A micro-enzymatic method to measure cholesterol and triglyceride in lipoprotein subfractions separated by density gradient ultracentrifugation from 200 microliters of plasma or serum

John D. Belcher, Jack O. Egan, George Bridgman, Richard Baker, and John M. Flack

Division of Epidemiology, School of Public Health, University of Minnesota, Minneapolis, MN 55455

Abstract A micro-enzymatic method was developed to measure total cholesterol (CHOL) and triglyceride (TG) in lipoproteins and their subfractions separated by density gradient ultracentrifugation. This method had a detection limit and sensitivity below 2 mg/dl and accuracy (bias to reference sera) and imprecision (coefficient of variation) of less than 3% between 2 and 30 mg/dl for both CHOL and TG. In addition, the method was in good agreement with standardized Abell-Kendall CHOL (r = 0.98) and enzymatic TG (r = 0.99) methods. Lipoproteins from 200 μl of plasma or serum were separated by either equilibrium (EQ)- or rate zonal (RZ)-density gradient ultracentrifugation and the resulting fractions were analyzed for CHOL and TG by the micro-enzymatic method. Lipoprotein measurements by these micro-enzymatic/density gradient methods were highly correlated with standardized Lipid Research Clinic (LRC) procedures and preparative ultracentrifugation. The EQ-density gradient procedure also allowed determination of CHOL and TG in LDL and HDL subfractions within any desired density interval. These methods will facilitate the measurement and study of lipoproteins and their subfractions especially in infants, children, the elderly, and small animals. In addition, the micro-enzymatic method may be adapted to other modes of lipoprotein separation such as liquid chromatography, electrophoresis, and precipitation. CHOL or TG determinations could be made on approximately 500 density gradient fractions per hour. —**Belcher, J. D., J. O. Egan, G. Bridgman, R. Baker, and J. M. Flack.** A micro-enzymatic method to measure cholesterol and triglyceride in lipoprotein subfractions separated by density gradient ultracentrifugation from 200 microliters of plasma or serum. *J. Lipid Res.* 1991. **32:** 359–370.

Supplementary key words low density lipoproteins • high density lipoproteins

Lipoprotein subfractions or subspecies exist within VLDL, LDL, and HDL (1). These subspecies have been shown to differ in size, density, charge, composition, metabolism, and risk for CHD (2–19). Differences in the concentration of these subspecies may be important in determining CHD risk. An increase of small, dense LDL particles has been associated with a greater risk for nonfatal myocardial infarction (14) and CAD (8). Also, changes in levels of large, less dense LDL (IDL or LDL_1) have been positively associated with progression of CAD (10). Levels of both large and small LDL subspecies appear to be positively linked to plasma TG levels (9, 14). Likewise, the HDL_2 subfraction has been reported to be more correlated with protection against CAD than the smaller, more dense HDL_3 subfraction (18). HDL_2 appears to be negatively linked to plasma TG levels (20).

LDL and HDL subspecies have been characterized by techniques such as polyacrylamide gradient gel electrophoresis (7, 9), density gradient ultracentrifugation (6, 7, 11, 12), precipitation (21), and preparative and analytical ultracentrifugation (2, 22). Some of these methods (particularly those used for measuring LDL subspecies) quantitate subspecies by indirectly measuring particle mass with densitometry or schlieren optical patterns. Measurement of particle mass is informative, but routine measurement of particle composition would be desirable. Further metabolic understanding of these subspecies has been hindered by the lack of easy and rapid micromethods for measuring their composition. This paper describes an accurate and precise micro-enzymatic method to measure the CHOL and TG concentrations of dilute lipoproteins and their subfractions.

Abbreviations: VLDL, very low density lipoproteins; IDL, intermediate density lipoproteins; LDL, low density lipoproteins; Lp[a], lipoprotein[a]; HDL, high density lipoproteins; VHDL, very high density lipoproteins; CHOL, cholesterol; TG, triglyceride; LRC, Lipid Research Clinics; EDTA, ethylenediamine tetraacetic acid; CHD, coronary heart disease; MI, myocardial infarction; CAD, coronary artery disease; EQ, equilibrium or isopycnic; RZ, rate zonal; CDC, Centers for Disease Control; ELISA, enzyme-linked immunosorbent assay; CV, coefficient of variation.

MATERIALS AND METHODS

Blood samples

Subjects were normo- and hyperlipidemic male and female volunteers (n = 77) ranging in age from 21 to 65 years. Venipuncture blood samples were collected after an overnight fast (12–14 h) in tubes containing either 1.5 mg of disodium EDTA per ml of blood (plasma) or no anticoagulant (serum). Serum was separated after being allowed to clot for 30 min at room temperature. The clot and/or cells were removed by centrifugation at 1,500 *g* for 30 min at 4°C. Plasma and serum were aliquoted and stored either at 5°C or −70°C. Plasma was used for analyses presented in Table 2 and Fig. 1. Serum was used for the analyses shown in Tables 1 and 3, and Figs. 2–4. Most analyses commenced within 3 days of blood collection using the unfrozen plasma or serum. Some of the EQ-density gradient lipoprotein separations were performed on frozen samples (2–3 months). Preliminary comparisons between fresh and frozen samples were unable to demonstrate any significant differences in their lipoprotein density gradient profiles (data not shown).

Equilibrium (isopycnic)-density gradient lipoprotein profile

Potassium bromide salt solutions of densities 1.020, 1.15, and 1.21 g/ml were prepared by dissolving anhydrous KBr in distilled water. All salt solutions contained 0.05%-EDTA (pH 7.0). Solution densities were checked gravimetrically and their refractive indices were measured by refractometry (Bausch and Lomb, Inc., Rochester, NY). A linear plot of refractance versus density was made for use in determining the density of gradient fractions. Two hundred microliters of plasma or serum in the gradient did not interfere with refractometry of the density gradient fractions except in the two fractions at the top (VLDL) and bottom (protein) of the gradient. The fraction densities were also determined by their conductance using a digital conductivity meter equipped with a 5-μl flow cell (Markson, Phoenix, AZ) placed on-line between the tube fractionator and the fraction collector. Conductance readings were recorded at the midpoint of each fraction. Density determinations by this method agreed well with those determined by refractometry (data not shown).

Gradients were constructed in 11 × 60 mm wettable Ultracote™ tubes (Seton Scientific, Sunnyvale, CA) using 21 gauge × 1.5 inch needles attached to 3-ml glass syringes. All six gradients for one rotor were poured at the same time using six syringes and needles. The following solutions were sequentially pipetted into each syringe and allowed to run down the side of the ultracentrifuge tube: 0.31 ml of d 1.21 g/ml KBr; 1.25 ml of d 1.15 g/ml KBr; 0.31 ml mixture of 0.20 ml plasma or serum plus 0.11 ml of 150 mM NaCl; and finally 2.13 ml of d 1.020 g/ml KBr. Total gradient volume was 4.0 ml. Tubes were centrifuged in an SW60 rotor in an L5-65 ultracentrifuge (Beckman Instruments, Palo Alto, CA) at 58,000 rpm for 22 h (45.6×10^7 g_{av}-min) at 15°C with an acceleration rate of 1 and the brake off. This gradient was adapted from the one described by Nilsson and coworkers (23) and seemed to give the best separation of LDL and HDL of many different gradients tested.

