papers on methodology

A low temperature flotation method to rapidly isolate lipoproteins from plasma

H. Tong, H. R. Knapp, and M. VanRollins[1]

Division of Clinical Pharmacology, Department of Internal Medicine, College of Medicine, The University of Iowa, Iowa City, IA 52242

Abstract To minimize oxidative modification, a low temperature, sequential flotation method was developed to isolate plasma lipoproteins in 18 h using a benchtop ultracentrifuge. The protein distributions were characterized using agarose and SDS-polyacrylamide gel electrophoresis, and an SDS-Lowry protein assay. The lipid distributions were assessed using a gas chromatography–mass spectrometric assay for cholesterol and an enzymatic assay for triglycerides. To validate the rapid flotation method, lipoproteins were also isolated from the same plasma samples using a modified Havel et al. flotation method (*J. Clin. Invest.* 34: 1345–1353, 1955). The same lipoproteins and apolipoproteins were present in fractions of comparable density, and the summed recoveries of protein, cholesterol, and triglyceride were also identical for the Havel et al. and rapid flotation procedures. Likewise, the amount of cholesterol and triglyceride in corresponding very low, intermediate, and low density lipoprotein (VLDL/IDL and LDL) fractions was the same for the two flotation procedures. The triglyceride and cholesterol levels in high density lipoprotein (HDL) isolated by rapid flotations, however, were 9–12% higher than in the HDL as isolated by Havel et al. Because a 9–12% increase in the HDL fraction reflects only 1–4% of the total triglyceride and cholesterol in plasma, we conclude that, while maintained at 4°C, lipoproteins were quantitatively isolated from human plasma in 1 day.—Tong, H., H. R. Knapp, and M. VanRollins. **A low temperature flotation method to rapidly isolate lipoproteins from plasma. *J. Lipid. Res.* 1998.** 39: **1696–1704.**

Supplementary key words ultracentrifugation • human plasma • very low density lipoproteins • low density lipoproteins • high density lipoproteins • mass spectrometry • cholesterol • triacylglycerides • autooxidation

We are interested in establishing a rapid preparative method to isolate lipoproteins in a manner that minimizes their oxidative modification. Ultracentrifugation methods have commonly been used to preparatively isolate lipoproteins from plasma or serum. One of the most used ultracentrifugation techniques involves sequential flotations in media of increasing densities, and was first employed by Havel, Eder, and Bragdon (1) to resolve lipoproteins into four classes: VLDL ($d \leq 1.006$ g/ml); IDL ($1.006 < d < 1.019$); LDL ($1.019 < d < 1.063$); and HDL ($1.063 < d < 1.21$). The Havel et al. flotation method is frequently used to isolate large amounts of lipoproteins for the purpose of biological testings, e.g., the generation of oxidized LDL. Unfortunately, the method requires several days to be completed. Studies have shown that apolipoproteins redistribute among different classes of lipoproteins during prolonged centrifugations; moreover, the integrity of the lipoprotein particles may be compromised because labile apolipoproteins located on lipoprotein surfaces can be preferentially degraded (2–4). The presence of free radicals in plasma (5) also raises the possibility that lipid peroxidation and autoxidation will occur at the relatively high temperature (20°C) traditionally used (6). When lipophilic anti-oxidants and free radical scavengers are prophylactically added to plasma, they can become sequestered by the plasma lipids, and render the lipoproteins inadequate for oxidation studies. Yet, without anti-oxidants, the use of prolonged centrifugations at 20°C favors the oxidation of native lipoproteins.

Common benchtop ultracentrifuges capable of generating very high *g* forces, have recently been used to isolate lipoproteins for both preparative and analytical purposes. To minimize the processing artifacts described above, Brousseau et al. developed a sequential flotation method for the preparative isolation of lipoproteins. By use of a Beckman TL 100 benchtop ultracentrifuge (up to 100,000 rpm) and a 100.2 fixed-angle rotor, they isolated serum lipoproteins at 15°C in 9.5 cumulative cen-

Abbreviations: VLDL, very-low density lipoproteins; IDL, intermediate density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; %T, total acrylamide content (w/v); $\%C_{bis}$, the ratio of *bis*-acrylamide to acrylamide monomer (w/w); GC/MS, gas chromatography–mass spectrometry; RSD, relative standard deviation.

[1]To whom correspondence should be addressed.

trifugation hours. However, their preparative method did not test the use of low temperatures; moreover, a second centrifugation step was required to minimize albumin contamination.

Recently, flotations with benchtop centrifuges have also been combined with precipitation techniques to isolate lipoproteins for analytical purposes. For example, Leonhardt et al. (8) described a procedure to float VLDL using a benchtop ultracentrifuge at 17°C, and used precipitations to separate LDL from HDL. The precipitation step eliminated the prolonged centrifugation times required for floating HDL. Similarly, Cathcart and Dominiczak (9) reported isolation of VLDL in 2.5 h at 4°C, and separated HDL from LDL by precipitating HDL. Fletcher, Barnes, and Farish (10) also reported a rapid semi-micro procedure for separating VLDL, IDL, and LDL at 20°C using a benchtop ultracentrifuge after HDL precipitation. The precipitated lipoproteins are commonly contaminated with plasma proteins; moreover, their lipid composition may differ somewhat from that of analogous lipoproteins obtained by flotation techniques (11). Finally, before precipitated lipoproteins can be used for biological studies, they must first be resolubilized so that precipitating agents may be completely removed by dialysis. For these reasons as well as their capacity to be readily automated, the hybrid flotation–precipitation methods are generally used for clinical analyses rather than for preparative purposes.

To date, no low temperature sequential flotation method using a benchtop ultracentrifuge to isolate lipoproteins for biological studies has been described. Maintaining lipoproteins at low temperature during processing will minimize their ex vivo oxidation. Currently, there is an intense effort to identify the oxidation products of phospholipids, sterols and apolipoproteins that are formed in vivo and promote atherosclerosis. Oxidized lipids such as lipid peroxides (12–14), oxysterols (15, 16) and apolipoproteins modified with aldehyde adducts (17, 18) exhibit biological activities that are of potential physiological, pathological or pharmacological importance (16, 19). Moreover, many studies have indicated the existence of lipid epoxides and oxysterols in plasma and bile, though their origins are unclear (5, 20–23). However, establishing the in vivo concentrations of lipoprotein epoxides and oxysterols has been rather difficult, because LDL is unstable and highly prone to oxidation during processing and storage (24). As the in vivo concentrations of lipid epoxides and oxysterols in plasma are likely to be very low, artifacts caused by oxidation during sample processing could present a serious problem for the interpretation of such studies.

In the present report, we describe a sequential flotation method to isolate lipoproteins at 4°C from 1.0 ml of plasma in 18 h. The VLDL/IDL, LDL and HDL fractions were almost freed of albumin contamination by optimizing both the centrifugation times and the sites for tube transections. To validate the method, the contents of the isolated fractions were compared to those generated by the Havel et al. method (1).

