Determination of kinetic parameters of apolipoprotein B metabolism using amino acids labeled with stable isotopes

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Abstract The use of amino acids labeled with stable isotopes represents a relatively new approach for determining kinetic parameters of apolipoprotein metabolism; thus, several aspects of experimental protocols need to be defined. The aims of the present study were to determine whether *a*) different amino acid tracers or *b*) different methods of tracer administration affected apolipopotein (apo) B kinetic parameters obtained by multicompartmental modeling, and *c*) to compare very low density lipoprotein (VLDL)-apoB metabolic parameters determined by multicompartmental modeling with those estimated by linear regression or by monoexponential analysis. [1-^{13}C]leucine and [^{15}N]glycine were given either as bolus injections or as primed constant infusions. A bolus of one amino acid was administered simultaneously with a primed constant infusion (8 h) of the other amino acid into four healthy normolipidemic subjects (age 23.0 ± 1.4 yr; BMI 20.9 ± 0.9 kg · m^{-2}). VLDL-, intermediate density lipoprotein (IDL)-, and low density lipoprotein (LDL)-apoB enrichments were followed over 110 h. For subsequent analysis these values were converted to tracer/tracee ratios. Using the multicompartmental model, the fractional catabolic rate (FCR) for VLDL-apoB was estimated to be 0.36 ± 0.09 h^{-1} after the administration of the tracer as a primed constant infusion and 0.35 ± 0.07 h^{-1} when the tracer was administered as a bolus. The values for VLDL-apoB production were 14.6 ± 6.5 mg · kg^{-1} · d^{-1} and 14.1 ± 5.4 mg · kg^{-1} · d^{-1}, respectively. The corresponding values for LDL-apoB were 0.027 ± 0.016 h^{-1} (0.026 ± 0.018 h^{-1}) for the FCR and 10.5 ± 3.7 mg · kg^{-1} · d^{-1} (10.4 ± 3.8 mg · kg^{-1} · d^{-1}) for the production following administration of the tracer as a primed constant infusion and a bolus, respectively. Approximately 47% of VLDL-apoB ultimately reached the LDL fraction via the VLDL-IDL-LDL pathway. Thirty-five percent of LDL-apoB did not originate from this cascade pathway, but was shunted from a rapidly turning over VLDL compartment directly into the LDL fraction. While there was some variation between individuals, VLDL-apoB and LDL-apoB parameters derived from the bolus and the primed constant infusions showed no significant differences and were closely correlated. Metabolic parameters were also independent of the two amino acids tested. Although values for FCRs of VLDL-apoB obtained from linear regression (0.36 ± 0.19 h^{-1}) or monoexponential analysis (0.50 ± 0.36 h^{-1}) did not differ significantly from those obtained by the multicompartmental model, there was considerable variation and no significant correlation in a given individual. While the monoexponential analysis provides a measure of VLDL-apoB FCR, its validity is questionable when applied to studies of a heterogenous population of particles, such as the VLDL fraction. Linear regression analysis is inappropriate because it lacks a physiological basis. Multicompartmental modeling takes the heterogenous nature of lipoprotein metabolism into account and provides information not obtainable with the other methods of analysis. We conclude that use of amino acids labeled with stable isotopes in combination with multicompartmental modeling comprise a very powerful tool for determining kinetic parameters for apoB. —**Parhofer, K. G., P. H. R. Barrett, D. M. Bier, and G. Schonfeld.** Determination of kinetic parameters of apolipoprotein B metabolism using amino acids labeled with stable isotopes. *J. Lipid Res.* 1991. **32:** 1311–1323.

Supplementary key words VLDL • LDL • IDL

Amino acids labeled with stable isotopes (SI) are being used with increasing frequency to study lipoprotein metabolism (1–11); in contrast to radioisotopes there are no known adverse effects associated with the use of SI. Various amino acids such as leucine, glycine, valine, lysine, arginine, and phenylalanine labeled with different SI have been used as tracers to study the metabolism of apolipoproteins (apo) A, B, C, and E (1–11).

In addition to different tracers, a number of different protocols and modeling techniques have been used to obtain metabolic parameters. SI-labeled amino acids are most often administered to subjects either as bolus injections (3, 4, 9, 10) or as primed constant infusions (1, 2, 5–8, 11). When looked at from a theoretical perspective, the bolus approach has advantages over the other method of administration (12). However, the primed constant in-

Abbreviations: apo, apolipoprotein; BMI, body mass index; FCR, fractional catabolic rate; GC-MS, gas chromatography-mass spectrometry; KIC, ketoisocaproic acid; SI, stable isotope; VLDL, very low density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein.

fusion approach has been used in combination with simple formulas to estimate kinetic parameters for VLDL-apoB. This includes a one-compartment model from which fractional catabolic rates (FCR) can be determined by fitting the observed VLDL-apoB enrichment data to a monoexponential function (2). Alternatively, linear regression analysis of the initial enrichment slope has been used to estimate FCRs of VLDL-apoB (1, 6–8) and LDL-apoB (6, 7). Both approaches assume that the VLDL fraction represents a homogeneous population of particles, an assumption known to be incorrect, since numerous studies (13–17), using exogenously labeled material, have shown that the VLDL pool is in fact heterogeneous. Multicompartmental models should therefore be more appropriate for the analysis of apoB tracer data. In addition to providing estimates of VLDL-apoB FCRs, the incorporation of VLDL, IDL, and LDL data into a multicompartmental model also yields information about other aspects of apoB metabolism, such as the kinetics of VLDL-apoB subfractions, fractions of VLDL-apoB removed directly, as well as kinetic parameters for IDL and LDL-apoB.

The present study was undertaken to clarify the following methodological aspects. Does *a*) the amino acid chosen as a tracer or *b*) the method of tracer administration affect estimates of metabolic parameters for VLDL-, IDL-, and LDL-apoB obtained by multicompartmental modeling? And *c*) how do VLDL-apoB metabolic parameters estimated by linear regression or monoexponential analysis compare with those obtained from multicompartmental modeling? We performed studies using [1-^{13}C]leucine and [^{15}N]glycine, an essential and a nonessential amino acid, respectively, both of which have been used frequently in lipoprotein studies. The tracers were administered either by bolus or by primed constant infusion. Multicompartmental modeling was used for the analysis of tracer data. In addition, VLDL-apoB tracer data following the primed constant infusion were analyzed by linear regression and by monoexponential analysis. The metabolic parameters for VLDL-, IDL-, and LDL-apoB derived from the use of the two amino acids, the two methods of tracer administration, and the different methods of analysis were compared. These results were also compared with previously published parameters on apoB metabolism.

METHODS

Subjects

Four healthy nonobese medical students (age 23.0 ± 1.4 years, body mass index (BMI) 20.9 ± 0.9 kg · m^{-2}) participated in this study after giving informed consent (**Table 1**). None of the subjects was taking any medication or hormones on a regular basis. One subject was studied twice, with the method of tracer application reversed. In this subject the two studies were approximately 5 months apart, during which time the subject had lost 2.5 kg. Other conditions were unchanged. All subjects recorded their dietary intake over 10–14 days prior to the study. They were then seen by a dietician and instructed to eat a diet consisting of 45% of daily caloric intake as carbohydrate, 35% as fat, and 20% as protein for at least 1 week prior to the study and during the study period. The daily cholesterol intake was 300 mg. The study protocol was approved by the Human Studies Committee of Washington University.