Rate zonal (RZ) density gradient lipoprotein profile

Potassium bromide salt solutions of densities 1.21 and 1.346 g/ml were prepared as described above. Gradients were constructed in 13 × 64 mm wettable Ultracote™ tubes using the same syringes and needles described above. The following solutions were successively pipetted into the syringe and allowed to run down the side of the ultracentrifuge tube: 1.35 ml of d 1.346 g/ml KBr; 0.50 ml of a d 1.21 g/ml solution containing 0.2 ml of plasma or serum plus 0.3 ml of d 1.346 g/ml KBr; and 3.75 ml of H_2O containing 0.05% EDTA, pH 7. The total gradient volume was 5.6 ml. Tubes were capped and centrifuged in a 50.3 fixed-angle rotor (Beckman Instruments) in an L5-65 ultracentrifuge at 48,000 rpm for 4 h ($39.8 \times 10^6 g_{av}$-min) at 15°C with an acceleration rate of 1 and the brake off.

Fractionation

The centrifuge tubes were punctured from the bottom and Fluorinert FC-40 (Sigma Chemical Co., St. Louis, MO) was pumped in at either 0.5 ml/min (EQ-density gradient) or 1.0 ml/min (RZ-density gradient). Each tube was fractionated into either 20 (EQ-density gradient) or 28 (RZ-density gradient) 0.2-ml fractions (Isco, Lincoln, NE). Fractions were collected in 0.6-ml polypropylene tubes (Sarstedt, Princeton, NJ) which were subsequently placed in a 96-well microtiter template (Sarstedt) to facilitate automated pipetting of fractions.

Micro-enzymatic cholesterol (CHOL) and triglyceride (TG) methods

The micro-enzymatic CHOL and TG reactions were performed in Dynatech Immulon 1 96-well microtiter plates (Chantilly, VA). The wells were pre-treated with 0.35 ml of 0.1% (v/v) Tween 20 (Sigma Chemical Co.) in 0.9% NaCl (saline) for 1 h at room temperature. Wells were emptied and allowed to dry before use. Pretreatment improved the precision and accuracy of subsequent absorbance readings.

Each microtiter plate contained one saline blank, four calibrators at 2, 6, 15, and 30 mg/dl of CHOL or TG (in the form of glycerol), a high (CHOL = 18, TG = 14.4 mg/dl) and a low (CHOL = 6.9, TG = 6.45 mg/dl) serum control in duplicate, three plasma or serum unknowns diluted 10-fold, and either 60 (three EQ analyses) or 84 (three RZ analyses) density gradient fractions. Cali-

brators were made by appropriate saline dilutions of Preciset Cholesterol Calibrator (Boehringer Mannheim Diagnostics, Indianapolis, IN) or gravimetrically prepared glycerol (Aldrich Chemical Co., Milwaukee, WI). Glycerol concentrations were corrected for the difference in molecular weights between glycerol (mol wt = 92) and triolein (mol wt = 885). Serum controls (CDC Lipid Standardization Program, Atlanta, GA) were carefully diluted either 10-fold (high control) or 40-fold (low control) with saline using volumetric flasks and pipets that were designed to be accurate within 0.05%. Controls were aliquoted and frozen at −70°C until use. Subsequent to this study, it was found that controls were more stable when diluted with 6% bovine serum albumin in saline instead of saline alone and when stored in Wheaton glass vials (Millville, NJ) sealed with a rubber septum and aluminum seal rather than plastic screw-top vials. Plasma samples were diluted 10-fold with saline before analysis to bring their values in range with the calibrators. The plasma dilution step was performed by adding 0.02 ml of plasma to 0.18 ml of saline using Hamilton syringes (Reno, NV).

Sample and reagent pipetting was automated with a Cetus-Perkin Elmer Propette liquid handling system (Norwalk, CT). Density gradient fractions, diluted plasma or serum, standards, controls, and a saline blank were pipetted (0.03 ml) into the plate wells followed by 0.15 ml of either enzymatic CHOL (High Performance) or TG (GPO) reagent (Boehringer Mannheim Diagnostics). The glycerol blank was not measured by the micro-enzymatic method. Plates were incubated at room temperature for 15 min. Plates were read immediately after this incubation; however the color was stable for at least 1 h. Each plate was read using a V_{max} microplate reader (Molecular Devices, Palo Alto, CA) in the end-point mode at 490 and 650 nm with the background absorbance at 650 nm subtracted to correct for well-to-well variation. The linear regression calibration lines and concentrations of CHOL and TG in each fraction were calculated by software (Softmax, Molecular Devices) on a microcomputer interfaced to the plate reader.

Calculation of lipoprotein lipid concentrations

After the initial data calculations by the Softmax program, the data were exported to Lotus 123 (Lotus Development Corp., Cambridge, MA) for further analysis. Both the EQ and RZ methods calculated VLDL CHOL or TG as the sum of the lipid concentrations in the first two density fractions at the top of the tube, i.e., the top of 0.4 ml of the gradient. Thereafter, the two density gradient procedures differed significantly in the methods used to calculate lipoprotein lipid concentrations.

The RZ-density gradient procedure calculated LDL and HDL CHOL and TG based on the shape of the density gradient CHOL profile. The cut-point between LDL and HDL was the minimum CHOL value between the LDL and HDL CHOL peaks. When no HDL peak was found (less than 5% of samples), then the average LDL/HDL cut-point was used (fraction 13). HDL CHOL was calculated as the sum of CHOL in the fractions after the cut-point. Lipoprotein TG concentrations were calculated using the CHOL-derived cut-points.

The EQ-density gradient procedure permitted the calculation of CHOL and TG concentrations within any desired density interval, including LDL and HDL subfractions. The density of each collected fraction was determined by refractometry. The fraction densities were computed in LOTUS-123^{tm} using the linear regression equation of density versus refractance derived from the stock KBr density solutions. The densities and the CHOL and TG concentrations of the density gradient fractions were then exported electronically to a third program written in Fortran on a VAX 8600 computer (Digital Equipment, Maynard, MA). This program calculated lipoprotein subfraction concentrations (as described below) in any specified density interval. The lipid concentrations of the lipoprotein subfractions were represented by the areas under the CHOL or TG concentration curves between a given density interval. Usually the cut-point for the density interval fell within a density gradient fraction rather than exactly between two fractions. In order to calculate how much of the lipid concentration to ascribe to the lipoprotein subfractions above and below the density cut-point, an interpolation technique was used which connects three adjacent points (fractions) on the concentration curve with a 2nd degree equation, i.e., parabola. This technique is a modified form of Simpson's rule used in calculus and is believed to be slightly more accurate than linear interpolation. The computer program allowed determination of CHOL and TG in LDL and HDL subfractions within any desired density interval. Lipoprotein subfractions were operationally defined as follows: VLDL (first two fractions at the top of the gradient; $d \leq 1.006$ g/ml), LDL_1 ($1.006 < d \leq 1.025$ g/ml), LDL_2 ($1.025 < d \leq 1.040$ g/ml), LDL_3 ($1.040 < d \leq 1.075$ g/ml), HDL_2 ($1.075 < d \leq 1.105$ g/ml), HDL_3 ($1.105 < d \leq 1.15$ g/ml), and VHDL (the last two fractions at the bottom of the gradient ($d > 1.15$ g/ml). A cut-point of 1.075 g/ml between LDL and HDL was used in calculating the LDL and HDL concentrations with the EQ density gradient method because this cutpoint was found on average to give the best agreement with the LRC methodology. This cut-point resulted in slightly lower HDL and higher LDL values compared to preparative ultracentrifugation (see methods below) which used the traditional d 1.063 g/ml cut-point between LDL and HDL.

The computer programs normalized the lipoprotein CHOL and TG concentrations to correct for the recovery of plasma CHOL and TG in the density gradient frac-

tions relative to whole plasma or serum; the mean percent recoveries (before normalization) and their standard deviations were: RZ CHOL = 101.3 ± 5.5%, TG = 98.0 ± 5.1; EQ CHOL = 104.6 ± 5.7%, TG = 104.8 ± 6.4%.

