# Direct measurement of apoprotein B specific activity in $^{125}$I-labeled lipoproteins[1]

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**Abstract** A method for determining apoprotein B specific activity in radioiodinated lipoproteins is described and validated. It utilizes organic solvents and tetramethylurea in the isolation of apoprotein B from other radiolabeled contaminants, both lipid and protein, in exogenously labeled VLDL. The contaminants are also removed from those lipoprotein classes subsequently derived from VLDL, namely IDL and LDL. The procedure requires approximately 50 $\mu$g of apoB per analysis, allowing specific activity determinations in triplicate on 3-ml plasma samples with a standard error of <6%. Finally, data from a study of apoprotein B turnover in VLDL, IDL, and LDL in a human subject is presented to demonstrate the potential of this method in further elucidating the kinetic interrelationships between these lipoprotein classes.

**Supplementary key words** lipoprotein turnover · very low density lipoproteins · intermediate density lipoproteins · low density lipoproteins · 1,1′,3,3′-tetramethylurea

Practical, suitable, and accurate methods for measuring specific activity of individual apoprotein components of the lipoproteins are necessary to increase our understanding of normal lipoprotein metabolism and the abnormalities that result in hypertriglyceridemia and/or hypercholesterolemia. Apoprotein B (apoB) represents approximately 40% of the protein mass of human very low density lipoproteins (VLDL, d < 1.006 g/ml) and is the major protein component of intermediate and low density lipoproteins (IDL, d 1.006–1.019 g/ml and LDL, d 1.019–1.063 g/ml) (1–4). Monitoring the specific activity of radiolabeled apoB in these lipoprotein classes provides a means for defining the kinetic parameters of apoB metabolism in normal individuals and in patients with hyperlipoproteinemia. Although limited by methodology, previous studies of apoB turnover have provided information concerning VLDL synthetic and catabolic rates (4–8) and have supported the concept that a majority of apoB in human VLDL eventually appears in LDL prior to final catabolism (4–6).

Radioiodination has successfully been used to label LDL for use as a tracer in LDL turnover studies. Since less than 5% of the label appears in the lipid moiety and more than 95% appears in apoB, the LDL apoB specific activity has been considered equivalent to whole LDL specific activity (9, 10). As a result, the plasma decay curve for this apolipoprotein possesses the same characteristics as the LDL decay curve. In contrast, radioiodination of VLDL results in labeling of other apoprotein components. In addition, a larger proportion of lipid labeling is found with VLDL than with LDL. Radioactivity in apoB may represent only 50% of the total label in VLDL (4–7). Therefore, studies of apoB metabolism after injection of $^{125}$I- or $^{131}$I-labeled VLDL require the isolation of apoB from the other labeled components to permit accurate specific activity measurements.

In this report, we refine and extend apoB turnover methodology by describing and validating a simple, reproducible procedure for determining apoB specific activity in VLDL, IDL, and LDL after injection of $^{125}$I-labeled VLDL. This procedure has the necessary characteristics of isolating apoB from other radiolabeled apoproteins and lipids and of permitting multiple apoB specific activity determinations on lipoproteins isolated from small plasma samples. This method utilizes the observation of Kane (11) that other VLDL apoproteins can be solubilized and quantitated in 1,1′,3,3′-tetramethylurea (TMU), leaving apoB behind as a precipitate. In previous reports, TMU has been used to *indirectly* quantitate apoB in VLDL and in other lipoproteins (2, 6) as the difference between soluble and total protein. We have

Abbreviations: ApoB, apoprotein B; VLDL, very low density lipoproteins; IDL, intermediate density lipoproteins; LDL, low density lipoproteins; TMU, 1,1′,3,3′-tetramethylurea; SDS, sodium decylsulfate; EDTA, disodium ethylenediamine tetraacetate.

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used this reagent to isolate apoB from delipidated lipoproteins for *direct* determination of radioactivity and protein mass and thereby to calculate apoB specific activity.

