High resolution plasma lipoprotein cholesterol profiles by a rapid, high volume semi-automated method

Byung H. Chung, Jere P. Segrest,[1] John T. Cone, Janice Pfau, Jack C. Geer, and Lois A. Duncan

Departments of Pathology, Biochemistry, and Microbiology, Institute of Dental Research and Comprehensive Cancer Center, University of Alabama in Birmingham Medical Center, Birmingham, AL 35294

Abstract A new rapid and sensitive method, the single vertical spin autoprofiler (VAP), has been developed for quantitative profiling of the major plasma lipoproteins. The method involves a combination of single vertical spin separation of plasma and continuous on-line analysis of cholesterol. Plasma lipoproteins are first separated by a 45-min spin in a vertical rotor, after which the amount of cholesterol in the effluent of each tube is monitored continuously by a modification of the BMC automated enzymatic cholesterol method; simultaneously, 80% of the sample is diverted by stream-splitting to a fraction collector for further analysis, if desired. VAP not only resolves very low density lipoprotein (VLDL), low density lipoprotein (LDL), and high density lipoprotein (HDL) peaks quantitatively but also detects the presence of intermediate density lipoprotein (IDL) and other lipoprotein variants. VAP was highly reproducible; the inter-run coefficient of variation for cholesterol concentration in VLDL, LDL, and HDL was 4.8%, 2.9%, and 2.4%, respectively. Cholesterol recovery using VAP was 98.5 ± 3.5%. Lipoprotein-cholesterol profiles of plasma from three major hyperlipoproteinemia phenotypes examined by VAP were qualitatively and quantitatively different from each other and from profiles of normolipidemic individuals. One significant finding was that IDL could be detected in the plasma of all type IV hyperlipoproteinemic subjects examined thus far. Several variant lipoprotein profiles which did not correspond to known phenotypes have also been detected by VAP using plasma from hyperlipidemic as well as from normolipidemic subjects. We submit that VAP is an accurate and rapid method for lipoprotein analysis, either for routine clinical screening or for detailed experimental studies. In addition, VAP provides a visual display of partially to completely resolved lipoprotein classes that is suitable for computer-assisted analysis.—**Chung, B. H., J. P. Segrest, J. T. Cone, J. Pfau, J. C. Geer, and L. A. Duncan.** High resolution plasma lipoprotein cholesterol profiles by a rapid, high volume semi-automated method. *J. Lipid Res.* 1981. **22:** 1003–1014.

Supplementary key words high density lipoproteins · low density lipoproteins · very low density lipoproteins · intermediate density lipoproteins · cholesterol · hyperlipoproteinemia

Epidemiological studies indicate that development of coronary artery disease correlates directly with plasma levels of low density lipoprotein (LDL) (1, 2) and inversely with plasma levels of high density lipoprotein (HDL) (3, 4); i.e., LDL and HDL are direct and inverse risk factors, respectively, for coronary artery disease. Further, there is increasing evidence that certain variant lipoprotein subclasses reportedly present in the very low density lipoprotein (VLDL) (5, 6), intermediate density lipoprotein (IDL) (6), LDL (7, 8), and HDL (9) density regions may represent additional risk factors for the development of atherosclerosis. Since the concentrations of VLDL, LDL, HDL, and lipoprotein variants may vary independently of one another, quantitation of each plasma lipoprotein, including variants, should prove valuable for assessment and management of patients with coronary artery disease. Currently, it is a major undertaking, inappropriate for most clinical laboratories, to quantitate the cholesterol content of VLDL, LDL, and HDL, not to mention that of lipoprotein variants, which also may be present. There is, in fact, some question as to the accuracy of any of the methods presently used. The Lipid Research Clinic method involves flotation ultracentrifugation to isolate VLDL, precipitation of LDL with heparin-$MnCl_2$, and subsequent measurement of HDL cholesterol in the remaining supernatant (10). A recent HDL cholesterol analysis proficiency survey indicated that the coefficient of variation in interlaboratory HDL cholesterol

Abbreviations: VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; IDL, intermediate density lipoprotein; "MDL", medium density lipoprotein; apo, apolipoprotein; SVS, single vertical spin method of lipoprotein fractionation; SF, sequential flotation method of lipoprotein fractionation; VAP, vertical (SVS) autoprofiler.

[1] To whom correspondence should be sent.

determinations by this method varied from 9 to 38% (11). There is no corresponding survey of coefficients of variation for VLDL and LDL cholesterol by the same method, but it seems likely that they, too, may be high. Presently, a method for quantitation of lipoprotein variants is not available. Since the relative risk for developing atherosclerosis changes appreciably with small differences in LDL/HDL cholesterol ratios (12, 13), an accurate, quantitative, high volume, high resolution method for cholesterol analysis of the major classes would be of significant benefit to clinical laboratories. Using our single vertical spin (SVS) method (14) for plasma lipoprotein separation, we have developed a semi-automated system, the vertical (SVS) autoprofiler (VAP), which quantitates the cholesterol content of the major lipoprotein classes as well as that of lipoprotein variants. In addition, the VAP system can provide triglyceride profiles and is suitable for computer-assisted data analysis.

EXPERIMENTAL METHODS

Reagents

Autoflo-cholesterol reagents, consisting of 400 mM phosphate buffer (pH 7.2), 3.8 mM 4-aminophenazone, 1.0 M methanol, 0.4% (w/v) hydroxypolyethoxydodecane, >64 mU/ml cholesterol esterase, >2.4 U/ml peroxidase, >70 mU/ml cholesterol oxidase, 6.4 mM phenol, and aqueous cholesterol standards were purchased from Bio-Dynamics/BMC Co., Indianapolis, IN.

A Technicon Autoanalyzer II (TAII), consisting of a proportioning pump, colorimeter, and recorder, was supplied by Dr. C. Andrew Robinson of the Division of Clinical Pathology. A cholesterol incubation manifold was supplied by Mr. Myron Myers of BMC Co. Brij 35 solution was obtained from Technicon Instruments, Tarrytown, NY. Sodium heparin (130–170 USP units/mg) was purchased from Sigma Chemicals, St. Louis, MO and Organon, Inc., West Orange, NJ.

Plasma samples

Blood samples from fasting volunteers and hyperlipidemic patients were collected in test tubes containing EDTA at a final concentration of 1 mg/ml blood. Blood from rat, dog, rabbit, and mouse was collected from animals housed in the University animal facilities in tubes containing 1 mg EDTA/ml blood. Fresh pooled plasma was obtained from the Alabama Regional Red Cross Blood Center, Birmingham, AL.

Single vertical spin centrifugation

A discontinuous NaCl/KBr density gradient was formed as previously reported (14), with a slight modification: 1.3 ml of plasma was pipetted into a 5-ml cellulose nitrate ultracentrifuge tube (Beckman Instruments) and the density was brought to 1.21 g/ml by adding solid KBr (0.43 g). Three and one-half ml of 0.15 M NaCl (d 1.006), pH 7.4, containing 0.01% EDTA, was layered over the sample using a Holter pump, Model 911 (Extra Corporeal Medical Specialties, King of Prussia, PA). Loaded tubes were immediately placed in a Sorvall TV 865 vertical rotor (Dupont Instruments, Wilmington, DE) and centrifuged in a Sorvall ODT-2 model ultracentrifuge at 370,000 *g* for 45 min (9.40×10^{10} ω^2t) at 10°C using the slow start setting on the ARC-1 automatic controller and the A rate setting on the reograd program.