MATERIALS AND METHODS

Isolation of human plasma

Six healthy volunteer adults (4 male and 2 females) were fasted for at least 12 h before having 100 ml of blood withdrawn from their antecubital veins. The blood was collected into two 60-ml syringes containing 0.8 ml of anticoagulant (0.2 m ethylenediaminetetraacetic acid (EDTA) in 150 mm NaCl, pH 7.4). After being divided equally between two plastic tubes, the 50-ml venous samples were gently inverted in the presence of 2.0 ml 150 mm NaCl that contained anti-bacterial agents (5 μg sodium azide, 4 mg chloroamphenicol, and 4.0 mg gentamicin sulfate) and the kallikrein inactivator aprotinin (200 units); the mixture was prepared fresh daily (25). Upon centrifuging the samples at 4000 g_{max} and 4°C for 30 min, the plasma was removed using plastic pipettes, mixed with benzamidine (proteolysis inhibitor) and phenylmethylsulfonyfluoride (serine proteases inhibitor), each to a final concentration of 1.0 mm, and transferred to Quick-Seal centrifuge tubes (Beckman, Palo Alto, CA) for the sequential flotation procedure. In the following studies, all the required centifugation equipment and accessories were purchased from Beckman.

Isolation of lipoproteins by the Rapid Flotation procedure

Lipoprotein fractions were isolated from four 1.0-ml aliquots of plasma using a benchtop ultracentrifuge (Optima TLX) and a fixed-angle rotor (TLA 120.2) operating at 120,000 rpm (627,000 g_{max}) and maintained at 4°C. The lipoproteins were separated in 2.0 ml dome-topped Quick-Seal tubes (Polyallomer, 11 × 32 mm) which have a sample capacity twice that of similarly sized open tubes. Appropriate centrifugation times for lipoproteins at 20°C were calculated using the equation $k_1/t_1 = k_2/t_2$ (*k*, factor; *t*, spin time), and a compensation of 12% to LDL and 9% to HDL was added for every 5 degree decrease in temperature (11). The increased centrifugation time was necessary to compensate for an increased solution viscosity that occurs at 4°C. Assuming a 12% compensation for VLDL/IDL, the appropriate times were calculated to be 4.93 h for VLDL/IDL, 4.93 h for LDL, and 9.04 h for HDL isolation at 4°C. In close agreement with the k factor and low temperature considerations, experimentally the VLDL/IDL was completely and reproducibly separated in 4 h, LDL in 5 h, and HDL in 9 h (vide infra).

Isolation of VLDL/IDL. One ml plasma and 0.119 ml of NaBr (38% w/v) were added with Hamilton syringes to a Quick-Seal tube. The volume and density of each plasma sample were adjusted to 2.0 ml and 1.0269 g/ml, respectively, at 20°C using 0.15 m NaCl containing 1.0 mm EDTA, and a digital balance. Each tube was heat-sealed (Quick-Seal Tube Sealer), centrifuged for 4.0 h at 627,000 g_{max} and 4°C, and transected 16.75 mm (10.5 setting) from the base using Teflon-coated blades and a graduated slicer (CentriTube Slicer). Reproducible transections were critical because the clear space that intervened between floated and nonfloated lipoproteins is much less than in the long centrifuge tubes used in the Havel et al. procedure. About 0.8 ml was present in the upper section; the floated VLDL/IDL contents were collected and combined with saline washes of the upper section.

Isolation of LDL. The remaining solution in the lower section was transferred to a new Quick-Seal tube. The final volume and density of the sample were adjusted to 2.0 ml and 1.0680 g/ml, respectively, at 20°C using 38% NaBr, 0.15 m NaCl, and a digital balance. Upon being centrifuged for 5.0 h at 627,000 g_{max} and 4°C, each tube was transected 17.5 mm mm (11.0 setting) from its base. About 0.75 ml was present in the upper section, and its floated LDL contents were collected and combined with saline washes of the upper section.

Isolation of HDL. Designated as the clear zone beneath the LDL, the topmost 0.1 ml in the bottom section was discarded to permit density adjustments. The remaining 1.15 ml in the bottom section was transferred to a new Quick-Seal tube, and its volume and density were adjusted to 2.0 ml and 1.21 g/ml, respectively, at 20°C using 38% NaBr and a digital balance. Each tube was centrifuged for 9.0 h at 627,000 g_{max} and 4°C. Upon transecting the tube 16.0 mm (10.0 setting) from the base, about 0.9 ml was isolated in the upper section, and its floated HDL content was collected and added to saline washes of the upper section.

Isolation of lipoproteins by the Havel et al. flotation procedure

Isolation of VLDL/IDL. Thirty-five ml plasma and 1.2391 ml of 38% NaBr were transferred to a dome-topped Quick-Seal tube (Polyallomer, 25 × 89 mm). The volume and density of each plasma sample were adjusted to 39 ml and 1.019 g/ml, respectively, using a digital balance and 0.15 m NaCl containing 1.0 mm EDTA. Upon being sealed, each tube was transferred to a fixed-angle rotor (Type 60), and centrifuged (L7-55) for 20 h at 50,000 rpm (176,200 g_{max}) and 20°C. Each tube was transected 58 mm from its base by use of a stainless steel blade; about 9.5 ml of the floated VLDL/IDL was collected from the top section. The upper surface of the blade and inside surface of the excised upper section were both washed three times with 0.15 m NaCl; the washes were added to the isolated VLDL/IDL fraction.

Isolation of LDL. The remaining sample present in the lower section was divided equally between two new Quick-Seal tubes. The volume and solution density in each tube were adjusted to 39 ml and 1.063 g/ml, respectively, using 38% NaBr, 0.15 m NaCl, and a digital balance. Upon being sealed, each tube was centrifuged for 20 h at 176,200 g_{max} and 20°C, and transected 51 mm from its base. About 19 ml was isolated in the upper tube section, collected, and combined with saline washes of the blade and upper tube section.

Isolation of HDL. Designated as the clear zone beneath the LDL, the topmost 10 ml in the bottom sections was discarded to permit density adjustments. The remaining infranatant in the two bottom sections was transferred to two new Quick-Seal tubes. The volume and density of each sample were increased to 39 ml and 1.21 g/ml, respectively, with 38% NaBr and 0.15 m NaCl, and the tubes were sealed and centrifuged for 40 h at 176,200 g_{max} and 20°C. After each tube was transected 51 mm from its base, the HDL was isolated in 19 ml, and combined with saline washes from the blade and upper tube section. Designated as the clear zone beneath the HDL, the topmost 10 ml in the bottom tube section was also collected to determine whether any cholesterol was inadvertently left behind.

Characterization of lipoprotein fractions

Agarose gel electrophoresis was used to identify the lipoprotein constituents of floated fractions. One ml of each VLDL/IDL, LDL, and HDL fraction was desalted and concentrated to about 30 μl by filtration (Centricon 50, Amicon, Beverly, MA) at 5000 *g*, 4°C for 60 min. The amount of protein or lipid lost during filtration from retained lipoproteins was assumed to be the same for comparable fractions. About 0.5–1.0 μl of each concentrated sample was loaded with a modified Hamilton syringe onto a pre-cast 1% agarose gel film (Universal Gel 8, Ciba-Corning). Human lipoprotein standards, generously provided by Dr. David Chappell, U. of Iowa, were similarly applied for identification purposes. At room temperature and under a constant 90 V field, the lipoproteins were separated in 35 min using 50 mm barbital buffer (pH 8.6; Universal PHAB buffer, Ciba-Corning). Upon being blotted, the gel was dried at 60°C for about 1 h, and stained for 30 min at room temperature with Fat Red 7B (2.25 mg dissolved in 10 ml methanol and 2 ml 0.1 N NaOH). The gel was destained in 70% methanol and dried overnight at room temperature.

