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papers and notes on methodology

Direct determination of human and rabbit apolipoprotein B selectively precipitated with butanol-isopropyl ether

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Abstract A method is described for the rapid, selective, and quantitative precipitation of apolipoprotein B from isolated hypercholesterolemic rabbit and human very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), and low density lipoproteins (LDL). Lipoprotein samples are heat-treated at 100°C in 1% SDS. The denatured apoprotein solutions are then mixed briefly with two volumes of butanol–isopropyl ether 45:55 (v/v) to precipitate the apoB. The supernatant solutions, containing the non-apoB proteins and lipids, are removed and the apoB pellet is washed once with water. To determine apoB specific activity, the apoB pellet is resolubilized in 0.5 M NaOH by heating for 30 min at 120°C. The hydrolyzed apoB protein is quantitated by fluorescence of a fluorescamine derivative. The precipitation of apoB is quantitative and selective: 99.5% of rabbit ^{125}I-labeled LDL-apoB and 97.5% of human ^{125}I-labeled LDL-apoB is precipitated and less than 5% of ^{125}I-labeled HDL added to unlabeled VLDL, IDL, or LDL is precipitated. Triglyceride and cholesteryl ester contamination of the apoB pellet is less than 2% of their original radioactivities.—**Klein, R. L., and D. B. Zilversmit.** Direct determination of human and rabbit apolipoprotein B selectively precipitated with butanol-isopropyl ether. *J. Lipid Res.* 1984. **25:** 1380–1386.

Supplementary key words VLDL • IDL • LDL • HDL • fluorescamine

Apolipoprotein B (apoB) is the major structural protein present in plasma very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), and low density lipoproteins (LDL). It is also the only lipoprotein component presently believed to be nonexchangeable, thus it is frequently used as a marker to facilitate the study of lipoprotein metabolism. ApoB may be separated quantitatively from human VLDL by treatment with tetramethylurea (1) or isopropanol (2). The apoB protein in lipoproteins has been estimated either by difference (1) or after resolubilization and direct measurement (3). Specific activities of apoB in metabolic studies have been determined after resolubilization of an apoB pellicle (3, 4).

In this report we have described a method to precipitate apoB from rabbit and human plasma VLDL, IDL, and LDL with butanol and isopropyl ether (BUT–IPE). The isolated apoB protein was quantitated by a fluorescence assay. The results show that the precipitation of apoB with BUT–IPE is rapid, quantitative, and selective, and offers several advantages over other methods.

MATERIALS AND METHODS

Lipoproteins

Blood samples were obtained from female New Zealand White rabbits (Beckens Research Animal Farm, Sanborn, NY) maintained on Purina Lab Rabbit Chow supplemented with 0.5% cholesterol (Nutritional Biochemicals, Cleveland, OH) and 2.5% Wesson Oil (Hunt-

Abbreviations: VLDL, very low density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; BUT, n-butanol; IPE, isopropyl ether; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; apoB, apolipoprotein B; EDTA, ethylenediaminetetraacetic acid.

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Wesson Foods, Inc., Fullerton, CA) or from a normolipidemic human male. A concentrated mixture of preservatives was added to the blood sample at the time of collection to obtain final concentrations of the antibiotics chloramphenicol (20 μg/ml) and gentamycin sulfate (50 μg/ml), the protease inhibitor ε-amino caproic acid (0.13%) (Sigma, St. Louis, MO), and the anticoagulant EDTA, pH 7.4 (0.1%).