Lipoprotein autoprofiler system

A schematic diagram of the vertical (SVS) autoprofiler (VAP) is shown in **Fig. 1**. Each centrifuge tube containing SVS-separated lipoproteins was placed in a gradient fractionator (Hoeffer Scientific Instruments, San Francisco, CA) and the tube was punctured at the bottom. The gradients remained stable for up to 3 hr when undisturbed and under refrigeration at 7°C. The sample effluent was removed downward by the TAII pump and stream-split. Twenty percent of the sample was continuously mixed with BMC enzymatic cholesterol reagent and incubated and the absorbance of the resulting color was measured by the 1.5-mm flow cell of the TAII colorimeter at 505 nm and plotted on a recorder. The other 80% of the sample was diverted into a fraction collector where samples were available for further analysis. Details of the VAP system, including the ratio of sample to reagent and the various pumping rates are shown in Fig. 1. The transit time in minutes was monitored by marking the recorder paper at the time of centrifuge tube puncture. (In the figures the term "retention time" is used instead of "transition time".) The presence of KBr had no effect on the BMC enzymatic assay of cholesterol (see Table 1). The linearity of the relationship between cholesterol concentration and peak area was calibrated using cholesterol standards, serially diluted pooled plasma, or lipoprotein aliquots. Cholesterol content was analyzed for each by quantitative injection of a 200-μl aliquot of an appropriate standard into the VAP, preceded and followed by a wash of 1 ml of 1% Brij solution. The peak area under the curve was measured by the use of a computer-aided graphics tablet (Apple II plus, Apple Computer Inc., Cupertino, CA). The

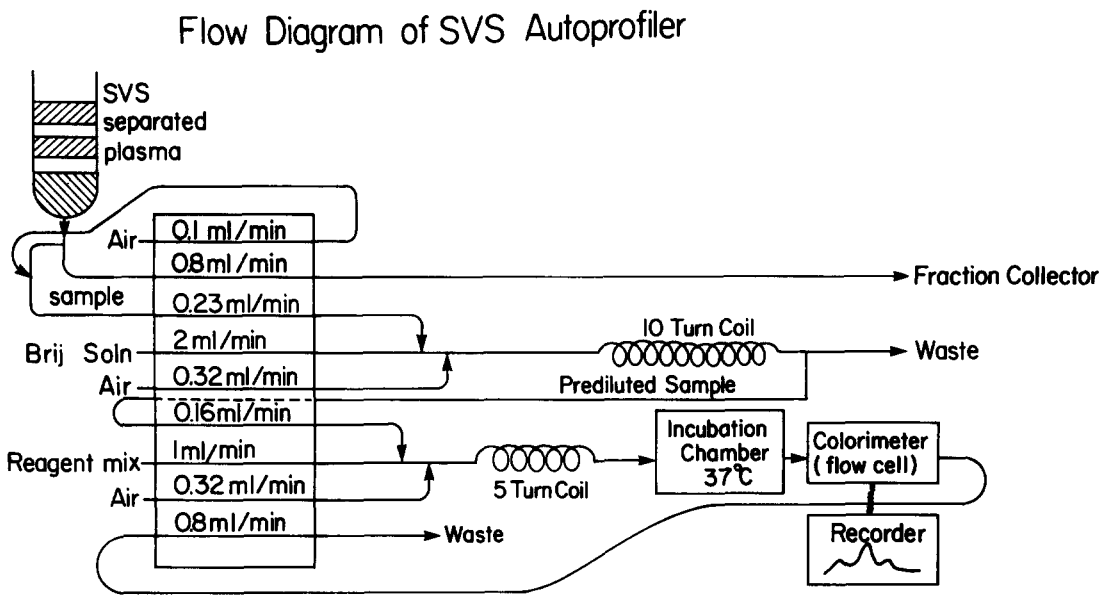

Fig. 1. A schematic flow diagram of the vertical autoprofiler (VAP).

recovery of plasma cholesterol following SVS separation of plasma was determined by subjecting 24 aliquots (1.3 ml) of a single normocholesterolemic plasma sample to VAP analysis: the total peak areas of 16 lipoprotein profiles and 8 nonfractionated plasma samples were compared.

Studies using authentic lipoprotein fractions

VLDL, IDL (d 1.006–1.020 g/ml), LDL (d 1.020–1.063 or 1.006–1.063 g/ml), HDL_2 (d 1.063–1.12 g/ml), and HDL_3 (d 1.12–1.21 g/ml) or HDL (d 1.063–1.21 g/ml) were isolated from pooled plasma by the sequential flotation method (15) and dialyzed against 0.15 M NaCl containing 0.01% EDTA, pH 7.4. The profile of each authentic lipoprotein fraction was determined by the VAP procedure. In addition, known amounts of lipoproteins were added to plasma and changes in lipoprotein cholesterol were determined by the VAP.

VLDL and LDL were precipitated by the heparin-manganese chloride method (16) using several different lots of heparin and by the phosphotungstate method (17) using the BMC (Bio-Dynamics/BMC Co.) HDL cholesterol kit. Profiles of the resulting supernatant solutions and an equivalent amount of untreated plasma were determined using the VAP procedure. Quantitation was accomplished using the computer-aided graphics tablet.

A representative SVS density gradient profile was determined by substituting d 1.21 g/ml KBr solution for density-adjusted plasma. The KBr solution was overlayered with d 1.006 g/ml NaCl solution containing 0.01% EDTA and the sample was subjected to SVS under conditions identical to those used for VAP analysis. The tube was punctured at the bottom, fractions were collected by the downward drop method, and the refractive index of each fraction was measured with a refractometer (Bausch and Lomb Co., Rochester, NY) and converted to density using a standard table from the CRC Handbook of Chemistry and Physics. Statistical analysis was performed by a linear least squares program with the Apple II computer.

Quantitation

The method used to quantitate the areas of each VAP profile represented by HDL, LDL, VLDL, and variant lipoproteins (IDL and "MDL") is the result of a three-step evolution. In the first approximation, areas were measured using an Apple Graphics Tablet (Apple Computer Inc.). The profile was traced from the graph paper on the graphics tablet and the total area, and thus the total cholesterol content, was obtained (Fig. 6B). HDL, LDL, and VLDL peaks were also drawn on the graphics tablet and their cholesterol content was calculated; overlap regions were estimated from the shapes of VAP profiles of isolated authentic lipoproteins. In the second approximation, once the Apple II Plus was interfaced with the VAP, a program was developed to perform the area calculations on-line; triangulation was the method used to resolve overlaps.

Since these methods contain inherent inaccuracies, not the least of which is their inability to quantitate variant lipoproteins, a more sophisticated on-line curve-fitting procedure recently has been developed.

This procedure was used for quantitations reported in the present communication in **Table 1.** The software approximates HDL, LDL, and VLDL curves as bicameral gaussians containing an exponential component on the trailing side, the parameters of which are derived from pre-isolated authentic lipoproteins. Similar procedures have been used successfully in the resolution of liquid chromatography peaks (18, 19). Curve beginnings and endings are sensed by a moving least squares method and the inflection points by a first derivative method. These techniques are used for on-line calculation and print out of lipoprotein cholesterol values, using difference calculations for IDL and "MDL." The software also controls injection of the sample and measures transit time to peak center. This on-line curve-fitting method has been published as an abstract (20) and will be published in detail elsewhere (manuscript in preparation).

Quantitation of VAP profiles for HDL, "MDL," LDL, IDL, and VLDL cholesterol by the on-line curve-fitting method is given in Table I.

