

# Computerized rapid high resolution quantitative analysis of plasma lipoproteins based upon single vertical spin centrifugation

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**Abstract** A method has been developed for rapidly quantitating the cholesterol concentration of normal and certain variant lipoproteins in a large number of patients (over 240 in one week). The method employs a microcomputer interfaced to the vertical autoprofiler (VAP) described earlier (Chung et al. 1981. *J. Lipid Res.* 22: 1003-1014). Software developed to accomplish rapid on-line analysis of the VAP signal uses peak shapes and positions derived from prior VAP analysis of isolated authentic lipoproteins HDL, LDL, and VLDL to quantitate these species in a VAP profile. Variant lipoproteins VHDL (a species with density greater than that of HDL<sub>3</sub>), MDL (a species, most likely Lp(a), with density intermediate between that of HDL and LDL), and IDL are subsequently quantitated by a method combining difference calculations with curve shapes. The procedure has been validated qualitatively by negative stain electron microscopy, gradient gel electrophoresis, strip electrophoresis, chemical analysis of the lipids, radioimmunoassay of the apolipoproteins, and measurement of the density of the peak centers. It has been validated quantitatively by comparison with Lipid Research Clinic methodology for HDL-, LDL-, and VLDL-cholesterol, and for MDL- and IDL-cholesterol by comparison of the amounts of MDL or IDL predicted to be present by the method with that known to be present following standard addition to whole plasma. These validations show that the method is a rapid and accurate technique of lipoprotein analysis suitable for the routine screening of patients for abnormal amounts of normal or variant lipoproteins, as well as for use as a research tool for quantitation of changes in cholesterol content of six or seven different plasma lipoprotein fractions.—Cone, J. T., J. P. Segrest, B. H. Chung, J. B. Ragland, S. M. Sabesin, and A. Glasscock. Computerized rapid high resolution quantitative analysis of plasma lipoproteins based upon single vertical spin centrifugation. *J. Lipid Res.* 1982. 23: 923-935.

**Supplementary key words** high density lipoprotein • low density lipoprotein • very low density lipoprotein • intermediate density lipoprotein • HDL cholesterol • computer analysis

The potential usefulness, both clinically and experimentally, of the single vertical spin autoprofiler (VAP) has been outlined (1). Practical use of the method, how-

ever, required the development of a means of accurate and reproducible peak quantitation. The similarity of a VAP profile to a gas or liquid chromatogram suggested that VAP profile quantitation could be achieved by on-line computer analysis, a common feature of contemporary gas and liquid chromatography systems. These systems, however, have the common feature that each peak must be separated from the next by a return to baseline for accurate analysis; treatment of fused peaks involves either a vertical drop to the baseline at the minimum point between the two peaks or a tangent method extrapolating the overlap slope of first peak as a straight line. Neither of these methods takes into account known information about the shape of a given chromatographic peak (2-4). Methods suggested in the literature for improving accuracy deal with varying the system parameters to improve the separation rather than derivation of mathematical ways to obtain more information from a fused peak (5).

Since significant improvement of the resolution of the VAP profile does not seem possible, quantitation of the separate peaks making up a VAP profile requires a mathematical procedure capable of deconvoluting the profile. We describe here a deconvolution method based upon the principle that the major lipoprotein species, HDL, LDL, and VLDL, have the same functional shape in a plasma VAP profile as they do when preisolated

Abbreviations: HDL, high density lipoprotein; VHDL, very high density lipoprotein; MDL, medium density lipoprotein; LDL, low density lipoprotein; IDL, intermediate density lipoprotein; VLDL, very low density lipoprotein; VAP, vertical autoprofiler; RIA, radioimmunoassay; compu-VAP, computerized vertical autoprofiler; LRC, Lipid Research Clinic.

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and run separately on the VAP. This communication details the on-line deconvolution program, briefly described elsewhere (1), and then uses a variety of qualitative and quantitative experiments for verification. The resultant computerized VAP increases processing speed to allow as many as 56 samples a day. Thus, subjects can easily be followed, with time to determine the effect of factors such as lipolysis or diet, on multivariant plasma lipoprotein levels. The rapidity of analysis also is useful in a routine clinical setting, giving, within a few hours, information requiring at least 24 hours to obtain by standard Lipid Research Clinic (LRC) methods, as well as giving information on variant lipoprotein concentrations, such as IDL and MDL, not available by routine methods.

## EXPERIMENTAL METHODS

### Vertical autoprofiler

The protocol for the treatment of the plasma samples has been published in detail elsewhere (1). Briefly, 1.3 ml of plasma is adjusted to a density of 1.21 g/ml by the addition of KBr. Normal saline (3.5 ml) is layered on top of the plasma in either 5-ml cellulose nitrate tubes (Model 305050, Beckman Spinco Division, Palo Alto, CA) or 5-ml Quik-Seal tubes (Beckman Spinco Model 342412). The former are run at 64,000 rpm for 45 min (Model OTD-2 ultracentrifuge, Dupont-Sorvall Inc., Newtown, CT) in a TV865 rotor (Dupont-Sorvall, Inc.) with reograd activated and ARC 3 controlled acceleration activated to cut off at an  $\omega^2t$  value of  $9.35 \times 10^{10}$  (a time setting of 45 min, total spin time of 57 min). The latter are run at 80,000 rpm for 44 min (Model L8-80, Beckman Instruments, Spinco Division, Palo Alto, CA) in a VTi 80 rotor (Beckman Instruments) with slow acceleration and breaking mode activated to cut off at an  $\omega^2t$  value of  $1.53 \times 10^{11}$  (a time setting of 44 min, total spin time of 48 min). After centrifugation the bottoms of the tubes are punctured and the effluent is fed into an AAI analyzer employing an enzymatic Autoflo cholesterol kit (Boehringer-Mannheim Diagnostics, Indianapolis, IN).

### Lipoprotein isolation

VLDL was isolated from plasma (6) and sent along with the whole plasma to the LRC, for analysis for total and HDL cholesterol. The LDL cholesterol value was obtained by subtracting the LRC value for HDL and VLDL from the LRC value for the total, after corrections for volume.

The HDL, LDL, and VLDL for peak shape determinations were isolated using standard sequential flo-

tation techniques (6). The VLDL isolated by this procedure has a density of 1.006 g/ml, the LDL has a density of 1.019–1.063 g/ml, and the HDL has a density greater than 1.063 g/ml. The LDL was repurified by the same technique to reduce the amount of an impurity of VLDL density found when the LDL sample was run on the VAP. These purified fractions were then run on the VAP and the data was used in the curve fits.

The MDL and IDL isolations were made by collecting fractions from a preparative single vertical spin isolation using the basic technique described earlier (7). Five ml of plasma was adjusted to a density of 1.21 g/ml and 12 ml of normal saline (density 1.006 g/ml) was layered on top in a 17-ml tube. The tube was centrifuged at 64,000 rpm for 90 min in a TV865B rotor (Dupont-Sorvall, Inc., Newton, CT) in the OTD-2 centrifuge. For MDL, fractions from the d 1.070–1.080 g/ml range were collected from a subject with high MDL; for IDL, fractions from the d 1.020–1.040 g/ml range were collected from a subject with high IDL. After pooling fractions from the region of interest, MDL or IDL was concentrated using ultrafiltration membrane cones (Model CF50A; Amicon Corp., Lexington, MA).