## METHODS

### Iodination of VLDL

After a 12–16 hr fast, volunteer subjects underwent plasmapheresis of 500 ml of blood, using disodium ethylenediamine tetraacetate (EDTA) (1.0 mg/ml) as anticoagulant. VLDL was separated from plasma by ultracentrifugation (Spinco, Model L2-65B) using an SW-27 rotor, followed by further purification and concentration using an SW-40 rotor. Each separation was effected at plasma density ($d < 1.006$ g/ml) at 100,000 *g* for at least 16 hr at 15°C. Approximately 4 ml of VLDL solution (4–8 mg VLDL protein/ml) was collected and dialyzed against four 1-liter volume changes of 0.9% NaCl (with EDTA 0.1 mg/ml, pH 7.4). Iodination was then performed according to the method of McFarlane (12) as utilized by Bilheimer, Eisenberg, and Levy (4). Efficiency of iodination averaged 15% (range: 7–35%). Less than 1% of the radioactivity was free $^{125}$I after 12 dialysis bath changes of the above saline solution. Three to 12% of the radioactivity could be extracted with chloroform-methanol 2:1. After dialysis the resulting $^{125}$I-labeled VLDL solution contained on the average 25 $\mu$Ci/mg of whole VLDL protein. Pre- and post-iodinated VLDL migrated as a single pre-beta band on agarose gel electrophoresis (13) and showed no qualitative differences by electron microscopic examination (14, 15). By using sequential ultracentrifugation of plasma at appropriate salt densities (16), LDL was similarly isolated for iodination and comparison in our apoB specific activity procedure.

### Specific activity measurement of apoB in $^{125}$I-labeled lipoproteins

*Lipid extraction.* Aliquots of the labeled lipoprotein preparations were transferred to 12 × 75 mm disposable glass tubes according to protein mass. In the standard procedure, 100–150 $\mu$g of VLDL, 50–100 $\mu$g of IDL, and 30–50 $\mu$g of LDL protein were used. The total volume of the aliquots did not exceed 500 $\mu$l. These quantities were selected to assure accurate specific activity determinations. The aliquots were sequentially extracted, using 2.0 ml of acetone and 2.0 ml of isopropanol. Each tube was thoroughly mixed and subjected to 1–2 min of sonic irradiation in an Ultrasonics System bath sonicator with fixed power setting. After centrifugation at 1000 *g* for 30 min at 10°C, the supernatant solvent was then removed, leaving about 0.5 ml of liquid and a visible protein precipitate.

*Isolation of Apo-B.* One ml of redistilled 1,1′,3,3′-tetramethylurea (Sigma Chemical Co., St. Louis, MO) (TMU) was added to each tube of delipidated protein. The contents of the tube were mixed and incubated overnight at room temperature to insure complete solubilization of the protein pellet. One ml of $H_2O$ was then added and mixed, producing a precipitate which was usually not visible. After centrifugation (1000 *g*) the supernatant TMU was aspirated, leaving approximately 0.5 ml of liquid overlying the pellet. A second addition of 1 ml of 9 M TMU was then made and the sample was mixed (Vortex mixer). After 30 min at room temperature, 1 ml of distilled water was added and centrifugation was repeated as above. The pellet remaining after careful aspiration of the supernatant was washed with 3.0 ml of distilled $H_2O$ to remove residual TMU which interferes with the Lowry protein estimation (11). In the case of VLDL, 15–40 $\mu$g of protein (50–75% of the initial apoB) remained in the tube at the end of the procedure. This working range of VLDL and LDL was selected so that the final protein pellet could be measured in the linear range of absorbance of bovine serum albumin (up to 40 $\mu$g). The protein pellet was completely dissolved after overnight incubation in 2.0 ml of "reagent C" of the procedure of Lowry et al. (17). Radioactivity was determined in a Nuclear Chicago gamma counter (Model #1085, 2″ sodium iodide crystal, dual channel) with 60% efficiency. Folin phenol reagent (0.2 ml) was then added and the absorbance was determined after 30 min at 750 nm for protein estimation, using bovine serum albumin as standard.