Standard methods

Lipoproteins were separated by standard LRC methods (24) which included centrifugation of 5 ml of plasma at d 1.006 g/ml to remove VLDL, followed by measurement of LDL + HDL CHOL in the d 1.006 g/ml infranatant. HDL CHOL was measured after heparin/manganese precipitation of VLDL and LDL from 2 ml of whole plasma (24) as modified by Bachorik, Walker, and Virgil (25) for enzymatic methods. Total and HDL CHOL were measured enzymatically using Autoflo High Performance Cholesterol Reagent (Boehringer Mannheim Diagnostics), SERCAL serum calibrator from CDC, and a COBAS FARA analyzer (Roche Diagnostic Systems, Montclair, NJ). Serum TG was measured with enzymatic Triglycerides GPO Reagent (Boehringer Mannheim) on a COBAS FARA analyzer using in-house glycerol standards prepared in 6% albumin. Serum glycerol was measured enzymatically with Triglyceride Blank Blend (Craig Bioproducts, Streamwood, IL) on the same analyzer. The reaction sequence for the glycerol blank assay was the same as the reaction sequence for the triglyceride assay except the glycerol blank reagent omitted the triglyceride lipase enzyme. The glycerol values were subtracted to obtain the final triglyceride values in Table 1 only. The serum glycerol values were not measured by the micro-enzymatic method, but were instead measured by the COBAS FARA, divided by the appropriate dilution factor, and subtracted from the micro-enzymatic triglyceride values in Table 1. Both the TG and the glycerol blank assays were validated using gravimetrically prepared glycerol standards and were standardized with the enzymatic CHOL and HDL CHOL assays by the CDC Lipid Standardization Program.

Abell-Kendall cholesterol determinations

For comparison purposes the plasma CHOL was measured by both the micro-enzymatic method and an in-house Abel-Kendall method (26). The in-house Abell-Kendall method was standardized as part of the National Reference System for Cholesterol to within 1% of the CDC Abell-Kendall method.

Preparative ultracentrifugation

Lipoproteins were isolated from plasma at d 1.006 and 1.063 g/ml essentially as described by Havel, Eder, and Bragdon (27). Plasma (5.0 ml) was either retained at d 1.006 g/ml or raised to a density of 1.063 g/ml with d 1.346 g/ml KBr solution. Samples were centrifuged in a 50.3 rotor (Beckman Instruments) at 104,000 *g* for 18 h at 15°C. Lipoproteins floating at the top of the tube were removed by tube slicing. The bottom fractions were reconstituted to their original plasma volume and CHOL was measured enzymatically as described above (see Standard methods). None of the enzymatic methods (CHOL or TG, standard or micro) were sensitive to interference by KBr salt solutions between the densities of 1.006 and 1.346 g/ml. VLDL CHOL was calculated as total plasma CHOL minus density > 1.006 g/ml CHOL; LDL CHOL was calculated as density > 1.006 g/ml CHOL minus density > 1.063 g/ml CHOL; HDL CHOL was calculated as density > 1.063 g/ml CHOL.

Lipoprotein[a] measurements

LP[a] was measured by ELISA using a monoclonal capture antibody and polyclonal detection antibodies specific for LP[a]. The reagents were supplied by Terumo Medical Corporation (Elkton, MD). The monoclonal antibody had specific reaction with apo[a] by Western blot and no cross-reactivity to plasminogen, apoB, LDL, VLDL, or HDL. In addition, the assay had no interference from triglycerides at concentrations up to 500 mg/dl and less than 10% diminution of values by hemoglobin at concentrations up to 5 mg/dl. Lp[a] standards were supplied by Terumo Medical Corporation and were calibrated to a reference preparation of human plasma obtained from Dr. John Albers of the Northwest Lipid Research Clinic, Seattle, WA. The Lp[a] value of the reference preparation was derived from a double antibody radioimmunoassay calibrated with purified Lp[a] (28). A standard curve was made by diluting the reference plasma sample at various dilutions and assigning Lp[a] values. An in-house calibrator plasma was also diluted at various dilutions and assayed along with the reference standard curve. An Lp[a] value for the in-house calibrator plasma was assigned by calculating the dose from the reference standard curve. The values reported are total Lp[a] lipoprotein mass.

RESULTS

Calibration of micro-enzymatic CHOL and TG assays

Micro-enzymatic CHOL and TG assays were routinely calibrated at 2, 6, 15, and 30 mg/dl of CHOL or TG (data not shown). The mean change in absorbance (slope) was 0.012 absorbance unit per mg/dl CHOL or TG (n = 50 for CHOL and n = 20 for TG). The Y intercept was 0.001 absorbance unit for CHOL and TG. The standard lines were linear with correlation coefficients (*r*) of 1.000. The standard lines were reproducible from assay to assay; the coefficients of variation averaged 3.9% for CHOL and 1.5% for TG calibrators. Both assays had a detection limit and sensitivity of less than 2 mg/dl and were linear to

TABLE 1. Accuracy and precision of the micro-enzymatic cholesterol and triglyceride methods

	Cholesterol						Triglyceride[c]					
			Accuracy		Precision				Accuracy		Precision	
Control[a]	n[b]	Reference Value	Mean	Bias	SD	CV	n[b]	Reference Value	Mean	Bias	SD	CV
		mg/dl	*mg/dl*	*%*	*mg/dl*	*%*		*mg/dl*	*mg/dl*	*%*	*mg/dl*	*%*
Q15	50	18.00	18.3	+1.7	0.48	2.6	20	14.40	13.64	−5.3	0.38	2.8
Q18	50	6.90	7.0	+1.4	0.15	2.1	20	6.45	6.57	+1.9	0.14	2.1

[a]Serum-based controls were provided by Centers for Disease Control (CDC) Atlanta, GA. Q15 and Q18 were diluted 10- and 40-fold, respectively, with saline, aliquoted, and frozen at −70°C. Reference values were assigned by CDC on the undiluted controls using the Abell-Kendall (26) and chromotropic acid methods.

[b]N is equal to the number of microtiter plates. Controls were measured in duplicate on each plate. The means of each plate were used to calculate the micro-enzymatic means and standard deviations.

[c]All triglyceride values have been corrected for serum glycerol. CDC corrects for glycerol by removing it with silicic acid. The micro-enzymatic triglyceride values were corrected for glycerol by measuring the glycerol in the undiluted controls with standard enzymatic methods on the COBAS FARA analyzer (see Methods). The values were then divided by the appropriate dilution factor and subtracted (diluted, Q15 = 1.00 and Q18 = 0.41 mg/dl, n = 96) from the micro-enzymatic values to obtain the triglyceride means.

greater than 50 mg/dl (data not shown). A concentration of 1 mg/dl (using 0.03 ml of sample) corresponded to a mass of 300 nanograms of CHOL or TG.

Accuracy and precision

The accuracy and precision of the micro-enzymatic method was determined by repetitive analysis of reference sera supplied by CDC. Reference CHOL or TG values were determined by CDC using the semi-automated Abell-Kendall (26) and the chromotropic acid (G. Myers, Centers for Disease Control, personal communication) methods, respectively. One reference serum (Q15) was diluted 10-fold to 18 mg/dl CHOL and 14.4 mg/dl TG. The other reference serum (Q18) was diluted 40-fold to 6.9 mg/dl CHOL and 6.45 mg/dl TG. The micro-enzymatic CHOL method had a bias of 1.4 and 1.7% and a CV of 2.1 and 2.6% at 6.9 and 18 mg/dl, respectively (**Table 1**). The micro-enzymatic TG method had a bias of 1.9 and −5.3% and a CV of 2.1 and 2.8% at 6.45 and 14.4 mg/dl, respectively. The glycerol blank was determined on undiluted plasma by the standard enzymatic method on the COBAS FARA (see Methods). This blank was subtracted from both the standard and the micro-enzymatic values after adjustment for dilutions.

