Isopropanol precipitation method for the determination of apolipoprotein B specific activity and plasma concentrations during metabolic studies of very low density lipoprotein and low density lipoprotein apolipoprotein B

Genshi Egusa, David W. Brady, Scott M. Grundy,[1] and Barbara V. Howard[2]

Phoenix Clinical Research Section, National Institute of Arthritis, Digestive, Diabetes and Kidney Diseases, National Institutes of Health, Phoenix, AZ

Abstract A method has been described for the measurement of apoB concentration and specific activity in very low density lipoprotein (VLDL) and low density lipoprotein (LDL) during metabolic studies. For measurement of specific activity, apoB was separated from other apolipoproteins and lipids by precipitation in, and subsequent washing with, isopropanol. For determination of apoB concentration, equal volumes of lipoprotein and isopropanol were mixed, and the protein content of the apoB precipitate was measured by the difference between total lipoprotein protein and the protein measured in the supernatant. Evidence that there was no apoB solubilization in isopropanol and that precipitated apoB was virtually free of soluble apolipoproteins was obtained by electrophoresis. Lipid contamination of the apoB precipitate was less than 1%. The methods were applicable to VLDL, intermediate density lipoprotein (IDL), and LDL from normolipemic patients with protein concentrations between 187 μg/ml and 1898 μg/ml. The data obtained using isopropanol were highly correlated with those using tetramethylurea, and recoveries of apoB were similar. Furthermore, the isopropanol method has been demonstrated to yield consistent data for apoB specific activities in a study of VLDL, IDL, and LDL metabolism.—**Egusa, G., D. W. Brady, S. M. Grundy, and B. V. Howard.** Isopropanol precipitation method for the determination of apolipoprotein B specific activity and plasma concentrations during metabolic studies of very low density lipoprotein and low density lipoprotein apolipoprotein B. *J. Lipid Res.* 1983. **24:** 1261–1267.

Supplementary key words apoB • VLDL • IDL • LDL

Apolipoprotein B (apoB) is the major structural protein for both very low density lipoproteins (VLDL) and low density lipoproteins (LDL). Because of its high molecular weight and insolubility it has proven difficult to quantify. An important advance in measurement of apoB was the report of Kane et al. (1, 2) that tetramethylurea (TMU) selectively precipitates apoB in the presence of other lower molecular weight apolipoproteins. Concentration can be estimated using the Lowry procedure either by difference or after resolubilization and direct measurement. Resolubilization has been employed for determination of specific activities of apoB in metabolic studies (3).

Despite the advantages of TMU, it is expensive and difficult to obtain in pure form, and it interferes with color development in the Lowry procedure. Recently Holmquist et al. (4, 5) reported that isopropanol can solubilize C and E apolipoproteins, and some investigators have used isopropanol to isolate apolipoproteins (6–10). In this report we have described methods using isopropanol to determine apoB concentration and specific activity in VLDL and LDL and compared them to methods using TMU. The results showed that isopropanol provides a convenient method equivalent to that using TMU for determining the concentration and specific activity of apoB in metabolic studies.

MATERIALS AND METHODS

Isolation of lipoproteins

Five hundred ml of venous blood (from a subject fasted overnight) was collected in plasmapheresis bags

Abbreviations: VLDL, very low density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; TMU, 1,1,3,3-tetramethylurea; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; apoB, apolipoprotein B; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid).

[1] Center for Human Nutrition, University of Texas Health Science Center, Dallas, TX 75235.

[2] Address reprint requests to Dr. Barbara V. Howard, Phoenix Clinical Research Section, NIADDK, National Institutes of Health, 4212 North 16th Street, Phoenix, AZ 85016.

