Studying apolipoprotein turnover with stable isotope tracers: correct analysis is by modeling enrichments

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Abstract Lipoprotein kinetic parameters are determined from mass spectrometry data after administering mass isotopes of amino acids, which label proteins endogenously. The standard procedure is to model the isotopic content of the labeled precursor amino acid and of proteins of interest as tracer-to-tracee ratio (TTR). It is shown here that even though the administered tracer alters amino acid mass and turnover, apolipoprotein synthesis is unaltered and hence the apolipoprotein system is in a steady state, with the total (labeled plus unlabeled) masses and fluxes remaining constant. The correct model formulation for apolipoprotein kinetics is shown to be in terms of tracer enrichment, not of TTR. The needed mathematical equations are derived. A theoretical error analysis is carried out to calculate the magnitude of error in published results using TTR modeling. It is shown that TTR modeling leads to a consistent underestimation of the fractional synthetic rate. In constant-infusion studies, the bias error percent is shown to equal approximately the plateau enrichment, generally <10%. It is shown that, in bolus studies, the underestimation error can be larger. ■ Thus, for mass isotope studies with endogenous tracers, apolipoproteins are in a steady state and the data should be fitted by modeling enrichments.—Ramakrishnan, R. **Studying apolipoprotein turnover with stable isotope tracers: correct analysis is by modeling enrichments.** *J. Lipid Res.* 2006. **47:** 2738–2753.

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Beginning in the 1970s, apolipoprotein kinetics were routinely studied with exogenous tracers, for instance by isolating VLDL or LDL from a subject, radioiodinating it, and injecting it back into the subject (1–4). Endogenous labeling, with a labeled precursor of the metabolite of interest, has the virtue of labeling the synthetic pathways and not altering tracer metabolic properties, as might happen with exogenous labeling. Whole body cholesterol metabolism was studied with tritiated water (5), and tritiated leucine has been used to study lipoprotein kinetics (6–8). Highly sensitive gas chromatography-mass spectrometry and refinements thereof, and the availability of synthetic amino acids and other molecules that are multiply labeled with mass isotopes, have altered the field to the point that endogenous labeling with mass isotopes is now the norm in human turnover studies (9).

An important aspect of mass isotopes is that the amount of tracer introduced is not negligible in relation to the amount in plasma of the tracee. Cobelli, Toffolo, and Foster (10) and Foster et al. (11) considered this problem and advocated the use of tracer-to-tracee ratio (TTR) in place of the previously standard use of tracer enrichment in atoms percent excess or moles percent excess (12–15). Since then, nearly all investigators have used TTR in analyzing mass isotope data to calculate lipoprotein turnover parameters. In what follows, we revisit this issue and derive the mathematical relationships needed for the analysis of tracer data from endogenous labeling. In particular, we show that the apolipoprotein system is in a steady state and that the correct formulation is in terms of tracer enrichment or concentration, not TTR. We show that compartmental models and the usual fractional synthetic rate (FSR) equations are valid provided that they are written for tracer enrichments but not for TTRs. The error in using TTR is shown for constant-infusion studies to be in the range of the plateau tracer enrichment, usually 5–10%; the error is shown to be higher for bolus studies.

The word "enrichment" (E) is used here solely to denote tracer concentration, defined as the amount of tracer divided by the sum of the amounts of tracer and tracee (16). The word is used sometimes to denote TTR, but not here.

CONSTANT TRACEE FLUX IMPLIES THAT PROTEIN AMOUNT CHANGES WITH TIME

That mass isotope tracers have nonnegligible mass has been recognized from early on. Matthews et al. (16) ad-

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Abbreviations: apoB, apolipoprotein B; E, enrichment; FCR, fractional catabolic rate; FSR, fractional synthetic rate; M, total (tracer and tracee) mass; m, tracer mass; P, amount or plateau level of precursor; R, total (tracer and tracee) flux; S, product synthetic rate; t, time; TG, triglyceride; TTR, tracer-to-tracee ratio; U, unlabeled or tracee mass.
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justed for it by assuming that the total flux is increased by a constant tracer infusion, so that a tracer balance can be written as:

$$(Q + i)E_p = iE_i \qquad (Eq.\ 1)$$

where Q is the tracee flux, i is the constant infusion rate of the tracer, E_p is the plasma enrichment of the traced molecule (amino acid in Ref. 16), and E_i is the infusion enrichment. With a radiotracer, because of its negligible mass, there would be no i on the left side of the equation, and the right side would be the infusion rate of radioactivity.

The question relevant to lipoprotein turnover is whether every component of the flux changes similarly. Foster et al. (11) assume that incorporation into specific proteins follows the same pattern. This approach is shown by them to lead to simple equations for protein kinetics. The tracee masses and fluxes are constant, and linear differential equations can be written for TTR, the equations identical in form to those written for radiotracers.

Figure 1 shows the essential part of their model (Fig. 1 in Ref. 11) for leucine incorporation into VLDL apolipoprotein B (apoB), leaving out other pathways. Before tracer infusion, there is U_{leu} of unlabeled leucine, being incorporated into VLDL apoB with a rate constant of k_{leu}; U_B is the tracee mass of VLDL apoB. During the tracer study, by the assumption of tracee steady state, U_{leu}, k_{leu}, and U_B do not change; at any time t, there is $m_{leu}(t)$ of tracer, being incorporated into labeled VLDL apoB with the same rate constant as the tracee, k_{leu}. [Since an apoB molecule has multiple leucine molecules, a labeled leucine combines with unlabeled leucine in the same apoB molecule, so this model is not precise, but it is easily rectified by combining the two apoB pools into one. None of the results below are affected, and the figure is drawn to be close to Fig. 1 in Foster et al. (11).] Thus, the constant tracee flux assumption predicts that, compared with the steady state before the study, more leucine should be

Fig. 1. Adapted from Ref. 11 to show the essential part of their Fig. 1. Leucine pools and synthesis of a protein of interest [e.g., VLDL apolipoprotein B (apoB)]. The larger pool is for the tracee, and the smaller pool is for the tracer. Under the assumption of tracee steady state, tracee incorporation into VLDL apoB is unaltered during the study, but tracer mass and incorporation change with time, indicated by (t); the tracer incorporation rate constant is the same as for the tracee. Other pathways for leucine and for VLDL apoB are not shown. The dashed rectangles denote total leucine and VLDL apoB.

incorporated into VLDL apoB during the tracer study and the total amount of VLDL apoB should increase correspondingly. If the TTR of leucine in VLDL apoB approaches 5%, which is typical, then, under the constant tracee flux assumption, VLDL apoB mass should be higher by 5% at the end of the constant infusion.

THE CONSTANT TRACEE FLUX ASSUMPTION LEADS TO A CONTRADICTION

There are two reasons why the assumption of constant tracee masses and fluxes may be invalid. One is that the assumption of constant tracee flux, applied simultaneously to multiple amino acids, leads to a contradiction. The other is that there is evidence that apolipoprotein synthesis is unaltered by tracer infusion. These reasons are elaborated on below.

Consider a study with a primed constant infusion of a tracer of leucine and a simultaneous bolus injection of a tracer of glycine, as in Parhofer et al. (17). **Figure 2A** shows three amino acids that go into the synthesis of apoB. The assumption that the tracee remains in a steady state can be applied to each of the three amino acids. The incorporation from each tracee pool is shown as constant, whereas the incorporation from each tracer pool varies with time, denoted by (t). Figure 2B shows the hypothetical total rate of incorporation of each precursor (tracer plus tracee) resulting from the assumption of tracee steady state. The values before tracer infusion are at the mol%s of the three amino acids in apoB. The total incorporation rate of leucine increases to a higher steady state, that of glycine increases sharply and declines with the clearance of the glycine tracer, and that of alanine remains unchanged. Thus, if the tracee fluxes are constant, the relative amounts of leucine, glycine, and alanine in newly synthesized apoB begin at the known values for apoB, but soon after the tracer study begins, newly synthesized apoB has 14% leucine and 9% glycine, the percentages changing every moment, a stoichiometric impossibility as this would mean a changing amino acid composition of the protein.