Gradient gel electrophoresis was used to identify apolipoprotein components of the floated fractions. Vertical slab-gels (0.75 mm × 11 cm × 14 cm) were prepared containing a 5–20% polyacrylamide gradient (26). In brief, 7.0 ml of 5% T, 0.8% C_{bis} was mixed with 7 ml of 20% T, 0.8% C_{bis} using a gradient maker (SG 50, Hoeffer Scientific, San Francisco, CA), and pumped at 7 ml/min into a sandwich chamber (Hoefer Scientific). Both solutions were previously fortified with 0.4% SDS plus 1.5 m Tris (pH 8.8), and had been degassed under house vacuum for at least 30 min. Complete polymerization occurred within 30 min after adding 50 μl of 10% ammonium persulfate and 5 μl N′,N′,N,N-tetramethylene-ethylenediamine. A stacking gel (0.75 mm × 2 cm × 14 cm) was similarly generated in 1–2 h using 4% T, 0.8% C_{bis}.

On the same or next day, samples from whole plasma and lipoprotein fractions were resolved using an electrophoresis chamber (SE 600, Hoefer) maintained at 15°C by a circulating cooler. Standards (SDS PAGE Molecular Weight Standards-High; Bio-Rad Laboratories, Richmond, CA) were applied to the outermost lanes to aid apolipoprotein identifications. All protein-containing samples (ca. 10 μg) were initially heated at 60°C for 1 h with 0.25 vol of 60 mm Tris-HCl (pH 6.8), 25% glycerol, 2% SDS, 14.4 mm 2-mercaptoethanol, and 0.1% bromophenol blue. Upon being diluted with deionized water to 50 μl, the denatured proteins were subjected to electrophoresis at a constant 60 mA for 4–5 h. The gel was transferred to a 0.1% Coomassie blue R-250 solution for overnight staining. Excess stain was removed by rinses with methanol–acetic acid–water 40:10:50 (v/v/v).

An SDS-Lowry protein assay was used to measure protein concentrations in each of the floated fractions (not previously de-salted by filtration). Preliminary results indicated that the NaBr concentrations used in the present study had no effect on protein determinations, and confirmed previous findings (27). After solublization with SDS and reaction with a Folin-Ciocalteu phenol reagent, the protein content of each lipoprotein fraction was determined spectrophotometrically as equivalents of bovine serum albumin (Pierce Chemical Co) (28). The mean relative standard deviation (RSD; n = 10) of ten albumin concentrations (10–60 μg) was 2.83%.

An isotope-dilution assay for cholesterol using gas chromatography–mass spectrometry (GC/MS) was used to assess lipoprotein recoveries. Cholesteryl esters were saponified using a low temperature modification of the Ishikawa micromethod (29). In brief, 10 μl of plasma or floated lipoprotein was added to a 1.0 ml Reactivial (Pierce Chemical Co., Rockford, IL) containing 10 μg [26,26,26,27,27,27-$^2H_6$]cholesterol (98.4% isotopic purity; Medical Isotopes, Pelham, NH). Tetramethylammonium hydroxide (6.25 mg in 100 μl of 2-propanol–methanol 3:1) was added, and the vials were flushed with argon for 30 sec, sealed with Teflon-lined caps, and periodically mixed at 4°C over a 6-h period. The saponified lipids were extracted by mixing with tetrachloroethylene (50 μl) and water (200 μl) for 1.0 min. After a 10-min centrifugation at 1250 g_{max} and 4°C, the lower organic phase containing unesterified cholesterol was collected and transferred to a 100-μl Reactivial.

Prior to quantitation by GC/MS, the unesterified cholesterol was converted to a trimethylsilylether derivative. In brief, the saponified samples were dried under a nitrogen stream, and suspended in 50 μl of N,O-*bis*(trimethylsilyl)trifloroacetamide (Pierce Chemical Co.) and 50 μl acetonitrile. Upon being flushed with argon for 30 sec, each vial was capped, shaken, and left overnight at room temperature. The next morning, the sample was dried under a stream of nitrogen and dissolved in 100 μl decane.

The trimethylsilyether of cholesterol was analyzed using capillary gas chromatography (5890, Hewlett-Packard). About one

hundredth of the total extract was deposited via a "duck-bill" injector (Hewlett-Packard) into a fused silica capillary column (0.25 mm (i.d.) × 30 m) containing a 0.25 μm film of 5% diphenyl–95%dimethylpolysiloxane (DB-5-HT, J&W, Rancho Cordova, CA). The linear velocity of the helium carrier gas was programmed to remain constant at 25 cm/sec. One min after injection, the oven temperature was increased 70°C/min from 170°C to a final constant temperature of 275°C. The injector temperature was programmed to be 3°C above that of the oven, while the interface remained at 280°C. Under these conditions, the retention time for cholesterol was 10.93 min.

Quantitation was performed using an isotope dilution technique and a quadrupole mass spectometer (5970B, Hewlett-Packard). Deuterated cholesterol was used as the internal standard to normalize for variability in recoveries during sample processing. To assess relative reponses, varying amounts of d_0-cholesterol (10–500 ng) were weighed and mixed with 100 ng of d_6-cholesterol. The d_0/d_6 mixtures were silylated (see above), and constant amounts of d_6-cholesterol (1.0 ng/1.0 μl) were injected into the GC/MS system. After the protium and deuterated cholesterols were ionized at 70 eV, the currents generated by *m/z* 458/464 (M) and 368/374 [M–$(CH_3)_3SiOH$] were integrated. Using regression analysis, the d_0/d_6 ratios were found to be highly linear ($r^2 = 0.9996$), i.e., for weight ratios varying between 1:10 to 5:1, the area ratio of m/z 458/464 was 1.0392 × (weight ratio of cholesterol/[d_6]cholesterol) − 0.007. Moreover, injection of the same sample ten times yielded a RSD of 0.64% for 500 mg/dl and 1.45% for 4.5 mg/dl cholesterol. Such low intraassay variability is critical for detecting small differences in cholesterol recoveries. Moreover, whole plasma cholesterol concentrations (mg/dl) determined by GC/MS (178, 244, 123, 168, 171, and 151) agreed within 3.3% of those determined enzymatically (181, 251, 126, 172, 174, and 156, respectively) (30). Thus, the preliminary studies indicated that the GC/MS assay had a 1.5% precision and 3.3% accuracy.