RESULTS

Development of plasma lipoprotein autoprofiler

SVS separation of plasma. We previously reported the complete separation of VLDL, LDL, HDL, and free protein by a single discontinuous density gradient ultracentrifugation in a vertical rotor (SVS) (14). In the present study, the gradient was modified to

TABLE 1. Quantitation of cholesterol in lipoprotein fractions

Figure	Experiment	Lipoprotein Fraction					
		HDL	"MDL"	LDL	IDL	VLDL	Total[a]
		cholesterol, mg/dl					
4 A–D	Controls	28.4 ± 3.1(A, B, C)		97.5 ± 6.4(A, B, D)		25.6 ± 1.1(A, C, D)	
	Supplemented	51.6(D)		154.2(C)		45.9(B)	
5 A	+VLDL+IDL+LDL	0	0	124.8	9.7	15.4	
	VLDL+IDL+LDL	0	0	50.7	29.3	16.8	
	VLDL+LDL	0	0	149.3	0	16.8	
5 B	$+HDL_3+HDL_2$	31.9	6.8				
	$+HDL_3$	53.4	16.6	91.0 ± 1.3	6.1 ± 3.5	17.5 ± 1.1	
	$+HDL_2$	30.5	6.8				
7 A	Control	26.8	0	93.7	16.8	22.7	160.0
	Frozen	27.4	0	90.6	14.8	20.3	153.1
7 B	Control	38.4	0	117.1	6.5	33.9	195.9
	Heparin-Mn^{+2}		0	50.6	6.5	13.9	110.8
		39.8 ± 2.1(mean)	0	13.7	3.6	3.4	60.5
	$P0_4$-tung		0	3.2	<0.1	<0.3	43.3
8 A	Left	43.0	1.8	106.9	0	6.9	158.6
B		66.8	0	100.0	12.0	3.5	182.2
C		35.2	0	96.7	3.7	18.8	154.4
A	Center	37.5	4.8	57.5	15.9	15.6	131.3
B		66.8	2.3	51.3	2.1	3.4	125.9
C		65.3	10.1	26.4	8.5	10.4	120.6
9 A	Type IIa	30.7	2.3	368.9	62.6	35.4	500.0
B	Type IV	23.0	3.2	85.8	36.0	102.0	250.0
C	Type V	12.8	3.5	35.2	0	298.5	350.0
10 A	Control	56.6	7.4	104.2	1.1	5.7	175.0
B		46.7	22.5	119.5	0	16.0	204.7
C		85.7	33.7	53.4	6.5	10.1	189.4
D		26.2	58.4	37.3	17.3	79.8	219.0
E		31.2	44.9	21	14.1	93.2	204.4
F	Type III	34.5	18.3	53.6	32.4	86.2	225.0
G		17.6	4.6	73.9	25.4	78.7	200.3
H		41.2	70.4	32.6	0.2	32.6	177.0
I		44.2	36.6	162.6	15.3	20.8	279.6
J	Type I	13.2	9.9	12.2	38.6	346.0	419.9

[a] Based upon total area under each complete VAP profile.

produce a wider separation of HDL, LDL, and VLDL in order to minimize the mixing produced as the sample flows through the autoanalyzer system. The banding positions of VLDL, LDL, and HDL using this modification are shown in **Fig. 2A**, and, it can be seen that HDL is not as well separated from free protein as it was using the original gradient (14). Fig. 2B shows the SVS density gradient profile produced when d 1.21 g/ml KBr is substituted for d 1.21 g/ml plasma.

Lipoprotein cholesterol profiles. A typical lipoprotein cholesterol profile of normocholesterolemic plasma is shown in **Fig. 3A**. Three separate peaks, representing the cholesterol present in HDL, LDL, and VLDL, are resolved. Transit times from tube puncture (t = 0) to the peak centers of HDL, LDL, and VLDL are 7.0–7.3, 8.7, and 9.8–10.1 min, respectively. Preisolated authentic lipoproteins, subjected to VAP under the same conditions, produce identical retention times (Fig. 3B). The partial separation of either HDL_2 from HDL_3 or IDL from LDL and VLDL is evident from the difference in transit times to the peak centers of the individual fractions: HDL_3 = 7.0 min, HDL_2 = 7.3 min, IDL = 9.3 min (Fig. 3B).

A mixture of preisolated authentic VLDL, LDL, and HDL gives a profile similar to that of plasma (**Fig. 4E**). Supplementation of plasma with authentic VLDL, LDL, or HDL increases the magnitude of the lipoprotein peak corresponding to the authentic lipoprotein added (Fig. 4A–D and Table 1).

Addition of IDL to a mixture of LDL and VLDL results in the appearance of a shoulder on the descending side of the LDL peak (**Fig. 5A**). Increasing

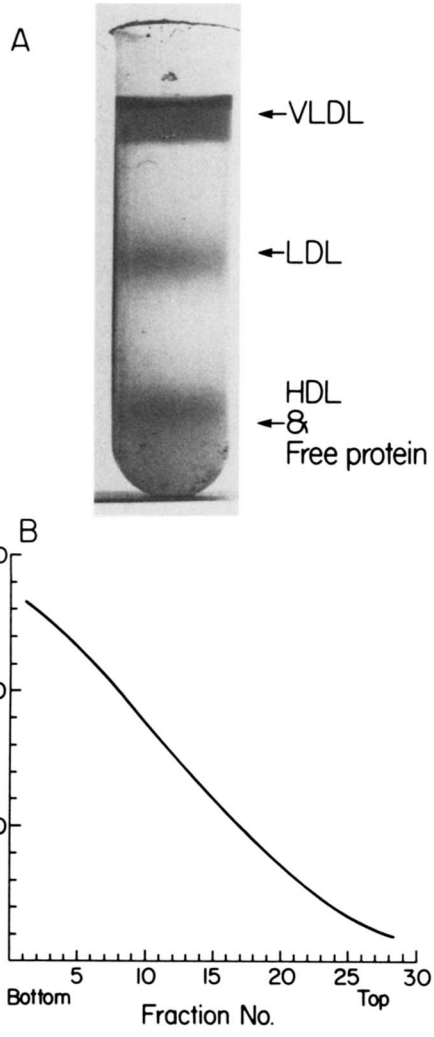

Fig. 2. A, Separation of plasma lipoproteins by single vertical spin (SVS) centrifugation. B, Density profile of gradient in A.

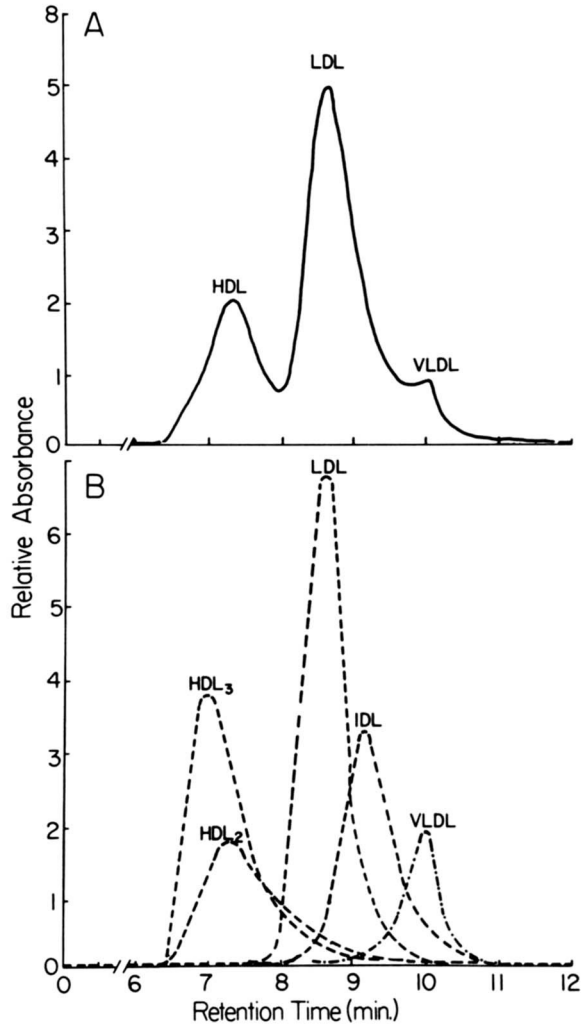

Fig. 3. VAP cholesterol profiles: A, A typical pattern for normocholesterolemic plasma. Quantitation of profile given in Table 1. B, Preisolated authentic lipoproteins.

