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Alternative equations for whole-body protein synthesis and for fractional synthetic rates of proteins

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Abstract

In a constant infusion study of a mass isotope of leucine, two alternative equations are commonly available to calculate amino acid oxidation rate and, thence, whole-body protein synthesis. One, developed by Matthews et al (Am J Physiol Endocrinol Metab. 1980;238: E473-E479), is shown here to require assuming a tracee steady state (TSS), namely, that tracee (unlabeled) amino acid concentrations and fluxes (rates of oxidation and incorporation into protein) are unaltered compared with the preinfusion state. The other, developed by Garlick and coworkers (Melville et al, *Metabolism* 1989;38:248-255), stems from a protein steady state (PSS) assumption, namely, that protein synthesis is unaffected by the tracer infusion. We derive here a simple expression for the relative difference in whole-body protein synthesis computed from the two assumptions, and a simple test of the validity of TSS in the form of an equality that must be satisfied by plasma measurements at all times. We also propose two experiments to discriminate between the two assumptions. Theoretical reasons and experimental evidence from the literature are offered to support PSS. The two assumptions result in different expressions for fractional synthetic rates (FSRs) of individual or organ proteins—TSS requires the use of tracer-to-tracee ratios and PSS the use of enrichments. An expression is derived here for the relative difference in FSR with TSS vs PSS. For both whole-body synthesis and for FSR, the TSS assumption consistently results in an underestimate, the relative bias roughly equal to the precursor amino acid enrichment.

1. Introduction

Stable isotopes have largely replaced radioisotopes in human studies of the metabolism of amino acids and proteins. Although stable isotopes have been with us for decades, there is disagreement on the appropriate equations to be used to calculate total body protein synthesis and fractional synthetic rates (FSRs) of individual proteins in plasma or in specific organs. Matthews et al [1], in a very early article, addressed a key distinction between radioactive and mass isotopes—tracers with stable isotopes have nonnegligible mass. They modified the classic equations, used with radioisotopes, for total amino acid flux, oxidation, and protein synthesis. Others, beginning with Garlick and coworkers [2], continued to use radiotracer equations for oxidation and protein synthesis even with mass isotopes. Both approaches remain in use by different investigators, although rarely with an explicit statement of the assumptions made [3]. The equation of Matthews et al is the more widely used for whole-body protein synthesis, whereas the approach by Garlick is the usual one for calculating FSRs of individual proteins or organs.

We show here that the equation of Matthews et al for protein synthesis is valid if and only if there is a tracee steady state (TSS), namely, that the concentrations and rates of oxidation and incorporation into protein of the tracee (unlabeled) amino acid remain unaltered by the tracer infusion. We also show that Garlick's oxidation equation is valid if and only if there is a protein steady state (PSS), namely, that the tracer infusion does not affect the synthesis rate of any protein.

Cobelli et al [4,5] were the first to formulate the TSS assumption. They used it to simplify the differential equations for tracer kinetics, especially when tracer amount was expressed as tracer-to-tracee ratio (TTR), allowing them to develop compartmental models to fit amino acid tracer

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data [6]. This was followed by their proposal of a new approach to study synthesis of individual proteins—Toffolo et al [7] for proteins in general and Foster et al [8] focusing on apolipoproteins. In both cases, modeling was for TTR, whether of the amino acid or of the protein. A consequence of TSS is that, during tracer infusion, if tracee incorporation into protein remains at the preinfusion rate, total protein synthesis must increase as some of the infused tracer gets incorporated into protein.

In contrast, Garlick et al [9] presented a different view, namely, that protein synthesis is unaltered by tracer infusion and proposed studying protein turnover with enrichments (typically, atoms percent excess or moles percent excess) instead of with TTR. Most investigators studying organ-specific protein synthesis use enrichments, whereas TTR is more common in apolipoprotein turnover studies.

None of the articles by Cobelli and coworkers or by Garlick and coworkers link the TTR-enrichment differences in their respective methods to the earlier differing equations for amino acid oxidation and whole-body protein synthesis.

We revisit the issue here and show that the equation of Matthews et al is consistent with Cobelli's TTR approach, both stemming from a TSS assumption, and that Garlick's oxidation equation is consistent with his enrichment approach, both stemming from a PSS assumption. The results here reveal an anomaly in the literature because the TSS-based equation of Matthews et al is more commonly used for whole-body protein turnover, whereas individual or organ-specific protein FSRs are calculated from PSS-based enrichments.

Theoretical considerations, as well as available experimental evidence, suggest that protein synthesis remains unaltered by the infusion of a single amino acid, supporting Garlick's approach.

2. Basic equations

2.1. A single equation for total amino acid flux

We consider a constant infusion experiment with a 1-carbon mass isotope of an amino acid. Fig. 1A shows a basic single-pool model for the 1-carbon amino acid before the infusion. It is assumed that the amino acid has only two fates—incorporation into protein and oxidation. (Extension to gluconeogenic and nonessential amino acids is straightforward and will not be done here.) The mass of the amino acid is M; the rate of incorporation into protein, termed *synthesis* for short, is S; the rate of release from protein breakdown is B; the oxidation rate is X; and the rate of exogenous entry is I. The total flux is Q, equal to $S + X$ (or $B + I$). If the constant infusion rate is i, there are two scenarios that lead to simple equations for X and S.