A modification of the above procedure permits the determination of apoB specific activity from larger amounts of VLDL or LDL total protein. After TMU extraction of as much as 500 $\mu$g of total VLDL protein or 400 $\mu$g of total LDL protein the residual apoB can be dissolved in reagent C (Lowry et al.) and the radioactivity can be measured. However, in this situation, each sample must be divided after the addition of reagent C into smaller aliquots for protein and radioactivity measurements. These aliquots are selected such that their protein content falls within the linear range of the protein standard curve (up to 40 $\mu$g equivalence with bovine serum albumin). Each aliquot requires individual determination of radioactivity, as significant internal quenching is noted with the larger masses of apoB. The sums of protein and radioactivity determinations in the aliquots from these larger samples provide the necessary data for accurate

TABLE 1. TMU extraction of apoB from radioiodinated VLDL and LDL: constant apoB specific activity over a range of lipoprotein protein masses[a]

| | Initial Protein Mass | Pellet Mass | Specific Activity | Estimated Recovery ApoB from Pellet[c] |
|---|---|---|---|---|
| | *μg* | *μg apoB*[b] | *decays/min/μg*[b] | |
| VLDL | 137.0 n = 8 | 42.91 ± 1.17 | 14,064 ± 185 | 70% |
| | 170.0 n = 8 | 54.81 ± 0.74 | 14,338 ± 191 | 72% |
| | 200.0 n = 10 | 56.80 ± 0.44 | 14,120 ± 97 | 63% |
| LDL | 60.0 n = 8 | 31.56 ± 1.83 | 389 ± 12 | 55% |
| | 75.0 n = 10 | 56.18 ± 1.42 | 383 ± 9 | 79% |
| | 90.0 n = 10 | 68.08 ± 1.93 | 392 ± 10 | 80% |

[a] Increasing amounts of $^{125}$I-labeled VLDL and $^{125}$I-labeled LDL total protein (μg) obtained from individually isolated and labeled VLDL and LDL were separately subjected to the TMU procedure. The mean values of apoB specific activity from different initial amounts of $^{125}$I-labeled VLDL (true also for $^{125}$I-labeled LDL) were statistically equivalent as determined by the nonpaired Student *t* test. This confirms that the TMU methodology provides a reproducible apoB specific activity value over the ranges of initial VLDL and LDL easily available from the turnover samples.

[b] Mean ± SE.

[c] Assuming that apoB comprises 0.45% of the protein mass of VLDL and 0.95% of the protein mass of LDL, the estimated apoB recovery (%) = ((μg protein from TMU pellet)/(fraction apoB × μg initial lipoprotein)) × 100.

specific activity measurements in these larger samples. However, the procedure becomes more laborious and difficult under these circumstances and is not necessary unless the VLDL apoB specific activity is very low or unless quantities of lipoprotein in excess of 40 μg are obtained after extraction.

*Other methods.* Electrophoresis was performed in 7.5% polyacrylamide (18) using buffers containing 8.0 M urea (pH 8.9). Gel permeation chromatography (Sephadex G-150) was done as previously described (19) using a column 1.2 × 110 cm. Ouchterlony radial immunodiffusion (19) was performed in 1% agarose gel using specific antisera to apoB prepared in sheep, and to apoE (arginine-rich apolipoprotein), apoC-II, and apoC-III polypeptides[3] prepared in goats. The final TMU pellet was solubilized in 100 mM sodium decylsulfate (SDS) and later dialyzed to 20 mM SDS (pH 8.2) prior to performing these procedures.

*Turnover studies.* After giving informed consent, male subjects, stabilized on weight maintenance diets on a metabolic ward, underwent plasmapheresis. $^{125}$I-labeled VLDL was prepared as previously described maintaining aseptic conditions. After filtration through a 0.22-μm Millipore filter, 60–80 μCi of $^{125}$I-labeled VLDL in 5% albumin was injected intravenously. Ten-ml volumes of venous blood were collected (up to 25 samples) in tubes containing EDTA (0.1 mg/ml) at frequent intervals for 48 hr. Three-ml volumes of plasma were adjusted to a total volume of 6.0 ml with NaCl solution d 1.006 g/ml and centrifuged at 100,000 *g* for 24 hr in a Beckman 40.3 rotor. The top 3.0 ml was removed by sequential aspiration into two fractions: 0.5 ml of concentrated lipoprotein (for specific activity determinations), and 2.5 ml of a clear middle phase. Both fractions were shown by agarose electrophoresis to contain only lipoprotein of pre-beta mobility. The sum of the radioactivity in the two fractions was taken to represent total VLDL counts in 3 ml of that plasma sample. The remaining infranate was adjusted to d 1.019 g/ml by the addition of 3.0 ml of NaCl–KBr solution (d 1.034 g/ml). After respinning, IDL was isolated and processed (16) in a fashion similar to VLDL. LDL was similarly collected after centrifugation at d 1.063 g/ml.