### Assays

Cholesterol assays were performed using a manual cholesterol kit (Boehringer-Mannheim Diagnostics, Indianapolis, IN). Apolipoprotein analyses were done by the solid phase radioimmunoassay procedure (8). Strip electrophoreses were done on Paragel 1% agarose gels (Worthington Diagnostics, Freehold, NJ) by the procedure of Papadopoulos (9). Gradient gel electrophoreses were performed by Dr. Ronald Krauss, the Donner Lab, University of California at Berkeley, using a method developed in his laboratory.<sup>4</sup> Lipid Research Clinic analyses for total and HDL cholesterol were done according to standard procedures (10). The electron micrographs were made on samples negatively stained by a variation of the procedure of Hamilton et al. (11). The microscope used was a Zeiss EM-10 (Carl Zeiss, Inc., New York, NY). Lipid Research Clinic methodology was performed at the Lipid Research Clinic Lab, Zeigler Research Building, University of Alabama in Birmingham.

### Computer hardware

An Apple II + computer containing 48K of RAM, an Apple disk drive, an Apple serial interface, and an Apple Silentyper printer (Apple Computer Inc., Cupertino, CA) was used for the VAP interface. This system was also equipped with an "Apple Clock" (Mountain Hardware, Santa Cruz, CA) a sixteen-channel, 8-bit analog to digital converter (Model AI02, Interactive Structures Inc.,

<sup>4</sup> Krauss, R. Unpublished data (manuscript in preparation).

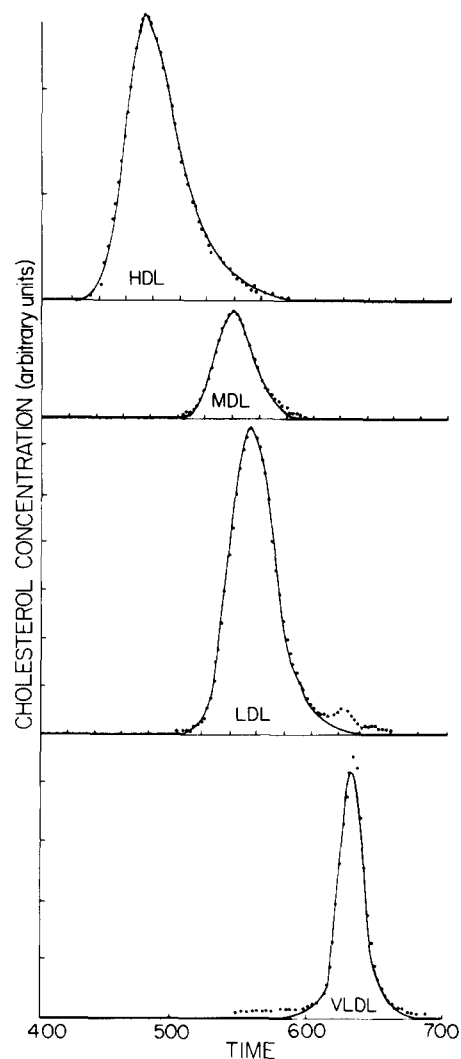
Bala Cynwyd, PA), a fast arithmetic processor (Model 7811C, California Computer Systems, Sunnyvale, CA), and a parallel triac output board (Electronic Systems Inc., San Jose, CA). A color television (Model XL100, R.C.A. Corporation, Indianapolis, IN) was connected to the video output of the Apple by means of a radio frequency modulator (Super Mod II, M. and R. Enterprises, Sunnyvale, CA). The connection to the colorimeter was made from the 5-volt telemetry output through a cable to the analog to digital converter.

### Computer curve deconvolution<sup>5</sup>

The algorithm for deconvolution of a profile is based on the assumption that the curve shape for HDL, LDL, and VLDL is the same in a VAP profile as it is when the preisolated authentic fractions are run on the VAP. The functional form of this curve has been assumed to be a bicameral gaussian with an exponential tail. The gaussian was chosen since in an equilibrium centrifugation experiment the concentration of a single lipoprotein species fully contained in the fluid part of the tube is a symmetric gaussian function of the distance from the center of the peak (12). When the tube is punctured, flow causes the leading edge of the gaussian to be foreshortened and the trailing edge to be broadened. The trailing edge is also broadened by wall drag, both in the tube and in the autoanalyzer, particularly prior to the insertion of bubbles into the flow lines. This latter effect is directly analogous to the dead volume effects analyzed by Lochmuller and Sumner (4). The net result of these factors is that an originally symmetrical gaussian peak will be convoluted by flow, drag, and mixing factors to a form that has a larger half-width on the trailing side than on the leading side and has an exponential modification to the trailing side. Authentic HDL, LDL, and VLDL were isolated as described in methods above and run on the VAP. The curves resulting were fit to this convoluted gaussian function. (The methods and parameters of this fit are detailed in the appendix.) The fits for these three species and MDL are shown in **Fig. 1**. The HDL fit clearly shows the effect of the exponential modification. Excepting the height, the parameters of the MDL fit were within 0.5% of the LDL parameters.

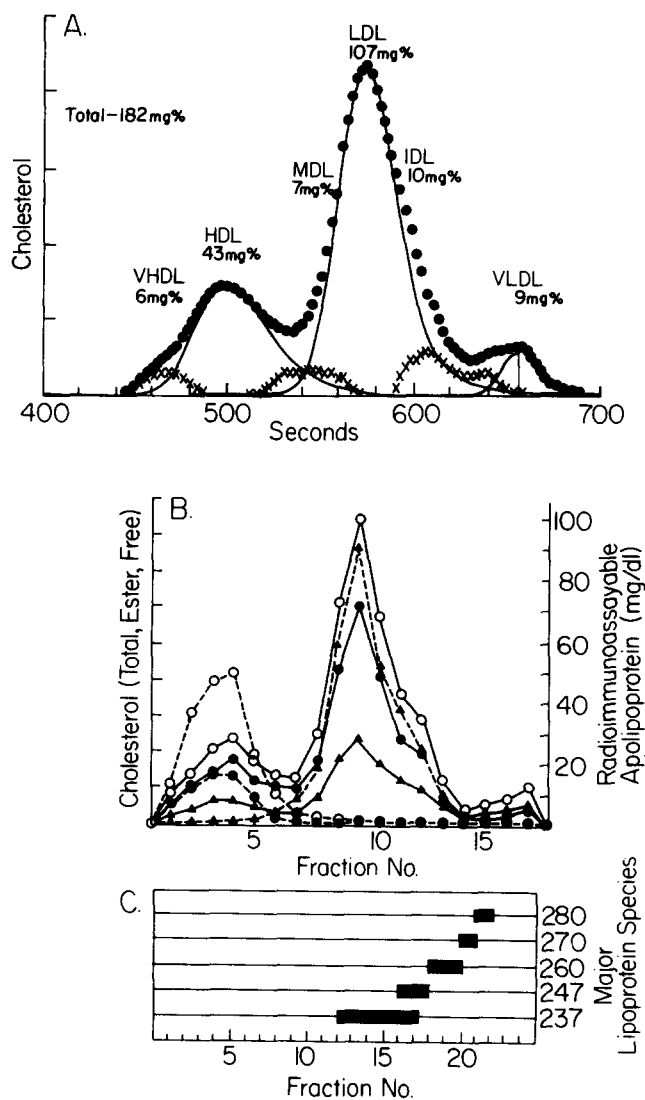
All data points are the average of 1,000 samples of the A/D converter and a time read from the clock. Curve start is detected by comparing the linear least squares slope of a data "slice" of seven consecutive points to a preset minimum. After curve start the area of the curve up to each point is measured using the trapezoidal rule.

<sup>5</sup> Complete instructions for setting up the compu-VAP, together with the deconvolution program on a floppy disk, will be made available to those interested. Write Dr. John T. Cone, Department of Pathology, University of Alabama in Birmingham Medical Center, Birmingham, AL 35294.



**Fig. 1.** Representation of the computer graphics pictures of the VAP curves for the authentic preisolated lipoprotein fractions HDL, MDL, LDL, and VLDL (···) and the least squares fit to those curves (—).

