Preparative and quantitative isolation of plasma lipoproteins: rapid, single discontinuous density gradient ultracentrifugation in a vertical rotor

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Abstract A rapid method has been developed for separation of the major plasma lipoproteins from up to 96 ml of plasma by a single ultracentrifugation step. This separation was achieved by a discontinuous density gradient centrifugation between the density range of 1.006 and 1.30 g/ml in Sorvall vertical rotors. Each lipoprotein fraction was sharply banded with VLDL at the top, LDL in the upper middle, and HDL in the lower middle portion of the tube. Use of authentic ^{125}I-labeled lipoproteins showed that complete separation of the three major classes and partial separation of HDL$_2$ and HDL$_3$ was achieved. The lipoprotein fractions prepared by this technique have properties indistinguishable from those isolated by the sequential flotation method in regard to their equilibrium banding density, electrophoretic mobility, and apolipoprotein composition. This method is suitable for the preparative isolation of lipoproteins as well as for quantitative clinical determinations of cholesterol and triglycerides in VLDL, LDL and HDL fractions of plasma. Used as an analytical tool this method allows samples as small as 1 ml of plasma and spin times as short as 45 min. Cholesterol levels in HDL fractions separated by this method have significantly lower values ($P < 0.05$) than those estimated by the heparin-manganese chloride precipitation method.—**Chung, B. H., T. Wilkinson, J. C. Geer, and J. P. Segrest.** Preparative and quantitative isolation of plasma lipoproteins: rapid, single discontinuous density gradient ultracentrifugation in a vertical rotor. *J. Lipid Res.* 1980. **21:** 284–291.

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Epidemiological studies indicate that development of coronary heart disease is directly correlated with serum levels of low density lipoprotein (LDL) (1, 2) or inversely correlated with serum levels of high density lipoprotein (HDL) (3, 4). However, isolation and quantitation of the plasma lipoproteins have remained tedious and time-consuming. A rapid procedure for lipoprotein isolation, which can be used for both preparative and analytical purposes, would be of benefit to both basic and clinical studies of the plasma lipoproteins.

A method for the separation of plasma lipoprotein fractions by single spin discontinuous density gradient ultracentrifugation in swinging bucket rotors has been reported as a method for the quantitation of lipoproteins, as well as a method for their small scale preparative isolation (5, 6). While this is a promising approach, the method, as originally described, required a minimum of 24 hr of centrifugation and was limited to a maximum of 24 ml of plasma per spin.

In the present communication we report a rapid, single spin ultracentrifugal method of plasma lipoprotein separation that is suitable both for quantitative chemical analysis and preparative isolation. The method employs a simple discontinuous density gradient in a vertical ultracentrifugal rotor.

EXPERIMENTAL METHODS

Plasma samples

Freshly collected plasma from normal individuals, obtained from the Red Cross, was pooled and used for the studies on the separation and characterization of lipoprotein fractions. For the studies on the distribution of cholesterol in VLDL, LDL, and HDL fractions, blood was collected from healthy young volunteers in tubes containing 0.1% EDTA; plasma from these samples were analyzed within 4 days of collection.

Density gradient centrifugation

A discontinuous NaCl/KBr density gradient was formed by adjusting the density (d) of the plasma to 1.30 g/ml with KBr and layering normal saline (d = 1.006 g/ml) over the adjusted plasma. The exact

conditions of gradient formation and centrifugation in various rotors are shown in **Table 1.**

Tubes loaded with sample and gradient were immediately placed in vertical rotors at room temperature and centrifuged in a Sorvall OTD-2 model ultracentrifuge at 10°C under conditions shown in Table 1 with the slow start setting on the ARC-1 automatic rate controller and at rate D on the reograd program setting. At the end of a run, the tubes were removed from the rotor and eighteen or twenty-one fractions were collected from each tube by puncturing the bottom using a gradient fractionator (Hoffer Scientific Instruments, San Francisco).

For the cholesterol quantitation experiments, plasma samples were centrifuged and the VLDL, LDL and HDL fractions were collected by monitoring the lipoprotein peaks with a continuous flow A_{280} UV detector. HDL cholesterol levels were also determined on the same plasma samples by the heparin–manganese chloride method of Warnick and Albers (7).

