Incorporation of a stable isotopically labeled amino acid into multiple human apolipoproteins

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Abstract Procedures are presented for the separation and determination of the isotopic enrichment of multiple human apolipoproteins labeled in vivo with a stable isotope amino acid. The isotopic enrichments of plasma lysine and plasma apolipoproteins were monitored for 16 days after a single intravenous dose of [4,4,5,5-$^2H_4$]lysine (5 mg/kg body weight). The use of a multiply deuterated amino acid enabled the measurement of isotopic enrichments above background over the entire 16-day time course in all proteins. Individual apolipoproteins were separated on a specially designed gradient sodium dodecyl sulfate polyacrylamide gel electrophoresis system cast in a conventional slab gel apparatus which resolved apoB-100, apoE, apoA-I, apoA-II, apoC-I, apoC-II, apoC-III-1, and apoC-III-2 on a single gel. After staining with Coomassie blue, proteins bands (containing 5 to 30 μg of individual apolipoprotein) were excised from the gel. Amino acids were recovered from hydrolyzed gel slices, derivatized, and analyzed by gas chromatography–mass spectrometry for determination of lysine isotopic enrichments. The utility of the method is demonstrated using examples of apolipoproteins B-100, A-I, A-II, C-I, C-II, and C-III from either total plasma d < 1.21 g/ml lipoproteins or selected lipoprotein subfractions. Lysine isotopic enrichments of proteins were generally determined with a precision of better than 5%. The isotopic enrichment profiles were consistent with literature reports of apolipoprotein metabolic kinetics based on the use of radioiodinated apolipoproteins. The procedures outlined can be used to separate and measure the isotopic enrichment of virtually any apolipoprotein from any chosen lipoprotein fraction. Thus, these procedures should find wide application in the study of apolipoprotein metabolic kinetics. —**Patterson, B. W., D. L. Hachey, G. L. Cook, J. M. Amann, and P. D. Klein.** Incorporation of a stable isotopically labeled amino acid into multiple human apolipoproteins. *J. Lipid Res.* 1991. **32:** 1063–1072.

Supplementary key words apoA-I • apoA-II • apoB-100 • apoC-I • apoC-II • apoC-III • [4,4,5,5-$^2H_4$]lysine

The regulation of lipid metabolism is achieved by controlling lipid absorption, biosynthesis, transport, and clearance from plasma. Multiple apolipoproteins are crucial to the formation and secretion of plasma lipoprotein particles and to the maintenance of their structural integrity; hence, apolipoproteins are critical in the regulation of lipid transport. In addition, specific apolipoproteins modify the activities of enzymes (e.g., lipoprotein lipase and lecithin:cholesterol acyltransferase) that are responsible for lipoprotein interconversions within plasma, and they also direct plasma lipoprotein clearance through receptor-mediated endocytotic processes. Thus, a comprehensive understanding of the coordinated metabolism of multiple apolipoproteins is essential to understand the regulation of lipid metabolism under different physiologic conditions.

Knowledge of apolipoprotein synthesis and turnover rates is central to understanding apolipoprotein metabolism. Most studies of apolipoprotein kinetics have relied on the analysis of the catabolism of apolipoproteins labeled with radioiodine. In addition, most of these studies have focused on single apolipoproteins (1–3), although simultaneous investigations of multiple radioiodinated apolipoproteins may be performed if they are separated before analysis (4–6) or initially labeled with different isotopes (7).

Exogenous labels measure apolipoprotein production rates indirectly as the product of fractional catabolic rates and steady-state pool size. These exogenous labels are particularly effective when used to examine details of metabolic conversions and catabolic pathways (4, 5, 8). A continuing concern, however, is that iodination itself may alter apolipoprotein physical properties, including apolipoprotein-lipid interactions, and thus may alter the metabolic kinetics of the tracer protein (9, 10).

Endogenous labeling with tracer amino acids avoids the potential complications of altered physical properties as a result of chemical modifications, and it has the potential

Abbreviations: VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; $NaDodSO_4$, sodium dodecyl sulfate; HFBA, heptafluorobutyric anhydride; GC–MS, gas chromatography–mass spectrometry; IDL, intermediate density lipoprotein.

to provide a more direct measure of biosynthesis through tracer incorporation rates. Furthermore, simultaneous labeling of all proteins is achieved using an endogenous labeling strategy, thereby facilitating investigations of multiple proteins. Extensive sample processing is necessary, however, to separate each labeled protein before isotopic enrichment determinations.

Both single doses of radioactive amino acids (11–14) and constant infusion of stable isotope amino acids (15–20) have been used to label apolipoproteins endogenously. With two exceptions (16, 20), these studies have focused on the metabolism of apoB, largely because the isolation of apoB from contaminating apolipoproteins is readily achieved by chemical extraction with isopropanol (21). Kilgore et al. (22) reported a $NaDodSO_4$ polyacrylamide gel electrophoresis technique suitable for the separation and analysis of apolipoprotein B-100 labeled with [^{3}H]leucine. This approach was laborious; it required electroelution of protein from the gel and a large amount (1–2 mg) of purified apolipoprotein for analysis of the tritium content. Cryer et al. (15) used a primed constant infusion of [^{15}N]glycine to monitor metabolism of apoB in very low density lipoprotein (VLDL), but their technique required 5-ml plasma samples to obtain sufficient VLDL for analysis, and isotopic dilution precluded determination of apoB isotopic enrichments in low density lipoprotein (LDL).