Fig. 1B shows one possibility—TSS. This assumption was introduced by Cobelli et al [4] as a way to simplify differential equations and later formalized by them [5] and adopted widely, especially in lipoprotein kinetics. Under this assumption, the tracee amino acid is unaltered with respect to masses, fluxes, and rate constants. The tracer is indistinguishable from the tracee and so the infusion expands the masses and fluxes proportionately and preserves the rate constants. A consequence is that incorporation into protein (synthesis) and oxidation both increase and by the same factor. By a simple mass balance, then, synthesis becomes $S(1 + i/Q)$, and oxidation increases to $X(1 + i/Q)$. The

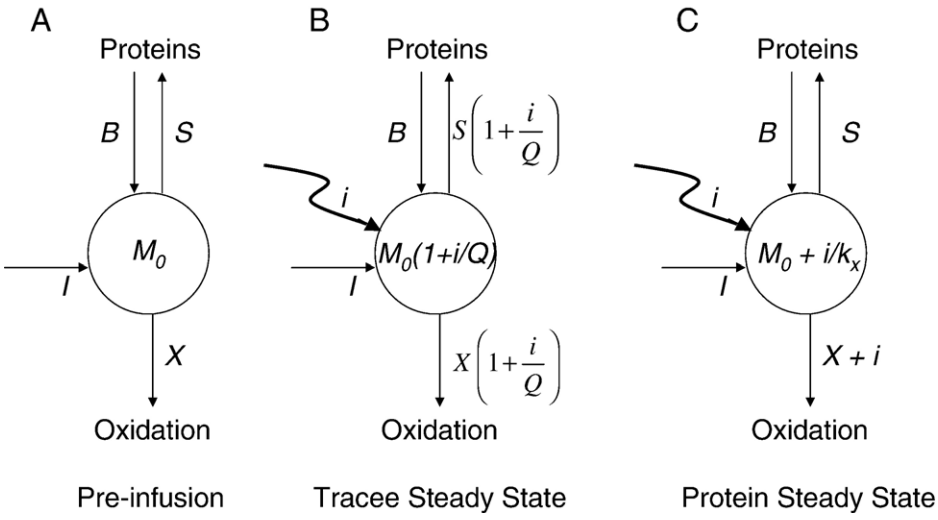

Fig. 1. A single-pool model for an amino acid before and during a constant tracer infusion. The symbol I indicates dietary intake, M_0 the preinfusion mass of the amino acid, X the rate of oxidation, S the rate of incorporation of the amino acid in whole-body protein synthesis, B the rate of amino acid release by protein breakdown, Q the total flux (equal to $S + X = B + I$), i the rate of constant tracer infusion, and k_X the rate constant for the oxidation of excess amino acid. A, The preinfusion condition. B, The model during the infusion under the TSS assumption; the fluxes out, S and X, and the mass all increase proportionately. C, The model during the infusion under the PSS assumption: protein synthesis, S, is unaltered while oxidation increases by i to balance the infusion. The mass increases by i/k_X.

mass increases from M to $M(1 + i/Q)$. Tracee steady state is equivalent to assuming first-order kinetics for protein synthesis and for oxidation with respect to the amino acid whose tracer is introduced.

Fig. 1C shows a second possibility—PSS. This was articulated first by Garlick et al [9] and is used by only a few groups [3,10]. Under this assumption, protein synthesis is unaltered by the infusion of a single amino acid because the levels of the other 19 amino acids involved in protein synthesis are not affected by the infusion. Therefore, the infusion i is balanced by an equal increase in oxidation from X to $X + i$, with the synthesis S remaining unchanged. Because some of the S is from the tracer, tracee incorporation into protein must necessarily decrease slightly with a corresponding increase in tracee oxidation during the infusion. Protein steady state is equivalent to assuming zeroth-order kinetics for protein synthesis with respect to the amino acid whose tracer is introduced, with the excess amino acid handled entirely by increased oxidation.

Whatever be the fate of the increased amino acid, total amino acid flux, Q, is calculated simply from a balance on labeled material at steady state. If the enrichment of the plasma amino acid pool is E_p, and the infusion enrichment is E_i, and if tracer recycling from protein breakdown is negligible so that the enrichment of amino acid from protein breakdown remains at the preinfusion background level of E_0, then the rate of label entry is $iE_i + QE_0$, which must equal the rate of label exit, which is $(Q + i)E_p$ under either TSS or PSS assumption, so that

$$Q = \frac{i(E_i - E_p)}{E_p - E_0} \tag{1}$$

If the background enrichment before infusion is negligible, that is, if E_0 is zero,

$$Q = i\left[\frac{E_i}{E_p} - 1\right] \tag{2}$$

This is the equation proposed without proof by Matthews et al [1]. It is seen that total amino acid flux is calculated identically under either assumption.

2.2. Two equations for amino acid oxidation

The oxidation rate of the carbon label, x, is calculated by multiplying carbon dioxide production rate by its enrichment (without background correction) and a factor for bicarbonate retention [1,11]:

$$x = \frac{V_{CO_2}E_{CO_2}}{0.81} \tag{3}$$

This must equal the amino acid oxidation rate multiplied by the enrichment of the amino acid pool where oxidation takes place. Because the two possibilities assume different levels for the total oxidation rate [$X(1 + i/Q)$ for TSS and $X + i$ for PSS] during the tracer infusion, the two assumptions lead to different calculated values for the preinfusion oxidation rate X and for the synthesis S.

Under TSS,

$$x = X_{TSS}\left[1 + \frac{i}{Q}\right]E_p \tag{4}$$

Substituting for Q from Eq. (1) and solving for X_{TSS},

$$X_{TSS} = \frac{x}{E_p}\frac{E_i - E_p}{E_i - E_0} \tag{5}$$

$$S_{TSS} = Q - X_{TSS} = \frac{i(E_i - E_p)}{E_p - E_0} - \frac{x}{E_p}\ \frac{E_i - E_p}{E_i - E_0} \tag{6}$$

If E_0 is zero, the equations simplify to the following:

$$X_{TSS} = x\left[\frac{1}{E_p} - \frac{1}{E_i}\right] \tag{7}$$

$$S_{TSS} = Q - X_{TSS} = i\left[\frac{E_i}{E_p} - 1\right] - x\left[\frac{1}{E_p} - \frac{1}{E_i}\right] \tag{8}$$

Eqs. (7) and (8) are the ones proposed by Matthews et al [1] for amino acid oxidation and protein synthesis, which are seen to follow from a TSS assumption. The converse can be proved as well. We can begin with Eq. (5), the equation of Matthews et al for oxidation, note that $(E_i - E_p)/(E_i - E_0)$ equals $(1 + i/Q)$ from Eq. (1), and derive Eq. (4), concluding that oxidation has increased by the factor $(1 + i/Q)$, which means TSS. Thus, the equations of Matthews et al hold if and only if TSS applies.