Lipoproteins were isolated at 10°C by sequential ultracentrifugation (5) at solvent densities of 1.006, 1.019, 1.063, and 1.21 g/ml to yield VLDL, IDL, LDL, and HDL, respectively. Salt solutions used to adjust plasma solvent densities contained the preservatives at the same final concentration as in plasma. Lipoproteins were iodinated with iodine monochloride (6), dialyzed against 0.9% NaCl with the above preservatives (hereafter referred to as saline), and passed through a 0.45-μm filter (Gelman, Ann Arbor, MI). The percentage of lipoprotein radioactivity present in lipid was determined after extracting the lipoprotein with chloroform–methanol 2:1 (v/v) (7). Human LDL were labeled with [^{14}C]cholesteryl oleate and [^{3}H]triolein (Amersham, Arlington Heights, IL) with cholesteryl ester transfer protein activity as described previously (8). Briefly, radiolabeled triolein and cholesteryl oleate were sonicated with phosphatidylcholine and were incubated with human plasma for 18 hr at 37°C. The radiolabeled LDL were then isolated at $1.019 < d < 1.063$ g/ml. All lipoprotein fractions were dialyzed against saline.

ApoB precipitation

Lipoprotein apoB was selectively precipitated by a modification of a previously described solvent system used for the delipidation of plasma or serum (9). The organic solvents, n-butanol and isopropyl ether (Fisher Scientific, Rochester, NY), were reagent grade. Isopropyl ether was passed over a column of Woelm basic aluminum oxide, activity grade 1 (Universal Scientific, Inc., Atlanta, GA) before use and was stored in a tightly capped amber bottle with 0.005% butylated hydroxytoluene added as a preservative. Both solvents were mixed in the given proportions immediately before use.

The precipitation of apoB and the subsequent determination of apoB protein mass were carried out in 1.5-ml polypropylene microcentrifuge tubes with caps (Brinkman, Westbury, NY). Each lipoprotein sample was added to the tube in a volume not exceeding 0.266 ml. Samples with smaller volumes were made up to 0.266 ml with saline. A 0.133-ml volume of a concentrated protein denaturing buffer was added to each sample. This buffer solution consisted of 150 mM Na-phosphate, pH 6.8, 3% (w/v) sodium dodecyl sulfate (Bio Rad, Rockville Centre, NY) and 0.003% (w/v) bromophenol blue, which facilitated visualization of the apoB pellicle and which highlighted any contamination of the pellicle with the aqueous layer containing the non-apoB protein. Each tube was tightly capped and the contents were mixed by vortexing and then incubated at 100°C for 5 min. After cooling, 0.8 ml of butanol–isopropyl ether (45:55, v/v) was added and the tubes were capped tightly. Each rack of samples was then inverted ten times within 15 sec to insure that both phases were mixed completely. Samples were then centrifuged at 2500 *g* for 4 min to separate the phases. Alternatively, the samples were agitated continuously for the stated period of time (see Table 2) with a gentle rocking motion (Labquake®, Labindustries, Berkeley, CA) and then centrifuged. After centrifugation, the precipitated apoB was found as a pellicle at the interface between the blue aqueous layer and the overlying organic solvent phase. The precipitated apoB was separated from the soluble apoproteins by tilting the centrifuge tube and slowly aspirating the two solvents with a Pasteur pipet. Positioning a high-intensity light behind and slightly above the tube aids in visualization of the pellicle during this process. The apoB pellicle becomes attached to the side of the tube during this procedure. While aspirating slowly, the organic solvent layer washes the pellicle and displaces the final traces of the blue aqueous layer containing the non-apoB proteins. The apoB pellicle was washed by adding 1.0 ml of distilled water. The tubes were capped and the contents were mixed gently by "finger-flicking" the tube. The apoB pellicle, which had been dislodged from the side of the tube during the above procedure, was sedimented after a brief centrifugation and the wash solution was aspirated. This step reduced the contamination of the apoB precipitate with non-apoB proteins, residual salt, SDS, and organic solvent. Radioactive iodine in each sample was determined with a gamma counter (Beckman Instruments, Inc., Irvine, CA).