Materials

[1-^{13}C]Leucine and [^{15}N]glycine were obtained from MSD Isotopes (Montreal, Canada). Isotopic purity was 99% for both compounds. For use the tracers were dissolved in sterile filtered saline (0.22 μm, Millipore Corporation, Milford, MA) and tested for presence of pyrogens (Scientific Associates, St. Louis, MO). Methylene chloride, triethylamine, and phenylenediamine were obtained from Sigma (St. Louis, MO); ethylacetate and isopropylether from EM-Science (Cherry Hill, NJ); 3.5 N HBr-propanol from Alltech Associates (Deerfield, IL); and BSTFA (N,O-bis-(trimethylsilyl)trifluoroacetamide) from Pierce (Rockford, IL). All other chemicals were obtained from Fisher Scientific (Fair Lawn, NJ). For isolation of plasma amino acids, cation exchange resin AG50W-X8 (Bio-Rad, Richmond, CA) was used in screening columns (Whale Scientific, Commerce City, CO).

TABLE 1. Clinical characteristics of study subjects

Study	Sex	Age	Weight	BMI	Chol	TG	Bolus	Primed Constant Infusion
		yr	*kg*	*kg · m^{-2}*	*mg · dl^{-1}*			
#1	F	22	61.5	21.5	160	142		[^{15}N]glycine
#2	M	25	73.8	20.7	169	78	[^{15}N]glycine	[1-^{13}C]leucine
#3	M	25	71.4	19.8	154	63	[1-^{13}C]leucine	[^{15}N]glycine
#4	M	22	63.5	19.4	131	75	[^{15}N]glycine	[1-^{13}C]leucine
#5	M	23	71.3	22.0	126	75	[^{15}N]glycine	[1-^{13}C]leucine

Studies #2 and #3 were performed in the same subject. Chol, plasma cholesterol; TG, plasma triglycerides.

Study protocol

After fasting for 10 h the subjects were admitted to the General Clinical Research Center at 6 PM. An intravenous line was placed in the cubital vein of each arm; one served for tracer injection, the other for blood sampling. At 8 PM a bolus of one amino acid ([^{15}N]glycine 10.0 mg · kg^{-1} or [1-^{13}C]leucine 5.0 mg · kg^{-1}) was administered simultaneously with a primed constant infusion of the other amino acid ([^{15}N]glycine 1.0 mg · kg^{-1} as prime immediately followed by 1.0 mg · kg^{-1} · h^{-1} as a constant infusion or [1-^{13}C]leucine 0.85 mg · kg^{-1} as prime immediately followed by 0.85 mg · kg^{-1} · h^{-1} as a constant infusion). After 8 h the tracer infusion was stopped and the subject remained fasting for another 8 h. Thereafter each subject followed the same diet as described above until completion of the study. Studies were undertaken for a period of 110 h in order to estimate metabolic parameters for LDL-apoB. Subject #1 received only [^{15}N]glycine as a primed constant infusion ([^{15}N]glycine 1.1 mg · kg^{-1} as prime immediately followed by 1.5 mg · kg^{-1} · h^{-1} as a constant infusion) over an 8-h period. No [1-^{13}C]leucine was injected and the duration of the study was only 16 h. Blood samples were collected into EDTA-containing tubes and plasma was separated by low speed centrifugation. Plasma samples were processed immediately for the isolation of lipoprotein fractions and aliquots of each were stored at −70°C until analysis. During the course of the study, 44 samples (every 5 min during the first hour, thereafter every 15 min, later half-hourly and hourly, and finally daily up to 110 h) were drawn for plasma amino acid enrichment and ketoisocaproic acid (KIC) enrichment. In addition, 32 samples (every 10 min during the first hour thereafter every 15 min, later half-hourly and hourly, and finally daily up to 110 h) were drawn for the determination of VLDL-, IDL-, LDL-apoB glycine and leucine enrichment. Aliquots for apoB pool sizes were drawn on eight occasions during the course of the study.

Analytical methods

Isolation of lipoproteins. VLDL (d<1.006 g · ml^{-1}), IDL (d 1.006–1.019 g · ml^{-1}), and LDL (d 1.019–1.063 g · ml^{-1}) were isolated from 4 ml plasma by sequential ultracentrifugation using standard methods (18). All lipoprotein fractions were isolated in Quick-Seal tubes (Beckman Instruments Inc., Palo Alto, CA) in a type 50.3 or 50.4 fixed-angle rotor (Beckman Instruments) at 170,000 *g* for 18 h at 10°C. IDL and LDL were dialyzed against EDTA saline (0.9% NaCl, 0.1 mM EDTA, pH 8.35) for 24 h with three changes of dialysate.

Measurement of lipids and apoB. ApoB concentrations were measured in VLDL, IDL, and LDL fractions by radioimmunoassay (19). Cholesterol and triglycerides were measured by commercially available tests (WAKO Pure Chemical Industries, Ltd., Osaka, Japan). VLDL-, IDL-, and LDL-apoB pool sizes were determined by multiplying the measured apoB concentrations by plasma volume (20).

Isolation and hydrolysis of apoB. ApoB was isolated from each lipoprotein fraction by precipitation with butanol-isopropylether as described previously (21). The precipitated fraction contained only apoB as established by SDS-polyacrylamide gel electrophoresis (22). The precipitated apoB was dried under nitrogen and then hydrolyzed in 12 N HCl for 16 h at 110°C. The hydrochloric acid was subsequently evaporated.

Isolation of plasma amino acids and KIC. Plasma amino acid enrichment was determined from 0.3 ml plasma after isolation by cation exchange chromatography (23). From these samples KIC was isolated and derivatized by the method of Schwartz, Karl, and Bier (24).

Determination of enrichment and calculation of tracer/tracee ratio. Amino acids obtained from the plasma samples or from the hydrolyzed apoB precipitates were derivatized to n-acetyl-n-propanol-esters (23). Leucine, glycine, and KIC enrichments were determined by gas chromatography–mass spectrometry (GC–MS) using 1.5 m × 2.0 mm glass columns (Supelco, Bellafonte, CA) packed with coated material (Amino Acid Packing, Alltech Assoc., Deerfield, IL) and a Finnigan 3300 quadropule mass spectrometer as described previously (25). Isotope ratios (R) were calculated from the observed ion current ratio (glycine *m/z* 161/160, leucine *m/z* 217/216, KIC *m/z* 276/275) using a standard curve. Enrichment was calculated using the method of Cobelli et al. (26). Because of the non negligible mass associated with SI tracers it is necessary to transform enrichment data to tracer/tracee ratios (26). Data in this format are analogous to specific activity in radiotracer experiments.

Modeling

Models for apoB metabolism. Different approaches were used to describe metabolism of apoB. Two of these, a linear regression analysis and a monoexponential approach, used the tracer/tracee ratios during the primed constant infusion to derive metabolic parameters for VLDL-apoB. The third approach, a multicompartmental model, shown in **Fig. 1,** provided estimates for metabolic parameters for VLDL-, IDL-, and LDL-apoB using tracer/tracee ratios derived from both the bolus and primed constant infusion studies. In contrast to the other methods, the multicompartmental model uses tracer information throughout the entire period of the study, i.e., during the rise and fall of the tracer/tracee ratios in all lipoprotein fractions. Implicit in the use of these models is the assumption that each subject remains in steady-state with respect to their apoB metabolism during the course of the study. Under this condition FCRs correspond to fractional synthetic rates.

