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Original Article

Whole-body protein kinetic models to quantify the anabolic response to dietary protein consumption

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SUMMARY

Determination of whole body rates of protein synthesis, breakdown and net balance in human subjects still has an important role in nutrition research. Quantifying the anabolic response to dietary protein intake is a particularly important application. There are different models with which to accomplish this goal, each with advantages and limitations.

The nitrogen (N)-flux method in which tracer is given orally has distinct advantages in terms of lack of invasiveness. In addition, the calculated results include all aspects of whole-body protein synthesis and breakdown. However, the prolonged timeframe of the method eliminates the possibility of the "pre-post" experimental design whereby each subject serves as their own control in the evaluation of the response to a meal.

Models based on the primed-constant infusion of an essential amino acid (EAA) tracer enable the determination of baseline whole-body protein kinetics within 2 h, and can quantify a dynamic change from the basal state. The greatest challenge when using an EAA model is distinguishing exogenous and endogenous sources of the tracee in the blood. One approach is to use an intrinsically-labeled protein. This method has the advantage that the exogenous tracee is clearly distinguished from endogenous tracee. On the other hand, the intrinsically-labeled protein method suffers from unmeasured dilution that is likely to cause the

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systematic underestimation of the rate of appearance of exogenous tracee and thus overestimate the rate of whole-body protein breakdown. Alternatively, the "bioavailability" approach estimates the contribution of exogenous tracee to the peripheral circulation from the amount of tracee ingested, the true ileal digestibility of the tracee, and the irreversible loss of tracee prior to entry into the peripheral circulation. Errors in assumed values with the bioavailability method can potentially be significant, but are not likely to result in the systematic over- or under-estimations of rates of whole-body protein synthesis and breakdown.
The optimal method depends on the degree of uncertainty regarding required assumptions in a particular circumstance. With all methods, it is advisable to calculate upper and lower bounds of whole body protein kinetics, in accord with reasonable maximal and minimal assumed values. Simultaneous use of two methods requiring different assumptions can also serve to confirm the validity of single approach.
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1. Introduction

Whole-body protein turnover refers to the pooled rates of protein synthesis and breakdown. Whole body protein turnover has been quantified by means of stable isotope tracer methodology in humans for close to 100 years [1]. The principal methodology used in the early studies has been criticized [2], but the major contribution of the early studies documenting the dynamic nature of the protein pool of the body was conceptual and did not require precise quantification. More recently, whole body protein kinetics have been determined in a variety of circumstances in which the accuracy of calculated rates of protein synthesis and breakdown has been important. In particular, quantification of the response of protein synthesis and breakdown to nutrient intake has played an important role in assessing optimal levels of protein intake, particularly in clinical circumstances, such as intensive care, in which long-term outcome studies are impractical (e.g., Ref. [3]). While methods to calculate rates of whole body protein kinetics have been analyzed in detail, (e.g. Refs. [2,4]), further analysis of methods to determine the response to nutrient intake is reasonable. All methods to determine the whole body protein kinetic response to nutrient intake require assumptions that can potentially affect the validity of the calculations. In this review we will focus on the challenging task of measuring the response of whole-body protein kinetics in response to consumption of dietary protein.

2. General principles of whole body protein models

Stochastic models ignore all of the various pools and components of whole body protein turnover, but rather focus on the overall process [2]. The tracer may label the entire nitrogen (N) pool (e.g., ^{15}N-glycine, ^{15}N-alanine), or trace the kinetics of an individual EAA that cannot be produced in the body (e.g., 1-^{13}C-leucine, $^{2}H_5$-phenylalanine). The EAA tracer enables quantification of the rate of appearance of the tracee, which in the fasted state is considered to be a direct function of whole-body protein breakdown [4]. Subtraction of the rate of irreversible loss (e.g., oxidation) of the tracee from its rate of appearance derives a value which is assumed to be a direct function of whole body protein synthesis. Net whole body protein balance is equal to protein synthesis minus breakdown [4]. EAA tracer can be administered intravenously as a continuous infusion until an isotopic equilibrium is reached in the primary tracee pool as well as in the end-product. A bolus of tracer, either consumed orally or injected intravenously, may also be used, provided that the area under the isotopic decay curve is used for the analysis of enrichment (see ref 4 for detailed discussion).