Once a curve has been expressed the curve is deconvoluted as follows. The maxima of the species in the profile three major lipoproteins (HDL, LDL, and VLDL) are corrected for overlap using the fitting functions. The HDL and LDL maxima are used together with the parameters to determine the area of these species. The left half of the VLDL function is used together with the measured area to the right of the VLDL maximum to obtain a first approximation to the VLDL area. The VHDL area is obtained by subtracting the area of the left-hand side of the calculated HDL function from the measured area up to the HDL maximum. The MDL is approximated by subtracting the area of the left-hand side of the LDL function and the VHDL and HDL areas from the measured area up to the LDL maximum. The IDL area is approximated by subtracting the areas



**Fig. 2.** A, Representation of the computer graphics picture generated by quantitated VHDL, HDL, MDL, LDL, IDL, and VLDL for a normal male subject (Subject A). B, Chemical analysis of the portion of the plasma not run through the autoanalyzer: (O—O), total cholesterol; (●—●), cholesterol ester; (▲—▲), free cholesterol; (○---○), apoA-I RIA; (●---●), apoA-II RIA; (▲---▲), apoB RIA. C, Major lipoprotein species of fractions 9–21 of the larger VAP tube as determined by gradient gel electrophoresis.

of the other five species from the total. (The results to this point represent compu-VAP deconvolution program A.) Since it was observed that the MDL often displays a bimodal shape with a leading shoulder to the left of the observed maxima, the MDL area is corrected by subtracting from it any area greater than that given by assuming the left-hand side of the MDL peak is a gaussian. This difference, assumed to be predominantly HDL<sub>2b</sub>, is then added back into the HDL curve. Likewise, the right-hand side of the IDL peak is assumed to be an exponentially modified gaussian similar to the right-hand side of LDL. The difference between the

exponentially modified gaussian IDL and the original is then added to IDL and subtracted from the VLDL (compu-VAP deconvolution program B). The details of these procedures are given in the appendix.

## RESULTS

In addition to a theoretical justification of the assumptions made in the deconvolution procedure outlined above (see appendix), we have undertaken an experimental validation based on qualitative and quantitative parameters. The qualitative validation sought to show that the variant lipoprotein species predicted by the VAP to be present are, in fact, there. The quantitative validation sought to demonstrate that the values of the concentrations obtained are accurate and reproducible. Comparisons were made with standard procedures as often as possible but in many cases modifications had to be made. For example, our comparison with Lipid Research Clinic methodology of quantitation had to take into account the fact that LRC methodology tends to lump in the LDL range all lipoprotein species with a density > 1.006 g/ml that are precipitated by heparin-manganese (13, 14) or phosphotungstate; by the VAP criteria this includes MDL and IDL as well as LDL (15).

### Qualitative validation

Gradient gel electrophoresis, strip electrophoreses, and negative stain electron micrographs were performed on fractions from tubes using a larger rotor (5 ml of plasma adjusted to d 1.21 g/ml with KBr, layered with 12 ml of normal saline, and centrifuged at 64,000 rpm for 90 min in a Dupont-Sorvall TV865B rotor in the OTD-2 centrifuge). Since the bands produced are essentially identical to the smaller tubes (7), the larger tubes were used in cases where a larger number of fractions might provide more detail. The other studies were done on fractions collected from the smaller 5-ml tubes. Four subjects were studied: Subject A, a normocholesterolemic control; Subject B, a patient with high MDL; Subject C, a patient with type III hyperlipoproteinemia; and Subject D, who had a previous history of coronary artery disease and was the father of a subject with high MDL (Fredrickson and Lees classification (16) type IV). Representations of the computer graphics display of the profiles are shown in Figs. 2A, 3A, 4A, and 5A, respectively. Also shown are the calculated values in mg/dl cholesterol for the six lipoprotein species deconvoluted by the program B. In these schematics, the raw MDL and IDL difference plots are shown (compu-VAP deconvolution program A), not the final difference plots resulting from the second approximation step contained in compu-VAP deconvolution program B. The computer-assisted VAP

(compu-VAP) predicts that subjects B, C, and D have higher IDL and MDL than the control. Subject B is seen to have the most MDL while subjects C and D have the highest IDL. The IDL peak also appears bimodal in all four cases.

### Apolipoprotein analyses

Radioimmunoassays (RIA) for apoA-I, apoA-II, and apoB (Figs. 2B, 3B, 4B, and 5B) were done on each of the fractions obtained from all four patients. The bottom of each tube was punctured and the effluent was collected by downward drop. The efflux rate of each tube was controlled by the constant rate of the proportioning pump and the fraction collector set in the time mode. Several features are of note. There is no contamination of the LDL region by apoA-I and apoA-II. The shapes of the apoA-I and apoA-II curves are very similar to the shape for HDL predicted by the compu-VAP, with the exception that the right-hand side of the apoA-I and apoA-II curves are slightly less skewed than that of the compu-VAP HDL curve. This is to be expected considering the

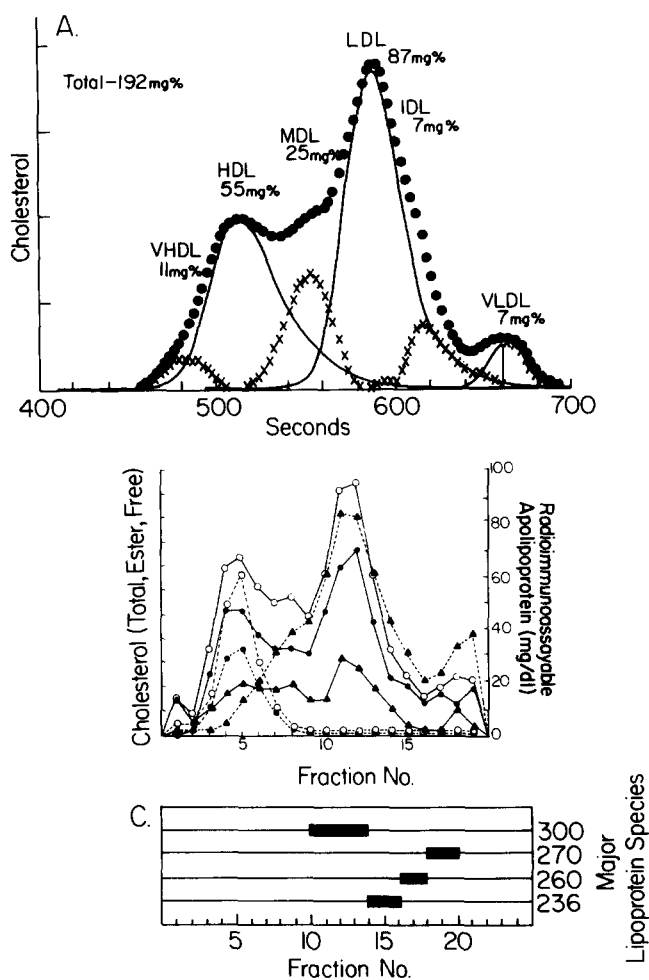


Fig. 3. Same as Fig. 2 for a subject with high MDL (Subject B).