Studies with authentic lipoprotein fractions

VLDL, LDL (d 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) were isolated from pooled plasma by the standard sequential flotation (SF) method (8). All isolated lipoprotein fractions were then dialyzed against 0.15 M NaCl containing 0.01% EDTA. Aliquots of LDL, HDL_2, and HDL_3 were labeled with $Na^{125}I$ by the iodine monochloride method of McFarane (9). ^{125}I-Labeled lipoproteins were added to whole plasma or to corresponding unlabeled authentic lipoproteins. The densities of the labeled whole plasma or lipoprotein fractions were adjusted to 1.30 g/ml and subjected to ultracentrifugation as already described. The distribution of radioactive label among the resultant fractions was measured by counting 100-μl portions of each fraction in a gamma counter.

Analysis of fractions

The levels of total cholesterol, free cholesterol, and/or triglyceride in lipoprotein fractions were determined by the respective enzymatic assay method (10, 11), using Boehringer test sets No. 124079 and 126012 (Bio-Dynamics/BMC, Indianapolis, IN). Phospholipid levels were determined from lipid extracted samples (chloroform–methanol 2:1) by phosphorus analysis (12). The level of apolipoproteins B and A-I in each fraction of plasma was measured by solid phase radioimmunoassay (13), after dilution of each aliquot in buffered saline containing 2% bovine serum albumin. The level of albumin in each fraction was estimated by both the bromcresol green method (14) and by immunoprecipitation (15). The final gradient shape was determined by forming a discontinuous density gradient with d 1.30 g/ml KBr solution in place of density adjusted plasma. The adjusted KBr solution was then overlaid with d 1.006 g/ml NaCl solution and subjected to vertical rotor centrifugation. The refractive index of each fraction was measured with a refractometer (Bausch and Lomb Co., Rochester, NY) and the refractive index converted to density with standard tables from the CRC Handbook of Chemistry and Physics. In order to eliminate the possibility that plasma might affect the gradient, the single vertical spin (SVS) method was applied to plasma serially diluted with d 1.30 g/ml KBr solution. No changes in lipoprotein band positions were noted.

Characterization of isolated lipoproteins

Lipoprotein fractions obtained by the single vertical spin and sequential flotation methods were characterized by electrophoresis and electron microscopy. Electrophoretic separation of intact lipoprotein fractions was done on 1% agarose gels by the procedure of Papadopoulos (16). Apolipoproteins of VLDL, LDL, and HDL fractions were characterized by polyacrylamide disc gel electrophoresis in urea (17). The size and distribution of lipoprotein particles in the lipoprotein fractions were determined by negative staining with 2% potassium phosphotungstate (18) and examining the grids on a Phillips 400 transmission electron microscope.

TABLE 1. Conditions for density gradient centrifugation in vertical rotors

Type of Rotor	Capacity of Tube	No. of Tubes per Rotor	Gradient Condition		Centrifugation Condition		
			Upper Layer 0.9% NaCl	Lower Layer Plasma (d = 1.30)[a]	Speed	Force	Time
	ml		ml	ml	rev/min	mean g	min
TV-865	5	8	3.5	1.5	65,000	369,548	45
TV-865B	17	8	12.0	5.0	65,000	339,632	90
TV-850	34	8	24.0	10.0	50,000	200,965	150