Under PSS,

$$x = (X_{PSS} + i)E_p \tag{9}$$

which does not involve Q. Solving,

$$X_{PSS} = \frac{x}{E_p} - i \tag{10}$$

$$S_{PSS} = Q - X_{PSS} = \frac{i(E_i - E_0)}{E_p - E_0} - \frac{x}{E_p} \tag{11}$$

If E_0 is zero, the equation gets simplified:

$$S_{PSS} = \frac{iE_i - x}{E_p} \tag{12}$$

This equation for S_{PSS} is the same as derived by Macallan et al [3] including Garlick, by Tauveron et al [12], and by Prod'homme et al [10]. One difference is that, in those publications, the expressions for Q and for X do not include a subtraction for the tracer infusion rate i: $Q = iE_i/E_p$ and $X = x/E_p$, identical to the formulas used in a radiotracer study, noting that iE_i corresponds to the radioactivity infusion rate.

Garlick's group [2,13], in their prior work with mass isotopes, used $Q = iE_i/E_p$ and $X = x/E_p$, apparently by analogy with radiotracer studies. Macallan et al [3] were the first to articulate the PSS assumption that the mass in the infusion increases oxidation and not protein synthesis. The reason that radiotracer infusion, where the infusion has negligible mass, leads to the same expression for protein synthesis as the PSS assumption with mass isotope infusion is that, in both cases, protein synthesis is assumed to be unaltered by the tracer infusion.

3. Comparing TSS and PSS assumptions

3.1. A simple expression for the difference in 2 formulas for synthesis

The difference in the protein incorporation rates calculated under the two assumptions is given by:

$$S_{PSS} - S_{TSS} = X_{TSS} - X_{PSS} = i - \frac{x}{E_p}\frac{E_p - E_0}{E_i - E_0} \tag{13}$$

The difference in synthesis as calculated from the two assumptions can be expressed relative to S_{PSS} to learn its magnitude and sign:

$$\frac{S_{PSS} - S_{TSS}}{S_{PSS}} = \frac{E_p - E_0}{E_i - E_0} \tag{14}$$

This very simple result appears to be novel. It is seen that the expression is always positive. Thus, the TSS assumption calculates a smaller preinfusion protein synthesis than does the PSS assumption; preinfusion oxidation is calculated to be larger under the TSS assumption.

It has been shown by Nissen and Haymond [14], Matthews et al [15], and others that the reciprocal pool enrichment is closer to the enrichment in the transfer RNA pools and in the pools where oxidation occurs. If the reciprocal enrichment (eg, α-ketoisocaproic acid for leucine tracer) E_k is used, it replaces E_p in Eqs. (1) to (14), with, for example, Eq. (14) for the relative error changing to the following:

$$\frac{S_{PSS} - S_{TSS}}{S_{PSS}} = \frac{E_k - E_0}{E_i - E_0} \tag{15}$$

Although the two assumptions are seen to lead to different values for protein synthesis, with PSS always leading to a larger value, the difference is small. In the two equations [Eqs. (14) and (15)] for the difference, the preinfusion enrichment, E_0, is nearly zero; the infusion enrichment, E_i, is typically close to and slightly smaller than 1; and the plasma amino acid or reciprocal enrichment, E_p or E_k, is less than 0.1 (10% atoms percent excess). Thus, the relative difference is a small percent.

As a numerical example, Matthews et al [1] infused 2.4 μm/kg per hour of ^{13}C-leucine at an enrichment of 92% and reported a mean plasma enrichment (E_p) of 0.021, a mean tracer oxidation rate (x) of 0.429 μm/kg per hour, and a mean preinfusion protein synthesis (S_{TSS}) of 80 μm/kg per hour. For $i = 2.4$ and $E_i = 0.92$, at the mean value of $x = 0.429$, the protein synthesis under PSS would be larger by 1.9 μm/kg per hour; the difference is less than 2.5%, and equals 0.021/0.92 as predicted by Eq. (14) for $E_0 = 0$.

3.2. Testing the validity of the TSS assumption

In trying to choose between the 2 assumptions, we first look at the plasma concentration, or equivalently the mass, of the amino acid. Under TSS, $M(t)$, the mass of the plasma pool at any time, is made up of 3 parts: (1) the mass of tracee remaining unchanged at its initial value M_0 (including the preinfusion label M_0E_0); (2) the amount of label from the infusion in the pool at that time, equal to $M(t)E_p(t) - M_0E_0$; (3) $(1 - E_i)/E_i$ times this label amount to account for the presence in plasma of unlabeled amino acid from the tracer infusion. Thus, at any time t,

$$\begin{aligned} M(t) &= M_0 + \left[M(t)E_p(t) - M_0E_0\right]\left[1 + \frac{(1 - E_i)}{E_i}\right] \\ &= M_0 + \frac{M(t)E_p(t) - M_0E_0}{E_i} \end{aligned} \tag{16}$$

Solving,

$$M(t) = M_0\frac{E_i - E_0}{E_i - E_p(t)} \tag{17}$$

This equation provides a simple test of the validity of the TSS assumption. All the terms in the above equation are measured in a typical experiment, and it should be possible to check if the equation is satisfied at each measurement. If there is a deviation, it would suggest that the TSS assumption may be invalid.

Eq. (17) holds for an arbitrary tracer infusion (bolus, primed constant infusion, flooding dose, etc) as well as for an arbitrarily complex model for leucine kinetics. The model may be a single pool, 4 pools to accommodate tissue amino acid and the reciprocal pools, and so on. Regardless of the model and regardless of the study design, if TSS applies, the plasma mass or concentration of the amino acid and its enrichment must satisfy Eq. (17) at all times.