Protein determination

The amount of precipitated apoB protein was determined by a fluorescence method (10). Protein standards (Pentex® Bovine Serum Albumin, Fatty Acid-Poor, Fraction V; Miles, Elkhart, IN) and the washed apoB pellets were dried in a forced-air oven for 1 hr at 110°C. The dried samples were hydrolyzed in 0.2 ml of 0.5 M NaOH by heating in a pressure canner (Presto, 16 qt) for 30 min at 120°C. After cooling, 0.2 ml of 0.5 M HCl and 1 ml of 0.5 M borate buffer, pH 8.5, were added successively to each tube and the contents were mixed vigorously after each addition. A 0.15-ml volume of fluorescamine reagent (20 mg/100 ml fluorescamine in reagent grade, anhydrous acetone) was added to each tube which was then capped quickly and mixed vigorously. With some batches of polypropylene

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tubes from a different distributor a precipitate appeared which was sedimented by centrifuging briefly. Fluorescence was determined with a spectrofluorometer (Perkin-Elmer Corp., Norwalk, CT) by excitation at 390 nm and emission was measured at 475 nm. In some instances, protein concentrations were determined by the method of Lowry et al. (11). Effects of light scattering on colorimetry of lipoprotein samples were eliminated by vigorously mixing each sample with 1 ml of chloroform after color development.

Lipid contamination

Lipids in the isolated apoB pellet were determined with human LDL labeled with [^{14}C]cholesteryl oleate and [^{3}H]triolein. The apoB was precipitated and the pellet was washed as described above. A 0.5-ml volume of methanol was added to each tube which was then sonicated for 15 sec, after which the dispersed protein was transferred to a scintillation vial. The above procedure was repeated twice. Each tube was washed with three 0.5-ml chloroform washes which were also transferred to the vial. The organic solvents were evaporated, scintillant was added (ACS®, Amersham, Arlington Heights, IL), and radioactivity levels were determined in a liquid scintillation counter (Beckman Instruments, Inc., Irvine, CA).

Apolipoproteins

To visualize the total apolipoprotein complement of a sample, lipoproteins were extracted with ethanol–ether 3:1 (v/v) and the apoproteins were separated by polyacrylamide gel electrophoresis in a 0.1% SDS as previously described (12), except that a 4.5–15% polyacrylamide gradient was used in the lower gel. Gels were stained with Coomassie Blue G-250 (0.2%) in 50% methanol, 7% acetic acid. Some samples were used for SDS-PAGE after precipitating the apoB and separating the apoB pellicle from the supernatant solutions exactly as described above. The overlying BUT–IPE layer was aspirated and the aqueous layer, containing the non-apoB proteins, was heated at 40°C to remove residual solvents. One drop of glycerol was then added to each sample to increase the solution density before samples were applied to the gel.

RESULTS

ApoB precipitation

The apolipoprotein composition of human and hypercholesterolemic rabbit lipoprotein samples before and after the precipitation of apoB with BUT–IPE is shown in the electrophoretogram in **Fig. 1.** Even-numbered lanes contain the soluble non-apoB protein of samples extracted with BUT–IPE (45:55, v/v) and the odd-numbered lanes contain the total apoprotein complement. Rabbit lipoprotein samples in the even-numbered lanes showed no large molecular weight proteins with electrophoretic mobilities similar to either the high molecular weight (B_H) or low molecular weight (B_L) apoB of the total apoprotein complement. The BUT–IPE-soluble apoproteins from human plasma show a trace of protein with mobility similar to that of the large molecular weight apoB. All samples contained the smaller molecular weight apoE, apoA-I, and apoC's suggesting that the apoB was precipitated selectively.