Fresh plasma was collected from 41 volunteers and analyzed in duplicate for CHOL and TG by the micro-enzymatic methods, the Abell-Kendall method, and a standardized enzymatic TG method. These results were plotted and analyzed to determine their correlation (**Fig. 1A and B**). The CHOL and TG micro-enzymatic methods had a correlation coefficient (r) of 0.98 and 0.99 with their standardized counterparts. The slopes were 1.008 and 0.899 for the CHOL and TG comparisons, respectively. TG concentrations were not corrected for

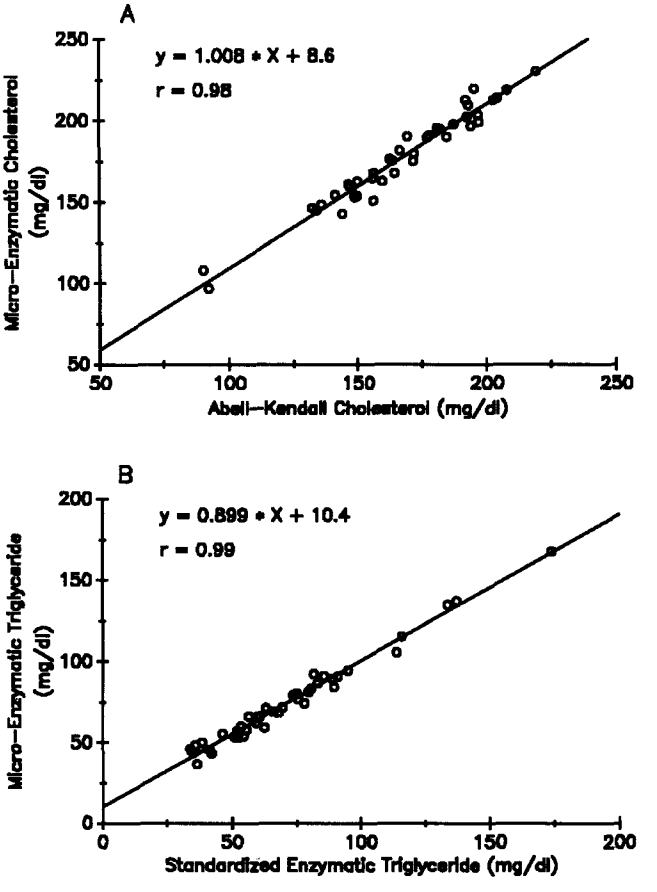

Fig. 1. Comparison of the micro-enzymatic cholesterol (A) and triglyceride (B) methods with the Abell-Kendall cholesterol method (A) and a standardized enzymatic triglyceride method (B). Plasma samples were diluted 10-fold before analysis by the micro-enzymatic cholesterol and triglyceride methods. The Abell-Kendall cholesterol and standardized enzymatic triglyceride measurements were made on undiluted plasma.

glycerol in plasma. The glycerol concentrations in these samples were 5.0 ± 3.6 mg/dl (mean ± SD).

Density gradient ultracentrifugation

The micro-enzymatic method was used to measure CHOL and TG in lipoprotein fractions separated by two different single-spin density gradient ultracentrifugation procedures. Plasma lipoproteins from eight different subjects with normal and abnormal (type IIa, type IIb, type IV, and elevated Lp[a]) lipoprotein phenotypes were separated by rate zonal (**Fig. 2**) or equilibrium (**Fig. 3** and **Fig. 4**) density gradient ultracentrifugation. Both density gradient centrifugation methods required only 200 μl of serum to generate a complete lipoprotein CHOL and TG profile. Subjects with hypertriglyceridemia (panels C and D in Figs. 2 and 3) typically had LDL peaks that were slower migrating or more dense than subjects with normal triglycerides (panels A and B in Figs. 2 and 3). One subject had an Lp[a] concentration of 70 mg/dl (determined immunochemically); in this subject an Lp[a] peak can be seen between LDL and HDL in the density region 1.050 to 1075 g/ml (Fig. 3B)

The CHOL and TG concentrations were converted from mg/dl to mM and expressed as the molar ratio of CHOL to TG (Figs. 2 and 4). Using this approach, one can see striking composition differences among these eight individuals as well as composition differences within LDL and HDL subclasses. The CHOL/TG ratio was much higher in LDL_2 than in LDL_1 and LDL_3 (Fig. 4). There was an additional increase in the CHOL/TG ratio in the HDL_3 of all subjects and in the LDL_3 region of the Lp[a]-enriched sample (Fig. 4). As expected, subjects with hypertriglyceridemia had a lower molar ratio of CHOL to TG in all lipoprotein fractions (Figs. 2 and 4). This ratio displayed a remarkable amount of inter-individual variability; the molar ratio of CHOL to TG in the LDL and HDL peak fractions varied about 3-fold in this study.

Comparisons between methods

In order to compare the density gradient separation methods, fasting plasma samples were collected from 12 volunteers. VLDL, LDL, and HDL were separated by four different methods: EQ-density gradient, RZ-density