containing citrate dextrose as anticoagulant (Fenwal Laboratories, Deerfield, IL). Plasma was immediately separated by centrifugation at 600 *g* for 20 min (10°C) and preservative (10% sodium azide, 0.2 M DTNB, 0.5% chloramphenicol) was added (10 μl per ml plasma). VLDL was isolated by ultracentrifugation at 40,000 rpm for 16 hr (15°C) in a Beckman 60 Ti rotor (Beckman Instruments, Inc., Palo Alto, CA) and recentrifuged through saline (d 1.006 g/ml) in a 40 Ti rotor. IDL was removed after adjusting the density to 1.019 g/ml using NaCl–NaBr (d 1.35 g/ml) and centrifuging at 40,000 rpm for 20 hr (15°C). LDL was isolated, after adjusting the density to 1.060 g/ml, in a type 50 Ti rotor at 50,000 rpm for 20 hr (15°C) and recentrifuged at d 1.070 g/ml at 40,000 rpm for 20 hr (15°C). Lipoprotein preparations were dialyzed against EDTA–saline (d 1.006 g/ml, 100 mM EDTA, pH 7.4) and ^{131}I-labeled VLDL and ^{125}I-labeled LDL were prepared according to the iodine monochloride method of McFarlane (11) as modified by Langer, Strober, and Levy (12) and dialyzed against 20 changes of EDTA–saline, pH 7.4.

Isolation of apoB for determination of concentration or specific activity

For the measurement of apoB concentration, 1 ml of lipoprotein sample was combined with 1 ml 100% isopropanol (analytical grade, J. T. Baker Chemical Co., Phillipsburg, NJ) in a 12 × 75 mm conical heavy-walled plastic centrifuge tube. After vigorous mixing (1 min) the samples were incubated overnight at room temperature (or at 4°C for samples containing high salt concentrations) and centrifuged at 1000 *g* for 30 min. ApoB concentrations in VLDL and LDL were determined by subtracting the absorbance (660 nm) of the supernatant from the absorbance of the initial lipoprotein preparation. If on occasion the precipitate was not completely sedimented by centrifugation, the supernatant was passed through a 0.22-μm filter.

For the measurement of apoB specific activity, the precipitation was performed as above and the supernatant was discarded. Two ml of 50% isopropanol was added to the pellet and the sample was mixed vigorously and centrifuged as above; this washing procedure was repeated one time. After this, 2 ml of 100% isopropanol was added to ensure complete lipid extraction. The mixture was agitated vigorously until the precipitate was completely dispersed, incubated 2 hr at room temperature, and then centrifuged as described above. After discarding the supernatant, aliquots of 1 N NaOH were added to the pellet; for VLDL or IDL, ca. 1 μl of NaOH per μg of initial protein; for LDL, ca. 2 μl of NaOH per μg of initial protein. Radioactivity of the NaOH suspension was determined immediately in an auto-gamma spectrometer (Packard Instrument Co., Inc., Downers Grove, IL). Then the NaOH suspension was incubated in a water bath at 37°C until the pellet was completely dissolved. This required approximately 1 day for VLDL and IDL and 2 to 3 days for LDL. Protein content of this solution was then measured. The proportion of radioactive iodine bound to lipid in the apoB precipitates was assessed by extraction with chloroform–methanol 2:1 (vol/vol) (13).

In order to compare the isopropanol methods to previous methodology using TMU, identical samples of VLDL and LDL were processed for apoB concentration