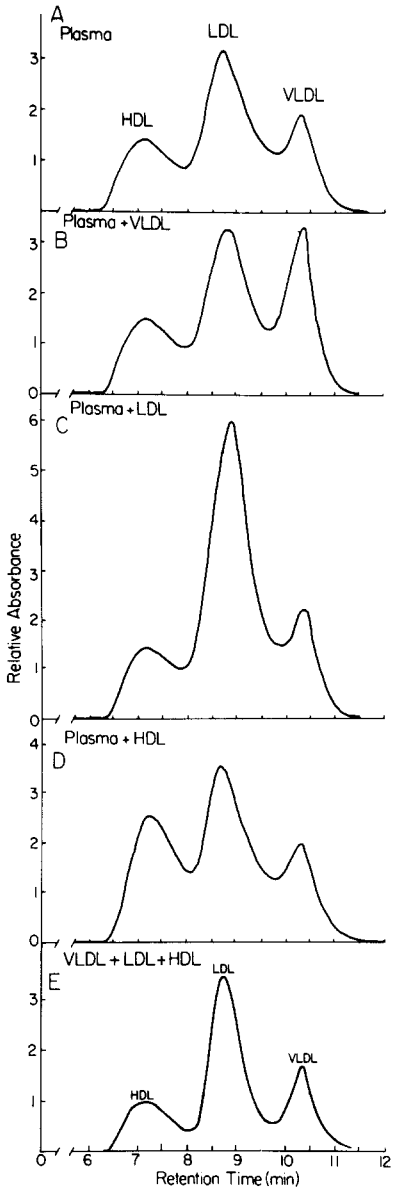

Fig. 4. Authentication of HDL, LDL, and VLDL peak positions. A–D, VAP cholesterol profiles of plasma and plasma supplemented with preisolated authentic lipoproteins. Normal saline (0.3 ml) or one of three preisolated lipoproteins containing 30–100 mg/dl cholesterol were added to 1-ml samples of plasma, and the final mixtures were subjected to the VAP procedure. E, VAP cholesterol profile of a mixture of preisolated authentic VLDL, LDL, and HDL. Samples (0.4 ml each) of VLDL (50 mg/dl cholesterol), LDL (100 mg/dl cholesterol) and HDL (50 mg/dl cholesterol) were mixed, the total volume of the mixture was brought to 1.3 ml with normal saline and the samples were subjected to the VAP procedure.

the ratio of IDL to LDL produces an IDL peak, although the two fractions still overlap (Fig. 5A).

The lipoprotein profiles of a mixture of LDL and VLDL, to which HDL_2, HDL_3 or a mixture of both was added, are shown in Fig. 5B. The ascending side of the HDL peak in the HDL_3 mixture is steeper than when HDL_2 was added, and the transit time peak centers are 7.0 and 7.3 min, respectively (Fig. 5B). When the mixture contains both HDL_2 and HDL_3 in equal amounts, a single peak at approximately 7.15 min is seen (Fig. 5B).

Calibration of the vertical autoprofiler. We evaluated the utility of the VAP system for quantitating the amount of cholesterol present in plasma or each lipoprotein class. In the first experiment, the area of a plasma peak (plasma was not subjected to SVS fractionation and was diluted serially) is shown to increase linearly with concentration (**Fig. 6A**).

When preisolated authentic VLDL, LDL, or HDL, each of known cholesterol concentration, is reisolated by SVS and analyzed by the VAP procedure, the

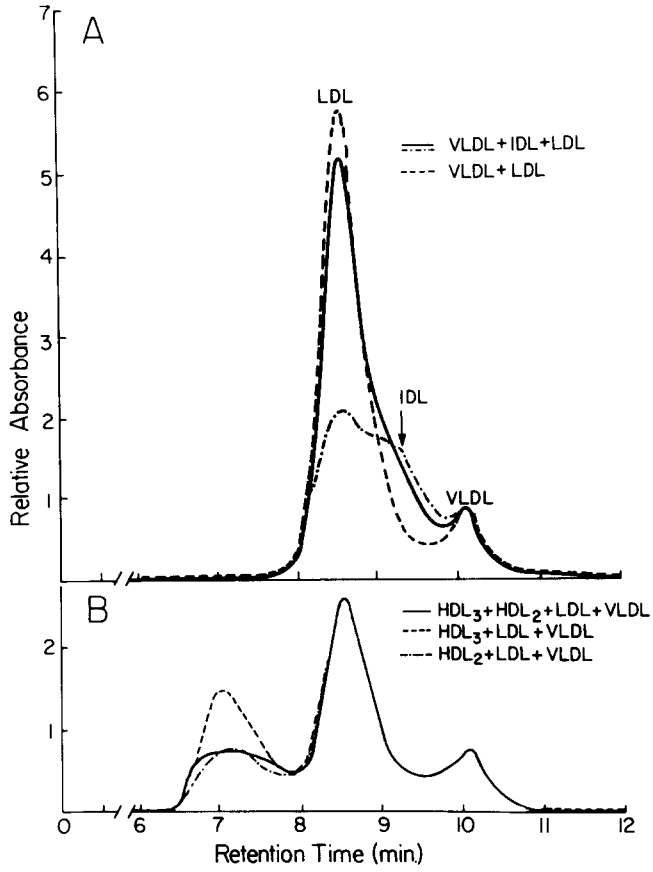

Fig. 5. Effects of IDL, HDL_3, and HDL_2 on VAP profiles. A, VAP fractionation of intermediate density lipoprotein (IDL): cholesterol profiles (– – – –) a mixture of authentic VLDL (d < 1.006 g/ml) and LDL (d 1.020–1.063 g/ml); (——) a mixture of VLDL, LDL, and IDL (d 1.006–1.020 g/ml); and (–·–·–) a mixture of VLDL, LDL, and IDL in which the concentration of IDL is increased and of LDL decreased relative to the previous sample. B, VAP fractionation of HDL_3 and HDL_2. VAP cholesterol profiles are shown of a mixture of pre-isolated authentic HDL_2 and/or HDL_3 with LDL and VLDL. For the HDL_2 + HDL_3 + LDL + VLDL mixture, equal concentrations of HDL_2 and HDL_3 cholesterol were used. Quantitation of each profile given in Table 1.

correlation coefficient (r) of peak area and cholesterol concentration for all VLDL, LDL, and HDL points is 0.997 (Fig. 6B). When r is calculated for VLDL, LDL, or HDL separately, the values are 0.998, 0.999, and 0.999, respectively. The standard deviation of fit for all the analyses is ±0.07 mg cholesterol.