An enzymatic assay was used to quantitate the triglyceride content of floated lipoproteins. Triglycerides were quantitated using a glycerol-3-phosphate oxidase method (31) modified for use with a microtiter plate reader ($THERMO_{max}$, Molecular Devices Corp., Sunnyvale, CA). In brief, 2–15 μl of lipoprotein fractions was diluted to 30 μl with 0.1 m sodium phosphate (pH 7.6) and mixed with 100 μl of solution A containing glycerol kinase (EC 2.7.1.30), adenosine triphosphate, glycerol-3-phosphate oxidase (1.1.3.21), peroxidase (EC 1.1.1.7), and 4-chlorophenol (Boehringer Mannhein, GmbH). After a 5-min incubation at 25°C to remove free glycerol, the mixture was shaken for 1 min with 100 μl solution B containing triglyceride lipase (EC 3.1.1.3) plus 4-aminoantipyrine (Boehringer Mannheim), and incubated 10 min at 25°C. Over a range of 50–1000 mg/dl glycerol, linear amounts of the chromogen 4-(*p*-benzoquinonemonoimino) phenazone were generated ($r^2 = 0.99826 \pm 0.1\%$ RSD; n = 6) as monitored by absorption at 490 nm against a reagent blank. On the same day, replicate assays of lipoprotein fractions containing 109, 122, and 307 mg glycerol/dl varied by 2.8–5.1% RSD (n = 10–12).

Statistics. The contents from corresponding fractions were analyzed as ratios, and evaluated using a one sample *t*-test. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Use of a high-speed, benchtop ultracentrifuge permitted isolation at 4°C of three lipoprotein fractions in ≤24 h

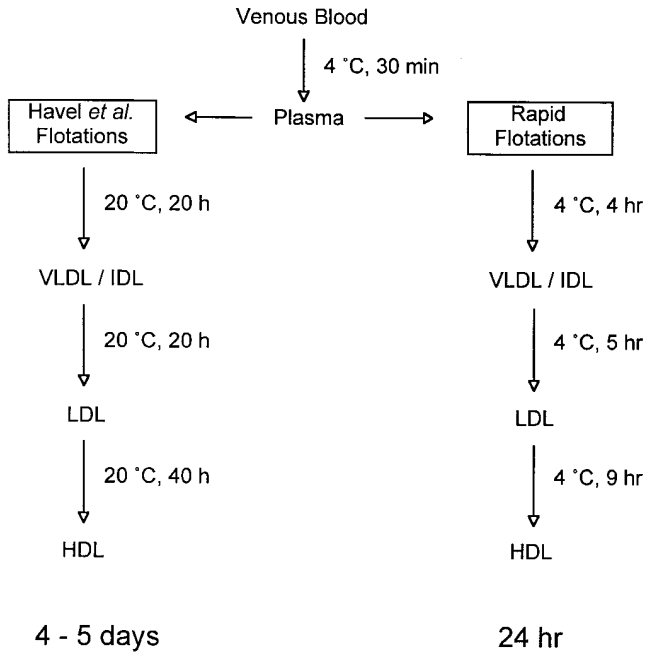

Fig. 1. Times and temperatures of Havel et al. and rapid flotation procedures. Abbreviations: VLDL, very low density lipoproteins; IDL, intermediate density lipoproteins; LDL, low density lipoproteins; and HDL, high density lipoproteins.

(**Fig. 1**). Enzymatic transformations and autooxidations of the lipid constituents should be minimized by the shorter centrifugations at 4°C as compared to the procedure used by Havel et al. (1). Because LDL and HDL fractions must first be desalted before their lipids can be quantitatively extracted by the Folch or Bligh-Dyer methods, it takes up to 24 h before all enzymatic activity is eliminated by extractive isolation. In contrast, similar extractions cannot be performed for up to 5 days when lipoproteins are isolated by the Havel et al. procedure (Fig. 1).

To determine whether the rapid flotations generated fractions with contents comparable to those of the Havel et al. procedure, six plasma samples were subjected to both flotation procedures. The contents of the resulting fractions were analyzed using electrophoretic techniques, and lipoprotein recoveries were assessed by protein, cholesterol, and triglyceride measurements. In addition, the summed recovery was compared to whole plasma values.

The types of lipoproteins present in the three fractions were identified by agarose gel electrophoresis; **Fig. 2** illustrates typical results from one of the six plasma samples. Neither of the two LDL fractions contained appreciable HDL when lipoproteins were identified by co-migrations with human standards; however, because of a pronounced tailing of the VLDL/IDL fraction (Fig. 2), it was uncertain as to whether significant amounts of VLDL/IDL occurred in the LDL fraction.

The types of proteins present in each fraction were examined using SDS polyacrylamide gel electrophoresis; **Fig. 3** illustrates typical results from the plasma samples. Intermediate-sized apolipoproteins and albumin were identified from semi-logarithmic plots of molecular weight ver-

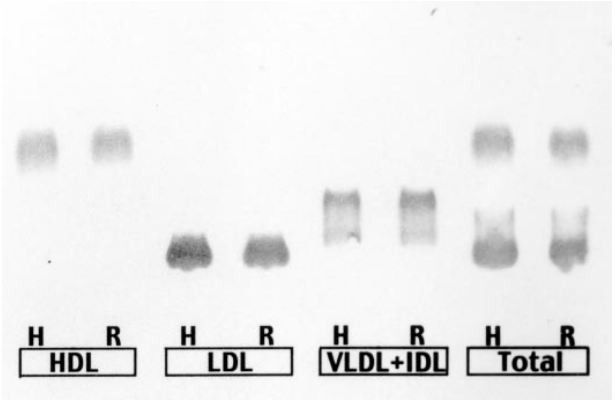

Fig. 2. Agarose gel electrophoresis of lipoprotein fractions isolated by the Havel et al. and rapid flotation methods. Fractions containing lipoproteins in an amount equivalent to that present in 1.5–3 μl of whole plasma were spotted in parallel lanes onto a pre-cast agarose film. The two HDL fractions were applied to lanes 1 and 2 (starting from the left side); the two LDL fractions to lanes 3 and 4; the VLDL/IDL fractions to lanes 5 and 6; and the total lipoprotein fractions (density ≤ 1.21 g/ml) were applied to lanes 7 and 8. Individual lipoproteins were resolved using a constant voltage, and visualized using a Fat Red 7B stain. Abbreviations: H, Havel et al.; R, rapid flotations.

sus R_f. ApoB-100 ($M_r > 200$ kd) was the major apolipoprotein in the VLDL/IDL and LDL fractions. Because no apoA-I ($M_r \approx 23$ kd, vs. expected 28 kd) was evident in the LDL fractions, the polyacrylamide gel electrophoresis studies confirmed that the HDL fraction was essentially free of LDL. Moreover, apolipoproteins E-1, E-2, and E-3 ($M_r \approx 34.9$, 33.3 kd vs. expected 34 kd) and apoC-II and apoC-III ($M_r < 14.4$ kd) were primarily in the VLDL/IDL fractions. Thus, in contrast to the agarose gel results, polyacrylamide gel electrophoresis data indicated that there was little contamination of the LDL fraction by dense IDL. Therefore, both electrophoresis studies indicated that similar types of lipoproteins had been isolated by the two flotation procedures.