The uppermost ultracentrifugal fraction (0.5 ml) of VLDL, IDL, and LDL were each diluted with water to a final volume of 1.0 ml. Protein determination (17) was performed on each sample. Similarly, aliquots of all samples were used for duplicate or triplicate specific activity determinations of apoB. The method of Kane (11) was used for quantitative determination of the percent of apolipoprotein represented by apoB.

## RESULTS

Increasing the VLDL total protein from 100 to 170 μg per sample and the LDL total protein from 30 to 90 μg per sample resulted in no change in apoB specific activity as determined by the procedure (**Table 1**). These results suggest that 25–50% of the apoB was lost from each sample during delipidation and TMU extraction. However, the isolation procedure provided adequate recoveries of apoB for specific activity measurement. Furthermore, the two-step delipidation removed $^{125}$I-labeled lipid contamination, because less than 1% of the radioactivity could be further extracted from the pellet with a methanol wash.

When $^{125}$I-labeled VLDL was added in increasing

[3] Several nomenclatures have been proposed for the other apolipoproteins of VLDL and IDL (19). These antisera were chosen for their availability and because they covered a spectrum of apoproteins other than apoB that might have remained after our TMU extraction procedure.

proportion to unlabeled VLDL while holding the total protein of the mixture constant, the predicted linear increase in specific activity was observed (**Fig. 1**). This clearly indicated that the extraction method does not distinguish between iodinated and native VLDL apoB. A similar result was obtained when apoB specific activity was determined in mixtures of labeled and unlabeled LDL.

The specificity of apoB isolation was demonstrated by several methods. First, the supernate from the first TMU wash (TMU-soluble fraction) was subjected to polyacrylamide gel electrophoresis, and the majority of the radioactivity entered the gel (**Fig. 2**). This is characteristic of all known apolipoproteins (2, 11) other than apoB. Two washes with TMU were required to remove all of the soluble apoproteins that entered the gel (Fig. 2*A*). This was confirmed by analysis of the SDS-solubilized pellet remaining after two TMU washes, because electrophoresis in the same system revealed no radioactive material entering the gel (Fig. 2*B*). All radioactivity remained at the interface of the stacking and running gel, a finding characteristic of apoB which is too large to enter 7.5% polyacrylamide gels (11). Second, gel filtration chromatography (Sephadex G-150) elution patterns of radioactivity remaining after two TMU washes of LDL and VLDL were the same. Third, Ouchterlony immunodiffusion patterns using the SDS-solubilized pellets (up to 100 $\mu$g of protein) and specific antisera to

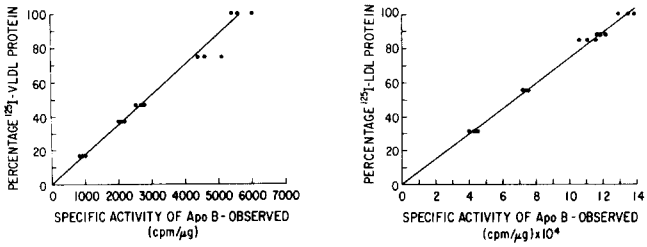


**Fig. 1.** Demonstration of effect on specific activity determination by addition of unlabeled to $^{125}$I-labeled lipoproteins. With the total lipoprotein protein content held constant (150 $\mu$g), $^{125}$I-labeled VLDL and unlabeled VLDL from the same individual were mixed in the percentage proportions noted on the ordinate, with 100% representing only labeled VLDL (left panel). Increasing the percentage of labeled to total VLDL protein shows a linear increase in VLDL apoB specific activity (correlation coefficient, $r^2 = 0.98$). The same relationship held true (right panel) when a constant amount of LDL total protein was made up of various percentages of labeled and unlabeled LDL ($r^2 = 0.99$). These data provide strong evidence that apoB in labeled and unlabeled VLDL and LDL is isolated with equal efficiency in the TMU procedure.