This can also been seen in the composition of the product, VLDL apoB. Under the assumption of constant tracee mass, the TTR in VLDL apoB leucine or glycine is equivalent to the change in total VLDL apoB. If leucine TTR is 4% at some time, then VLDL apoB at that moment is 4% higher than before the study. Likewise, if glycine TTR in VLDL apoB is 2%, then VLDL apoB is up by 2% at that moment. For an amino acid such as alanine, there is no tracer and so total VLDL apoB should be unchanging. Figure 2C shows the VLDL apoB TTR data from Parhofer et al. (17), with the TTR values presented here, under the tracee constancy assumption, as changes in total VLDL apoB mass. The horizontal line indicates that an untraced amino acid such as alanine would imply no change in VLDL apoB. The three curves in Fig. 2C show very different behaviors for total VLDL apoB. Thus, the data of Parhofer et al. (17) are inconsistent with the tracee steady state assumption. The data of Demant et al. (18), with
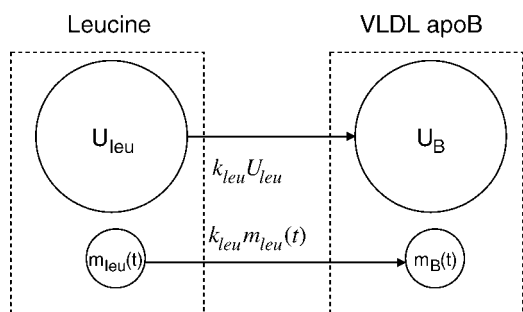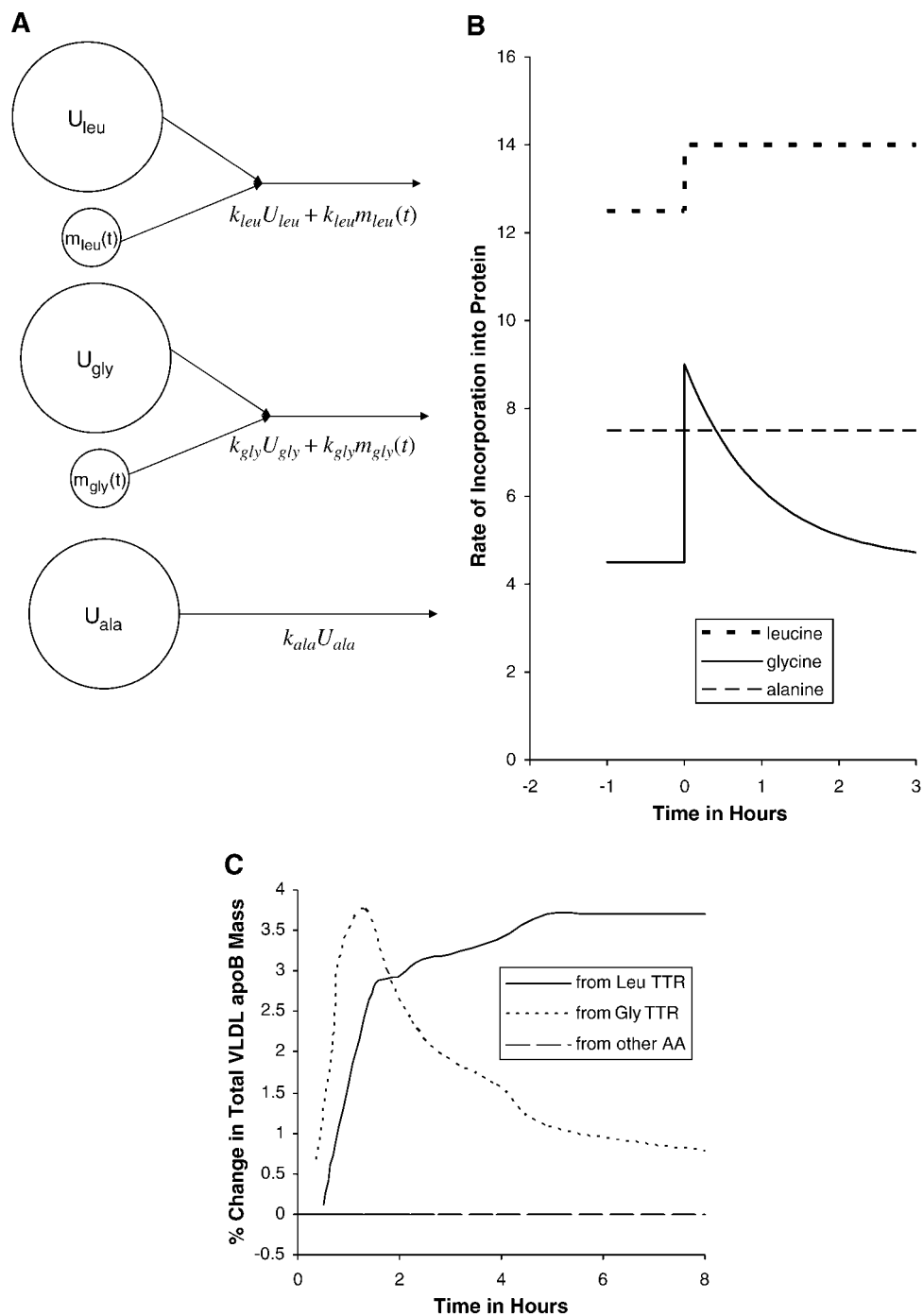
Fig. 2. Schemata of three amino acid precursors, and their rates of incorporation into a specific apolipo-protein in a hypothetical study with a primed constant infusion of a tracer of leucine and a simultaneous bolus injection of a tracer of glycine. Under the assumption of tracee steady state, A shows the precursor pools, with the small pools representing tracers. If the tracee (unlabeled) masses U_{leu}, U_{gly}, and U_{ala} are constant, the rates of incorporation vary with time, as given by the formulas under the arrows and as shown in B. The numbers along the y axis are in arbitrary units. The curves begin at the leucine, glycine, and alanine contents of apolipoprotein B before tracer infusion. Soon after 0 h, leucine is at 14 instead of 12.5, and glycine is at 9 instead of 4.5, resulting in a changing stoichiometry of the apoB product, an impossibility. C shows the tracer-to-tracee ratio (TTR) data from Fig. 4 of Parhofer et al. (17), presented here, under the assumption of tracee steady state, as the change in total mass of VLDL apoB. If tracee apoB were constant, total apoB increased by nearly 4% to a new steady level, according to the leucine TTR data, whereas the glycine TTR data would suggest that total apoB increased quickly by nearly 4% and then declined. The dashed horizontal line indicates that an untraced amino acid should be interpreted as no change in VLDL apoB. Thus, the assumption of a tracee steady state is contradicted by the data of Parhofer et al. (17). AA, amino acid.

simultaneous leucine bolus and phenylalanine constant infusion, would also support this conclusion. Indeed, any double-tracer study in which the TTRs of the two tracers in VLDL apoB are not identical contradicts the assumption of tracee steady state.

APOLIPOPROTEIN SYNTHESIS UNAFFECTED BY AMINO ACID INFUSION

The other reason for the likely invalidity of a constant tracee flux to apolipoproteins is that there is no evidence that infusion of a single amino acid affects apolipoprotein synthesis. Apolipoprotein synthesis is regulated by many factors affecting transcription, mRNA stability, translation, and posttranslational degradation, but a single amino acid availability is not known to be such a factor.

Cohn et al. (14) and Lichtenstein et al. (19) measured VLDL apoB a number of times during a 15 h constant-infusion study. No time trend in VLDL apoB mass was seen, whereas VLDL apoB TTR increased to ∼6%. This constancy has been replicated in a number of studies by that group (20–23), in which multiple VLDL apoB fractions were obtained during the study. Other studies (24–31) have also found no change in apolipoprotein concentrations during constant-infusion studies. There appear to be no reports of an increase in VLDL apoB mass during a constant-infusion study.