We present a method with which to determine the isotopic enrichment of multiple individual human plasma apolipoproteins. Apolipoproteins were separated on a specially designed gradient $NaDodSO_4$ polyacrylamide gel electrophoresis system cast in a conventional slab gel apparatus. Isotopic enrichments were determined on only 5–30 μg protein. We have applied the technique to a novel protocol whereby a single dose of a stable isotopically labeled amino acid ([4,4,5,5-$^2H_4$]lysine) was administered. The sensitivity associated with using a multiply deuterated amino acid enabled isotopic enrichments above baseline to be monitored over a 16-day period for all apolipoproteins examined in VLDL, LDL, and high density lipoprotein (HDL). The utility of the technique is demonstrated by the measurement of isotopic enrichments of apoB-100, apoA-I, apoA-II, apoC-I, apoC-II, and apoC-III. Portions of this work have appeared in abstract form (23, 24). A compartmental kinetic analysis of the isotopic enrichment of apolipoproteins B-100, A-I, and A-II, plus albumin, transferrin, haptoglobin, and transthyretin isolated from the subjects of this study has also appeared in abstract form (25).

MATERIALS AND METHODS

Clinical protocol

Three adult males were studied. Their total plasma cholesterol concentrations ranged from 120 to 260 mg/dl and their total apoB levels ranged from 38 to 84 mg/dl, increasing with total cholesterol levels. Their apoA-I and A-II levels were similar, averaging 90 ± 6 and 30 ± 3 mg/dl, respectively.

Subjects were fasted overnight and for 4 h after the isotope was administered. A baseline blood sample (7 ml) was obtained by venipuncture immediately before isotope administration. Each subject received an intravenous dose (5 mg/kg body weight) of [4,4,5,5-$^2H_4$]lysine-HCl (Merck Sharp & Dohme, St. Louis, MO) at time zero. Venous blood samples (7 ml) were taken at approximately 15-min intervals for the first hour, 30-min intervals for the next 3 h, and then every 4 h over the next 20 h. Samples were then obtained every 24 h for 3 days, and thereafter every 48 or 72 h. A total of 22 to 33 samples was collected over a 16-day period. Subjects consumed their usual diets ad libitum throughout the study.

Stock solutions (concentrated 100-fold) were immediately added to blood samples to produce final concentrations of 0.02% soybean trypsin inhibitor, 0.01% merthiolate, 0.02% sodium azide, and 0.1% disodium EDTA. Plasma was obtained by centrifugation at 1500 *g* at 4°C, divided into 0.5-ml aliquots, and stored at −70°C.

Lipoprotein and apolipoprotein isolation

To demonstrate the application of our methods to various lipoprotein fractions, two lipoprotein isolation procedures were followed. For the first, total lipoproteins were isolated from plasma by ultracentrifugation at a density of 1.21 g/ml. Two ml of phosphate-buffered KBr (d 1.23 g/ml) was added to 200 μl of plasma. Plasma samples were then spun at 80,000 rpm at 5°C for 16 h in a TL-100 table-top ultracentrifuge (Beckman Instruments, Palo Alto, CA) using a TLA-100.3 rotor. The upper 3 mm containing total lipoprotein was removed. For the second approach, a VLDL fraction and a fraction containing intermediate density lipoprotein (IDL) + LDL + HDL were obtained. Phosphate-buffered KBr (d 1.006 g/ml, 1.0 ml) was gently overlaid on 1.0 ml plasma; the sample was spun at 100,000 rpm, 5°C for 2 h in a TLA-100.3 rotor. After recovery of VLDL in the upper 0.7 ml, the infranatant was adjusted to a density of 1.21 g/ml by the addition of solid KBr, the sample was overlaid with 1.0 ml of phosphate-buffered KBr (d 1.21 g/ml), and a combined IDL + LDL + HDL fraction was recovered after ultracentrifugation at 100,000 rpm at 5°C for 6 h. To reduce potential exchange of apoC proteins between VLDL and HDL, blood samples were immediately chilled on ice and processed rapidly; VLDL recovery by ultracentrifugation was started within 2 h of venipuncture for all time points. Recovered lipoproteins were washed 3 times with phosphate-buffered saline and concentrated with Centricon 30 (Amicon, Danvers, MA) centrifugational concentrators. Protein contents of the concentrates were

determined with the BCA assay from Pierce (Rockford, IL).

Apolipoproteins were separated from the total lipoprotein density fraction by $NaDodSO_4$ polyacrylamide gel electrophoresis. Gels (1.5 mm thick) were cast in 20-cm plates (Protean II, Bio-Rad, Richmond, CA) containing the buffers of Laemmli (26). A 6-cm linear gradient from 2.5–18% total acrylamide was pumped into the bottom of the plate and immediately underlaid with a 10-cm zone of 18% total acrylamide (bisacrylamide:acrylamide, 1:20). After polymerization, this separating gel was overlaid with a stacking gel (4 cm) of 3% total acrylamide (bisacrylamide:acrylamide, 1:38). Total apolipoproteins (approximately 100 μg loaded per sample) were electrophoresed at 40 mA/gel for 1.5 h after the tracking dye had eluted from the gel (approximately 5.5 h total run time). Gels were stained for a maximum of 3 h in a solution of 0.02% Coomassie blue, 45% methanol, 10% acetic acid, and were destained with frequent changes of 7% acetic acid until the background was clear. Protein bands were identified by comparison to known standards. Bands of interest were excised from the stained gel and frozen at − 10°C for subsequent determinations of isotopic enrichment. Identification of apoC-III isoforms was assisted by variable sample exposure to neuraminidase.