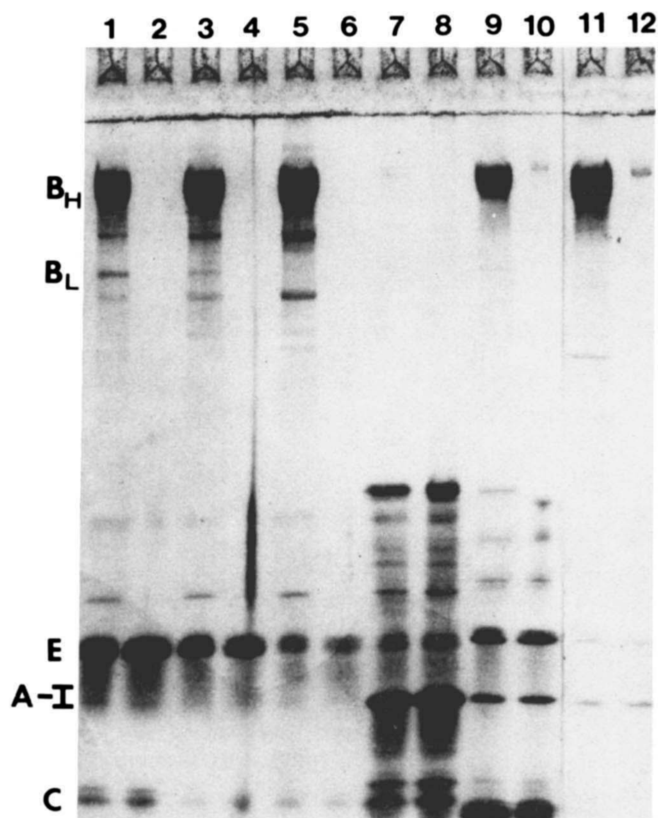

Fig. 1. The 4.5–15% polyacrylamide-SDS gel electrophoresis patterns of apoproteins isolated from hypercholesterolemic rabbit and normal human lipoproteins. Even-numbered lanes show soluble apoproteins of samples extracted with butanol–isopropylether 45:55 (v/v); odd-numbered lanes contain the total apoprotein complement of non-extracted samples. Samples were obtained from hypercholesterolemic rabbit VLDL (lanes 1, 2), IDL (lanes 3, 4), LDL (lanes 5, 6), HDL (lanes 7, 8), and human VLDL (lanes 9, 10) and LDL (lanes 11, 12). Approximately 10 μg of protein was applied to each sample lane. The apoproteins identified are the high molecular weight form of apoB (B_H), the low molecular weight form of apoB found in lymph chylomicrons from rabbits (B_L), apoprotein E (E), apoprotein A-I (A-I), and the low molecular weight C apoproteins (C).

To determine the amount of apoB precipitated with BUT–IPE, hypercholesterolemic rabbit VLDL and human LDL samples were iodinated and aliquots of these lipoproteins were mixed with BUT–IPE to precipitate

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the apoB. Apoproteins in the supernatant solution and in aliquots of lipoproteins not extracted with BUT–IPE were separated by SDS-PAGE. Radioactivities in the apoB bands of the SDS gels were determined in triplicate. Recovery of radioactivity applied to the gel averaged 88%. ApoB radioactivity in the BUT–IPE-soluble rabbit VLDL protein was 0.5% of the radioactivity in total apoB, whereas in the human LDL sample, 2.5% of the initial apoB radioactivity was found in the BUT–IPE-soluble fraction.

In one experiment we compared the precipitation of low and high molecular weight rabbit apolipoprotein B obtained from a rabbit fed a single 150-g portion of rabbit chow containing 13% lard, and injected subsequently with 200 mg/kg Triton WR 1339 to reduce chylomicron and VLDL clearance. A triglyceride-rich fraction from plasma was labeled with ^{125}I and total apoB was precipitated by the BUT–IPE procedure. From the amounts of ^{125}I in $apoB_H$ and B_L in the pellet and that present in the original lipoprotein fraction, both determined by SDS gel electrophoresis, it was evident that both forms of apoB were precipitated with equal efficiency.

Effect of solvent proportions

The ratio of butanol to isopropyl ether in the organic solvent phase is critical to obtain the complete selective precipitation of apoB. This is shown by the data in **Table 1.** In these studies, iodinated LDL and HDL were used to monitor apoB and non-apoB protein, respectively. Human ^{125}I-labeled LDL apoB and normal rabbit ^{125}I-labeled LDL apoB were 95 and 84%, respectively, of the total radioactivity in protein as determined by SDS-PAGE. The percentage distribution of radioactivity in hypercholesterolemic rabbit ^{125}I-labeled HDL protein was determined after SDS-PAGE and averaged 35, 9, 44, and 13% for the regions of the gel corresponding to albumin, apoE, apoA-I, and apoC's. The recovery of radioactivity applied to the gel averaged 95%.

