Measurement of muscle protein fractional synthesis and breakdown rates from a pulse tracer injection

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Zhang, Xiao-Jun, David L. Chinkes, and Robert R. Wolfe. Measurement of muscle protein fractional synthesis and breakdown rates from a pulse tracer injection. Am J Physiol Endocrinol Metab 283: E753–E764, 2002.—We have developed a new method to determine the fractional synthesis rate (FSR) and breakdown rate (FBR) of muscle protein. This method involves a pulse tracer injection and measurement of enrichment in the arterial blood and muscle at three time points. The calculations of FSR and FBR are based on the precursor-product principle. To test this method, we gave a pulse injection of [ring-13C6]phenylalanine of 4–6 mg/kg in five rabbits. The measured FBR value (0.233 ± 0.060%/h) was almost identical (P = 0.35) to that (0.217 ± 0.078%/h) estimated from a leg arteriovenous balance model (Biolo G, Chinkes D, Zhang X-J, and Wolfe RR. J Parenter Enteral Nutr 16: 305–315, 1992). The measured FSR value tended to be lower than that estimated from the leg model (0.125 ± 0.036% vs. 0.185 ± 0.086%/h; P = 0.14), possibly because the new method measures only muscle FSR, whereas the leg balance model also includes skin and bone contributions. The pulse tracer injection did not affect muscle protein kinetics as measured by leucine and phenylalanine kinetics in the leg. In another five rabbits, we demonstrated that sampling could be reduced to either one or two muscle biopsies when multiple pulse injections were used. This method can be completed in 1 h with one muscle biopsy and has technical advantages over currently used methods.

Stable isotopes; gas chromatograph-mass spectrometer; arteriovenous balance; rabbits

The metabolic status of protein in a tissue is determined by the relative rates of synthesis and breakdown. Changes in protein balance can be caused by changes in synthesis or breakdown or both. Thus, to obtain a complete knowledge of the protein metabolism in a tissue, it is necessary to measure the rates of both synthesis and breakdown. The arteriovenous (a-v) balance methods, with the use of either a two-compartment model (3, 10) or a three-compartment model (5) for calculations, are a common choice for measurement of protein kinetics in a tissue because the rates of synthesis, breakdown, and net balance can be obtained or inferred. A potential drawback of these methods is that the a-v unit usually includes several tissues. For example, the leg has been frequently used as an a-v unit to represent muscle protein metabolism, but in some circumstances the metabolic contribution from nonmuscle tissue (mainly skin) may be significant (7, 18). The tracer incorporation methods are based on the rate of incorporation of amino acid tracer into the target tissue protein. The tracer may be given either as a constant infusion or as a bolus, either alone or with a significant amount of tracee added (flooding dose method) (11). These methods reflect protein metabolism in a particular tissue, or even a particular protein, but do not provide a measure of breakdown. Thus there is considerable information about the regulation of protein synthesis in a variety of tissues such as muscle (15), liver (2), and intestinal mucosa (1), as well as specific proteins such as albumin and fibrinogen (12), but few measurements of breakdown.

We previously described the measurement of protein breakdown by a method that corresponds to fractional synthesis rate (FSR) and therefore is called the fractional breakdown rate (FBR) (25). This measurement requires infusion of a tracer to achieve isotopic plateau in the arterial blood and in the intracellular free amino acid pool in tissue and then measurement of decay in enrichment after the tracer infusion is stopped. Thus this FBR measurement can be combined with the primed-constant infusion to obtain both FSR and FBR simultaneously from the same tissue samples (9, 17). Whereas the above procedure provides a feasible approach to measure tissue protein FSR and FBR, it requires at least 4–5 h to complete, and usually three tissue biopsies are necessary. The goal of the present experiment was to develop a new method to determine both FSR and FBR of tissue protein within a shorter time frame (e.g., 1 h) and from only one biopsy. We selected the skeletal muscle as the target tissue for development of this novel method because the protein kinetics in muscle can be concomitantly determined by the three-compartment a-v model (5), thereby providing reference rates of synthesis and breakdown with which to evaluate the validity of the new method.

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METHODS

Rationale and Equations

Protein synthesis and breakdown are two opposing processes that function simultaneously. The movement of amino acids from the muscle intracellular free (MIF) pool into the protein-bound pool reflects protein synthesis, and the movement of amino acids from the protein-bound pool into the MIF pool represents protein breakdown if the amino acid is not synthesized in the tissue. The measurements of FSR and FBR are both based on the precursor-product principle except that the definitions of precursor and product are reversed for the two processes. For protein synthesis the MIF pool is a good approximation of the precursor (i.e., aminoacyl-tRNA) (4) and the product is the protein-bound pool. For protein breakdown the protein-bound pool is one of the precursors (the other is the arterial blood) and the MIF is the product pool. Thus the FSR is determined by the rate of tracer incorporation from the MIF pool to the bound pool, and the FBR is determined by the rate at which tracer release from the bound pool dilutes the enrichment in the MIF pool.

The precursor-product principle commonly has been applied to the movement of tracer from the MIF pool to the bound pool for determination of FSR. However, the information of FBR reflected by the movement of tracer from the bound pool to the MIF pool has been neglected. In fact, the rationale for the use of the precursor-product principle to measure FBR is as solid as its use to measure FSR. The intracellular protein breakdown is the only factor that causes enrichment gradient between the arterial blood and MIF pool; again an amino acid tracer is used that is not synthesized in the tissue. Without protein breakdown the enrichment in the MIF pool will equal that in the arterial blood. When the rate of inward transport from the arterial blood into the MIF pool is constant, the greater the rate of FBR, the lower the enrichment in the MIF pool, and vice versa. Thus the enrichment difference between the arterial blood and MIF pool reflects the rate of tracer movement from the bound pool to the MIF pool. Under the physiological steady state, in which the concentrations of amino acids in the blood and in the MIF pool are constant, the FBR of muscle protein can be calculated from the following equation (25)

$$ FBR = \frac{E_M(t_2) - E_M(t_1)}{P \int_{t_1}^{t_2} E_A(t) dt - (1 + P) \int_{t_1}^{t_2} E_M(t) dt} \cdot (Q_{M/T}) \quad (1) $$

where $P = E_{AB}/(E_A - E_M)$ at isotopic plateau, and $E_A$ and $E_M$ are isotopic enrichments in the arterial and MIF pools, respectively; $E_M(t_2) - E_M(t_1)$ is the change of enrichment in the MIF pool from time ($t$) $t_1$ to $t_2$ and $Q_{M/T}$ is the ratio of intracellular free tracee content vs. protein-bound tracee content in the muscle.

