semi-dry condition.

4. A 40 mL solution of $NaHCO_3$ buffer, pH 9.5, containing 2.32 g NaCl and 0.50 g heparin, was added immediately to the activated sepharose. The mixture was shaken vigourously for one minute and then shaken overnight at 4°C.

5. The gel was then filtered and unbound heparin was washed off with 200 mL of 1 mol/L $NaHCO_3$, pH 8.0. The gel was then washed with one liter of 0.1 mol/L sodium acetate at pH 5.0, to hydrolyze residual reactive groups on the sepharose. Finally, the gel was washed with 0.05 N NaCl, 0.005 mol/L tris, pH 7.4.

c. Column Preparation and Elution Procedures

BioRad Econo-Columns (inner diameter 1 cm, length 20 cm) were used for all heparin-sepharose affinity chromatography procedures. Before the affinity medium was packed in the column, it was thoroughly degassed. All elution solutions were also degassed prior to use. Absorbance of eluates was monitored at 280 nm. One milliliter fractions of the column were collected with the fraction collector. The sample applied to the top of the gel was 1.0 mL of HDL isolated from density gradient ultracentrifugation. The sample was first eluted with 0.05 N NaCl, 0.025 mol/L $MnCl_2$, 0.005 mol/L tris, pH 7.4. After the first eluted peak was collected, the elution buffer was changed to 0.095 N NaCl, 0.005 mol/L tris, pH 7.4. Once the second peak was completely eluted, the column was then washed with 1.0 N NaCl, and then re-equilibrated with 0.05 N NaCl, 0.025 mol/L $MnCl_2$, 0.005 mol/L tris, pH 7.4.

8. HDL-Cholesterol Quantification

The method of Burstein et. al. (29), as modified by Lopes-Virella (30) was used for the quantification of HDL cholesterol. Essentially this procedure involves the precipitation of VLDL and LDL by phosphotungstic acid and magnesium, and then quantifying the cholesterol present in the supernatant. As previously mentioned, the heparin-Mn^{+2} precipitation procedure was not used since it may precipitate HDL containing apoprotein E, and since Mn^{+2} interferes with enzymatic methods for cholesterol quantitation (31).

a. Precipitation Reagents: 0.05 mol/L $MgCl_2 \cdot 6H_2O$, 5 mmol/L phosphotungstic acid.

b. Precipitation Procedure: 0.5 mL of serum and 0.5 mL of

precipitation reagent were thoroughly mixed by vortex action. The precipitate was then separated from the supernatant by centrifugation at 1500 x g for 15 minutes. The supernate was immediately removed following centrifugation and subsequently analyzed for cholesterol by use of the Technicon Simultaneous Multiple Analyzer Computer Control (SMAC).

The SMAC was an enzymatic methodology to quantify cholesterol. Cholesterol esterase converts all cholesterol esters to free cholesterol. Cholesterol oxidase oxidizes cholesterol to cholest-4-en-3-one and hydrogen peroxide. The hydrogen peroxide, in the presence of 4-amino-antipyrene and phenol, is acted upon by peroxidase, and a quinonemine dye and water and produced by this reaction. The quinonemine dye is pink in color, and its absorbance at 510 nm is monitored (36).

9. Statistical Methods

Mean values and standard deviations of the data were calculated using standard formulas. Parametric analysis of data was accomplished by using Pearson's correlation coefficient. Nonparametric statistical evaluation of the data was performed using Spearman's rank order correlation test, and calculating a correlation coefficient, rho. Spearman's rho measures the correspondence between the relative rankings of values obtained for two variables, rather than using the numerical values of those variables. The formula used to calculate Spearman's rho is:

$$\text{rho} = 1 - \frac{6(\Sigma(d^2))}{n(n^2-1)} \quad ,$$

where n is the total number of subjects examined, and d is the difference in rankings of the two variables. If two values are tied for a ranking,

each tied value is assigned the average rank available to the tied measurements. That is each observation tied for a given rank is assigned the average of the ranks they would ordinarily occupy (37).

D. Results

1. Isolation of HDL by Density Gradient Ultracentrifugation

As previously noted, a control tube containing a NaCl-KBr solution of d = 1.210 g/mL was run parallel with serum samples. Figure 1 is a plot of density versus fraction number for 18 of these control tubes. One can observe that a linear density gradient was formed following ultracentrifugation of the discontinuous gradient.

Following ultracentrifugation, four yellow bands were visible to the eye. By monitoring the absorbance of ultracentrifuged samples at 280 nm during fractionation four absorption peaks were observed, as shown in Figure 2. The first of these peaks corresponds to the density range in which VLDL is found (d = 0.96-1.006 g/mL), the second LDL (d = 1.006-1.063 g/mL), and the third peak represents HDL (d = 1.063-1.210 g/mL). The fourth broad peak corresponds to a yellow-orange layer found at the bottom of the ultracentrifuge tube, and contains albumin, bilirubin, very high density lipoproteins, and other serum proteins (38-40).