Hydrolysis of apolipoproteins

Electrophoresis gel slices (approximately 1.5 × 3 × 5 mm) containing a desired apolipoprotein were dried in vacuo for 2 to 3 h. Desiccated gel slices were transferred to 13 × 100 mm Pyrex culture tubes, 0.5 ml of Ultrex 12 N HCl (J. T. Baker, Inc., Phillipsburg, NJ) was added, and the tubes were sealed with Teflon-lined caps. The samples were hydrolyzed at 110°C for 24 h. The tubes were cooled and the contents were carefully transferred to clean 3.5-ml vials. Care was taken to minimize the transfer of gel degradation products. The hydrolysis tubes were washed once, and the wash was added to the hydrolysate solution. The hydrolysates were evaporated under nitrogen, and 1.0 ml of 1 N acetic acid was added to each vial. The free amino acids were separated from gel degradation products by cation exchange chromatography.

Purification and derivatization of amino acids

Plasma samples (250 to 500 μl) were buffered with 1.0 ml of 1 N acetic acid. Protein hydrolysate or plasma samples were applied to a 1-ml AG-50W-X8 (H^+ form) cation exchange column (Bio-Rad #143-5441). The resin was washed to neutrality with deionized water. The amino acids were eluted with 2 ml of 3 M ammonium hydroxide, then with 1 ml deionized water. The samples were then evaporated under nitrogen.

Fresh n-propanol–HCl was prepared by the addition of 5 ml of acetyl chloride to 25 ml anhydrous n-propanol at 0°C. The esterification reagent (0.5 ml) was added to each dried sample. The vials were sealed with a Teflon cap and heated at 110°C for 1 h. The samples were evaporated under nitrogen, and 100 μl of heptafluorobutyric anhydride (HFBA) was added to each vial. The samples were heated at 60°C for 20 min. Excess HFBA was evaporated gently under a stream of nitrogen to minimize loss of volatile amino acid derivatives. The samples were dissolved in 400 μl ethyl acetate and sealed in an autosampler vial.

Isotopic abundance measurements of lysine

Isotopic abundances were measured by methane negative chemical ionization gas chromatography–mass spectrometry (GC–MS) using a Hewlett-Packard 5988 instrument (Hewlett-Packard Scientific Instrument Division, Palo Alto, CA). The gas chromatography used a 30 m × 0.32 mm DX-4 capillary column (J & W Scientific, Folsom, CA) with a temperature gradient from 150 to 230°C at 10°C/min. The retention time of the lysine derivative was approximately 7.8 min at a He flow rate of 1.7 ml/min. The isotopic abundance measurements were determined using the [M–HF] ion at *m/z* 560. The instrument was calibrated with suitable standards, and routine isotopic analyses were performed automatically using the instrument autosampler. Data reported here are the mean of triplicate isotopic abundance measurements. The natural [m + 4]/m ratio at *m/z* 564 (0.033 ± 0.003%) served as the standard with which the enriched samples were compared. Data are reported as the molar ratio of the enriched [$^2H_4$]lysine samples, obtained by solving a set of simultaneous linear equations that described the isotopic contributions to each *m/z* value from pure analytical standards (27). The [m + 1]/m and [m + 2]/m ratios were monitored to detect instrument drift during extended periods of unattended operation. Isotopic measurements were repeated when the [m + 1]/m and [m + 2]/m ratios drifted more than ± 1% of their baseline values.

Analytical quality control procedures

The small amount of amino acids anticipated in these studies (1 to 10 nmol) required stringent controls to prevent contamination by bacterial or fungal proteins. Electrophoresis reagents were prepared daily and stored for no more than 24 h to minimize microbial growth. Water quality was monitored frequently to detect excessive amino acid levels. Blank samples were inserted into the analytical scheme at various points to monitor potential contamination at the electrophoresis, hydrolysis, ion exchange, and derivatization stages. Samples from an analytical run were discarded if the amount of lysine in these blanks exceeded 5% of the lysine levels in the protein hydrolysate, determined by the area of the selected ion recording at *m/z* 560. The isolations were repeated when

the source of contamination was identified and eliminated. The relative contamination rarely exceeded 2% for a 10-μg protein hydrolysate.

Reagents and assays

Unless otherwise noted, all reagents were purchased from Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Fairlawn, NJ) and were of standard reagent grade. Agarose-conjugated neuraminidase was purchased from Sigma. Purified marker proteins of apoC-I, apoC-II, and apoC-III-1 were the generous gifts of Dr. Henry Pownall. Purified water from a Milli-Q system (Millipore Corp., Bedford, MA) was used in all procedures. Apolipoproteins were assayed by rocket immunoelectrophoresis using antisera and standards from Calbiochem (San Diego, CA).