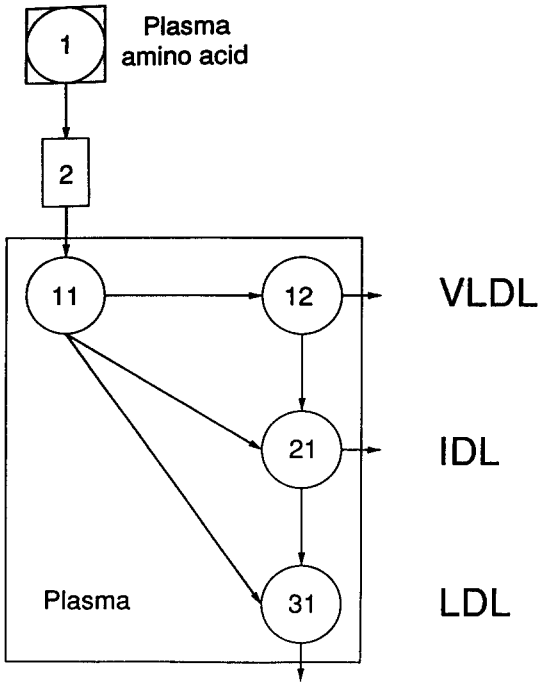

Fig. 1. Multicompartmental model for apoB metabolism. Compartment 1: plasma leucine or glycine tracer/tracee ratio (forcing function); compartment 2: delay compartment (synthesis of apoB and secretion of VLDL particles); compartments 11 and 12: plasma VLDL-apoB; compartment 21: plasma IDL-apoB; compartment 31: plasma LDL-apoB. All apoB enters the plasma as VLDL-apoB via compartment 11. ApoB in this rapidly turning over compartment has one of the following fates: conversion to another VLDL compartment (compartment 12), conversion to IDL-apoB (compartment 21), or shunting to LDL-apoB (compartment 31). ApoB can be removed from any compartment except compartment 11.

Although there is no physiological basis for a linear regression analysis, this method was included because of its frequent use in previous studies (1, 6–8). Use of monoexponential analysis assumes that the VLDL fraction contains a homogeneous class of particles that have similar physicochemical and metabolic properties. In both approaches it is also assumed that a primed constant infusion provides a constant level of tracer in the precursor. In addition, it is assumed that the existing intracellular pool of apoB is negligible in size compared to the plasma VLDL-apoB pool. Furthermore, it is assumed that the VLDL-apoB glycine or leucine tracer/tracee ratio curve reaches a plateau that reflects the tracer/tracee ratio of the precursor.

Based upon previously published models (13–17), a simple multicompartmental model, shown in Fig. 1, was developed to describe VLDL-, IDL-, and LDL-apoB tracer/tracee ratios. This model represents the simplest structure consistent with both previously published studies and our experimental data. The model consists of a precursor compartment (compartment 1) and a delay compartment (compartment 2) accounting for the synthesis and assembly of apoB into lipoproteins. Compartments 11 and 12 are used to account for the kinetics of the VLDL-apoB fraction and represent a minimum delipidation chain. Compartment 11 represents a rapidly turning over pool of VLDL particles some of which are converted to the other, more slowly turning over VLDL compartment, compartment 12. ApoB from both VLDL compartments can be converted to IDL-apoB. LDL in turn was derived from the IDL fraction or directly from the rapidly turning over VLDL compartment through a shunt pathway. The kinetics of the IDL-apoB and LDL-apoB fractions are accounted for by single compartments, compartment 21 and compartment 31 respectively. It is assumed that all apoB enters the plasma as VLDL via compartment 11.

Calculation of kinetic parameters. For the analysis of the VLDL-apoB tracer data after a primed constant infusion, a monoexponential function was used to fit the data. The function: $A(t) = A_p\ (1 - e^{-k(t-d)})$, where A(t) is the tracer/tracee ratio at time t, A_p is the precursor tracer/tracee ratio, d is the delay time until the appearance of labeled apoB in the VLDL fraction, and k represents the FCR of VLDL-apoB (2).

VLDL-apoB FCR was also estimated by an alternate and simpler approach, a linear regression analysis. The initial slope of the tracer/tracee ratio curve was estimated by linear regression analysis using VLDL-apoB data during the first 2 h of the primed constant infusion. This value was divided by the level of precursor tracer/tracee ratio to provide an FCR. As in previously published studies (1, 6–8) using this approach, it was assumed that the VLDL-apoB tracer/tracee ratio at the end of the primed constant infusion (usually the highest value) corresponds to the tracer/tracee ratio of the precursor.

CONSAM/SAAM (27) programs were used to determine the parameters of the multicompartmental model in Fig. 1. For this analysis it was assumed that the source of amino acid that is incorporated into apoB was from the plasma pool, compartment 1. Plasma amino acid tracer/tracee ratios were described by a triexponential function (see Appendix) which was used as a forcing function (28) in the model. The use of a forcing function obviates the need for a complex model to describe the kinetics of the amino acid because this mathematical description embodies the recycling processes of the tracer. In study #1 the turnover rate of LDL-apoB could not be determined accurately because of the duration of the study. The average value of the four other studies (FCR for LDL-apoB = 0.027 h^{-1}) was therefore assumed. All other parameters were adjustable. Because of the uncertainties associated with measuring the IDL-apoB, the mass associated with the IDL compartment was not constrained to the measured value. After fitting the model to the tracer/tracee ratios using a least squares approach, production rates, FCRs, and conversion rates were determined for VLDL-, IDL-, and LDL-apoB. The FCR of VLDL-apoB is the

weighted (related to mass distribution) average of the turnover rates of the two VLDL pools. The turnover rate of each pool is the sum of individual rate constants. Another way to calculate VLDL-apoB FCR is to divide the transport of apoB into the VLDL by the pool size of VLDL-apoB. The FCR of IDL-apoB corresponds to the sum of individual rate constants of compartment 21 (rate of irreversible loss and rate of conversion to LDL-apoB). The FCR of LDL-apoB corresponds to the rate of irreversible loss from compartment 31.

Statistics

All data are expressed as mean ± standard deviation (SD). The paired *t*-test was used to compare parameters derived from the two methods of tracer application. Bolus and primed constant infusion data were also correlated. An analysis of variance with repeated measures was used to compare the three different methods of analysis (linear regression, monoexponential, multicompartmental). All statistical analyses were calculated using InStat software, Graph PAD, San Diego, CA.

RESULTS

Plasma concentrations of VLDL-, IDL-, and LDL-apoB are presented in **Table 2.** These values represent means of eight separate samples taken during the 110-h study period. Five of these values were obtained during the period of fasting. ApoB concentrations remained constant in all lipoprotein fractions during this period, indicating that each subject remained in a steady state during the course of the study.

TABLE 2. ApoB concentration in different lipoprotein fractions

Study	VLDL-ApoB	IDL-ApoB	LDL-ApoB	Plasma-Volume
		mg · dl⁻¹		*l*
#1	4.38 ± 0.63	2.36 ± 0.48	42.8 ± 3.6	2.76
#2	4.64 ± 0.58	1.09 ± 0.36	56.0 ± 8.6	3.32
#3	4.07 ± 0.76	3.15 ± 0.38	30.2 ± 2.3	3.21
#4	3.26 ± 0.47	2.49 ± 0.57	26.9 ± 2.8	2.86
#5	2.66 ± 0.23	3.89 ± 0.31	59.5 ± 4.6	3.21

ApoB concentrations represent mean values ± SD from eight measurements.

Plasma leucine and glycine tracer/tracee ratios from a representative study (study #2) are shown in **Fig. 2.** In this study [^{15}N]glycine was given as a bolus and [1-^{13}C]leucine was administered as a primed constant infusion. The plasma amino acid tracer/tracee ratio curve after a bolus showed an initial rapid fall followed by several more slowly disappearing components. In contrast, the shape of the amino acid tracer/tracee ratio curve following a primed constant infusion showed an initial rapid fall followed by a rise until the infusion was stopped, thereafter the shape was similar to that following a bolus. The features of these curves were independent of which amino acid was injected. Plasma amino acid tracer/tracee ratio curves were fitted with triexponential functions (see Appendix). These functions were subsequently used to define the forcing functions used in multicompartmental modeling.