Davis and coworkers (32) have shown in pigs that, once the neonatal phase is over, amino acid infusion or protein intake increases muscle protein synthesis, but the effect on liver protein synthesis is quite modest, as was found earlier in rats (33, 34). Of particular relevance to apolipoproteins, Motil et al. (35) studied two different protein intakes in five women and found that leucine oxidation increased with protein intake but lysine incorporation into apoB did not. These animal and human studies are consistent with cell culture studies showing that a significant fraction of newly synthesized apoB is degraded, with secretion determined largely by lipid availability (36). Intracellular apoB degradation has been estimated by compartmental modeling to be ∼90% in HepG2 cells (37, 38) and >30% in primary hepatocytes (39).

Thus, there is no reason to expect that a tracer amino acid infusion alters apolipoprotein synthesis. Assuming that apolipoprotein synthesis is unaltered by tracer infusion avoids the contradiction implied by the constant tracee flux assumption and is consistent with the published data cited above.

PRECURSOR ENRICHMENT IS FORCING FUNCTION FOR APOLIPOPROTEIN KINETICS

We next show that the introduction of tracer alters tracee incorporation (as opposed to the total of tracer and tracee) into apolipoproteins. Consider **Fig. 3**, which shows, for amino acid 1, the tracer with mass $m_1(t)$ and tracee with mass $U_1(t)$ at the site of synthesis of the apolipo-
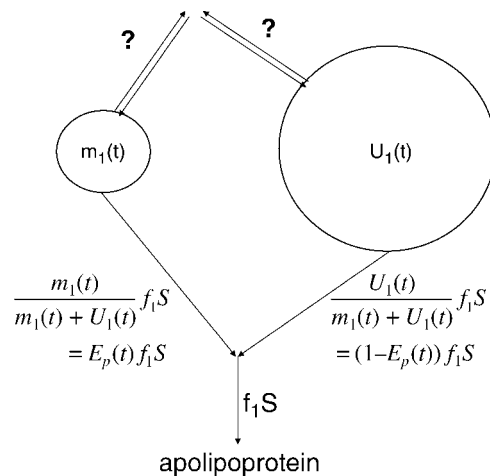

Fig. 3. The tracer and tracee precursor pools for an amino acid whose tracer is introduced. Both pool sizes can vary with time, indicated by (t), but the total (tracer plus tracee) incorporation rate into an apolipoprotein is unchanged. The separate incorporation rates of tracer and tracee are proportional to their respective masses, as given by the formulas. The fluxes on other pathways, such as oxidation, clearance, or other storage pools, may bear different relationships to the masses, as indicated by question marks to mean "unknown."

protein of interest. The rate of incorporation into the protein remains at f_1S before and after tracer is introduced, where S is the protein synthesis and f_1 is the fraction from amino acid 1. Since the tracer and tracee are indistinguishable, the tracer incorporation rate is obtained simply by multiplying f_1S by the tracer fraction of the pool, $\dfrac{m_1(t)}{m_1(t) + U_1(t)}$, which is the enrichment $E_P(t)$ of the precursor pool. The tracee incorporation rate, therefore, is $(1 - E_P(t))f_1S$, compared with f_1S in the absence of tracer.

Thus, for any apolipoprotein, after tracer infusion: *1)* the apolipoprotein synthesis and the rate of incorporation from each amino acid remain unaltered; *2)* tracee flux to the apolipoprotein is altered; and *3)* tracer flux to the apolipoprotein equals precursor pool enrichment multiplied by the incorporation rate of that amino acid.

These points are illustrated by the bar graphs in **Fig. 4**; tracer content is shown by hatched areas. Two experimental designs are considered. The left panels are for a primed constant infusion. The top left panel shows the mass of free amino acid in the precursor pool at four different times. There is no tracer at time zero; with the introduction of tracer, the total mass increases and remains at a constant level from 1 to 15 h, as does the mass of tracer. The middle left panel shows the rate of apolipoprotein synthesis at the four times. The total synthesis, S, remains constant, whereas a fraction of the synthesis is labeled after time zero. By the principle of isotopic indistinguishability (40), the tracer fraction of the synthesis equals the tracer fraction in the precursor pool:

$$\frac{S_{LC1}}{S} = \frac{P_{LC1}}{P_{TC1}}, \; \frac{S_{LC8}}{S} = \frac{P_{LC8}}{P_{TC8}}, \; \frac{S_{LC15}}{S} = \frac{P_{LC15}}{P_{TC15}} \quad (Eq.\ 2)$$
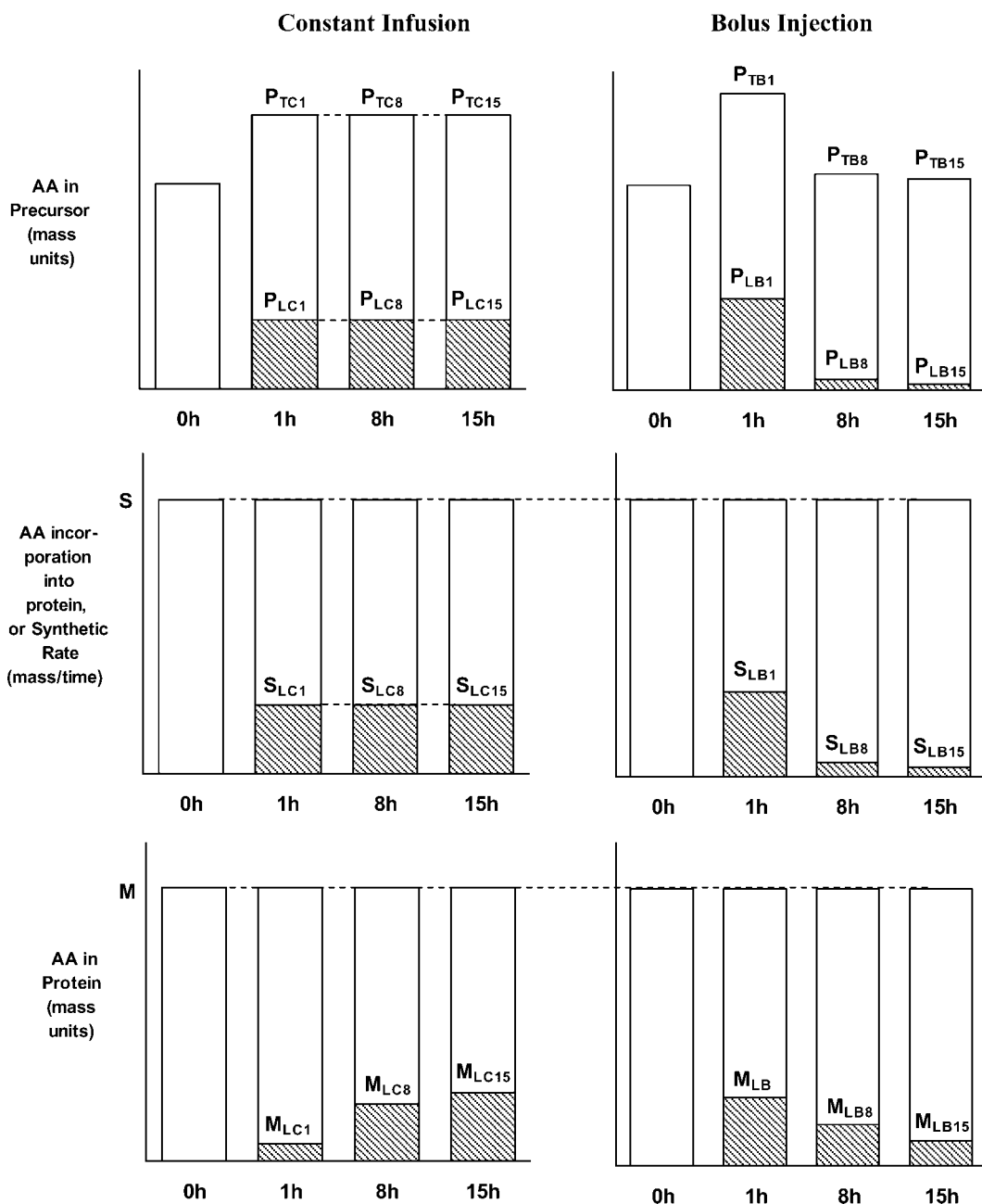
Constant Infusion

