

Fractionation of human serum lipoproteins by single-spin gradient ultracentrifugation: quantification of apolipoproteins B and A-I and lipid components

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Abstract A sensitive and reproducible method has been developed for separation of the major serum lipoproteins from 1 ml or less of human serum by isopycnic density gradient ultracentrifugation. The serum, applied to a step gradient (total volume 12.8 ml), was spun for 48 hr at 38,000 rpm at 10°C and, in each of the fractions, apolipoproteins B and A-I were quantified by the respective radioimmunoassays. The markers for lipid distribution used were [4-¹⁴C]cholesterol and [U-¹⁴C]lecithin, each incubated with an aliquot of serum at 20°C for 75 min prior to ultracentrifugation. In control sera, three main fractions, very low density (VLDL), low density (LDL), and high density (HDL) lipoproteins were clearly separated from a bottom fraction. Their flotation, electrophoretic, and chemical properties were in good agreement with those reported for the corresponding lipoproteins separated by conventional ultracentrifugation. Both apo B and apo A-I were fully recovered. Essentially all of the apo B was found in VLDL (9.3 ± 3.5%) and LDL (87 ± 4.6%); of the apo A-I, 81.0 ± 5.7% was in HDL and the remainder (17.0 ± 5.8%) was in the bottom fraction. The peak activities of [¹⁴C]cholesterol coincided with the peak of apo B in both LDL and VLDL, and with the peak of apo A-I in HDL. The results with the radiolabeled cholesterol were in good agreement with those obtained by chemical analyses. Carbon 14-labeled lecithin, although fully recovered, was not an accurate marker of phospholipid distribution because, under our experimental conditions, a significant amount of the lecithin was converted into its lyso derivative. The mechanism of the conversion was not established; it appeared to be unrelated to the activities of either lecithin-cholesterol acyl transferase or a Ca²⁺-dependent phospholipase. Besides its validity in the study of control sera, our method also proved successful in the separation of the serum lipoproteins of the few patients with dyslipoproteinemia (abetalipoproteinemia and familial hypercholesterolemia) who were examined. However, the applicability of the method to all dyslipoproteinemias was not assessed. Taken together, the results indicate that the single-spin method could be useful in clinical studies as a complement to other established techniques.

Supplementary key words isopycnic density gradient ultracentrifugation · radioimmunoassay · abetalipoproteinemia · familial hypercholesterolemia

An accurate description of the distribution and quantitation of all of the serum lipoproteins requires a method that ensures complete recoveries, separates the major lipoprotein classes satisfactorily, and provides sufficient yields to permit analyses of the lipoprotein constituents. These requirements are not easily satisfied by the commonly adopted sequential flotation techniques in angle-head rotors (1). One reason is that some lipoproteins or their components may be lost during the series of preparative steps. Secondly, these techniques rely on conventional density limits for solutions or solvents that, although adequate for normolipidemic sera, may not necessarily apply to hyperlipidemic states. Density gradient ultracentrifugation in swinging-bucket rotors has been applied to the study of serum lipoproteins and to the subfractionation of major lipoprotein classes (2-4). However, this method has received comparatively little attention as a means for quantita-

Abbreviations: HDL, high density lipoproteins; LDL, low density lipoproteins; VLDL, very low density lipoproteins; BF, bottom fraction; ABL, abetalipoproteinemia; LCAT, lecithin-cholesterol acyltransferase; Apo A-I, the major apoprotein of HDL; Apo A-II, the minor apoprotein of HDL; Apo B, major apoprotein of LDL; SDS, sodium dodecylsulfate; EDTA, ethylene diamine tetraacetic acid; DTNB, (5,5'-dithiobis [2-nitrobenzoic acid]); PAGE, polyacrylamide gel electrophoresis; HDL₂, HDL of d 1.063-1.125 g/ml; HDL₃, HDL of d 1.125-1.21 g/ml.

¹ Operated by the University of Chicago for the Energy Research and Development Administration under Contract EY-76-C-02-0069.

tive separation of lipoproteins from whole serum. As an outgrowth of the germinal studies by Oncley, Walton, and Cornwell (5), Light and Gurd (6) gave a preliminary account of the separation of serum lipoproteins in a single nonlinear gradient by use of an angle-head rotor. The staining of the lipoproteins made possible quantitative measurements by densitometric scanning. While the present work was being completed, Redgrave, Roberts, and West (7), using a high-speed swinging-bucket rotor, reported on the separation of serum very low density lipoproteins (VLDL), low density lipoproteins (LDL), and high density lipoproteins (HDL) by a single nonlinear density gradient. These lipoprotein classes were characterized by cellulose acetate electrophoresis and immunoelectrophoresis and by the distribution of cholesterol. No studies were reported on the apolipoproteins.