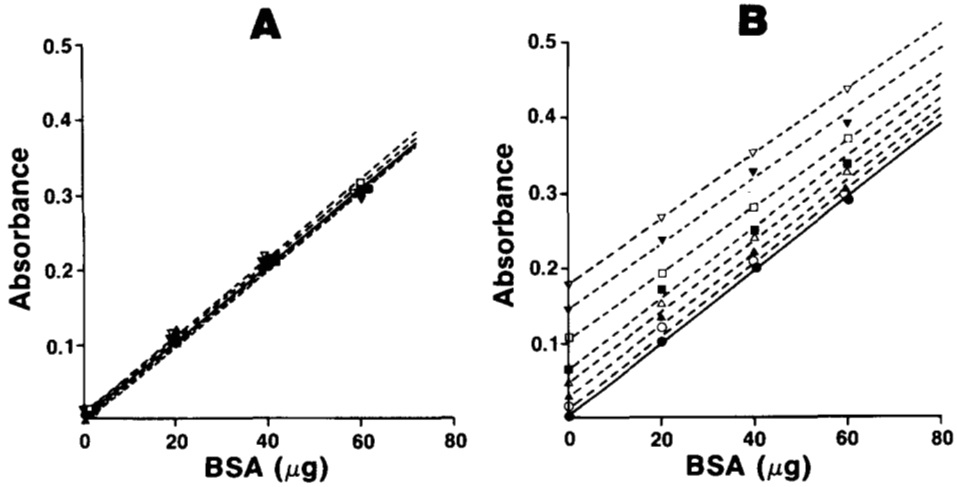

Fig. 1. Effect of TMU and isopropanol on protein measurement by the Lowry method (14). A. Isopropanol: standard bovine serum albumin (BSA) (0 ~ 60 μg) was mixed with isopropanol and water at the following final concentrations: 0% (●), 5% (▲), 10% (■), 15% (□), 20% (▼), 25% (▽). Each standard line was linear with $r > 0.99$. B. TMU: TMU was added to standard BSA (0 ~ 60 μg) at the following final concentrations: 0% (●), 0.5% (○), 1% (▲), 1.5% (△), 2% (■), 3% (□), 4% (▼), and 5% (▽). Each standard line was linear with $r > 0.99$.

as described by Kane et al. (2) and for specific activity according to the method described by Le et al. (3).

Protein measurement

Protein concentrations in lipoprotein fractions and NaOH digests were determined by a modified Lowry procedure that employed 1% SDS in reagent A (14). In all assays the reference bovine serum albumin solution was standardized using an extinction coefficient at 280 nm of $A_{1\ CM}^{1\%} = 6.6$ in order to be independent of water of hydration. The influence of TMU and isopropanol on color development was assessed by comparing assays containing from 0.5% to 25% solvent (**Fig. 1**). With increasing TMU concentration, absorbance of the blank rose from 0 to 0.177, and the slope of the standard line decreased. In contrast, isopropanol in concentrations up to 25% had no effect on color development. For all subsequent experiments, Lowry assays were performed using standards containing the same amount of TMU, NaOH, or isopropanol as that of the samples. Since it has been shown that apolipoproteins are not all equivalent to albumin in the Lowry procedure, the chromogenicity factor for apoB compared to a bovine albumin standard was taken to be 1.0, and a factor of 1.16 was used for the isopropanol supernatant solutions (2).

Electrophoresis

The isopropanol supernatants and protein precipitates were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) (Bio-Rad Lab, Richmond, CA) (15). PAGE was performed in a discontinuous buffer system (15) with the addition of 0.1% SDS to the upper chamber buffer. Gels were cast in 13 × 0.5 cm glass tubes. Running gels were 7.5% acrylamide, 0.2% N,N′-methylene-bisacrylamide, and stacking gels were 1.25% acrylamide and 0.3% N,N′-methylene-bisacrylamide. Whole lipoprotein samples were delipidated using chloroform, and all samples were suspended in 0.01 M phosphate buffer (pH 7.0) containing 1% SDS and 1% β-mercaptoethanol; electrophoresis was conducted at 2 mamp/gel until the tracking dye (bromophenol blue) reached the end of the gels. Gels were stained using Coomassie Brilliant blue G250, destained in 10% acetic acid, and scanned at 595 nm using a Gilford 250 spectrophotometer (Gilford Instruments Inc., Walnut Creek, CA). Relative migration (R_f) was calculated as distance migrated/distance of tracking dye.

Metabolic study

Volunteer subjects admitted to a metabolic ward consumed a weight-maintaining diet (40% fat, 15% protein, 45% carbohydrate) for a week. Sterile and pyrogen-free autologous ^{131}I-labeled VLDL (25 μCi) was prepared as

TABLE 1. Comparison of apoB concentration and specific activity determined using isopropanol and tetramethylurea