The derivation of Eq. 1 has been described in detail in our previous work (25), and the following is a brief explanation of this equation. Equation 1 is simply a precursor-product equation (29) if one ignores the variable $Q_{M/T}$. The variable $P$ in this equation is necessary because there are two precursor pools: the arterial blood and protein-bound amino acids within the cell. Therefore, the relative contribution of these two sources to product pool (i.e., MIF) needs to be included in the calculation, which is accomplished by using the variable $P$. The variable $P$ can be calculated from isotope enrichments in the arterial blood ($E_{AB}$) and MIF pool ($E_M$) at isotopic plateaus: $P = E_{AB}/(E_A - E_M)$. The variable $P$, as defined above, is equal to the ratio of fractional tracee from artery vs. fractional tracee from breakdown, and $1 + P$ is the ratio of total tracee from both artery and breakdown to fractional tracee from breakdown. After introduction of the variable $P$, the denominator of the equation calculates the change in the tracer MIF pool size divided by fractional tracee from breakdown, and the numerator is the change in the tracer MIF pool size divided by the tracer MIF pool size from $t_1$ to $t_2$. When the change in the tracer MIF pool size is canceled out, the equation becomes (fractional tracee from breakdown)/(tracee MIF pool size). However, the FSR calculates the rate of tracer incorporation into protein vs. protein-bound pool size. To be consistent with the unit of FSR, Eq. 1 has to be multiplied by the ratio of $Q_{M/T}$. Then Eq. 1 can be rearranged as (fractional tracee from breakdown)/$T$, which is exactly the definition of FBR.

This original method requires infusion of tracer to reach isotopic plateaus in the arterial blood and in the MIF pool and then observation of decay after stopping the tracer infusion. If we eliminate $P$ from Eq. 1, we remove the requirement of isotopic plateaus. The above equation holds for any two time points, so if we chose time points $t_2$ and $t_3$ rather than $t_1$ and $t_2$, then the equation becomes

$$ FBR = \frac{E_M(t_3) - E_M(t_2)}{P \int_{t_1}^{t_2} E_A(t) dt - (1 + P) \int_{t_1}^{t_2} E_M(t) dt} \cdot (Q_{M/T}) \quad (2) $$

Thus we have two equations and two unknowns, i.e., FBR and $P$. If we solve Eq. 2 for $P$ and substitute it into Eq. 1, we obtain the equation

$$ \frac{\int_{t_1}^{t_2} [E_M(t) - E_M(t_2)] dt \int_{t_1}^{t_2} [E_M(t) - E_M(t_1)] dt}{\int_{t_1}^{t_2} E_M(t) dt \int_{t_1}^{t_2} E_A(t) dt} \cdot (Q_{M/T}) $$

$$ - \frac{\int_{t_1}^{t_2} E_M(t) dt \int_{t_1}^{t_2} E_A(t) dt}{\int_{t_1}^{t_2} E_M(t) dt \int_{t_1}^{t_2} E_A(t) dt} $$

$$ + \frac{\int_{t_1}^{t_2} E_M(t) dt \int_{t_1}^{t_2} E_A(t) dt}{P} $$

$$ + \frac{\int_{t_1}^{t_2} E_M(t) dt \int_{t_1}^{t_2} E_A(t) dt}{(1 + P)} $$

$$ - \frac{\int_{t_1}^{t_2} E_M(t) dt \int_{t_1}^{t_2} E_A(t) dt}{Q_{M/T}} \quad (3) $$

Therefore, a measurement at isotopic plateau is not required if the arterial and intracellular enrichments are measured at three time points. The detailed proof in mathematics is addressed in the Appendix.

The FSR measures the rate of tracer incorporation from the MIF pool into the protein-bound amino acid pool. According to the precursor-product principle, the equation for FSR (23) is

$$ FSR = \frac{E_M(t_2) - E_M(t_1)}{P \int_{t_1}^{t_2} E_A(t) dt} \cdot (Q_{M/T}) \quad (4) $$
where $E_F$ and $E_B$ are the enrichments of free and bound amino acid, respectively. Because of the difficulty and impracticality of obtaining enrichment of the actual immediate precursor (i.e., aminoacyl-tRNA), the free amino acid pool is often used as an acceptable surrogate of the precursor for muscle protein synthesis (4). Thus the numerator calculates the increment of enrichment in the bound pool over time period $t$, and the denominator is the average enrichment in the free pool.

Equation 3 indicates that if we administer a pulse injection of an amino acid tracer that is not synthesized in the tissue and measure the enrichment decay in the arterial blood and tissue MIF pool at three time points and the ratio of the tracer amino acid in the free and bound pools, we can calculate FBR. Equation 4 indicates that if we measure the free and bound enrichment over the decay period, we can calculate muscle FSR.

**Experimental Procedures**

**Animal.** We used male New Zealand white rabbits (Myrtle's Rabbitry, Thompson Station, TN), each weighing ~4.5 kg. This study was approved by the Animal Care and Use Committee of The University of Texas Medical Branch at Galveston.

**Isotopes.** $L$-[ring-$^{13}$C$_6$]phenylalanine ($L$-[ring-$^{13}$C$_6$]Phe; 99% enriched) and $L$-[1-$^{13}$C]leucine ($L$-[1-$^{13}$C]Leu; 99% enriched) were purchased from Cambridge Isotope Laboratories (Woburn, MA). $L$-[ring-$^{2}$H$_5$]Phe (98% enriched) and $L$-[1,2-$^{13}$C$_2$]Leu (99.3% enriched) were purchased from Tracer Technologies (Somerville, MA). $L$-[15N]Phe (99% enriched) was purchased from Isotec (Miamisburg, OH).

**Design.** There were two groups of five rabbits each. Group 1 was used to establish the method for measuring FSR and FBR of muscle protein from a pulse injection of Phe tracer. The measured FSR and FBR values were compared with the corresponding values estimated from the three-pool model for validation. To assess whether the pulse tracer injection affected muscle protein kinetics, we also used the three-pool model to measure Phe and Leu kinetics in the leg before and after the Phe tracer injection.

Group 2 was used to test the hypothesis that if three stable isotopomers of Phe were injected at the same dose but at different times, muscle protein FSR and FBR could be measured from one muscle sample at 60 min after the first tracer injection. The rationale is as follows: $L$-[ring-$^{13}$C$_6$]Phe, $L$-[ring-$^{2}$H$_5$]Phe, and $L$-[15N]Phe are injected at 0, 30, and 55 min, respectively. Because the three Phe tracers have similar metabolic fate after being administered intravenously (13), at 60 min the free enrichments of $L$-[15N]Phe, $L$-[ring-$^{2}$H$_5$]Phe, and $L$-[ring-$^{13}$C$_6$]Phe in muscle represent 5, 30, and 60 min of decay, respectively. By the same rationale, muscle protein FBR and FSR can also be measured from two biopsies at 5 and 60 min or 30 and 60 min if two Phe tracers are injected at 0 and 30 min or at 0 and 55 min.

The anesthetic and surgical procedures were described in our previous publications (24, 27, 28). In brief, after an overnight fast with free access to water, the rabbits were anesthetized with ketamine and xylazine. Catheters were placed in the right jugular vein and left carotid artery. The venous line was used for infusion of anesthetics and saline and also for the primed-constant infusion of isotopes (group 1). The arterial line was for collection of arterial blood and monitoring of heart rate and mean arterial blood pressure. The femoral vein on the left leg was exposed via a groin incision, and a catheter was inserted into the left femoral vein for pulse injection of the Phe tracers (groups 1 and 2) and withdrawal of venous blood drawing from the leg (group 1). In group 1 the left femoral artery was exposed at the inguinal level, and a 1.5-RB flow probe (Transonic Systems, Ithaca, NY) was placed on the artery for measurement of blood flow rate on a small animal blood flowmeter (T106; Transonic Systems). A tracheal tube was placed via tracheotomy in both groups. The tracheal tube was placed in an open hood, which was connected to an oxygen line so that the rabbit inhaled oxygen-enriched room air.