A description of both the distribution and the quantitative recovery of the apolipoproteins and lipids in biological fluids would be valuable for studies involving the physiological functions of these lipoproteins. To this end, we have developed a sensitive method which uses 1 ml of serum, or even less, and separates VLDL, LDL, and HDL from the other serum proteins. The isopycnic banding of the lipoproteins is achieved by a nonlinear gradient, formed after a single ultracentrifugation in the swinging-bucket rotor. Apolipoproteins B and A-I are then assayed by their respective radioimmunoassays; for cholesterol, radioactive isotope techniques are used. In this report, we describe the development and validity of this technique and its application to the separation of lipoproteins in normolipidemic and in some dyslipoproteinemic sera. A preliminary account of this work has appeared (8).

MATERIALS AND METHODS

Source of sera

The studies were carried out on fresh sera which were obtained from five fasting, healthy, normolipidemic individuals [four males and one female, with cholesterol ($m \pm SD$) = 177 ± 44 mg/dl and triglycerides = 69 ± 42 mg/dl]. Aliquots of sera from one male and from the female subject were stored at 4°C and -20°C for three weeks before use.

Plasma samples from two previously described (9) patients (A.M.V. and M.S.) with abetalipoproteinemia (ABL) were kept at 4°C for 6 days prior to application to the gradient. The serum from a patient homozygous for familial type II hyperlipoproteinemia was stored at 4°C for 4 days before use.

Incubation of serum with radioactively labeled lipids

Each serum sample was divided into two 1-ml aliquots. Before centrifugation, one aliquot was incubated for between 10 and 75 min with either 10 μ l/ml of [7-³H]cholesterol (sp act 9.4 Ci/mmol) or 50 μ l/ml of [4-¹⁴C]cholesterol (sp act 53.7 mCi/mmol) (Amersham-Searle). The [¹⁴C]cholesterol was preferred because of its higher counting efficiency. The second aliquot of serum was incubated with 50 μ l/ml of [U-¹⁴C]lecithin (sp act 1.765 Ci/mmol) (Nuclear Chicago). The labeled lipids, dissolved in benzene, were first dried by blowing with purified nitrogen. The residue was promptly redissolved in 0.1 ml of 95% ethanol before incubation with serum. The purity of the labeled lecithin was checked by thin-layer chromatography (10). Incubation for 75 min at 20°C insured maximal incorporation (95%) of the radioactive lipid into the lipoprotein complexes, as shown by the fact that longer incubation times did not change the results.

In some experiments, the incubation was carried out after inactivation of lecithin-cholesterol acyl transferase (LCAT), either by heating the serum at 56°C for 30 min or by the addition of DTNB to the systems (11). To prevent phospholipase A₂ activity, 1% EDTA was added to the serum (see Results) (12).

Application of serum to gradient and collection of fractions

A discontinuous NaCl density gradient was formed in a 12.8-ml cellulose nitrate tube, 9/16" \times 3-3/4" (Spinco #331101), containing 500 mg of sucrose on the bottom. The gradient was obtained by layering, from bottom to top, 5 ml of 4 M NaCl, 1 ml of serum, and 6.8 ml of a 0.67 M NaCl solution containing 0.05% EDTA, pH 7.0. Each tube was immediately placed in a Spinco titanium swinging-bucket rotor, Model SW40 (total capacity, 6 tubes) and spun in a Spinco Model L2-65B ultracentrifuge at 38,000 rpm for 48 hr at 10°C using no brake at the end of the run. In the initial phase of the study, ultracentrifugation for 48 hr was found to be important to ensure isopycnic banding, since prolongation of the run did not influence the lipoprotein distribution. At the end of each run, the top 0.2 ml, containing VLDL and, if appropriate, other very low density fractions, was removed by a micropipette to prevent specimen losses. Each tube was then punctured at the bottom and its contents were pumped out, by a continuous ascending flow, with a Sage Syringe Pump (Model 351, Orion Research Inc., Cambridge, MA) adjusted to a speed of 1 ml/min. Twenty-four fractions, 15 drops each, were collected with a Gilson

microfractionator (Model FC-8OH, Gilson Medical Electronics, Inc., Middleton, Wi).