	Initial[a] Protein	Isopropanol					Tetramethylurea				
		ApoB[b] Concentration	ApoB Ppt. after Washes	ApoB[c] Recovery	ApoB[d]	Specific activity	ApoB[b] Concentration	ApoB Ppt. after Washes	ApoB[c] Recovery	ApoB[d]	Specific Activity
	μg/ml	μg/ml	μg/ml	%	%	dpm/μg	μg/ml	μg/ml	%	%	dpm/μg
^{131}I-VLDL	249.8	109.5 ± 6.8[e]	80.6 ± 2.2	74	44	411 ± 10	91.0 ± 12.0	65.9 ± 8.1	72	36	404 ± 11
	489.6	208.2 ± 4.9	158.5 ± 3.3	76	43	411 ± 12	186.4 ± 5.2	155.0 ± 11.1	83	38	405 ± 8
	709.3	293.5 ± 1.1	215.2 ± 1.0	73	41	437 ± 8	298.4 ± 8.0	266.1 ± 6.6	89	42	405 ± 13
	938.3	394.3 ± 3.0	310.3 ± 9.8	79	42	410 ± 10	402.8 ± 12.3	319.5 ± 15.3	79	43	419 ± 10
	1231.7	498.7 ± 8.7	406.8 ± 7.6	82	40	424 ± 12	522.4 ± 10.4	476.3 ± 13.7	91	42	418 ± 11
				76.8 ± 1.7	42.0 ± 0.7				82.8 ± 3.4	40.2 ± 1.4	
^{125}I-LDL	187.6	180.8 ± 11.2	156.3 ± 8.0	86	96	2581 ± 86	170.5 ± 8.9	106.5 ± 9.4	62	91	2632 ± 128
	398.0	384.5 ± 7.8	326.4 ± 7.3	85	97	2552 ± 118	365.8 ± 18.1	276.1 ± 12.5	75	92	2571 ± 86
	606.2	576.2 ± 4.2	436.2 ± 13.1	76	95	2566 ± 75	563.7 ± 7.3	363.3 ± 8.4	64	93	2622 ± 151
	977.1	914.6 ± 13.8	859.7 ± 9.4	94	94	2601 ± 102	883.6 ± 14.1	718.7 ± 11.6	81	90	2639 ± 61
	1394.4	1313.9 ± 14.4	1203.8 ± 17.5	92	94	2556 ± 93	1287.0 ± 20.3	990.7 ± 10.1	77	92	2601 ± 73
	1898.0	1819.3 ± 18.5	1514.5 ± 14.4	83	96	2585 ± 84	1778.1 ± 31.5	1446.0 ± 15.7	81	94	2620 ± 131
				86.0 ± 2.6	95.3 ± 0.5				73.3 ± 3.4	92.0 ± 0.6	

[a] Calculated as the sum of apoB plus the protein content of the supernatant.
[b] ApoB concentration was determined by subtracting the absorbance of the supernatant from the absorbance of the initial lipoprotein preparation.
[c] Percent apoB recovery = [μg apoB precipitate after washes/μg apoB (*b*)] ×100.
[d] Percent apoB = [μg apoB (*b*)/μg initial protein (*a*)] ×100.
[e] Values are mean ± SEM of six determinations.