In particular, for a constant infusion study, at infinite time, combining Eqs. (1) and (17),

$$M_{\infty TSS} = M_0(1 + i/Q) \tag{18}$$

Eq. (18) is more specific than Eq. (17) in that it is for the particular study design of constant infusion with a single-pool model.

3.3. Testing the validity of the PSS assumption

The PSS assumption merely keeps the synthesis S unchanged. For a constant infusion study, the increase in oxidation equals the tracer infusion rate, i. If the increased

oxidation is by the same mechanism as the preinfusion oxidation, then, in the pool where the oxidation takes place,

$$\frac{X}{M_{X0}} = \frac{X+i}{M_{X\infty}} \tag{19}$$

where the subscript X denotes the oxidation pool. Solving,

$$M_{X\infty} = M_{X0}(1 + i/X) \tag{20}$$

This equation does not provide a test of the validity of PSS because we do not have access to the oxidation pool. If the single-pool model is assumed to hold, then Eq. (20) can be compared to Eq. (18). Because the oxidation rate, X, is always smaller than the total flux, Q, Eq. (20) predicts a larger final mass than Eq. (18) does under TSS. Therefore, if the amino acid amount is measured to be larger than predicted by Eq. (18), PSS would be preferred.

However, the converse is not true. If the amino acid amount is different from the prediction from Eq. (20), it does not invalidate the PSS assumption. A discrepancy could be because oxidation, as is likely, takes place outside the plasma pool. It is also possible that the excess amino acid from the infusion is oxidized by a different mechanism than before the infusion.

The TSS assumption is strong because it asserts that the tracee system remains unaltered by the tracer study. Serial measurements of plasma concentrations and enrichments can be used to test if Eq. (17) is satisfied at each point in time. On the other hand, the PSS assumption merely constrains protein synthesis and makes no assertion otherwise; in particular, it postulates no specific mechanism for the oxidation of the infused tracer, which may be the same or different from oxidation before tracer infusion. Furthermore, the oxidation takes place in an inaccessible pool. Thus, a PSS model is consistent with a broad range of data.

3.4. Two experiments to discriminate between TSS and PSS

It is possible to discriminate between TSS and PSS by repeat studies that vary the flux of the tracer or of the tracee. Consider two tracer infusions at rates i_1 and i_2. With either assumption, the basal oxidation rate, X, which can be calculated with either infusion, must be the same for internal consistency. Under TSS, we get, from Eq. (5) applied to the two infusions:

$$X_{\mathrm{TSS}} = \frac{x_1}{E_{p_1}}\frac{E_\mathrm{i} - E_{p_1}}{E_\mathrm{i} - E_0} = \frac{x_2}{E_{p_2}}\frac{E_\mathrm{i} - E_{p_2}}{E_\mathrm{i} - E_0}$$
$$\text{Or, } \frac{x_2}{E_{p_2}} - \frac{x_1}{E_{p_1}} = \frac{x_2 - x_1}{E_\mathrm{i}} \tag{21}$$

On the other hand, under PSS, we get, from Eq. (10) applied to the two infusions:

$$X_{\mathrm{PSS}} = \frac{x_1}{E_{p_1}} - i_1 = \frac{x_2}{E_{p_2}} - i_2$$
$$\text{Or, } \frac{x_2}{E_{p_2}} - \frac{x_1}{E_{p_1}} = i_2 - i_1 \tag{22}$$

Thus, the differences in the oxidation rates at the two infusions are different under the two assumptions. Because the tracer oxidation rate is typically a small fraction of the infusion rate (less than a fifth in Matthews et al [1]), the right side of Eq. (22) is expected to be much larger than the right side of Eq. (21), which means PSS would predict a much larger difference in the oxidation rates.

A second experiment to discriminate between PSS and TSS would be a study at two different levels of unlabeled leucine intake, say I and I', while the intakes of other amino acids are unaltered. If the basal oxidation rate at intake I is X and the total flux Q, the oxidation rate at intake I' is different under the two assumptions. Under TSS, the increased unlabeled leucine intake would be partitioned between protein incorporation and oxidation, so oxidation increases to:

$$X' = X\left[1 + \frac{I' - I}{Q}\right] \tag{23}$$

Under PSS, on the other hand, the increased unlabeled leucine intake would be balanced by a matching increase in oxidation:

$$X' = X + I' - I \tag{24}$$

Protein steady state would predict a much larger increase in the calculated basal oxidation rate, X', when the intake of unlabeled leucine is increased.

3.5. Theoretical support for PSS

3.5.1. Protein synthesis unaltered by infusion of single amino acid

Consider the synthesis of a protein, before the infusion of a tracer, as shown in Fig. 2A. The pools represent 20 amino acids, with the mass of the ith amino acid equal to M_i. The arrows are for the synthesis of one specific protein. The synthetic rate is S, and the mole fraction of the ith amino acid in the particular protein is f_i. Now, suppose a tracer of amino acid 1 is introduced as a bolus that doubles the mass of that amino acid, as shown in Fig. 2B. Three possibilities can be envisaged for the synthetic rate of the protein of interest, as shown under each arrow. Under the TSS assumption, which postulates first-order kinetics for protein synthesis with respect to amino acid 1, the tracee part of amino acid 1 would continue to be incorporated in synthesizing that protein at the same rate. Under this assumption, the tracer, being indistinguishable from the tracee, would be incorporated at the same rate, thus doubling the total incorporation rate of amino acid 1 to $2f_1S$. But this requires, for the protein to preserve its amino acid composition, that the incorporation rate of every other amino acid be doubled as well. However, the masses of the other amino acids have not been altered by the tracer infusion, and there is no reason for their incorporation rates to be altered; we assume here that the mass of amino acid 1