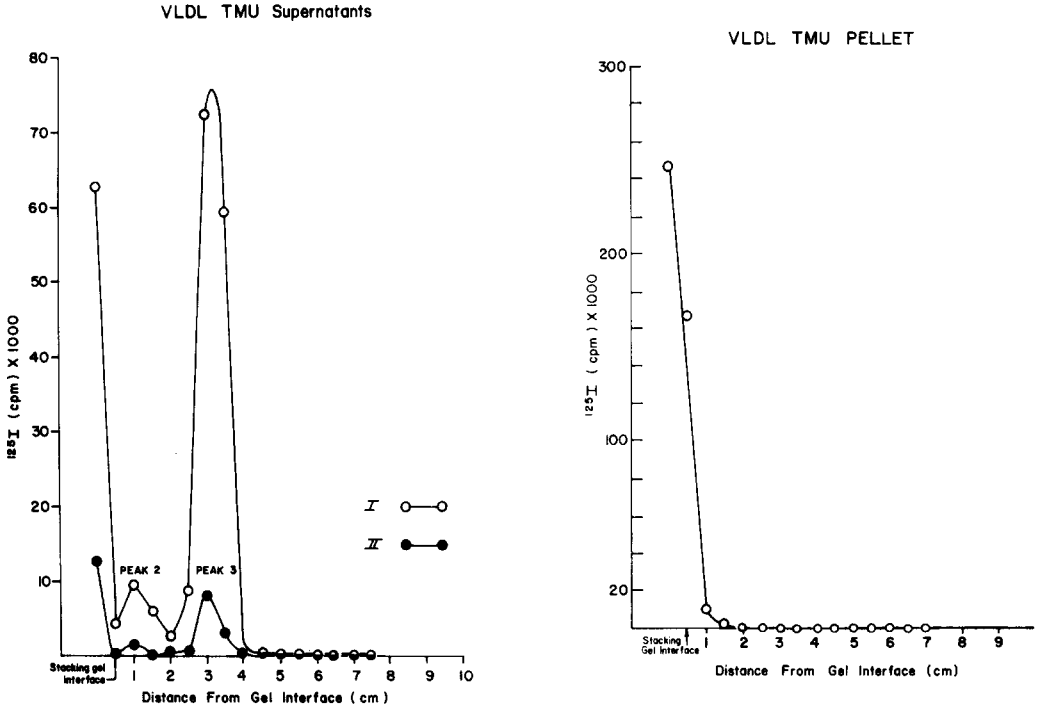


**Fig. 2.** The movement (abscissa) of radioactivity (ordinate) as labeled apoproteins from $^{125}$I-labeled VLDL into a 7.5% polyacrylamide gel (containing 8.0 M urea) after TMU extraction. The pattern of radioactivity from two successive TMU washes of lipid-extracted $^{125}$I-labeled VLDL (first wash, open circles; second wash, closed circles) is shown in the left panel. The majority of the counts enter the running gel, a characteristic of apoE and apoC. As a small number of counts entered the gel from the second wash, two TMU washes were included in our procedure to assure complete removal of all apoproteins except apoB. The SDS-solubilized residual pellet after two TMU washes (right panel) was electrophoresed in an identified system and no radioactivity was found to enter the running gel, a characteristic of apoB.