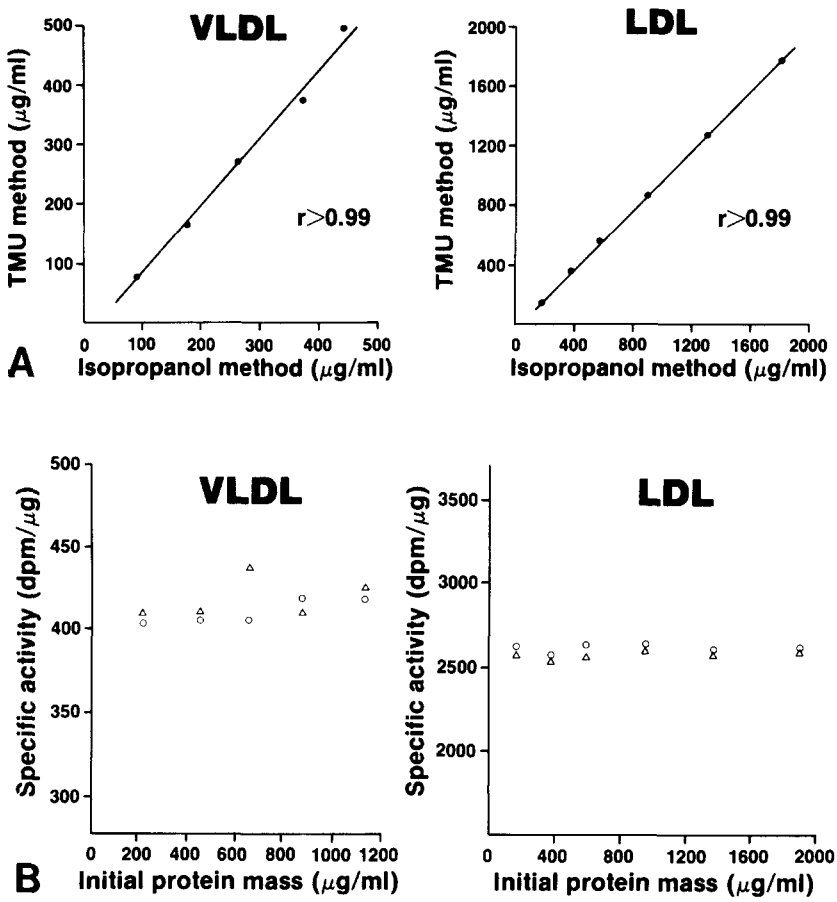

Fig. 2. A. Relationships between apoB concentration of VLDL and LDL determined using isopropanol and TMU. B. Comparison of apoB specific activity in VLDL and LDL measured using isopropanol (Δ) and TMU (O) over a wide range of initial protein concentrations.

described above (except that preservative was omitted from the plasma) and injected at time 0. At the indicated times, 4 ml of plasma was obtained and VLDL, IDL, and LDL were isolated by ultracentrifugation at d 1.006, 1.019, and 1.063 g/ml, respectively. ^{131}I-labeled apoB specific activity of VLDL, IDL, and LDL was determined as described.

RESULTS AND DISCUSSION

Concentrations and specific activities of ^{131}I-labeled VLDL-apoB and ^{125}I-labeled LDL-apoB determined using isopropanol and TMU were compared (**Table 1, Fig. 2**). Estimates of apoB concentration by the two methods were highly correlated (Fig. 2A). Slightly lower values were obtained by TMU when the protein mass of VLDL was low (<600 μg/ml); somewhat lower values also were noted for LDL-apoB measured by the TMU procedure. This could be because the TMU procedure requires greater dilution of the TMU supernatant to eliminate the effect of TMU on the color development of the Lowry procedure (2).

Specific activities of apoB in VLDL and LDL by the two methods were virtually identical using the two solvents (Table 1). Furthermore, when using isopropanol, specific activities of VLDL apoB and LDL apoB were constant over a wide range of apoB concentrations (187 to 1898 μg/ml) (Fig. 2B). With isopropanol, the recovery of apoB after solubilization of the precipitates averaged 76.8% for VLDL and 86.0% for LDL. Recovery of apoB in VLDL appeared to increase in both methods with increasing initial protein mass. It is, thus, likely that the loss represents loss of precipitated apoB during the extensive washing procedure. Solubilization of the apoB precipitate in LDL after isopropanol treatment usually required 2–3 days incubation with 1 N NaOH. For this reason, the radioactivity measurements were made directly after mixture with NaOH; this was done because of the rapid decay of isotopes generally employed in