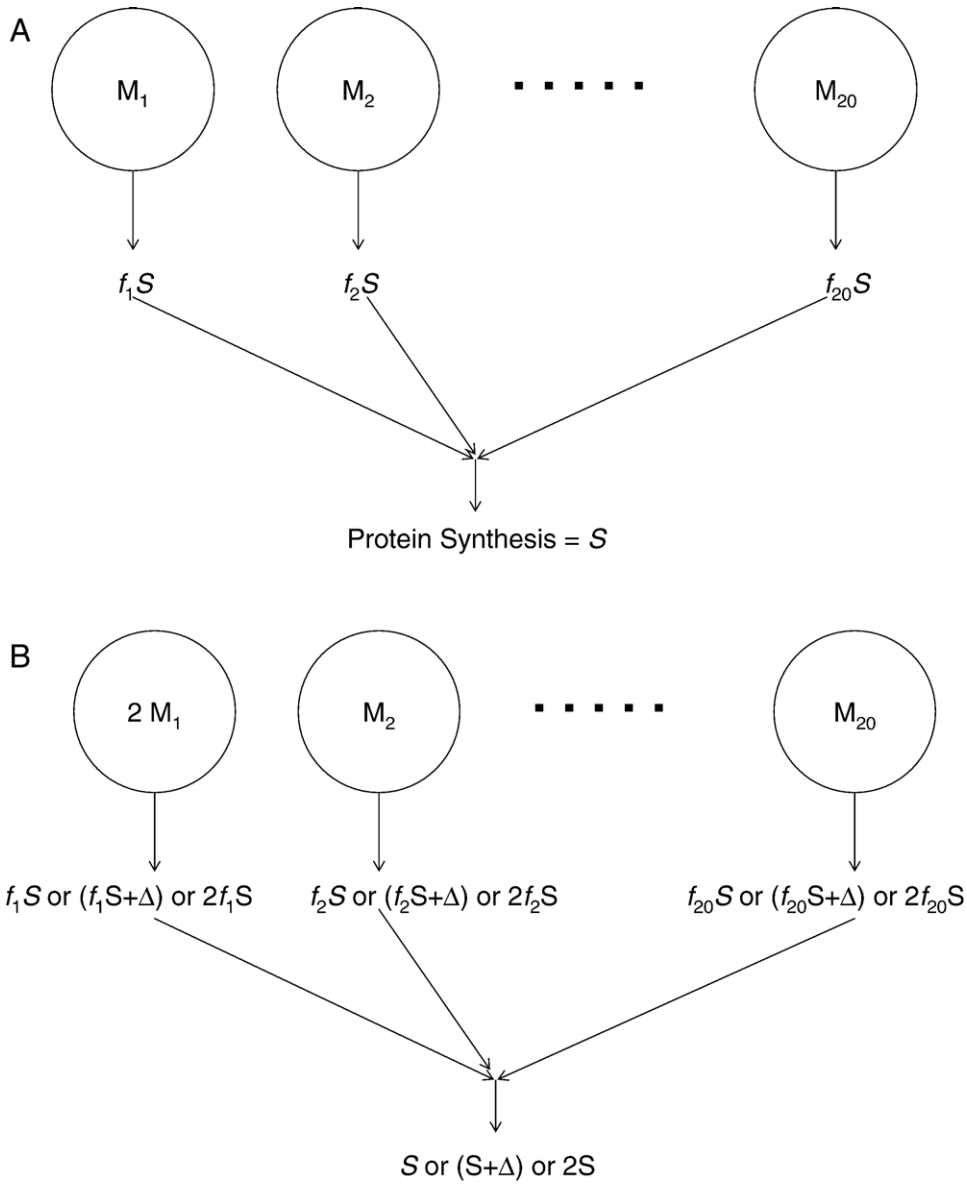

Fig. 2. A schematic of the synthesis of a specific protein from 20 amino acids. (Pathways to other proteins, transamination, and oxidation are left out for simplicity.) A, The condition before tracer infusion: the rate of synthesis is S, the mole fraction of amino acid 1 is f_1, etc. B, The condition after the tracer infusion has doubled the mass of amino acid 1. Three possibilities are given for the total synthetic rate and for the incorporation rates from each amino acid. Under PSS, the synthesis is unaltered, and individual incorporation rates are unaltered as well. Alternatively, under TSS, the synthesis is doubled in response to the doubling of the mass of amino acid 1, and the incorporation rate of every other amino acid is doubled as well, which is unlikely for the amino acids whose masses were unaltered. The third possibility, of an increase short of doubling, is indicated as well.

has no extended effect on protein synthesis. A second possibility is that the total incorporation rate of amino acid 1 increases short of doubling, say to $f_1S + \Delta$. But this, too, requires, for proper stoichiometry, that the incorporation rate of every other amino acid increase.

The third possibility is the PSS assumption, which postulates zeroth-order kinetics for protein synthesis with respect to a single amino acid—that protein synthesis is unaltered by the tracer, that the incorporation rate of each amino acid remains where it was before the tracer was introduced. This is the simplest and most straightforward, requiring no change in the kinetics of any other amino acid.

This reasoning can be applied to each protein to say that the synthesis rate of any protein is unaltered by infusing a single amino acid. It follows, then, that total protein synthesis, as well, is unaltered by a constant infusion. The concept of PSS was first advanced by Garlick et al [9]. The development here follows one we used in a recent article [16] concerning apolipoprotein kinetics.

4. Two equations for protein FSR

It is possible to study protein turnover with multicompartmental models, as is commonly done in