2. Concentration Gradient Electrophoresis

Concentration gradient electrophoresis using a linear 4% to 15% acrylamide gradient was performed on several HDL samples isolated by ultracentrifugation to determine their purity (that is, to check for the presence of VLDL and LDL). Linearity of the acrylamide gradient was established by monitoring the absorbance of the acrylamide at 295 nm in a 1.0 mL flow cell as the solution passed out of the gradient mixer.

Figure 1. Density plotted as a function of fraction number following fractionation of density gradient (n=18).

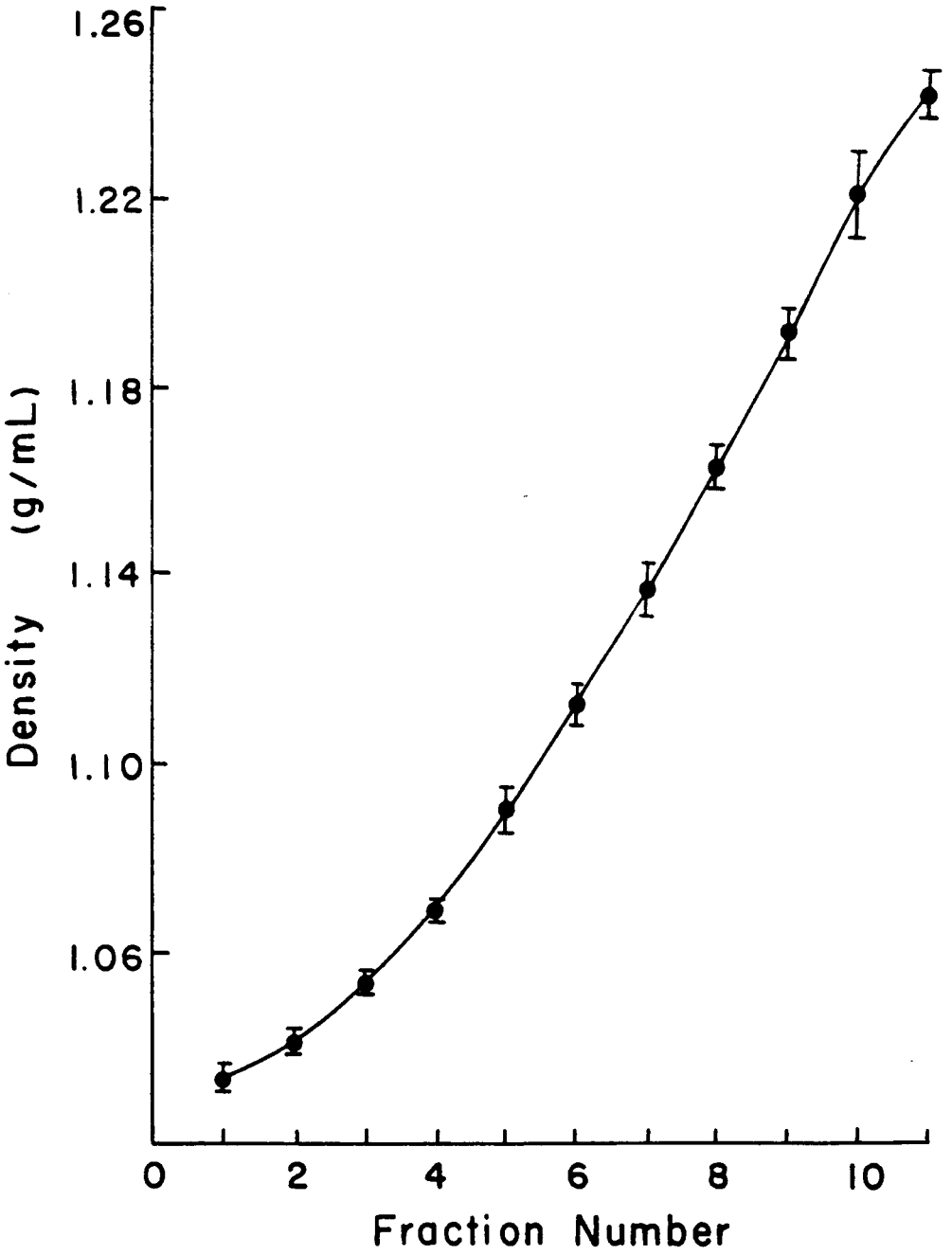

1.26
1.22
1.18
1.14
1.10
1.06
Density (g/mL)
0
2
4
6
8
10
Fraction Number

Figure 2. Lipoprotein profile pattern in density gradient following ultracentrifugation. The solid line (——) indicates lipoprotein profile and the dotted line (-o-o-o-) indicates the density gradient along the tube.