3. N-flux model of protein kinetics

^{15}N-glycine, and, to a lesser extent, ^{15}N-alanine, have been popular choices of tracers for more than 50 years [2] to measure the turnover of the entire N pool of the body. Tracer can be given as either a continuous infusion or sip feeding to achieve an isotopic equilibrium throughout the entire N pool, or as a bolus. Theoretically, either urinary urea or ammonia enrichment can be used to calculate N-flux, although urea has generally been the end-product of choice. In the case of constant tracer intake to achieve isotopic equilibrium, the plateau enrichment in urinary urea is used to calculate N-flux [5]. When a bolus of tracer is given, all of the^{15}N in urea is recovered over time. This approach requires determining not only the total amount of ^{15}N excreted in urine, but also the residual ^{15}N label in body at the end of the collection period. Since urea constitutes about 90% of excreted N [6], it is usually sufficient to estimate the residual label in urea at the end of the study period (i.e., enrichment times concertation), multiplied by volume of distribution of urea (total body water, TBW). TBW can be directly measured, either by stable isotope dilution or by impedance. Alternatively, TBW can be assumed as 0.764 x lean body mass, if lean body mass is known [7], or as 0.6 x body weight if lean body mass is not known [8]. The impact on the calculated rates of whole body protein synthesis and breakdown of using an assumed value for total body water is generally small. Table 1 shows that calculated rates of whole body protein synthesis, breakdown and net balance were similar in a recent study using the ^{15}N-alanine approach [9], regardless of whether the measured or an assumed value for TBW was used. While extreme conditions of body hydration, such as dehydration or severe edema, might result in a greater variance between calculated whole body protein kinetics when different approaches are used to estimate TBW, under most circumstances measurement of TBW is not necessary. There is also residual ^{15}N in ammonia in the TBW, but it is generally not a sufficient amount to significantly affect the calculated rates of protein kinetics.

Table 1
Effect of value for total body water on calculated protein kinetics.

	Measured (Ref 9)	LBM (Ref 7)	Calculated from Anthropometrics
Protein Synthesis	4.95 (4.34–5.56)	5.13 (4.51–5.75)	5.07 (4.47–5.68)
Protein Breakdown	4.72 (4.09–5.35)	4.90 (4.27–5.54)	4.85 (4.23–5.47)
Net Balance	0.225 (0.040–0.409)	0.225 (0.040–0.409)	0.225 (0.040–0.409)

Mean (95% confidence interval) of protein synthesis, protein breakdown, and net balance in grams per kilogram of body mass per day (data from Hirsch 2020) when measuring total body water by bioelectric impedance analysis (InBody 770, BioSpace, Seoul, South Korea), or calculating total body water based off Watson 1980 AJCN and St-Onge 2004 AJPEM.

Determination of whole body N-flux requires certain assumptions. The assumption that the entire N pool is equally labeled with the N in the tracer via transamination reactions is fundamental to this model. Unfortunately, the lack of uniform distribution of ^{15}N throughout its entire volume of distribution when^{15}N-glycine is used as tracer is proven by the discrepancy between urea and ammonia enrichment at isotopic plateau during a tracer constant infusion. ^{15}N-urea enrichment is generally approximately 50% higher in excreted urea than ammonia in the post-absorptive state. This difference has led to the thesis that glycine is preferentially converted to urea as opposed to other amino acids [10,11]. Nonetheless, there is a strong empirical support for the concept of a single body N pool and the ability of ^{15}N-glycine to trace that pool in the post-absorptive state. Stein, et al. [12]. compared the rates of protein synthesis calculated by analyzing the enrichment of the nitrogen end-products to the incorporation of ^{15}N-glycine into the entire homogenized carcass of rats. Their data indicated a good agreement between the indirect (end-product) and direct (tissue incorporation) methods in the calculation of protein synthesis, thereby providing support for the assumption of a single N pool. Nonetheless, the discrepancy between urea and ammonia enrichments raises concern that ^{15}N-glycine is less than an optimal tracer for measuring turnover of the body N pool. ^{15}N-alanine more appropriately reflects the body's nitrogen pool than does ^{15}N-glycine [13]. Alanine plays an important role in the interorgan transfer of amino nitrogen from the muscle, gut, and kidney for hepatic urea production, and is extensively involved in transamination reactions [14,15].

There are conceptual advantages to deriving values for protein synthesis and breakdown from the determination of whole-body N-flux, particularly if the tracer is given as an oral dose of ^{15}N-alanine. Importantly, most dietary guidelines have been based on N-balance data and measuring N-flux adds a dynamic aspect to traditional N-balance method. Further, N-flux can be determined over a shorter time interval than N-balance. Since the oral bolus of ^{15}N-alanine methodology involves pooling of all excreted ^{15}N over the sampling time (usually about 10 h), the method is well-suited for determining integrated effects of interventions, including assessing the response to different amounts of dietary protein in multiple meals throughout the day.

There are also technical advantages to the oral bolus of ^{15}N-alanine method. If ^{15}N enrichment in excreted urea can be measured by isotope-ratio mass spectrometry (IRMS), gas-chromatography mass spectrometry (GCMS), or liquid-chromatography mass spectrometry (LCMS), depending on the dose of tracer (and thus enrichment of urea). An oral dose of as little as 300 mg of ^{15}N-alanine is sufficient if analysis is by IRMS, while about 2 g of ^{15}N-alanine is adequate if enrichment is to be measured by GCMS. The oral bolus-dose method is ideally suited for field studies, as it does not require an intravenous infusion and all urine samples are pooled, which simplifies collection. Providing the tracer dose orally is not only convenient for free-living subjects, but also avoids the rigor and expense of pharmacy-prepared infusions. Consequently, oral ingestion of a bolus of ^{15}N-alanine and subsequent determination of the labeled end-product in urea has proven efficacious in both clinical [15] and field trial studies [16].