The isotopic infusion protocols in the two groups are illustrated in Fig. 1. In group 1 (Fig. 1A), after collection of a blood sample and a muscle specimen from the left leg for measurement of background enrichment, a primed-constant infusion of $L$-[1-$^{13}$C]Leu (rate 0.35 mmol·kg$^{-1}$·min$^{-1}$; prime 21 mmol/kg) and $L$-[15N]Phe (rate 0.15 mmol·kg$^{-1}$·min$^{-1}$; prime 6 mmol/kg) was started. During the basal period of 180 min, five pairs of simultaneous arterial and femoral venous blood samples (0.25 ml each) were collected. At 180 min a muscle specimen was taken from the adductor muscle of the left leg, and $L$-[ring-$^{13}$C$_6$]Phe (4–6 mg/kg) in 3 ml of 0.45% saline was injected into the left femoral vein within 20 s and the line was flushed with 2 ml of saline. The selection of the dose for the pulse injection was based on our pilot studies, in which varying amounts of $L$-[ring-$^{13}$C$_6$]Phe ranging from 4–12 mg/kg were injected. We found that a dose of 4 mg/kg was minimal for measurement of a significant change in enrichment in the muscle protein-bound pool over a 60-min period. Over the following 90 min after the pulse tracer injection, another five pairs of simultaneous a-v blood were collected. The blood flow rate was recorded from the blood flowmeter at each a-v blood collection. Additional arterial blood (0.25 ml each) and muscle samples (~70 mg each from the adductor muscle of the right leg) were taken at 5, 10, 30, 60, and 90 min (see details in Fig. 1A).

In group 2 (Fig. 1B), the background blood and muscle samples were collected after completion of the surgical procedures. During the basal period of 180 min, only the anesthesia and saline were infused into the jugular vein to match the treatment of group 1. $L$-[ring-$^{13}$C$_6$]Phe (5.56 mg/kg), $L$-[ring-$^{2}$H$_5$]Phe (5.53 mg/kg), and $L$-[15N]Phe (5.40 mg/kg) in 3 ml of 0.45% saline were injected into the femoral venous catheter at time 0 and 30 and 55 min after the end of the basal period, respectively. The three doses of Phe tracer were each 32.5 mmol/kg. Frequent arterial blood samples (0.25 ml each; see Fig. 1B) were taken over a 115-min time period to measure the enrichment decay in arterial blood of each tracer for 60 min. Adductor muscle samples from the right leg were taken at 5, 30, and 60 min (~70 mg for 5- and 30-min samples and ~200 mg for 60-min sample). The three muscle biopsies allowed us to measure enrichment decay in the MIF pool from $L$-[ring-$^{13}$C$_6$]Phe at 5, 30, and 60 min; from $L$-[ring-$^{13}$C$_6$]Phe and $L$-[ring-$^{2}$H$_5$]Phe at 5 and 60 min; from $L$-[ring-$^{13}$C$_6$]Phe and $L$-[15N]Phe at 30 and 60 min; and from $L$-[ring-$^{13}$C$_6$]Phe, $L$-[ring-$^{13}$C$_6$]Phe, and $L$-[15N]Phe using one biopsy at 60 min. Therefore, we were able to calculate muscle FBR and FSR from either one or two muscle biopsies as well as from three muscle biopsies. At the end of the sampling for kinetic measurements, additional blood was taken from the arterial line to measure blood gas and hematocrit.

After collection, the muscle samples were either gently blotted or quickly washed in ice-cold saline to remove visible blood. The 60-min muscle sample in group 2 was processed...
Fig. 1. Experimental protocols in group 1 (A) and group 2 (B); X indicates sampling of blood or muscle. In group 1, during the 180 min of the basal period, 5 pairs of arterial (A) and femoral venous (V) blood samples were collected at 120, 135, 150, 165, and 180 min. After a muscle sample was taken from the adductor muscle of the left leg, the pulse tracer injection was performed. Over the following 90 min, another 5 pairs of arterial and venous blood samples were collected at 10, 20, 30, 60, and 90 min after the pulse tracer injection. Additional arterial blood and muscle samples were taken at 5, 10, 30, 60, and 90 min. In group 2, only the 120 min of the injection period are illustrated, and the 3-h basal period without isotope infusion is not included. Thus 0 min in group 2 is equal to 180 min in group 1. L-[ring-2H5]phenylalanine (L-[ring-2H5]Phe), L-[ring-2H5]phenylalanine (L-[ring-2H5]Phe), and L-[15N]phenylalanine (L-[15N]Phe) were injected at 0, 30, and 55 min of the injection period. Arterial blood was taken at 5, 10, 15, 30, 35, 40, 45, 60, 65, 70, 85, and 115 min after the injection of L-[ring-13C6]Phe. Muscle samples were taken at 5, 30, and 60 min.

A

B

differently because from this single muscle biopsy the following information was obtained: enrichments of three Phe tracers, content of unlabelled Phe, and percentage of dry protein in the sample. To conveniently accomplish these analyses, we took a relatively large muscle sample (~200 mg) and cut the muscle into two pieces. One piece was washed in ice-cold saline for measurement of Phe enrichment; the other piece was gently blotted only, for measurement of free Phe content and percent protein in muscle. This was because washing in saline may change free amino acid content and water content in the muscle. The muscle samples were immediately frozen in liquid nitrogen and stored at −80°C for later processing. The muscle samples were processed immediately after collection as described in Sample analysis.

Heart rate, mean arterial blood pressure, and rectal temperature were maintained stable by adjustment of the doses of anesthetics, saline, and heating lamps. These vital signs were monitored through the experiments and recorded every 30 min.

Sample analysis. After collection, the blood samples were transferred to tubes with 1 ml of 7.5% sulfosalicylic acid for deproteinization. In group 1, 50 μmol of the internal standard solution, which contained L-[1-13C2]Leu (70 μmol/l) and L-[ring-2H5]Phe (30 μmol/l), were added to each tube for calculation of Phe and Leu concentrations in the blood. The exact amounts of the internal standard solution and blood were obtained by weighing on a scale. After centrifugation, the supernatant was processed to make the t-butyldimethylsilyl (TBDMS) derivatives of amino acids (16).

To measure both free Phe enrichment and content in the muscle, a tissue internal standard solution, which contained L-[ring-2H5]Phe at 6 μmol/l, was added to ~30 mg of muscle (1 μl for each mg of tissue). After homogenization in 10% perchloric acid, three times at 4°C, the pooled supernatant was processed for the TMDMS derivatives (16). The 60-min muscle samples in group 2 were processed as follows. The piece of muscle that had been gently blotted, but not washed, was cut into two aliquots (~30 mg each): one was processed without the internal standard solution for measurement of L-[ring-2H5]Phe enrichment from injected L-[ring-2H5]Phe; the other was added with the tissue internal standard solution (1 μl for each mg of tissue). The enrichment difference of L-[ring-2H5]Phe was used to calculate the content of unlabeled Phe in muscle.