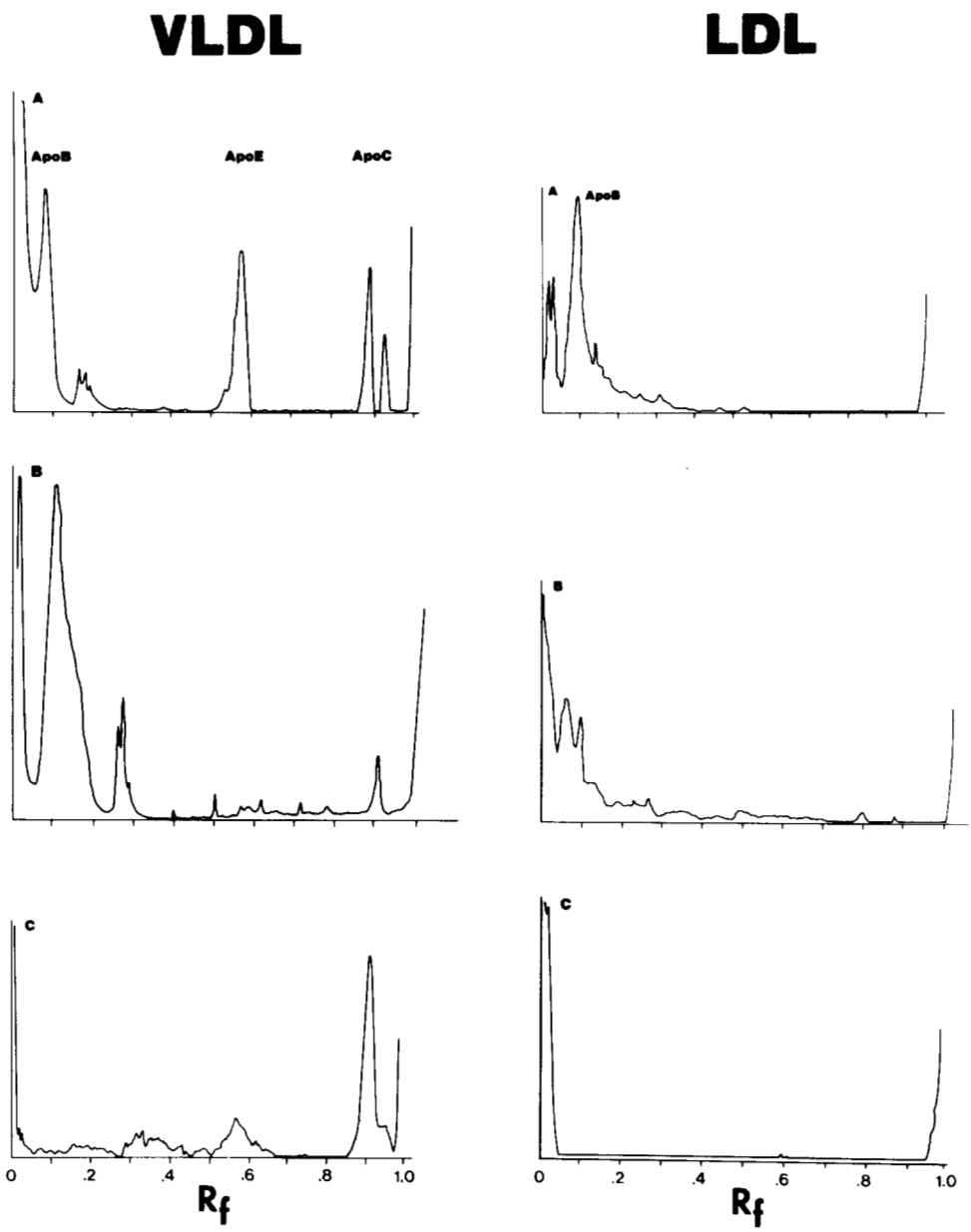

Fig. 3. Densitometric scan of SDS-polyacrylamide gel electrophoresis of isopropanol supernatants and washed precipitates: VLDL, left panel; LDL, right panel. Top of gel is at the left; the abscissa is the relative migration compared to the tracking dye (R_f). A. Intact VLDL and LDL preparations. B. ApoB precipitates obtained using isopropanol. C. Supernatants after isopropanol precipitation of apoB.

metabolic studies. The apoB precipitated by isopropanol was more readily soluble in both NaOH and electrophoresis buffer than precipitates obtained with TMU. This appears to be a definite advantage of isopropanol.