Tracer amounts of human ^{125}I-labeled LDL and rabbit ^{125}I-labeled LDL and ^{125}I-labeled HDL were mixed with nonradiolabeled rabbit VLDL, IDL, and LDL. Samples were mixed with BUT–IPE in varying proportions and the percentage of radioactivity precipitated was determined. The percentage of ^{125}I-labeled LDL apoB precipitated increased as the proportion of butanol to isopropyl ether was increased. The percentage of non-apoB rabbit ^{125}I-labeled HDL precipitated also increased, but the percentages were substantially lower than those for LDL apoB. ^{125}I-labeled LDL apoB precipitation was only slightly increased when more than 45% BUT was used in the solvent system, but the precipitation of ^{125}I-labeled HDL increased markedly.

TABLE 1. Effect of the proportion of butanol to isopropyl ether on the precipitation of apoB and non-apoB apoprotein

BUT:IPE	% ^{125}I-ApoB Precipitated[a] Human LDL[c]	% ^{125}I-ApoB Precipitated[a] Rabbit LDL[c]	% ^{125}I-Non-ApoB Precipitated[b] Rabbit HDL[c]
25:75	NP[d]	NP	NP
40:60	56.0 ± 11.0[e]	64.0 ± 11.0	1.4 ± 0.1
41:59	65.0 ± 12.0	83.0 ± 10.0	1.9 ± 0.2
42:58	73.0 ± 11.0	90.0 ± 4.0	3.1 ± 0.4
43:57	86.0 ± 4.0	95.1 ± 0.7	3.5 ± 0.4
44:56	94.5 ± 2.0	96.9 ± 0.7	4.0 ± 0.9
45:55	97.4 ± 0.4	99.6 ± 0.9	4.7 ± 0.7
47:53	98.6 ± 0.3	100.2 ± 0.8	11.4 ± 4.4
50:50	99.1 ± 0.4	100.5 ± 1.0	9.7 ± 2.6

[a] Determined as [cpm precipitated/(cpm in protein × fraction of radioactivity in apoB)] × 100.

[b] Determined as [cpm precipitated/(cpm precipitated + cpm in supernatant)] × 100. Percentage distribution of radioactivity in ^{125}I-labeled HDL protein was determined after SDS-PAGE and averaged 35, 9, 44, and 13% for regions of the gel corresponding to albumin, apoE, apoA-I, and apoC's, respectively.

[c] Each incubation contained 40 μg protein from non-radioactive rabbit VLDL, IDL, or LDL plus tracer amounts of human ^{125}I-labeled LDL, normocholesterolemic rabbit ^{125}I-labeled LDL, or hypercholesterolemic rabbit ^{125}I-labeled HDL. Lipoprotein sample plus tracer was mixed with denaturing buffer solution, the sample was heat-denatured, the apoB was precipitated with the indicated BUT–IPE (v/v), and the organic:aqueous solvent ratio was 2:1. Precipitated protein was isolated, washed, and counted as described in Methods and Materials.

[d] No pellicle formed. No defined pellicle formed even when 80 μg of protein was used.

[e] Mean ± SD of triplicate determinations. Data from VLDL, IDL, and LDL incubations are combined.

Effect of lipoprotein solution composition

Lipoproteins were dialyzed routinely against 0.9% NaCl solution containing a mixture of lipoprotein preservatives (see Materials and Methods). To determine whether the preservatives mixture influenced the precipitation of apoB, human ^{125}I-labeled LDL was added to unlabeled rabbit LDL that had been dialyzed against 0.9% NaCl plus preservatives, 0.9% NaCl, or distilled water. The apoB was precipitated with BUT–IPE and apoB radioactivity in the isolated and washed apoB pellet was determined. The preservatives mixture did not influence the apoB precipitation as 98.3 ± 2.2% and 98.1 ± 2.6% of the apoB radioactivities were precipitated when the samples had been dialyzed against saline plus preservatives or saline, respectively. In contrast, when the lipoprotein samples had been dialyzed against distilled water, only 78.8 ± 1.0% of the apoB radioactivity was precipitated, suggesting that the ionic strength of the solution influences the precipitation of apoB.