Quantification of the response to dietary protein by the N-flux method requires accounting for the contribution of exogenous tracee to the total measured rate of appearance of tracee. Thus, protein breakdown in the post-prandial state is calculated as the total N-flux minus the amount of dietary N consumed. While all N consumed may not be absorbed, the amount of error from this assumption is not likely to be large. Further, the N-flux approach to the determination of the response of endogenous protein kinetics to dietary protein consumption has the great advantage that no assumption is required regarding splanchnic clearance of ingested N; the N-flux value includes all splanchnic protein metabolism. The advantage of this aspect of the N-flux method will be evident when considered in the context of the assumptions required in other models of whole-body protein kinetic that will be discussed below.

The most limiting feature of the whole body N-flux determination is that rapid changes in protein kinetics, such as occur after dietary protein consumption, cannot be determined. Isotopic equilibration throughout the body nitrogen pool requires too much time for the method to be useful in acute studies. For this reason, determining protein kinetics using a single essential amino acid tracer have become popular.

4. Single essential amino acid (EAA) model of protein kinetics

The general model calls for a labeled EAA to be infused into a subject, and the rates of appearance (Ra) and of irreversible loss of the unlabeled counterpart of the tracer (the tracee) are determined by traditional tracer methodology [4]. The rate of whole body protein breakdown in the post absorptive state is calculated from the rate of appearance (Ra) of the tracee and the amount of the tracee per gram of protein. The difference between the Ra and the rate of irreversible loss of the tracee (e.g., oxidation) equals the amount of the tracee reincorporated into protein, and this rate can be extrapolated to the rate of whole body protein synthesis by taking account of the contribution of the tracee amino acid to the whole body protein pool. In the fed state account must be taken of the contribution of absorbed tracee to the measured Ra. There are a variety of permutations of the general model in which different tracers and end products are used.

The principal advantage of EAA models, as compared to the N-flux model, is that, with appropriate priming of the relevant pools, it is possible to collect reliable data within 90 min of the start of the tracer infusion. Because of the short time required and rapid tracer responses, evaluation of the acute response to various perturbations, including meal feeding, is possible. A further advantage of the EAA models is that each subject can serve as their own control. Expressing data as change from baseline is particularly useful when the clinical condition of each subject may differ markedly, and changes in baseline turnover in any given patient may occur over time as well.

A variety of EAA tracers with different end products have been used with this basic model, but the most popular have been 1-^{13}C-leucine as infused tracer and $^{13}CO_2$ as the end product of oxidation, and

$^{2}H_{5}$-phenylalanine as infused tracer and hydroxylation of phenylalanine to tyrosine as the product of the irreversible loss of phenylalanine. The leucine model has the advantage of being able to approximate the precursor enrichment for oxidation by measuring the plasma enrichment of alpha-ketoisocaproic acid (KIC), due to the intracellular equilibrium between KIC and leucine [17]. The leucine model has the technical disadvantage of having to collect breath samples in order to measure both the rate of total CO_2 excretion as well as expired CO_2 isotopic enrichment. The phenylalanine model has the advantage of not requiring any breath samples, but presents the disadvantage that there is no analogous surrogate for the intracellular enrichment of phenylalanine in the liver (where hydroxylation occurs).

The various assumptions underlying the EAA models have been discussed extensively (e.g., Ref. [4]). Among the most important assumptions, the kinetics of a single EAA is assumed to be representative of all EAAs when account is taken of the respective contributions to the composition of whole-body protein. This assumption has proven valid in the basal state [18], but in some circumstances may become problematic. For example, the oxidation of leucine increases disproportionately during exercise as compared to the oxidation of other essential amino acids [19]. Simultaneously infusing two different EAA tracers can identify a potential problem with the assumption that one EAA is representative of all EAAs, but in the case of divergent responses, it is usually difficult to ascertain which amino acid (if either) is representative of whole-body protein metabolism.

The validity of the assumption that the tracer kinetics of one EAA is representative of all EAAs, as well as all other assumptions implicit in the EAA model in the post-absorptive state, is supported by the empirical evidence that the basal rates are similar with different EAA tracers and end products. This includes ^{15}N-lysine and urea as the end product [20]; 1-^{13}C-lysine and $^{13}CO_2$ as the end product [21]; 1-^{13}C-leucine and CO_2 as the end product [21]; and $^{2}H_{5}$-phenylalanine and hydroxylation to tyrosine as the end product [22].