Analysis of the fractions

1) *Quantitation and/or distribution of the radioactive cholesterol and phospholipids.* In the initial phase of our studies, attempts were made to quantify the cholesterol and phospholipids in each gradient fraction by chemical methods. The available techniques, although applicable to the peak areas, were found to be insufficiently sensitive to detect the small amounts of lipids present in the other fractions. In the case of cholesterol, however, satisfactory results were obtained by counting the various gradient fractions of serum samples that had been pre-incubated with the radiolabeled sterol. One-hundred microliter aliquots of whole sera and of each fraction were diluted 1:4 with distilled water, and counted in 10 ml of Instagel (Packard, cat. #6002174) in a Packard Tri-Carb liquid scintillation unit (Model 3002, Packard Industries, Downers Grove, IL). Diluting of the fractions with distilled water totally eliminated quenching due to sucrose and salts in the gradient. Recoveries were determined by measuring the total radioactivity in all fractions and comparing it with the radioactivity in the whole serum. The percent radioactivity in each fraction was expressed as a percentage of the total counts in the whole gradient.

In the case of phospholipids, labeled lecithin was found to be unsatisfactory for quantification, but proved to be a useful marker for the localization of the lipoprotein peaks (see Results). The [^{14}C]lecithin present in each fraction was counted and calculated according to the methods described for the radiolabeled cholesterol. Further analysis of the [^{14}C]lecithin in the lipoprotein peaks and bottom fractions was carried out by thin-layer chromatography (Baker-flex Silica gel 1B2, J. T. Baker Chemical Co., Phillipsburg, NJ), with chloroform-methanol-acetic acid-water 25:15:4:2 as the developing solvent. Before this step, the bottom fractions BF (tubes 2-4), HDL (tubes 8-10), LDL (tubes 14-16), and the whole serum were dialyzed against 0.9% saline and delipidated in ethanol-ether 3:2 (13). The lipids were dried under nitrogen, dissolved in 0.2 ml of chloroform-methanol 9:1, and applied to the thin-layer plates; unlabeled lecithin and lysolecithin were used as standards. The lysolecithin was prepared in the laboratory by cleavage of HDL lipid by phospholipase A_2 (14). The areas corresponding to the lipids, identified by I_2 vapors (10), were cut out, suspended in Instagel, and counted. This procedure gave over 95% recovery of the radioactivity applied to the plate.

2) *Quantitation and distribution of apolipoproteins.*

The apolipoproteins B and A-I in each fraction were measured by radioimmunoassay² (15). Briefly, aliquots of each fraction were appropriately diluted in buffer (0.133 M borate, pH 8, 0.5% bovine serum albumin (BSA), and 0.05% EDTA). The diluted samples were used directly in the apo B assay,² but those for the apo A-I radioimmunoassay were heated at 52°C for 3 hr prior to being measured (15). For each dilution, four different aliquots, covering an 8- to 10-fold range, were assayed in duplicate. This ensured that a minimum of two dilutions would lie in the optimal range of the assay. In addition, the parallelism of the apolipoprotein in each fraction could be compared to the standard.

We determined the recovery of the apolipoproteins by comparing the sum of the apolipoprotein in the fractions to that in the unfractionated serum. The distribution of the apolipoprotein in the different lipoprotein classes was calculated by comparison of the sum of the apolipoprotein from the respective fractions to the total amount recovered in the gradient.

Other techniques

1) *Determination of the densities of the fractions.* Density measurements were carried out in a Digital precision density meter (Model DMA02C, Anton Paar K.G., A-8054, Graz, Austria) on each fraction at 20°C. A nonlinear gradient was obtained between the densities of 1.246 g/ml (bottom) and 1.018 g/ml (top), Fig. 3. The densities of the fractions from control gradients without serum and of those with the experimental samples were comparable and were found to be highly reproducible.

2) *Characterization of the lipoproteins.* The lipoproteins separated by density gradient ultracentrifugation were subjected to physical and chemical studies so that their properties could be compared with those obtained by sequential flotation according to the method of Havel, Eder, and Bragdon (16). Electrophoresis of the intact lipoproteins on 1% agarose³ and polyacrylamide gel electrophoresis in 1% SDS were carried out as described previously (17). In addition, 8 M urea polyacrylamide gel electrophoresis was conducted on samples pretreated with tetramethylurea (18). Flotational studies were carried out in a Beckman Model E analytical ultracentrifuge at 25°C and 44,000 rpm, with Schlieren optics and double-sector cells. Before analysis, the specimens

² Karlin, J. B., D. J. Juhn, A. M. Scanu, and A. H. Rubenstein. 1977. Radioimmunoassay of human apolipoprotein B. Submitted to *Eur. J. Clin. Invest.*

³ Agarose electrophoresis was performed according to the directions of the manufacturer with an ACI Agarose Film/Cassette System (Corning, Palo Alto, CA 94303).