Specificity of the isopropanol precipitation procedure was confirmed by comparing SDS-PAGE of isopropanol precipitates and supernatants with those of whole VLDL (**Fig. 3,** left) and LDL (Fig. 3, right). Electrophoresis of isopropanol supernatants on SDS-PAGE revealed no presence of apoB when either LDL or VLDL were processed (Fig. 3C). In addition, no obvious Coomassie blue-stainable bands (which would indicate the presence of large aggregates) were noted in the stacking gel. Thus, apoB precipitation was complete. In the sample shown on the gel, the apoE peak was small, indicating some possible loss or degradation during processing. Electrophoresis after resolubilization of the isopropanol precipitate disclosed no apoE and very little contami-

TABLE 2. Proportion of radioactivity bound to lipid in apoB precipitates

	Isopropanol Method	TMU Method
VLDL apoB	1.02 ± 0.11%	0.46 ± 0.07%
LDL apoB	0.72 ± 0.07%	0.12 ± 0.04%

VLDL apoB and LDL apoB precipitates obtained as described in Methods for specific activity measurement were extracted with chloroform–methanol 2:1 for 30 min. Values are mean ± SEM (N = 6) of lipid radioactivity/total radioactivity.

nation (4%) by apoC (Fig. 3B). The TMU precipitates contained similar proportions of apoC (data not shown).

Amounts of lipid-bound radioiodine found in the apoB precipitate were quantified (**Table 2**). In all cases lipids accounted for less than 1% of total radioactivity. In addition there was no detectable lipid mass after isopropanol extractions. To determine whether triglyceride was co-precipitated with apoB when isopropanol techniques were employed on VLDL from patients with hypertriglyceridemia, a patient with VLDL-triglyceride of 2229 mg/dl was studied. [^{3}H]Triolein in a small volume of ethanol was added to the VLDL preparation (2470 μg of protein/ml) and the apoB was precipitated using isopropanol as described above. Less than 1% of ^{3}H-radioactivity was associated with the apoB pellet after the usual washing procedure. Efficient delipidation required dispersing the pellet completely (using a glass rod if necessary) and agitating frequently during incubation. This suggests that efficient removal of triglyceride can be accomplished using these procedures, even in patients with high triglyceride. However, the apoB precipitation using isopropanol has not been verified using lipoproteins from patients with a variety of hyperlipemias or disorders of apoprotein composition.

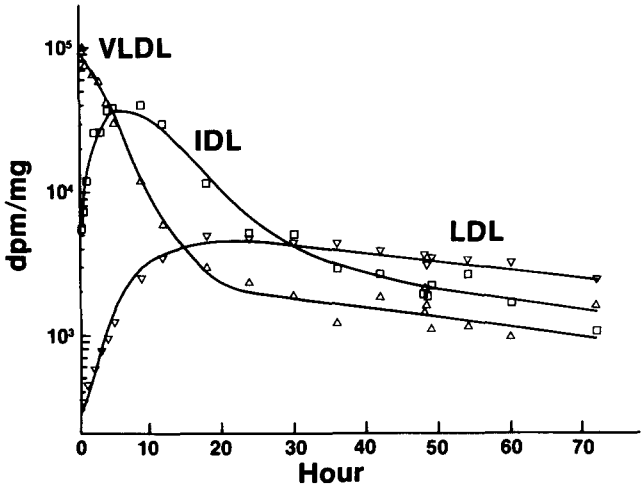

Fig. 4. ApoB specific activity of VLDL, IDL, and LDL after injection of ^{131}I-labeled VLDL. Data were obtained from a normolipemic volunteer, and the curves were generated for VLDL (△ – – – △), IDL (□ – – – □), and LDL (▽ – – – ▽) by a multicompartmental model as described by Phair et al. (16).

The time courses of specific activities of ^{131}I-labeled apoB in VLDL, IDL, and LDL in a representative metabolic study using isopropanol are shown in **Fig. 4.** Although confirmation of apoB pool size requires radioimmunoassay or other direct quantitative techniques, the isopropanol method yields specific activity and concentration data suitable for metabolic studies. The high ionic strengths in the LDL and IDL fractions made efficient precipitation of apoB more difficult; this could be overcome by lowering the incubation temperature to 4°C. No difference was found in contamination of apoB with other apolipoproteins when precipitates obtained at 25°C or 4°C were examined using SDS-PAGE (data not shown).