Evaluation of the vertical autoprofiler. Lipoprotein cholesterol profiles for aliquots of the same plasma sample separated in the same rotor were highly reproducible. The intrarun and interrun coefficients of variation (CV) of total cholesterol for a series of 24 whole profiles were 1.6 and 0.7%, respectively, and the intrarun CV for the VLDL, LDL, and HDL cholesterol peaks was 4.8%, 2.6%, and 2.2%, respectively. The recovery of plasma cholesterol following separation of plasma into individual lipoprotein fractions by SVS was 98.5 ± 3.5% (n = 16). When a normocholesterolemic plasma was frozen and thawed prior to VAP analysis, there were only minor changes in the cholesterol profile as compared to the fresh plasma (**Fig. 7A** and Table 1).

Precipitation of apoB-containing lipoproteins with either heparin-$MnCl_2$ (16) or sodium phosphotungstate-$MgCl_2$ (17) and subsequent VAP analysis of the supernatant indicates that precipitation is not complete (Fig. 7B). The degree of precipitation, however, depends on the lot of heparin and the method used. Results using two different heparin batches are shown in Fig. 7B. Although the phosphotungstate kit produces the better result, 2% of the LDL and VLDL cholesterol remains (Table 1).

Use of VAP to evaluate plasma lipoprotein cholesterol profiles. Lipoprotein cholesterol profiles of plasma from three normocholesterolemic human subjects are shown in **Fig. 8,** left panel. The profile patterns are similar, yet the ratios and total HDL, LDL, and VLDL cholesterol vary somewhat (Table 1). Fig. 8, center panel, shows the profiles of plasma from normocholesterolemic subjects with unusually low LDL/HDL ratios. The increased concentration of HDL in female subjects B and C presumably represents mostly HDL_2, since the transit time for both HDL peak centers is 7.3 min as compared to 7.0 min for male subject A. Profiles from a number of otherwise normal subjects consistently reveal shoulders on either the ascending or the descending side of the HDL peak (Fig. 8, right panel); positive identification of these lipoprotein variants has not been made.

Plasma profiles from type IIa, IV, or V hyperlipoproteinemic patients, identified by standard Lipid Research Clinic methodology, are shown in **Fig. 9.** A marked increase in LDL and a decrease in HDL cholesterol characterize the type IIa profile (plasma

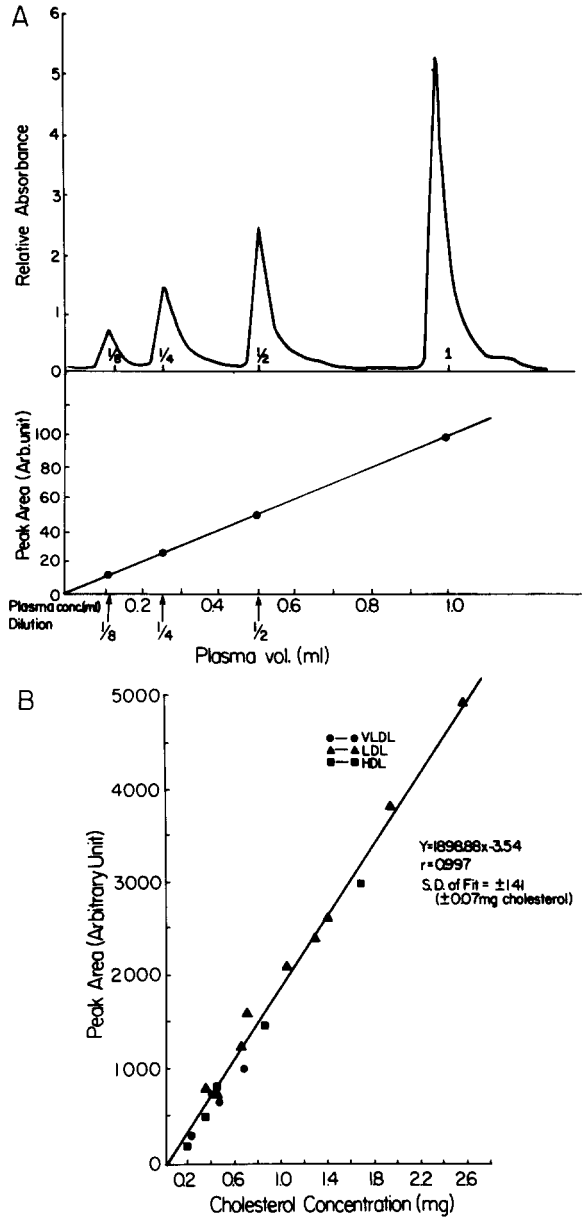

Fig. 6. A, Linear relationship between peak area and concentration of plasma cholesterol: (upper) autoanalysis of 200 μl of serially diluted (0, 1/2, 1/4, 1/8) plasma which has not been subjected to SVS fractionation; (lower) peak area measured by a computer-aided graphic device and plotted versus relative cholesterol concentration. B, Linear relationship between peak area and lipoprotein cholesterol of pre-isolated, serially diluted VLDL, LDL, and HDL analyzed by the VAP procedure. Aliquots, 0.2 ml, 0.4 ml, 0.6 ml, 0.8 ml, 1.0 ml or 1.2 ml of pre-isolated authentic VLDL, LDL, or HDL, of known cholesterol concentration, were pipetted into centrifuge tubes, the total volume brought to 1.3 ml with normal saline, and the samples subjected to VAP analysis. Regression lines were then obtained by a linear least square program with the Apple II plus computer.

cholesterol = 500 mg/dl, Fig. 9A and Table 1); increases in both VLDL and IDL and a decrease in HDL cholesterol are found consistently in numerous type IV profiles (Fig. 9B and Table 1); increased

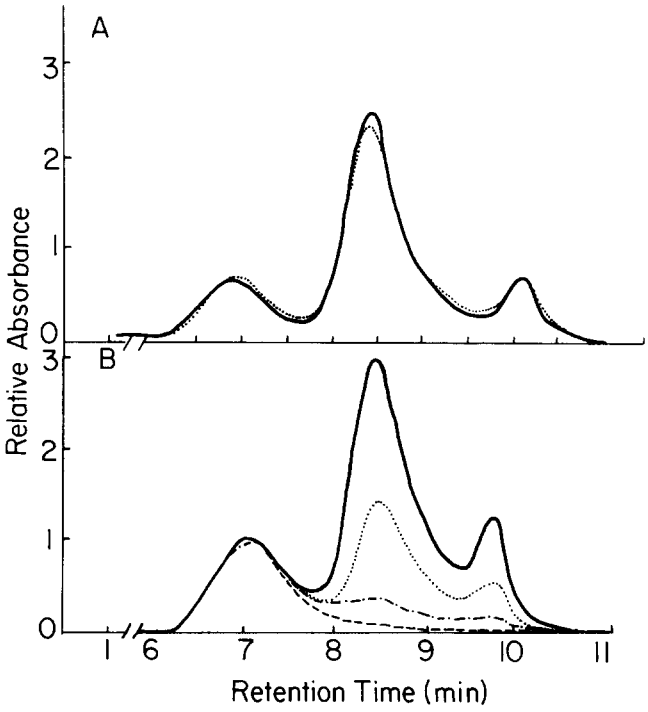

Fig. 7. A, VAP cholesterol profiles of fresh and frozen-thawed plasma: fresh plasma was frozen at −70°C and left overnight. Frozen plasma was then thawed at 7°C and subjected to VAP analysis along with identical plasma stored overnight in a refrigerator at 7°C; (——), fresh plasma; (· · · · · · ·), frozen-thawed plasma. B, VAP cholesterol profiles of the supernatant of plasma treated with heparin-$MnCl_2$ (prepared from two different sources of heparin) or with sodium phosphotungstate-$MgCl_2$ as compared with untreated hyperlipidemic plasma. Sodium heparin (0.12 ml) (5000 USP unit/ml, 130–170 unit/mg) and 0.15 ml of 1.0 M $MnCl_2$ or 0.3 ml of 4% sodium phosphotungstate and 0.075 ml of 2 M $MgCl_2$ were added to 3 ml of plasma. The samples were allowed to stand 30 min at 4°C, then centrifuged at 1500 *g* for 30 min at 4°C. Supernatants and untreated plasma, appropriately diluted with normal saline, were subjected to VAP analysis: (——), untreated plasma; (–·–·–) and (· · · · · · ·), heparin-$MnCl_2$-treated plasma; (– – –), sodium phosphotungstate-$MnCl_2$-treated plasma. Quantitation of each profile given in Table 1.