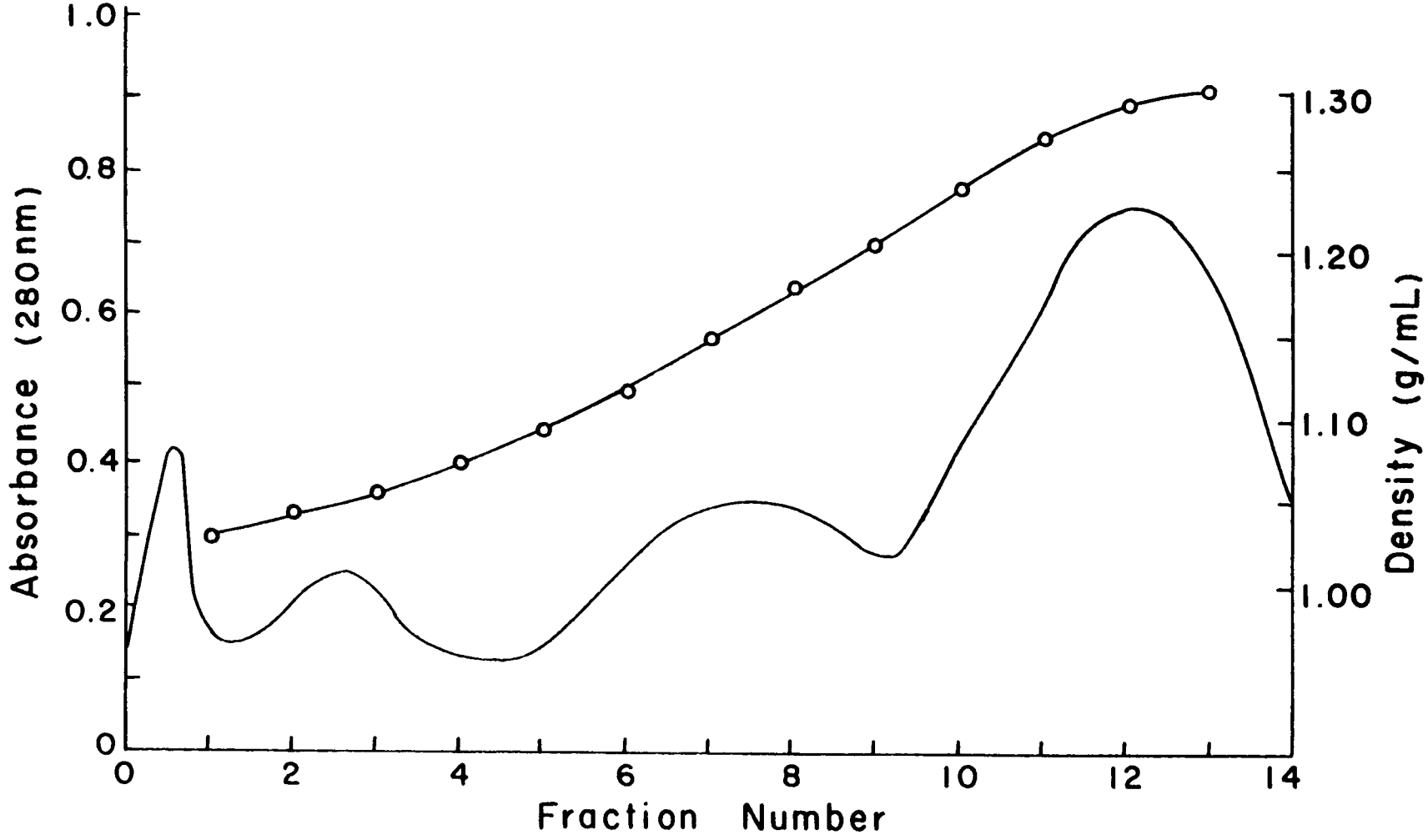

1.0
0.8
0.6
0.4
0.2
0
Absorbance (280nm)
0
2
4
6
8
10
12
14
Fraction Number
1.30
1.20
1.10
1.00
Density (g/mL)

Figure 3 is a photograph of a typical slab gel, stained for protein. The HDL was distributed in the middle of the concentration gradient gel.

3. Heparin-Sepharose Affinity Chromatography

HDL isolated by density gradient ultracentrifugation was applied to a heparin-sepharose affinity column so that the relative amounts of HDL with and without apoprotein E could be calculated. HDL with apoprotein E was bound to the affinity column, while HDL without apoprotein E was not (24). The affinity column was equilibrated with elution buffer for twenty minutes prior to application of sample. After the unbound HDL was eluted, a buffer of higher sale concentration, and without a divalent metal cation, was used to elute the bound HDL. Figure 4 demonstrates a typical elution profile, monitoring absorbance at 280 nm. Relative amounts of HDL present were determined by calculating the area under the absorption peaks.