Fig. 3 shows the VLDL-apoB leucine tracer/tracee ratio in study #2 during the 8-h period of primed constant infusion. In panel A the linear regression analysis to the observed VLDL-apoB data is shown. Panel B shows the

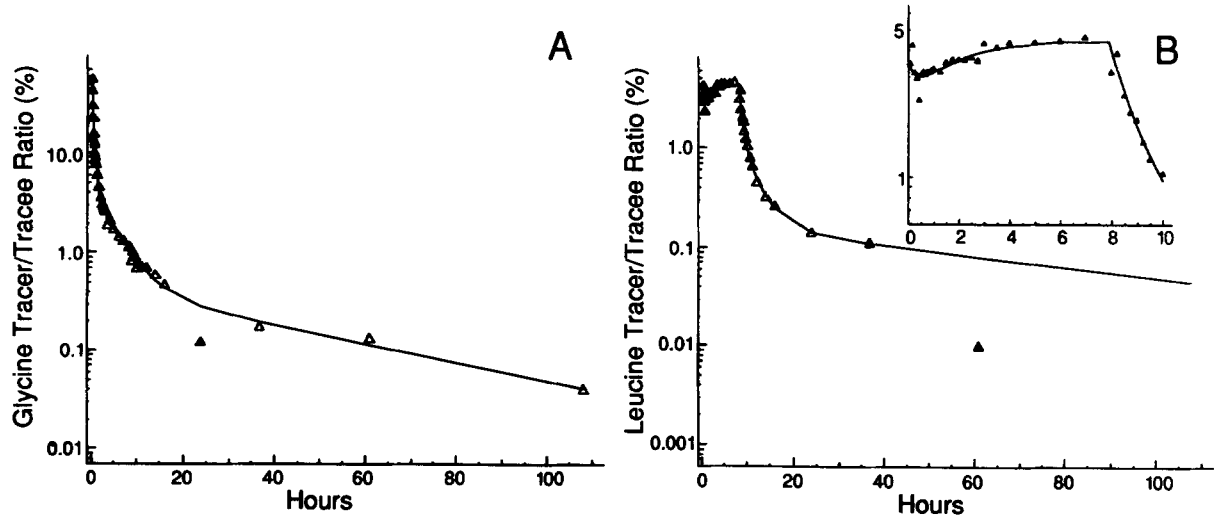

Fig. 2. Plasma glycine (A) and plasma leucine (B) tracer/tracee ratio following a bolus of [^{15}N]glycine and a primed constant infusion of [1-^{13}C]leucine, respectively, in a representative study (#2). A triexponential function (see Appendix) (line) was fit to observed values (symbols). Two data points (glycine tracer/tracee ratio at 24 h and leucine tracer/tracee ratio at 60 h) were excluded from the fitting process.

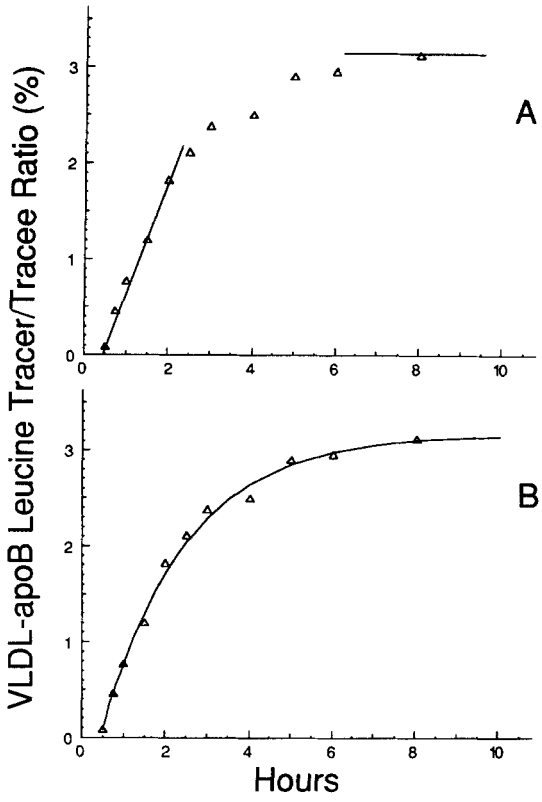

Fig. 3. VLDL-apoB leucine tracer/tracee ratio following a primed constant infusion of [1-^{13}C]leucine over 8 h (study #2). Panel A shows the fit to the initial slope and the assumed ratio in the precursor used to calculate the VLDL-apoB FCR by the linear regression analysis. It is obvious that the calculated parameter depends on the number of time points used for the regression analysis. Panel B shows the fit of the monoexponential model.

monoexponential analysis of the same data. Note that during the infusion period the tracer/tracee ratio of VLDL-apoB continues to rise, a plateau is not attained. **Fig. 4** shows the observed values (symbols) and fits to the VLDL-, IDL-, and LDL-apoB tracer/tracee ratio data (lines) using the multicompartmental model (Fig. 1) for study #4. Panels A and B show VLDL-, IDL-, and LDL-apoB data following a bolus of [^{15}N]glycine. Panels C and D show data in the same fractions after a primed constant infusion of [1-^{13}C]leucine. **Fig. 5** shows rate constants for all compartments for study #4. Values represent rate constants derived from the bolus data after application of [^{15}N]glycine, while values in parentheses are derived from the [1-^{13}C]leucine primed constant infusion data.

Table 3 shows estimates of VLDL-apoB production and FCRs derived by the different methods used for the analysis of VLDL-apoB tracer/tracee ratios after a primed constant infusion. Except in study #5, VLDL-apoB production rates estimated using the monoexponential method were higher than those using the linear regression analysis (14.0 ± 16.0 mg · kg^{-1} · d^{-1} vs. 14.6 ± 7.6 mg · kg^{-1} · d^{-1}, mean ± SD). Mean VLDL-apoB production rates using the multicompartmental model (14.8 ± 5.7 mg · kg^{-1} · d^{-1}) were comparable to those estimated by the other methods of analysis and there was no significant difference among the three methods of analysis (P = 0.32). When FCRs derived from the different methods of analysis were compared to each other, values from the monoexponential approach showed a slightly better correlation with those using the multicompartmental approach than did those from linear regression analysis (r = 0.53 vs. r = 0.47). However, none of the correlations were significant, highlighting the considerable variation seen within individuals. Studies #2 and #3 were performed in the same subject approximately 5 months apart. During study #3 VLDL-apoB production was significantly lower than that in study #2. This decrease in VLDL production was evident regardless of the method of analysis.