[a] Density of plasma was adjusted to d 1.30 g/ml by adding solid KBr.
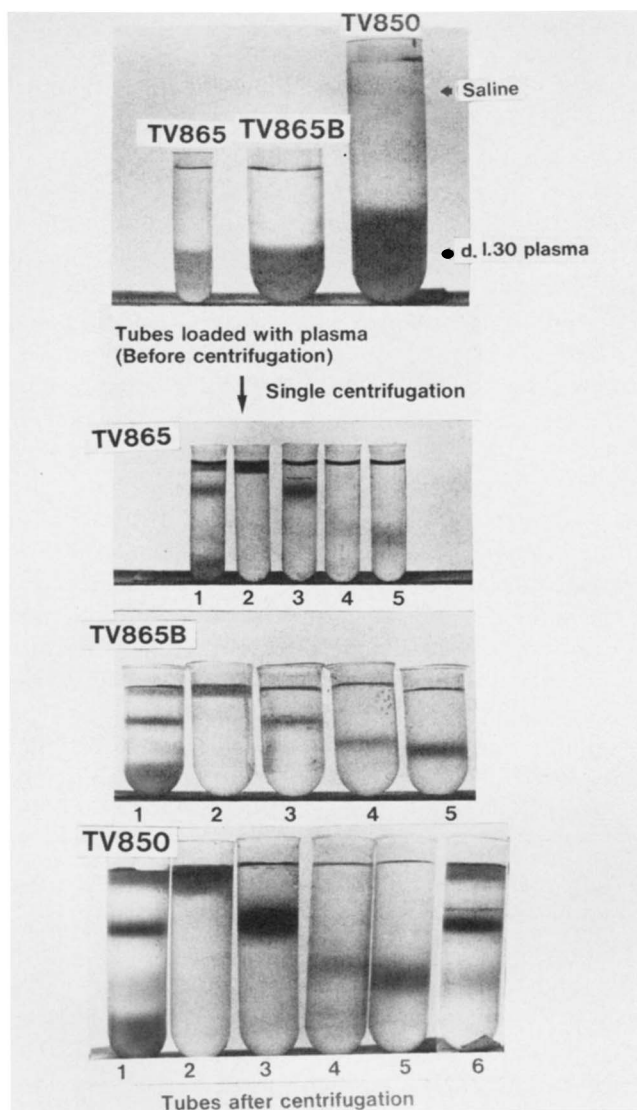
Fig. 1. Separation of plasma lipoproteins and the banding of preisolated VLDL, LDL, HDL₂, and HDL₃ by SVS centrifugation. Plasma (1), authentic VLDL (2), authentic LDL (3), authentic HDL₂ (4), authentic HDL₃ (5), and mixture of VLDL, LDL and HDL (6).

RESULTS

Separation and recovery of plasma lipoproteins

Using the SVS method, separation of the three major lipoprotein fractions (VLDL, LDL, HDL) is achieved (**Fig. 1**). This separation is accomplished with a 45-min spin time with a TV865 rotor, a 90-min spin time with a TV865 B rotor, and a 150-min spin time with a TV850 rotor (Table 1). Rotor shutdown adds approximately 20 min to the total run time.

After an SVS step, lipoprotein fractions preisolated by the SF method form discrete bands with VLDL at the top, LDL in the upper middle, and HDL (HDL₂ and HDL₃) in the lower middle portion of the tubes. Each of these fractions corresponds to one of the three

major bands resulting from SVS fractionation of whole plasma samples (Fig. 1). The broad band at the bottom corresponds to free plasma protein (Fig. 1). By the SVS method, preisolated HDL₂ is partially separated from preisolated HDL₃.