Bolus Injection

Fig. 4. Schematic bar graphs showing the changes with time in precursor amino acid (P), incorporation rate into apolipoprotein (S), and amino acid (AA) in apolipoprotein (M) for two types of tracer studies. The full height of each bar represents the total of tracer and tracee, whereas the hatched portion is for the tracer. The subscript T stands for total (tracer plus tracee), L stands for tracer (label), C stands for constant infusion, B stands for bolus, and 1, 8, and 15 indicate 1, 8, and 15 h. The left panels are for a primed constant infusion. The upper left panel shows that the tracer in the precursor increases and stays at a constant fraction from 1 to 15 h. The middle left panel shows that apolipoprotein incorporation is unchanged, with the tracer contributing that same fraction from 1 to 15 h. The bottom left panel shows that the apolipoprotein mass remains the same while the amount of label increases from 0 to 1 to 8 h, approaching a plateau at 15 h. The right panels are for a bolus study. The upper right panel shows that the tracer in the precursor increases and then declines from 1 to 15 h. The middle right panel shows that the apolipoprotein incorporation is unchanged, with the tracer contributing a fraction equal to its fraction in the precursor. The bottom right panel shows that the apolipoprotein mass remains the same while the amount of label increases from 0 to 1 h and then decreases to 15 h.

where S stands for synthetic rate (mass/time), P stands for the amount of precursor (free amino acid), the subscript L refers to the label, T refers to the total mass, C refers to the constant-infusion design, and 1, 8, and 15 refer to the time. The bottom left panel shows the mass of amino acid

in the apolipoprotein of interest at the four times. The total amount, M, remains constant, whereas an increasing fraction of the mass is labeled over 15 h, approaching the fraction in the precursor pool (and the synthetic pathway) given in Eq. 2.

The right side of Fig. 4 is for a bolus injection. The top right panel shows the mass of free amino acid in the precursor pool at the four times. The difference from constant infusion is that there is a much greater perturbation at 1 h, with a high tracer content that decreases with time. The middle right panel shows the rate of apolipoprotein synthesis at the four times. As with constant infusion, total synthesis, S, remains constant, whereas the labeled fraction increases and decreases with the precursor pool:

$$\frac{S_{LB1}}{S} = \frac{P_{LB1}}{P_{TB1}}, \frac{S_{LB8}}{S} = \frac{P_{LB8}}{P_{TB8}}, \frac{S_{LB15}}{S} = \frac{P_{LB15}}{P_{TB15}} \quad (Eq.\ 3)$$

where the subscript B refers to the bolus injection design. This is analogous to Eq. 2. The bottom right panel shows the mass of amino acid in the apolipoprotein of interest at the four times. As with constant infusion, the total amount, M, remains constant, whereas the labeled fraction changes over 15 h.

Figure 4 illustrates how, with a constant-infusion or bolus study, the precursor amino acid pool can be in an unsteady state while apolipoprotein synthesis and mass remain constant, with the rate of tracer incorporation proportional to tracer enrichment in the precursor.

UNSTEADY-STATE PRECURSOR/ STEADY-STATE APOLIPOPROTEIN

These ideas are summarized in the schematic model in **Fig. 5** for the kinetics of an amino acid and an apolipo-protein of interest. The model is similar to that used by many researchers for apoB (41–44). The details, regarding the number of pools and their connectivity, may vary with the apolipoprotein being studied and with the mode of tracer administration. The key element here is the dashed horizontal line that separates the free amino acid model from the apolipoprotein model. Before the tracer study, the study subject is in a steady state: that is, the masses and fluxes of the free amino acid and of the protein are constant with time. When the mass isotope tracer is introduced, it clearly increases the total amount of the free amino acid in plasma. This may or may not lead to changes in the rate constants and alter the masses and fluxes of the unlabeled free amino acid as well as of the total (labeled plus unlabeled) amino acid. Modeling amino acid turnover has been described by Cobelli et al. (40). Clearly, the free amino acid part of the system is in an unsteady state.

We next consider the double arrows crossing the horizontal line, indicating synthesis or incorporation of the amino acid into the apolipoprotein of interest (two arrows are given to allow for the possibility of synthesis into multiple lipoprotein fractions). This flux (mass per unit of time) is not limited by the amount of a single amino acid in the precursor pool. Whether the mass of that pool increases by 5% or even by 100%, it is not rate-limiting for apolipoprotein synthesis; hence, the total synthetic flux (labeled plus unlabeled) is not affected.
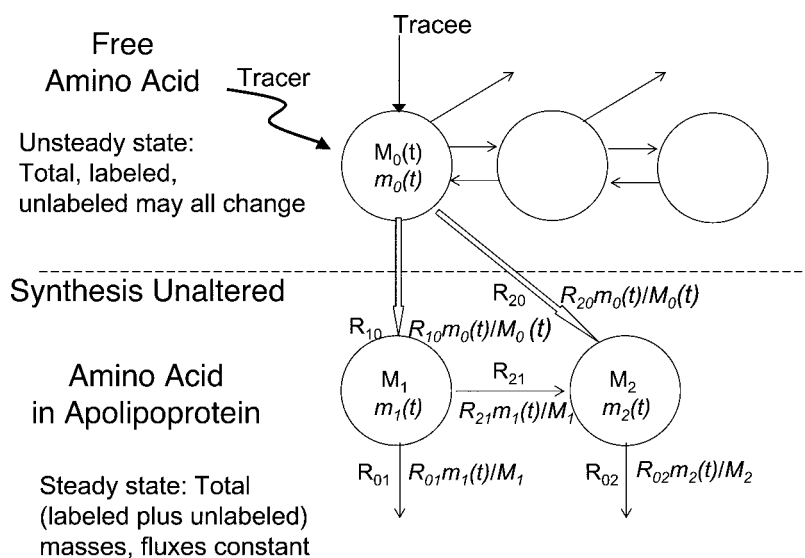

Fig. 5. Scheme of the amino acid and apolipoprotein systems. When a tracer is introduced, that amino acid is in an unsteady state, but the apolipoprotein remains in a steady state. The dashed line separates the unsteady amino acid system from the steady apolipoprotein system. The number of pools is for illustrative purposes. The unaltered synthesis paths are shown by double arrows. Total masses are denoted by M, and fluxes are denoted by R. Tracer masses are denoted by m; labeled fluxes, which equal the corresponding total fluxes multiplied by the source pool enrichments, are given in terms of total fluxes and tracer-to-total mass ratios or enrichments. For instance, the total flux from protein pool 1 to pool 2 is R_{21}, and the corresponding tracer flux is $R_{21}m_1(t)/M_1$, or R_{21} multiplied by the tracer enrichment in pool 1. Tracer quantities are shown in italics, below or to the right of the corresponding total quantities.

Moving down to the apolipoprotein part of the model, since the synthetic rates are not affected, the total (labeled plus unlabeled) masses and fluxes of the apolipoprotein remain constant during the tracer study. Thus, there is a steady state for total apolipoprotein. As labeled amino acid is incorporated into protein, the tracer content of the protein masses will change, much as with exogenous tracers.

KEY FEATURES OF ENDOGENOUS LABELING

Thus, the key features of endogenous labeling, with radioactive or mass isotopes, are as follows.