Using the multicompartmental model shown in Fig. 1, VLDL-, IDL-, and LDL-apoB FCRs and production rates were estimated (**Table 4** and **Table 5**). Comparisons between bolus and primed constant infusion derived parameters as well as comparisons between the two tracers included only the four studies in which both approaches and both amino acids were used (studies #2–#5). VLDL-apoB production rates and FCRs estimated from the primed constant infusion data (14.6 ± 6.5 mg · kg^{-1} · d^{-1}, 0.36 ± 0.09 h^{-1}, respectively) and from the bolus data (14.1 ± 5.4 mg · kg^{-1} · d^{-1} and 0.35 ± 0.07 h^{-1}, respectively) were very similar. When compared on an individual basis these parameters were also highly correlated (r = 0.98, P < 0.02). The delay time until labeled apoB was first detectable in the VLDL fraction showed little variation in all studies (0.47 h ± 0.02 h) and was not dependent upon the amino acid used or the method of tracer administration. The ratio of the VLDL-apoB mass in compartments 11 and 12 varied between 2.4 and 8.3; in studies #1 and #2, however, no slowly turning over VLDL compartment could be identified. IDL-apoB FCRs and production rates showed a wider range and a greater variability. While there was no significant difference between the FCRs for IDL-apoB derived from the bolus and those derived from the primed constant infusion, the production rate for IDL-apoB was significantly lower for the bolus data, when a paired analysis was performed (P < 0.05). Nonetheless, parameters derived from the bolus and those derived from the primed constant infusion were strongly correlated (r = 0.98, P < 0.03). The IDL-apoB pool sizes predicted by the model did not differ significantly from the measured values. A comparison of metabolic parameters for LDL-apoB showed no difference between the data derived from the primed constant infusion and the bolus (FCR: 0.027 ± 0.016 h^{-1} and 0.026 ± 0.018 h^{-1}; production rates 10.5 ± 3.7 mg · kg^{-1} · d^{-1} and 10.4 ± 3.8 mg · kg^{-1} · d^{-1}, respectively). There was

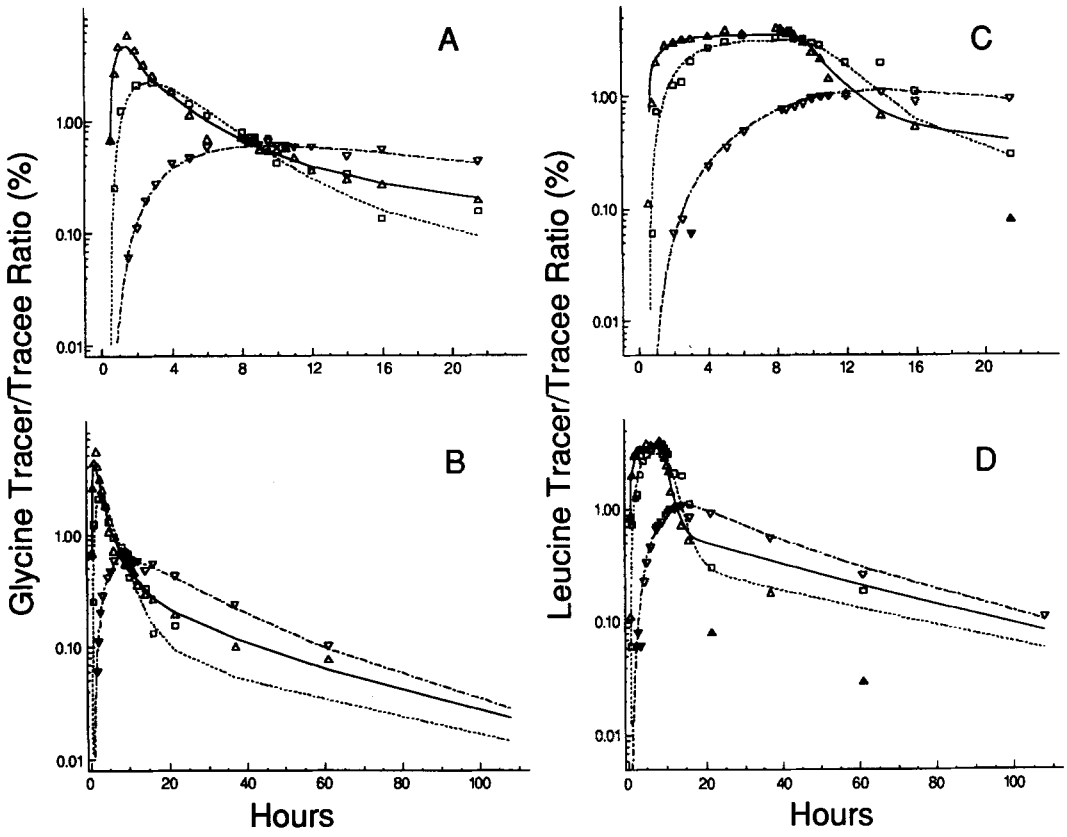

Fig. 4. Observed values (symbols) and calculated fits (lines) to the VLDL-, IDL-, and LDL-apoB tracer/tracee ratio using the multicompartmental model in a representative study (#4). Panels A and B show VLDL- (△, solid line), IDL- (□, dotted line), and LDL-apoB (▽, dashed line) glycine data after a bolus of [^{15}N]glycine; panels C and D show VLDL- (△, solid line), IDL- (□, dotted line), and LDL-apoB (▽, dashed line) leucine data in the same subject following a primed constant infusion of [1-^{13}C]leuine. Three points (LDL-apoB leucine tracer/tracee ratio at 3 h and VLDL-apoB leucine tracer/tracee ratios at 24 h and 60 h were excluded from the fitting process.

also no significant difference between the data derived from the two tracers for VLDL-, IDL-, and LDL-apoB. Finally, the FCRs of all three lipoprotein fractions and the production rates for VLDL-apoB and IDL-apoB showed a strong correlation between parameters derived from [^{15}N]glycine and [1-^{13}C]leucine ($r > 0.95$; $P < 0.05$, for all correlations).

In addition to providing estimates of apoB production rates and FCRs, the compartmental model provides information about the conversion of VLDL to IDL and subsequently to LDL (**Table 6**). Approximately 75% of VLDL-apoB production was converted to IDL either via compartment 12 or directly from compartment 11. Excluding the shunt pathway, approximately 47% of VLDL-apoB was ultimately converted to LDL. In addition to this pathway, approximately 24% of the rapidly turning over VLDL (compartment 11) was shunted directly to the LDL fraction. This latter input mechanism accounted for approximately 35% of LDL-apoB production. ApoB production from the VLDL-IDL-LDL cascade combined with that from the shunt pathways could not account for approximately 7% of total apoB mass in the LDL fraction. Although there was considerable variation between individuals, a comparison of these parameters derived from the bolus and the primed constant infusion showed no significant difference.

When endogenously incorporated tracers are used, the calculation of metabolic parameters is dependent upon assumptions concerning the kinetics of the precursor. Application of linear regression or monoexponential analysis to primed constant infusion derived data assumes a constant precursor tracer/tracee ratio. Several indirect methods to establish the level of tracer in the precursor have been proposed (1, 2, 6–8). For the linear regression analysis this is thought to be reflected by the tracer/tracee ratio in VLDL-apoB at the end of the primed constant infusion. When a monoexponential function is used the asymptote, A_p, is assumed to represent precursor tracer/tracee ratio. In studies using leucine as a tracer, labeled plasma KIC (the transamination product of leucine) has been used as an alternate measure for the level of tracer in the precursor (29–31). In **Table 7** the level of tracer/tracee ratio in the plasma amino acid pool and that in VLDL-apoB at the end of the primed constant infusion

