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In Vivo Measurement of Muscle Protein Synthesis Rate Using the Flooding Dose Technique

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Abstract

Skeletal muscle mass is determined by the balance between rates of protein synthesis and degradation. Protein synthesis rates can be measured in vivo by administering an amino acid as a tracer that is labeled with an isotope (radioactive or stable) of C, H, or N. The rate at which the labeled amino acid is incorporated into muscle protein, as a function of the amount of labeled amino acid in the precursor pool at the site of translation, reflects the rate of protein synthesis. There are a number of approaches for performing this measurement depending on the question being addressed and the experimental system being studied. In this chapter, we describe the “flooding dose” approach using L-[³H]-phenylalanine as the tracer and that is suitable for determining the rate of skeletal muscle protein synthesis (total and myofibrillar proteins) over an acute period (ideally less than 30 min) in any size animal; details for working with mice are presented. The method describes how to administer the tracer without anesthesia, the tissue collection, and the preparation of muscle and blood samples for analysis of the tracer and tracee amino acids in the precursor pool and in muscle proteins.

Keywords

Protein synthesis; Translation; Protein degradation; Skeletal muscle; Amino acid tracer; Phenylalanine

1. Introduction

Protein mass is determined by the balance between protein synthesis and degradation (1–3). Over a relatively chronic time-frame (days), the two processes are in balance in the skeletal muscles of the full grown adult at steady state and muscle mass remains constant. However, the balance in vivo shifts from positive to negative during a day due to the responsiveness of protein synthesis and breakdown to changes in nutrients, activity, hormones, and growth factors. Net gain of muscle protein occurs when the rate of protein synthesis is greater than the rate of protein breakdown, whereas muscle mass is lost when the rate of protein degradation exceeds synthesis rates. Therefore, the quantification of these processes in vivo, and how the balance changes over time, is critical for understanding how muscle protein mass is regulated by biological variables.

There are a number of approaches for quantifying the rates of skeletal muscle protein synthesis and degradation in vivo, and the one that is most suitable depends on the question

being addressed and the experimental system being studied. The differences among these approaches and their advantages and disadvantages have been addressed in a number of reviews (4–8). As is evident from these reviews, it is not possible to cover the technical details for all of these in a single chapter as they can entail quite different *in vivo* protocols and subsequent analytical techniques. The majority of the techniques involve the administration of an amino acid as a tracer that is labeled with an isotope (radioactive or stable) of C, H, or N. The rate at which the labeled amino acid is incorporated into muscle protein, as a function of the amount of labeled amino acid in the precursor pool at the site of translation, reflects the rate of protein synthesis. The approaches for measuring skeletal muscle protein degradation *in vivo* (5, 9–12) are more limited, technically difficult, and not practical to perform in small animals such as rats or mice. They also require the use of amino acid tracers either to estimate arteriovenous differences across a muscle bed, or to label muscle proteins, and then measure the rate at which the label is lost from the muscle due to degradation. An alternative approach that can be used in experimental animals and measures protein degradation rate averaged over a period of days will be presented in this chapter (13, 14). Additionally, it should be remembered that at steady state, the rates of synthesis and degradation are equal.

In this chapter, the protein synthesis technique known as the “flooding” or “large” dose technique developed originally by Garlick et al. (15) is presented. It is suitable for measuring protein synthesis in any size animal, and to determine the synthesis rate of both constitutive and secreted proteins in any tissue (4). The basic procedure involves administering relatively rapidly (<30 s), as a single bolus, a large dose of the tracee amino acid along with the tracer amino acid. The total amount of the labeling amino acid administered should be 5 to 10 times the body’s total free pool of the essential amino acid chosen as tracee, and ideally, the amino acid should not be metabolized by the tissue of primary interest to minimize the introduction of error from the recycling and dilution of the label; the use of amino acids that are posttranslationally modified in skeletal muscle (e.g., histidine, lysine) also could introduce errors and are best avoided. The amount of tracer administered (i.e., the specific radioactivity or enrichment) depends on the synthesis rate of the protein (higher amounts for slower rates), and the sensitivity of the detection system. The objective of using a large dose is to “flood” the precursor amino acid pool with the tracer amino acid so that the specific radioactivity or enrichment (in the case of stable isotope-labeled tracers) of the extracellular, intracellular, and acylated tRNA pools come into equilibrium instantaneously (ideally), and remain in equilibrium throughout the labeling period (16). To ensure that this condition is satisfied, the labeling period is restricted to less than 30 min, ideally, during which time the organism should be in a relatively physiological “steady state.” The proteins to be assessed are then isolated and the amount of tracer incorporated into the protein is determined. The data are expressed as fractional rates of protein synthesis (FSR or Ks), which represent the fraction of the total protein (TP) pool synthesized per unit time. This provides a measure of the rate at which translation is occurring in the cells, and is independent of the TP mass. Absolute synthesis rate (ASR) is the product of FSR and the total mass of the protein; because TP mass reflects the product of the long-term protein balance of the tissue, ASR does not reflect the activity of the protein synthetic process in the tissue at that moment in time. When all proteins in a muscle are analyzed, the synthesis rate of mixed muscle