TABLE 1. Properties of the lipoproteins isolated by single-spin ultracentrifugation from normolipidemic sera

Frac-tions	Densities	Desig-nations	Flotation Coefficients ^a		Apparent Hydrated Density	Chemical Properties ^{a,b}			PAGE in SDS and 8 M Urea	Apoprotein Distribution		Cholesterol Distribution	
			$S_{(1.068)}$	$S_{(1.21)}$		Protein	Total Cholesterol	Phospho-lipids		Triglyc-erides	Apo A-I ^c		Apo B ^d
	g/ml				g/ml (mean ± SD)	%	%	%		% of total (mean ± SD)			
22-24	<1.020	VLDL	N.D.	N.D.	N.D.	9.9	16.0	29.5	44.7	N.D.	9.3 ± 3.5	11.0 ± 2.7	4.9
13-19	1.026-1.060	LDL	6.2		1.039 ± .002	29.1	38.9	24.8	3.2	Apo B	1.6 ± 0.4	83.6 ± 4.6	54.9
6-12	1.069-1.120	HDL		1.59	1.101 ± .010	51.5	15.7	27.5	1.8	Apo A-I, A-II, and C-peptides	81.0 ± 5.7	1.4 ± 0.9	26.0 ± 2.8
1-5	1.130-1.246	BF	N.D.	N.D.	N.D.						17.0 ± 5.8	5.0 ± 0.6	6.0

N.D., Not determined due to the small amounts of lipoprotein available.

^a Determined on fractions 15-17 (LDL) and 7-9 (HDL).

^b Determined on pooled specimens from six fractions.

^c Average data from gradients of six normolipidemic males.

^d Average data from gradients of eight normolipidemic males.

^e The radioactively labeled lipids were incubated with serum for 75 min at 20°C. Since the results with [³H] and [¹⁴C]-cholesterol were identical, the results from a total of five gradients were averaged.

^f Cholesterol values determined by chemical analysis (see Methods).

were dialyzed against the desired solvent densities, d 1.21 g/ml for HDL and d 1.063 g/ml for LDL. The final dialysate was used as a blank. Flotation coefficients were calculated as previously described (19). For each lipoprotein, total protein (20), total cholesterol (21), total phospholipids (22), and triglycerides (23) were determined.

RESULTS

Normolipidemic sera

After centrifugation for 48 hr at 38,000 rpm, the step gradient containing the 1 ml serum sample was equilibrated into a smooth nonlinear gradient ranging in density from 1.018 to 1.246 g/ml (Fig. 3, top). In the top of the tube (fractions 22–24), there was a milky white band of d 1.018 g/ml, with the chemical properties of VLDL (Table 1). A second, orange-yellow band, present approximately in the middle of the tube (fractions 13–19), banded between d 1.026 and 1.060 g/ml in the region of LDL and had the apparent hydrated density of 1.039 ± 0.002 g/ml and the chemical composition reported for this lipoprotein (Table 1). A third, clear yellow band, located near the bottom of the tube (fractions 6–12), banded between d 1.069 and 1.130 g/ml and had the apparent hydrated density of 1.101 ± 0.010 g/ml and the chemical properties of HDL (Table 1). The last fraction (1–5), to which we refer as bottom fractions (BF), contained the other serum proteins as well as some apo A-I and some lipid (see below).

The electrophoretic migration in 1% agarose gel for VLDL, LDL, and HDL obtained by the single-spin procedure compared favorably with that of the

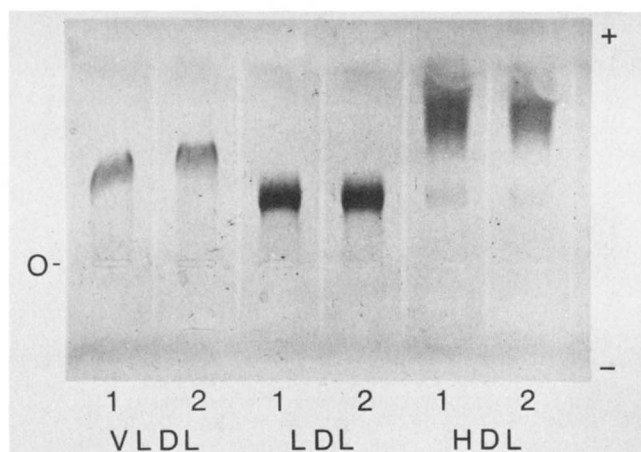


Fig. 1. Electrophoretic patterns of VLDL, LDL, and HDL on 1% Agarose. 1, Lipoproteins separated by the single-spin method; 2, lipoproteins separated by conventional preparative ultracentrifugation.