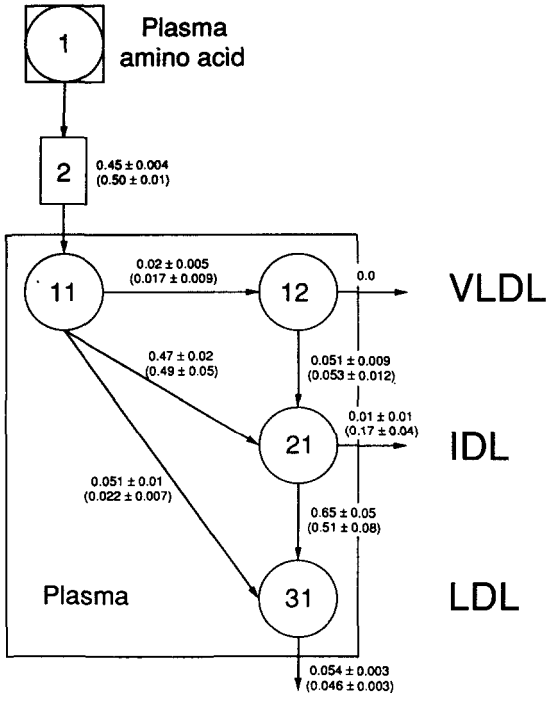

Fig. 5. Turnover rates of individual pools for study #3. Shown are best estimates ± SD in h^{-1} for rate constants derived from the bolus of [^{15}N]glycine and those derived from the primed constant infusion of [1-^{13}C]leucine (in parentheses). The numbers next to the delay compartment (compartment 2) represent the time in hours necessary for the synthesis and secretion of apoB. The FCR of VLDL-apoB is the weighted (related to mass distribution) average of the turnover rates of the two VLDL pools. The turnover rate of each pool is the sum of individual rate constants. The FCR of IDL-apoB corresponds to the sum of individual rate constants of compartment 21 (rate of irreversible loss and rate of conversion to LDL-apoB). The FCR of LDL-apoB corresponds to the rate of irreversible loss from compartment 31.

period are given. In addition, values for the asymptote, A_p, and the level of tracer/tracee ratio in plasma KIC are presented. In all studies VLDL-apoB tracer/tracee ratio was significantly less than that of the plasma amino acid (36% and 75% for glycine and leucine, respectively). The value of A_p was comparable to VLDL-apoB tracer/tracee ratio and KIC tracer/tracee ratio was approximately 70% that of plasma leucine.

DISCUSSION

Because amino acids labeled with SI are being used more frequently for the study of apoB metabolism, it is important to establish the optimal tracers, protocols, and methods of data analysis for such studies. We compared metabolic parameters derived from studies using two different amino acids as tracers, [^{15}N]glycine and [1-^{13}C]leucine. We also studied the effect of two different methods of tracer administration and three different methods of data analysis. We conclude that apoB metabolic parameters are independent of the amino acid used as a tracer and the method by which the tracer is administered. Although VLDL-apoB metabolic parameters derived from linear regression, monoexponential analysis, and multicompartmental modeling were not significantly different from each other, the three methods of analysis showed considerable variability and no significant correlation when compared within a subject. Of the three methods, multicompartmental modeling provides the most detailed information on apoB metabolism.

The complex nature of apoB metabolism has been demonstrated in many studies, largely based upon the use of exogenous tracers (13–17, 32). In these studies compartmental models were used to describe VLDL-apoB kinetics to take the heterogeneous nature of the VLDL fraction into account. Although little has been reported on the metabolism of IDL, its kinetics are usually described by a one- or two-pool model. LDL-apoB metabolism is generally described by a two-pool model, where one compartment is assumed to reflect plasma LDL, while the other represents an extravascular exchange compartment (see reference 15 for review). A more recent study however has shown the need for several plasma LDL pools (33). Initial findings suggested that all LDL-apoB was

TABLE 3. Comparison of kinetic parameters for VLDL-apoB estimated by linear regression, monoexponential, and by multicompartmental analysis after a primed constant infusion of either [^{15}N]glycine or [1-^{13}C]leucine

	Linear Regression		Monoexponential		Multicompartmental	
Study	FCR	Production	FCR	Production	FCR	Production
	h^{-1}	$mg \cdot kg^{-1} \cdot d^{-1}$	h^{-1}	$mg \cdot kg^{-1} \cdot d^{-1}$	h^{-1}	$mg \cdot kg^{-1} \cdot d^{-1}$
#1	0.15	7.1	0.29	13.7	0.33	15.7
#2	0.39	19.5	0.50	25.1	0.47	23.6
#3	0.41	18.0	0.43	18.9	0.29	12.6
#4	0.64	22.5	1.10	36.7	0.40	14.1
#5	0.21	6.0	0.16	4.6	0.28	8.1
Mean ± SD	0.36 ± 0.19	14.6 ± 7.6	0.50 ± 0.36	14.0 ± 16.0	0.35 ± 0.08	14.8 ± 5.7

FCR or production of VLDL-apoB was not significantly different for the three methods of analysis when compared with an analysis of variance for repeated measures ($P = 0.32$); production of VLDL-apoB determined by monoexponential analysis correlated slightly better ($r = 0.53$) than those from linear regression analysis ($p = 0.47$) with parameters obtained by multicompartmental modeling.

TABLE 4. FCRs of VLDL-, IDL-, and LDL-apoB estimated by multicompartmental modeling

Study	FCR of VLDL-ApoB		FCR of IDL-ApoB		FCR of LDL-ApoB	
	Infusion	Bolus	Infusion	Bolus	Infusion	Bolus
	h^{-1}		h^{-1}		h^{-1}	
#1	0.33 ± 0.12		0.54 ± 0.07		0.027[a]	
#2	0.47 ± 0.14	0.43 ± 0.06	0.42 ± 0.16	0.29 ± 0.04	0.021 ± 0.003	0.017 ± 0.002
#3	0.29 ± 0.04	0.28 ± 0.03	0.43 ± 0.15	0.40 ± 0.04	0.032 ± 0.002	0.022 ± 0.002
#4	0.40 ± 0.02	0.39 ± 0.02	0.68 ± 0.21	0.66 ± 0.04	0.046 ± 0.003	0.054 ± 0.003
#5	0.28 ± 0.01	0.30 ± 0.03	0.21 ± 0.01	0.16 ± 0.01	0.008 ± 0.003	0.012 ± 0.001
Mean ± SD[b]	0.36 ± 0.09	0.35 ± 0.07	0.44 ± 0.19	0.38 ± 0.21	0.027 ± 0.016	0.026 ± 0.018

Parameters were determined using the multicompartmental model shown in Fig. 1; values for individual studies represent best estimates ± SD.
[a]FCR of LDL-apoB could not be established in this study; the mean value of studies #2–#5 was assumed.
[b]Mean ± SD of best estimates of studies #2–#5; no significant differences (paired *t*-test) were apparent when the parameters derived from the bolus were compared to those derived from the primed constant infusion or those derived from glycine were compared to those derived from leucine.

derived from sequential conversion of VLDL through IDL to LDL (34). Further studies, however, have demonstrated the need for an input into the LDL fraction other than that from the classical VLDL–IDL–LDL pathway. Either direct secretion of LDL particles or a rapidly turning over VLDL compartment shunting apoB directly into the LDL fraction could account for this input (17).

The model presented here represents the simplest structure consistent with both previously published studies and with our experimental data. The kinetics of the VLDL fraction were described by two compartments, one of which turned over rapidly. These two compartments represent a minimal delipidation chain. Other models have used longer delipidation chains. In contrast to those models where parameters for individual compartments were constrained, all parameters in our model were adjustable. IDL and LDL kinetics were accounted for by single compartments, because it was not possible to define the kinetics of more than one compartment in each of these fractions. The conversion of VLDL-apoB to IDL-apoB can occur from both VLDL compartments. IDL-apoB can either be converted to LDL-apoB or removed from plasma. In addition to the input of apoB into the LDL fraction via this classical pathway, our model included a shunt pathway from the rapidly turning over VLDL compartment. Consistent with findings from other studies (1, 35, 36), the inclusion of this pathway was necessary to account for the rapid appearance of labeled apoB in the LDL fraction.