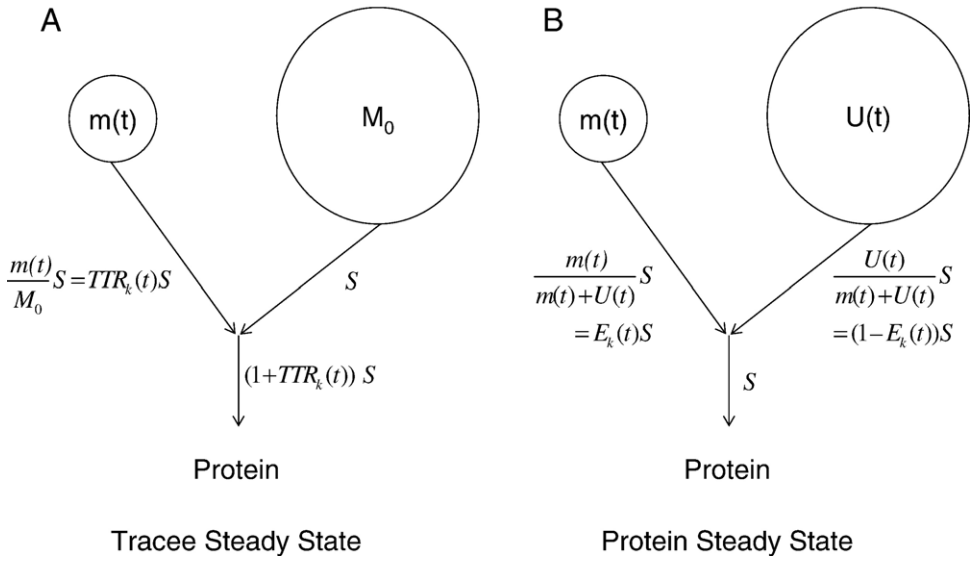

Fig. 3. The tracer and tracee precursor pools for an amino acid whose tracer is introduced, and the incorporation into a protein, at time t during a tracer infusion study when the amount of precursor tracer is $m(t)$. Before the infusion, the precursor pool has a mass M_0 and incorporation rate S. A, The precursor tracee pool mass and protein incorporation remaining unchanged while the tracer incorporation equals the tracee incorporation times $TTR_k(t)$. The total incorporation at time t is $[1 + TTR_k(t)]S$. B, Total protein incorporation remaining unchanged at S. The precursor tracee pool may be changing with time, its mass denoted by $U(t)$. The incorporation rates of tracer and tracee are proportional to their respective masses, as given by the formulas.

apolipoprotein studies. Barrett et al [17] have reviewed modeling TTR; this author [16] has provided the framework for modeling enrichment. We confine the analysis here to single-pool models (ignoring any tracer recycling due to protein breakdown) used commonly to calculate protein FSRs.

We look at the traced amino acid in detail, with the unlabeled form and the tracer in the precursor considered separately, as shown in Fig. 3A for the TSS assumption and in Fig. 3B for the PSS assumption. In each, the large circle represents the unlabeled form and the small circle the tracer. The preinfusion mass is M_0, the preinfusion rate of incorporation into the protein of interest S, and the mass of tracer at time t equals $m(t)$. Under TSS, the tracee mass remains at the preinfusion level of M_0, and the tracee protein incorporation remains at S. The tracer incorporation, which is additional, equals the tracee incorporation S multiplied by $TTR_k(t)$, the precursor TTR (the subscript "k" denotes the precursor). On the other hand, under PSS, the total protein incorporation remains at the preinfusion level of S, so the incorporation rates of both tracer and tracee change with time. At any time t, if the amount of unlabeled amino acid is $U(t)$ and that of the tracer $m(t)$, the incorporation rate of each part into the protein of interest equals the unaltered total incorporation rate, S, multiplied by the respective mass fractions, as shown in Fig. 3B. Thus, the instantaneous incorporation rate of the tracer is $TTR_k(t)S$ under TSS and $E_k(t)S$, as shown in the figure.

Toffolo et al [7], after the earlier proposal by Cobelli et al [5] to model TTRs to study protein turnover under the TSS assumption, have derived expressions for the calculation of protein turnover, which can be written as:

$$\mathrm{FSR}_{\mathrm{TSS}} = \frac{\mathrm{TTR_P}(T)}{\int_O^T \mathrm{TTR_k}(t)dt} \tag{25}$$

where the subscript "P" denotes the protein of interest, the subscript "k" denotes the appropriate precursor pool, and T is the point in time when the protein enrichment is measured. In a primed constant infusion experiment, if the precursor pool can be assumed to be at a constant enrichment for the entire study duration, the equation simplifies to:

$$\mathrm{FSR}_{\mathrm{TSS}} = \frac{\mathrm{TTR_P}(T)}{\mathrm{TTR_k}T} \tag{26}$$

Under the assumption of PSS, protein synthesis is unaltered by tracer infusion. Consequently, the masses and fluxes of protein pools are unaffected as well. The tracer incorporation rate must equal the rate of accumulation of tracer in the protein pool, noting that the total mass, M_P, is constant from the PSS assumption:

$$\frac{d}{dt}(M_P E_P) = M_P \frac{dE_P}{dt} = E_k(t)S \tag{27}$$

Integrating the equation on both sides,

$$M_P \int_0^T \frac{dE_P}{dt}dt = S\int_0^T E_k(t)dt \tag{28}$$

The FSR is readily obtained:

$$\mathrm{FSR_{PSS}} = \frac{S}{M_\mathrm{P}} = \frac{E_\mathrm{P}(T)}{\int_O^T E_\mathrm{k}(t)dt} \tag{29}$$

In a primed constant infusion experiment, if the precursor pool can be assumed to be at a constant enrichment for the entire study duration, the equation simplifies to:

$$\mathrm{FSR_{PSS}} = \frac{E_\mathrm{P}(T)}{E_\mathrm{k}T} \tag{30}$$

For both TSS and PSS, if the product protein pool is sampled at two time points, the numerator in (Eqs. (25), (26), (29), and (30) would be replaced by the difference in TTR or E of the product pool at the two time points; the integrals in the denominators of Eqs. (25) and (29) would be evaluated between the two time points; and the denominators in Eqs. (26) and (30) would contain the time difference instead of T.