A single vertical spin of a control discontinuous starting gradient creates a nonlinear gradient which covers a density range from 1.014 to 1.25 g/ml. The lower half of the gradient is approximately three times steeper than the upper half (**Fig. 2A**). On the basis of this control gradient, VLDL bands in the 1.014–1.016 g/ml density range, LDL in the 1.020–1.062 g/ml density range, and HDL in the 1.062–1.185 g/ml density range. HDL₂ has its peak centered at d 1.10 g/ml and HDL₃ at d 1.155 g/ml (Figs. 2A and B).
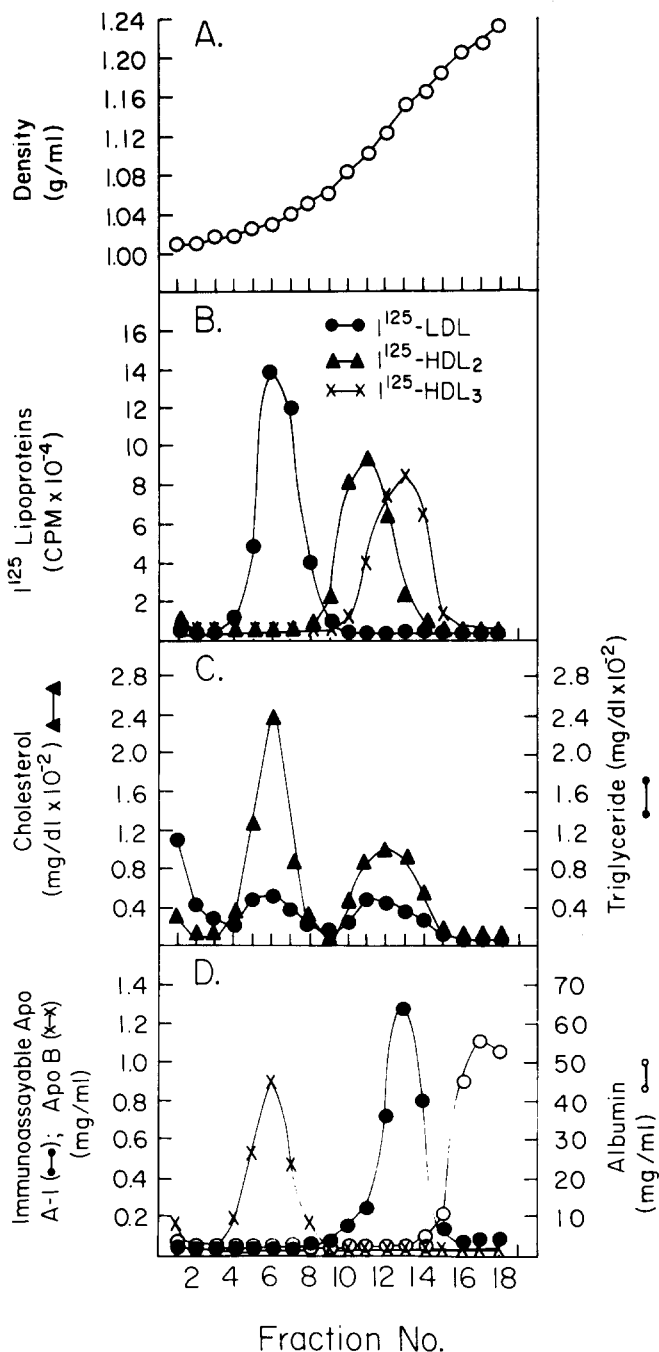
[125]I-Labeled lipoproteins (LDL and HDL) added to plasma and subjected to SVS fractionation, are recovered in the corresponding lipoprotein peak (Fig. 2B). When I[125]-labeled lipoproteins are added to plasma, their recoveries are slightly less than those in which the labeled lipoproteins are added to the corresponding unlabeled lipoprotein fractions (2% for LDL and HDL₃, and 6% for HDL₂). We interpret this as resulting from transfer to other lipoprotein fractions.

[125]I-Labeled HDL₂ bands on the low density side of ^{125}I-labeled HDL₃ after a single spin of whole plasma or corresponding unlabeled carrier lipoprotein. However, there is considerable overlap between the two fractions, such that HDL₂ appears as a low shoulder on the low density side of the complete HDL peak (Figs. 2B and 2D). This partial separation of HDL₂ from HDL₃ from whole plasma is best seen in **Fig. 3.**

The distributions of cholesterol and triglyceride in the separated lipoprotein fractions after SVS fractionation are shown in Fig. 2C. The peaks of cholesterol and triglyceride are coincident with the peaks of VLDL, LDL and HDL (determined by A₂₈₀, ^{125}I and radioimmunoassay). As expected, the highest cholesterol peak corresponds to the LDL fraction and the highest triglyceride peak to the VLDL fraction. There is no detectable cholesterol in the fractions of d > 1.21 g/ml. In contrast, when the same gradient was used in a Sorvall swinging bucket rotor (AH 650) and plasma centrifuged at 50,000 rpm for 60 hrs, detectable quantities of cholesterol were found in the d > 1.21 g/ml fractions.

Use of either the TV-865 or the TV-850 rotor (Figs. 3A and B) gives quantitatively similar separations of VLDL, LDL and HDL to that given by the TV-865B rotor (Fig. 2). Fig. 3A shows the separation achieved between ^{125}I-labeled LDL and ^{125}I-labeled HDL on the TV-865 rotor. Fig. 3B shows the separation achieved among VLDL, LDL, and HDL on the TV-850 rotor; the positions of the lipoprotein fractions are indicated

by measurements of free cholesterol, cholesteryl ester (determined by subtracting total from free cholesterol) and phospholipid. Again note the partial separation