proteins is determined; this includes the synthesis rate of all cell and protein species in the tissue including both extracellular and intracellular proteins. Alternatively, individual proteins, or groups of proteins (e.g., the myofibrillar, sarcoplasmic, and stromal fractions in muscle), or organelles (e.g., mitochondria) can be isolated and the FSR of their component proteins determined (14, 17–20).

Over the years, the “flooding dose” technique has received criticism for a number of reasons (reviewed in ref. (21)). However, as with all indirect approaches for measuring a process, it is difficult to be certain of the approach that gives the “correct” value. Nonetheless, there is general agreement that if the technique is used adhering strictly to conditions that ensure that the underlying assumptions are not violated, the results obtained are valid. Thus, the measurement described provides a brief snapshot of the in vivo FSR. In this chapter, the described protocol uses L-[4³H]-phenylalanine as the tracer and it is geared toward measurements of skeletal muscle proteins (total and myofibrillar) in rats or mice.

2. Materials

The procedure is divided into two parts: the in vivo animal labeling component, and the analytical component. The latter describes the preparation of the samples and it is suitable for the measurement of both specific radioactivity and enrichment (if the amino acid tracer is labeled with a stable-isotope) of the amino acid tracer incorporated into muscle proteins. The actual determination of specific radioactivity or enrichment is not described here as there are a number of possible options depending on the technology available to the investigator.

Care should be taken to ensure that all solutions, glassware, and disposables that come into contact with the proteins to be analyzed are protein and amino acid-free. Thus, high purity water (18 MΩ cm at room temperature) should be used for everything; all glassware for making up solutions should be soaked in 5% HNO₃ for at least 6 h and rinsed thoroughly before using. Glassware that comes into contact with samples from the animals should be soaked overnight in Chromerge® solution and then rinsed thoroughly. *Extreme precaution* should be taken when handling this solution that contains concentrated H₂SO₄; i.e., use only in a chemical fume hood; wear a rubber apron over lab coat, heavy duty acid-resistant rubber gloves over regular latex gloves, eye protection, and preferably handle glassware with tongs. The solution should be stored in an acid-proof cabinet and can be reused until it turns green at which point it should be disposed as biohazard waste. When handling radioactive materials, take all standard precautions and ensure that you have biosafety approval for using the radioactive materials in the lab and in the animals.

2.1. In Vivo Protein Synthesis Protocol

1. Isotope injection solution: Each animal is administered 1.5 mmol phenylalanine/kg body weight. The amount of radioactive tracer added depends on the FSR of the proteins of interest, the duration of the labeling period, and the sensitivity of the detection system, and requires a rough idea a priori of what these values are (see Notes ¹ and ²).

For an experiment with 16 adult mice (approximately 30 g body weight each) and a labeling time of 30 min (note that this is just an example and is unlikely to be appropriate for all applications or the same application in different labs), a total volume of 5.3 mL will be needed ($[30 \times 16]/100 = 4.8 \text{ mL} + 10\%$ for losses), containing 600 μCi L-[^3H]-phenylalanine/mL in 0.9% NaCl. The final concentration is 150 mM with a specific radioactivity of 4 mCi/mM; each mouse receives approximately 200 μCi .

In a 10 mL flat-bottomed clean, screw-cap, glass vial weigh 47.7 mg NaCl (analytical grade); and 131.175 mg L-phenylalanine (TraceCERT[®], see Note ³). Pipette in 2.12 mL water and 3.18 mL L-[^3H] phenylalanine (1 mCi/mL; see Note ⁴). Add a magnetic stir bar, cap, and Parafilm[®] the vial, and place on a stir plate to mix until the phenylalanine is completely dissolved (see Note ⁵). Unless you are going to use it immediately, store the solution at 4°C. Before using it, bring it to room temperature and ensure that any phenylalanine that has precipitated out is redissolved. For long-term storage (>4 weeks), the injection solution should be frozen at -20°C.