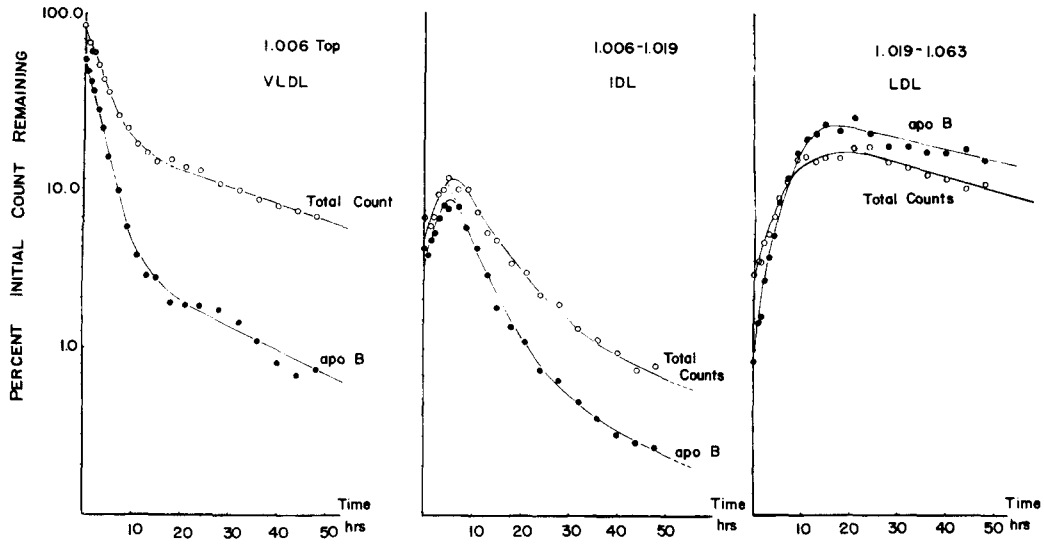


**Fig. 3.** A representative $^{125}$I-labeled VLDL turnover study in a 55-year-old male subject with phenotypic type IV hyperlipoproteinemia (patient R.R.). From left to right, these panels present data related to VLDL, IDL, and LDL. The decay in radioactivity in total plasma VLDL, IDL, and LDL (open circles), as well as the radioactivity of the total mass of apoB in these classes (closed circles) is plotted against time and is expressed as a percentage of whole plasma radioactivity at 5 min after injection of a tracer amount of $^{125}$I-labeled VLDL. These total apoB counts in each lipoprotein class equal apoB specific activity × total mass of protein × fraction apoB. The total mass in each lipoprotein fraction is calculated by multiplying the plasma volume by the average lipoprotein concentration during the study. The fraction apoB is determined by the standard Kane procedure: ((total lipoprotein protein) − (TMU-soluble apoprotein))/(total lipoprotein protein). The lipoproteins containing the least apoB (VLDL and IDL) show the most difference between the decay of total radioactivity and apoB radioactivity. At least two exponentials in the decay of VLDL apoB and IDL apoB are easily seen.

apoB, apoC-II, apoC-III, and apoE polypeptides showed precipitin bands only against apoB. Studies with purified apolipoproteins and with whole VLDL solubilized in identical concentrations of sodium decylsulfate (20 mM) indicate that only 1 $\mu$g of apoE and apoC-II were required for immunoprecipitin lines. Thus, the results with the solubilized TMU pellets indicate that less than 1% contamination was present in these peptides. Finally, when $^{125}$I-labeled VLDL was delipidated with organic solvents and then chromatographed on Sephadex G-150 to isolate the apoB (19), the specific activity of the protein in 12 aliquots of the void volume fraction was 6273 ± 129 cpm/$\mu$g. This compared to a specific activity of 6120 ± 247 cpm/$\mu$g (n = 11) for the TMU pellet obtained from the same VLDL sample.

**Fig. 3** depicts the results from a representative $^{125}$I-labeled VLDL turnover study in a subject with phenotypic Type IV hyperlipoproteinemia. The specific activity values for apoB were based on determinations made in triplicate on each lipoprotein fraction from each plasma sample. For comparison purposes, the decay in radioactivity in whole VLDL, IDL, and LDL, as well as the total counts in the apoB mass in each of these lipoprotein fractions, are plotted against time and expressed as a percentage of total plasma counts at 5 min after injection. These total apoB counts are derived from apoB specific activity data, the total mass of protein, and the proportion of apoB protein in each lipoprotein fraction. It is clear