VLDL with markedly decreased LDL and HDL are characteristic of the type V profile (Fig. 9C and Table 1).

We have determined the lipoprotein cholesterol profiles of plasma from a large number of individuals (over 2000 as of this writing) and have observed anomalous peaks in lipoprotein patterns from many hyperlipoproteinemic as well as normolipidemic subjects (**Fig. 10** and Table 1). The identity and significance of these peaks are not known for certain but are under active investigation.

The plasma lipoprotein cholesterol profiles of several animal species are shown in **Fig. 11.** The density range of a given lipoprotein peak is often shifted or broadened as compared to the corresponding human peak; the peak center of rabbit LDL is

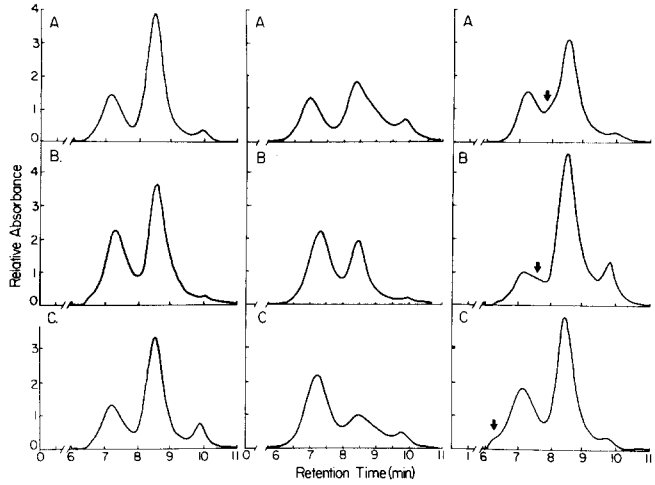

Fig. 8. VAP cholesterol profiles of plasma from normocholesterolemic subjects which illustrate variation. Left panel, three "normal" profiles. Center panel, VAP cholesterol profiles of plasma from three subjects with high HDL/LDL ratios; A, young male; B and C, young females. Right panel, VAP cholesterol profiles of plasma from normocholesterolemic subjects showing reproducible lipoprotein shoulders in the HDL density range. Quantitation of profiles in left and middle panels given in Table 1.

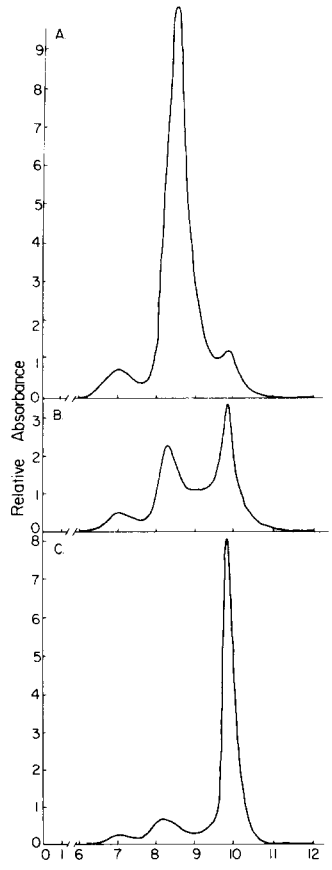

Fig. 9. VAP cholesterol profiles of plasma from three patients with hyperlipoproteinemia, authenticated by Lipid Research Clinic methodology. A, Familial type II-a (diluted 2:1); B, type IV; C, type V. Quantitation of profiles given in Table 1.

at a lower density than that of human; the HDL fraction in the mouse is in the density range of human HDL_2 and rat HDL is bimodal and broad.

DISCUSSION

The procedure used most often for separation and quantitation of plasma HDL involves polyanion precipitation of VLDL and LDL (22). In the Lipid Research Clinic modification of this method (10), VLDL is separated by flotation ultracentrifugation prior to precipitation. Either method has a number of weaknesses: *a*) incomplete precipitation of apoB-containing lipoproteins leads to overestimation of HDL cholesterol (16); *b*) precipitation of apoE-containing HDL_2 leads to underestimation of HDL cholesterol (23); *c*) VLDL cannot be quantitated directly by the polyanion precipitation method; *d*) VLDL flotation is time-consuming and limits the number of analyses to a few per day; and *e*) neither method allows for quantitation of lipoprotein subfractions and/or variants. Based on the results presented in Fig. 7B, the heparin-$MnCl_2$ method seems to vary, presumably due to variation in the effectiveness of the heparin preparations used. Electrophoresis is another method used to quantitate plasma lipoprotein cholesterol (24). There is some question, however, as to whether it is truly quantitative; e.g., values for HDL cholesterol measured by this method are poorly reproducible (21). Since the classic means of defining lipoprotein classes is based on their hydration densities (26), quantitation on this basis would seem desirable. Unfortunately, methods currently in use are time-consuming and not amenable to large-scale clinical use; further, there is evidence that extended ultracentrifugation produces artifacts in lipoprotein composition and, possibly, density, due to dissociation of apolipoprotein and/or lipid (25).

Use of SVS ultracentrifugation for density separation, followed by continuous flow autoanalyzer analysis of lipoprotein cholesterol, has a number of advantages over present methods and makes possible a rapid, quantitative, high resolution, high volume analysis of plasma lipoprotein cholesterol profiles.

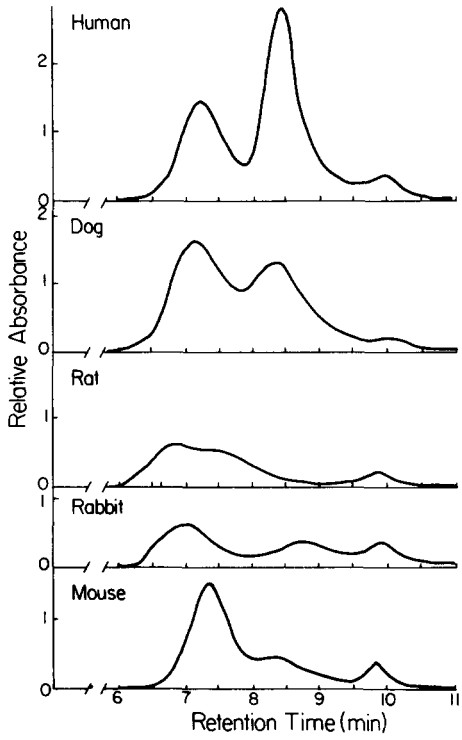

Fig. 11. Comparison of VAP cholesterol profiles of plasma from a normocholesterolemic human subject with plasma from four animal species. The details of diet for each animal are unknown.