1) By using a labeled precursor, not rate-limiting for the synthesis of the apolipoprotein of interest, even in nontracer amounts (whether as a bolus or as a primed constant infusion), the synthetic pathways are labeled without affecting the steady state of the apolipoprotein: the masses, fluxes, and rate constants of apolipoprotein turnover are not affected.

2) In contrast, the precursor itself is in an unsteady state, with the introduction of the label altering the total masses and possibly fluxes and/or rate constants.

3) The kinetics of the apolipoprotein differ in a subtle way from those of an exogenous radiolabel, in which the amount of tracer is so small that the tracee (unlabeled) masses, fluxes, and rate constants may be assumed to be constant. With endogenous labeling with a mass isotope, the total masses, fluxes, and rate constants do not change but the amounts of tracer and tracee do change in the course of the study.

MATHEMATICAL MODELING OF APOLIPOPROTEIN KINETICS

Since mass isotopes, unlike radiotracers, have significant mass, it is possible that conventional pool models may not apply. We first show that, since the apolipoprotein system is in a steady state, with constant total masses and fluxes, it is possible to use pool models for tracer enrichment. Consider Fig. 5, which shows two protein pools both receiving newly synthesized protein. Total (labeled plus unlabeled) masses are denoted by M, total fluxes by R, and tracer masses by m. As in Fig. 3, tracer fluxes equal total fluxes multiplied by the source pool enrichments. For instance, the total flux from protein pool 1 to pool 2 is R_{21}, and the corresponding tracer flux is $R_{21}m_1(t)/M_1$, or R_{21} multiplied by $y_1(t)$, the tracer enrichment in pool 1. (Tracer quantities are shown in italics, below or to the right of the corresponding total quantities.) The rate of change of $m_1(t)$, tracer mass in pool 1, is given by a differential equation:

$$\frac{dm_1(t)}{dt} = R_{10}w(t) - (R_{01} + R_{21})\frac{m_1(t)}{M_1} \qquad (Eq.\ 4)$$

where w(t) is the precursor enrichment, equal to $m_0(t)/M_0(t)$. The equation can be rewritten in terms of tracer enrichment, since $m_1(t)$ equals $M_1y_1(t)$, and M_1 is constant:

$$\frac{dy_1(t)}{dt} = \frac{R_{10}}{M_1}w(t) - \frac{R_{01} + R_{21}}{M_1}y_1(t) \qquad (Eq.\ 5)$$

where L values are rate constants, with L_{ij} defined as the flux into pool i from pool j divided by the mass of pool i. Proceeding similarly, a differential equation for tracer enrichment in pool 2 is obtained:

$$\frac{dy_2(t)}{dt} = \frac{R_{20}}{M_2}w(t) + \frac{R_{21}}{M_2}y_1(t) - \frac{R_{02}}{M_2}y_2(t) \qquad (Eq.\ 6)$$

It is seen that the differential equations 5 and 6 are linear and stationary with constant coefficients, identical to what would be obtained with exogenous tracers. Thus, although mass isotopes introduce a nonnegligible mass into the precursor pools, the apolipoprotein system is modeled as with exogenous tracers (45–49).

$$\frac{dy}{dt} = Ay(t) + sw(t) \qquad (Eq.\ 7)$$

is the classic pool model in matrix-vector notation, where y(t) is the tracer enrichment vector in moles percent excess, A is the matrix of rate constants (A_{ii} is the negative of the total flux out of pool i divided by the mass of that pool, and A_{ij} is the flux into pool i from pool j divided by the mass of pool i), s_i is the direct synthetic flux into pool i divided by the mass of pool i, and w(t) is the precursor tracer enrichment function, which may not be describable by a pool model since the precursor system is in an unsteady state.

To clarify, consider a single pool for a protein, as is done in modeling apolipoprotein [a] (50) or apoC-III (51). The mass balance for the tracer is written as:

$$\frac{d}{dt}(My) = Sw - Ry \qquad (Eq.\ 8)$$

where S is the synthetic rate, equal to R, the flux out of the pool. Since the protein is in a steady state, M is constant, and so the equation can be rewritten as:

$$\frac{dy}{dt} = k(w - y) \quad \text{where } k = FCR = FSR$$
$$= \frac{\text{synthetic rate}}{\text{protein mass}} = \frac{S}{M} \qquad (Eq.\ 9)$$

With a primed constant infusion, if the precursor enrichment w can be assumed constant, an analytical solution is available:

$$y = w(1 - e^{-kt}) \qquad (Eq.\ 10)$$

Both w and y are tracer enrichments.

It is not possible to write a linear differential equation for TTR because tracee mass and flux are not constant. The equation for TTR analogous to equation 8 is the following:

$$\frac{d}{dt}(M_u(t)TTR) = S_u(t)\frac{w}{1-w} - R_u(t)TTR \qquad (Eq.\ 11)$$

$$M_u(t) \frac{d}{dt}(TTR) + TTR \frac{d}{dt}(M_u(t)) = S_u(t) \frac{w}{1-w}$$
$$- R_u(t)TTR \qquad (Eq.\ 12)$$

This is a nonlinear differential equation, which has to be solved along with a separate differential equation for $M_u(t)$, the subscript u denoting the unlabeled tracee. In general, there is no simple solution.

Thus, the linear differential equations with constant coefficients used in various modeling programs such as SAAM (49) and Poolfit (52) are valid only for tracer enrichments, not for TTR.

MATHEMATICAL MODELING OF PRECURSOR KINETICS

As seen in the top panels of Fig. 4, the precursor is not in a steady state. Total and tracer masses and fluxes are varying with time. Cobelli et al. (40) have discussed various possibilities. We have seen that tracee fluxes to proteins are affected, since the total apolipoprotein synthesis is not altered while part of it gets labeled, as seen in the middle panels of Fig. 4. The modeling of the precursor is quite complex, and data may not be available to do justice to the problem. As Barrett et al. (53) stated, "The kinetics of amino acids are complex, so a possible approach to incorporating plasma amino acid data into the development and fitting of a compartmental model to tracer data is to use a forcing function. In this way, the system can be decoupled and the plasma amino acid data can be used as the source of tracer." Following their reasoning, the precursor is merely a forcing function. So any model (or even no model) is adequate as long as the observed data for the precursor are fitted well. The data fitted can be TTR or enrichment.

As seen above, the model for apolipoprotein turnover requires precursor tracer enrichment. Therefore, regardless of how the precursor data are fitted, they have to be converted to enrichments for use as $w(t)$ in equation 7 for any apolipoprotein model.

ERROR IN USING TTR

Since most investigators have calculated kinetic parameters from TTR data, and not enrichments, the question arises of the magnitude of error in reported results. Some theoretical analysis is provided here to give an idea of the magnitude of the error.

Since TTR equals $E/(1 - E)$, it is always greater than E. We can also compare the shapes of the two curves by looking at the time derivative of TTR:

$$\frac{d}{dt}TTR(t) = \frac{d}{dt}\left(\frac{E(t)}{1 - E(t)}\right) = \frac{1}{(1 - E(t))^2} \frac{d}{dt}E(t)$$
$$(Eq.\ 13)$$

Since the denominator on the right side is always <1, the slope of TTR is always greater in magnitude than that of E: if E is increasing, TTR increases faster; if E is decreasing, TTR decreases faster. **Figure 6A** shows an illustration of this for a single pool receiving a bolus injection (the rest of the figure will be described below).

The error in using TTR instead of enrichment E is given by: error = TTR − E. The error is a positive bias, which can be expressed as a fraction:

$$\text{bias fraction} = \frac{TTR - E}{E} = \frac{TTR}{TTR/(1 + TTR)} - 1 = TTR$$
$$(Eq.\ 14)$$

Thus, the TTR curve is greater than the E curve by a fraction that equals TTR. If TTR is 10%, then E is 9.09%, and the error is 0.91%, which is 10% of the enrichment. The relative error increases from zero at zero enrichment to a maximum at the peak.