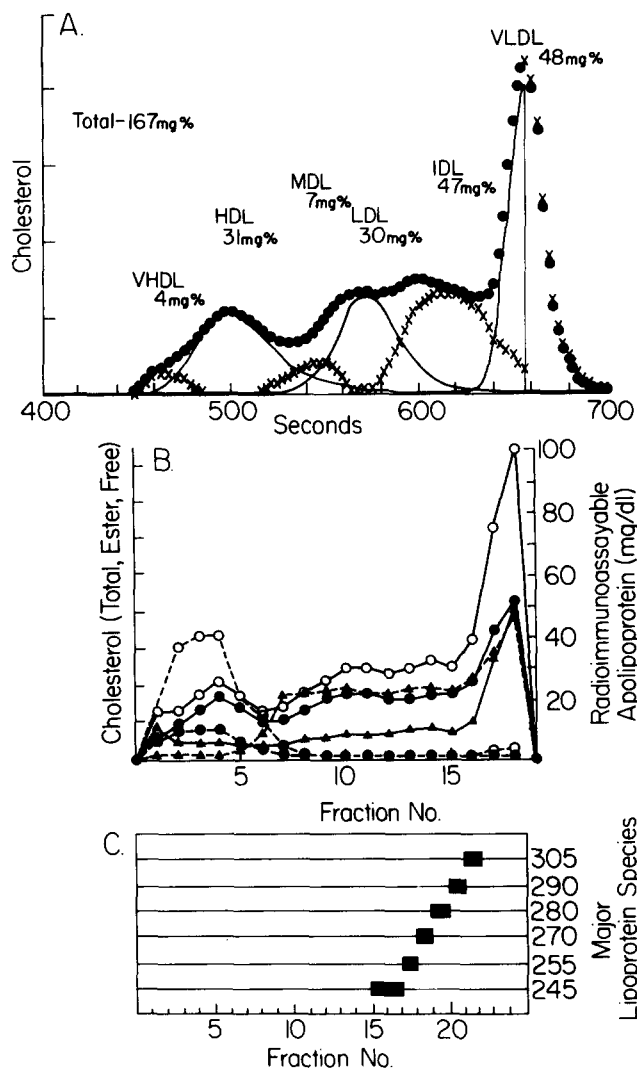


Fig. 4. Same as Fig. 2 for a type III subject (Subject C).

7-min period that the HDL species is in the analyzer and subject to skewing forces.

There is a clearly definable shoulder on the left-hand side of the apoB RIA curves of subjects B, C, and D and a suggestion of one in the normal patient (Fig. 2B). The shoulder occurs in the region where the compu-VAP predicts the presence of MDL. This suggests that one of the apolipoproteins contained in the fractions in the MDL region of the profile is apoB. Also, the Lipid Research Clinic analysis of a pure sample of MDL (53 mg/dl total cholesterol and 0 mg/dl HDL cholesterol) indicated that this lipoprotein species is precipitated completely by the heparin-manganese reagent, providing further evidence that the predominant apolipoprotein in MDL is apoB (although an apoE-containing lipoprotein, such as HDL<sub>c</sub>, cannot be ruled out on this basis).

The right-hand side of the radioimmunoassayable apoB RIA curve from all four subjects also has a shoul-

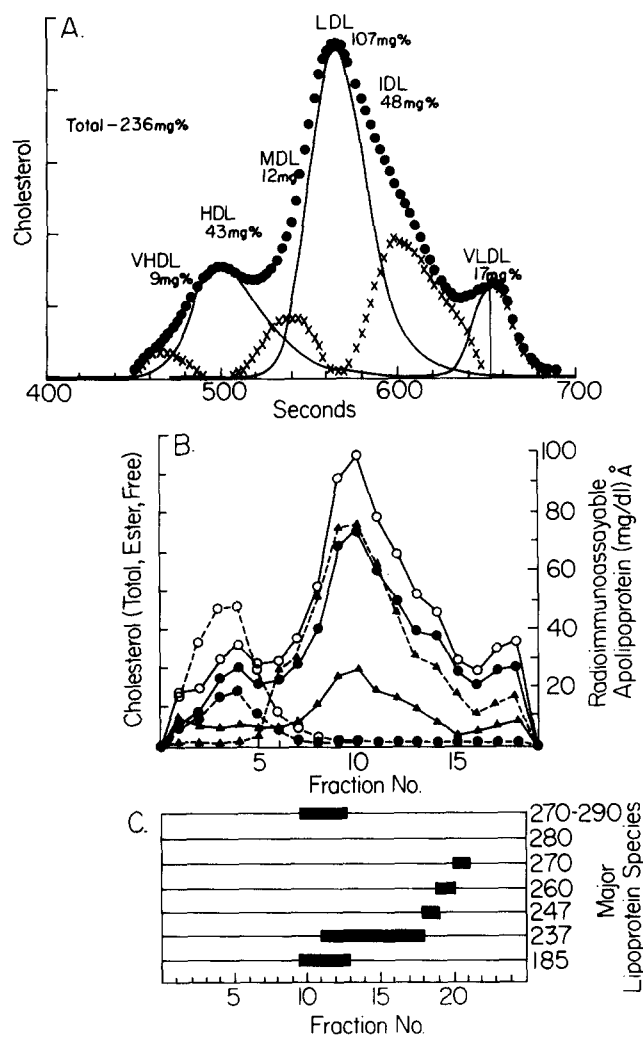


Fig. 5. Same as Fig. 2 for a type IV subject (Subject D), who had a history of coronary artery disease and was the father of a subject with elevated MDL.

der. In the type III subject this shoulder is defined as a separable peak falling between LDL and VLDL. The compu-VAP predicts IDL in this region in all four subjects (Figs. 2A, 3A, 4A, and 5A).

### Cholesterol analysis

The total cholesterol curve obtained by individual analysis of each VAP fraction shows somewhat more resolution than the corresponding VAP profile since the fractions were not subjected to the degree of skewing and mixing forces to which a continuous flow VAP analysis is. Reflective of this higher resolution, a distinct shoulder is seen on the right-hand side of the LDL peak of all four profiles. This, together with the shoulder on the right-hand side of each apoB RIA curve, gives strong evidence for the existence of an IDL species. There are also distinct shoulders on the left-hand side of the LDL

cholesterol peak in all but the normocholesterolemic subject; in the latter there is only a hint of a shoulder. This agrees with the VAP prediction of significant amounts of MDL in the subjects B-D and a minimal amount of MDL in subject A.

### Gradient gel electrophoresis

The fractions taken from the IDL section of the profiles show a variety of different sized lipoprotein species seen in Figs. 2C, 3C, 4C, 5C, all of them intermediate between LDL and VLDL. This agrees with the compu-VAP prediction of IDL in all of the patients. For subjects B, C, and D, fractions taken from the MDL region of the VAP curve are seen to contain lipoprotein particles in the 270–300 Å range. This is the size assigned to the Lp(a) lipoprotein particle by Dr. Ronald Krauss.<sup>6</sup> This, together with the presence of apoB, precipitation by heparin-manganese, and the strip electrophoretic data given below, strongly implies that MDL largely represents Lp(a) in the four subjects examined here. Also, fractions from the MDL region of several subjects rich in this lipoprotein show a precipitation reaction with anti-Lp(a) (graciously supplied by J. J. Albers); subjects essentially negative for MDL show no precipitation.

### Negative stain electron microscopy

Negative stain electron micrographs of selected fractions are shown in Fig. 6. The prints were subjected to morphometric analysis and the results are shown in Fig. 7. Fraction 10 in each subject is seen to contain not only an HDL<sub>2</sub> diameter particle but also a lipoprotein particle in the 260–300 Å range. This is in qualitative agreement with the compu-VAP which predicts MDL to be present in all four profiles, and the size is the same as that predicted by the gradient gels.

Fractions 18 and 21 are seen to contain lipoprotein particles larger than the LDL particles found in fraction 15. Thus all four subjects have lipoprotein particles intermediate in size between LDL (fraction 15) and VLDL (fraction 24), in agreement with the prediction of the compu-VAP. The size predicted for these particles by the electron microscopy is also in agreement with the size predictions of the gradient gels.

### Electrophoresis

Periodic fractions from all four subjects were subjected to strip electrophoresis. Fractions 2 and 4 contained a lipoprotein specie (VHDL) migrating faster than HDL in a pre- $\alpha$  position. Fraction 10 is taken from subject B with high MDL and contains a lipoprotein species that migrates between LDL ( $\beta$ ) and VLDL (pre- $\beta$ ). This is the same mobility found by Albers and Hazzard (17) for

<sup>6</sup> Personal communication. Dr. Ronald Krauss.