2. Injection line: 1 mL syringe (with 0.01 mL calibration markings); 5" piece of PE 50 tubing; 23G Luer-stub adaptors; 26G $\times$ 5/8" hypodermic needles with hub removed (see Note ⁶). Attach the Luer-stub adapter to one end of the tubing, and the white end of the hypodermic needle to the other. At both ends, the tubing should be pushed until it abuts the white flare. This will be attached to the syringe and used for tail injection.

¹The tracer should be administered in the smallest feasible volume. This is dictated by the solubility of phenylalanine, which is close to 150 mM. Thus, administration of 0.1 mL/10 g body weight of a 150 mM solution (15 $\mu\text{mol}/10 \text{ g}$ body weight) will constitute a flooding dose. Alternatively, a greater volume of a more dilute solution can be administered (e.g., 0.2 mL/10 g body weight of a 75 mM solution). This may be necessary if a final specific radioactivity activity higher than 6.67 mCi/mmol phenylalanine is needed.

²The amount of radioactive tracer to add is a compromise between the cost of the isotope-labeled tracer and the counting precision for the incorporated ^3H -phenylalanine. ^{14}C -ring labeled phenylalanine could be used but is much more expensive. It is useful to keep the following facts in mind in estimating how much tracer to use: (a) on average phenylalanine constitutes 6% of body proteins by weight; (b) when using a radioactive ^3H -labeled tracer, the largest source of error usually comes from the uncertainty in counting the ^3H associated with the phenylalanine incorporated into the protein(s); the error is the square root of the total net counts accumulated. For example, the uncertainty in a net (total - background) value of 400 counts is ± 20 counts, i.e. $\pm 5\%$. Thus, administering more tracer to the animal, increasing the amount of sample analyzed, reducing background activity, increasing the counting time of the samples (e.g., if the 400 counts were collected over 20 min, increasing this to 60 min would reduce the error to $\pm 3\%$), and factors that affect the efficiency of counting ^3H (the scintillation counter itself, the scintillation fluid, the composition and preparation of the sample analyzed) all factor into the final decision of the specific radioactivity of the injection solution. Ideally, one should aim for a counting error of the incorporated ^3H -phenylalanine of less than $\pm 2-3\%$.

³It is important that the "cold" phenylalanine used is as close to 100% pure L-phenylalanine. It should be stored desiccated.

⁴The solution should be certified as being >98% pure L-phenylalanine. Unless one has a chiral column to independently verify the R-phenylalanine content, one has to take the manufacturer's word for it. Ideally, the solution should be run over an HPLC column and counted to verify that all the ^3H counts are in phenylalanine and that the amount specified is present (we find that it is usually approximately 93-95%). However, this is not the case with every batch, in which case it should be either returned for another batch, purified in-house, or take into account the activity present, provided it is not also present in another amino acid (such as tyrosine, as is often the case). The amount of "cold" carrier phenylalanine in the solution is negligible and can be ignored in the calculations. The stock tracer solution is vulnerable to radiolysis and should be stored at 4°C for periods of <1-2 months or frozen for longer periods.

⁵The phenylalanine takes a while to dissolve (hours). To facilitate the process, warm the solution in a water bath at 37°C before putting the vial on the stir plate. Spin the stir bar as fast as possible (tape the vial to the plate so it does not fall over). The solution must be completely clear.

⁶This procedure is for the acute intravenous administration of the amino acid tracer solution. If the animal has a catheter already implanted, it can be used to administer the tracer. To remove the hub from the needle, hold the needle with a pair of forceps and flame the hub with a Bunsen burner (5-10 s), enough to soften the plastic material. Use hemostats to pull the hub off the needle. This should leave the "hub-end" of the needle with a white coating and slightly flared at the point where the needle emerged from the hub. If the white coating is removed, you cannot use the needle as it will be too small for the tubing. Prepare one needle per animal.