Fig. 2. A. Distribution of density (g/ml) in the fractions from a gradient of d 1.006 g/ml NaCl and d 1.30 g/ml KBr after SVS centrifugation in a TV 865B rotor at 65,000 rpm for 90 min. B. Distribution of I^{125}-labeled authentic lipoproteins added to plasma in the fractions after SVS centrifugation in a TV 865B rotor at 65,000 rpm for 90 min. C. Distribution of cholesterol and triglyceride of plasma in the fractions after SVS centrifugation in a TV 865B rotor at 65,000 rpm for 90 min. D. Distribution of albumin and radioimmunoassayable apo A-I and apo B of plasma in the fractions after SVS centrifugation in a TV 865B rotor at 65,000 rpm for 90 min.
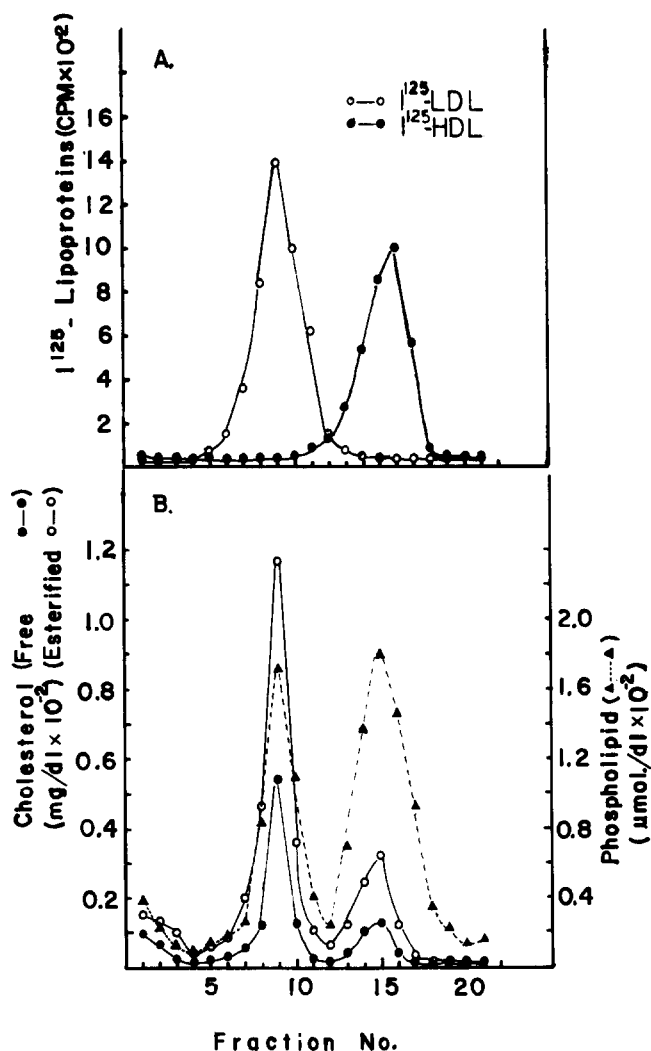

Fig. 3. A. Distribution of ^{125}I-labeled authentic lipoproteins added to plasma in the fractions after SVS centrifugation in a TV 865 rotor at 65,000 rpm for 45 min. B. Distribution of free cholesterol, cholesteryl ester (total cholesterol minus free cholesterol), and phospholipid of plasma in the fractions after SVS centrifugation in a TV 850 rotor at 50,000 rpm for 150 min.

achieved between HDL$_2$ and HDL$_3$ in both Figs. 3A and B.

## Purity of lipoprotein fractions

The distributions of apo A-I and apo B after an SVS fractionation of whole plasma are shown in Fig. 2D. Apo B is primarily located in the LDL fraction, with a small amount also located in the VLDL fraction. Apo A-I is entirely localized in the HDL fraction. There is no measurable overlap between the apo B and A-I fraction. However, there is some minor overlap between apo A-I and free protein (determined by chemical analysis for albumin) after a single spin fractionation. If the HDL peak is pooled and subjected to a second single spin fractionation step, however, no albumin can be detected in the subsequent HDL frac-
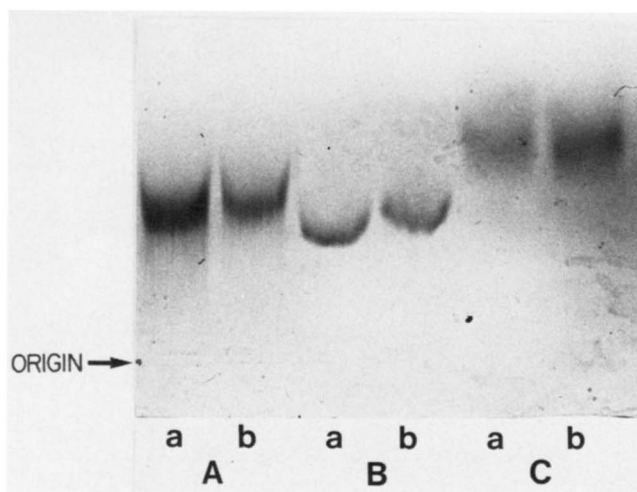
Fig. 4. Electrophoretic patterns of VLDL (A), LDL (B), and HDL (C) isolated by SVS (a) and SF (b) methods.