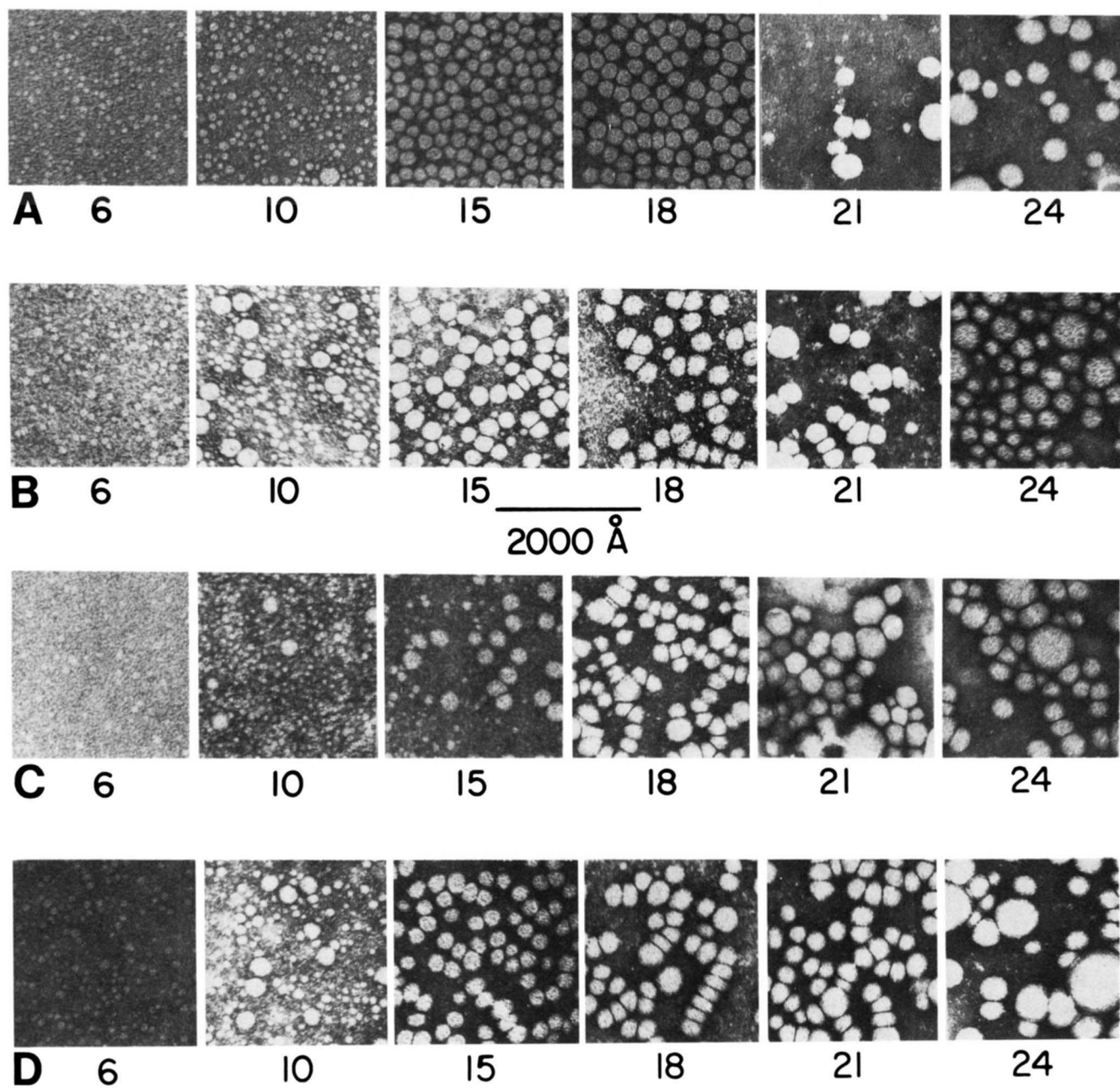


Fig. 6. Negative stain electron micrographs of selected fractions taken from VAP profiles of subjects A–D.

the lipoprotein Lp(a), again suggesting that MDL largely represents Lp(a) for the four subjects examined.

#### Cluster analysis

In a final qualitative experiment, all 24 fractions taken from the region indicated in **Fig. 8** were rerun on the compu-VAP. This produced a series of peaks of varying sizes and positions; the maxima of each peak is plotted below the profile with the computer-derived deconvolution shown for reference. The peak center of fraction 1 is seen to be lower on the profile than HDL, indicating

that it contains a lipoprotein species of higher density than HDL (VHDL); fractions 9, 10, and 11 are seen to contain lipoproteins intermediate in density between HDL and LDL (MDL); and fractions 18 and 19 contain lipoproteins intermediate between LDL and VLDL (IDL).

#### Quantitative validation

*VAP reproducibility.* The linearity of the VAP method for authentic isolated lipoproteins was demonstrated earlier (1). In order to verify the reproducibility of the VAP

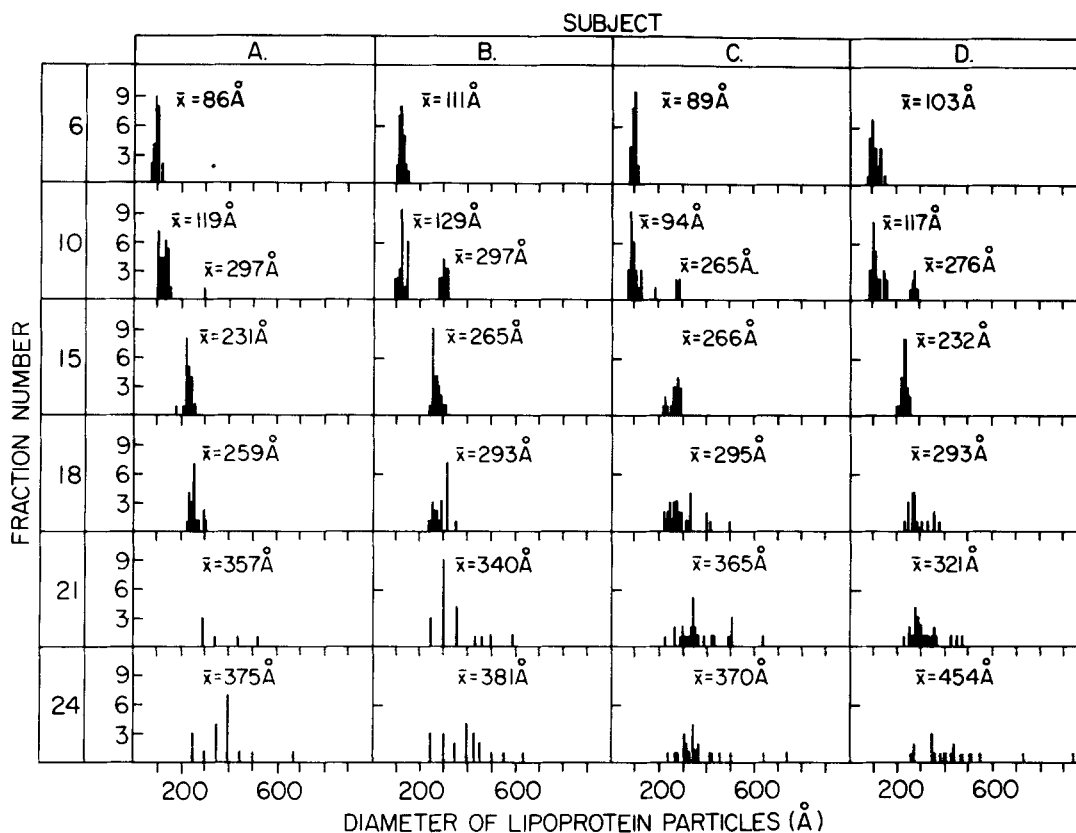


Fig. 7. Morphographic analysis of the electron micrographs in Fig. 6.