4.1. Quantifying protein kinetics in the fed state with the EAA model

Account must be taken of the contribution of absorbed amino acids from dietary protein to the total flux rate of the tracee in order to calculate whole body protein breakdown using the EAA model. The Ra of exogenous tracee must be subtracted from the total Ra as measured in the peripheral plasma to derive the rate of endogenous protein breakdown. The calculated rate of whole body protein synthesis, on the other hand, does not require the Ra's of endogenous and exogenous to be distinguished, so an error in the determination of the exogenous Ra will affect only the accuracy of the calculated rate of protein breakdown. Consequently, an error in the determination of exogenous Ra will translate to an error not only in the value of protein breakdown but also net balance as well.

There are two basic approaches for quantifying exogenous Ra in the context of the EAA model: the intrinsically labeled protein method and the bioavailability approach. We have recently discussed both approaches extensively [23,24]. The following discussion highlights the most salient points.

4.2. Intrinsically-labeled protein approach for quantifying exogenous Ra

The seemingly most elegant approach to distinguishing exogenous Ra (from digested protein) from endogenous Ra (from protein breakdown) is to use a dietary protein containing an isotopically labeled amino acid, called an intrinsically-labeled dietary protein. The intrinsically-labeled protein contains the same tracer amino acid as is infused, but labeled differently, thereby enabling clear-cut differentiation of the ingested source of the labeled amino acid from the infused tracer. Regardless of the means of labeling or the specific protein to be labeled, the process of producing intrinsically-labeled proteins in sufficient quantities to be used in human experiments is difficult and expensive, and the variety of proteins that can be labeled is limited. Nonetheless, the conclusions drawn from experiments using intrinsically-labeled proteins have formed a basis for our current understanding of the physiological responses of endogenous protein metabolism to dietary protein consumption. However, as compared to the other approaches to quantifying whole-body protein kinetics, the intrinsically-labeled protein approach has received relatively little critical evaluation.

The intrinsically-labeled protein method is predicated on the assumption that the appearance of the ingested tracer amino acid in the peripheral blood quantitatively reflects the appearance of its

unlabeled counterpart. Determining the amount of an absorbed radioactive tracer in an intrinsically-labeled protein would be relatively straight-forward. The total amount of radioactivity appearing in the circulation over time, regardless of whether it is contained in its original form or in a metabolite, divided by the specific activity (decays per minute/g amino acid) of the labeled amino acid in the dietary protein, would give an estimate of the amount of exogenous amino acid (tracee) absorbed. Another approach that may give an accurate estimate of dietary amino acid uptake is the recently developed dual-isotope method [21]. This approach relates plasma isotopic enrichments of the same amino acid originating from two differently labelled proteins fed together; one of the labeled proteins, for which the true ileal digestibility of the amino acid is well-characterized, serves as a baseline.

The most popular use of intrinsically-labeled proteins has been with a single dietary protein labeled with a stable isotope tracer. This approach is complicated as compared to the use of a radioactive tracer by the fact that the stable isotope enrichment is measured as a ratio of the tracer/tracee, rather than as an absolute amount of tracer. The accurate determination of the amount of tracee absorbed requires that the enrichment of the labeled amino acid in the intrinsically-labeled protein must not be diluted from the point of ingestion to the point of sampling in the peripheral blood by any process other than protein breakdown. The assertion has been made that this assumption is valid [26]. However, the isotopic enrichment of ingested tracer in an intrinsically labeled protein is diluted in the gastrointestinal tract (GIT) by the digestive process. We have estimated that an intrinsic label is diluted approximately 25% in the GIT as a result of the digestion of endogenous (of body origin) proteins, such as digestive enzymes, mucus and cells that enter the GIT during the digestion of a protein meal [27]. The amount of endogenous protein secreted into the GIT is similar to the amount of dietary protein ingested [28]. Much of this secreted protein is rapidly digested and reabsorbed, while some (about 20–30%) is lost into the colon and does not reappear in the circulation as amino acids [29]. The synthesis of GIT proteins (without distinction between tracer and tracee) roughly matches the rate of secretion of endogenous protein, thereby approximately maintaining GIT protein balance [30]. Thus, the ingested tracer enrichment is diluted an amount related to the digestion and reabsorption of secreted endogenous proteins.

It could be argued that since the isotopic dilution of intrinsically-labeled protein in the GIT represents the breakdown of digestive enzymes, etc., that process should be included as part of whole body protein breakdown. Although digestion of proteins that have been secreted into the GIT is a different process than the metabolic degradation of endogenous intracellular protein (i.e., protein breakdown), secreted proteins are lost from the body protein pool, so it is reasonable to include that process in the breakdown side of the net balance equation. However, the quantitative interpretation of the dilution of the tracer from the intrinsically-labeled protein as arising entirely from digestion and reabsorption of endogenously secreted gut proteins is complicated by the rapid turnover of proteins in the GIT. The rapid turnover of GIT proteins means that within the time frame of an experiment there is potential for recycling of the absorbed tracer and unlabeled tracee that is incorporated into GIT proteins and secreted into the GIT [29,30]. The amount of label recycling is dependent on how close the body pool is to isotopic equilibrium. The enrichment of recycled tracer will reflect the intracellular enrichment from which the synthetic precursors for GIT synthesis were drawn, which will be lower than the absorbed enrichment until an isotopic equilibrium is achieved. If the isotopic enrichment of the tracer amino acid in the newly-synthesized GIT proteins is less than the enrichment of its counterpart in the intrinsically-labeled protein, the process of tracer recycling will (somewhat paradoxically) dilute the absorbed tracer enrichment even further. The further dilution of the absorbed tracer by recycling of tracee and tracer will cause a further overestimation of the dilution already resulting from digestion and reabsorption of endogenously-secreted proteins.