RESULTS

Enrichment of plasma lysine

In this study, the appearance of tracer after a single intravenous dose of labeled lysine was monitored over 16 days in multiple apolipoproteins. **Fig. 1** shows the change in the plasma lysine isotopic enrichment during this time period for subjects 2 and 3. A similar kinetic profile was observed for subject 1. Lysine molar ratio enrichments for the earliest time points ranged from 0.25 to 0.58. Lysine enrichment fell rapidly during the first few hours, which reflected the exchange of plasma lysine with freely exchangeable pools of free lysine. Thereafter the rate of fall diminished for 2 to 4 days, as plasma lysine exchanged with less rapidly turning over pools of lysine. After 2 to 4 days, the rate of fall in enrichment was much reduced. Long-term tracer recycling was apparent during this phase, because labeled lysine was returned to the plasma from slowly turning over protein systems. The coefficient of variation for triplicate GC–MS analyses was typically under 5%, smaller than the graph symbols used in this figure.

Apolipoprotein isolations

A special NaDodSO$_4$ polyacrylamide gel electrophoresis procedure was developed (**Fig. 2**) to separate multiple individual apolipoproteins from a given lipoprotein fraction for isotopic enrichment analyses. All of the major and minor apolipoproteins were resolved on a single electrophoretic system with the exception of apoC-II and apoC-III-0. VLDL (lane 1) contained apoB-100, apoE, apoC-III-2, apoC-III-1, apoC-II/C-III-0, and faint traces of apoA-I and apoA-II, but no apoC-I. The combined IDL + LDL + HDL fraction (lanes 5–7) contained apoB-100, apoA-I, apoA-II, apoC-III-2, apoC-III-1, apoC-I, and traces of apoE and apoC-II/C-III-0. Small amounts of albumin and other plasma proteins were present in the combined LDL + HDL fraction, but did not affect apolipoprotein resolution. Digestion by neuraminidase resulted in a progressive loss of apoC-III-2 and apoC-III-1 bands and an increase of apoC-III-0 bands. ApoA-II separated into a doublet. When fully reduced, the doublet disappeared and reappeared between apoC-I and apoC-II. A small amount of the reduced apoA-II was apparently present in nonreduced HDL samples.

Isotopic enrichments of recovered apolipoproteins

Measures of the isotopic enrichment of multiple apolipoproteins isolated by this electrophoretic system are demonstrated in the following figures. Results obtained for the three most prevalent apolipoproteins (B-100, A-I, and A-II) are shown in **Fig. 3**. The proteins for this

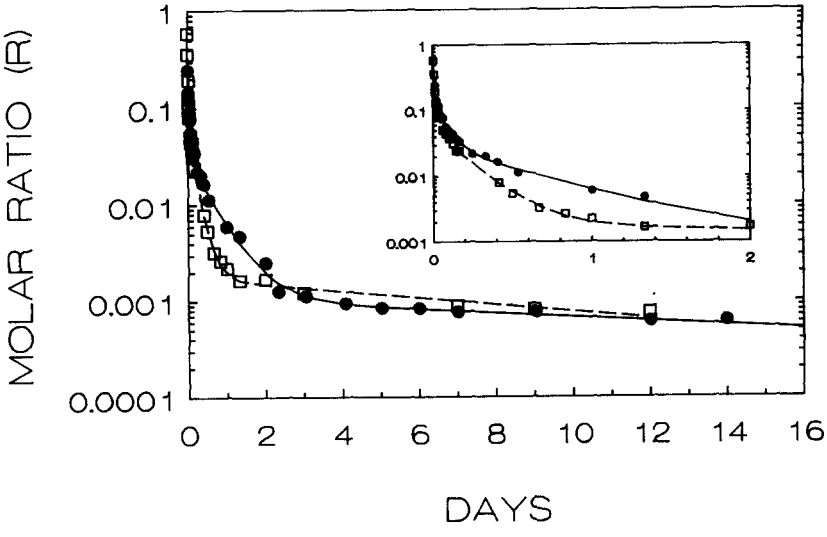

Fig. 1. Isotopic enrichment of plasma lysine. The tracer:tracee molar ratio (R) of plasma lysine was measured up to 16 days after a single intravenous administration of [$^2H_4$]lysine (5 mg/kg body weight). Results shown for subjects 2 (circles + solid line) and 3 (squares + dashed line). Inset: first 2 days only.