methodology, three rotors, each containing eight aliquots of the same plasma, were subjected to VAP analysis. The average total cholesterol concentration was 148 mg/dl. The intrarotor coefficient of variance ranged from 0.9 to 1.7% while the interrotor and total coefficient of variance were 1.6%. These values compare favorably with the minimum standard of 3% set by the Center for Disease Control for participation in their standardization program.<sup>7</sup> Linearity was checked by running known dilutions of two different samples of plasma on the VAP. A plot of the cholesterol concentration calculated from the dilution and the value predicted by the compu-VAP had a slope of  $1.0027 \pm 0.0086$ , intercept of  $-0.18 \pm 1.02$  mg/dl, and correlation coefficient ( $r$ ) of 0.997 indicating that the method is linear with respect to concentration.

**Total cholesterol.** Plasma from 50 individuals was subjected to compu-VAP analysis and standard LRC procedures. A plot of the VAP values versus the LRC values (data not shown) had a slope of 0.91 and an intercept of 8.3 mg/dl with a correlation coefficient ( $r$ ) of 0.96.

<sup>7</sup> The Center for Disease Control-National Heart, Lung, and Blood Institute High Density Lipoprotein Cholesterol Standardization Program. Adrian Hainline, Jr., Chief, Clinical Chemistry Standardization Section, Metabolic Biochemistry Division, Clinical Chemistry Division.

The 95% confidence interval on the parameters of the population regression line are ( $0.88 < \beta < 0.95$ ) for the slope and ( $1.7 < \alpha < 14.9$ ) for the intercept. A slope slightly different from one and a non-zero intercept in

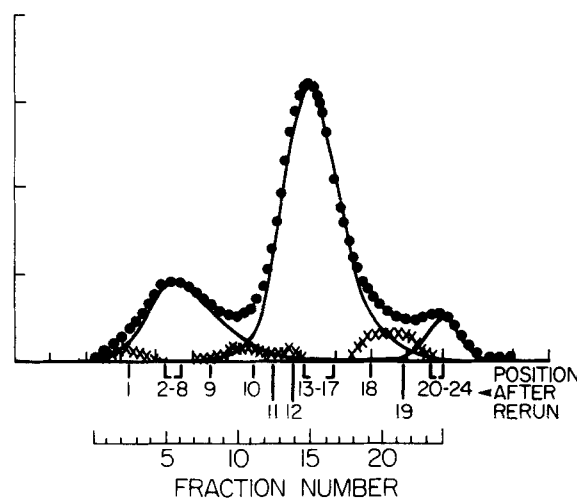


Fig. 8. Positions of the fractions from a VAP profile before and after rerun. Fractions collected during a VAP run were rerun on the VAP. The positions before collection are shown in the lower bar while the position of the peak after rerun is shown under the deconvoluted compu-VAP profile.



this type of plot using different techniques is not unusual (e.g., Robinson, Hall, and Vasiliades (18)). We conclude, therefore, that differences between compu-VAP and LRC total cholesterol could be primarily ascribed to the differences between the types of method, i.e., enzymatic versus chemical.

**HDL cholesterol.** A scatter diagram comparison of HDL measurements of LRC and compu-VAP methodologies (data not shown) has a slope (0.77) significantly different from 1, although the correlation coefficient ( $r$ ) is good (0.95). A slope of 0.77 is consistent with the values predicted by the compu-VAP exceeding those of the LRC by as much as 20%. To investigate this difference, supernate remaining after heparin-manganese precipitation of LDL and VLDL, defined by the LRC as HDL, was run on the compu-VAP. Three representative LRC HDL peaks thus obtained have been superimposed on the deconvoluted VAP HDL peaks of the corresponding whole plasma in Fig. 9. The area of the LRC HDL supernate peak is noticeably smaller than the computer-predicted HDL peak for the whole plasma, with the majority of the difference in an area to the right of the HDL maximum, i.e., in the HDL<sub>2</sub> region.

**LDL and VLDL cholesterol.** The results of a comparison of the analysis of HDL, LDL, and VLDL by LRC and compu-VAP methodologies are shown in Table 1. In making this comparison, VHDL and HDL measured by the VAP were summed to obtain an HDL

TABLE 1. Comparison of HDL, LDL, and VLDL concentrations measured by the compu-VAP and LRC (N = 4)

	HDL	LDL	VLDL
	mg/dl		
VAP	35.0 ± 15.8	85.4 ± 35.3	14.0 ± 12.3
LRC	28.2 ± 12.5	92.9 ± 34.7	13.3 ± 10.9
VAP-LRC	6.9 ± 3.8	-7.5 ± 3.7	0.8 ± 1.4
P(VAP-LRC ≠ 0)	0.01	0.01	NS

value for comparison with LRC methodology, and MDL and IDL were added to each compu-VAP LDL total to obtain an adjusted LDL value for comparison, since both MDL and IDL are precipitated by heparin-manganese; VLDL values were compared directly. The value of the VLDL predicted by the compu-VAP is quite close to the LRC value (average deviation  $0.8 \pm 1.7$  mg/dl cholesterol) even for one subject with a high VLDL cholesterol. Following the pattern seen in Fig. 9, compu-VAP HDL is higher and, consequently, compu-VAP LDL is lower than LRC HDL and LDL, respectively.

**MDL and IDL cholesterol.** There are no methods routinely available for quantitating MDL or IDL cholesterol. One can make crude quantitative inferences from the sizings of the electron micrographs summarized in Fig. 7. The relative amounts of the 260–300 Å particles (MDL) seen in fraction 10 are highest in subjects B and D and lowest in subject A, with subject C, the type III patient, being intermediate. Likewise, the relative amounts of the 320–370 Å particles seen in fraction 21 are most plentiful in subjects C and D with somewhat less in subjects A and B. Because of the small sample size, however, these quantitative inferences are quite crude. Since one advantage to lipoprotein analysis by the compu-VAP method would be the possibility of quantitation of MDL and IDL species, an enrichment method was used to judge the ability of the compu-VAP to quantitate these species. Measured aliquots of these variant lipoprotein species, isolated as described in methods and ranging in volume from 0.10 ml to 0.70 ml were mixed with 0.70 ml of plasma; the density of each sample previously had been adjusted to 1.21 g/ml. The aliquots of each variant lipoprotein were run in a separate rotor with one tube per rotor being pure variant for reference. The known concentration of the variant, computed from the known amount of MDL or IDL added, was plotted against the compu-VAP prediction (data not shown). The slope of the MDL line is  $0.918 \pm 0.023$  with an intercept of  $0.23 \pm 0.97$  mg/dl and a correlation coefficient ( $r$ ) of 0.94; the slope of the IDL line is  $1.033 \pm 0.034$ , with an intercept of  $0.28 \pm 0.98$  mg/dl and a correlation coefficient ( $r$ ) of 0.88. The standard deviations of the fits are 4.2 mg/dl and 4.5 mg/dl cholesterol, respectively.