An additional complication in the calculation of net protein balance by the intrinsically-labeled protein approach is that the reincorporation into GIT proteins of amino acids from the digested endogenous proteins that were secreted (or the equivalent amount from other sources) will not be detected as part of the total rate of appearance of tracee, measured peripherally by means of the infused tracer. Since the rate of whole-body protein synthesis is based on the rate of appearance of tracee into the peripheral circulation, the direct reincorporation (in a net sense) into GIT proteins of the amino acids resulting from the digestion and absorption of endogenous proteins will not be included in the calculation of whole-body protein synthesis. Thus, while under the inherent assumptions the

dilution of ingested tracer by the digestion of endogenous protein secreted into the GIT is considered to be a direct reflection of protein breakdown, the corresponding reincorporation of absorbed amino acids into GIT proteins is not included in the protein synthesis side of the net balance equation. The net anabolic effect of dietary protein will therefore inevitably be underestimated by the intrinsically-labeled protein approach. In contrast to the process of recycling of tracer, synthesis of GIT proteins from amino acid derived from the plasma will be included in the whole body rate of protein synthesis.

Once the labeled amino acids in intrinsically-labeled protein are absorbed, the assertion that further dilution of the tracer occurs only as a result of protein breakdown [26] is valid only when an isotopic steady state has been achieved in the intracellular pools of the cells of the intestines and splanchnic bed, as well as the recycled amino acids. Many hours of a steady rate of absorption of a labeled amino acid are required to achieve isotopic equilibrium in the free intracellular pools of those tissues and organs, and in GIT proteins, and this approach is not compatible with normal dietary patterns of meal feeding. Non-steady state calculations have been used to attempt to account for a variable rate at which the exogenous tracer appears in peripheral blood where sampling occurs (e.g. Ref. [31]), but these calculations do not fully address the issue of the dilution of absorbed tracer in the intracellular pools of the intestine and splanchnic bed before appearance in the peripheral circulation.

A simple schematic diagram (Fig. 1) representing the intracellular compartment of an intestinal cell through which absorbed amino acids pass before reaching the blood illustrates the problem with the assumption that there is no dilution of absorbed tracer other than by protein breakdown. The figure illustrates a basic principle of tracer methodology that applies following ingestion of an intrinsically-labeled protein. The same principles apply to other tissues and organs of the splanchnic bed through which blood passes before reaching the hepatic vein and entering the peripheral circulation, where sampling occurs.

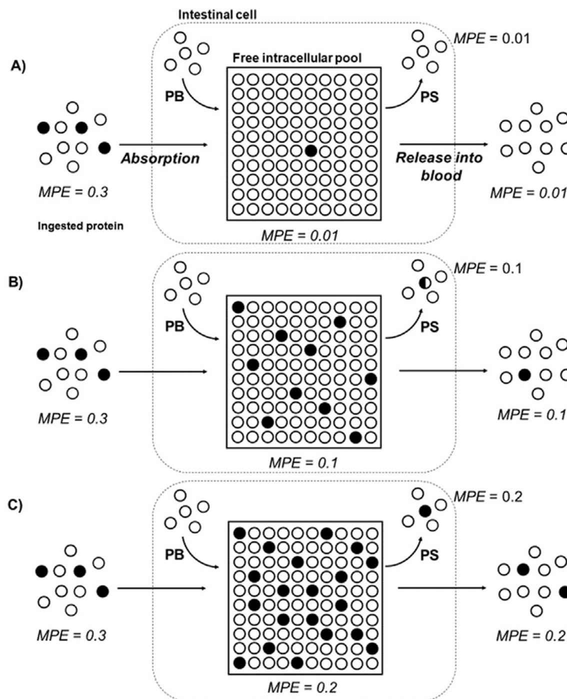

Fig. 1. Schematic representation of an intestinal cell through which absorbed dietary amino acids pass before entering the blood. For simplicity, we assume that the rate of appearance of tracee into the cell from protein breakdown (PB) and disappearance of tracee from the intracellular pool into protein (i.e., protein synthesis, PS) are constant and equal. We also assume that protein breakdown constitutes one-third of the entry of tracee into the cell and that the absorbed tracer + tracee constitutes two thirds of the entry into the intracellular compartment. Unit of enrichment is mole percent excess (MPE), calculated as (tracer/tracee)/[1 + (tracer/tracee)] x 100.