3. For injection, large pieces of cheese cloth (triple layer, approximately $8 \times 8''$); water in a beaker heated to 47–49°C; lamp; timer.
4. Large scissors or a guillotine with very sharp blades (see Note ⁷); various sized sharp scissors and fine tipped forceps for dissection.
5. Polystyrene and aluminum weigh dishes (small); 3'' $\times$ 3'' aluminum foil pouches (see Note ⁸); 1.5 mL microfuge tubes.
6. 0.2M chilled perchloric acid (PCA, 2.2 mL 60% PCA and 97.8 mL mQ water; see Note ⁹).
7. 95 $\times$ 12 mm Pyrex Petri dish bottoms filled with black dissecting wax; pins.
8. Liquid nitrogen, styrofoam containers.
9. Analytical and pan balance.
10. Screw capped tissue storage tubes suitable for liquid nitrogen.
11. One data recording worksheet per mouse.
12. Metric Vernier calipers.

2.2. Preparation of Tissue and Blood for Measurement of L-[4³H]-Phenylalanine Specific Radioactivity

1. 4M KOH; use chilled.
2. 1M acetic acid: 57 mL of glacial acetic acid (HPLC grade)/L (with mQ water).
3. 3M NH₄OH: 48 mL of 14.8M NH₄OH/200 mL (with mQ water).
4. 1M HCl: 16.6 mL of 6M HCl (ULTREX II Ultrapure)/100 mL (with mQ water).
5. Dowex[®]-50 resin (AG-50W-X8; 100–200 mesh, H⁺ form) cleaned and stored in 1M NaOH (see Note ¹⁰).
6. X2 low salt/sucrose homogenizing buffer: 100 mM K₂HPO₄ (17.42 g/L); 100 mM KH₂PO₄ (13.61 g/L); 0.5M sucrose; Triton X-100. Mix 61.5 mL of K₂HPO₄ with 38.5 mL of KH₂PO₄ and pH to 7.0 with KOH or phosphoric acid. To a 100 mL volumetric flask add 68.4 g sucrose, add approximately 90 mL of phosphate buffer and dissolve sucrose. Add 2 mL of Triton X-100 and bring to volume with phosphate buffer; good for <2 weeks; use chilled.

⁷If the animal does not have an indwelling catheter to permit the rapid administration of a large dose of anesthesia, decapitation without the use of anesthesia is used to euthanize rodents and collect trunk blood. This requires detailed justification and special approval from the IACUC committee. Its use is warranted because the prior use of anesthesia or sedation will alter the rate of protein synthesis to variable and not always predictable extents (24). See Note 18 for alternatives.

⁸Make these from heavy duty aluminum foil.

⁹PCA is hazardous and should always be used with caution.

¹⁰To prepare the Dowex[®] resin, suspend the entire bottle of resin in mQ water in a 2 L beaker. Mix with a spatula; never use a magnetic stirrer. Pour off water and repeat the wash 3 $\times$. Suspend the resin in mQ water again, and allow it to sit for 30 min with occasional stirring. Decant the water and repeat this last step 6 $\times$. Suspend the resin in 1M NaOH and allow it to sit overnight with occasional stirring. Decant and resuspend in the resin in 0.4 L of 1M NaOH. Allow it to sit for 4 h with occasional stirring. Repeat the last step 6 $\times$, leaving overnight when necessary. Decant and resuspend the resin in 2 volumes of 1M NaOH (verify that it is very basic) and store at 4°C.

7. X1 low salt/sucrose homogenizing buffer, pH 7: dilute X2 buffer 1:1 with mQ water; store at 4°C; use chilled.
8. 1.2M PCA: 13 mL of 60% PCA/100 mL (with mQ water); use chilled.
9. 0.2M PCA; use chilled.
10. 0.1M and 0.3M NaOH.
11. 6M HCl: concentrated HCl (ULTREX II Ultrapure) diluted 1:1 with mQ water (see Note ¹¹); use fresh.
12. BCA protein assay reagents (22).
13. pH paper tape.
14. Centrifugal concentrator (e.g., Savant Speedvac) with rotors that will accept all tube sizes to be used in the analyses (see Note ¹²).
15. Syringe filters (0.22 or 0.45 µm).
16. Disposable filter columns for ion exchange resin (individually fitted with funnel) and racks.
17. Glass vials (17 × 60 mm, flat-bottomed) with screw caps.
18. Steel mortar and pestle for powdering frozen muscles.
19. Homogenizer (e.g., an Ultra-Turrax[®]T-25) with stainless steel dispersion probes (S25N-8G) chilled on ice.
20. Glass rods (4 × 125 mm).
21. Polypropylene tubes (12 × 75 mm).
22. Tube shaker (e.g., Tomy shaker).
23. Kimble KIMAX[®] borosilicate glass screw top tubes (13 × 100 mm) with Teflon[®]-lined caps.
24. Evaporator with heating blocks that accommodate glass tubes and with a gassing manifold equipped with removable stainless steel needles.
25. Oven set at 110°C.
26. N₂ gas.