Lipoproteins used in these studies were isolated by sequential ultracentrifugation at increasing solvent densities. To determine whether solutions with densities greater than 1.006 g/ml would influence the precipita-

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tion reaction, tracer amounts of human ^{125}I-labeled LDL or rabbit ^{125}I-labeled HDL were mixed with unlabeled rabbit VLDL. The solvent density of the solutions was increased to d = 1.019 or d = 1.063 g/ml with KBr. The apoB was precipitated with BUT-IPE and apoB radioactivity levels in the washed apoB pellets were determined. The percentages of human ^{125}I-labeled LDL apoB precipitated averaged 95.1 ± 0.3, 96.2 ± 0.5, and 95.7 ± 2.0% for solutions with solvent densities equal to d = 1.006, d = 1.019, and d = 1.063 g/ml, respectively. In contrast, the percentages of rabbit ^{125}I-labeled HDL precipitated averaged 4.2 ± 0.2, 6.2 ± 0.2, and 7.7 ± 0.2% for solution densities of d = 1.006, d = 1.019, and d = 1.063 g/ml, respectively. Thus, sample dialysis is not necessary when apoB mass determinations are to be made on samples composed primarily of apoB. However, if apoB radioactivity is to be determined and the majority of the radiolabel originated in non-apoB protein, serious errors in the determination of apoB radioactivity may be incurred if the samples are not first dialyzed against saline.

Protein recovery

We wished to determine whether apoB initially precipitated with BUT-IPE was lost when the apoB pellicle was washed with water. In these samples at least 96.5 ± 1.5% of the precipitated ^{125}I-labeled LDL apoB was recovered after one wash. When tracer amounts of ^{125}I-labeled HDL were added to nonradiolabeled lipoprotein and the sample was mixed with BUT-IPE (45:55), only 6% of the initial ^{125}I-labeled HDL was precipitated. The wash step further reduced ^{125}I-labeled HDL radioactivity contamination of the pellet to 4.7% of that present initially (Table 1). This wash step also removed residual organic solvent and SDS.

Effect of protein concentration

This method may be used to precipitate quantitatively the apoB from lipoproteins over a wide range of lipoprotein protein amounts as shown in **Table 2.** Tracer amounts of human ^{125}I-labeled LDL were added to between 20 and 250 μg of nonradiolabeled rabbit lipoprotein. The samples were mixed with BUT-IPE to precipitate the apoB. Irrespective of the amount or type of lipoprotein carrier, human ^{125}I-labeled LDL apoB was quantitatively precipitated.

Lipid contamination

Since triacylglycerol and cholesteryl ester are lipids present in high concentrations in hypercholesterolemic rabbit plasma, we determined the extent of contamination of the apoB pellet with these lipids. Human LDL were labeled with [^{3}H]triolein and [^{14}C]cholesteryl oleate and tracer amounts of the radiolabeled LDL were mixed with hypercholesterolemic rabbit VLDL, IDL, and LDL. The apoB was precipitated and the radioactivity in the pellet was determined. As shown in **Table 3,** less than 2% of the radioactive lipids initially present in the sample were found in the apoB pellet.

TABLE 2. Effect of amount of sample protein on the precipitation of apoB

Lipoprotein Protein[a]	Percent of ^{125}I-labeled LDL ApoB Precipitated[b]		
	VLDL	IDL	LDL
μg			
250	96.7	97.3	97.4
160	96.6	97.4	97.5
80	96.8	97.3	97.6
40	97.2	97.5	97.7
20	97.2	97.3	97.8
Mean ± SD	96.9 ± 0.2	97.4 ± 0.1	97.6 ± 0.1

[a] Lipoprotein protein concentration was determined according to method of Lowry et al.