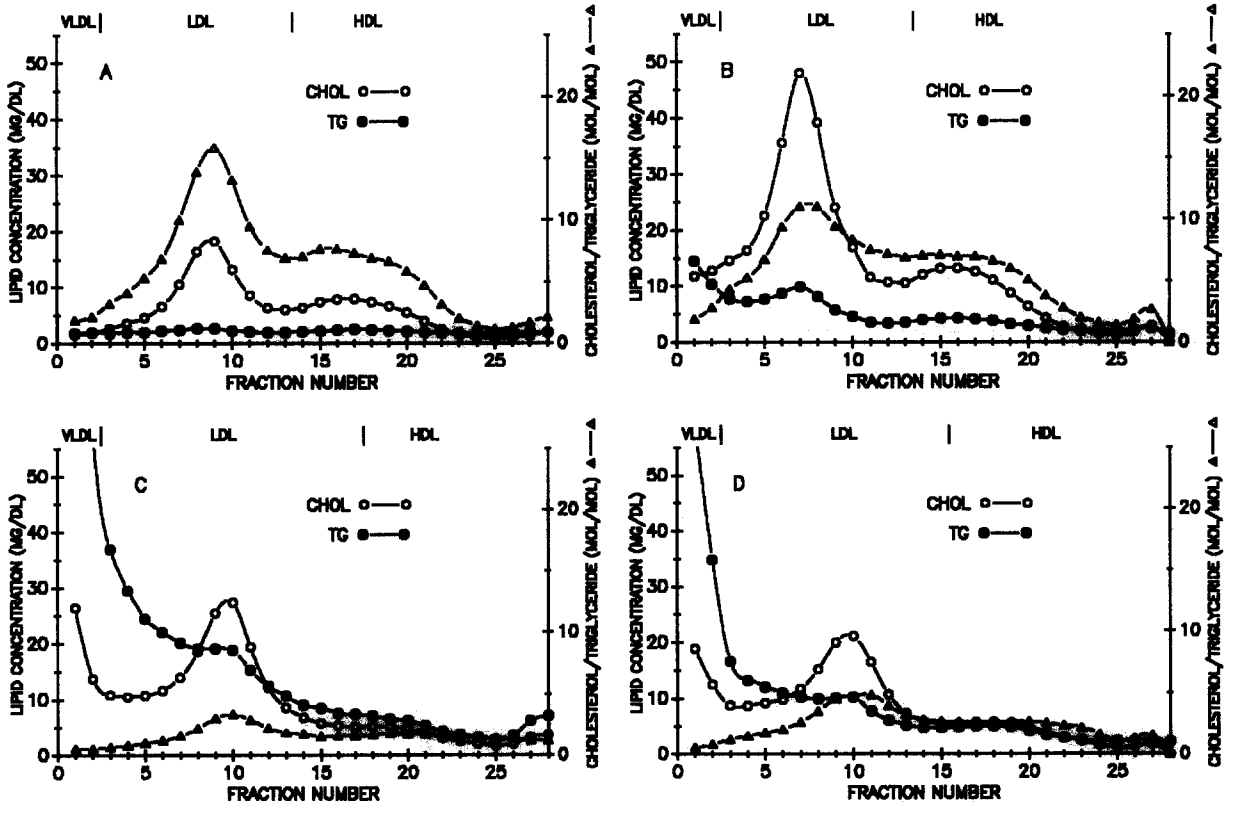

Fig. 2. Rate zonal density gradient lipoprotein profile generated from 200 μl of human serum from four subjects with the following lipoprotein phenotypes: (A) normal; (B) type IIa; (C) type IIb; and (D) type IV. The total plasma cholesterol (CHOL) and triglyceride (TG) concentrations, determined by the micro-enzymatic method, were as follows: CHOL (mg/dl) A = 163, B = 391, C = 270, D = 224; TG (mg/dl) A = 58, B = 139, C = 564, and D = 268. The cholesterol (open circles) and triglyceride (closed circles) concentrations of each density gradient fraction are expressed as mg/dl. The sum of all 28 fractions equals the total plasma lipid concentration. The ratio of cholesterol to triglyceride (triangles) in each fraction is expressed as a molar ratio. Lipoprotein boundaries are marked by vertical lines at the top of each panel.

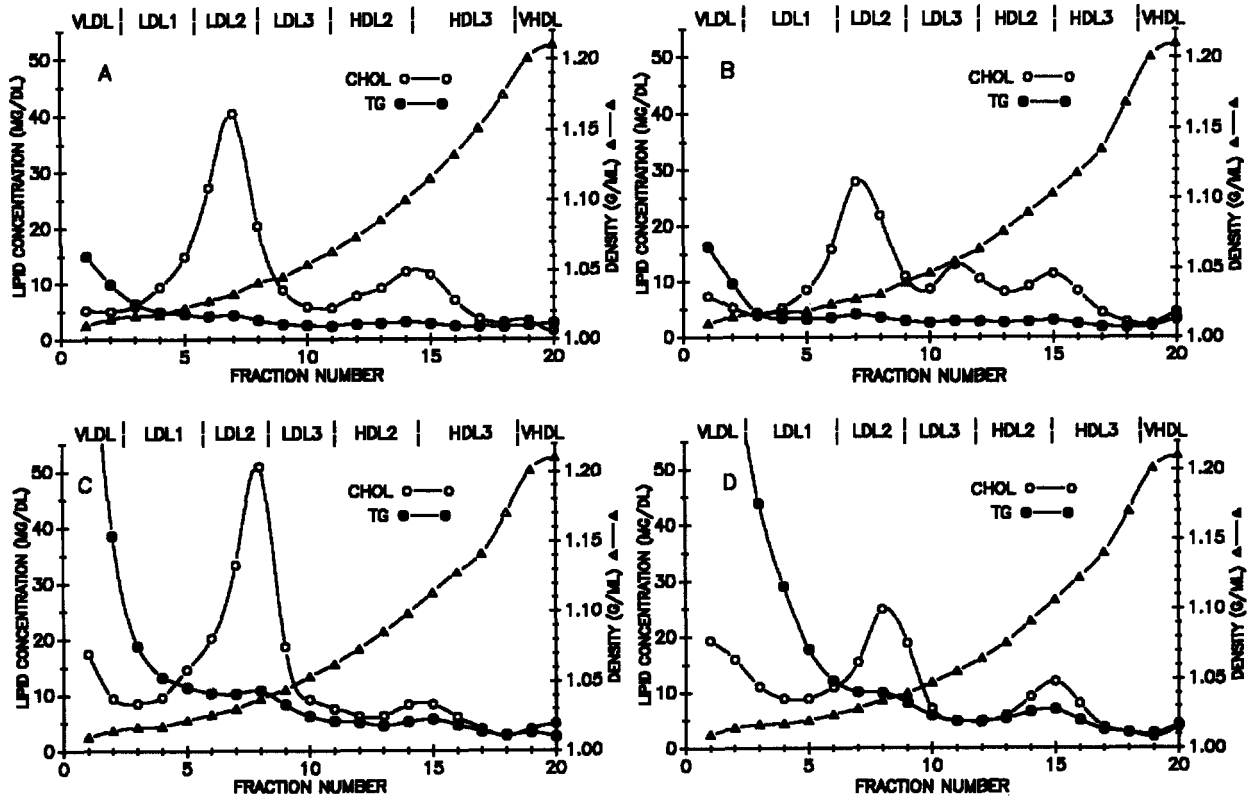

Fig. 3. Equilibrium density gradient lipoprotein profile generated from 200 μl of human serum from four subjects with the following lipoprotein phenotypes: (A) normal; (B) normal with elevated Lp[a] (70 mg Lp[a]/dl); (C) type IIb; and (D) type IV. The total plasma cholesterol (CHOL) and triglyceride (TG) concentrations, determined by standardized enzymatic methods (see Methods), were as follows: CHOL (mg/dl) A = 208, B = 192, C = 250, D = 199; TG (mg/dl) A = 80, B = 71, C = 250, D = 329. The cholesterol (open circles) and triglyceride (closed circles) concentrations of each density gradient fraction are expressed as mg/dl. The sum of all 20 fractions equals the total plasma lipid concentration. The density of each fraction (triangles) is expressed as g/ml. Lipoprotein boundaries are marked by vertical lines at the top of each panel. Subjects are different from those used for Fig. 2.

gradient, standardized LRC procedures, and preparative ultracentrifugation. The CHOL concentration was measured in each lipoprotein fraction by either the micro-enzymatic method (EQ- and RZ-density gradient) or the standardized enzymatic method (LRC procedure and preparative (PREP) ultracentrifugation). All values were normalized to the total plasma CHOL determined by the standardized enzymatic method. Included in the group were individuals with type IIa, IIb, and IV hyperlipoproteinemia. The overall means and standard deviations of the four different methods were similar despite the marked differences in method procedures. **Table 2** summarizes the between-method comparisons for VLDL, LDL, and HDL CHOL. Each comparison shows the linear regression slope, Y intercept, correlation coefficient (r) and standard deviation of the residuals of y on x ($Sy \cdot x$). The lowest correlation coefficients and slopes were seen when comparing VLDL and HDL by the RZ-density gradient method with the other three methods. However, the RZ HDL method had a correlation coefficient of 0.87, a slope of 1.03, and a Y intercept near zero with the LRC precipitation method. All of the methods were highly correlated for LDL CHOL. LDL and HDL concentrations determined by the EQ-density gradient method had correlation coefficients between 0.91 and 0.99 with the LRC and preparative ultracentrifugation methods for all three lipoproteins.

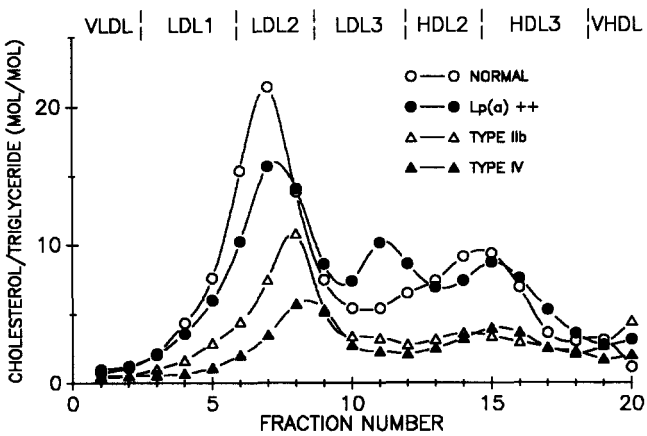

Fig. 4. Molar ratio of cholesterol to triglyceride of lipoprotein profiles shown in Fig. 3. See Fig. 3 for details.