Before consumption of the intrinsically-labeled protein the pre-existent intracellular pool of free tracee amino acid is derived from protein breakdown and inward transport from the blood. For simplicity, in our example we will assume that throughout the absorption period no (recycled) label appears in the intracellular pool from whole body protein breakdown, and that the rate of protein synthesis equals the rate of protein breakdown. We will also assume a steady rate of tracer absorption, and that the rate of whole body protein breakdown is one-third the rate of absorption of tracee into the cell.

When tracer first appears in the cell from the digestion of an intrinsically-labeled protein (**panel A in** Fig. 1), there is a very small amount of tracer relative to pre-existent tracee in the cell. The efflux of molecules from the cell does not distinguish between tracer and tracee molecules. Since the intracellular tracer to tracee ratio is much less than the tracer to tracee ratio entering the cell, the tracer to tracee ratio leaving the intracellular compartment (i.e., efflux into blood and incorporation into protein) is significantly diluted below the tracer to tracee ratio in the intrinsically-labeled protein. Most of this dilution arises because of the pre-existent intracellular pool of (unlabeled) tracee. As tracer continues to be absorbed into the cell over time, the tracer to tracee ratio of the intracellular pool rises (**panel B in** Fig. 1). As entry of tracer into the cell continues, (the point represented in panel B), there is a progressive increase in the intracellular tracer to tracee ratio, and the contribution of tracee from protein breakdown accounts for a greater proportion of the dilution of absorbed tracer than at the outset of tracer absorption. With continued steady absorption of tracer amino acid an isotopic steady state will eventually be achieved, at which time there will be no further change in the tracer to tracee ratio of either intracellular amino acids or of amino acids leaving the cell into the blood, as long as tracer absorption remains constant (**panel C in** Fig. 1). At isotopic equilibrium any dilution in the tracer to tracee ratio of the amino acid derived from the intrinsically-labeled protein will reflect the rate of protein breakdown in the cell.

Fig. 1 illustrates that in an isotopic non-steady state the true rate of absorbed tracee will be underestimated because of dilution of absorbed tracer by the pre-existent tracee. Since the calculated rate of protein breakdown is the measured total Ra of tracee minus the exogenous Ra, an underestimation of exogenous Ra will result in an overestimation of the rate of protein breakdown. When an isotopic equilibrium is reached there will no longer be any dilution of the tracer by the pre-existent tracee (panel C, Fig. 1). At isotopic equilibrium, any dilution of the tracer leaving the cell will reflect the appearance of tracee from protein breakdown within the cell. However, if that source of dilution of absorbed tracer is added to the whole body rate of protein breakdown, the net balance between protein synthesis and breakdown at the whole body level will be underestimated by an amount equal to the intracellular recycling of tracee back into protein without appearing in the peripheral blood. The EAA model does not include intracellular recycling of tracee from protein breakdown into synthesis, as illustrated in Fig. 1, in the calculation of either breakdown or synthesis. The rate of whole body protein synthesis is calculated as the rate of appearance of tracee into peripheral plasma minus irreversible loss, and the tracee arising from whole body protein breakdown in Fig. 1 never enters the circulation. The dilution of the absorbed tracer by breakdown thus represents only half of the intracellular recycling of tracee, and if that component of intracellular recycling is included in the calculation of whole body net protein balance without also including the rate of synthesis from intracellular recycling, the net anabolic response to dietary protein ingestion will be underestimated. Unfortunately, the intracellular recycling of tracee cannot be measured in a human experiment.

An isotopic steady state can be accomplished by frequent sip feeding of intrinsically labeled protein, thereby avoiding the problem of dilution by pre-existent tracee in the intracellular pool, if the sip feeding is given for a long enough time. An isotopic steady state also minimizes the problems discussed above regarding the recycling of tracee and tracer via the synthesis of GIT proteins and secretion into the GIT. Sip feeding may also provide less of a stimulus for the secretion of digestive enzymes that dilute the enrichment of the tracer amino acid in intrinsically-labeled protein. However, sip feeding will likely blunt the anabolic effect of a dietary protein as compared to meal feeding [32]. More problematic, even in an isotopic steady state the dilution of absorbed tracer by intracellular recycling of tracee from breakdown back into synthesis will result in the underestimation of the true anabolic response to dietary protein.

A large enough bolus dose of intrinsically-labeled protein would be analogous to the "flooding dose" technique to measure protein synthesis [33]. In this case, panels A and B in Fig. 1 would be avoided. On the

other hand, consumption of a large bolus of intrinsically labeled protein will trigger the release of a correspondingly large amount of endogenous protein, particularly digestive enzymes [28], which will dilute the enrichment of the tracer amino acid before it is absorbed. In addition, the intracellular recycling of tracee from breakdown directly into synthesis will still cause an underestimation of the net balance response. Most problematic, all sources of error with the intrinsically-labeled protein approach result in the underestimation of the net protein balance response to dietary protein by an amount that is likely physiologically significant [24]. Providing a large bolus of intrinsically-labeled protein will circumvent some of the issues discussed above, but some underestimation of the net anabolic response to dietary protein is inevitable. It is possible that integrating the total area under the curve of the enrichment of absorbed tracer vs time would lessen the complications inherent in interpreting the data from the intrinsically-labeled protein method, but to our knowledge this approach has not been previously used.