3. Methods

3.1. In Vivo Protein Synthesis Measurement

1. Values for skeletal muscle protein FSR are influenced by whether the animal is in the postprandial or fasted state (23). Because different information is conveyed by the FSR values in these two conditions, careful thought should be given to

¹¹This is expensive; only make up as much as you need. Prepare in the fume hood.

¹²As concentrated HCl will be evaporated, the system must have an acid trap, and the refrigeration component must attain –104°C to prevent HCl fumes from reaching and destroying the vacuum pump.

determining which is most appropriate, and the protocol should then be standardized to ensure that all mice are in the same condition. Access to food should be denied for 4–8 h for FSR measurements performed in the fasted state, whereas measurements should be made within 3 h of the start of the light cycle to ensure that FSR values reflect the “fed” or postprandial condition. Alternatively, animals can be fasted for 6–8 h during the dark cycle, and then given access to food for 90–120 min preceding the FSR measurement (see Note 13).

2. Before beginning the experiment, the lab should be set up with four work stations: (a) an injection station: this should have a pan balance for weighing the mouse, a lamp, the isotope injection solution, the injection line, and the supply of hub-less needles; (b) a euthanasia station: this should have an ice bucket into which three labeled blood collection tubes, one aluminum foil pouch, and a beaker with chilled ice water are placed before each mouse is injected; a large polystyrene weigh boat, large and medium scissors, and forceps; (c) a dissection station: with a shallow rectangular filled ice tray containing the dissection dishes (keep surface in ice until ready to use); dissection instruments and pins; a styrofoam bucket containing liquid nitrogen on which one labeled aluminum weigh boat per type of muscle is floated; (d) a weighing and storage station: with an analytical balance, prelabeled blood, and tissue storage tubes; liquid nitrogen in a styrofoam box for temporary sample storage; a microfuge (ideally refrigerated) for processing blood samples.
3. At least 2 or, ideally, 3 people are required for performing the intravenous injection. Weigh the animal and determine the volume of isotope solution to be injected (0.1 mL/10 g body weight); record both on worksheet. For the example used here, a 31 g mouse would receive 0.31 mL. Draw this volume into the syringe through the injection line ensuring that there are no air bubbles. Make a small slit in the center of the cheese cloth and thread the mouse tail through it. Fold the cheese cloth over the mouse and roll the mouse up so that it is totally restrained in the cloth with only the tail protruding (see Note 14). Holding the body of the mouse, dip the entire tail in the warm water for 90 s to dilate the tail veins. Lay the mouse on the bench on its side with the tail protruding under a bright light. One person should hold the mouse in this position and simultaneously hold the base of the tail firmly to prevent the mouse from jerking it during the injection. While also holding the tail, a second person inserts the needle into a lateral tail vein about half way along its length. The needle should be held in line with the vein and enter the skin at as flat an angle as possible, bevel side up. As soon as the needle looks like it is in the vein, a little suction is applied from the syringe by the third person, and some blood should be visible in the tubing if the needle is in the vein. If no blood returns, the needle is not in the

¹³If the fasting and refeeding approach is taken to ensure that postprandial FSR values are obtained, the animal should be weighed before and after refeeding, and/or stomach contents recorded to ascertain that the animal had eaten.

¹⁴This is often named the “mouse burrito” because of the similar appearance. If done correctly, the mouse is lightly restrained and stays quite still.

vein; in this case remove the needle and attempt again proximal to the previous attempt (see Note ¹⁵). Once the needle is in the vein, the third person injects the solution over approximately 10 s (proportionally less or more time for smaller or bigger volumes), while the second person holds the tail and the needle, and the first holds the mouse and the tail. A timer is started (counting up) when half the solution has been injected. Once the tail is warmed, the injection can be completed in 30–45 s (see Note ¹⁶). After the entire volume is injected, the needle is removed, and pressure applied to the tail until it stops bleeding. The mouse is unwrapped and returned to its cage (ideally in a separate room).