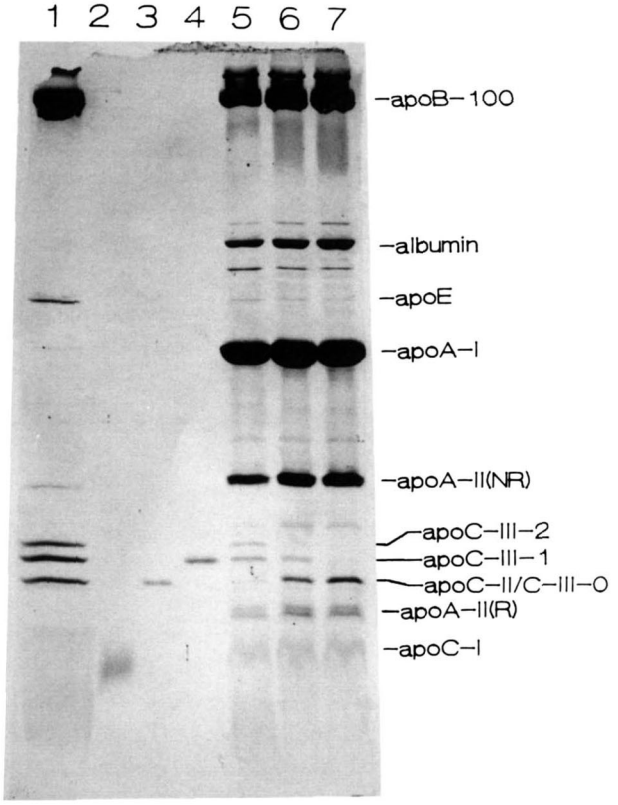

Fig. 2. $NaDodSO_4$ polyacrylamide gel electrophoresis of plasma apolipoproteins. Apolipoproteins were resolved under nonreducing conditions on a hybrid gel containing a 6-cm gradient of 2.5–18% polyacrylamide above a 10-cm zone of 18% acrylamide. See text for additional details. Lane 1: VLDL fraction, d < 1.006 g/ml; lane 2: apoC-I standard; lane 3: apoC-II standard; lane 4: apoC-III-1 standard; lanes 5–7; combined IDL + LDL + HDL fraction, d 1.006–1.21 g/ml. Digestion with neuraminidase: lane 5, none; lane 6, partial; lane 7, complete. ApoA-II was present in both nonreduced (NR) and reduced (R) forms.

demonstration were isolated from the plasma total d < 1.21 g/ml lipoproteins from subject 2. Similar profiles were observed for the other two subjects. Each curve was characterized by a rapid rise to peak isotopic enrichment molar ratios near 0.002 for apoA-I and apoA-II and between 0.004 and 0.008 for apoB for the different subjects. Thereafter, tracer enrichment decreased in a bi-exponential manner. The terminal slopes of all proteins approached the terminal slope of plasma lysine enrichment. The coefficient of variation for triplicate GC–MS analyses of these and all protein samples recovered from polyacrylamide gels was typically less than 5%, although coefficients of variation approached 10% at very low enrichments.

Since apoB-100 is kinetically heterogeneous, it is customary to subfractionate lipoproteins rather than study a single d < 1.21 g/ml fraction. **Fig. 4** demonstrates the isotopic enrichment profiles of apoB isolated from VLDL and a combined IDL + LDL + HDL subfraction. VLDL-apoB achieved a very rapid peak isotopic enrichment, with the highest level of enrichment (0.06) attained of all apolipoproteins in all fractions studied. ApoB enrichment in IDL + LDL rose as the VLDL-apoB enrichment decreased. These profiles demonstrated a typical precursor/product relationship (28), as the (IDL + LDL)-apoB enrichment curve crossed over the VLDL-apoB enrichment curve at the peak of (IDL + LDL)-apoB enrichment. The peak VLDL-apoB enrichment was 8-fold higher than (IDL + LDL)-apoB enrichment.

To demonstrate the application of our procedures to less prevalent apolipoproteins, Figs. 5 and 6 illustrate isotopic enrichment profiles obtained with various apolipoproteins C. **Fig. 5** shows the enrichment of apolipoproteins C-II and C-III-1 recovered from VLDL, in comparison with the VLDL-apoB enrichment which was

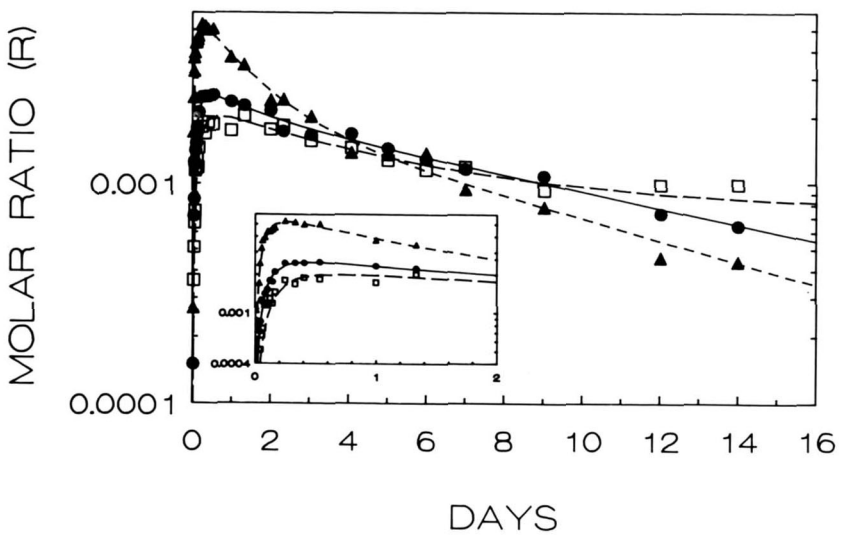

Fig. 3. Isotopic enrichment of apolipoprotein B-100, A-I, and A-II. Individual apolipoproteins were isolated by $NaDodSO_4$ polyacrylamide gel electrophoresis (Fig. 2) from plasma total d < 1.21 g/ml lipoproteins. Protein bands (containing 5–30 μg protein) were visualized by Coomassie blue stain, excised, and hydrolyzed. Amino acids were recovered and derivatized for the determination of molar ratio isotopic enrichment (R) by GC/MS. Results shown for subject 2. Inset: first 2 days only. ApoA-I: solid line with solid circles, apoA-II: long dash line with open squares; apoB-100: short dash line with solid triangles.