TABLE 2. Summary of comparisons between lipoprotein separation methods[a,b]

Lipoprotein	n	Mean Cholesterol Method X	Mean Cholesterol Method Y	Y Intercept	Slope	Correlation Coefficient (r)	Sy · x[c]
		mg/dl					
		LRC	EQ[d]				
VLDL	12	24	23	0.6	0.95	0.91	6.2
LDL	12	149	148	8.4	0.93	0.99	6.9
HDL	12	47	49	−2.2	1.09	0.92	3.3
		PREP[e]	EQ[d]				
VLDL	12	24	23	0.6	0.95	0.91	6.2
LDL	12	145	148	11.9	0.94	0.99	6.5
HDL	12	51	49	5.7	0.85	0.95	2.7
		LRC	RZ				
VLDL	12	24	19	2.7	0.69	0.88	5.3
LDL	12	149	152	0.1	1.02	0.99	8.1
HDL	12	47	49	0.7	1.03	0.87	4.1
		PREP[e]	RZ				
VLDL	12	24	19	2.7	0.69	0.88	5.3
LDL	12	145	152	3.8	1.02	0.99	7.3
HDL	12	51	49	15.4	0.66	0.74	5.7
		EQ[d]	RZ				
VLDL	12	23	19	4.7	0.62	0.83	6.2
LDL	12	148	152	−6.6	1.07	0.98	10.9
HDL	12	49	49	13.5	0.72	0.73	5.8
		PREP[e]	LRC				
VLDL	12	24	24	0	1.00	1.00	0.0
LDL	12	145	149	4.1	1.00	1.00	3.1
HDL	12	51	47	10.5	0.72	0.95	2.3

[a]Abbreviations: LRC, Lipid Research Clinic (method); EQ, equilibrium (density gradient method); RZ, rate zonal (density gradient method); PREP, preparative (method); VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins.

[b]EQ and RZ methods were performed using 0.2 ml of plasma and LRC and PREP methods were performed using 7.0 and 10.0 ml of plasma, respectively. All lipoprotein values were normalized to the same total cholesterol value determined by the standard enzymatic method on the COBAS FARA (see Methods).

[c]Standard deviation of the residuals about the regression line. The differernce, measured in the Y direction, between a given data point and the regression line is called the residual for that point. Sy · x is the standard deviation of those residuals.

[d]HDL defined as particles with density > 1.075 g/ml (see Methods).

[e]HDL defined as particles with density > 1.063 g/ml.

The standard deviation of the residuals about the regression line (Sy · x) was calculated for each method comparison of each lipoprotein (Table 2). The difference, measured in the Y direction, between a given data point and the regression line is called the residual for that point. Therefore a lower Sy · x value generally indicates better agreement between methods. The lowest Sy · x values for all lipoproteins were seen when comparing the PREP and LRC methods. The same VLDL values were used in calculations with the PREP and LRC methods since both methods used the same procedure. This resulted in an Sy · x value of zero for VLDL. The RZ density gradient method had slightly lower Sy · x values than the EQ density gradient method when comparing VLDL values with the LRC and PREP methods. However, the EQ method had slightly lower Sy · x values than the RZ method when comparing LDL and HDL values with the LRC and PREP methods. Overall, the agreement of the density gradient methods with the LRC and PREP methods was acceptable.

Analytical precision of the density gradient methods.

The analytical precision (**Table 3**) was determined using either blind duplicate samples (EQ) or repetitive analyses of the same sample (RZ). Duplicate samples analyzed by the EQ method received fake identifiers to prevent detection of duplicate samples by the laboratory staff. The CHOL concentrations were measured in all density gradient fractions by the micro-enzymatic method and normalized to the total serum CHOL concentration. The total serum CHOL was determined by either the standard enzymatic method (EQ analyses) or by the micro-enzymatic method after a 10-fold dilution (RZ analyses). The precision of both methods was acceptable; however,

TABLE 3. Analytical precision[a] of the equilibrium (EQ) and rate zonal (RZ) density gradient methods

Method	Run Type[b]	n[c]	Analytical Precision (%)									
			Total CHOL[d]	VLDL	LDL_1	LDL_2	LDL_3	Total LDL	HDL_2	HDL_3	VHDL	Total HDL
EQ	WR & BR[e]	20	1.3	13.3	12.5	4.8	7.6	2.7	9.3	8.0	32.2	4.0
RZ	WR	10	2.0	7.5				4.2				8.0
RZ	BR	10	2.3	12.3				3.9				11.9

[a]Analytical precision for the EQ method was determined from blind duplicate pairs and was defined as the mean absolute difference between duplicates divided by the mean of the duplicates times 100. Analytical precision for the RZ method was determined from repeated measures of the same normolipidemic sample and was defined as the standard deviation divided by the mean times 100 (coefficient of variation).

[b]WR, within run; BR, between run.

[c]For EQ, n = the number of duplicate pairs analyzed. For RZ, n = the number of repeat determinations of the same sample.

[d]Total CHOL was determined by a standard enzymatic method for EQ samples and by the micro-enzymatic method (after 10-fold dilution) for RZ samples. All lipoprotein CHOL determinations were by the micro-enzymatic method.

[e]Total CHOL precision was determined within run (WR) and lipoprotein CHOL precision was determined between run (BR).

the EQ method had better precision (except for VLDL) which partially reflects the increased precision of the standard enzymatic method relative to the micro-enzymatic method for measuring total cholesterol. The 10-fold dilution required for measurement of total CHOL by the micro-enzymatic method causes some of the additional variability, so the standard enzymatic method is now used for measuring total CHOL whenever sample volume permits. In general, analytic precision in measuring lipoprotein CHOL was better in fractions with higher CHOL levels (total LDL, total HDL, and LDL_2) than in the fractions with lower CHOL levels (VHDL and VLDL).

DISCUSSION

Several existing technologies have been adapted into a reliable micro-method for determining the CHOL and TG concentrations of lipoproteins and their subspecies. There are several advantages to these methods. First, only 200 μl of serum are needed; 50 μl are sufficient in situations where the sample is extremely difficult to obtain such as from some finger sticks or mice. Secondly, CHOL and TG can be measured in VLDL, LDL, and HDL and in their subspecies within any desired density interval. These techniques have also been used to measure choline-containing phospholipid and unesterified cholesterol concentrations in density gradient fractions (data not shown). The third advantage is the direct measurement of VLDL and LDL as opposed to estimating them using the serum TG concentration (29). This obviates the need for fasting blood samples and provides a flexible way to study lipoprotein metabolism in post-prandial plasma.