The quantity of protein in comparable fractions was very similar (**Table 1**). Although albumin (observed $M_r \approx$ 69 kd, vs. expected 66 kd) was variably present in the three fractions (Fig. 3), its contributions were minimal in the Rapid Flotation procedure. Serum albumin is considered to be the predominant protein contaminant of lipoproteins isolated by ultracentrifugation methods (7, 32). After repeatedly experiencing difficulty in removing albumin from the HDL fraction by the Havel et al. procedure, we divided the VLDL/IDL and LDL infranatants between two tubes before subjecting them to prolonged centrifugations. Using this dilution technique, we obtained the highest quality lipoproteins from Havel's method for reference purposes. As judged by agarose gel electrophoresis and SDS 5–20% PAGE, lipoprotein fractions isolated using our rapid method were of purity equal to those obtained by the slightly modified Havel et al. method.

Under these conditions, the total protein recovered from 1.0 ml plasma by the Havel et al. procedure was approximately 2.1 mg/ml. Very close to the same amount of total protein was recovered by the rapid flotation method; replicate determinations had 3% variability (Methods section). Likewise, the amount of protein was virtually the same in comparable subfractions. Thus, both flotation methods produced fractions with matching amounts of apolipoproteins and protein contaminants.

To precisely assess lipoprotein recoveries, the amount of total cholesterol in each fraction was measured using GC/MS methods. However, small amounts of cholesterol were unavoidably lost prior to these analyses. After each LDL fraction was collected, a volume was discarded from the top of the remaining solution, the so-called LDL clear zone. Without removing this amount of solution, the total

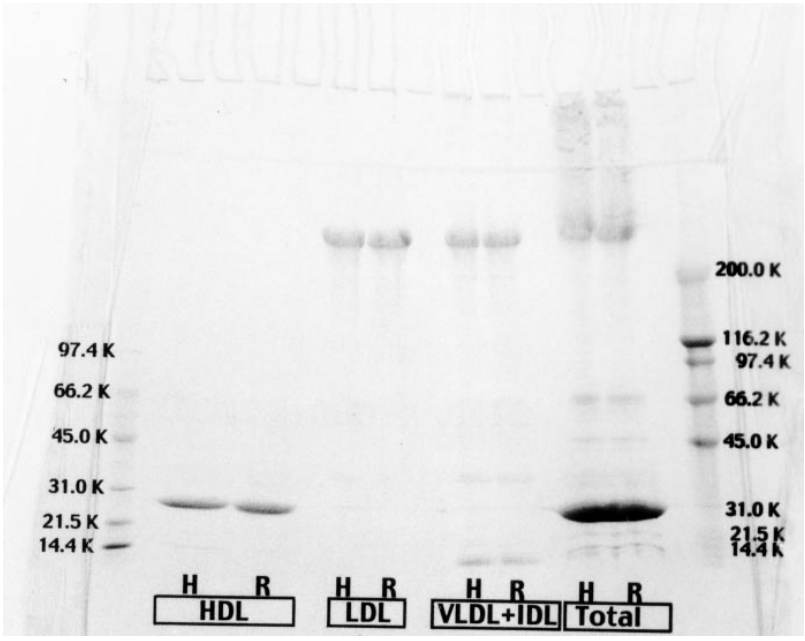

Fig. 3. SDS-PAGE (5–20%) gradient gel electrophoresis of denatured lipoproteins isolated by the Havel et al. and rapid flotation methods. Standards with molecular weights ranging from 14,400 to 200,000 were applied to the outermost lanes of a 5–20% SDS-PAGE. Excepting the total lipoprotein fractions (density ≤ 1.21 g/ml), about 10 μg protein from the fractions isolated by Havel et al. and rapid flotation methods was applied in the remaining lanes: HDL to lanes 2 and 3; LDL to lanes 4 and 5; and VLDL/IDL to lanes 6 and 7. In addition to the major apolipoprotein B_2 ($M_r > 200$ kD), the small apolipoproteins E_{1-3} ($M_r \approx 34$ kD) and C_{2-3} ($M_r <$ 14.4 kD were also evident on the 5–20% SDS-PAGE gradient (lanes 6 and 7). To lanes 8 and 9, more than 10 μg protein of the total lipoprotein fractions was applied in order to permit ready visualization of the minor apolipoproteins. The major and minor apolipoproteins were resolved by applying a constant current, and visualized by staining with Coomassie blue. Abbreviations: H, Havel et al.; R, rapid flotations.

TABLE 1. Protein content of fractions isolated by Havel et al. (1) and rapid flotation procedures

Lipoprotein Fraction	Havel et al. Flotation Procedure[a]	Rapid Flotation Procedure[b]
VLDL/IDL	0.151 ± 0.053	100.8 ± 4.7%
LDL	0.577 ± 0.228	99.3 ± 1.9%
HDL	1.363 ± 0.112	102.4 ± 1.1%
Summed protein in all fractions	2.090 ± 0.308	101.8 ± 0.6%

[a] Data represent mean mg ± SD (n = 6) of protein isolated from 1.0 ml of plasma. The protein contents were directly assayed without concentrating or desalting the isolated fractions.

[b] To more readily show differences from the reference method, the data are presented as mean percent ± SEM (n = 6) of the protein value in the corresponding Havel et al. fraction.

volume of the sample after the density adjustment would be larger than the volume of centrifuge tube. The amount of cholesterol lost to the LDL clear zone represented 2.1% ± 1.1 and 0.4% ± 0.2 of the plasma cholesterol level (161 mg/dl ± 37, n = 6), respectively, in the Havel et al. procedure (1) and the current rapid flotation procedure, respectively. An additional 0.7% ± 0.7% of cholesterol was lost to the HDL clear zone in the Havel et al. procedure; however, no cholesterol was detectable in the HDL clear zone in the rapid flotation procedure. In summary, 0.4–2.8% of the total plasma cholesterol was discarded before the cholesterol content of individual lipoprotein fractions could be analyzed.

Cholesterol recoveries for the two procedures represented 96% of the total amount available in whole plasma (**Table 2**). Because the experimental error for cholesterol determinations was less than 1.5% (Methods section), these results indicated that the cholesterol recoveries were indeed less than 100%. However, as 0.4% and 2.8% of the total cholesterol had been discarded with the clear zones, both procedures accounted for 96.4–98.8% of the lipoprotein cholesterol originally present in plasma.

Analysis of the cholesterol distributions revealed that comparable fractions had similar cholesterol contents. Both flotation procedures yielded similar amounts of cholesterol in the VLDL/IDL fraction, as well as in the LDL fraction (Table 2). With such variability in the six plasma samples, only differences of 12% (VLDL/IDL) and 6% (LDL) would have been detected with a power of 0.83. Surprisingly, the HDL fraction isolated by the rapid flotation procedure had 12% more cholesterol than the same fraction isolated by the Havel et al. procedure. However, because the additional cholesterol represented only 3.4% (12.2% × 28.6) of the plasma total cholesterol, the total recovery and distribution of cholesterol were very similar for the two flotation methods.