4. SDS Electrophoresis

HDL obtained by ultracentrifugation, HDL not bound by the affinity column, and HDL bound by the column, were isolated and delipidated by ether-ethanol extraction. It can be seen in Figure 5 that total HDL and HDL bound to the affinity column contained three different types of proteins, corresponding to apoproteins AI, AII, and E, whereas unbound HDL contained only apoprotein AI and AII (24, 27).

Separation of proteins by SDS polyacrylamide gel electrophoresis is dependent upon the molecular weight of the proteins. A plot of log molecular weight versus distance migrated in the gel should yield a straight line (41). Such a plot is shown in Figure 6 using the data from Figure 5. The molecular weights of apoproteins E, AI, and AII

Figure 3. Photograph of concentration gradient slab gel stained for protein (1) whole serum, (2) HDL sample, (3) molecular weight marker, (4) HDL sample, (5) whole serum.

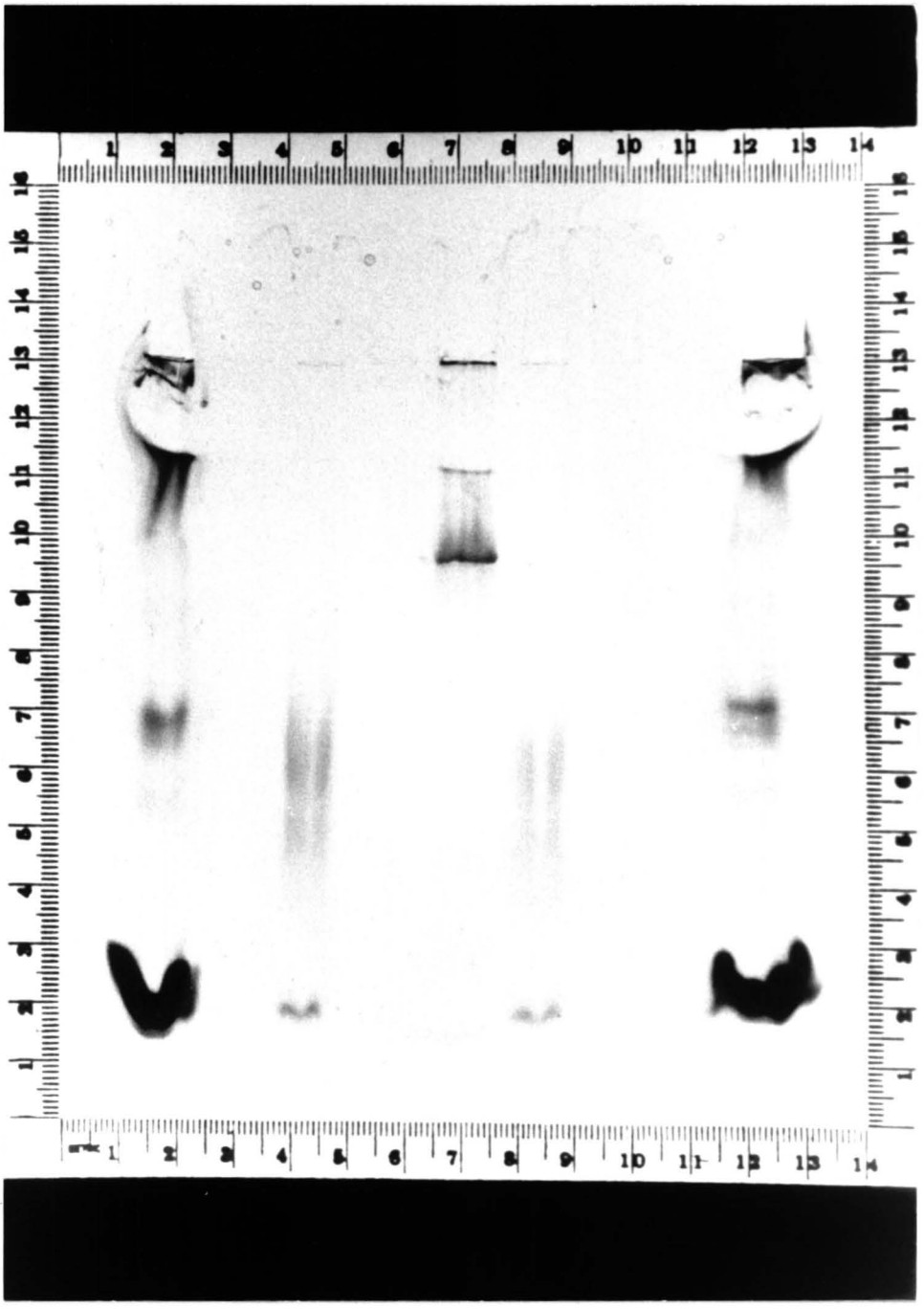


Figure 4. Typical elution profile of HDL from heparin-sepharose affinity column. Arrow indicates change of elution buffer.