4.3. Bioavailability method for estimating exogenous Ra

The bioavailability approach can also be used with EAA models to estimate the amount of dietary protein that is absorbed into the body and detected in the peripheral circulation. The exogenous rate of appearance using the bioavailability approach is based on the amount of protein ingested, the true ileal digestibility (TID) of the dietary protein, and the irreversible loss of the tracee in the splanchnic bed. The approach can be used with any EAA model of protein kinetics, but the phenylalanine method is advantageous because the fraction of absorbed tracee cleared by the splanchnic bed can be directly measured as the hydroxylation of phenylalanine to tyrosine in the liver [23]. One advantage of the bioavailability approach is that the response to a combination of a variety of proteins can be quantified. In addition, a physiological steady state is not required, meaning that the method is well suited for quantifying the response to a meal. Further, the complication of the intrinsically-labeled protein approach caused by the secretion and digestion of endogenous protein is avoided because this process does not cause any change in net tracee absorption. On the other hand, only the total anabolic response can be determined because only the total contribution of exogenous phenylalanine to the peripheral circulation can be estimated, not the rate at which it is absorbed.

There are three significant assumptions underlying the estimation of the total appearance of ingested phenylalanine in the peripheral circulation. First, it is assumed that the fraction of absorbed phenylalanine that is irreversibly cleared by the splanchnic bed before appearing in peripheral blood can be accurately determined. Second, it is assumed that the rate of synthesis of GIT proteins balances the rate of digestion of endogenous protein secreted into the GIT (i.e., no net synthesis or loss of GIT proteins). Amino acids arising from the breakdown of GIT proteins that are released into the blood, and GIT proteins that are synthesized from amino acid precursors delivered to the GIT cells by arterial blood flow, are included in the measurement of whole body protein synthesis and breakdown. Third, the TID of the tracee in the protein meal must either be assumed or measured independently.

The irreversible loss of phenylalanine (i.e., hydroxylation to tyrosine) occurs only in the liver [34]. Determination of the fraction of phenylalanine uptake that is irreversibly hydroxylated to tyrosine at the whole body level therefore enables a direct measurement of the irreversible loss of absorbed tracee (phenylalanine absorbed x fraction of uptake hydroxylated). The measurement of the fraction of phenylalanine hydroxylated requires the precursor (i.e., phenylalanine) enrichment at the site of hydroxylation to tyrosine (i.e., liver). Since the intracellular pool of the liver is not directly accessible in human studies, the plasma enrichment of phenylalanine is generally measured, and the intrahepatic precursor enrichment in the fed state estimated to be the plasma phenylalanine enrichment x 0.8, based on the study of Reeds and associates [35]. The assumed value of 0.8 may be in error in any given study, but the error would be random and not large, owing to the relatively rapid transmembrane transit of phenylalanine [36].

The bioavailability method includes all absorbed Phe that enters the blood and is not hydroxylated in the liver on the first pass as being incorporated into protein, including splanchnic protein synthesis. Similarly, whatever amino acids released into the blood as a result of splanchnic protein breakdown that are not directly hydroxylated in the liver are included as part of whole body protein breakdown. Since the fraction of Phe passing the liver that is hydroxylated is directly measured, these aspects of protein turnover are included as part of the whole body response. In contrast, the bioavailability

method does not account for the amount of endogenous protein secreted into the GIT, or the amount of GIT proteins synthesized from amino acids taken up by the enterocytes that never enter the blood. These two processes are assumed to be equal. There are no data from human subjects directly addressing this assumption. The fractional synthetic rate (FSR) of intestinal proteins was found to not significantly change in human subjects after a protein meal, but the measurements were quite variable [37]. A more recent study found a significant increase in the FSR of duodenal mucosal cells with feeding [38], but the relation of the increase in FSR to the amount of endogenous protein secreted into the gut is unclear. Additional data from human subjects addressing this issue would be helpful.

In any experiment, the amount of dietary protein consumed and the phenylalanine content of the ingested protein are known. The amount of ingested protein that is absorbed must account for true ileal digestibility (TID) (corrected for gut endogenous amino acids). TID can be determined experimentally, but more commonly, it is estimated from literature values. Although abundant true ileal digestibility data are not available for humans, the pig has proven to be an excellent model (second choice), and if neither human nor pig data are available, values from the rat can be used [39]. In order to assess the importance of precise values for TID, we have calculated protein kinetic values from several protein foods using a range of values for TID. In each case, we calculated protein kinetics using the best literature value for TID, then recalculated the protein kinetic values using the same tracer data and an assumed TID of plus and minus 5% of the originally assumed value [40]. For example, if the best evidence reports a value for TID is 80%, then we have calculated protein kinetics using values of TID of 75%, 80%, and 85%. The results are shown in Fig. 2. In theory, using the wrong TID value could result in an incorrect conclusion; however, Fig. 2 demonstrates that, over a wide range of protein food sources, a TID within reasonable upper and lower bounds will have minimal effect on the calculated anabolic effect. If, in contrast to the data shown in Fig. 2, the upper and lower bounds overlap significantly, it is possible to measure the TID in order to avoid assumptions. The dual isotope tracer approach described above [25], wherein the absorption of a labeled test protein is compared to the absorption of labeled spirulina protein, could be performed at the same time as the protein kinetic measurements are made. Measurement of TID with the dual tracer approach would be particularly useful if a reliable literature value is not available, or if the test protein has a very low TID, such that reasonable upper and lower bounds would be difficult to estimate. The major limitation of the concurrent measurement of TID and protein kinetics is the availability of appropriately labeled test proteins.