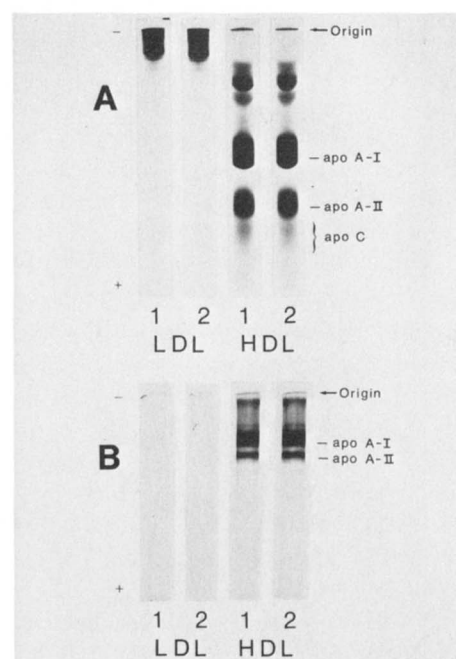


Fig. 2. A, Polyacrylamide gel electrophoresis (10% acrylamide), in 0.1% SDS, of LDL and HDL. B, Polyacrylamide gel electrophoresis (7.5% acrylamide, pretreated with tetramethylurea), in 8 M urea, of LDL and HDL. Amount of protein applied to each gel: 75 μ g. 1, Lipoproteins separated by the single-spin method; 2, lipoproteins separated by conventional preparative ultracentrifugation.

corresponding density classes separated by sequential flotation (Fig. 1). The VLDL obtained from the single-spin method was not studied further because too little material was available. The polypeptide distribution, as assessed by SDS- and 8 M urea-polyacrylamide gel electrophoresis, for both the LDL and HDL isolated by the single-spin method gave profiles similar to those of the respective lipoproteins isolated by sequential flotation (Figs. 2A and 2B; Table 1). The peak fractions of LDL and HDL had values of $S_{f(1.063)} = 6.2$ and $S_{f(1.21)} = 1.59$, respectively (Table 1).

The validity of the separation of the major classes of lipoproteins by the single-spin technique was also supported by the distribution of apo B and apo A-I in the fractions (Fig. 3 and Table 1). The recovery of apo B was $92 \pm 3\%$. Essentially all of the apo B, $9.3 \pm 3.5\%$ and $87 \pm 4.6\%$, was found in VLDL and LDL, respectively (Table 1). HDL contained $1.4 \pm 0.9\%$ apo B; this was mainly due to the apo B in fraction 12 (Fig. 3). Fractions 1–11, comprising HDL and BF, contained less than 0.2% of total apo B (Fig. 3).

The recovery of apo A-I from fractionated normolipidemic sera was $98 \pm 12\%$. The apo A-I was distributed in a symmetrical peak, with HDL containing $81.0 \pm 5.7\%$ of the total apo A-I (fractions 6–12, Fig. 3, Table 1). The remaining apo A-I ($17 \pm 5.8\%$)

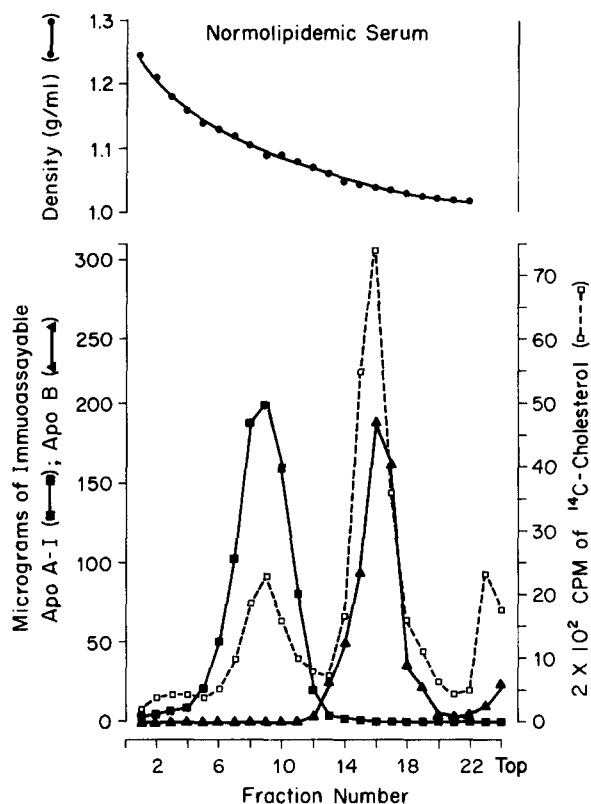


Fig. 3. Distribution of immunoassayable apo A-I (■—■), apo B (▲—▲), and [^{14}C]cholesterol (□---□) in the fractions from 1 ml of serum from a normolipidemic human male separated by the single-spin method. The density (g/ml) of each fraction is plotted on the upper scale (●—●).

was present in fractions 1–5 which comprised the BF (Table 1). Less than 0.1% of the apo A-I was in fractions 20–24; the small amount ($1.6 \pm 0.4\%$) in the LDL region was due to the apo A-I in fractions 13 and 14 (Table 1 and Fig. 3).