4. Approximately 10 min later, the mouse is again taken from the cage, and using a 20G hypodermic needle, the opposite lateral tail vein to the one injected is punctured and 25 μ L of blood is collected with a pipette and immediately mixed with 0.25 mL of 0.2M chilled PCA, vortexed, and set on ice. The exact amount of time elapsed at the time the blood was collected is recorded on the worksheet (T_1). The tail bleeding is stopped by applying pressure and the mouse is returned to its cage (see Note ¹⁷).
5. After 29.75 min from the injection time, the animal is removed from the cage, and at exactly 30 min, the head of the mouse is excised (see Note ¹⁸) and the time is noted (T_2). From the head, 50 μ L of blood is collected and mixed with 0.5 mL chilled 0.2M PCA, vortexed well and placed on ice. The trunk blood is collected into another tube and kept on ice to clot; this is used for measuring hormones, metabolites, etc., and not pertinent to the protein synthesis measurement itself. Twenty to 30 s after the decapitation, both hind limbs are severed at the joint between the femur and the pelvis using scissors, the skin is peeled off, and the limbs are placed in the prechilled foil pouch, wrapped up, and buried in the ice to chill. This is recorded as the “chill time” (T_3) for the hind limb muscles and represents the duration of the protein labeling period; no more than 40–50 s should elapse from decapitation to chilling. If the diaphragm is to be measured, the skin and abdominal wall muscles are cut, the organs below the diaphragm are rapidly removed and the carcass is submerged in the chilled water; the time of submersion is recorded (as T_3 for the diaphragm). Similarly, if front limb muscles are needed, the arms are detached, skinned, and chilled as described for the hind limbs (see Note ¹⁹-very important).

¹⁵Before inserting the needle, always make sure that the isotope solution is all the way to the tip and that no air bubbles have been inadvertently introduced.

¹⁶With experience, it is possible for the same person to place the needle and do the injection. It is possible to use this approach to reliably inject mice as small as 10 g. For mice smaller than 10 g, or if the tail vein injection cannot be performed, the isotope tracer can be administered intraperitoneally using a 30G needle. This has the disadvantage that one cannot be sure until after the analysis if any of the isotope solution was injected into the intestine, and the equilibration time within the precursor free pool is not immediate as when the dose is administered intravenously (25, 26). Additionally, some of the isotope solution may leak out of the hole left by the needle, especially in smaller animals. It is important to keep precise notes on each animal to establish if any technical issues encountered are associated with FSR values that deviate significantly from others in the same treatment group and, therefore, are most likely erroneous.

¹⁷If the total duration of the labeling period is shortened to 15 min or less, the intermediate time point blood sample can be omitted.

¹⁸If the animal has an indwelling catheter, an overdose of anesthetic can be used to rapidly euthanize it (<15–30 s). Once the animal is unconscious, it is decapitated, and the same procedures followed.

¹⁹Once the tissue has been chilled, it must *NEVER* be allowed to warm up until the isolated protein has been treated with PCA. This is important to prevent proteolysis. Proteolysis will result in the dilution of the precursor pool with unlabeled phenylalanine and, thus,

6. One minute after the limbs are chilled, they are individually removed from the pouch (they should not be wet), and pinned out on the chilled and dried dissection dish in the ice tray. The muscles needed for measuring protein FSR are rapidly and quantitatively dissected using dry, chilled dissection instruments, and then dropped on the foil boats floating on the liquid nitrogen where they freeze instantaneously (see Note ²⁰). After one limb is completely dissected, the second is removed from the pouch and the process repeated. The carcass is pinned out and the diaphragm dissected quantitatively, mopped rapidly with a Kimwipe® to remove surface water, and frozen. The muscles should not be on the chilled dissection dish for more than 2–3 min. The bones that subtend the muscles can be reserved and their lengths determined with Vernier calipers when time permits.
7. Once all muscles have been dissected and frozen, they are rapidly weighed on the analytical balance, transferred to chilled storage vials, and held in liquid nitrogen or dry ice until they can be stored at –80°C. After sitting on ice for at least 10 min, the PCA-treated blood samples are microfuged at $13,200 \times g$ for 3 min and the clear supernatant is transferred to a microfuge tube, and stored frozen at –80°C. The whole blood is allowed to clot for >30–40 min, centrifuged, and the serum is recovered and stored frozen at –80°C.
8. To avoid cross-contamination, all instruments and dishes are washed and rechilled between mice. The needle on the injection line is replaced, and the process is repeated for the next animal. With experience and three people, the process from the time the mouse is euthanized to completion can be accomplished in less than 10 min.