There are also several disadvantages to these methods. First, the cost of the equipment required to perform these analyses is expensive. One needs an ultracentrifuge, rotor(s), fractionator, and microtiter plate reader. In addition, a computer and automated liquid pipetting devices are desirable for faster sample throughput. Secondly, the visual resolution of discrete LDL and HDL subspecies by the EQ density gradient method is not as apparent as with native gradient gel electrophoresis (7, 9) or as with other density gradient centrifugation methods using larger rotors with longer sample separation zones (11, 12, 23). The EQ method can be scaled-up to a larger rotor with an appropriate increase in sample volume and centrifugation time. A 22-h spin at full speed in an SW60 rotor (used by the EQ method) is equivalent to a 61-h spin in an SW41 rotor (30). The 22-h spin used in our EQ method is clearly in excess of the time required for the lipoproteins to reach equilibrium. Therefore, centrifugation times less than 22 h are possible with the EQ method using the SW60 rotor. In the RZ separation, lipoproteins did not reach their density equilibrium during centrifugation and the resolution of lipoproteins was not as good as with the EQ method. Therefore, we did not attempt to determine the concentration of LDL and HDL subspecies using the RZ method. However, compared to the EQ method, the RZ method has a time advantage (4 h of centrifugation versus 22 h) and a sample throughput advantage (18 samples per run versus 6 samples per run). The density gradient methods are suitable for the specialized research laboratory but perhaps not for the routine clinical laboratory.

The existence of LDL and HDL subspecies is documented in the literature by the existence of heterogeneity of size, density, charge, composition, metabolism, and atherogenicity (2–19). In this study, the LDL-containing fractions consistently displayed heterogeneity in the CHOL/TG molar ratio across the density gradient

(Figs. 2 and 4). The least dense (LDL_1) and most dense (LDL_3) regions have much lower CHOL/TG molar ratios than the LDL CHOL peak in the LDL_2 region. The ratios within LDL subfractions varied among individuals according to their lipid status. Subjects with hypertriglyceridemia had much lower CHOL/TG ratios in all lipoproteins and also tended to have their LDL peak fractions at higher densities. The mean density of the LDL CHOL peak in 20 male volunteers measured 8 times each over 10 weeks was d 1.0318 g/ml ± 0.0019 (SE). These data are consistent with the findings of others (7, 11, 12).

Kraus (31) has proposed a model to explain the metabolic interrelationships of the LDL subclasses. This model incorporates TG metabolism as a major determinant of LDL subspecies formation. Subjects with elevated plasma TG tend to have smaller, more dense LDL subspecies and lower HDL CHOL (9). In hypertriglyceridemic subjects, VLDL TG may replace LDL and HDL cholesteryl ester. The LDL and HDL TG can then be removed by hepatic lipase or lipoprotein lipase which would result in smaller, more dense LDL and HDL particles (31, 32).

Krauss and Burke (7) and McNamara and colleagues (9) both report the presence of seven major LDL size species in humans using native gradient gel electrophoresis; most humans have either one or two major LDL subspecies. These seven LDL subspecies are found between the densities of 1.025 and 1.063 g/ml. In addition, Krauss (31) reports two IDL subspecies present between the densities of 1.008 and 1.028 g/ml. Chapman and colleagues (12) have isolated and characterized eight major LDL subspecies between the densities of 1.024 and 1.050 g/ml using density gradient ultracentrifugation. These LDL subspecies exhibited size, density, hydrodynamic, charge, and composition heterogeneity. All 40 normolipidemic male subjects in that study displayed a single, well-defined symmetric or asymmetric peak in their density gradient and analytical ultracentrifugal profiles, similar to that reported by Fisher (33) in 86 normolipidemic subjects. The EQ CHOL profiles are very similar to those reported by these investigators.

Two studies have related small dense LDL to increased risk for CAD (8) and non-fatal MI (14). Krauss and colleagues (10) have also related IDL (called LDL_1 in this paper) to progression of CAD. Based on this information about CHD risk, the LDL subspecies have been lumped (in this paper) into three broad LDL subclasses. The LDL_1 subclass was defined as all LDL between the densities of 1.006 and 1.025 g/ml; this corresponds approximately to Krauss's (31) IDL_1 and IDL_2 fractions. In men, the LDL_1 subclass contained, on average, 10% of the plasma CHOL and 24% of the LDL CHOL. The LDL_2 subclass was defined as LDL between the densities of 1.025 and 1.040 g/ml; this corresponds approximately to Krauss and Burke's (7) LDL-I and LDL-II subclasses and McNamara and colleagues' (9) LDL-1 to LDL-3 subclasses. In men, the LDL_2 subclass contained, on average, 35% of the total plasma CHOL and 52% of the total LDL CHOL. The LDL_3 subclass was defined as LDL between the densities of 1.040 and 1.075 g/ml; this density region should contain Krauss and Burke's LDL-III A + B and IV A + B and McNamara and colleagues' LDL_4, LDL_5, LDL_6, and LDL_7. In men, the LDL_3 subclass contained, on average, 10% of the total plasma CHOL and 24% of the total LDL CHOL. The LDL_3 subfraction corresponds to the small dense LDL subfraction that has been linked to increased cardiovascular disease (8, 14). The LDL_3 subclass described here also contained Lp[a] (Fig. 3). Lp[a] can often be present in the 1.050–1.075 g/ml density region (7, 12, 34). If the LRC heparin/manganese procedure precipitates Lp[a] with densities greater than 1.063 g/ml, then this may explain why LDL and HDL values determined by the EQ density gradient method were in better agreement with the LRC heparin/manganese precipitation method when 1.075 g/ml was used as the cut-point between LDL and HDL rather than 1.063 g/ml. However, we did not test this hypothesis.

Lp[a] migrates as a large molecular weight particle on native gradient gel electrophoresis (35) and therefore appears as a band distinct from the dense LDL subspecies described by those using that technique (7, 9, 14, 35). Thus the LDL_3 fraction described here, since it contains Lp[a], might be more atherogenic than the one described by Austin and colleagues (14). Since the EQ density gradient method cannot unequivocally distinguish between LDL and Lp[a], future studies using the EQ density gradient procedure should measure Lp[a] immunochemically to differentiate the cardiovascular risks associated with Lp[a] from those associated with LDL subfractions.

In summary, an accurate and reliable method for measuring the CHOL and TG concentration of lipoproteins and their subspecies has been described that requires only 200 μl of plasma or serum. Future studies will report the concentration and the intra- and inter-individual variability of lipoprotein subspecies, the relationship of lipoprotein subspecies to CHD risk, and the effect of prescribed blood pressure medications on subfraction levels.

The authors are deeply appreciative of Jennifer Hayes, Veronica Hill, Don Koehler, Janet Kopp, Ivan Mills, and Peggy Neibling for their expert technical assistance and Drs. Ivan Frantz and Brian Ishida for their helpful discussions and support. This work was supported by the Biochemical Epidemiology and Lipid Research Core (BELRC) Laboratory and by BRSG 2 SO7 05 448 awarded by the Biomedical Research Support Grant Programs, Division of Research Sources, National Institutes of Health.

Manuscript received 11 June 1990 and in revised form 30 August 1990.