In the normolipidemic sera, the distribution of the [^{14}C]- or [^3H]cholesterol coincided with the apo B peaks of VLDL and LDL and with the apo A-I peak of HDL (Fig. 3). The percentages of [^{14}C]- or [^3H]cholesterol in VLDL, LDL, HDL, and BF were 11 ± 2.7 , 56 ± 2.2 , 26 ± 2.8 , and $5.0 \pm 0.6\%$, respectively (Table 1). The percent of radiolabeled cholesterol in LDL and HDL was in good agreement with the distribution of the total unlabeled cholesterol that was measured in the pooled fractions of LDL and HDL from six gradients (total unlabeled cholesterol in LDL, 54.9%; in HDL, 33.3%).

The recoveries and distribution of apo B, apo A-I, and [^{14}C]cholesterol which were observed in the two fresh normolipidemic sera were indistinguishable from those observed in the same sera stored for 3 weeks at 4°C or -20°C .

In sera incubated at 20°C with [^{14}C]lecithin for 20–75 min and then subjected to single-spin ultra-

centrifugation, the radioactivity was found in VLDL ($4.6 \pm 1.6\%$), LDL ($16 \pm 0.7\%$), HDL ($31 \pm 0.6\%$), and BF ($47 \pm 1.5\%$). However, the measured radioactivity in the various fractions did not correspond with the chemical determinations of the total phospholipids; only the latter results closely reflected the lipid distribution reported for serum lipoproteins. In a preliminary investigation, we found that (1) the high peak of radioactivity observed in the BF ([^{14}C]lecithin incubated with serum at 20°C , 75 min, ratio 1:20, v:v) comprised about 67% lecithin and 33% lysolecithin; (2) essentially all of the radioactivity in LDL and HDL (91.4% and 94.4%, respectively) was due to [^{14}C]lecithin; (3) the generation of lysolecithin could not be prevented by heating the serum at 56°C for 30 min, or by the addition of DTNB, or by 1% EDTA.

Dyslipoproteinemic sera

Immunoreactive apo B was not detected in fasting sera from the two subjects with ABL (M.S. and A.M.V.). The apo A-I concentrations, 40 mg/dl and 32 mg/dl in M.S. and A.M.V., respectively, were significantly reduced compared to control values. Both sera were subjected to single-spin ultracentrifugation, and the recovery of apo A-I was 91%. In comparison to the symmetrical apo A-I distribution in normolipidemic sera (Fig. 3), apo A-I in ABL sera exhibited a distinctive and reproducible profile (Fig. 4), characterized by its broad distribution and by two overlapping peaks in the HDL and BF regions (Fig. 4). The majority (84%) of the [^{14}C]cholesterol was found in the HDL area, and 12%

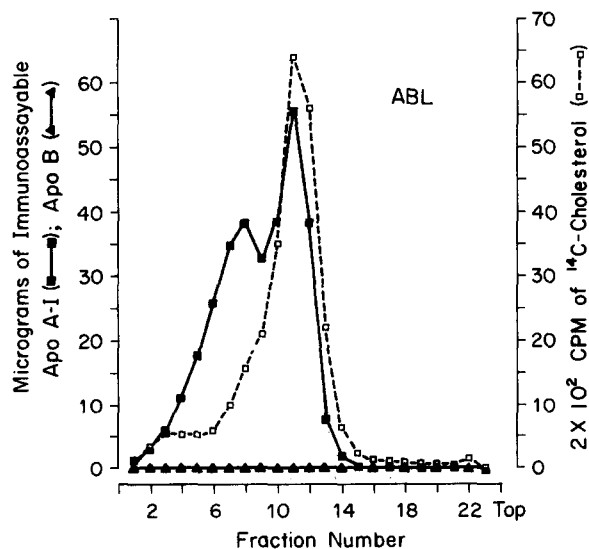


Fig. 4. Distribution of immunoassayable apo A-I (■—■), apo B (▲—▲), and [^{14}C]cholesterol (□---□) in the fractions from 1 ml of serum from an ABL human male (M.S.) separated by the single-spin method.

was in BF (Fig. 4). No apo B, apo A-I, or [^{14}C]cholesterol was found in the LDL and VLDL regions (Fig. 4).