The rapid flotation procedure yielded similar total amounts of triglyceride as the Havel et al. procedure (**Table 3**). Because the recovery in the Havel et al. procedure was 103.6% of the total triglycerides originally present in plasma, this meant that 101% of the total plasma cholesterol was recovered by the rapid flotation procedure. As these determinations are within the 5.1% experimental error (Methods section), the present results indicate that, like cholesterol, the triglycerides were quantitatively recovered from plasma by both flotation procedures.

As with cholesterol, the triglyceride contents of fractions generated by the two flotation procedures were similar but not identical. Both flotation procedures yielded similar amounts of triglycerides in the VLDL/IDL fraction, as well as in the LDL fraction (Table 3). With the variability described in Table 3, only a triglyceride difference of 8% (VLDL/IDL) and 11% (LDL) would have been detected with a power of 0.85. In contrast, the HDL fraction isolated by the rapid flotation procedure had significantly more triglyceride (9%) than the HDL fraction in the Havel et al. procedure. The extra triglyceride represented only 1.0% (8.9% × 10.8) of the total triglyceride present in whole plasma. In conclusion, both the total recovery and distribution of triglyceride between the lipoprotein fractions were similar for both flotation procedures.

The two flotation methods also generated lipoprotein fractions with matching triglyceride to cholesterol molar ratios (**Table 4**). Closely resembling that found in whole plasma, the ratio of summed recoveries confirmed that neither flotation procedure produced differential losses of the major neutral lipids. Perhaps more importantly, both flotation procedures generated HDL fractions with the same molar ratio of triglycerides to cholesterol. Thus, the HDL isolated by the rapid flotation procedure

TABLE 2. Lipoprotein recoveries as assessed by GC–MS quantitation of cholesterol

Lipoprotein Fraction	Havel et al. Flotation Procedure[a]	Rapid Flotation Procedure[b]
VLDL/IDL	9.6 ± 3.9%	96.0 ± 3.3%
LDL	57.8 ± 7.3%	97.6 ± 1.7%
HDL	28.6 ± 9.4%	112.2 ± 3.3%[c]
Summed cholesterol recovery from all fractions	96.0 ± 3.0%	101.1 ± 1.3%

[a] Cholesterol contents were directly assayed without concentrating or desalting the isolated fractions. Data represent mean percent ± SD (n = 6) of the total plasma cholesterol concentration (4.17 mm ± 0.96).

[b] Data represent mean percent ± SEM (n = 6) of cholesterol values in the corresponding Havel et al. fraction.

[c] This value was different ($P = 0.014$) from that found for the HDL cholesterol isolated by the Havel et al. procedure (1).

TABLE 3. Lipoprotein recoveries as assessed by enzymatic assay of triglycerides

Lipoprotein Fraction	Havel et al. Flotation Procedure[a]	Rapid Flotation Procedure[b]
VLDL/IDL	76.0 ± 6.9	96.5 ± 2.1
LDL	16.8 ± 4.2	93.2 ± 3.0
HDL	10.8 ± 5.7	108.9 ± 1.1[c]
Summed triglycerides recovered from all fractions	103.6 ± 6.9	97.7 ± 2.8

[a] Triglyceride contents were directly assayed without concentrating or desalting the isolated fractions. Data represent mean percent ± SD (n = 6) of plasma triglyceride concentrations (0.89 mm ± 0.52).

[b] Data represent mean percent ± SEM (n = 6) of triglyceride values in corresponding Havel et al. fraction.

[c] This value was different ($P = 0.0005$) from that found for HDL isolated by the Havel et al. procedure.

TABLE 4. Triglyceride to cholesterol molar ratios in fractions isolated using Havel et al. (1) and rapid flotation procedures

Lipoprotein Fraction	Havel et al. Flotation Procedure[a]	Rapid Flotation Procedure[b]
VLDL/IDL	1.68 ± 0.48	100.9 ± 2.8
LDL	0.06 ± 0.02	95.6 ± 3.4
HDL	0.07 ± 0.02	97.5 ± 3.0
(Summed triglycerides)		
(Summed cholesterol)	0.22 ± 0.10	96.7 ± 1.7
Whole plasma	0.21 ± 0.10	

[a]Data represent the mean ± SD (n = 6) of the triglyceride to cholesterol molar ratios.

[b]Data represent the mean percent ± SEM. (n = 6) of values in the corresponding Havel et al. fraction.

did not have a selective increase in cholesterol relative to triglycerides.

DISCUSSION

In vivo oxidations of the phospholipid, cholesterol, and apolipoprotein constituents of lipoproteins may play an important role in the pathogenesis of atherosclerosis (16, 19, 33, 34). To study lipoprotein oxidations in vitro, the Havel et al. (1) flotation procedure is the preparative method most commonly used, and requires several days for completion (Fig. 1). During this period, the apolipoproteins may change or transfer among different classes of lipoproteins, lipoproteins may be degraded by enzymes or microorganisms, and lipid autooxidation may occur (34). Recently, more rapid methods for preparing plasma lipoprotein fractions have been proposed that use tabletop ultracentrifuges with higher *g* force capacities (7–10). These brief reports described the details of conditions to achieve faster separations; however, data demonstrating the completeness and reproducibility of the plasma lipoprotein separations were not presented. Moreover, most of the studies with tabletop ultracentrifuges were carried out at room temperature, which may allow significant autooxidation to occur; free radicals are readily formed from unsaturated fatty acids at room temperature as well as at physiological temperature (35, 36).

Recently, Cathcart et al. (9) reported that VLDL could be isolated in 2.5 h at 4°C using a tabletop centrifuge and rotor. However, they also concluded that the isolation of VLDL under these conditions was not very reproducible, with an RSD of 50% and 34.2% for 0.38 and 0.76 mm cholesterol, respectively. If one considers the temperature effects, the k factors in the Havel et al. (1) (k = 90) and Cathcart et al. (9) (k = 14) procedures, plus the time required to isolate VLDL in the Havel procedure (20 h), it can be calculated from $k_1/t_1 = k_2/t_2$ that at least 4.3 h are required to isolate VLDL with the centrifuge and rotor used by Cathcart et al. In agreement with these calculations, we were unable to isolate VLDL at 4°C in less than 4.0 h. To ensure a reproducible isolation of VLDL/IDL, we elected to routinely use 4-hr centrifugations.

In the present study, three lipoprotein fractions, VLDL/IDL, LDL and HDL, were isolated at 4°C in 4, 5, and 9 h, respectively. The summed recovery of cholesterol and triglycerides by this rapid flotation procedure was essentially identical to that determined with the Havel et al. (1) method, and was very close to that of whole plasma. In early studies with the rapid flotation procedure, two factors were found to be critical for minimizing sample losses and obtaining quantitative recoveries. First, a continued sharpness of the Teflon-covered blade in the tube slicer was important because leaks around the blade frequently developed within 10 to 15 cuts. Second, the inner surface of the cut tubes and the surface of the blade had to be thoroughly washed after each transection. Close attention to these two details was critical in achieving quantitative recoveries.