The protein precipitates were washed thoroughly to remove free amino acids and fat and were dried in an oven at 80°C overnight to obtain the dry protein pellets (25). The percentage of dry protein in the muscle was calculated from the wet and dry weights. The dry protein pellets were hydrolyzed and then processed for the N-acetyl, N-propyl ester (NAP) derivatives of amino acids (23).
The isotopic enrichments in the blood and muscle supernatant were determined on a Hewlett-Packard 5988B gas chromatograph-mass spectrometer (GC-MS); ions were selectively monitored at mass-to-charge (m/z) ratios of 234, 235, 239, and 240 for Phe enrichment and at m/z ratios of 302, 303, and 304 for Leu enrichment. Isotopic enrichments were expressed as molar percent excess for the three-pool model and for the tracer incorporation method and as tracer-to-tracer ratio for the enrichment decay in the arterial blood and MIF pool and for the internal standard method. The enrichments were corrected for the contribution of the abundance of isotopomers of lower weight to the apparent enrichment of isotopomers with larger weight. A skew correction factor was also used to calculate l-[ring-13C6]Phe enrichment in the blood and muscle supernatant (21). l-[ring-13C6]Phe enrichment in the muscle protein hydrolysate was measured on a gas chromatograph-combustion-isotope ratio mass spectrometer (GC-C-IRMS; Finnigan, MAT, Bremen, Germany). The measured 13CO2 enrichment was converted to Phe enrichment by multiplying by 14/6 to account for the dilution of six labeled carbons with the total 14 carbons in the derivatized Phe.

Calculations. FBR was calculated by Eq. 3. In this equation, Qa/T is the ratio of free to bound Phe in muscle. In practice, we measured the amount (in μmol) of free Phe in the piece of muscle that was homogenized and normalized to micromoles of free Phe per gram of muscle. In our previous study (26), we reported that 1 g of dry muscle protein contains 250 μmol Phe. The content of protein-bound Phe in 1 g of muscle was therefore calculated by [(250 μmol/g) × (% dry protein in muscle)]. Because both the free and bound Phe contents were expressed in micromoles per gram of muscle, Qa/T is equal to free Phe content divided by bound Phe content. FSR was calculated from Eq. 4, in which the free enrichment in muscle was used as a surrogate of precursor enrichment.

Leu and Phe kinetics in the leg were calculated from a three-compartment model that we published previously (5). The following are equations for calculating the rate of disappearance (Rd), rate of appearance (Ra), and net balance (NB):

\[ R_d = [(E_a \times C_a) - (E_v \times C_v)]/E_m \times BF \]  

\[ NB = (C_a - C_v) \times BF \]  

\[ R_a = R_d - NB \]  

Here Eₐ, Eᵥ, and Eₘ are enrichment in the arterial blood, venous blood, and MIF pool, respectively; Cₐ and Cᵥ are concentration in the arterial blood and venous blood, respectively; and BF is blood flow rate in the femoral artery. Because Phe is neither synthesized nor degraded in the limb, the Rₐ represents protein synthesis and the Rₐ represents protein breakdown. Because Leu is not synthesized in muscle, its endogenous Rₐ comes exclusively from breakdown.

However, because Leu can be oxidized in muscle, its Rₐ is the sum of incorporation into protein (synthesis) and oxidation. To convert Phe Rₐ and Ra calculated from the three-pool model to FSR and FBR, we need to know the muscle mass in the hindlimb and the amount of Phe in leg muscle protein, which are calculated by the following equations:

\[ \text{leg muscle mass} = (\text{BF in ml} \cdot \text{leg}^{-1} \cdot \text{min}^{-1})/(0.0783 \text{ ml} \cdot \text{g}^{-1} \cdot \text{min}^{-1}) \]  

\[ \text{Phe in leg muscle protein} = \text{leg muscle mass} \times (\% \text{protein}) \times (250 \mu\text{mol/g}) \]  

Here BF is the blood flow rate in the femoral artery. The value of 0.0783 ± 0.0385 ml·g⁻¹·min⁻¹ is that 1 g of leg adductor muscle receives 0.0783 ml of blood per min, which we measured in five rabbits using the microsphere technique (unpublished data). In Eq. 9 the assumed value that 1 g of muscle protein contains 250 μmol Phe was calculated from the internal standard method in our previous experiment (26); the percent protein was calculated from the wet and dry weight of muscle samples. Knowing the Phe amount in leg muscle protein, we converted Phe Ra and Rₐ to FSR and FBR.

FSRc = Rₐ/Phe in leg muscle protein  

FBRc = Rₐ/Phe in leg muscle protein

Here, FSRc and FBRc are converted from Rₐ and Ra of Phe, which are distinguished from FSR and FBR measured from the pulse tracer injection.

Statistical analysis. Data are expressed as means ± SD. Differences between two groups or between FSR and FBR were evaluated using the Student’s t-test. P < 0.05 was considered statistically significant.

RESULTS

The general characteristics of the rabbits in the two groups are presented in Table 1. In group 1, after 2 h of primed-constant infusion, the enrichments of l-[12N]Phe and l-[13C]Leu in the arterial blood reached plateaus (Fig. 2A). The arterial Phe concentration of unlabeled Phe plus l-[15N]Phe was constant during the 120–270 min, although the pulse tracer injection caused the concentration of l-[ring-13C₆]Phe to transiently increase and fall (Fig. 2B). The isotope enrichments in the arterial blood, femoral venous blood, and muscle free pool and the concentrations of Phe and Leu in the arterial and femoral venous blood are presented in Table 2. These data, along with the blood flow rates in

**Table 1. General characteristics**

<table>
<thead>
<tr>
<th>Group</th>
<th>Body Wt, kg</th>
<th>Rectal Temp, °C</th>
<th>Heart Rate, beats/min</th>
<th>MAP, mmHg</th>
<th>Hct, %</th>
<th>Dose of Tracer, mg/kg</th>
<th>BF, ml·leg⁻¹·min⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>Basal</td>
<td>Injection</td>
</tr>
<tr>
<td>Group 1</td>
<td>4.3 ± 0.1</td>
<td>39.0 ± 0.3</td>
<td>168 ± 14</td>
<td>84 ± 3</td>
<td>33.9 ± 0.6</td>
<td>5.6 ± 0.9</td>
<td>12.2 ± 1.6</td>
</tr>
<tr>
<td>Group 2</td>
<td>4.5 ± 0.1</td>
<td>38.9 ± 0.3</td>
<td>169 ± 7</td>
<td>81 ± 4</td>
<td>33.7 ± 4.0</td>
<td>5.6 ± 0.3</td>
<td>Not measured</td>
</tr>
</tbody>
</table>