tion by the chemical assay and only a faint band is detected by an immunoprecipitation assay.

The VLDL, LDL, and HDL fractions isolated by the SVS method have electrophoretic mobilities identical to the corresponding lipoproteins isolated by the
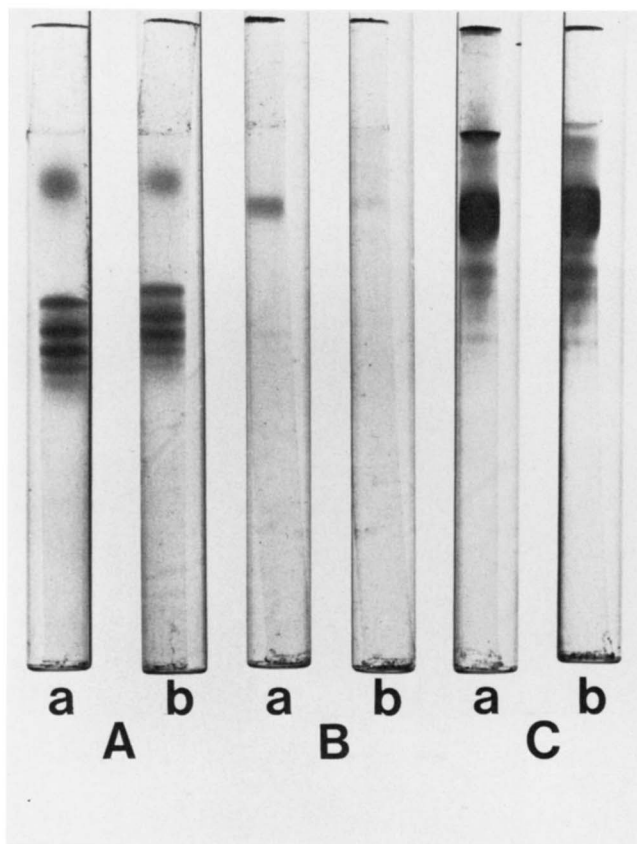
Fig. 5. Urea gel electrophoresis on VLDL (A), LDL (B), and HDL (C) isolated by SVS (a) and SF (b) methods.
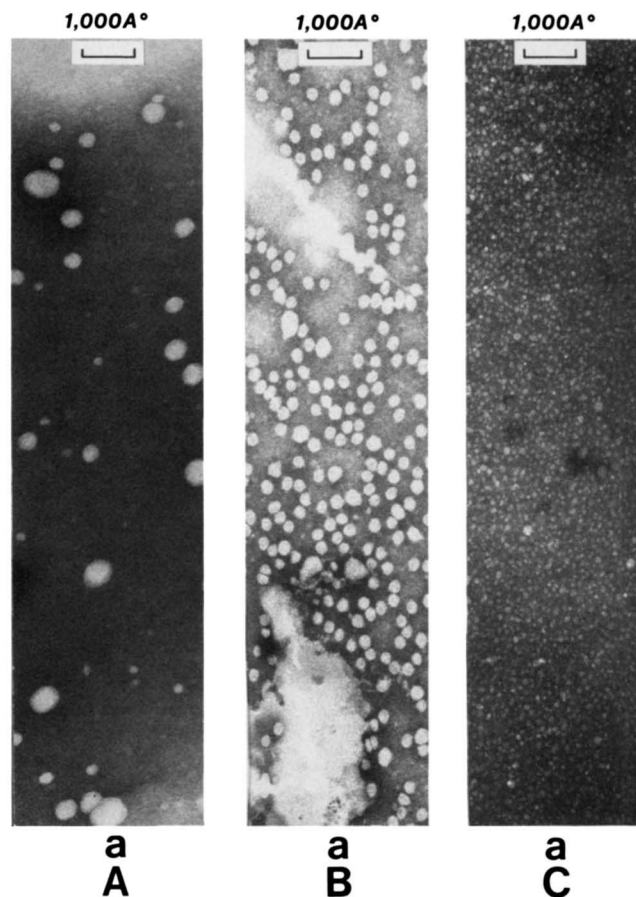
Fig. 6. Electron micrograph of VLDL (A), LDL (B), and HDL (C) isolated by the SVS method.