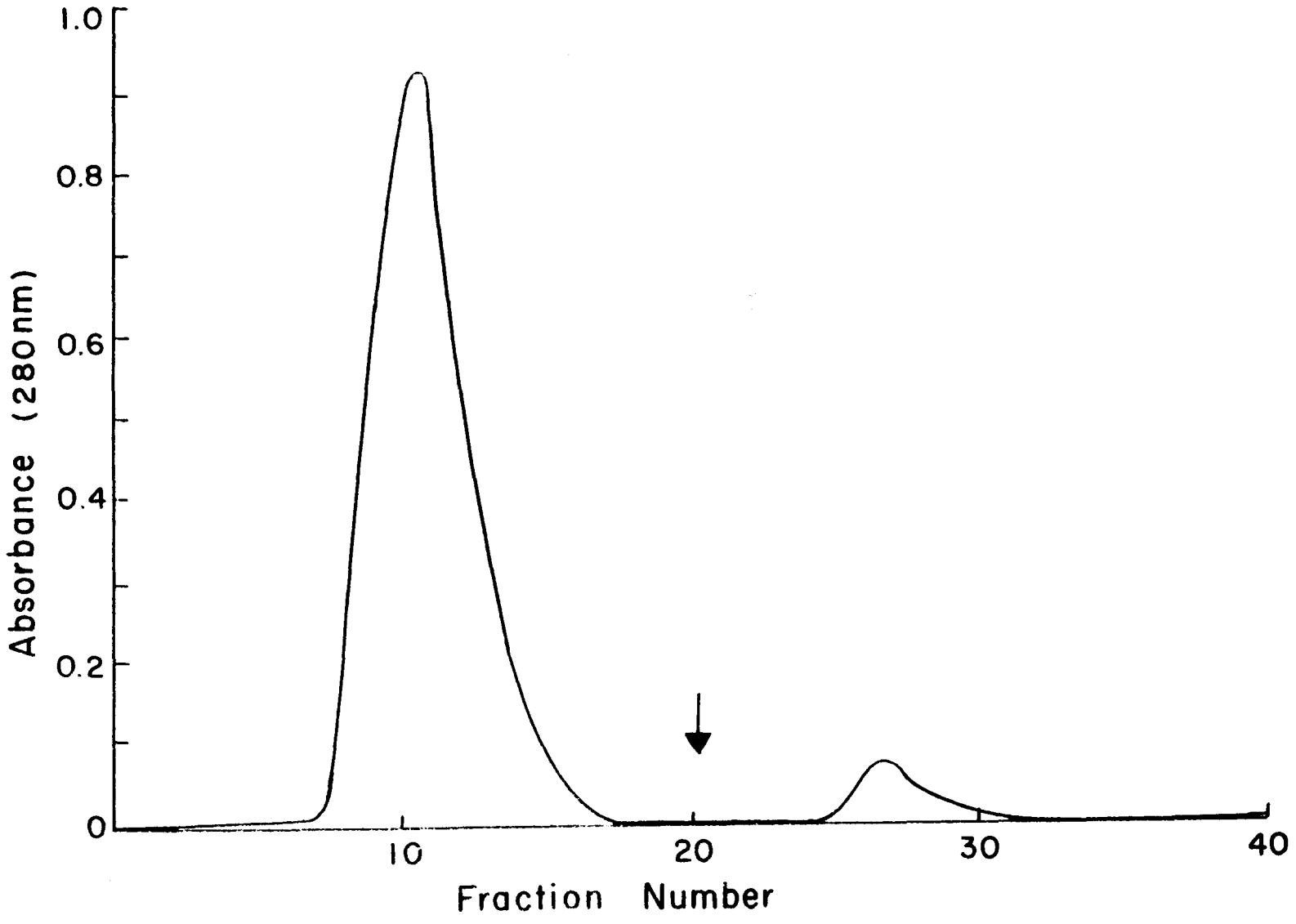

1.0
0.8
0.6
0.4
0.2
0
Absorbance (280nm)
10
20
30
40
Fraction Number

Figure 5. Densiometric scan of SDS electrophoresis of delipidated lipoproteins: (A) total HDL, (B) HDL not bound to affinity column, (C) HDL bound to affinity column. Peak 1, running gel-stacking gel interface; peak 2, apoprotein E; peak 3, apoprotein AI; peak 4, apoprotein AII.