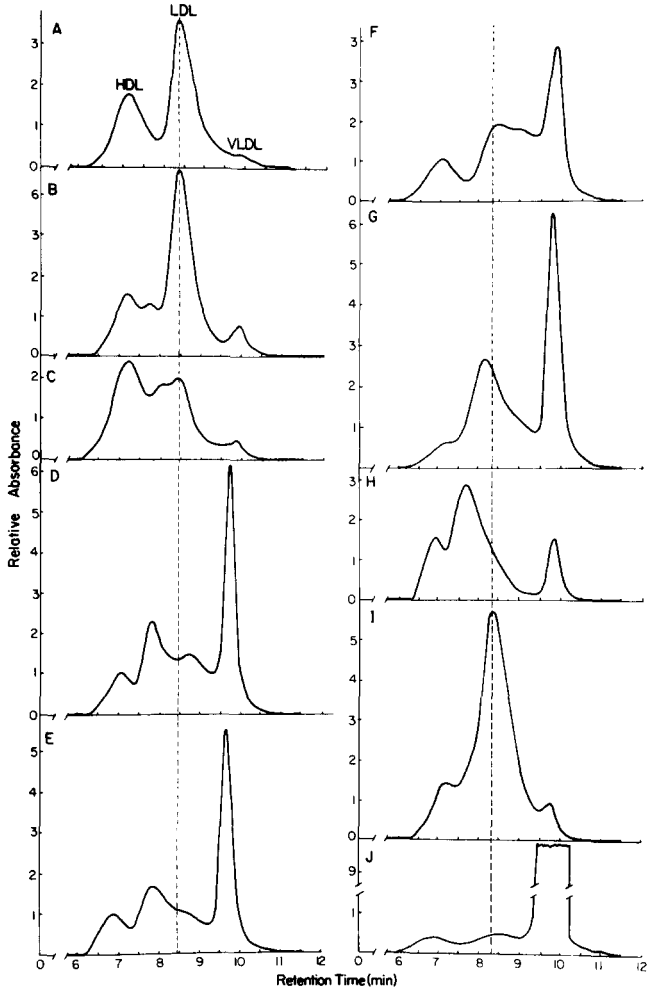

Fig. 10. VAP cholesterol profiles of normal and hyperlipidemic subjects showing variant lipoprotein peaks: A, Normocholesterolemic control pattern; B–J, Variant lipoprotein patterns. Quantitation of each profile given in Table 1.

TABLE 2. Advantages of the SVS lipoprotein autoprofiler

1. Provides a rapid, high resolution, high volume lipoprotein profile (8 profiles/2 hr and up to 50 analyses/8-hr day) using only ultracentrifugation for fractionation.
2. Provides a quantitative analysis of cholesterol and triglyceride content of HDL, LDL, IDL, and VLDL.
3. Provides computer-assisted curve deconvolution and analysis of data.
4. Short spin time of 45 min minimizes artifacts due to dissociation and/or transfer of apoprotein and lipid.
5. Variant lipoproteins appear as reproducible peaks or shoulders.
6. Apolipoprotein profiles are available on HDL, LDL, IDL, and VLDL, as well as variant lipoprotein fractions.
7. HDL, LDL, IDL, and VLDL, as well as variant lipoproteins, are available for additional analyses, such as cholesteryl ester and phospholipid.
8. Storage of plasma up to 10 days with a single freeze-thaw does not affect reproducibility of VAP cholesterol analysis.

Table 2 lists the advantages of the VAP procedure compared with other methods of lipoprotein quantitation. The VAP procedure visually displays a complete lipoprotein profile in one step and quantitation of each lipoprotein cholesterol peak is achieved by computer-assisted data analysis (20). We are currently comparing values obtained using VAP with those obtained by the standard Lipid Research Clinic method. A preliminary scatter diagram comparison of total cholesterol measured by VAP versus Lipid Research Clinic methods indicates good fit (r = 0.902, n = 45).

Since the lipoprotein profiles of plasma from hyper- or hypolipoproteinemic patients examined thus far with VAP are distinctly different from each other and from those of normolipidemic individuals, the system is proving useful for lipoprotein phenotyping of the standard Fredrickson, Levy, and Lees (27) types (Fig. 9). As we noted elsewhere (14), chylomicrons must be removed from chylemic plasma by low speed centrifugation, before SVS ultracentrifugation, to prevent inboard layering of this fraction along the length of the tube wall. In our hands there is no layering of VLDL on the inboard wall or free protein on the outboard wall of the centrifuge tube. Of particular interest is the clear and reproducible presence of intermediate density lipoprotein, representing greater than 5% of total plasma cholesterol, in the plasma of all type IV hypertriglyceridemics examined. The presence of IDL in type IV patients has not been generally recognized and warrants reevaluation, although the polydisperse LDL previously reported for this disorder likely represents IDL (28).

As shown in Fig. 10, there is considerable variation among plasma lipoprotein profiles in the general population. Many of these profiles contain variant peaks falling in a density range between HDL and LDL (Fig. 10B, C, D, E, H): We have given the operational designation, medium density lipoprotein ("MDL") to these peaks. Because several of the profiles containing "MDL" also show markedly decreased or even absent LDL (Fig. 10D, E, H), we presume that "MDL" is a variant of LDL, probably Lp(a), since "MDL" has an electrophoretic mobility midway between LDL and VLDL, is precipitated by heparin-$MnCl_2$, and contains apoB (data not shown).

The profile shown in Fig. 10F is representative of a type III pattern. Fig. 10J is a profile derived from a young child with severe hypertriglyceridemia and, presumably, represents a type I pattern. Interestingly, the very small cholesterol peak to the left of the VLDL and chylomicron peak in Fig. 10J has an IDL rather than an LDL transit time. This is consistent with the finding that type I hyperlipoproteinemia is due to a deficiency in lipoprotein lipase.

Many otherwise normal VAP profiles show a distinct shoulder on the dense side of the HDL peak (Fig. 8, right C). We suspect that this shoulder may correspond to the HDL subfraction HDL_{3D} (or VHDL) described by Patsch, et al. (29).

We recently completed the assembly of a companion triglyceride autoanalyzer, which enables us to determine cholesterol and triglyceride lipoprotein profiles simultaneously (**Fig. 12**). We are in the process of calibrating and evaluating the triglyceride VAP and will report results of these studies later.

Since the VAP system uses approximately 40% of the total SVS separated lipoprotein sample for combined cholesterol and triglyceride analysis, the remaining sample is routinely directed into a fraction collector (Fig. 1) and used for other analyses such as apolipoprotein determination by radioimmunoassay

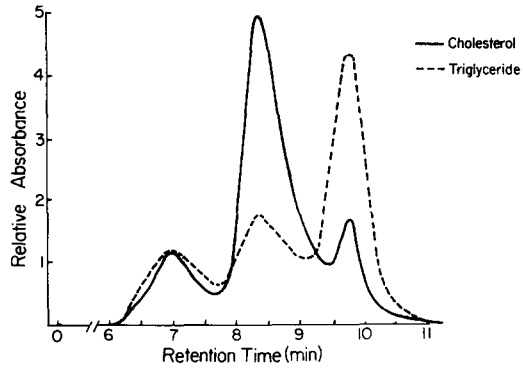

Fig. 12. Simultaneous VAP profiling of lipoprotein cholesterol and triglyceride from plasma of a subject with total plasma triglyceride in the upper normal range.

and lipid analyses for cholesteryl ester, phospholipid, and fatty acids.

The VAP procedure should prove to be an accurate and reproducible means for evaluating plasma levels of HDL cholesterol and for determining the LDL/HDL and VLDL/HDL cholesterol ratios. We have preliminary evidence to suggest that the VLDL/HDL cholesterol ratio and level of IDL are markedly increased in a population of patients with coronary artery disease compared with normal controls. If these prove to be true risk indicators for coronary artery disease, the VAP method should find wide clinical use, since no alternative high volume procedure for determining these factors is presently available. In the same way, "MDL" (Lp(a)?) measurements also may prove to be clinically relevant (30).