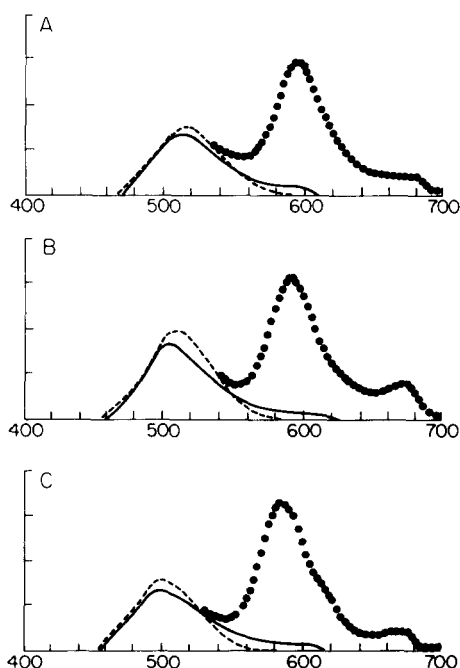


Fig. 9. Comparison of the compu-VAP HDL curve (---) with the curve obtained by running the HDL supernate from the LRC HDL cholesterol determinations on the VAP (—). The total compu-VAP profile is shown for comparison (· · ·).

TABLE 2. Intersample reproducibility of the compu-VAP; average cholesterol concentration of specified fractions of a single sample (N = 24)

Total	VHDL	Total HDL	MDL	LDL	IDL	VLDL
<i>mg/dl</i>						
148 ± 23	6.7 ± 0.7	41.8 ± 1.0	9.6 ± 1.2	84.7 ± 2.2	3.5 ± 3.4	8.7 ± 1.3

The reproducibility of compu-VAP predictions of the concentrations for the separate lipoprotein species of 24 aliquots of a single plasma sample contained in three separate rotors with each rotor holding 8 separate aliquots is shown in **Table 2**. None of the variations are greater than 3% of the total cholesterol with the largest variations seen in IDL and LDL. The reproducibility of the HDL measurements (1 mg/dl S.D.) are within the limits for its measurement set by the CDC. The small coefficients of variation for each lipoprotein species suggests that statistical fluctuations in peak shape are minimal.

## DISCUSSION

The results of the validation experiments outlined in this communication show that the compu-VAP can analyze the signal it receives to quantitate on-line accurately and reproducibly the major lipoproteins HDL, LDL, and VLDL as well as the variant lipoprotein species MDL and IDL and, although not yet examined, presumably also VHDL. All of the measurements are completed and printed out within 30 sec of the expression of the profile, requiring no tedious or time-consuming calculations as in the method of Nilsson et al. (19). The algorithm developed for curve analysis follows logically from theoretical considerations of the design and hydrodynamic flow of the system. It is significant that even those lipoprotein species whose concentrations are derived by calculating a difference of areas are predicted with satisfactory accuracy and reproducibility. As an example, the compu-VAP procedure predicts the presence of MDL in concentrations greater than 1 mg/dl in about 90% of the individuals examined so far. This is in qualitative agreement with several references (17, 20–23) which indicate the presence of measurable Lp(a) in nearly all subjects (20).

The VHDL species has been widely reported (19, 24, 25) but no quantitation had been previously attempted. One reason for lack of information on VHDL is that its density makes it difficult to separate from the free protein fraction. While a possibility to be considered is that VHDL is a dense degradation product of other lipoprotein species, we feel this is an unlikely possibility on the basis of disappearance of VHDL following *in vitro* lipolysis by lipoprotein lipase (26). Also Blanche et al. (27) have reported two species “HDL<sub>3b</sub>” and “HDL<sub>3c</sub>”

with average densities of 1.154 g/ml and 1.171 g/ml, respectively. Both of these species would float in the VHDL region of our gradient which has a maximum density of 1.129 g/ml.

The significant quantitative difference between the compu-VAP method and previous methods is in the HDL concentration. Because the area missing from the LRC supernate is in the HDL<sub>2</sub> portion of the HDL region, it is reasonable to assume that some HDL<sub>2</sub> is being precipitated by the heparin-manganese method, as suggested by Srinivasan (28), and that the compu-VAP HDL value is a more accurate indicator of HDL concentration than the LRC value.

There have been a number of references to heterogeneity of HDL (24, 25), LDL (29–32) and VLDL (33–36). No attempt is made in this communication to deconvolute the HDL peak into more than two fractions (HDL and VHDL), although we have recently developed a modification of the compu-VAP that effectively separates and quantitates five HDL subfractions (37). Studies on HDL samples richer in HDL<sub>2</sub> or HDL<sub>3</sub> have indicated that there is no detectable difference in the shape of the right-hand side of the curve for either type, although the peak center varies, and thus the only modification made to the HDL peak is the addition of any non-gaussian leading portion of MDL.

Six different subfractions of LDL have been reported (29, 32) ranging in density from 1.024 g/ml to 1.060 g/ml (29) or 1.006 g/ml to 1.063 g/ml (32). In our gradient (7), many of these subfractions would fall in the IDL range. It is possible that the bimodal shape of the IDL curve that we often observe is caused by the presence of varying amounts of what have been called “LDL” subfractions. The density of the LDL measured by our method varies between 1.050–1.064 g/ml, which corresponds to the densities of LDL fractions 5 and 6, respectively, described by Shen et al. (29). The LDL function derived from the classical density cut for LDL (d 1.019–1.063 g/ml), however, fits the curve whether the density is at the lower or higher ends of this range.

The densities measured in this paper are based on the density of the gradient at the position of the lipoprotein. Since in the conditions of the experiment the lipoproteins are not in equilibrium with the gradient,<sup>8</sup> this tends to overestimate the density. Since the position from profile

<sup>8</sup> Segrest, J. P. Unpublished observation.

TABLE 3. Average compu-VAP lipoprotein concentrations for a population in the 20–39 years age group

Lipoprotein Species	20–29 Years				30–39 Years			
	Males (n = 60)		Females (n = 22)		Males (n = 14)		Females (n = 10)	
	Mean ± S.D.	Range	Mean ± S.D.	Range	Mean ± S.D.	Range	Mean ± S.D.	Range
	<i>mg/dl</i>		<i>mg/dl</i>		<i>mg/dl</i>		<i>mg/dl</i>	
VHDL	6 ± 2	1–10	6 ± 2	3–9	5 ± 2	3–10	7 ± 3	4–14
HDL	43 ± 10	23–68	48 ± 9	28–63	45 ± 13	23–70	49 ± 8	36–64
VHDL + HDL	49 ± 11 (45) <sup>a</sup>	26–77	54 ± 10 (54) <sup>a</sup>	31–70	50 ± 14 (44) <sup>a</sup>	26–78	56 ± 11 (55) <sup>a</sup>	41–74
MDL	8 ± 8	0–20	5 ± 5	0–12	5 ± 7	0–19	7 ± 8	3–19
LDL	84 ± 20	45–142	83 ± 19	35–125	90 ± 23	59–133	102 ± 23	60–133
IDL	22 ± 9	12–47	16 ± 5	8–26	22 ± 14	6–53	18 ± 9	7–33
MDL + LDL								
+ IDL	114 ± 24 (110) <sup>a</sup>	72–173	104 ± 20 (102) <sup>a</sup>	56–147	117 ± 26 (130) <sup>a</sup>	67–170	127 ± 23 (114) <sup>a</sup>	79–142
VLDL	7 ± 4 (16) <sup>a</sup>	1–18	4 ± 2 (12) <sup>a</sup>	1–10	8 ± 6 (23) <sup>a</sup>	3–25	4 ± 3 (12) <sup>a</sup>	1–11
Total	168 ± 27 (171) <sup>a</sup>	121–240	162 ± 22 (168) <sup>a</sup>	113–214	177 ± 28 (197) <sup>a</sup>	132–227	186 ± 23 (181)	135–218

<sup>a</sup> Values taken from Heiss et al. (38).

to profile for a particular lipoprotein of a particular individual is essentially invariant, this overestimation is reproducible and we are in the process of calibrating the system to eliminate this overestimation.