We consider three simple situations that are amenable to theoretical analysis. In what follows, FSR_{TTR} is used to denote the FSR computed by TTR modeling, and FSR_E denotes the correct FSR from modeling enrichment.

ERROR IN FSR$_{TTR}$ OF A SINGLE RAPID POOL WITH A CONSTANT INFUSION

Many investigators have used primed constant infusion and modeled the apolipoprotein of interest by a single pool (29, 30, 41, 50, 54–70). The FSR is calculated by fitting the TTR data by an increasing exponential:

$$TTR(t) = \text{plateau}(TTR)[1 - e^{-rt}] \qquad (Eq.\ 15)$$

where r is the FSR of interest. However, the modeling should be done for enrichment, which leads to:

$$E(t) = P[1 - e^{-kt}] \qquad (Eq.\ 16)$$

where k is the true FSR, E is the enrichment, and P is the plateau enrichment of the precursor, E and P expressed as fractions. TTR is given by:

$$TTR(t) = \frac{E(t)}{1 - E(t)} = \frac{P(1 - e^{-kt})}{1 - P(1 - e^{-kt})} \qquad (Eq.\ 17)$$

This is not a simple increasing exponential, but for the usual values of P (<0.1), it can be fitted by an increasing exponential quite adequately, which may be a reason that this error has not been reported. A general result for the error is not available; individual data sets have to be fitted both ways to calculate the errors. However, if we assume that the initial increase of the TTR data is well fitted by an increasing exponential, the initial slope of the fitted curve (equation 15) must equal that of the true TTR response (equation 17). By equating the time derivatives of the two equations, we get:

$$\frac{P}{1 - P}re^{-rt} = \frac{Pke^{-kt}}{[1 - P(1 - e^{-kt})]^2} \qquad (Eq.\ 18)$$
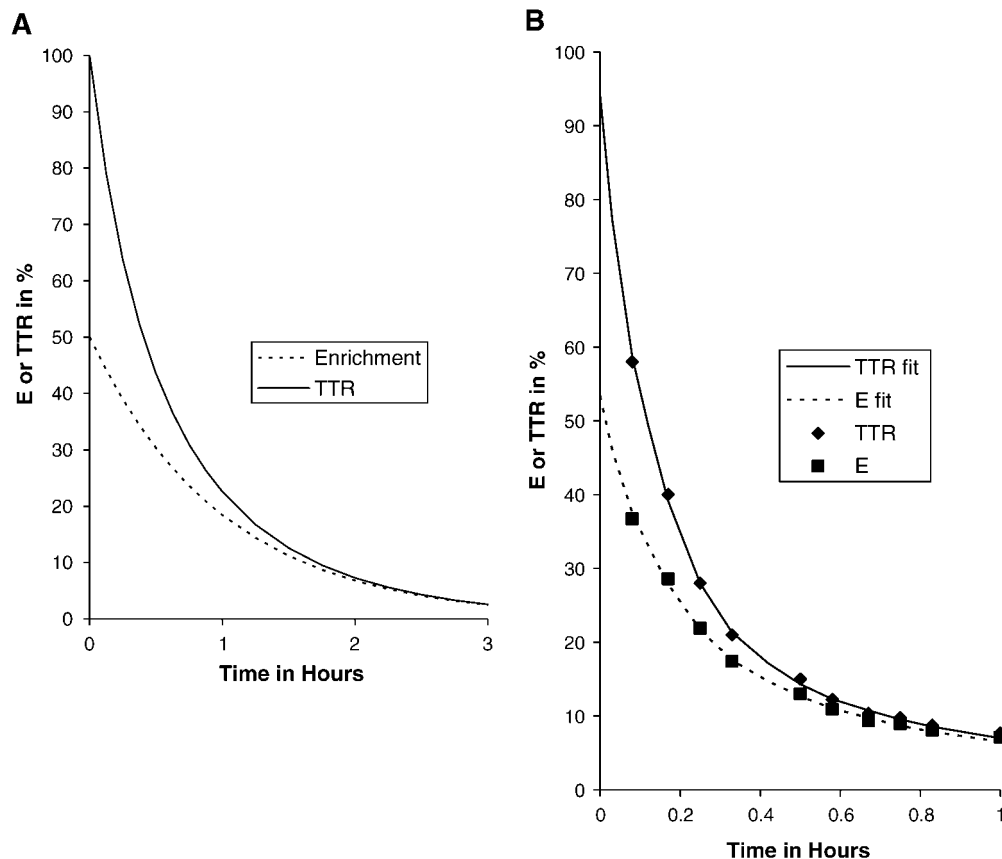
Fig. 6. In a bolus study, precursor TTR is higher and sharper than precursor tracer enrichment (E). A: Theoretical situation with a single precursor pool whose enrichment declines monoexponentially. B: TTR data taken from Parhofer et al. (17) and enrichments calculated from their TTR data, along with fitted curves. The area under the TTR curve is 39% higher in A and 35% higher in B than the corresponding area under the enrichment curve. This overestimation of the forcing function leads to an underestimate of fractional synthetic rates with TTR modeling.

At very early times, this becomes, approximately

$$\frac{P}{1 - P}r = Pk \qquad (Eq. 19)$$

which leads to $r = k(1 - P)$.

Thus, the FSR calculated by fitting the TTR data by an increasing exponential roughly equals the true FSR multiplied by $(1 - P)$, an underestimate by a fraction roughly equal to the plateau enrichment.

ERROR IN FSR$_{TTR}$ OF SLOWLY TURNING-OVER APOLIPOPROTEINS WITH A CONSTANT INFUSION

Some studies have used primed constant infusions and looked at slowly turning-over apolipoproteins such as LDL apoB, apoC-III, and apolipoprotein [a], proteins whose enrichments show a linear increase with time during the course of the study; the FSR has been calculated in a "model-free" approach by fitting the early TTR data by a straight line and dividing the slope by the precursor TTR (19, 71–73):

$$FSR_{TTR} = \frac{slope(TTR)}{plateau(TTR)} \qquad (Eq. 20)$$

The correct model-free method is to use the enrichment plateau and slope:

$$FSR_E = \frac{slope(enrichment)}{plateau(enrichment)} \qquad (Eq. 21)$$

As shown above, the TTR denominator has a positive bias equal to plateau(TTR). The numerator, being a slope estimated by fitting a number of points, is more complicated to analyze. The ideal situation, in which protein enrichment data are available continuously from zero until the maximum on the linear portion, is solved in Appendix 1:

$$\frac{FSR_{TTR}}{FSR_E} \approx (1 - P)(1 + 3g/4) \qquad (Eq. 22)$$

where g is the highest enrichment reached in the protein. **Table 1** shows the error in a slow FSR calculated from TTR modeling. It is seen that the protein enrichment has little influence on the error, which is primarily determined by the error in the plateau. Thus, FSR from TTR data underestimates the true FSR of a slow pool by roughly the plateau enrichment (e.g., if the plateau enrichment is 10%, FSR from TTR underestimates the true FSR of a slow pool by ~10%).

TABLE 1. Bias error in FSR using TTR slope/plateau instead of enrichment values

g, Top of Line	Plateau Enrichment			
	5	8	10	15
%			%	
0.1	−4.93	−7.93	−9.93	−14.94
0.5	−4.64	−7.65	−9.66	−14.68
1.0	−4.28	−7.30	−9.32	−14.36

FSR, fractional synthetic rate; TTR, tracer-to-tracee ratio. The column heads give four typical plateau enrichment values. The left column gives three possible values for the top enrichment on the linear portion of the apolipoprotein enrichment data. The other columns give the bias error in percent in computing FSR from TTR data for each combination of plateau enrichment and g. Thus, if the enrichment plateau is 10% and the linear portion increases from 0 to 0.5%, FSR calculated from the TTR plateau and the slope of the TTR data will be 9.66% lower than the correct FSR (from the enrichment plateau and the slope of the enrichment data).