In addition to possessing clear cut advantages in clinical settings, the VAP method, because of its rapidity and high volume capability, is proving to be remarkably useful in investigations of lipoprotein metabolism. We have already performed a number of kinetic studies that show complex and coordinated alterations in lipoprotein concentration and density over short time intervals.

We would like to thank Dan Gianelloni for providing the BMC cholesterol and triglyceride manifolds and for helpful suggestions. This work was supported in part by NIH grant HL 19551 and by National Research Service Award T32 CA09128, awarded by the National Cancer Institute, DHHS.

Manuscript received 30 September 1980 and in revised form 14 April 1981.

REFERENCES

1. Kannel, W. B., W. P. Castelli, T. Gordon, and P. M. McNamara. 1971. Serum cholesterol, lipoproteins and risk of coronary heart disease; the Framingham Study. *Ann. Intern. Med.* **74:** 1–12.
2. Castelli, W. P., G. R. Cooper, J. T. Doyle, M. Garcia-Palmieri, T. Gordon, H. C. Hames, S. B. Hulley, A. Kagan, M. Kuchmak, D. McGee, and W. J. Vicic. 1977. Distribution of triglyceride and total LDL, and HDL cholesterol in several populations: a cooperative lipoprotein phenotyping study. *J. Chronic Dis.* **30:** 147–169.
3. Castelli, W. B., J. T. Doyle, T. Gordon, C. G. Homes, M. C. Hjortland, S. B. Hulley, A. Kaganiard, and W. J. Zukel. 1977. HDL cholesterol and other lipids in coronary heart disease. The cooperative lipoprotein phenotyping study. *Circulation.* **55:** 767–772.
4. Kannel, W. B., W. P. Castelli, and T. Gordon. 1979. Cholesterol in the prediction of atherosclerotic disease. *Ann. Intern. Med.* **90:** 85–91.
5. Zannis, V. I., and J. L. Breslow. 1980. Characterization of unique human apolipoprotein E variant associated with type III hyperlipoproteinemia. *J. Biol. Chem.* **255:** 1759–1762.
6. Hazzard, W. R., and E. L. Bierman. 1971. Impaired removal of very low density lipoprotein (VLDL) remnants in the pathogenesis of broad-beta disease (type III hyperlipoproteinemia). *Clin. Res.* **19:** 476.
7. Dahlen, G., K. Berg, U. B. Ramberg, and A. Tamm. 1974. Lp(a) lipoprotein and pre-β_1-lipoprotein in young adults. *Acta Med. Scand.* **196:** 327–331.
8. Snidermann, A., S. Shapiro, D. Marpole, B. Skinner, B. Teng, and P. O. Kwiterovich, Jr. 1980. Association of coronary atherosclerosis with hyperapobetalipoproteinemia (increased protein but normal cholesterol levels in human plasma low density (β) lipoproteins). *Proc. Natl. Acad. Sci. USA.* **77:** 604–608.
9. Mahley, R. W., T. L. Innerarity, T. P. Bersot, A. Lipson, and S. Margolis. 1978. Alteration in human high density lipoproteins, with or without increased plasma cholesterol, induced by diets high in cholesterol. *Lancet.* **1:** 807–809.
10. Manual of Laboratory Operation, Lipid Research Clinic Program. Lipid and Lipoprotein Analysis. 1974. U.S. Department of HEW, publication No. (NIH) 75-628. 56–58.
11. Warnick, R. G., J. J. Albers, and E. T. Leary. 1980. HDL cholesterol: results of interlaboratory proficiency tests. *Clin. Chem.* **26:** 169–170.
12. Gordon, T., W. P. Castelli, M. Hjortland, W. B. Kannel, and T. C. Dawber. 1977. High density lipoprotein as a protective factor against coronary heart disease; the Framingham Study. *Am. J. Med.* **62:** 707–714.
13. Heldenberg, D., I. Tamir, O. Levtow, Y. Burstein, and B. Werbin. 1979. Lipoprotein measurements—a necessity for precise assessment of risk in children from high risk families. *Arch. Dis. Child.* **54:** 695–698.
14. Chung, B. H., T. Wilkinson, J. C. Geer, and J. P. Segrest. 1980. Preparative and quantitative isolation of plasma lipoproteins: rapid, single discontinuous density gradient ultracentrifugation in a vertical rotor. *J. Lipid Res.* **21:** 284–291.
15. 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.
16. Warnick, G. R., and J. J. Albers. 1978. A comprehensive evaluation of the heparin-manganese precipitation procedure for estimating high density lipoprotein cholesterol. *J. Lipid Res.* **19:** 65–76.
17. Lopes-Virella, M. F., P. Stone, S. Ellis, and J. A. Colwell. 1977. Cholesterol determination in high density lipoproteins separated by three different methods. *Clin. Chem.* **23:** 882–884.
18. Metzger, H. D. 1970. True peak area separation of overlapping peaks in a gas chromatogram by a process computer. *Chromatographia.* **3:** 64–70.
19. Lochmuller, C. H., and M. Sumner. 1980. Estimation of extra-column dead volume effects using a mixing cell model. *J. Chromatogr. Sci.* **18:** 159–165.
20. Cone, J. T., J. P. Segrest, B. H. Chung, A. Glasscock, and M. Hutchinson. 1981. Computerized rapid high resolution quantitative analysis of plasma lipoproteins including HDL_2, HDL_3 and IDL. *Federation Proc.* **40:** 345 (Abstract 641).
21. Kohl, H. H., W. Johnson, and L. L. Rogers. 1980. Intercomparison of four precipitation methods and electrophoresis of high-density lipoprotein cholesterol determination. *Clin. Chem.* **26:** 672–673.

22. Burstein, M., H. R. Scholnick, and R. Martin. 1970. Rapid method for the isolation of lipoproteins from human serum precipitation with polyanions. *J. Lipid Res.* **1:** 583–595.
23. Srinivasan, S. R., T. A. Foster, and G. S. Berenson. 1980. Comparability of results by simplified methods for measuring serum lipoprotein cholesterol in a pediatric population. *Clin. Chem.* **26:** 1548–1553.
24. Conlon, D. R., L. A. Blankenstein, P. A. Pasakarnis, C. M. Steinberg, and J. E. Damelia. 1979. Quantitative determination of high density lipoprotein cholesterol by agarose gel electrophoresis. *Clin. Chem.* **25:** 1965–1969.
25. Fainaru, M., R. J. Havel, and K. Imaizumi. 1977. Apoprotein content of plasma lipoproteins of the rat separated by gel chromatography or ultracentrifugation. *Biochem. Med.* **17:** 347–355.
26. Gofman, J. W., F. T. Lindgren, and H. Elliot. 1949. Ultracentrifugal studies of lipoproteins of human serum. *J. Biol. Chem.* **179:** 973–979.
27. Fredrickson, D. S., R. I. Levy, and R. S. Lees. 1967. Fat transport in lipoproteins—an integrated approach to mechanism and disorder. *N. Engl. J. Med.* **276:** 34–44, 94–103, 148–150, 218–225, 273–281.
28. 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.
29. 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.
30. Kostner, G. M., P. Avagaro, G. Cazzolato, E. Marth, G. Bittolo Bon, and G. B. Quinci. 1981. Lipoprotein Lp(a) and the risk for myocardial infarction. *Atherosclerosis.* **38:** 51–61.