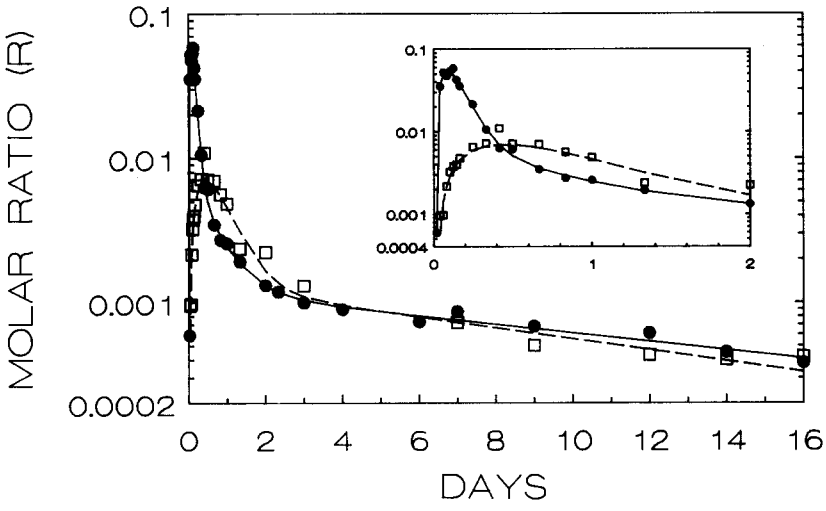

Fig. 4. Isotopic enrichment of apoB-100 in VLDL and IDL + LDL. VLDL was isolated at d < 1.006 g/ml; a combined IDL + LDL + HDL fraction was recovered at d 1.006–1.21 g/ml. ApoB-100 was recovered and isotopic enrichment was determined as described for Fig. 3. Results shown for subject 3. Inset: first 2 days only. VLDL apoB: solid line with solid circles; IDL + LDL apoB: dashed line with open squares.

shown in Fig. 4. Although recovered from the same lipoprotein subfraction, the VLDL-apoC proteins peaked at considerably lower enrichments and at later times than did that of VLDL-apoB, and their decrease from peak enrichments occurred at a slower rate than did that of VLDL-apoB. ApoC-II, apoC-III-1, and apoC-III-2 (data not shown) had parallel isotopic enrichment profiles; however, the enrichment of apoC-III-1 averaged 58% greater than apoC-II throughout the time course (Fig. 5). The terminal slopes of these enrichment time profiles were roughly parallel to that of VLDL-apoB.

Results obtained with apolipoproteins C isolated from the combined IDL + LDL + HDL fraction are shown in **Fig. 6**. ApoC-II and apoC-III-1 within HDL had roughly parallel time course profiles, with an average of 38% higher enrichment in apoC-III-1, similar to results for these proteins recovered from VLDL. Although not presented on the same graph, the enrichment profiles for either apoC-II or apoC-III-1 were virtually superimposable whether the proteins were isolated from either VLDL (Fig. 5) or the combined IDL + LDL + HDL fraction (Fig. 6). The peak isotopic enrichment of apoC-I in HDL was identical to that of apoC-II, although its decrease from peak enrichment was slightly slower and its final enrichment after 16 days was lower than was observed for apoC-II.

DISCUSSION

All apolipoproteins are labeled simultaneously when tracer amino acids are used. Thus, it is potentially possible to assess the metabolic kinetics of multiple apolipoproteins simultaneously in a single study, provided a sufficient quantity of each protein can be conveniently isolated for analysis. Isopropanol extraction (21) is rapid and convenient if apoB is the only protein of interest;

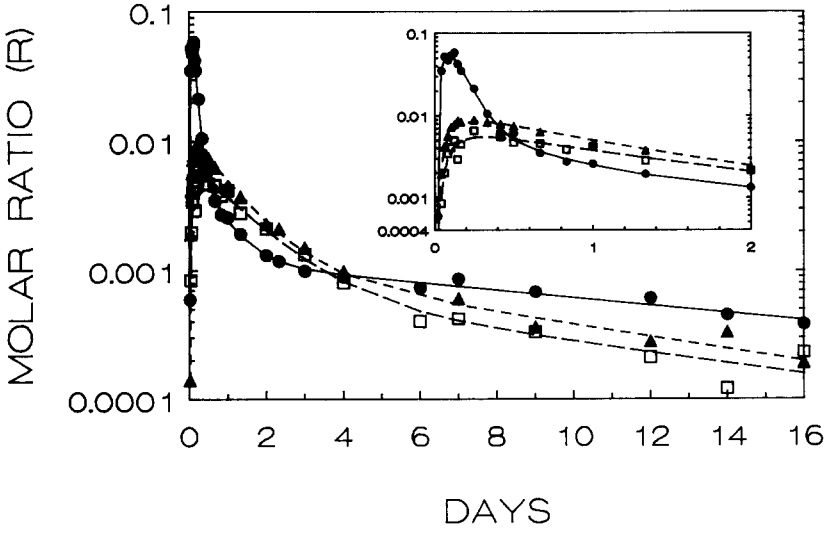

Fig. 5. Isotopic enrichment of VLDL apolipoproteins. VLDL was isolated at d < 1.006 g/ml. ApoB-100, apoC-II, and apoC-III-1 were recovered and isotopic enrichments were determined as described for Fig. 3. Results shown for subject 3. Inset: first 2 days only. ApoB-100: solid line with solid circles (same data as Fig. 4); apoC-II: long dash line with open squares; apoC-III-1: short dash line with solid triangles.