4.1. An expression for the difference in two formulas for FSR

The FSR from the 2 assumptions can be contrasted by comparing Eqs. (26) and (30) for the simplified case. Noting that TTR = $E/(1 - \mathrm{E})$, we get from Eq. (26):

$$\begin{aligned}\mathrm{FSR_{TSS}} &= \frac{\dfrac{E_\mathrm{P}(T)}{1-E_\mathrm{P}(T)}}{T\frac{E_\mathrm{k}}{1-E_\mathrm{k}}} = \frac{E_\mathrm{P}(T)}{E_\mathrm{k}T}\frac{1-E_\mathrm{k}}{1-E_\mathrm{P}(T)} \\ &= \mathrm{FSR_{PSS}}\frac{1-E_\mathrm{k}}{1-E_\mathrm{P}(T)}\end{aligned} \tag{31}$$

Typically, $E_\mathrm{P}(T)$ is under 0.01 and only a fraction of E_k, and so, approximately,

$$\mathrm{FSR_{TSS}} \simeq \mathrm{FSR_{PSS}}(1 - E_\mathrm{k}) \tag{32}$$

Fractional synthetic rate computed with TTR, under the TSS assumption, underestimates the FSR by a fraction that is approximately the precursor enrichment. Thus, for instance, if the precursor enrichment is 5%, the underestimation is by approximately 5%.

In the more general case, from Eqs. (25) and (29),

$$\mathrm{FSR_{TSS}} \simeq \mathrm{FSR_{PSS}}\frac{\displaystyle\int_0^T E_\mathrm{k}(t)dt}{\displaystyle\int_0^T \mathrm{TTR_k}(t)dt} \tag{33}$$

Because TTR is always greater than the enrichment, the integral in the denominator is larger than that in the numerator and, hence, TTR modeling underestimates the FSR.

Elsewhere [16], we have analyzed single-pool models for apolipoprotein turnover where tracer loss is not ignored. The extent of FSR underestimation with TTR is similar to the result above.

With the flooding-dose protocol, the precursor pool is thought to be at a constant enrichment for the short duration of the study, in which case the results obtained here should apply. If there is a modest decline in precursor enrichment, Eq. (32) should still hold, provided a mean enrichment for the duration is used for E_k.

5. Discussion

Matthews et al [1] first proposed equations for total amino acid flux and oxidation for use with mass isotope infusions. The authors provide the same justification for both equations. As we have seen, whereas the equation of Matthews et al for total amino acid flux [Eq. (2)] is valid for a range of assumptions concerning the fate of the tracer infusion, the equations of Matthews et al for oxidation [Eq. (7)] and for protein synthesis [Eq. (8)] require an assumption of TSS or first-order kinetics for both oxidation and protein synthesis with respect to the infused amino acid; thus, they are consistent with the later work of Cobelli et al [4,5], which show that TTR modeling is indicated under this assumption. Wolfe [18] derived equations for leucine flux and whole-body protein synthesis in terms of TTR. These can be shown to be identical to the equations of Matthews et al except that Wolfe does not account for incomplete enrichment of the tracer infusion.

The alternative approach has been to assume a PSS, that protein synthesis is unaltered by the infusion of a single amino acid, or, equivalently, that protein synthesis is a zeroth-order process with respect to any single amino acid. This assumption leads to a different expression for total body protein synthesis, very interestingly identical to that for radiotracers, which introduce negligible mass.

We have derived equations that are more general than those reported in the literature by accounting for preinfusion enrichment in the study subject. Cobelli et al [4] have attempted to account for preinfusion label and for nonlabeled infusion, but the approach seems to be unnecessarily convoluted. The results derived here are seen to be straightforward extensions of the equations due to Matthews and Garlick.

The equation of Matthews et al is widely used to calculate whole-body protein synthesis, but the FSR of individual proteins or in specific organs is typically calculated with enrichments and not TTR. This inconsistency appears to be due to nonrecognition of the TSS assumption behind the equations of Matthews et al. Several articles that calculated both whole-body protein synthesis and FSR used the equation of Matthews et al for the former but enrichments and not TTR to calculate FSR [19-25]. An early example of this inconsistency can be found in Rennie et al [26], who, soon after the equations of Matthews et al were published, used them to determine whole-body protein synthesis but calculated muscle protein synthesis using enrichments and not TTR. Balagopal et al [27,28] used the equation of

Matthews et al for leucine oxidation in one article but enrichments for FSR soon thereafter.

A few publications report equations for oxidation that differ from both TSS [Eq. (7)] and PSS [Eq. (10)]. Kalhan et al [29] multiply the formula in Eq. (7) by the infusion enrichment, whereas Battezzati et al [30] use the formula in Eq. (10) without subtracting the infusion; neither is consistent with TSS or PSS.

We have shown here that the TSS assumption results in smaller values for total body protein synthesis than does the PSS assumption and derived a very simple expression for the relative underestimation by the TSS assumption: $(E_k - E_0)/(E_i - E_0)$, which is the ratio of the protein/oxidation precursor enrichment to the infusion enrichment.

We have also compared the formulas for FSR of individual or organ proteins under the two assumptions. The TSS assumption results in an underestimation of the FSR; the relative bias, interestingly, is approximately equal to the amino acid precursor enrichment E_k, quite close to the relative underestimation by TSS of whole-body protein synthesis.

We have also derived a simple test of the validity of TSS—the tracee amino acid concentration should remain constant throughout the experiment, which means the total amino acid concentration in plasma at any moment should equal the preinfusion concentration multiplied by the infusion enrichment (above background) and divided by the difference between infusion and plasma enrichments, as given by Eq. (17). This validity test for TSS can be done with the data from any study (constant infusion, bolus, flooding dose, etc), provided plasma amino acid concentrations are measured at multiple times during the study, as for instance by Hovorka et al [31]. If Eq. (17) is not satisfied at every measurement time, the assumption of TSS is invalid.

The PSS assumption is much weaker and not invalidated by amino acid concentration measurements, whatever they may be. We have proposed experiments that can compare the two assumptions directly, either with two different tracer infusion rates in the same subject [Eqs. (21) and (22)] or with two different unlabeled amino acid intakes in the same subject [Eqs. (23) and (24)]. These experiments can falsify one assumption or the other, or even both.