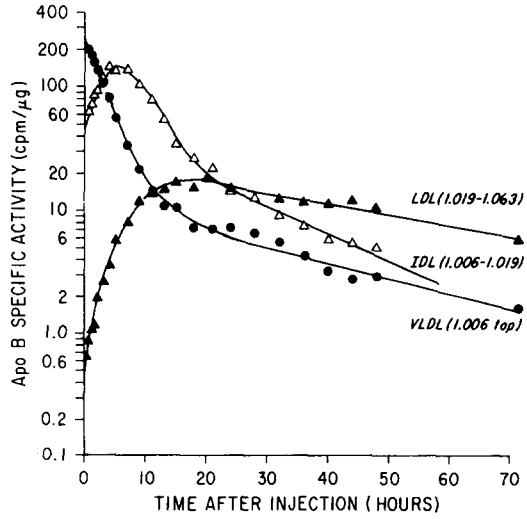


**Fig. 4.** ApoB specific activity curves of VLDL (open circles), IDL (open triangles), and LDL (closed triangles) obtained in the turnover study depicted in Fig. 3.) There is more than a 3-hr difference between the time of the maximum specific activity for IDL apoB and the time of the identical value for VLDL apoB specific activity.

that at least two exponential components are seen in the curve describing the decay of apoB radioactivity in both VLDL and IDL. In addition, the lipoproteins containing proportionately the least apoB (VLDL and IDL) showed the largest difference between the decay in total radioactivity and in apoB radioactivity. In contrast, the curves for radioactive decay in total LDL and LDL apoB were, as expected, quite similar.

In **Fig. 4,** the relationship between plots of specific activity for apoB in VLDL, IDL, and LDL obtained during the same study are shown. A difference of more than 3 hr between the time of the maximum specific activity of IDL apoB and the time of corresponding value for VLDL apoB specific activity was noted in this study. Similar delays have been observed in preliminary analysis of subsequent studies and have been reported previously by other investigators (20). This delay is not compatible with a simple precursor–product relationship between apoB in VLDL and in IDL (21).

## DISCUSSION

In previously reported VLDL apoB turnover studies in humans, several procedures have been applied to the isolation of apoB from exogenously labeled VLDL and to the determination of mean percent VLDL apoB composition. For example, Bilheimer et al. (4) used SDS–polyacrylamide gel electrophoresis and gel chromatography in these measurements. More recently, Sigurdson, Nicoll, and Lewis (5, 6) employed Sephadex G-150 column chromatography in both measurements. Both groups were able to demonstrate the appearance of radioactive apoB in LDL after injection of labeled VLDL, implying a precursor–product relationship between these lipoprotein classes. However, the disadvantages of these methods are considerable, including the large sample volume required as well as the extensive time and effort necessary to analyze each sample.

It would be attractive to directly apply the Kane (11) TMU procedure to turnover studies using radioiodinated VLDL. In recently reported studies (7), apoB specific activity was obtained by counting whole $^{131}$I-labeled VLDL, subtracting the radioactivity counts in the TMU-soluble material, and dividing this difference by the apoB estimated to be in the insoluble fraction. However, this methodology essentially neglects the contamination by iodinated lipid. In our experience and in that of Bilheimer et al. (4), up to 14% of the total VLDL radioactivity may be found in iodinated lipid. Consequently, an overestimate of apoB specific activity may result. Contamination to this extent may have a significant impact on mathematical modeling of apoB kinetics. To circumvent lipid contamination, other investigators have endogenously labeled VLDL with $^{75}$Se-labeled methionine (22–24) or [$^{3}$H]-labeled lysine (8). However, the interpretation of such data from human studies to date is quite difficult to analyze mathematically because of the inherent complexity of endogenous labeling. Furthermore, such data do not allow complete analysis of the source of apoB input into the higher density classes, IDL and LDL. Thus, precursor–product relationships between VLDL apoB and apoB in the other lipoprotein density classes cannot be easily studied with these methods.

The methodology presented in this report specifically isolates apoB without contamination from labeled lipid. It is quite reproducible, permitting multiple determinations of apoB specific activity on small quantities of lipoprotein. Thus, frequent small plasma samples, desirable for mathematical modeling of complex turnover kinetic parameters, can be obtained without significantly reducing the plasma pool size of the lipoprotein. Since there is no recycling of $^{125}$I-labeled VLDL apoB and since injected $^{125}$I-labeled VLDL serves as the only source of tracer, the precursor–product relationships between the apoB-containing lipoproteins can be studied. The methodology thus satisfactorily fulfills requirements for detailed apoB turnover studies in human subjects using exogenously labeled autologous radioiodinated VLDL. Finally, this technique provides for the recovery of the VLDL lipids and the TMU-soluble apoproteins in each sample of plasma obtained, thus allowing for further studies of these components.

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