The total serum apo B concentrations (333 mg/dl) was elevated threefold in the subject with familial type II hyperlipoproteinemia, total serum apo A-I was 81 mg/dl. Both apo B and apo A-I were fully recovered after ultracentrifugation (90 and 102%, respectively). The apo B peak of LDL was sharp, symmetrical, and banded in the same region as normolipidemic LDL (Fig. 5 versus Fig. 3). Ninety-three percent of the apo B was in the LDL and 4% in the VLDL region. In contrast to the normolipidemic sera, the apo A-I in the HDL region was more broadly distributed (Fig. 3 versus Fig. 5): 68% was in HDL, and 29% formed a shoulder extending into the BF region (Fig. 5).

[^{14}C]Cholesterol was fully recovered (95% of the total). The distribution of [^{14}C]cholesterol paralleled that of apo B: 79% in LDL and 10% in VLDL (Fig. 5). However, 8% of the total [^{14}C]cholesterol appeared in the HDL region and had a profile that was distinct from that of apo A-I (Fig. 5). Time of incubation did not change the results.

DISCUSSION

The present studies have demonstrated that the separation of the major serum lipoproteins from a small volume of serum by isopycnic density gradient ultracentrifugation is convenient and reproducible. The flotation, electrophoretic, and chemical properties of the major serum lipoproteins separated by this single-spin ultracentrifugation method are in good agreement with those reported for the corresponding lipoproteins separated by conventional ultracentrifugation procedures. This method, which can be applied to as little as 1 ml of serum, is similar to that described by Redgrave (7), but has the additional advantages of providing quantitative distributions of apolipoproteins B and A-I in the individual fractions by radioimmunoassay techniques, and of increasing the sensitivity of the cholesterol determination by the use of [^{14}C]cholesterol as an effective marker.

The discontinuous gradient is simple to prepare, and the preparative and analytical procedures that follow are easily carried out. Although time and care are required in the appropriate dilution of each fraction for the radioimmunoassays, the use of automated pipettes and the calculation of the results by computer programs are efficient. Some technical details deserve special emphasis. First, the sera can

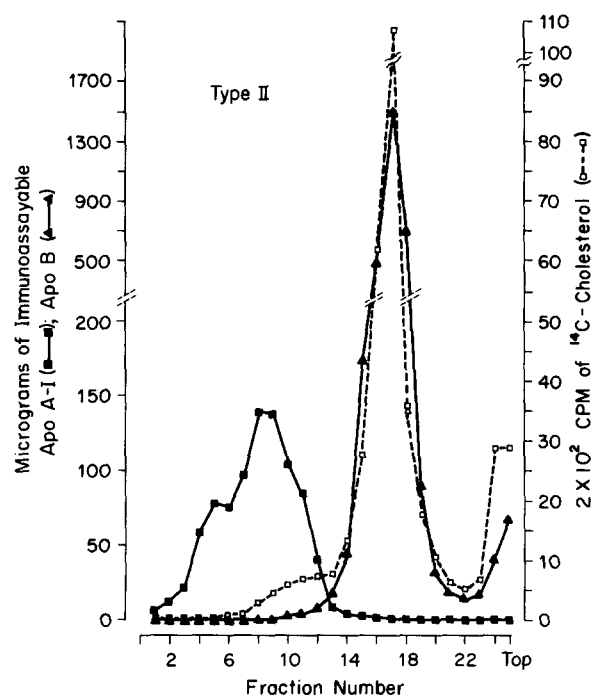


Fig. 5. Distribution of immunoassayable apo A-I (■—■), apo B (▲—▲), and [^{14}C]cholesterol (□---□) in the fractions from 1 ml of serum from a human female homozygous for familial hypercholesterolemia separated by the single-spin method.

be stored between -20°C and 4°C without any effect on the results. It should be noted, however, that the immunoreactivity of apo B diminishes upon storage of the fractions obtained from single-spin ultracentrifugation (unpublished observations); therefore, the assay of apo B must be done within 48 hr after these fractions are collected. Second, in the apo A-I radioimmunoassay, and in agreement with a previous study (15), pre-heating of the fractions is necessary for maximal exposure of the immunoreactivity of this apoprotein, and for its full recovery. Third, the use of sucrose permits a sharper resolution of the various bands and greater stability of the gradient (6). Finally, the discontinuous gradient which we developed does not provide for the separation of VLDL from chylomicrons, a fact that must be taken into consideration when hypertriglyceridemic sera are under study.