The distribution of lipoproteins, apolipoproteins, protein, cholesterol, triglycerides, and proteins among rapidly floated lipoproteins was similar to that of the Havel et al. method. Qualitatively, the same lipoproteins and apolipoproteins were present in comparable fractions subjected to electrophoresis; thus, the distribution of lipoproteins between the various fractions did not undergo major alterations due to the use of very high centrifugal forces. However, 9–12% more triglycerides and cholesterol were present in the rapidly floated HDL than in the Havel et al. floated HDL. It is possible that this increase resulted from a redistribution of a small amount of LDL to the HDL fraction, because both fractions possessed a similar triglyceride to cholesterol molar ratio (Table 4). Moreover, Leonhardt, Pietzsch, and Nitzsche (37) noted that use of an increased centrifugal force decreased the recovery of LDL cholesterol by 9%. Although the amount of protein in comparable fractions was similar for the two flotation procedures (Table 1), the LDL fraction contained five times more cholesterol per mg of protein than did the HDL fraction (Tables 1, 2). Therefore, a small amount of LDL cholesterol and triglyceride may have been redistributed to the HDL fraction, and been accompanied by an amount of apolipoprotein too small to be quantified (Table 1) or visualized (apolipoprotein A-I in Fig. 2). Alternatively, the increased cholesterol and triglyceride levels found may have reflected an improved recovery of HDL in the rapid flotation procedure. During the isolation of lipoprotein using the Havel et al. flotation procedure, 2.8% of the total cholesterol was discarded from the LDL and HDL infranatants; in contrast, only 0.4% of the total cholesterol was discarded in the rapid flotations. Thus, 2.4% more cholesterol was available to be isolated as HDL by the rapid flotation method. This difference accounts for over two-thirds (8.4% = 2.4/28.6) of the 12% increase in rapidly floated HDL (Table 2). Thus, although both possibilities may have contributed to the observed increase in HDL cholesterol and triglycerides with the rapid flotation procedure, most of the increase was probably due to an improved recovery of HDL. Perhaps more importantly, a 9–12% increase in HDL lipids reflects less than 1–4% of the cholesterol and triglyceride levels in whole plasma.

TABLE 5. Comparison of cholesterol and triglyceride concentrations in lipoproteins isolated by flotation procedures

	Ref.	VLDL/IDL	LDL	HDL	1.2 (g/ml)	Summed Recovery
Cholesterol						
Rapid flotation, n = 6 plasmas		0.41 ± 0.28	2.38 ± 0.77	1.26 ± 0.20		4.05 ± 0.95
Havel et al. flotation, n = 20 sera	1	0.58 ± 0.22	2.65 ± 0.56	1.25 ± 0.27[a]		4.48 ± 0.74
Brousseau et al. flotation, n = 10 sera	7 VLDL	0.21 ± 0.13	2.37 ± 0.62	1.60 ± 0.39	0.21 ± 0.15	4.80 ± 1.11
	IDL	0.41 ± 0.21				
		0.62				
Karpe et al. flotation, n = 24 plasmas	38	0.26 ± 0.14	3.32 ± 0.63	1.21 ± 0.22		4.77 ± 0.38
Triglycerides						
Rapid flotation, n = 6 plasmas		0.68 ± 0.46	0.13 ± 0.06	0.08 ± 0.02		0.89 ± 0.51
Brousseau et al. flotation, n = 10 sera	7 VLDL	0.41 ± 0.32	0.21 ± 0.07	0.10 ± 0.07	0.02 ± 0.01	0.91 ± 0.51
	IDL	0.17 ± 0.08				
		0.58				
Karpe et al. flotation, n = 24 plasmas	38	0.67 ± 0.34	0.27 ± 0.07	0.10 ± 0.03		1.05 ± 0.06

Data represent mean mm ± SD.
[a] Density > 1.063 g/ml.

Concerning accuracy, the rapid flotation procedure produced lipoprotein fractions with cholesterol concentrations comparable to values published in the Havel et al. study, as well as to those in more recent flotation procedures (**Table 5**). The summed 97% total recovery fell within the 92–101% range observed for these three studies. Moreover, the 59% LDL and 31% HDL values were almost identical to the Havel et al. values, and within the 49–70% and 25.4–33% range observed for healthy French and Swedish volunteers (7, 38). Similar to our findings with the Havel et al. procedure, Brousseau et al. (7) also reported that 4.4% of the total cholesterol was present in a fraction with density greater than 1.2 g/ml. The 10% cholesterol in rapidly floated VLDL/IDL was close to the corresponding Havel et al. 13% value, and between the 5.5 –12.9% range reported in the two other studies. Thus, based on cholesterol determinations, the rapid flotation procedure produced reasonable estimates of lipoprotein concentrations.

The rapid flotation procedure also generated lipoprotein fractions with triglyceride concentrations comparable to recent published values (Table 5; triglyceride concentrations were not determined in the original Havel et al. study). A summed triglyceride recovery of 100% by the rapid flotation procedure was within the 94–102% range observed for the two recent studies. Moreover, the 9.0% HDL and 76% VLDL/IDL were comparable to the 9.5–11.0% and 64% values observed for samples from healthy French and Swedish volunteers (7, 38). However, the 15% LDL isolated by the rapid flotation procedure was barely more than half the 23–26% observed for European and Swedish volunteers (7, 38). Thus, with the possible exception of the LDL fraction, the rapid flotation procedure yielded lipoprotein fractions with reasonable triglyceride distributions.

In conclusion, the lipoprotein profiles derived by rapid flotations were very similar qualitatively and quantitatively to those generated by the Havel et al. procedure (1). Thus, by use of a simple benchtop centrifuge, lipoproteins from up to 10.0 ml of plasma (or ten 1.0-ml plasma samples) could be isolated in 1 day for chemical characterization and biological testing. Because lipoprotein autooxidation and redistributions during processing were not assessed in the present study, future studies are needed to determine the extent to which rapid flotations at low temperatures reduce autooxidation, and whether specific lipid classes, such as phospholipids with high levels of polyunsaturated fatty acids, are protected.

This work was partially supported by an American Heart Association grant, GIA 96012380, and by NIH grants HL-48877, HL-49264, and DK-07663. The authors also wish to acknowledge the helpful advice of David Chappel (University of Iowa) regarding the qualitative analyses. We would further like to acknowledge the College of Medicine Gas Chromatography–Mass Spectrometry Facility for their gracious help.

Manuscript received 24 December 1997 and in revised form 23 March 1998.