The fitting of the multicompartmental model described above to the observed tracer/tracee ratios resulted in an FCR for VLDL-apoB of 0.35 h^{-1} (mean of bolus and infusion studies). The corresponding production rate for VLDL-apoB was approximately 14 $mg \cdot kg^{-1} \cdot d^{-1}$. These values are in the upper range of those previously reported for VLDL-apoB metabolism, most of which were derived from exogenous labeling studies. Our finding is consistent with a recently published study by Beltz et al. (35) who compared apoB metabolic parameters using endogenously ([^{3}H]leucine) and exogenously (radioiodinated VLDL) labeled apoB in hypertriglyceridemic subjects. Compared to the FCR of VLDL-apoB derived from the radioiodinated data, the FCR for the endogenously labeled material was approximately 1.4-times greater. VLDL-

TABLE 5. Production rates of VLDL-, IDL-, and LDL-apoB estimated by multicompartmental modeling

Study	Production of VLDL-ApoB		Production of IDL-ApoB		Production of LDL-ApoB	
	Infusion	Bolus	Infusion	Bolus	Infusion	Bolus
	$mg \cdot kg^{-1} \cdot d^{-1}$		$mg \cdot kg^{-1} \cdot d^{-1}$		$mg \cdot kg^{-1} \cdot d^{-1}$	
#1	15.7 ± 3.0		11.1 ± 5.7		12.2[a]	
#2	23.6 ± 2.8	21.5 ± 3.9	16.5 ± 3.7	15.9 ± 2.3	12.8 ± 1.8	10.1 ± 1.2
#3	12.6 ± 0.5	12.6 ± 0.4	9.7 ± 9.3	9.3 ± 1.0	10.5 ± 0.7	7.3 ± 0.7
#4	14.1 ± 0.5	13.7 ± 0.4	13.5 ± 1.1	12.4 ± 1.0	13.5 ± 0.8	15.8 ± 0.9
#5	8.1 ± 0.2	8.5 ± 0.2	6.3 ± 0.6	5.1 ± 0.5	5.3 ± 0.8	8.3 ± 1.0
Mean ± SD[b]	14.6 ± 6.5	14.1 ± 5.4	11.5 ± 4.4	10.6 ± 4.6	10.5 ± 3.7	10.4 ± 3.8

Parameters were determined using the multicompartmental model shown in Fig. 1; values for individual studies represent best estimates ± SD.
[a]FCR of LDL-apoB could not be established in this study; the mean value of studies #2–#5 was assumed to calculate LDL-apoB production.
[b]Mean ± SD of best estimates of studies #2–#5; no significant differences were apparent for VLDL-apoB or LDL-apoB production when the parameters derived from the bolus were compared (paired *t*-test) to those derived from the primed constant infusion. For IDL-apoB, however, there was a small but significant difference ($P < 0.05$); parameters derived from the two different tracers were not significantly different from each other.

TABLE 6. Conversion rates of VLDL-, IDL-, and LDL-apoB estimated by multicompartmental modeling

Study	% of VLDL-ApoB Production Converted to IDL-ApoB		% of VLDL-ApoB Production Converted to LDL-ApoB via IDL		% of LDL-ApoB Derived from Shunt Pathway	
	Infusion	Bolus	Infusion	Bolus	Infusion	Bolus
#1	70.7 ± 9.3		51.0 ± 31.4		37.5 ± 19.2	
#2	69.9 ± 21.2	73.9 ± 15.2	18.4 ± 8.4	30.8 ± 5.7	55.0 ± 17.2	55.5 ± 12.5
#3	71.0 ± 24.1	73.5 ± 3.5	46.6 ± 12.3	9.3 ± 4.6	33.5 ± 5.2	45.9 ± 8.9
#4	95.9 ± 10.3	90.5 ± 5.0	71.8 ± 9.8	88.9 ± 7.0	4.2 ± 2.6	8.2 ± 2.1
#5	78.0 ± 7.4	59.9 ± 5.5	47.1 ± 9.2	60.0 ± 7.6	32.2 ± 5.7	41.9 ± 1.3
Mean ± SD[a]	78.9 ± 11.8	74.5 ± 12.5	46.0 ± 21.8	47.3 ± 34.71	31.3 ± 20.8	37.9 ± 20.6

Parameters were determined using the multicompartmental model shown in Fig. 1. Values for individual studies represent best estimates ± SD.
[a]Mean ± SD of best estimates of studies #2–#5; no significant differences were apparent when the parameters derived from the bolus were compared (paired *t*-test) to those derived from the primed constant infusion.

apoB FCR (which in steady-state is equivalent to fractional synthetic rate reported in other studies) determined in previously published studies using [^{15}N]glycine (1, 2) also compare favorably with the results presented here. On the other hand, Cohn et al. (6) and more recently Lichtenstein et al. (7) reported significantly lower values (0.21 h^{-1} and 0.13 h^{-1}, respectively) using [^{2}H]leucine (6) and [^{2}H]leucine, [^{2}H]valine, and [^{2}H]lysine (7). Similar VLDL-apoB FCRs were derived for those three amino acids (7). In those studies (1, 2, 6, 7) the amino acids were administered as primed constant infusions and data were analyzed by linear regression (1, 6, 7) or monoexponential (2) analysis. In none of those studies were enrichment values converted to tracer/tracee ratios, the correct form in which data should be analyzed (26). It is further worth noting that in those studies the longer infusion periods (6, 7) were associated with lower VLDL-apoB FCRs. This may be expected since FCRs determined by either linear regression or monoexponential analysis are dependent on the level of tracer/tracee ratio at the end of the infusion period. Failure to reach a plateau will necessarily result in incorrect parameters for VLDL-apoB metabolism.

Another drawback of the linear regression analysis can be seen in Fig. 3. Because there is no physiological basis for a linear regression analysis, the number of time points used for the linear fit is entirely arbitrary. As a consequence both the sampling schedule and the number of time points used for the linear fit will affect the steepness of the slope and therefore the calculated metabolic parameters.

Although little information has been published, IDL-apoB FCRs and production rates measured in the present study were higher than those reported by Kesaniemi, Beltz, and Grundy (36). The poorly defined nature of the IDL fraction, however, makes comparison of such parameters difficult. Although IDL-apoB FCRs were not different, there was a small but significant difference for IDL-apoB production estimated using the bolus and the primed constant infusion data. We believe that this difference may be accounted for by uncertainty associated with IDL-apoB pool size determination. Alternately, we cannot exclude the possibility that IDL-apoB metabolism is more complex than described here. However, there is insufficient information in our data to model this lipoprotein fraction in a more complex way.