The result is similar to that obtained for a fast apolipoprotein pool in equation 19.

ERROR IN FSR$_{TTR}$ OF SLOWLY TURNING-OVER APOLIPOPROTEINS WITH A BOLUS INJECTION

For a slowly turning-over apolipoprotein, we can ignore the clearance of tracer from that protein during the study so that the amount of tracer in the protein at the end of the study can be equated to the amount incorporated from the precursor over the duration of the study:

$$M\,E(T) = S\int_0^T E_p(t)dt \qquad (Eq.\ 23)$$

The equation can be solved for the true FSR:

$$FSR_E = \frac{S}{M} = \frac{E(T)}{\int_0^T E_p(t)dt} \qquad (Eq.\ 24)$$

(The equation simplifies to equation 21 when the precursor is at a constant level.) With TTR modeling, which assumes that the tracee mass and flux are constant, the equation above is derived with TTR in place of E:

$$FSR_{TTR} = \frac{TTR(T)}{\int_0^T TTR_p(t)dt} \qquad (Eq.\ 25)$$

It is shown in Appendix 2 that, for a single pool for the precursor, it is possible to get an estimate of the error in FSR from TTR data:

$$\frac{FSR_{TTR}}{FSR_E} = \frac{1}{1 - E(T)}\frac{c}{\log[1/(1-c)]} \qquad (Eq.\ 26)$$

where c is the initial precursor enrichment. The error can be quite significant. As the magnitude of the bolus increases, the value of c increases and the integral ratio gets smaller, so the error is worse. For a small bolus, c may be 0.1, the integral ratio is 0.95, which means a small error from using TTR modeling. For a larger bolus, c may be 0.5 (TTR of 1), which is quite common (17, 18, 74), the integral ratio is 0.72, which means TTR modeling will underestimate the true FSR of a slowly turning-over apolipoprotein by >25%.

The underestimation error with TTR modeling appears to be larger in a bolus study than in a primed constant-infusion study. The reason is that the precursor TTR is generally <10% during a constant-infusion study, so the error in considering it as enrichment is <10% as well; on the other hand, in the early part of a bolus study, the precursor TTR can exceed 100%, which means an error of >100% if TTR is used as the forcing function.

ERRORS IN RATE CONSTANTS IN A MULTICOMPARTMENTAL MODEL

With more complex models, a theoretical error analysis appears impossible. There is no simple formula for the errors that arise from TTR modeling. The data from an individual study have to be refitted by modeling enrichments and the new model parameters compared with the earlier results from TTR modeling.

One published study (75), from the author's group, was available for reanalysis. This was a paired study in eight subjects of VLDL triglyceride (TG), plasma apoC-III, and VLDL/intermediate density lipoprotein (IDL)/LDL apoB turnover using a bolus of radiolabeled glycerol, a primed constant infusion of isotopic leucine, and a bolus of isotopic phenylalanine. The reanalysis by fitting enrichments instead of TTR resulted in slightly higher fractional catabolic rates (FCRs), as expected: 46 of 48 apoB FCRs were higher, the difference ranging from −3% to +11%. The mean pretreatment and posttreatment apoB FCRs increased from 3.92 to 3.95 and from 4.53 to 4.69 pools/day, respectively, for VLDL; from 4.06 to 4.27 and from 3.1 to 3.23 pools/day, respectively, for IDL; and from 0.36 to 0.367 and from 0.366 to 0.371 pool/day, respectively, for LDL. Changes in VLDL TG FCRs were similar to those in VLDL apoB FCR, since a combined model for apoB and TG was used to fit apoB enrichment and TG specific radioactivity data simultaneously. Plasma apoC-III FSRs, calculated from straight-line fits (equations 20 and 21 above), increased in every case, the differences ranging from 2.4% to 6.1% and averaging 4.2%, quite close to the prediction from equation 22 for a mean plateau enrichment (P) of 5.9% and peak apoC-III enrichment (g) of 1–2%. The mean pretreatment and posttreatment apoC-III FSRs increased from 0.397 to 0.413 and from 0.294 to 0.308 pool/day, respectively. Treatment effects were essentially unaltered by reanalyzing the data with enrichments. The most significant kinetic parameter P values changed from 0.015 to 0.012 for VLDL TG FCR,

changed from 0.052 to 0.027 for VLDL apoB FCR, and stayed at 0.06 for plasma apoC-III FSR.

If one were to extrapolate from the theoretical results developed above, since precursor enrichment is overestimated by using TTR in place of enrichment, rate constants are underestimated by TTR modeling, and the errors are larger in bolus studies. Also, in bolus studies, as was shown theoretically in equation 13 and exemplified in Fig. 6, precursor TTR declines much faster than does precursor enrichment. Thus, using precursor TTR as the forcing function will lead to underestimation of apolipoprotein FSR in fitting protein enrichment data.

TRANSFORMING THE DATA OR THE MODEL

The primary mass spectrometry data are in the form of TTR, whereas the model is in terms of enrichment. From a statistical point of view, it is best if the fitting program can transform model-generated values to TTR and fit to the data. But many programs do not have such a facility. In that case, the primary data, after background correction (16), need to be converted to enrichment before fitting by the model.

DISCUSSION

Most modeling of mass isotope tracer data to study apolipoprotein kinetics is based on two original papers by Cobelli, Toffolo, and Foster (10) and Foster et al. (11). These papers were extremely influential. Nearly all lipoprotein studies with mass isotopes since then, including by this author's group (75), have modeled TTR. Research groups that modeled enrichments (14, 76–81) switched to TTR modeling (56, 59, 82–87). Only a few groups have continued to model enrichments (88–94); however, one group that modeled TTR (95) has switched recently to modeling enrichments (96, 97).

Although the studies by Cobelli, Toffolo, and Foster (10) and Foster et al. (11) both deal with the nonnegligible mass of mass isotope tracers, they fail to recognize the crucial distinction, illustrated in Fig. 5, between the unsteady state of a precursor and the steady state of the product. Those authors develop their models as if the tracer introduced is the same molecule as the molecule of interest. In fact, the molecule of interest is synthesized from multiple precursor molecules, usually not limited by precursor availability (e.g., cholesterol or a macromolecule such as an apolipoprotein). So the unsteady state of a precursor, caused by the introduction of the tracer, does not alter the steady state of the product. Although Cobelli, Toffolo, and Foster (10) consider multicompartmental models without explicitly considering distinct precursor and product, Foster et al. (11) apply Cobelli's theory to a precursor-product system, in which the product is an apolipoprotein, but assume incorrectly that the masses and fluxes of the unlabeled tracee, whether as precursor or as product, remain constant during the study. Their stated first assumption is that "The

tracee system remains in a steady state during the experiment." This assumption is clearly stated in their Fig. 2 as well as in the constant tracee masses and fluxes in all of their equations. We have shown here that this assumption leads to a contradiction, seen in Fig. 2B, C. The apolipoprotein steady state is for the total of tracer and tracee.

Parhofer et al. (17) and Demant et al. (18) show the equivalence of bolus and primed constant-infusion studies. Demant et al. (18) also studied three different boluses in a single subject and found that the TTR data, both in plasma and in apolipoproteins, were nearly identical for boluses differing by a factor of 10, after suitable normalization. Their plasma data provide strong support to tracee constancy in the precursor state, as has been assumed in amino acid kinetics and whole body protein turnover (16, 98). Their apoB data are consistent with total apoB or just tracee apoB being constant; the low TTR values cannot distinguish between the two. To establish tracee constancy in an apolipoprotein, it would be necessary to measure the apolipoprotein mass during a constant-infusion study and show that the mass increases along with TTR.