REFERENCES

1. 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.
2. Schaefer, E. J., S. Eisenberg, and R. I. Levy. 1978. Lipoprotein apoprotein metabolism. *J. Lipid Res.* **19:** 667–687.
3. Kunitake, S. T., and J. P. Kane. 1982. Factors affecting the integrity of high density lipoprotein in the ultracentrifuge. *J. Lipid Res.* **23:** 936–940.
4. Castro, G. R., and C. J. Fielding. 1984. Evidence for the distribution of apolipoprotein E between lipoprotein classes in human normocholesterolemic plasma and for the origin of unassociated apolipoprotein E (LpE). *J. Lipid Res.* **25:** 58–67.
5. Brooks, C. J. W., R. M. Mckenna, W. J. Cole, J. MacLachlan, and T. D. V. Lawrie. 1983. Profile analysis of oxygenated sterols in plasma and serum. *Biochem. Soc. Trans.* **11:** 700–708.
6. Ray, B. R., E. O. Davidson, and H. L. Crespi. 1954. Experiment on the degradation of lipoproteins from serum. *J. Phys. Chem.* **58:** 841–846.
7. Brousseau, T., V. Clavey, J-M. Bard, and J-C. Fruchart. 1993. Sequential ultracentrifugation micromethod for separation of serum lipoproteins and assay of lipids, apolipoproteins, and lipoprotein particles. *Clin. Chem.* **39:** 960–964.
8. Leonhardt, W., J. Pietzsch, U. Julius, and M. Hanefield. 1994. Recovery of cholesterol and triacylglycerol in very-fast ultracentrifugation of human lipoproteins in a large range of concentrations. *Eur. J. Clin. Chem. Clin. Biochem.* **32:** 929–933.
9. Cathcart, S., and M. H. Dominiczak. 1990. The measurement of

lipoprotein subfractions in plasma using a tabletop ultracentrifuge. *Ann. Clin. Biochem.* **27:** 459–464.
10. Fletcher, C. D., J. F. Barnes, and E. Farish. 1994. A rapid semimicro method for the separation of lipoprotein fractions that uses a benchtop ultracentrifuge. *Clin. Chim. Acta.* **226:** 95–99.
11. Mills G. L., P. A. Lane, and P. K. Weech. 1984. The isolation and purification of plasma lipoproteins. *In* Laboratory Techniques in Biochemistry and Molecular Biology. R. H. Burdon and P. H. van Knippenberg, editors. Elsevier, New York, NY. 18–116.
12. Spiteller, G. 1993. On the chemistry of oxidative stress. *J. Lipid Mediat.* **7:** 199–221.
13. Lenz, L., H. Hughes, and J. L. Mitchell. 1990. Lipid hydroperoxy and hydroxy derivatives in copper-catalyzed oxidation of low density lipoprotein. *J. Lipid Res.* **31:** 1043–1050.
14. Smith, W. L. 1989. The eicosanoids and their biochemical mechanisms of action. *Biochem. J.* **259:** 315–324.
15. Smith, L. L. 1987. Cholesterol autooxidation 1981–1986. *Chem. Phys. Lipids.* **44:** 87–125.
16. Smith, L. L. 1996. Review of progress in sterol oxidations: 1987–1995. *Lipids.* **31:** 453–487.
17. Haberland, M. E., M. A. Fogelman, and P. A. Edwards. 1982. Specificity of receptor-mediated recognition of malondialdehyde-modified low density lipoproteins. *Proc. Natl. Acad. Sci. USA.* **79:** 1712–1716.
18. Haberland, M. E., D. Fong, and L. Cheng. 1988. Malondialdehyde-altered protein occurs in atheroma of Watanabe heritable hyperlipidemic rabbits. *Science.* **241:** 215–218.
19. Hwang, L. P. 1991. Biological activities of oxygenated sterols: physiological and pathological implications. *Bioessays.* **13:** 583–589.
20. Knapp, H. 1991. Diols of arachidonic (AA) and eicosapentaenoic acid (EPA) acids esterified in human biliary lecithins. *Clin. Res.* **39:** 717A.
21. Koopman, B. J., J. C. Vandermolen, and B. C. Wolthers. 1987. Determination of some hydroxycholesterols in human serum samples. *J. Chromatogr.* **416:** 1–11.
22. Hodis, H. N., D. W. Crawford, and A. Sevenian. 1991. Cholesterol feeding increases plasma and aortic tissue cholesterol oxide levels in parallel: further evidence for the role of cholesterol oxidation in atherosclerosis. *Atherosclerosis.* **89:** 117–126.
23. Sevanian, A., R. Seraglia, P. Trald, F. Rossato, F. Ursini, and H. Hodis. 1994. Analysis of plasma cholesterol oxidation products using gas- and high-performance liquid chromatography / mass spectrometry. *Free Radical Biol. Med.* **17:** 397–409.
24. Kleinveld, H. A., H. M. Hak-Lemmers, A. H. Stalenhoef, and P. M. Demacker. 1992. Improved measurement of low-density-lipoprotein susceptibility to copper-induced oxidation: application of a short procedure for isolating low-density lipoprotein. *Clin. Chem.* **38:** 2066–2072.
25. Edelstein, C., and A. M. Scanu. 1986. Precautionary measures for collecting blood destined for lipoprotein isolation. *Methods Enzymol.* **128:** 151–155.
26. Bollage, D. M., and S. J. Edelstein. 1991. Protein Methods. Wiley-Liss, New York, NY. 95–142.
27. Blanchard, R. F., S. D. Blas, and P. J. Davis. 1978. Effect of NaI on protein determination by the Lowry method and by absorption spectroscopy. *Anal. Biochem.* **87:** 521–526.
28. Lees, M. B., and S. Paxman. 1972. Modification of the Lowry procedure for the analysis of proteolipid protein. *Anal. Biochem.* **47:** 184–192.
29. Klansek, J. J., P. Yancey, R. W. St. Clair, R. T. Fischer, W. J. Johnson, and J. M. Glick. 1995. Cholesterol quantitation by GLC: artifactual formation of short-chain steryl esters. *J. Lipid Res.* **36:** 2261–2266.
30. Allain, C. C., L. S. Poon, C. S. G. Chan, W. Richmond, and P. C. Fu. 1974. Enzymatic determination of total cholesterol. *Clin. Chem.* **20:** 470–475.
31. Kohlmeier, M. 1986. Direct enzymic measurement of glycerides in serum and in lipoprotein fractions. *Clin. Chem.* **32:** 63–66.
32. Schumaker, V. N., and D. L. Puppione. 1986. Sequential flotation ultracentrifugation. *Methods Enzymol.* **128:** 155–170.
33. Steinberg, D., S. Parthasarathy, T. E. Carew, J. C. Khoo, and J. L. Witztum. 1989. Beyond cholesterol: modifications of low-density lipoprotein that increase its atherogenicity. *N. Engl. J. Med.* **320:** 915–924.
34. Steinbrecher, U. P., H. Zhang, and M. Longheed. 1990. Role of oxidatively modified LDL in atherosclerosis. *Free Radical Biol. Med.* **9:** 155–168.
35. Krilov, D. 1991. Spin traps initiate free radical formation in unsaturated fatty acids. *Acta Pharmacol. Jugosl.* **41:** 345–350.
36. Krilov, D. 1995. Formation of free radicals in oxidation of human low-density lipoprotein. An EPR spin trapping study. *Crotica Chem. Acta.* **68:** 409–415.
37. Leonhardt, W., J. Pietzsch, and S. Nitzsche. 1994. Very-fast ultracentrifugation of human plasma lipoproteins: influence of the centrifugal field on lipoprotein composition. *Clin. Chim. Acta.* **224:** 21–31.
38. Karpe, F., M. Bell, J. Bjorkegren, and A. Hamsten. 1995. Quantification of postprandial triglyceride-rich lipoproteins in healthy men by retinyl ester labeling, and simultaneous measurement of apolipoproteins B-48 and B-100. *Arterioscler. Thromb. Vasc. Biol.* **15:** 199–207.