SF method (**Fig. 4**). The apoproteins of the VLDL and HDL fractions isolated by SVS were identical to those of the authentic SF lipoproteins on the basis of urea polyacrylamide gel electrophoresis (**Fig. 5**). However, a protein band in addition to apo B appears in the LDL isolated by SVS. This band has an electrophoretic mobility similar to that of apo E.

Negative stain electron microscopy (**Fig. 6**) of the SVS lipoprotein fractions indicates that the average sizes of VLDL, LDL and HDL are 427 ± 92 Å, 222 ± 32 Å and 110 ± 15 Å, respectively, which fall into the size ranges of these lipoproteins reported elsewhere (19).

Comparison of the quantitation of HDL cholesterol by the SVS and precipitation methods

The distribution of cholesterol in the VLDL, LDL, and HDL fractions isolated by the SVS method from normal healthy human subjects is shown in **Table 2.** The percent mean distribution of cholesterol in VLDL, LDL, and HDL fractions is 7.7%, 63%, and 29%, respectively. The recovery of cholesterol in the three

TABLE 2. Plasma cholesterol distribution in VLDL, LDL, and HDL fractions separated by SVS and precipitation methods and collected from normal, healthy volunteers

Samples	No. of Samples	Separation Method	Mean ± S.D.[a]	Range	% Distribution ± S.D.	P Value
			mg/dl	mg/dl		
Plasma	20		173.2 ± 28.9	126.0–251.5		
VLDL	20	Single spin	14.5 ± 10.7	4.6–41.7	7.7 ± 4.7	
LDL	20	Single spin	117.7 ± 25.9	81.0–161.1	63.0 ± 6.3	
HDL	20	Single spin	50.5 ± 12.4	38.4–86.5	29.0 ± 6.0	P < 0.05
HDL[b]	20	Precipitation	54.2 ± 15.1	43.2–99.7	31.0 ± 7.9	

[a] Determined by enzymatic assay kit (Bio-dynamics/BMC, Indianapolis, IN).
[b] Heparin–Mn^{2+} precipitation procedure (7).

lipoprotein fractions combined was 99.2 ± 1.5% of plasma cholesterol. HDL cholesterol measured by the heparin–manganese chloride precipitation method (7) represents 31% of the plasma cholesterol and is significantly higher ($P < 0.05$) than HDL cholesterol estimated by SVS fractionation.

DISCUSSION

A rapid single spin fractionation of the three major classes of plasma lipoproteins (VLDL, LDL, and HDL) has been achieved by use of a discontinuous density gradient with vertical ultracentrifuge rotors. The properties of the lipoproteins prepared by this technique are indistinguishable on the basis of apoprotein composition, electrophoretic mobility, and mean particle size and equilibrium banding density from comparable authentic lipoprotein fractions prepared by sequential flotation. No measurable cross contamination between classes can be detected using radioimmunoassay and ^{125}I-labeled lipoproteins. This single spin method is highly reproducible and appears to be well suited for the separation of lipoprotein fractions. However hyperlipidemic disorders involving elevated chylomicrons will need a preliminary centrifugation step to remove the chylomicrons, since this lipoprotein fraction tends to stick onto the inboard wall of the tube during centrifugation.

The treatment of plasma with KBr during the preparation of the density gradient has no measurable effect on the stability of plasma lipoproteins. For example, the recovery of ^{125}I-labeled LDL and HDL in an almost quantitative fashion coincident with corresponding authentic unlabeled lipoprotein indicates that the association of apo LDL and apo HDL proteins with the intact lipoprotein particles is not affected by the presence of KBr up to d 1.30 g/ml.