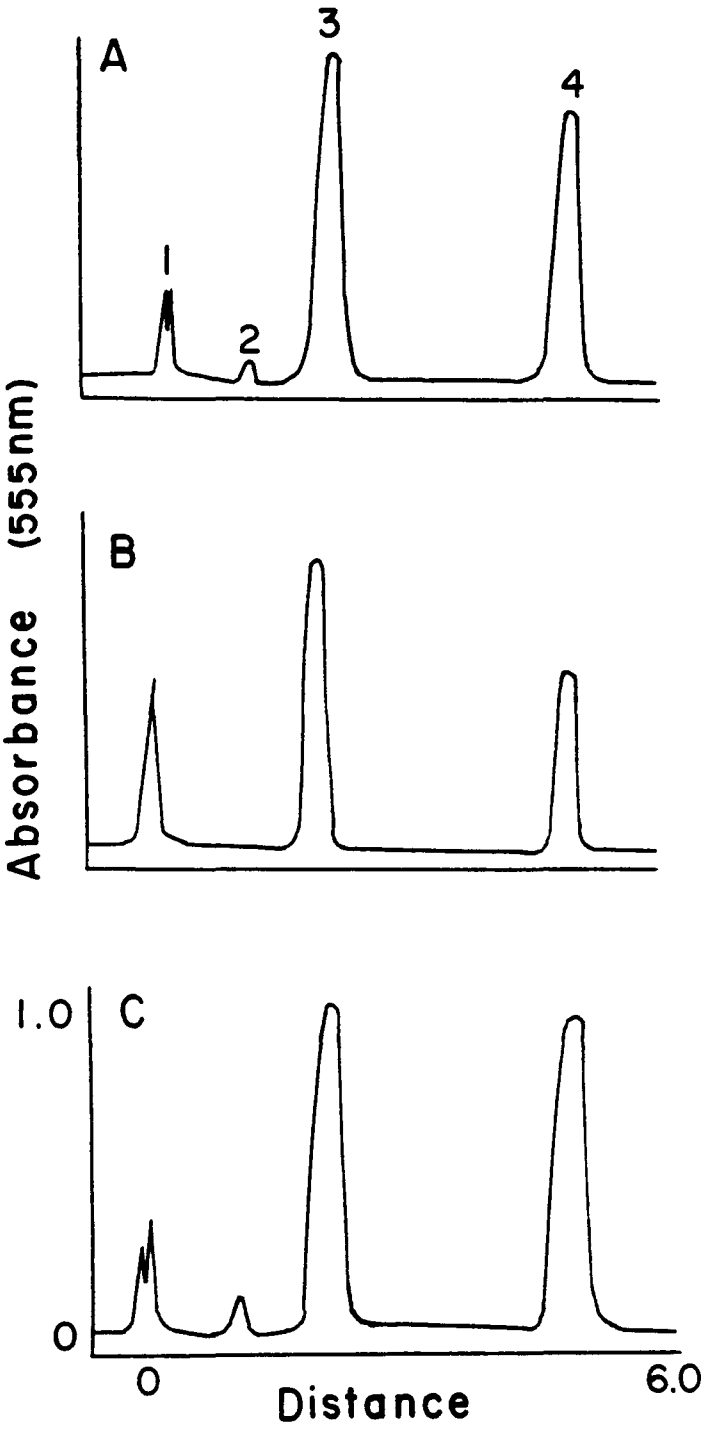
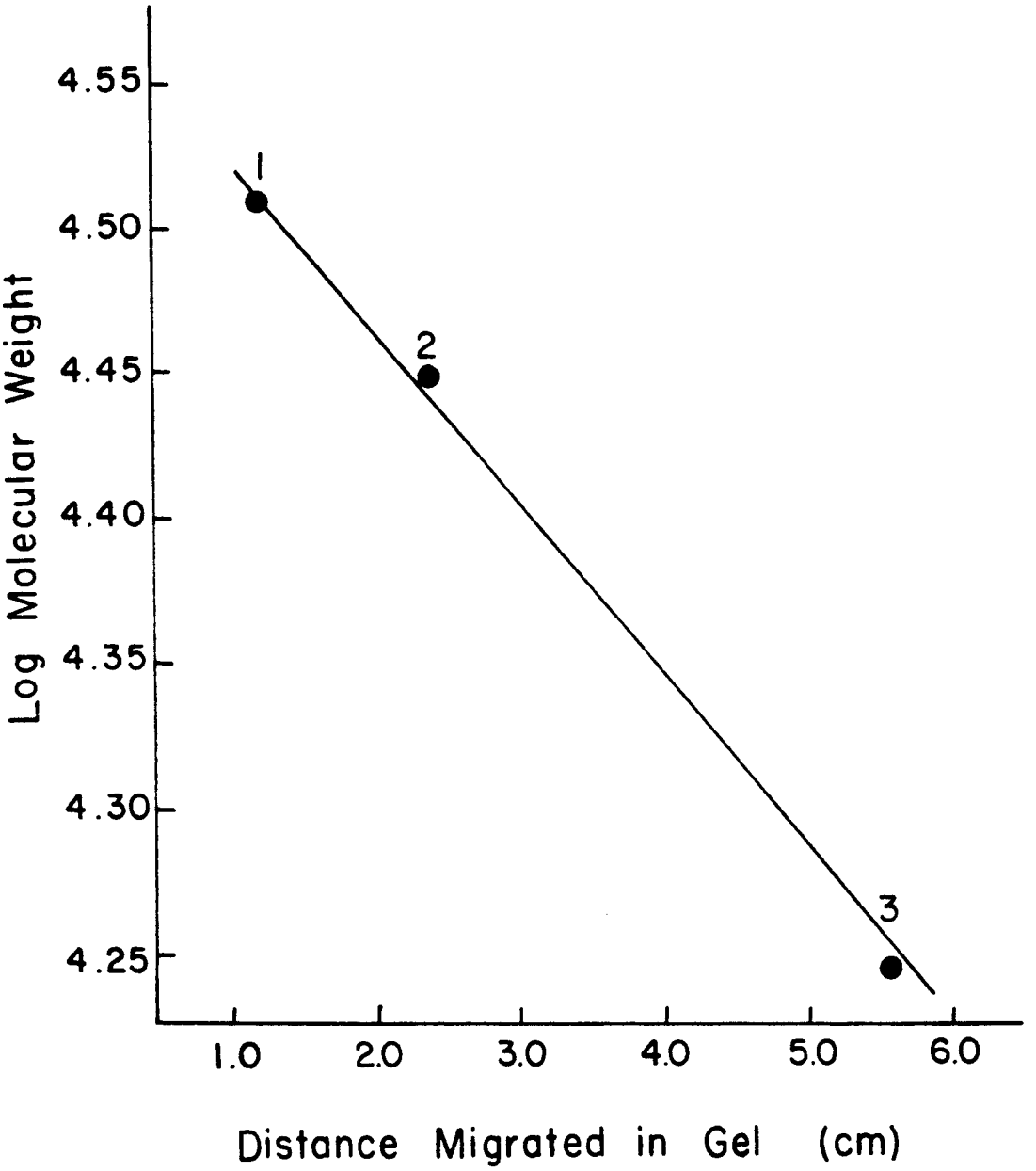