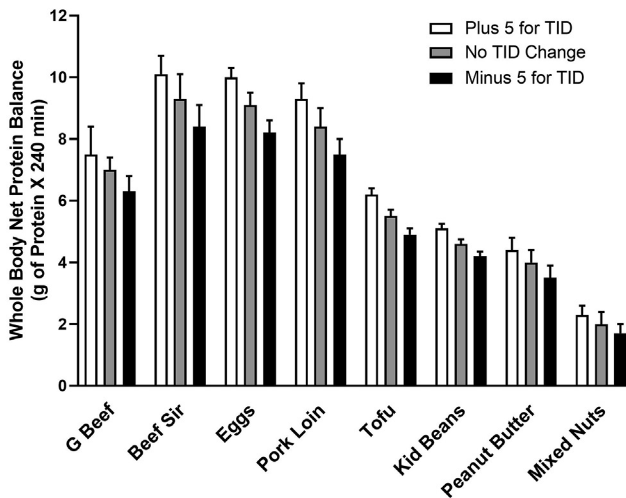

Fig. 2. Whole body net protein balance (protein synthesis minus breakdown) in a variety of protein food sources calculated by the bioavailability approach using the phenylalanine method. Values were calculated using the best available literature value for TID, and TID values 5% greater and 5% less than the best available value. The serving sizes were consistent with the “ounce equivalents” in MyPlate from the Dietary Guidelines for Americans [41]. The reasonable upper and lower bounds of the assumed TID did not affect the ranking of anabolic effect.

5. Clinical applications of whole body protein models

All of the methods for measuring the responses of whole body protein kinetics to dietary consumption of protein meals have advantages and limitations that may impact the selection of the best approach in any given clinical state. The measurement of N-flux is an attractive method for determining overall rates of whole body protein synthesis and breakdown over the course of the day. Few assumptions are required, and the method is advantageous because the tracer can be given orally and sample analysis is straight-forward. However, since the method does not distinguish the post-absorptive and post-prandial states, the method is best suited to circumstances in which there is little variation in the physiological state of the experimental subjects. The EAA methods, on the other hand are well-suited for clinical studies, even in critically ill or severely injured patients. A more dynamic assessment of protein kinetics enables an experimental design in which each subjects serves as their own control. Thus, the fed values for each subject can be compared to their own baseline values. This aspect of the EAA methods makes them particularly well-suited to the measurement of the responses to a single meal in patients with widely varying clinical status, and correspondingly different baseline values.

All EAA methods share similar assumptions. In the post-absorptive state the rate of whole body protein breakdown is calculated from the total rate of appearance of tracee amino acid into blood, and the rate of whole body protein synthesis is calculated from the rate of whole body protein breakdown minus the rate of irreversible loss of tracee. Whole body protein synthesis is calculated in the post-prandial state in the same way as in the post-absorptive state. The differentiation of EAA models is entirely due to the calculation of whole body protein breakdown in the post-prandial state. The differing approaches to the calculation of whole body protein breakdown with EAA models impact the calculation of net whole body protein balance as well, since whole body protein synthesis is calculated in the same way with all EAA models. The intrinsically-labeled protein definitively differentiates endogenous and exogenously-derived tracee. However, the small number of dietary proteins that can be conveniently labeled limits the method. In addition, experimental designs that fail to account for tracer dilution in the processes of digestion and absorption will systematically overestimate the rate of protein breakdown relative to the rate of protein synthesis. EAA models that use the bioavailability method for estimating the contribution of exogenous tracee to total appearance of tracee in the blood usually rely on assumptions based on literature values for protein digestibility to calculate protein breakdown. Whereas reasonably accurate values for true ileal digestibility are available for most dietary proteins, clinical conditions that alter digestibility will affect the accuracy of the assumed values. Incorporation of the dual tracer approach [25] to measuring digestibility to both the intrinsically-labeled protein and the bioavailability method may improve the accuracy of calculated protein kinetics.

From this summary it should be evident that all whole body protein methods have some limitations. It should therefore be routine to consider how potential errors in assumptions might affect the interpretation of results by calculating upper and lower bounds, based on reasonable ranges of assumed values.

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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