Hsu et al [32] conducted tracer studies at two different daily leucine intakes: 5 subjects at 45 mg/kg and 5 at 65.5 mg/kg (3 subjects were common). These intakes correspond to roughly 14 and 21 μmol/kg per hour, respectively. They found leucine oxidation to be around 20 and 30 μmol/kg per hour, respectively; the difference was quite close to the difference in the intakes, as would be predicted by PSS from Eq. (24). If TSS were correct, oxidation, which was only 15% to 20% of the total flux, would have increased by only 15% to 20% of the increased intake, according to Eq. (23). This work provides the strongest evidence that increasing leucine intake results in a corresponding increase in leucine oxidation, supporting PSS and invalidating TSS.

If neither assumption is correct, other alternatives would need to be considered. It is possible that, if tracer infusion exceeds some level, other pathways are up-regulated to handle the excess amino acid—for instance, increased excretion in the urine or bile. It is also possible that high levels of the infused amino acid affect the transport or oxidation of other amino acids, thus possibly affecting their availability for protein synthesis.

5.1. Other experimental evidence against TSS

A number of investigators have infused two amino acids simultaneously, sometimes both as constant infusions [1] and sometimes with one as a constant infusion and the other as a bolus [33-35]. The enrichment curves for the two amino acids are clearly different in the latter case. Even when both are constant infusions, the final enrichment is not likely to be the same for the two amino acids. Suppose the prestudy incorporation rates of the two amino acids into a particular protein are f_1S and f_2S, respectively. During the study, if the TTR of one amino acid is $TTR_1(t)$ and of the other amino acid $TTR_2(t)$, then, under TSS, the instantaneous incorporation rates of the two amino acids become $f_1S[1 + TTR_1(t)]$ and $f_2S[1 + TTR_2(t)]$, respectively. The stoichiometric ratio changes from the preinfusion $\frac{f_1}{f_2}$ to $\frac{f_1(1+TTR_1(t))}{f_2(1+TTR_2(t))}$, which is changing every moment because the time courses of enrichments of the two amino acids are generally different due to different fractional clearance rates. Such a change in protein composition is clearly impossible. Elsewhere, we [16] have presented data from the work of Parhofer et al [33] with two tracers to explain this contradiction in detail. There is no such contradiction under the PSS assumption. Thus, dual tracer studies show the invalidity of TSS.

Synthesis rates of specific proteins have been measured and found to be similar at different infusions of amino acids, supporting the PSS assumption. Ballmer et al [36] found no change in albumin synthesis. Garlick [37] has reviewed the evidence for the influence of increased leucine levels on protein synthesis; it appears that an increase within the physiological range has little effect on muscle protein synthesis in the absence of insulin stimulation.

5.2. Evidence against PSS

There are situations where PSS may not apply. If the amino acid being infused, and only that one, is limiting for protein synthesis, then increasing its concentration can lead to a proportionate increase in protein synthesis, consistent with TSS. However, it is generally assumed that the study subject is in a state of sufficiency with respect to the amino acid infused. A second possibility is that the infused amino acid performs a signaling function [37-41]. Although PSS may not apply, TSS may also not apply because the relationship between amino acid level and translation may not be first order. A third possibility is that the transamination preceding oxidation of the infused amino acid leads to the amino group transferring to other amino acids limiting for protein

synthesis, thus leading to increased synthesis. It is not clear how this process can be modeled under either assumption.

A flooding dose increases the plasma pool several-fold, much higher than is achieved during a constant infusion study. There is no unanimity in the literature about the effect of a flooding dose on protein synthesis. Smith et al [38,42] found that flooding with one amino acid can influence the protein incorporation of another. On the other hand, Caso et al [43] used a flooding dose of approximately 260 μmol/kg or a primed constant infusion of 6 μmol/kg per hour of phenylalanine and obtained very similar protein synthesis rates. If TSS were to hold, a much higher synthesis rate should be computed with a flooding dose. Garlick et al [9] summarize the evidence in favor of the view that, even in the flooding dose protocol where there is a very significant addition of amino acid, PSS is the more reasonable assumption.

It is possible that a mixed approach is indicated, especially if the equations of Matthews et al can be shown to hold. Even if PSS does not apply to all proteins, it is certainly the correct assumption for a number of proteins such as apolipoproteins whose synthesis is not limited by amino acid availability [16].

5.3. Prior debate in the literature

There has been little debate in the literature about the two assumptions. Toffolo et al [7], while advocating the TTR approach, did analyze the PSS assumption and stated, correctly, that the PSS "condition can be tested by measuring the total mass or concentration in B [the protein pool], which should remain constant during the experiment." This author has previously summarized available data from studies of apolipoprotein B turnover [16], which did in fact show that the total protein mass was constant during the experiment.

Toffolo et al also point out that an unaltered protein synthesis, in the face of changing amino acid amount, implies a tremendous variation in the rate constant for the protein incorporation of the amino acid, which they find unreasonable, thus warranting a rejection of the PSS assumption. However, as Garlick et al [9] have pointed out, this criticism is only applicable if a first-order kinetic model applies. If the protein synthesis is determined by many other factors, including the levels of the other 19 amino acids, the rate constant of incorporation is not a meaningful biological quantity.

6. Conclusion

Modeling necessarily involves assumptions, and we have contrasted two for studying amino acid kinetics. We have seen that the differences in calculated total body protein synthesis or individual FSR are modest, less than 10% for a typical constant infusion study. Because many studies compare repeated measurements in the same subjects, using the wrong equation is unlikely to lead to incorrect conclusions. Still, recognizing the assumptions behind the different equations in use for whole-body protein synthesis and for FSR of individual proteins or organs can help in having a consistent approach.

To conclude, investigators who assume a TSS should use the equations of Matthews et al for oxidation and total body synthesis and use TTR to calculate FSR of proteins [Eqs. (5), (6), and (25)]. Investigators who assume a PSS should use the equations from Garlick and coworkers instead [Eqs. (10), (11), and (29)]. Both sets of equations, as derived here, account for preinfusion enrichment. Based on the theoretical reasons depicted in Fig. 2, and the experimental evidence from dual tracer studies as well as results from multiple leucine intakes, we believe theory and experimental data favor PSS.

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