The distribution of cholesterol and triglyceride following an SVS fractionation coincides with the position of authentic VLDL, LDL, and HDL. No detectable cholesterol is found in the fraction d > 1.21 g/ml, in contrast to results of swinging bucket centrifugation in our hands and to previous reports (6). This finding supports an artifactual dissociation of lipoproteins during long ultracentrifugation as suggested by Levy and Fredrickson (20). Compatible with this possibility is the apparent presence of apo E in LDL isolated by the SVS method (Fig. 5).

After SVS fractionation there is some contamination of albumin in all three lipoprotein fractions, as measured by chemical assay. Albumin, determined by immunoprecipitation, was removed from VLDL and LDL by one SVS wash; however there was still a faint albumin precipitation band in the HDL fraction after a comparable wash.

There are a number of reports of the separation of lipoproteins by density gradient centrifugation, (5, 6, 21–23), including the use of zonal rotors (24). However this method has not been widely used as a means for routine lipoprotein isolation owing to tedious sample loading and a limited sample volume. The SVS fractionation method described here has several advantages over previously reported methods: a short centrifugation time, a simple gradient preparation, and a larger maximum sample volume. Further, this method partially separates HDL_2 from HDL_3 and the separation of HDL from free plasma protein is cleaner than previously reported (21, 22). As a tool for preparative isolation, the optimal maximal sample volume is 80 ml per rotor, although 96 ml can be separated with only minimal cross contamination.

The SVS method for lipoprotein fractionation is potentially a very useful tool for the quantitative analysis of lipid and apolipoprotein components of plasma lipoproteins. In the analytical mode, lipoproteins can be fractionated from 1 ml or less of plasma with a 45 min centrifugation time. In this regard we have developed a method of stable storage of individual plasma density gradients utilizing coils of narrow
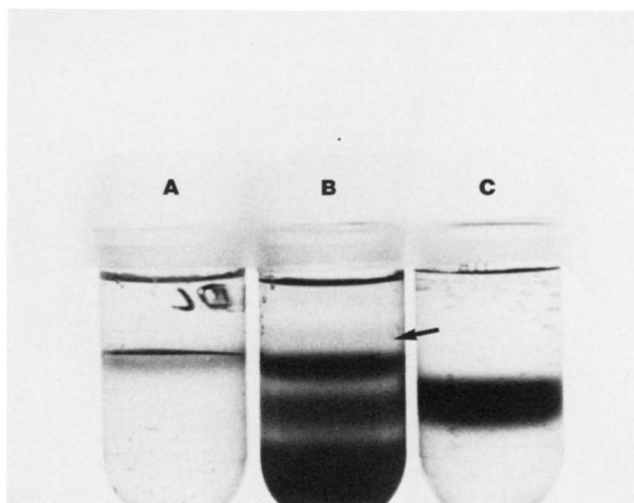
Fig. 7. Separation of an abnormal plasma lipoprotein by SVS centrifugation. Authentic LDL, (A), abnormal plasma, (B), and authentic HDL, (C). Arrow indicates an extra band running in the intermediate density range.

diameter plastic tubing, that should be of value in the ultimate development of an automated plasma lipoprotein analyzer based on the SVS method for clinical use. Preliminary work using this technique for the quantitative analysis of cholesterol in VLDL, LDL, and HDL fractions produces values comparable to those measured by conventional Lipid Research Clinic methods (25). Our observation that HDL cholesterol estimated by the heparin–manganese chloride precipitation method is significantly higher than that estimated by the SVS method is compatible with the previous suggestion that the precipitation method overestimates HDL cholesterol (7). The 4 mg/dl overestimation of the precipitation versus the SVS method is twofold greater than suggested by Warnick and Albers (7), and the difference could be greater in hyperlipidemic patients.

The SVS fractionation method should prove to be a strong diagnostic tool for detecting abnormal hyperlipoproteinemia; we have observed lipoprotein bands in addition to VLDL, LDL, and HDL in occasional samples of fresh pooled sera obtained from the Red Cross. For example, we have observed bands in the intermediate density range between VLDL and LDL (**Fig. 7**). Finally, the single spin fractionation method should be an excellent tool for investigating lipoprotein metabolism, as the rapid separation may provide information regarding lipoprotein exchange, fate, and origin not obtainable by more time-consuming methods.∎

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