A
B
C
1
2
3
4
Absorbance (555nm)
1.0
0
0
Distance
6.0

Figure 6. Plot of log molecular weight versus distance traveled in SDS gel. Point 1, apoprotein E; point 2, apoprotein AI, point 3, apoprotein AII.


4.55
4.50
4.45
4.40
4.35
4.30
4.25
Log Molecular Weight
1
2
3
1.0
2.0
3.0
4.0
5.0
6.0
Distance Migrated in Gel (cm)

are 33,000, 28,000, and 17,500 daltons respectively, and it can be seen that their migration in the gel is linearly related to their log molecular weight.

5. HDL-Cholesterol Determination

The method of Burstein (29), as modified by Lopes-Virella (30), employing phosphotungstic acid and Mg^{+2}, was used to precipitate apoprotein B containing lipoproteins. After precipitation and centrifugation, the cholesterol in the supernatant was quanitated through use of a SMAC, employing an enzymatic cholesterol methodology. Values determined in this manner represented HDL-cholesterol.

Results for HDL-cholesterol and precentage of HDL containing apoprotein E for a group of 24 hospital patients are given in Table 4. As previously stated the group comprising this sample cannot be considered a healthy, normal population.

6. Statistical Analysis

The mean percentage of HDL containing apoprotein E in this group of hospital patients was 11.9 $\pm$ 3.4%. This agrees well with results obtained by Mahley and coworkers (24), who found that typically 5 to 17% of HDL contains apoprotein E.

The average value of HDL-cholesterol, obtained by the phosphotungstate precipitation technique, was 0.44 $\pm$ 0.15 g/mL. Lopes-Virella (30), employing the same method observed a mean HDL-cholesterol value of 0.495 $\pm$ 0.129 g/mL (n = 47).

The relationship between the percentage of HDL containing apoprotein E and HDL-cholesterol was then examined for correlation, using both parametric and nonparametric statistics. Parametric evaluation of the data was performed by calculation of Pearson's

Table 4

Percentage of HDL containing apoprotein E and HDL-cholesterol for 24 hospitalized patients.