[b] Approximately 250,000 cpm in 3.9 μg human LDL protein added to non-radiolabeled rabbit VLDL, IDL, or LDL for each assay. ApoB was precipitated as described in Methods and Materials using BUT-IPE 45:55.

Rate of apoB precipitation

This procedure for the precipitation of apoB is rapid. However, if large numbers of samples are to be analyzed and the precipitated apoB pellet cannot be isolated immediately, samples may be held overnight without significant loss of the pelleted apoB (**Table 4**). ApoB was precipitated from rabbit lipoprotein fractions containing tracer amounts of human ^{125}I-labeled LDL. At the times indicated the precipitated apoB was isolated and washed and the radioactivity was determined as described in Materials and Methods. The zero-time sample was the apoB pellet isolated immediately after the precipitation procedure. In samples isolated at later times, both the aqueous and organic solvent phases were continuously agitated with a rocking motion until the apoB pellet was isolated. The percentage of ^{125}I-labeled LDL radioactivity found in the precipitated apoB was similar at all times assayed, suggesting that the apoB is irreversibly precipitated. These data also indicate that the reaction is rapid and complete, thus eliminating the need for any delay in isolating the apoB pellet.

ApoB specific activity

The amount of apoB precipitated in each sample may be determined rapidly and easily by a fluorescence assay. The apoB was selectively precipitated from ^{125}I-labeled hypercholesterolemic rabbit VLDL and ^{125}I-labeled human LDL. The apoB pellets were dried at 110°C and were solubilized and hydrolyzed with NaOH as described in Materials and Methods. The hydrolyzed protein was

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TABLE 3. Lipid contamination of precipitated apoB pellets

Sample	Percent of Initial Lipid Radioactivity Present in ApoB Pellet[a]	
	[^{3}H]Triacylglycerol	[^{14}c]Cholesteryl Ester
VLDL	2.1 ± 0.4[b]	1.9 ± 0.4
IDL	1.2 ± 0.7	1.4 ± 0.2
LDL	1.3 ± 0.3	1.3 ± 0.3

[a] Eighty micrograms of lipoprotein protein was used in each assay. ApoB was precipitated as described in Methods and Materials. Samples were agitated by rocking overnight before apoB pellets were isolated. Each sample contained 62,996 cpm of [^{3}H]triolein and 26,402 cpm of [^{14}C]cholesteryl oleate.

[b] Mean ± SD of three observations.

reacted with fluorescamine and fluorescence was determined. The results are shown in **Table 5.** The amount of apoB precipitated from a lipoprotein solution was reproducible irrespective of the class of lipoprotein donor or the amount of lipoprotein protein reacted, as indicated by an average coefficient of variation of 3.4% and 3.6% for the amount of apoB protein and radioactivity precipitated, respectively, for the eight sets of samples. The amount of apoB, expressed as a percentage of the total protein initially present in the reaction mixture, was similar irrespective of the amount of apoB assayed. This suggests that the protein solubilization procedure was complete.

DISCUSSION

We have developed a procedure to precipitate apoB selectively from isolated plasma lipoproteins. The method is rapid in that the apoB is quantitatively precipitated immediately after mixing the SDS-denatured apoB fraction with two volumes of butanol–isopropyl ether 45:55. The method is selective in that, while the apoB is quantitatively precipitated, only minimal amounts of non-apoB protein are co-precipitated.

This contrasts with the results obtained when isopropanol (2) or tetramethylurea (1) are used to precipitate apoB. Incubation periods of 30 min (1) to overnight (3, 4) are recommended when these procedures are used. In addition, these methods require multiple washes of the precipitated apoB, some of which utilize further incubation periods. These wash steps also result in the loss of 14–45% of the precipitated apoB. The single brief wash utilized in the BUT–IPE precipitation method results in the loss of less than 4% of the apoB initially precipitated. When tetramethylurea or isopropanol are used to precipitate apoB from human lipoproteins, the non-apoB protein contamination of the apoB pellicle is minimal (1, 4). However, when these methods are used with hypercholesterolemic rabbit lipoproteins, substantial amounts of apoE are co-precipitated (unpublished observations).