Most lipoprotein turnover studies with mass isotopes have used primed constant infusions (27, 29, 41, 50, 68, 70, 99–103). Our results suggest that the reported FSRs and production rates are underestimates, with the percent error roughly equal to the plateau enrichment percent. In paired studies, it is likely that this underestimation is equal in two studies in the same subject, so conclusions from paired studies are likely to be unchanged when the data are analyzed correctly by modeling enrichments. In studies in which each subject is studied only once and two or more groups are compared, if different subjects' plateaus are different, the corrections to be made will be different, thus changing the group standard deviations and possibly group differences as well. The effects are expected to be modest.

Bolus studies are popular for the good reason that kinetics are more clearly delineated compared with a constant-infusion study (17, 18, 43, 95). Our results suggest that the errors from using TTR are likely to be greater in bolus studies, since the area under the precursor forcing function can be overestimated considerably, as shown in equation A13 below. For a single pool precursor, an initial precursor TTR of 1.0 can mean an overestimation of the area by 39%. As a second example, Fig. 6B shows the precursor TTR data from Parhofer et al. (17) and also the precursor enrichment data calculated from their TTR data. Each set of data was fitted by a sum of three exponentials and the area calculated to 1 h. The area under the TTR curve was 35% higher than the area under the E curve.

The relatively large errors in the forcing function derived here (equations A10 to A12 below) are for the case in which the plasma amino acid is treated as the immediate precursor of apolipoproteins. After the early publications (17, 104), bolus studies generally invoke an intermediate pool between plasma and liver synthesis (pool 1 in Refs. 9, 18, 43, pool 2 in Ref. 95). It is likely that the error from using precursor TTR is diminished considerably by the very presence of the intermediate

pool and/or by making the intermediate pool turn over more slowly than it actually does; the intermediate pool is only a mathematical construct and is not observed. The investigators may find their models simplified considerably with amino acid enrichment.

Some researchers have modeled apolipoprotein enrichment and also used α-ketoisocaproic acid instead of plasma leucine as the precursor (28, 105–107), which should approximate the liver tRNA pool better than does plasma leucine (91, 93). However, they use the precursor TTR and not enrichment as the forcing function. We have shown here that the major source of error when using TTR is from precursor TTR.

Although some of the formulation here is general, the steady state of the product was most clearly demonstrated when the product was an apolipoprotein, as in Fig. 2. For other product molecules, a careful analysis is necessary for each product.

Patterson and coworkers have studied TG kinetics in a wide range of human subjects (31, 108–112) and found that plasma TG levels remain unchanged after a bolus injection of isotopic glycerol, leading them to propose a nonlinear function for the incorporation of glycerol in VLDL TG synthesis, which "ensures that the amount rather than fraction of plasma glycerol or palmitate used for VLDL-TG synthesis remains constant over time" (113). Thus, the VLDL TG system is in a steady state after a glycerol tracer injection, making the results of this paper applicable.

More generally, if the precursor whose tracer is introduced is not rate-limiting for the product of interest, then the results of this paper would be valid: that the precursor is in an unsteady state but the product is in a steady state.

CONCLUSION

The correct method of analyzing lipoprotein turnover data from mass isotope infusions is to express the data as tracer enrichments or moles percent excess and fit by compartmental models for apolipoprotein enrichments, with precursor enrichment as the forcing function. Modeling TTRs results in a consistent underestimate of FSRs. The error is a function of the precursor enrichment: the higher the precursor enrichment, the larger the error. Determining the extent to which this error may have affected results in previous publications that modeled TTR requires a reanalysis of the data.

APPENDIX 1

We derive here the error in calculating FSR from TTR data for a slowly turning-over apolipoprotein in a primed constant-infusion study. We assume that enrichment is available at all times until time T and that the data are linear over this time range:

$$E(t) = bt \qquad (Eq.\ A1)$$

TTR is then given by:

$$TTR(t) = \frac{E(t)}{1 - E(t)} = \frac{bt}{1 - bt} \qquad (Eq.\ A2)$$

Fitting TTR(t) by a linear function, ct, by linear regression requires the minimization of the integrated squared deviation between $\frac{bt}{1 - bt}$ and ct, a generalization of the usual least squares:

$$Min\ I = \int_0^T \left(\frac{bt}{1 - bt} - ct \right)^2 dt \qquad (Eq.\ A3)$$

At the minimum, the partial with respect to c is zero:

$$\frac{\partial I}{\partial c} = 0 = -2b \int_0^T \frac{t^2}{1 - bt} dt + 2c \int_0^T t^2 dt \qquad (Eq.\ A4)$$

Solving for c, we get for the ratio of the TTR slope to the enrichment slope:

$$\frac{c}{b} = \frac{\displaystyle\int_0^T \frac{t^2}{1 - bt} dt}{\displaystyle\int_0^T t^2 dt} \qquad (Eq.\ A5)$$

The integrals can be evaluated analytically (114) to yield:

$$\frac{c}{b} = \frac{3}{g^3} \left[\log \frac{1}{1 - g} - g - \frac{g^2}{2} \right] \qquad (Eq.\ A6)$$

where g equals bt, the enrichment at the upper end of the linear portion.

The formula for the ratio of the two slopes is complex. By applying the infinite series expansion for the logarithm, it can be shown that

$$\frac{c}{b} = 1 + \frac{3}{4}g + \frac{3}{5}g^2 + \frac{3}{6}g^3 + \dots \qquad (Eq.\ A7)$$

or, to an approximation,

$$\frac{slope(TTR)}{slope(enrichment)} = \frac{c}{b} \approx 1 + \frac{3}{4}g \qquad (Eq.\ A8)$$

Thus, the TTR slope is very slightly larger than that of the enrichment, since g seldom exceeds 0.02 (2%) for slow pools. Combining the result shown above with equations 20 and 21, and noting that TTR = E/(1 − E), it is possible to derive an approximate expression for the error in FSR from TTR:

$$\frac{FSR_{TTR}}{FSR_E} \approx (1 - P)(1 + 3g/4) \qquad (Eq.\ A9)$$

where P is the precursor enrichment plateau.

APPENDIX 2

We derive here the error in calculating FSR from TTR data for a slowly turning-over apolipoprotein in a bolus study. Dividing equation 25 by equation 24, we get

$$\frac{FSR_{TTR}}{FSR_E} = \frac{1}{1 - E(T)} \frac{\int_0^T E_p(t)\,dt}{\int_0^T \frac{E_p(t)}{1 - E_p(t)}\,dt} \qquad (Eq.\ A10)$$

where T is the duration of the study. The first factor on the right side is clearly >1, but since $E(T)$ seldom exceeds 0.01 or 0.02 for a slow pool, it is a small effect. The two integrals, on the other hand, can be quite different. Since TTR is always larger than enrichment (equation 14), the integrand is larger in the denominator, so the integral in the denominator is also larger than that in the numerator. Examples are seen in Fig. 6: the numerator is the area under the enrichment curve, and the denominator is the area under the TTR curve. The TTR curve is always above the enrichment curve; therefore, its area is always larger.

There is no simple formula for the magnitude of the error in general. It is possible, however, to derive it for the case of a single pool for the precursor, illustrated in Fig. 6A:

$$\frac{FSR_{TTR}}{FSR_E} = \frac{1}{1 - E(T)} \frac{\int_0^T ce^{-kt}\,dt}{\int_0^T \frac{ce^{-kt}}{1 - ce^{-kt}}\,dt} \qquad (Eq.\ A11)$$

where c is the initial precursor enrichment. Solving (114),

$$\frac{FSR_{TTR}}{FSR_E} = \frac{1}{1 - E(T)} \frac{c(1 - e^{-kT})}{\log[(1 - ce^{-kT})/(1 - c)]} \qquad (Eq.\ A12)$$

It can be shown that the integral ratio comes closest to unity, which is best for TTR modeling to have the smallest error, when T is very large; this can also be seen in Fig. 6, where the biggest difference between TTR and E is at early times. At very large T values, equation 31 simplifies to:

$$\frac{FSR_{TTR}}{FSR_E} = \frac{1}{1 - E(T)} \frac{c}{\log[1/(1 - c)]} \qquad (Eq.\ A13)$$

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