Values are means ± SD. Data for rectal temperature (rectal temp), heart rate, and mean arterial blood pressure (MAP) are averages of 8 measurements during the experiments. The blood flow rates (BF) in the leg are averages of 5 measurements before and after the pulse tracer injection. The values of hematocrit (Hct) are measured at the end of infusion. *P = 0.03 vs. basal.
Fig. 2. A: arterial enrichments of L-[15N]Phe and L-[1-13C]leucine (Leu) reached isotopic plateaus from 120 to 270 min of the arterial-venous (a-v) sampling period. This indicates that the pulse injection of L-[ring-13C6]Phe did not have a significant effect on the isotopic steady state of L-[15N]Phe or L-[1-13C]Leu. B: the concentration of unlabeled Phe plus L-[15N]Phe in the arterial blood was constant from 120 to 270 min of a-v sampling period. The change in concentration of total Phe (i.e., sum of unlabeled plus 15N- or 13C6-labeled Phe) was due to the pulse injection of L-[ring-13C6]Phe.

the femoral artery (see Table 1), were used to calculate Leu and Phe kinetics using Eqs. 5–7. Because the calculated Phe kinetics during the injection period did not include L-[ring-13C6]Phe, the contribution of L-[ring-13C6]Phe could be estimated. After the pulse injection, L-[ring-13C6]Phe could incorporate into protein at a rate proportionate to its enrichment in the MIF pool. Thus Phe Rd was the sum of Ra from the three-pool model and Rd = C × 8.4 ± 2.5%, where 8.4 ± 2.5% was the average L-[ring-13C6]Phe enrichment in the MIF pool over the 90-min time period. Because the pulse injection should not affect Phe Ra calculated from the three-pool model, Phe Ra did not need correction for L-[ring-13C6]Phe kinetics. The values of Rd, Ra, and net balance of Phe and Leu before and after the pulse tracer injection are presented in Table 3.

To calculate FBR, we measured the enrichment of L-[ring-13C6]Phe in the arterial blood and in the MIF pool as well as the ratio of free Phe to protein-bound Phe (i.e., QM/T in Eq. 4) in muscle. These data from group 1 are presented in Table 4. The FBR values calculated from five combinations of three sampling times, namely 5, 30, and 60 min, 10, 30, and 60 min, 5, 30, and 90 min, 10, 30, and 90 min, and 30, 60, and 90 min, are presented in Table 5. To assess whether the variability of the FBR values from the five measurements was due to an interindividual or intermeasurement variation, we compared the standard deviations between rabbits and within rabbits. The standard deviation within rabbits calculated from the five measurements (0.0279 ± 0.0136) was significantly (P < 0.05) smaller than that of measurements between the five rabbits (0.0498 ± 0.0074), indicating that the measurements of the FBR value were consistent within rabbits. We selected the 5, 30, and 60-min data as a representative measurement.

In group 2, the arterial enrichments of the three Phe tracers followed the same decay pattern (Fig. 3A), confirming that they have a similar metabolic fate in the body (13). The decay curve of L-[ring-13C6]Phe in

Table 3. Leu and Phe kinetics in leg in group 1

<table>
<thead>
<tr>
<th></th>
<th>Ra</th>
<th>R4</th>
<th>Net Balance</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Leucine tracer</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal period</td>
<td>71.1 ± 12.7</td>
<td>68.3 ± 12.0</td>
<td>4.8 ± 2.4</td>
</tr>
<tr>
<td>Injection period</td>
<td>74.4 ± 16.7</td>
<td>71.4 ± 16.6</td>
<td>3.2 ± 2.6</td>
</tr>
<tr>
<td><strong>Phenylalanine tracer</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal period</td>
<td>21.3 ± 7.0</td>
<td>26.2 ± 5.9</td>
<td>-4.9 ± 1.5</td>
</tr>
<tr>
<td>Injection period</td>
<td>20.2 ± 7.2</td>
<td>23.8 ± 6.1</td>
<td>-3.6 ± 1.5</td>
</tr>
</tbody>
</table>

Data are means ± SD in μmol·kg·h⁻¹, measured by the primed-constant infusion of L-[15N]Phe and L-[1-13C]Leu and the 3-pool model. Ra, rate of disposal; Rd, rate of appearance. The Rd of Phe in the injection period has been corrected to include the contribution from L-[ring-13C6]Phe.

Table 2. Enrichments and concentrations of Phe and Leu in group 1

<table>
<thead>
<tr>
<th></th>
<th>EA</th>
<th>EV</th>
<th>EM</th>
<th>CA</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Basal period</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phe</td>
<td>0.1058 ± 0.0116</td>
<td>0.0789 ± 0.0080</td>
<td>0.0432 ± 0.0074</td>
<td>0.0675 ± 0.0135</td>
<td>0.0742 ± 0.0140</td>
</tr>
<tr>
<td>Leu</td>
<td>0.1144 ± 0.0115</td>
<td>0.0926 ± 0.0069</td>
<td>0.0527 ± 0.0061</td>
<td>0.2312 ± 0.0396</td>
<td>0.2227 ± 0.0391</td>
</tr>
<tr>
<td><strong>Injection period</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phe</td>
<td>0.1077 ± 0.0104</td>
<td>0.0791 ± 0.0074</td>
<td>0.0452 ± 0.0053</td>
<td>0.0702 ± 0.0128</td>
<td>0.0790 ± 0.0118</td>
</tr>
<tr>
<td>Leu</td>
<td>0.1250 ± 0.0106</td>
<td>0.0970 ± 0.0065</td>
<td>0.0518 ± 0.0067</td>
<td>0.1971 ± 0.0273</td>
<td>0.1940 ± 0.0273</td>
</tr>
</tbody>
</table>

Data are means ± SD. EA, EV, and EM are enrichments (in mole % excess) in the arterial blood, femoral venous blood, and muscle free pool, respectively. Phenylalanine (Phe) enrichment refers to L-[15N]Phe from the primed-constant infusion. Ca and CV are concentrations (in μmol/ml) in the arterial and femoral venous blood, respectively. Phe concentration includes unlabeled Phe and L-[15N]Phe. The values are averages of 5 measurements. The contribution of injected L-[ring-13C6]Phe to the leg Phe kinetics is estimated separately (see RESULTS in text). Leu, leucine.
MUSCLE PROTEIN SYNTHESIS AND BREAKDOWN

Table 4. Phe enrichment and content in the muscle in group 1

<table>
<thead>
<tr>
<th></th>
<th>Arterial blood</th>
<th>MIF pool</th>
<th>Content, μmol/g wet muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Enrichment</td>
<td></td>
<td>Free pool</td>
</tr>
<tr>
<td>5 min</td>
<td>0.6855 ± 0.1240</td>
<td>0.1943 ± 0.0882</td>
<td>0.1047 ± 0.0319</td>
</tr>
<tr>
<td>10 min</td>
<td>0.3299 ± 0.0672</td>
<td>0.1719 ± 0.0384</td>
<td>0.1094 ± 0.0263</td>
</tr>
<tr>
<td>30 min</td>
<td>0.1349 ± 0.0305</td>
<td>0.1271 ± 0.0372</td>
<td>0.1106 ± 0.0157</td>
</tr>
<tr>
<td>60 min</td>
<td>0.0723 ± 0.0198</td>
<td>0.0755 ± 0.0233</td>
<td>0.1191 ± 0.0122</td>
</tr>
<tr>
<td>90 min</td>
<td>0.0430 ± 0.0116</td>
<td>0.0458 ± 0.0136</td>
<td>0.1238 ± 0.0202</td>
</tr>
</tbody>
</table>

Data are means ± SD. Isotope enrichment is expressed as tracer-to-tracee ratio (TTR). Phe contents in the free and bound pools are unlabeled Phe. The content of free Phe was measured by the internal standard and normalized to μmol/g wet muscle. The content of protein-bound Phe was calculated from 250 μmol/g × % of dry protein in muscle.