An unexpected difficulty in our work came from the use of [^{14}C]lecithin as a marker for phospholipid distribution. The distribution of the radioactivity did not coincide with the chemical data, and a significant portion of the radiolabeled lecithin was converted into its lysoderivative. According to our preliminary results, the latter process may not be attributable either to the action of lecithin-cholesterol acyl transferase (lack of inactivation by heat and DTNB) or to a Ca^{2+} -dependent phospholipase (activity not prevented by

1% EDTA). A more detailed examination of the lecithin-lysolecithin conversion is needed before firm conclusions can be drawn; studies are now in progress in our laboratory. In spite of these uncertainties, the current results are of interest because they show that, in our system, the radioactive lecithin is distributed among the major lipoprotein classes, and that essentially all of the lysolecithin generated is confined to the bottom fraction in a still undefined physical state.


The demonstration of the presence of [^{14}C]lysolecithin in the bottom fraction is in agreement with the studies of Phillips and Wille (24), who showed that lipoproteins separated by either agarose gel electrophoresis or ultracentrifugation contain a significant amount of lysolecithin in the pre-albumin region or the $d > 1.21$ g/ml fraction, respectively. On the other hand, they detected very little lysolecithin in VLDL, LDL, or HDL. These results were taken to suggest that the presence of lysolecithin in the $d > 1.21$ g/ml fraction is not an artifact of ultracentrifugation (24), a conclusion that appears supported by our data.

In contrast to the labeled lecithin, radioactive cholesterol was found to equilibrate readily among all of the serum lipoproteins, and to provide a convenient quantitative marker for the distribution of this sterol among them. Besides its practical importance, the fact that cholesterol equilibrates readily among all serum lipoproteins also has structural significance in that it supports the unitary model of lipoprotein structure recently proposed by this laboratory. This model is based on physical and chemical data (25) that favor the existence of a thermodynamic equilibrium among all cholesterol molecules in plasma.

With respect to apolipoproteins, it is important to note that both apo B and apo A-I were fully recovered in all normolipidemic and dyslipoproteinemic sera studied. Moreover, in both normolipidemic and hypercholesterolemic sera, apo B and apo A-I were the main components of LDL and HDL, respectively. The concurrent elevation of apo B and [^{14}C]cholesterol in the LDL of the type II hyperlipoproteinemic subject studied is in agreement with data in the literature (26). On the other hand, the observed broader distribution of apo A-I in the HDL of this subject, compared to the symmetrical distribution in normolipidemic sera, remains unexplained.

In the ABL sera, we found the anticipated absence (9) of LDL. For apo A-I, the profile was reproducible and distinctive; it was similar to that previously reported for the ABL HDL₂ fractionated by density gradient ultracentrifugation with a linear

C_2Cl_2 gradient and spun in a SW-40 rotor at 38,000 rpm, 20°C, for 66 hr (9).

Based on our results, although still limited in number, the single spin method appears to be well suited for the separation of lipoproteins from normo- and dyslipoproteinemic sera. However, its true applicability in all dyslipoproteinemias must wait further investigation. This technique can be complemented in the future by development of radioimmunoassays for human apo A-II, arginine-rich peptide, and C-peptides. It also readily provides information on the hydrated density of a given lipoprotein, which is extremely valuable when unknown samples are examined and decisions are to be made on the optimal medium density required when the conventional sequential flotation methods are used. Moreover, the method as now developed identifies a "bottom fraction" of d 1.143 g/ml, which permits the examination of either lipid-improverished or lipid-free apolipoproteins, thus providing a favorable system for the investigation of their mode of origin. By suitable modification of the discontinuous gradient, further resolution of subcomponents within each lipoprotein class should be possible, for example, the resolution of HDL₂ from HDL₃. The sensitivity and reproducibility of this method, its applicability to small-scale samples, and the potential application of automated procedures make it very well suited for clinical studies. Systematic use of this method is likely to provide a better understanding of the serum lipoprotein distribution in normolipoproteinemic and dyslipoproteinemic subjects as a function of time, dietary manipulations, and drug therapy. 

The authors wish to thank Dr. Gunther Fless for his assistance in the flotation analyses, Mr. Peter Lagocki for preparing the lysolecithin, and Mrs. Elisabeth Lanzl and Mrs. Joey Czerwonka for preparation of the manuscript. The authors also wish to express their gratitude to Dr. M. S. Brown from Texas Allied Health Science Center, Dallas, Texas for providing the serum sample from a patient with familial type II hyperlipoproteinemia and to Dr. H. Kayden from New York University Medical Center for the plasma specimens of the two patients with abetalipoproteinemia. This research was supported by USPHS Grants HL-15062-06, HL-08727, and HL-18577. Dr. Angelo M. Scanu was a recipient of USPHS Career Development Award HL-24,867.

Manuscript received 2 February 1977 and accepted 20 June 1977.

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