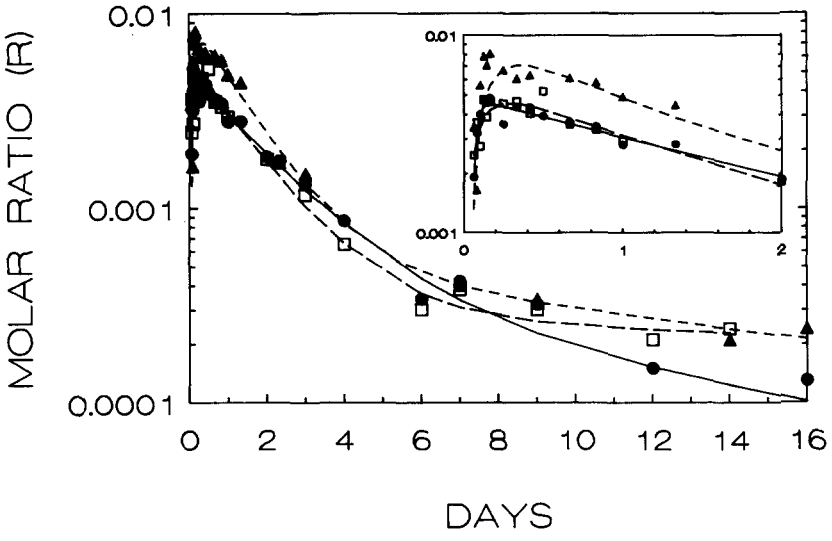

Fig. 6. Isotopic enrichment of apoC proteins from HDL. Combined IDL + LDL + HDL at d 1.006–1.21 g/ml was recovered. ApoC-I, apoC-II, and apoC-III-1 were recovered and isotopic enrichments were determined as described for Fig. 3. Results shown for subject 3. Inset: first 2 days only. ApoC-I: solid line with solid circles; apoC-II: long dash line with open squares; apoC-III-1: short dash line with solid triangles.

however, this method does not readily enable the subsequent separation of other apolipoproteins. The $NaDodSO_4$ polyacrylamide gel electrophoresis system reported here is capable of resolving all the apolipoproteins of interest from any given lipoprotein fraction.

Maguire, Lee, and Connelly (29) reported a $NaDodSO_4$-glycerol gel system for the resolution of many apolipoproteins. However, their system did not resolve apoA-II from apoC proteins, apoC-III isoforms were not resolved, and apoC-I was not identified. The $NaDodSO_4$ polyacrylamide gel electrophoresis system outlined here provided simultaneous resolution and recovery of all but two of the apolipoproteins, apoC-II and apoC-III-0. These proteins have nearly identical molecular weights and isoelectric points, making their separation difficult even by isoelectric focusing (30). There is much more apoC-II present than apoC-III-0 in native lipoproteins (301); thus, the apoC-II/C-III-0 band on our gel should primarily contain apoC-II. ApoC-III isoforms were well separated; thus differences in only one sialic acid residue significantly altered $NaDodSO_4$ binding and electrophoretic properties.

We have demonstrated isotopic labeling of multiple apolipoproteins using total plasma d < 1.21 g/ml lipoproteins, or subfractions of VLDL and combined IDL + LDL + HDL. Note that the acrylamide gel method would, however, be suitable for the separation and recovery of all apolipoproteins present in any chosen lipoprotein subfraction, such as obtained by more refined ultracentrifugational or immunoaffinity procedures. We have not demonstrated isotopic labeling of apoE because, in general, there was insufficient protein present in the lipoprotein fractions examined. This could in principle be overcome by loading more protein on the gel, although a thicker acrylamide gel may be needed to accomodate a larger sample volume.

We have found that the determination of isotopic enrichment of proteins separated by $NaDodSO_4$ polyacrylamide gel electrophoresis is reliable. The GC–MS analysis of derivatized amino acids recovered from hydrolyzed gel slices was not affected by hydrolysis products of polyacrylamide or Coomassie blue due to selective ion monitoring. The quantity of amino acids recovered from 5–30 μg of protein was sufficient for at least 50 replicate GC–MS analyses per sample. The sample sizes required for this analysis sharply contrast with the 1–2 mg of protein needed when 5 μCi/kg body weight of [^{3}H]leucine is administered in human studies (22). The lower limit on sample size (5 μg) is set by background contamination; the amount of lysine obtained from blank gel slices approached an unacceptable level of 5% when 5 μg of protein was analyzed.

We previously used a single intravenous dose to examine the metabolic kinetics of stable isotopically labeled lysine over 6 hours (31). We have now extended the utility of this single dose administration approach to obtain data suitable for metabolic kinetic studies of multiple apolipoproteins. The isotopic enrichment of plasma lysine can be monitored for at least 16 days after a single dose of [$^2H_4$]lysine is given. Tracer recycling from a pool that turned over very slowly (perhaps muscle) was dominant after 2 to 4 days (Fig. 1). This systemic whole-body tracer recycling was not observed in our earlier study (31) because of its short duration.