The MIF pool was either measured from the L-[ring-13C6]Phe tracer using the three muscle samples (5, 30, and 60 min) or taken from L-[15N]Phe and/or L-[ring-2H3]Phe enrichments using two or one muscle biopsy (Fig. 3B). For example, when the 60-min muscle sample was used for FBR, the enrichment decay in the MIF pool at 5 min was taken from L-[15N]Phe, which was injected at 55 min (i.e., 5 min before the 60-min biopsy); the enrichment decay at 30 min was taken from L-[ring-2H3]Phe, which was injected at 30 min (i.e., 30 min before the 60-min biopsy); and the enrichment decay at 60 min was measured directly from L-[ring-13C6]Phe tracer, which was injected at 0 min (i.e., 60 min before the 60-min biopsy). The same rationale was used to determine the decay curves when two muscle biopsies were taken at 5 and 60 min or at 30 and 60 min. The dry protein in muscle was 21.7 ± 0.6%, the free and bound Phe contents in muscle were 0.1059 ± 0.052 and 54.27 ± 1.60 μmol/g, respectively, and the QsoT ratio was 526 ± 96. The calculated FBR values with three muscle samples (5, 30, and 60 min), two muscle samples (5 and 60 min or 30 and 60 min), and one muscle sample (60 min) were almost identical (P > 0.05; Fig. 4).

The values of muscle protein FSR, calculated from the pulse injection of L-[ring-13C6]Phe, are presented in Table 6. In group 1, the FSR was calculated from 5 to 60 min after the Phe tracer injection; in group 2, the FSR was calculated from 0 to 60 min. Because in group 2 we measured the FSR value from a 60-min muscle sample, we used the blood background sample to estimate the background enrichment of L-[ring-13C6]Phe in the muscle. The FSR values were not significantly different between the groups (P > 0.05). For each rabbit, the value of FBR was consistently greater than the value of FSR, indicating a negative balance of muscle protein.

To evaluate the validity of the FSR and FBR values from the pulse tracer injection, we converted the rates of synthesis and breakdown from the three-pool model to FSR and FBR using Eqs. 8–11. The total amount of Phe in leg muscle was 11,330 ± 1,656 μmol. The values of FSR and FBR were 0.185 ± 0.086%/h and 0.217 ± 0.078%/h, respectively, which were not significantly (P = 0.14 and 0.35 by one-tail paired t-test) different from the values of FSR (0.125 ± 0.036%/h) and FBR (0.233 ± 0.060%/h) measured from the pulse injection method.

**DISCUSSION**

Our goal was to develop a stable isotope method to determine muscle protein FSR and FBR within the shortest time period and using the least number of muscle biopsies. The results demonstrate that by the pulse Phe tracer injection and measurement of enrichment decay at three time points, muscle protein FSR and FBR can be determined over a 60-min time frame. Furthermore, when two or three Phe tracers were used for multiple pulse injections, the number of muscle biopsies could be reduced to two or even one. Thus the pulse tracer injection method satisfactorily achieves our goal.

To evaluate the validity of the pulse injection method, we converted the Rα and Rα of Phe, measured from the three-compartment leg model, to FSR and

Table 5. FBR of muscle protein in group 1

<table>
<thead>
<tr>
<th>Sampling Times</th>
<th>Rabbit 1</th>
<th>Rabbit 2</th>
<th>Rabbit 3</th>
<th>Rabbit 4</th>
<th>Rabbit 5</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-30-60</td>
<td>0.216</td>
<td>0.323</td>
<td>0.230</td>
<td>0.156</td>
<td>0.239</td>
<td>0.233 ± 0.060</td>
</tr>
<tr>
<td>10-30-60</td>
<td>0.225</td>
<td>0.248</td>
<td>0.284</td>
<td>0.154</td>
<td>0.250</td>
<td>0.232 ± 0.049</td>
</tr>
<tr>
<td>5-60-90</td>
<td>0.243</td>
<td>0.292</td>
<td>0.160</td>
<td>0.198</td>
<td>0.260</td>
<td>0.230 ± 0.052</td>
</tr>
<tr>
<td>10-60-90</td>
<td>0.247</td>
<td>0.240</td>
<td>0.189</td>
<td>0.191</td>
<td>0.281</td>
<td>0.230 ± 0.039</td>
</tr>
<tr>
<td>30-60-90</td>
<td>0.223</td>
<td>0.251</td>
<td>0.195</td>
<td>0.170</td>
<td>0.296</td>
<td>0.230 ± 0.049</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>0.231 ± 0.013</td>
<td>0.271 ± 0.036</td>
<td>0.212 ± 0.048</td>
<td>0.174 ± 0.020</td>
<td>0.265 ± 0.023</td>
<td></td>
</tr>
</tbody>
</table>

Sampling was at 5, 30, and 60 min (5-30-60); 10, 30, and 60 min (10-30-60); 5, 60, and 90 min (5-60-90); 10, 60, and 90 min (10-60-90); and 30, 60, and 90 min (30-60-90). FBR, fractional breakdown rate.
FBRc. The FBR values from these two independent methods were almost identical (0.233 ± 0.060%/h vs. 0.217 ± 0.078%/h; P = 0.35). The FSR value from the pulse injection method (0.125 ± 0.036%/h) tended to be lower than that converted from Phe Ra (0.185 ± 0.086%/h; P = 0.14). This is likely because the pulse injection method directly measures the protein metabolism in the adductor muscle, whereas the three-pool model measures protein metabolism in the total leg, including not only muscle but also skin, bone, and so forth. Thus the three-pool model may overestimate muscle protein synthesis compared with breakdown. In humans or dogs, nonmuscle tissue (mainly skin) is estimated to be 10–15% of total leg protein kinetics (6, 7). However, in some species the contribution may increase. Preedy and Garlick (18) reported that the rat hemicorpus contains 39% by weight of nonmuscle tissue. Because skin has a faster protein synthesis rate than muscle, the metabolic contribution by the nonmuscle tissue could be a considerable portion of the measured protein kinetics in the hemicorpus (18, 19). In our previous experiment (24), we estimated that the limb skin accounted for 26–42% of the total limb tracer uptake in the rabbit. Using the primed-constant infusion method, we reported that the FSR of ear skin was 0.30%/h (28). Because the skin is able to maintain its protein mass in the postabsorptive state (24), its FSR equals its FBR. If the leg skin has a similar protein turnover rate to that of the ear skin, the FBR in leg skin (~0.30%/h) should be close to the FBR in leg muscle (0.23%/h; see Table 5). Therefore, the inclusion of skin protein breakdown should have little import on the FBRc values converted from the three-pool model; hence, the FBR and FBRc values measured from these two independent methods were close. In contrast, because the synthesis rate of skin protein is greater than that of muscle protein, the inclusion of skin protein synthesis could cause overestimation of the FSRc value converted from the three-pool model. Thus we would expect the FSR from muscle tissue to be slightly lower than synthesis calculated from the balance method.