3.2. Preparation of Tissue and Blood for Measurement of L-[4³H]-Phenylalanine Specific Radioactivity

Keep in mind that all samples and waste are radioactive and need to be handled appropriately.

1. Blood: Thaw out blood PCA-supernatants on ice. Lay out a strip of pH paper on the bench. To each sample, add 10–20 μ L chilled 4M KOH, vortex, and test pH. Repeat this process until pH 7 is attained (see Note ²¹). At neutral pH, potassium perchlorate is insoluble and precipitates out as white crystals. Repeat for all samples. Once samples have all been on ice for at least 30 min, centrifuge at $8,000 \times g$ for 20 min at 2–4°C. Carefully remove supernatant and place in a clean microfuge tube and discard the precipitate. Place samples in the Speedvac to dry overnight. Cap and store at 4°C until they are purified over the ion-exchange column.

the specific radioactivity values for the precursor pool will be inaccurate (too low), and the estimate of FSR will be wrong (overestimated).

²⁰An alternative approach is to use one limb to take pieces of muscle rapidly (not quantitatively) and freeze for measuring protein FSR. The second leg is then used to quantitatively dissect out the muscles with less concern about them warming up. This is possible with larger animals/muscles where sufficient muscle for performing all the analyses can be obtained from one side.

²¹Care should be taken to ensure that the KOH does not become contaminated. If the samples become too alkaline, they can be back titrated with PCA.

2. Purification of blood and muscle free precursor pool (see Note ²²): Prepare ion exchange resin in H⁺ form as follows: add 1.25 mL of Dowex[®]-50 resin (prepared as in Note ¹¹) to each column, and wash resin with 100 mL mQ water; then use pH paper to check that the pH of the eluant from the column is the same as the water. Add 5.0 mL of 1M HCl to each column; use pH paper to check that the final column eluant is acidic. Wash each column with ~75 mL mQ water and check that the eluant pH is the same as the water. Now columns are ready to use (see Note ²³). Dissolve dried blood (from Subheading 3.2, step 1) or muscle free pools (from Subheading 3.2, step 4) in 1.25 mL of 1M acetic acid; vortex to dissolve and apply to prepared Dowex[®]-50 column. Wash the sample vial with 1 mL 1M acetic acid and apply to the same column; repeat this 3× so that a total of 5.25 mL is applied to the column. Check that the pH of the eluant is acidic using pH paper. Rinse Dowex[®]-50 with 75 mL mQ water; then check that the pH of the eluant from the column is the same as water using pH paper. Put a clean (Chromerge[®]-washed), labeled glass vial under each column and elute amino acids with 3 mL of 3M NH₄OH. Apply a further 3 mL of water to the column and collect eluant into vial. Place vials in the evaporator with the heating block at 65°C; direct nitrogen gas into each vial using the needle manifold assembly and reduce the volume by half at which point no ammonia smell should be detectable. Then transfer the vials to the Savant Speedvac to complete the drying. Dissolve each sample in 1.0 mL of mQ water and then filter into a microfuge tube using a syringe and syringe filter. Dry the filtered samples in the Savant Speedvac and reconstitute in a volume of water or reagent as required by the technique that will be used to isolate the phenylalanine and determine the radioactivity or stable isotope enrichment associated with it (see Note ²⁴).
3. Muscle homogenization: Using the steel pestle and mortar chilled in liquid nitrogen, powder all muscles (see Note ²⁵). Very rapidly weigh out approximately 50 mg of powdered muscle into a chilled, labeled, polypropylene tube. Record the exact weight on a worksheet. With the tube on the balance, tare, and add 1 mL of chilled mQ water. Record weight of water on worksheet and return the tube immediately to ice. Dry the chilled dispersion probe on the homogenizer by running it briefly in air while wiping with a Kimwipe[®]. Holding the sample tube in an ice jacket, homogenize the muscle for 3 × 30 s with 15 s rests in between. Cap the tube and place it on ice. Wash, chill, and dry the

²²Purification of the free pool amino acids over the ion exchange column is necessary to obtain a “clean” phenylalanine peak when the samples are subjected to HPLC using an anion exchange column (PA-1 column, DIONEX). If an alternative technique is used, the need for this clean up step may be unnecessary. We have found that it is rarely necessary to perform this step on samples from rats and pigs, but essential for mouse samples.