The above results indicate that isopropanol is equally as effective as TMU for precipitating apoB and for measurements of its concentration and specific activity. Compared to TMU, isopropanol is cheaper and more easily maintained in pure form; this assures more consistent results in long term studies. Furthermore, isopropanol has two other distinct advantages over TMU: it does not interfere with the protein measurement by the Lowry procedure, and the apoB precipitated by isopropanol is solubilized in NaOH or electrophoresis buffer more easily.

We wish to express our appreciation to Dr. Gloria Vega for supplying hyperlipidemic plasma, to Dr. Julian Marsh for discussions of apoB techniques, and to Dr. Barbara Vasquez for her advice on electrophoresis procedures. We also thank Ms. Verna Kuwanhoyioma for help in preparation of the manuscript.

Manuscript received 26 November 1982 and in revised form 6 May 1983.

REFERENCES

1. Kane, J. P. 1973. A rapid electrophoretic technique for identification of subunit species of apoproteins in serum lipoproteins. *Anal. Biochem.* **53:** 350–364.
2. Kane, J. P., T. Sata, R. L. Hamilton, and R. J. Havel. 1975. Apoprotein composition of very low density lipoproteins of human serum. *J. Clin. Invest.* **56:** 1622–1634.
3. Le, N. A., J. S. Melish, B. C. Roach, H. N. Ginsberg, and W. V. Brown. 1978. Direct measurement of apoprotein B specific activity in ^{125}I-labeled lipoproteins. *J. Lipid Res.* **19:** 578–584.
4. Holmquist, L., and K. Carlson. 1977. Selective extraction of human serum very low density apolipoproteins with organic solvents. *Biochim. Biophys. Acta.* **493:** 400–409.
5. Holmquist, L., K. Carlson, and L. A. Carlson. 1978. Comparison between the use of isopropanol and tetramethylura for the solubilization and quantitation of human

serum very low density apolipoproteins. *Anal. Biochem.* **88:** 457–460.
6. Sparks, C. E., and J. B. Marsh. 1981. Analysis of lipoprotein apoproteins by SDS-gel filtration column chromatography. *J. Lipid Res.* **22:** 514–518.
7. Sparks, C. E., and J. B. Marsh. 1981. Metabolic heterogeneity of apolipoprotein B in the rat. *J. Lipid Res.* **22:** 519–527.
8. Huff, M. W., N. H. Fidge, P. J. Nestel, T. Billington, and B. Watson. 1981. Metabolism of C-apolipoproteins: kinetics of C-II, C-III_1, and C-III_2, and VLDL-apolipoprotein B in normal and hyperlipoproteinemic subjects. *J. Lipid Res.* **22:** 1235–1246.
9. Nestel, P. J., T. Billington, and B. Smith. 1981. Low density and high density lipoprotein kinetics and sterol balance in vegetarians. *Metabolism.* **30:** 941–945.
10. Nestel, P., N. Tada, T. Billington, M. Huff, and N. Fidge. 1982. Changes in very low density lipoproteins with cholesterol loading in man. *Metabolism.* **31:** 398–405.
11. McFarlane, A. S. 1958. Efficient trace-labelling of proteins with iodine. *Nature (London).* **182:** 53.
12. Langer, T., W. Strober, and R. I. Levy. 1972. The metabolism of low density lipoprotein in familial Type II hyperlipoproteinemia. *J. Clin. Invest.* **51:** 1528–1536.
13. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226:** 497–509.
14. Markwell, M. A. K., S. M. Haas, L. L. Bieber, and N. E. Toebert. 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal. Biochem.* **87:** 206–210.
15. Bamburg, J. R., E. M. Shooter, and L. Wilson. 1973. Assay of microtuble protein in embryonic chick dorsal root ganglia. *Neurobiology.* **3:** 162–173.
16. Phair, R. D., M. Hall III, D. W. Bilheimer, R. I. Levy, R. Goebel, and M. Berman. 1976. Modeling lipoprotein metabolism in man. *Proc. Summer Simulation Conference.* July 1976.