The pulse Phe tracer injection did not affect protein metabolism in the muscle, which was supported by the almost identical values of Leu and Phe kinetics in the muscle intracellular free pool (see Fig. 1A). This was most likely due to the frequent blood sampling during the injection period (see Fig. 1A), although the volume of blood for each sample was minimized to 0.25 ml. The hematocrit value (33.9 ±

![Fig. 3. A: enrichment decay curves of L-[ring-13C6]Phe, L-[ring-2H3]Phe, and L-[15N]Phe in the arterial blood after the pulse injection of these 3 tracers. The comparable decay curves indicate that these 3 Phe tracers have the same metabolic fate after intravenous injection. B: the L-[ring-13C6]Phe enrichment decay in the muscle intracellular free pool is expressed in 4 ways. The curve of 3 biopsies represents values of L-[ring-13C6]Phe enrichment at 5-, 30-, and 60-min muscle samples; the curve of 2 biopsies A represents values from L-[ring-13C6]Phe enrichment at 5 and 60 min and L-[ring-2H3]Phe at 30 min; the curve of 2 biopsies B represents values from L-[ring-13C6]Phe enrichment at 30 and 60 min and from L-[15N]Phe at 5 min; and the curve of 1 biopsy represents L-[ring-13C6]Phe enrichment at 60 min; and the values at 5 and 30 min were the enrichments of L-[15N]Phe and L-[ring-13C6]Phe, respectively.

![Fig. 4. The value of muscle protein fractional breakdown rate (FBR) in group 2 calculated from 3 muscle biopsies at 5, 30, and 60 min was almost identical (P > 0.05) with values from 2 muscle biopsies at 5 and 60 min or at 30 and 60 min and from 1 muscle biopsy at 60 min.](image-url)
Table 6. Muscle protein FSR

<table>
<thead>
<tr>
<th>Free IE</th>
<th>Bound IE</th>
<th>Time, min</th>
<th>FSR, %/h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>0.0979 ± 0.0298</td>
<td>0.0001113 ± 0.0000488</td>
<td>55</td>
</tr>
<tr>
<td>Group 2</td>
<td>0.1045 ± 0.0143</td>
<td>0.0001181 ± 0.0000263</td>
<td>60</td>
</tr>
</tbody>
</table>

Enrichment is expressed as mole % excess. FSR, fractional synthesis rate; Free IE, L-[ring-13C6]Phe enrichment in the muscle free pool, calculated from the area under the decay curve of 5, 30, and 60 min; bound IE, the increase in L-[ring-13C6]Phe enrichment in the protein-bound pool.

0.6%; see Table 1) measured at the end of the experiments indicated that the volume of blood withdrawn was acceptable. In both human and rabbit experiments in our laboratory, we found that a 20% reduction of leg blood flow rate by mechanical clamping of the femoral artery had no detectable effects on the rates of protein synthesis and breakdown in leg muscle measured by both the leg a-v model and the tracer incorporation method (unpublished data). Thus a 13% reduction of leg flow rate should not have a significant impact on muscle protein kinetics in the leg.

The pulse tracer injection method has certain advantages over the currently used primed-constant infusion or flooding dose method to measure muscle protein FSR. Because the enrichment in the muscle free pool is a good approximation of the true precursor (4, 20), the primed-constant infusion method commonly has been selected for measurement of muscle FSR. However, this method may require several hours of stable isotope tracer use to achieve sufficient enrichment in the product for accurate measurement. Even after a successful prime, it usually takes 1 h or more to reach an isotopic plateau in intracellular enrichment, and at least 2–3 h more may be required to increase the enrichment in the protein-bound pool to the level that can be accurately determined. Thus the minimal time to complete the measurement in muscle may be 3–4 h. Depending on the experiment, it may be difficult to maintain a physiological steady state over that time period, particularly in small animals. This is one reason that the flooding dose method has been an option of methods in small animals. However, the use of the flooding dose method is subject to the risk of stimulating muscle protein synthesis because of the large amount of amino acid injected (20), and this large bolus may obscure the treatment effect, particularly if the treatment involves amino acids (14). The pulse injection method combines the advantages of the primed-constant infusion (no effect on synthesis) and flooding dose (relatively short time interval) methods while limiting the disadvantages of the other methods.

The pulse tracer injection method has advantages over the original FBR technique (25). Most importantly, the pulse tracer method does not require an isotope plateau and therefore can be performed without tracer infusion. In addition, by use of two or three Phe tracers, the pulse tracer method can be used with only two or even one biopsy. The use of multiple stable isotopomers of an amino acid to reduce the number of tissue biopsies is based on the fact that these isotopomers have similar metabolic fates (13). This concept has been used previously. For example, Dudley et al. (8) reported a method of staged infusion of six stable isotopomers (2 isotopomers of Leu and 4 isotopomers of Phe) to measure the FSR of lactase phlorizin hydrolase from frequent blood samples and one tissue (intestinal mucosa) biopsy in pigs. FSR values measured from the multiple-tracer, single-sample approach compared well with values measured from the conventional isotope infusion approach with tissue samples collected at timed intervals during the infusion (8). In the present experiment, the similar curves of Phe enrichments in the arterial blood and muscle free amino acid pool (Fig. 3, A and B) support the validity of the timed pulse injections of the Phe tracers as an alternative to multiple muscle biopsies.

We have expressed the enrichment as mole percent excess for calculation of FSR and tracer-to-tracer ratio for FBR. The protein breakdown releases unlabeled Phe into the MIF pool. Thus the enrichment decay was expressed as tracer-to-tracer ratio, which reflects dilution of tracer in the MIF pool by the unlabeled Phe released from proteolysis. For the same reason, the value of $Q_{MT}$, the ratio of free vs. bound Phe in muscle, was also referred to as unlabeled Phe. In contrast, the movement of amino acids from the MIF pool to protein-bound pool included both labeled and unlabeled Phe. Accordingly, the enrichment values for FSR calculation were expressed as mole percent excess, which accounts for not only $L$-[ring-13C6]Phe but also $L$-[15N]Phe in group 1 and all three Phe tracers injected in group 2. To be consistent with the FSR calculation, the enrichment used for the three-pool model was also expressed as mole percent excess, which was derived from $L$-[15N]Phe infusion.