REFERENCES

1. Lippel, K., S. Gianturco, A. Fogelman, P. Nestel, S. M. Grundy, W. Fisher, A. Chait, J. Albers, and P. S. Roheim. 1987. Meeting Summary. Lipoprotein Heterogeneity Workshop. *Arteriosclerosis.* **7:** 315–323.
2. Lindgren, F. T., L. C. Jensen, R. D. Wills, and N. K. Freeman. 1969. Flotation rates, molecular weights, and hydrated densities of the low density lipoproteins. *Lipids.* **4:** 337–344.
3. Adams, G. H., and V. N. Schumaker. 1970. Equilibrium banding of low density lipoproteins. III. Studies on normal individuals and the effects of diet and heparin-induced lipase. *Biochim. Biophys. Acta.* **210:** 462–472.
4. Lee, D. M., and P. Alaupovic. 1970. Studies of the composition and structure of plasma lipoproteins. Isolation, composition, and immunochemical characterization of low density lipoprotein subfractions of human plasma. *Biochemistry.* **9:** 2244–2252.
5. Hammond, M. G., and W. R. Fisher. 1971. The characterization of a discrete series of low density lipoproteins in the disease, hyper-pre-β-lipoproteinemia. *J. Biol. Chem.* **246:** 5454–5465.
6. Shen, M. M. S., R. M. Krauss, F. T. Lindgren, and T. M. Forte. 1981. Heterogeneity of serum low density lipoproteins in normal human subjects. *J. Lipid Res.* **22:** 236–244.
7. Krauss, R. M., and D. J. Burke. 1982. Identification of multiple subclasses of plasma low density lipoproteins in normal humans. *J. Lipid Res.* **23:** 97–104.
8. Crouse, J. R., J. S. Parks, H. M. Schey, and F. R. Kahl. 1985. Studies of low density lipoprotein molecular weight in human beings with coronary artery disease. *J. Lipid Res.* **26:** 566–574.
9. McNamara, J. R., H. Campos. J. M. Ordovas, J. Peterson, P. W. F. Wilson, and E. J. Schaefer. 1987. Effect of gender, age, and lipid status on low density lipoprotein subfraction distribution. Results from the Framingham offspring study. *Arteriosclerosis.* **7:** 483–490.
10. Krauss, R. M., P. T. Williams, J. Brensike, K. M. Detre, F. T. Lindgren, S. F. Kelsey, K. Vranizan, and R. I. Levy. 1987. Intermediate-density lipoproteins and progression of coronary artery disease hypercholesterolaemic men. *Lancet.* 62–66.
11. Swinkels, D. W., H. L. M. Hak-Lemmers, and P. N. M. Demacker. 1987. Single spin density gradient ultracentrifugation method for the detection and isolation of light and heavy low density lipoprotein subfractions. *J. Lipid Res.* **28:** 1233–1239.
12. Chapman, M. J., P. M. Laplaud, G. Luc, P. Forgez, E. Bruckert, S. Goulinet, and D. Lagrange. 1988. Further resolution of the low density lipoprotein spectrum in normal human plasma: physicochemical characteristics of discrete subspecies separated by density gradient ultracentrifugation. *J. Lipid Res.* **29:** 442–458.
13. Swinkels, D. W., P. N. M. Demacker, H. L. M. Hak-Lemmers, M. J. T. M. Mol, S. H. Yap, and A. van't Laar. 1988. Some metabolic characteristics of low-density lipoprotein subfractions, LDL-1 and LDL-2: in vitro and in vivo studies. *Biochim. Biophys. Acta.* **960:** 1–9.
14. Austin, M. A., J. L. Breslow, C. H. Hennekens, J. E. Buring, W. C. Willett, and R. M. Krauss. 1988. Low-density lipoprotein subclass patterns and risk of myocardial infarction. *JAMA.* **260:** 1917–1921.
15. Marzetta, C. A., D. M. Foster, and J. D. Brunzell. 1989. Relationships between LDL density and kinetic heterogeneity in subjects with normolipidemia and familial combined hyperlipidemia using density gradient ultracentrifugation. *J. Lipid Res.* **30:** 1307–1317.
16. Weisgraber, K. H., and R. W. Mahley. 1980. Subfractionation of human high density lipoproteins by heparin-Sepharose affinity chromatography. *J. Lipid Res.* **21:** 316–325.
17. Patsch, W., G. Schonfeld, A. M. Gotto, Jr., and J. R. Patsch. 1980. Characterization of human high density lipoproteins by zonal ultracentrifugation. *J. Biol. Chem.* **255:** 3178–3185.
18. Miller, N. E., F. Hammett, S. Saltissi, S. Rao, H. Van Zeller, J. Coltart, and B. Lewis. 1981. Relation of angiographically defined coronary artery disease to plasma lipoprotein subfractions and apolipoproteins. *Br. Med. J.* **282:** 1741–1744.
19. Cheung, M. C., J. P. Segrest, J. J. Albers, J. T. Cone, C. G. Brouillette, B. H. Chung, M. Kashyap, M. A. Glasscock, and G. M. Anantharamaiah. 1987. Characterization of high density lipoprotein subspecies: structural studies by single vertical spin ultracentrifugation and immunoaffinity chromatography. *J. Lipid Res.* **28:** 913–929.
20. Davis, C. E., D. Gordon, J. LaRosa, P. D. S. Wood, and M. Halperin. 1980. Correlations of plasma high-density lipoprotein cholesterol levels with other plasma lipid and lipoprotein concentrations. The Lipid Research Clinics Program Prevalence Study. *Circulation.* **62, Suppl.IV:** 24–30.
21. Gidez, L. I., G. J. Miller, M. Burstein, S. Slagle, and H. A. Eder. 1982. Separation and quantitation of subclasses of human plasma high density lipoproteins by a simple precipitation procedure. *J. Lipid Res.* **23:** 1206–1223.
22. Lindgren, F. T., H. A. Elliot, and J. W. Gofman. 1951. The ultracentrifugal characterization and isolation of human blood lipids and lipoproteins with applications to the study of atherosclerosis. *J. Phys. Colloid Chem.* **55:** 80–87.
23. Nilsson, J., V. Mannickarottu, C. Edelstein, and A. M. Scanu. 1981. An improved detection system applied to the study of serum lipoproteins after single-step density gradient ultracentrifugation. *Anal. Biochem.* **110:** 342–348.
24. Warnick, R., and J. J. Albers. 1982. Quantitation of Lipoproteins. *In* Manual of Laboratory Operations, Lipid Research Clinics Program, Lipid and Lipoprotein Analysis, Second Edition. A. Hainline, Jr., J. Karon, and K. Lippel, editors. National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD. 63–77.
25. Bachorik, P. S., R. E. Walker, and D. G. Virgil. 1984. High density lipoprotein cholesterol in heparin-$MnCl_2$ supernates determined with the Dow enzymatic method after precipitation of Mn^{++} with HCO_3^-. *Clin. Chem.* **30:** 839–842.
26. Abell, L. L., B. B. Levy, B. B. Brodie, and F. E. Kendall. 1952. Simplified method for the estimation of total cholesterol in serum and demonstration of its specificity. *J. Biol. Chem.* **195:** 357–366.
27. Havel, R. J., H. A. Eder, and J. H. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **34:** 1345–1353.
28. Albers, J. J., J. L. Adolphson, and W. R. Hazzard. 1977. Radioimmunoassay of human plasma Lp[a] lipoprotein. *J. Lipid Res.* **18:** 331–338.
29. Friedewald, W. T., R. I. Levy, and D. S. Fredrickson. 1972. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin. Chem.* **18:** 499–502.

30. Rotors and Tubes for Preparative Ultracentrifuges. An Operator's Manual. 1985. Beckman Instruments, Inc. Palo Alto, CA. Section 1, 1-10.
31. Krauss, R. M. 1987. Relationship of intermediate and low-density lipoprotein subspecies to risk of coronary artery disease. *Am. Heart J.* **113:** 578-582.
32. Deckelbaum, R. J., E. Granot, Y. Oschry, L. Rose, and S. Eisenberg. 1984. Plasma triglycercide determines structure-composition in low and high density lipoproteins. *Arteriosclerosis.* **4:** 225-231.
33. Fisher, W. R. 1983. Heterogeneity of plasma low density lipoproteins. Manifestations of the physiologic phenomenon in man. *Metabolism.* **32:** 283-291.
34. Utermann, G. 1989. The mysteries of lipoprotein[a]. *Science.* **246:** 904-910.
35. McNamara, J. R., H. Campos, J. L. Adolphson, J. M. Ordovas, P. W. F. Wilson, J. J. Albers, D. C. Usher, and E. J. Schaefer. 1989. Screening for lipoprotein[a] elevations in plasma and assessment of size heterogeneity using gradient gel electrophoresis. *J. Lipid Res.* **30:** 747-755.