The flatness of the gradient in the VLDL region (7) would seem to preclude any subfractionation of this lipoprotein species under present conditions and thus a single gaussian is a reasonable approximation to the VLDL curve shape. A possibility that cannot be ruled out at present, however, is that, due to non-equilibration conditions, the lighter IDL shoulder is, in fact, what has been called a dense VLDL subspecies by others.

The second approximations incorporated into the VAP deconvolution algorithm for MDL and IDL (subtraction of putative HDL<sub>2b</sub> from MDL and addition of putative VLDL overlap to IDL-compu-VAP deconvolution program B) have not as yet been validated. One approach will be to compare compu-VAP results with results from VAP analysis of expanded HDL and IDL single vertical spin separations (37), where HDL<sub>2b</sub>-MDL and IDL-VLDL overlaps are minimized. Chemical and physical comparisons will also be made.

The average cholesterol concentration of the various lipoprotein species for a group of subjects separated by age and sex measured by the compu-VAP is shown in Table 3. Also shown are the mean values obtained for HDL, LDL, and VLDL by Heiss et al. (38). The similarities (with the apparent exception of VLDL) of the mean values measured by the two separate methods is further testimony for the accuracy of the compu-VAP procedure.

The compu-VAP can also be used in a routine clinical setting. It can handle 245 patients in a week with controls in each rotor, significantly more than any reported method, such as that of Nilsson et al. (19), which provides

even a portion of the information provided by the VAP. The growing weight of evidence implicating variant lipoproteins as risk factors for coronary artery disease indicates that the compu-VAP method can play a major role in lipoprotein analysis in the future, from both a research and clinical standpoint. ■

## APPENDIX

### Data handling

The set of points comprising the curves for the pure preisolated lipoprotein species HDL, LDL, and VLDL, collected as described above, was analyzed by a non-linear least squares program developed in our laboratory for the Apple computer which is basically a modification of one used earlier by the senior author (39). The functional form, together with final parameters, is shown in Table 1A and Table 2A. Because of correlation between the five parameters, minimization had to be carried out in two steps, first minimizing residuals for P1 and P2, and then using these to obtain P3, P4, and P5. P3, P4, and P5 were then used to obtain P1 and P2 and the procedure was repeated until the variation in all parameters between consecutive iterations was less than  $1 \times 10^{-6}\%$ . The functional form is a combination of the gaussian with unequal width factors on each side as used by Metzgar (40) and the exponential modification required by the convoluting factors

TABLE 1A. Functional form used in fitting the authentic preisolated HDL, LDL, and VLDL cholesterol curves

$$\begin{aligned}
 & T < T_0 \\
 & A = A_{\text{MAX}} \times \text{Exp}((-P1) \times (T - T_0)^2) \\
 & T \geq T_0 \text{ and } T < T_0 + P3 \\
 & A = A_{\text{MAX}} \times \text{Exp}((-P2) \times (T - T_0)^2) \\
 & T \geq T_0 + P3 \\
 & A = A_{\text{MAX}} \times (\text{Exp}((-P2) \times (T - T_0)^2) \\
 & \quad + P4 \times (T - T_0 - P3) \times \text{Exp}((-P5) \times (T - T_0 - P3))) \\
 & A_{\text{MAX}}, \text{ Observed maximum absorbance; } T_0, \text{ time at which } A \\
 & = A_{\text{MAX}}.
 \end{aligned}$$

TABLE 2A. Parameters used in the fit of the HDL, LDL, and VLDL cholesterol curves

Parameter	HDL	LDL	VLDL
P1	2.128E - 03	2.508E - 03	5.961E - 03
P2	9.289E - 04	1.535E - 03	6.019E - 03
P3	3.356E + 01	3.356E + 01	1.602E + 01
P4	1.184E - 02	5.996E - 03	2.145E - 02
P5	5.939E - 02	5.939E - 02	1.389E - 01

described above and theoretically justified in many places (41-43). The actual form chosen was a compromise between theoretical validity and computational ease and efficiency. The form chosen accurately described the data without requiring too much time for calculation.

#### Data collection

To obtain the curves described above, and all VAP profiles, dedicated software was developed that has the advantage of not requiring programming knowledge to operate it. The A/D converter is sampled more than once to enhance its accuracy. The 1,000 samples take 80 milliseconds. The method of curve-start detection is similar in principle to the increasing tangent method of Woerlee and Moi (5) except that all points in the slice are considered at once, rather than considering the first point paired with each of the subsequent ones. This makes the method of this paper less sensitive to noise. After a curve-start is detected, the extrapolated least squares value of the first point in the slice is taken as the initial baseline. Curve-end is detected by monitoring whether the slope falls below a certain preset value and the linear least squares value for the midpoint of that slice is smaller than a second preset value. The least squares value of the last point in the slice is taken as the final baseline. The baseline of the curve is taken as the simple average of the two values and the baseline correction is the product of this number and the length of time required for the curve. After curve-start is detected, the trapezoidal rule is used to calculate the area, relative to zero, from the third from the last point in the slice in which curve-start is detected to the last data point in which curve-end is detected. If the area at this point is less than a preset minimum, the area is erased and processing continues. If not, then the program prints out the six lipoprotein values, the total area, the time the sample was put in, the starting and ending time for the profile, and the baseline corrections, both on the printer and onto the disk. All of the program except for the sample of the analog to digital converter and a subroutine generating a warning tone is in Applesoft Basic. The other two subroutines are in 6502 assembly language. Since an interpretative language is used there is a moderately long time (about 2 sec) between points. The characteristic that allows this slow sampling rate is the long time (over 4 min) required for the expression of the profile. A second factor is that there are approximately 30-45 sec between profiles, so that this time can be used for the information to print out as well as for sophisticated calculations. Because of the long time required for a profile, the sampling rate is sufficiently fast to make the differences between trapezoidal and Simpson's integration negligible and so the computationally simpler one is used.

#### Data analysis

Fits of the equation of Table 1A to the data for the preisolated authentic HDL, LDL, and VLDL at several concentrations showed that  $A_{max}$  was the only parameter whose variation is required that needed to keep the other parameters within 0.5% for all concentrations. In a profile, the maximum of these three main species is corrected for overlap by the two other species, using the fitting functions. The overlap is generally less than 0.5% of full scale and in no case is this correction sufficiently great to cause a change any greater than this in the corrected  $A_{max}$ 's, consequently only a first order correction is used. Knowledge of the positions of HDL, LDL, and VLDL is used to set ranges of values where these species may be found. The non-gaussian component of MDL is computed by using the computed height of the MDL obtained by subtracting the HDL, LDL and VLDL functions from the observed profile and parameters obtained from the analysis of pre-isolated MDL to calculate the gaussian component of the left-hand

side. This is then subtracted from the measured area of the left-hand side to get the gaussian component. The IDL is first approximated by subtracting the area of VHDL, HDL, MDL, LDL, and VLDL from the measured total area. The gaussian correction to the IDL is computed by extrapolating the peak or shoulder on the IDL at about 630 sec, using the functional form of the right-hand side of the LDL. The difference between the area of this computed extrapolation and the actual measured area is added to the previously determined area of IDL and subtracted from VLDL. All computed lipoprotein areas are corrected for a non-zero baseline by multiplying the total correction by the ratio of the width of the base of each lipoprotein species to the width of the profile. The areas are then multiplied by a calibration factor obtained by running a sample of known cholesterol concentration as the first sample in a set of eight in a centrifuge rotor. The total concentration as well as the concentration of each fraction, along with the peak timing, is printed out between curves. In addition, an off-line program can use the printer to form a representation of the profile similar to those in Figs. 2A, 3A, 4A, and 5A. The outer dots represent the data points, the solid lines are the major lipoprotein areas (HDL, LDL, and VLDL from left to right) and the X's trace the variant lipoprotein areas.

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