LDL-apoB production rates compare favorably with previously published results (15, 16); however, estimates for LDL-apoB FCRs were marginally higher in our

TABLE 7. Comparison of different measures to estimate the level of precursor tracer-tracee ratio during a primed constant infusion of either [1-^{13}C]leucine or [^{15}N]glycine

Study	Tracer Used for Primed Constant Infusion	Plasma Glycine or Leucine Tracer/Tracee Ratio[a,b]	VLDL-ApoB Glycine or Leucine Tracer/Tracee Ratio[a,b]	Asymptote of VLDL-ApoB Glycine or Leucine Tracer/Tracee Ratio[b,c]	Plasma KIC Tracer/Tracee Ratio[a,d]
		%	%	%	%
#1	[^{15}N]glycine	11.2	4.0	4.5	
#2	[1-^{13}C]leucine	4.5	3.1	3.2	3.2
#3	[^{15}N]glycine	7.4	2.7	4.4	
#4	[1-^{13}C]leucine	5.5	4.0	3.8	3.4
#5	[1-^{13}C]leucine	5.3	4.3	4.0	3.8

[a]Values represent tracer/tracee ratios at the end of the primed constant infusion.
[b]Depending on which amino acid was administered as a prime constant infusion.
[c]Derived from the fit of the monoexponential analysis (A_p).
[d]Only in studies where [1-^{13}C]leucine was administered as a primed constant infusion.

studies. This may in part be due to our use of a single compartment to model the LDL fraction. Although LDL-apoB is known to be kinetically heterogenous (33), we were able to fit the LDL-apoB data with a one-pool model in these normolipidemic subjects. The analysis of LDL data in hyperlipidemic subjects will undoubtedly require a more sophisticated model. Although there was considerable variation between individuals, approximately 47% of VLDL-apoB was converted to LDL, via the VLDL-IDL-LDL pathway. This is consistent with previous findings (16, 36–38) reporting conversion rates between 41 and 87%. In addition, 37% of LDL-apoB was derived directly from the rapidly turning over VLDL compartment. In both normals and hyperlipidemics an input mechanism independent of the classical cascade has been proposed to account for that fraction of LDL-apoB not derived from the VLDL-IDL-LDL pathway (16, 35–37). Our estimates for this input are slightly higher than previously published findings on normolipidemics in whom the reported range is between 0 and 28% of total LDL-apoB production (36, 37). In some of our studies we were unable to account for a small fraction (7%) of LDL-apoB production by the VLDL-IDL-LDL cascade and the shunt pathway. We believe this is due to errors associated with the determination of apoB pool sizes.

Confounding the determination of LDL-apoB metabolism is the problem associated with the kinetics of the precursor. In these studies it was difficult to distinguish with confidence the kinetic characteristics of the LDL-apoB from those of the amino acid precursor because of recycling of the tracer. A similar finding was also recently reported by Beltz et al. (35). Thus, while the use of endogenous tracers provides good evidence for the rapid appearance of apoB into the LDL fraction (35), this methodology may not be optimal to determine the metabolism of LDL-apoB or any other slowly turning over protein pool. Although the use of tracers with higher abundance (such as deuterium$_4$ compounds) or analysis of samples by isotope ratio mass spectrometry will increase the quality of the data, this is not likely to eliminate the problem associated with amino acid recycling. Exogenously labeled material may be superior in such situations despite the potential problems associated with this approach (35, 39).

Irrespective of which kinetic model is used, the calculation of kinetic parameters will be dependent upon assumptions concerning the kinetics of the amino acid precursor. In linear regression and monoexponential analysis it is assumed that a primed constant infusion provides a constant level of tracer in the precursor. There is evidence from this study and others (1, 7), that no constant plasma amino acid tracer/tracee ratio is reached during an equilibration period of 1–2 h. The obvious failure to achieve such a constant level of tracer during a period of the study, a condition that must be satisfied, may be another reason for the variability seen between the three different methods of analysis. After an equilibration period the level of plasma amino acid tracer/tracee ratio continues to increase, usually until the infusion is stopped. This phenomenon is a function of the turnover rate of large protein pools from which amino acids recirculate. In a similar fashion, VLDL-apoB tracer/tracee ratios continue to rise until the infusion is stopped. This was seen in our studies and in studies where amino acids were infused for up to 15 h (1, 2, 6, 7). It is unclear whether this rise can be attributed to the increasing amount of tracer in the precursor alone or whether this is compounded by the presence of a slowly turning over compartment in the VLDL fraction. In using the multicompartmental approach, however, it is assumed that the shape of the plasma amino acid curve is the same as that of the precursor at the site of apoB synthesis. The fact that the apoB metabolic parameters derived from the bolus and primed constant infusion are similar supports this assumption.

Several other methods have been proposed to establish the level of tracer in the precursor during a primed constant infusion. Enrichment in hippurate (1, 2) and in plasma KIC (29–31) have been used as measures of intracellular glycine and leucine enrichment, respectively. Matsushima et al. (40) recently showed in rat liver perfusion experiments that the level of glycyl-tRNA enrichment was 20–30% lower than that of hippurate after single pass perfusion. This indicates that labeled hippurate does not directly reflect the level of labeled glycine in the precursor, when glycine is used as a tracer. In muscle protein synthesis studies, KIC enrichment has been proposed as a measure of intracellular leucine enrichment (29–31). In two of our studies, however, KIC tracer/tracee ratio was lower than that of leucine in VLDL-apoB at the end of the primed constant infusion, again indicating that labeled KIC may not directly reflect the level of labeled leucine in the precursor, when leucine is used as a tracer. Thus, based upon a sparsity of information, it is unclear whether the tracer/tracee ratios of either hippurate or KIC provide better estimates than those of plasma amino acids as a measure for the tracer/tracee ratio in the precursor.

In conclusion, metabolic parameters derived for apoB in these studies appear to be independent of the amino acids used and of the method by which the tracer is administered. Because the linear regression analysis has no physiologic basis, this methodology is inappropriate for the analysis of apoB metabolism. Under certain conditions, however, some metabolic parameters for VLDL-apoB can be estimated by monoexponential analysis. It is questionable, however, whether this method of analysis can be applied to the study of a heterogenous population of particles such as the VLDL fraction. Multicompartmental modeling, because of its ability to account for the heterogenous nature of lipoprotein metabolism and inte-

gration of VLDL, IDL, and LDL data into a single model, provides information not obtainable by the other methods of analysis.

APPENDIX

Fit of triexponential function to plasma amino acid tracer/tracee ratio data. Plasma amino acid tracer/tracee kinetics were fit using a triexponential function. This function was subsequently used as a forcing function to describe the input of tracer into the apoB synthesis compartment of the model. It is the shape of the function rather than the absolute values which drive the apoB model. To fit plasma amino acid data following a bolus, a sum of three exponentials was used:

$$y(t) = \sum_{i=1}^{3} A_i e^{-a_i t} \qquad Eq.\ 1)$$

where A_i are the coefficients of the exponential terms a_i.

For the primed constant infusion, the following sum of three exponentials was used to fit the plasma amino acid tracer/tracee ratio data:

$$y(t) = A_0 + \sum_{i=1}^{3} A_i e^{-a_i t} \qquad Eq.\ 2)$$

with $0 \leq t \leq T$, where T is the time at which the infusion stops, and A_0 is the plateau tracer/tracee ratio. What makes this function different from functions used to describe exponential decays is the presence of the constant term A_0 and the fact that some of the A_i will be negative. Function *2* describes only data until the infusion stops (time T), then a second function, similar to the function *1* used for the bolus data, must be used to describe the washout period. At time T the tracer/tracee ratio is given by function *2*. The equation used to account for the washout period is therefore a three exponential function with an initial amount of y(T). The expression is:

$$y(t) = y(T) \sum_{i=1}^{3} B_i e^{-a_i(t-T)} \qquad Eq.\ 3)$$

with $T < t$. Although the exponential terms a_i are the same as in equation *2*, the coefficients B_i are numerically different from A_i. Notice also in the exponential terms (t-T) appears instead of t; this is to account for the time shift necessary to describe the decay starting at time T, the time at which the infusion was stopped.

We thank Dr. J. Arends for advice and assistance concerning the GC-MS methods and the staff of the General Clinical Research Center at Washington University for help in the human studies. This work was supported by grants from the National Institutes of Health (RR02176, HL15308, RR00954, HD20805). Klaus Parhofer is a Fellow of the American Heart Association, Missouri Affiliate. Hugh Barrett is a recipient of a travel grant of the National Heart Foundation of Australia.

Manuscript received 25 February 1991 and in revised form 23 May 1991.

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