Such whole-body tracer recycling through the plasma can be incorporated in a compartmental analysis of plasma protein metabolic kinetics (11–14, 23, 25). It is expected that such recycling would be dominant in plasma protein subsystems after sufficient protein turnover occurs. Indeed some proteins were observed to have long terminal slopes which were essentially monoexponential and parallel to the plasma lysine. This was particularly

noted for VLDL-apoB, and for LDL-apoB in subject 3 (Fig. 4) who appeared to have a high apoB fractional catabolic rate (23) suggesting that the low levels of plasma cholesterol and apoB (120 and 38 mg/dl, respectively) observed in this subject were due to increased plasma clearance rates. As a general feature, however, the apolipoproteins did not present long monoexponential terminal slopes exactly parallel to the plasma lysine enrichment (Figs. 3, 5, 6). The shapes of these enrichment curves and their terminal slopes mainly result from the rates of clearance and exchange with the non-plasma space unique to each protein. The long time course of 16 days was used in this study in order to resolve these kinetic parameters for a variety of proteins that spanned a wide range of turnover rates. The protocol could be considerably shortened for studies of faster proteins such as VLDL-apoB. A detailed kinetic analysis of these apolipoproteins, plus albumin, transferrin, transthyretin, and haptoglobin recovered from the same study has been prepared (25) and will be presented elsewhere.

It is of interest to compare the kinetic profiles that result from endogenous tracer amino acid labeling against literature reports of the catabolic kinetics of radioiodinated apolipoproteins since iodination can alter physical (and thus perhaps the metabolic) properties of apolipoproteins (9, 10). The use of endogenous and exogenous labels have been directly compared for apoB (12). Results with the two labels were generally consistent, although differences in VLDL metabolism appeared; they were reconciled by a compartmental analysis incorporating both sets of tracer data simultaneously (12). Direct comparisons of endogenous and exogenous labels used for apoA-I, A-II, and apoC metabolism have not been reported.

In our studies, apoA-I attained a higher and earlier peak isotopic enrichment and had a faster decrease from peak enrichment compared with that of apoA-II (Fig. 3), consistent with reports that radioiodinated apoA-I is catabolized at a faster rate than apoA-II (7, 8). ApoB-100 in VLDL and IDL + LDL showed a typical precursor/product relationship (28) (Fig. 4). This was expected based on studies using radioiodinated apoB (5, 12, 21) and has been further validated with radioactive amino acid tracers (11, 12, 14, 22).

Like apoA-I, apoC proteins undergo large conformational changes while interacting with phospholipid bilayers through hydrophobic interactions (32, 33), and rapidly exchange between lipoprotein particles both in vitro and in vivo (34, 35). Because of their smaller size, it is possible that chemical modification with a large hydrophobic atom such as iodine could cause a greater perturbation to physical and metabolic properties of apoC than apoA-I (9, 10). All apoC kinetic studies to date have used radioiodinated tracers; the present study is the first to demonstrate endogenous labeling of apoC proteins with a tracer amino acid.

Enrichments of a given apoC isolated from VLDL and HDL were virtually superimposable throughout the study (compare Fig. 5 to Fig. 6), consistent with the kinetic equivalency of these pools (5, 36). The enrichments of apoC-II and apoC-III-1 (Figs. 5 and 6) and apoC-III-2 (data not shown) were parallel throughout the time course, confirming reports that these proteins follow identical kinetics (36). ApoC-I, however, did not exhibit an enrichment profile that was parallel to the other apoC proteins (Fig. 6); this may result from its exclusive residence within HDL. We found that both peak isotopic enrichments and rates of decrease from peak enrichments for apoCs were intermediate between apoA-I and VLDL-apoB (compare Figs. 3, 5, and 6), consistent with reports that apoC proteins are catabolized at a rate intermediate between apoA-I and VLDL-apoB (5–8, 36). ApoC-II attained a lower peak enrichment than did apoC-III-1 (Figs. 5 and 6); this would result if a significant proportion of apoC-II (but not of apoC-III) was synthesized in the intestine (37), since intestinal pools of amino acids are expected to be of lower enrichment than hepatic pools due to dietary intake. Thus, our observations with an endogenous amino acid tracer suggest that previous apoC kinetic studies are not grossly in error due to a potential alteration of metabolic properties as a result of radioiodination.

In this report, we have presented many examples to demonstrate the utility of obtaining tracer kinetic data for multiple apolipoproteins using a single dose administration of a stable isotopically labeled amino acid. The principal advantages of the method presented are its ability to conveniently separate and determine the enrichment of any apolipoprotein of interest from any chosen lipoprotein fraction, the ability to monitor isotopic enrichments of proteins that span a wide range of turnover rates, and the very small amounts of protein needed (5 to 30 μg) for analysis. The technique is not without its disadvantages: the amino acid derivatizations and quality control procedures are laborious and time-consuming; special instrumentation is required for precise GC–MS measurements; and a large number of time points are needed to accurately define the protein kinetics following a single-dose tracer administration. Nonetheless, with suitable modification to lipoprotein isolation procedures, adaptation of the methods outlined here should be useful in the elucidation of metabolic kinetics of multiple apolipoproteins within lipoprotein subclasses.

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