Percentage of HDL Containing Apoprotein E	HDL-cholesterol (g/L)
6	0.28
7	0.26
7	0.48
7	0.64
8	0.28
9	0.48
11	0.36
11	0.60
11	0.62
11	0.72
12	0.22
12	0.32
12	0.40
12	0.44
13	0.34
13	0.38
13	0.60
14	0.30
15	0.52
15	0.56
16	0.32
16	0.32
16	0.62
19	0.56

correlation coefficient which is dependent upon a bivariate normal distribution. A value of 0.323 was obtained for r, indicating no significant correlation.

Calculation of Spearman's rho, a rank order correlation coefficient, was employed for nonparametric analysis of the data. Nonparametric statistics make no assumptions about the distribution of a group of numbers. Instead, nonparametric statistics rely principally on the order of observations with respect to each other, rather than their exact numerical values (37). No correlation was observed, rho for the data equals 0.098, whereas the critical value for rho, at the 90% confidence level is 0.343 (42).

DISCUSSION

HDL has been the topic of intensive investigation due to its protective role against atherosclerosis. In this study HDL were isolated by density gradient ultracentrifugation and then subdivided into HDL with and without apoprotein E by heparin-sepharose affinity chromatograpgy. HDL-cholesterol determination was also performed and the statistical relationship between the amount of HDL containing apoprotein E and HDL-cholesterol was examined.

Isolation of HDL by a single ultracentrifugation on a linear salt density gradient yielded results of similar to those obtained by other researchers (38-40). Although ultracentrifugation requires a great deal of time, the initial discontinuous gradient is rapidly constructed. Also, use of a dendity gradient allows for the quanitation of all lipoprotein classes after a single ultracentrifugation, rather than the multiple ultracentrifugations needed for sequential floating techniques.

Polyacrylamide concentration gradient gel electrophoresis was used to examine the HDL obtained by density gradient ultracentrifugation. Concentration gradient electrophoresis is a powerful technique for the separation of proteins since it exploits not only differences in charge density, but also differences in protein size. As the concentration of acrylamide in the gel is increased, the pore size of the gel decreases. Therefore, during electrophoresis there is a progressive decrease in protein mobility as the decreasing pore size increases resistance to the migration of proteins (43).

The appearance of HDL as a broad band after concentration gradient electrophoresis can be explained by the size heterogeneity of HDL.

HDL range in size from 7.5 nm to 15.0 nm (1). The larger HDL are slowed and trapped in the gel first, while the smaller HDL can migrate further. At the end of the electrophoresis, the lipoproteins are virtually stationary. Therefore their position in the gel depends predominatly on their molecular diameter (43). Comparable results have been obtained by many other researchers (24, 40, 43, 44).

Heparin-sepharose affinity chromatography was effective in separating HDL containing apoprotein E from HDL without this apoprotein, as evidenced by SDS electrophoresis of delipidated lipoproteins. In this study, the percentage of HDL containing apoprotein E ranged from 6% to 19%. Mahley and Weisgraber (24, 44) found that typically 5% to 17% of HDL contained apoprotein E. SDS electrophoresis of total HDL yielded an apoprotein AI to AII ratio of 1:1, which agrees well with previously reported values (45, 46). The exact types and ratios of apoproteins in HDL can vary considerably for as yet unexplained reasons. The exact apoprotein composition may be dependent upon the rate of synthesis or degradation of lipoproteins (11). Also, it has been shown that there is an exchange of apoproteins between HDL particles, and among HDL and other lipoproteins (3).

HDL-cholesterol quantification by the Lopes-Virella method has several advantages over other precipitation techniques. This method is simple, fast, and solutions of phosphotungstate are stable over long periods of time (30). Also, if the heparin-Mn^{+2} method were employed, HDL containing apoprotein E may be precipitated. The specific mechanisms of lipoprotein precipitation by polyanions and divalent cations have not been established with certainty. Interaction between negatively charged groups on the polyanion and positively charged groups on the protein moiety

of the lipoprotein are probably very important. Also, divalent metal cations can interact with negatively charged groups of lipoproteins, such as phospholipids, to facilitate formation of large insoluble complexes (46).

Twenty four hospital serum specimens were analyzed for the percentage of HDL containing apoprotein E and HDL-cholesterol using the previously described methods. Comparison of these two parameters for correlation by parametric and nonparametric statistics yielded no significant correlation.

Future studies of these parameters in a healthy, normal population, of much larger number, using the procedures detailed in this paper, should be performed. Also, a thorough comparison of male and female HDL apoprotein E content and HDL-cholesterol concentration could provide further insight into the reasons behind the lower occurrence of atherosclerosis in females. Additional studies should also be undertaken in examining the apoprotein E concentration as a function of age. This may provide valuable information, especially with regards to females, whose chances of developing atherosclerosis increase following menopause (23).

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