In summary, the pulse tracer injection method is satisfactory in measurement of muscle protein FSR and FBR. This method requires only 1 h and one or two muscle biopsies for both FSR and FBR. The dose of tracer does not stimulate muscle protein synthesis. Thus this new method has advantages over both the currently used primed-constant infusion and the flooding dose methods. The principle of this method is applicable to tissues other than muscle.
APPENDIX

We have previously presented a method of calculating protein FBR in muscle. The original method required the infusion of a labeled amino acid and the measurement of arterial amino acid enrichment and intracellular free amino acid enrichment during isotopic steady state and during isotopic nonsteady state. The formula used to calculate FBR is

\[
FBR = \frac{E_M(t_2) - E_M(t_1)}{P \int_{t_1}^{t_2} E_A(t) \, dt - (1 + P) \int_{t_1}^{t_2} E_M(t) \, dt} \cdot (Q_{M/T}) \tag{A1}
\]

where \(E_M(t)\) is the intracellular free enrichment at time \(t\), \(E_A(t)\) is the arterial enrichment at time \(t\), \(Q_{M/T}\) is the ratio of intracellular free content to protein bound content in muscle, and \(P = E_M/(E_A - E_M)\), where \(E_M\) and \(E_A\) are the intracellular and arterial enrichments, respectively, at isotopic steady state.

If we eliminate \(P\) from the above equation, we can remove the requirement of obtaining steady-state measurements. The above equation holds for any two time points, so if we choose time points \(t_2\) and \(t_3\) rather than \(t_1\) and \(t_2\), then the equation becomes

\[
FBR = \frac{E_M(t_3) - E_M(t_2)}{P \int_{t_2}^{t_3} E_A(t) \, dt - (1 + P) \int_{t_2}^{t_3} E_M(t) \, dt} \cdot (Q_{M/T}) \tag{A2}
\]

Thus we have two equations and two unknowns, i.e., \(FBR\) and \(P\). If we solve Eq. A2 for \(P\) and substitute it into Eq. A1, we obtain the equation

\[
FBR = \frac{[E_M(t_2) - E_M(t_1)] \cdot \int_{t_1}^{t_2} [E_A(t) - E_M(t)] \, dt - [E_M(t_3) - E_M(t_2)] \cdot \left[ \int_{t_1}^{t_2} E_A(t) \, dt - \int_{t_1}^{t_3} E_M(t) \, dt \right]}{\int_{t_2}^{t_3} E_M(t) \, dt \cdot \int_{t_1}^{t_2} E_A(t) \, dt - \int_{t_1}^{t_2} E_M(t) \, dt \cdot \int_{t_2}^{t_3} E_A(t) \, dt} \cdot (Q_{M/T}) \tag{A3}
\]

Therefore, a measurement at isotopic steady state is not required if the arterial and intracellular enrichments are measured at three time points.

Note that if we define

\[
P_1 = \frac{\int_{t_1}^{t_2} E_M(t) \, dt}{\int_{t_1}^{t_2} E_A(t) \, dt - \int_{t_1}^{t_2} E_M(t) \, dt}
\]

and

\[
P_2 = \frac{\int_{t_1}^{t_2} E_M(t) \, dt}{\int_{t_2}^{t_3} E_A(t) \, dt - \int_{t_2}^{t_3} E_M(t) \, dt}
\]

then Eq. A2 can be written

\[
FBR = \frac{E_M(t_3) - E_M(t_2)}{p_2 \cdot \int_{t_2}^{t_3} E_A(t) \, dt - (1 + p_2) \cdot \int_{t_1}^{t_2} E_M(t) \, dt + p_1 \cdot \int_{t_1}^{t_2} E_A(t) \, dt - (1 + p_1) \cdot \int_{t_1}^{t_2} E_M(t) \, dt} \cdot (Q_{M/T})
\]

Proof

If we rearrange the denominator in Eq. A2, we get

\[
FBR = \frac{E_M(t_3) - E_M(t_2)}{P \int_{t_2}^{t_3} [E_A(t) - E_M(t)] \, dt - \int_{t_2}^{t_3} E_M(t) \, dt}
\]

Rearranging Eq. A4 further yields

\[
P \int_{t_2}^{t_3} [E_A(t) - E_M(t)] \, dt - \int_{t_2}^{t_3} E_M(t) \, dt = \frac{E_M(t_2) - E_M(t_3)}{FBR} \cdot (Q_{M/T}) \tag{A5}
\]

so

\[
P \int_{t_2}^{t_3} [E_A(t) - E_M(t)] \, dt = \int_{t_2}^{t_3} E_M(t) \, dt + \frac{E_M(t_2) - E_M(t_3)}{FBR} \cdot (Q_{M/T}) \tag{A6}
\]

and

\[
P = \frac{\int_{t_2}^{t_3} E_M(t) \, dt + \frac{E_M(t_2) - E_M(t_3)}{FBR} \cdot (Q_{M/T})}{\int_{t_2}^{t_3} [E_A(t) - E_M(t)] \, dt} \tag{A7}
\]
If we substitute Eq. A7 into Eq. A1, then

\[
FBR = \frac{E_{3d}(t_2) - E_{3d}(t_1)}{\int_{t_2}^{t_1} E_{3d}(t) \ dt + \frac{E_{3d}(t_2) - E_{3d}(t_1)}{\text{FBR}} \cdot (Q_{3d}/T) \cdot \left[ \int_{t_1}^{t_2} E_{3}(t) - E_{3d}(t) \ dt \right] - \int_{t_1}^{t_2} E_{3d}(t) \ dt}
\]

so

\[
FBR \cdot \left[ \int_{t_2}^{t_1} E_{3}(t) dt \right] \cdot \left[ \int_{t_1}^{t_2} E_{3}(t) dt \right] = \left[ E_{3d}(t_2) - E_{3d}(t_1) \right] \cdot (Q_{3d}/T)
\]

and thus

\[
\int_{t_2}^{t_1} E_{3}(t) dt \cdot \left[ \int_{t_1}^{t_2} E_{3}(t) dt \right] - \int_{t_1}^{t_2} E_{3d}(t) dt \cdot \left[ \int_{t_1}^{t_2} E_{3}(t) dt \right] = \left[ E_{3d}(t_2) - E_{3d}(t_1) \right] \cdot (Q_{3d}/T)
\]

thus

\[
\int_{t_2}^{t_1} E_{3}(t) dt \cdot \left[ \int_{t_1}^{t_2} E_{3}(t) dt \right] - \int_{t_1}^{t_2} E_{3d}(t) dt \cdot \left[ \int_{t_1}^{t_2} E_{3}(t) dt \right] = \left[ E_{3d}(t_2) - E_{3d}(t_1) \right] \cdot (Q_{3d}/T)
\]

Hence we get Eq. A3 as desired

\[
FBR = \frac{\left[ E_{3d}(t_2) - E_{3d}(t_1) \right] \cdot \left[ \int_{t_1}^{t_2} E_{3}(t) dt \right] - \left[ E_{3d}(t_3) - E_{3d}(t_2) \right] \cdot \left[ \int_{t_1}^{t_2} E_{3}(t) dt \right] - \left[ E_{3d}(t_2) - E_{3d}(t_1) \right] \cdot \left[ \int_{t_1}^{t_2} E_{3d}(t) dt \right]}{(Q_{3d}/T)}
\]
REFERENCES


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