The concentration of apoB precipitated has been estimated with the procedure of Lowry et al. (11) either by the difference between soluble and total protein (1, 2) or after resolubilization of the isolated apoB and direct measurement (3, 4). The latter approach has been employed for determination of specific activities of apoB in metabolic studies. The time required for resolubilization was lengthy and ranged from 1 to 3 days. Gentle resolubilization techniques were used presumably because the Lowry procedure was used for protein quantitation and harsher conditions would result in decreased color development (11).

In this study, the precipitated apoB was quantitated by a protein fluorescence assay, which resulted in a substantial savings in time and increase in sensitivity. The isolated apoB was resolubilized in only 30 min. In addition, the protein was hydrolyzed, which resulted in an average fourfold increase in the sensitivity of the assay. This eliminated the need for the large blood samples that were utilized in previous metabolic studies involving humans (3, 4) and are prohibitive in studies involving smaller animals such as rabbits, rats, or non-human primates.

Iodinated human or normal rabbit LDL were used as markers for apoprotein B in these studies, because these fractions contain predominantly apoB and minimal correction for radiolabel in non-apoB protein was necessary. This necessitated the assumption that the behavior of apoB from LDL would be similar to that from VLDL or IDL. This assumption is probably valid, because the first step of this procedure is to treat the entire protein complement of the lipoprotein with SDS under denaturing conditions. Under these conditions, any potential differences in charge or configuration of the proteins are masked by the SDS-binding and unfolding of the

TABLE 4. Time course of the precipitation of apoB

Time of Incubation	Percent of Zero-time ^{125}I-labeled LDL Radioactivity in Precipitated ApoB		
	VLDL	IDL	LDL
hr			
0	100.0[a]	100.0	100.0
1	100.1	100.0	100.2
2	99.5	99.8	100.2
3	99.1	99.6	100.1
24	97.9	97.9	99.5

[a] ApoB was precipitated as described in the text. The ^{125}I-labeled LDL radioactivity present in apoB at t = 0 was normalized to 100%. The radioactivity in apoB isolated at later time points was expressed as a percentage of the zero-time value.

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TABLE 5. Determination of amount and specific activity of apoB precipitated with butanol-isopropyl ether

Lipoprotein	Initial Protein Assayed[a]	ApoB Mass[b]	ApoB[c]	cpm Precipitated	ApoB Specific Activity
	μg	μg	%	cpm	cpm/μg
^{125}I-labeled human LDL	182.0	172.4 ± 2.1[d]	94.7	170,570 ± 4,890[d]	990 ± 41[d]
	130.0	114.7 ± 3.5	88.3	114,460 ± 3,400	998 ± 16
	75.3	69.9 ± 4.3	92.8	65,356 ± 3,380	936 ± 30
	27.4	23.7 ± 1.3	86.7	23,020 ± 1,120	972 ± 79
^{125}I-labeled rabbit VLDL	131.8	102.7 ± 3.6	78.0	937,960 ± 13,180	9140 ± 408
	94.1	70.5 ± 3.5	74.9	633,980 ± 34,310	8990 ± 242
	54.5	39.0 ± 0.6	71.6	365,500 ± 10,390	9370 ± 295
	24.8	18.2 ± 0.2	73.7	171,310 ± 4,820	9380 ± 189

[a] Amount of protein reacted with BUT–IPE 45:55.
[b] Amount of apoB precipitated; determined by fluorescence assay after resolubilization and hydrolysis of apoB pellet with 0.5 N NaOH.
[c] (Amount apoB precipitated/initial amount of protein in assay) × 100.
[d] Mean ± SD for three determinations.

protein. The selective insolubility of SDS-solubilized apoB in the presence of BUT–IPE may be due to the large molecular size of apoB relative to that of the other apoproteins, to the aggregability of the protein due to its sulfhydryl content (13), to carbohydrate content and composition of apoB (14, 15), or the anomalous binding of SDS to glycoproteins (16).

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