²³The columns can be prepared ahead of time and then capped with mQ water and stored in the cold room (for a day or so). The water is drained from the columns before the sample is applied. It is not difficult to process up to 24 samples at once.

²⁴The amount of free tracee amino acid in the sample can be roughly estimated. For example, in the present protocol the mice are administered 1.5 mmol phenylalanine/kg body weight which equilibrates in the body fluid compartment. If we assume the latter to be approximately 60% of body weight, the concentration will be approximately 2.5 μmol/mL water, or 40 nmol in 50 μL of blood, or 35 nmol in a 400 μL aliquot of the 1:20 muscle homogenate. The sensitivity of the detection system will dictate how much the samples should be diluted, and the appropriate sample size that should be analyzed in the first place.

²⁵It is best to powder all samples before proceeding to the analysis step. To avoid cross-contamination of samples, the mortar and pestle should be warmed to room temperature, washed, and chilled in liquid nitrogen between every sample.

dispersion probe thoroughly between samples. Homogenize all samples before proceeding to the next step.

4. Analyzing for TP: Gently invert each tube several times to mix the homogenate, take an aliquot and make a 1:200 dilution with 0.1M NaOH for measuring TP using the BCA assay (see Note ²⁶). Remove 400 μ L of homogenate into another prechilled polypropylene tube; add 66 μ L chilled 1.2M PCA, vortex immediately, and incubate on ice for >20 min. Centrifuge at $8,000 \times g$ at 4°C for 15 min. Transfer the supernatant which contains the muscle free precursor pool into a 1.5 mL microfuge tube; cap and freeze at -20°C. These samples will then be processed in the same way as the blood free pool described in Subheading 3.2, step 1. Wash the remaining precipitate with 1 mL 0.2M PCA using a glass rod to help break up the pellet; vortex thoroughly. Incubate on ice for >15 min before centrifuging at $8,000 \times g$ at 4°C for 15 min. Discard the supernatant. Repeat this wash step 4 more times (see Note ²⁷). Before the last centrifugation, transfer the protein slurry quantitatively to a KIMAX[®] hydrolysis tube, using additional 0.2M PCA to rinse and ensure complete transfer. Centrifuge the tube at $1,500 \times g$ at 4°C for 30 min. Discard the supernatant and invert the tube to drain off any remaining PCA. Then cap (with Teflon[®] insert) and store at -20°C until the hydrolysis is performed (see Subheading 3.2, step 6).
5. Isolation of myofibrillar proteins (MP, see Note ²⁸): After taking the TP homogenate aliquot, add an equal volume of the 2 $\times$ low salt/sucrose buffer to the remainder of the homogenate, cap, mix well, and place the tubes on the shaker in the cold room for 60 min to solubilize membranes. Then, centrifuge the homogenate at $1,500 \times g$ at 4°C for 10 min to separate the soluble proteins (sarcoplasmic, hemoglobin, plasma proteins, and soluble extracellular proteins) from the insoluble MP pellet. Place the tubes on ice after centrifugation being careful not to shake them as the pellet is fragile. Carefully remove and discard the supernatant. Wash the MP pellet with 1 mL of the 1 $\times$ low salt/sucrose buffer. Vortex well, and then centrifuge as in the previous step. Discard the supernatant and keep the pellet. Repeat the MP pellet wash in 1 $\times$ low salt/sucrose buffer. To the resulting MP pellet, add 1 mL of ice cold mQ water and vortex well to resuspend myofibrils. Centrifuge the suspension at $1,500 \times g$ at 4°C for 10 min. Discard the supernatant and repeat the wash in ice cold mQ water. Resuspend the MP pellet in 1 mL of cold mQ water (vortex thoroughly) and quantitatively transfer the slurry to a large prechilled, polypropylene tube. Centrifuge at $8,000 \times g$ for 15 min at 4°C. Gently remove supernatant and discard (see Note ²⁹). To the MP pellet add 2.0 mL of 0.3M NaOH; vortex well and incubate in a water

²⁶For an adult muscle, this will yield a solution of approximately 50 μ g protein/mL. After incubating the samples at 37°C for 1 h to solubilize the proteins, the exact protein content is determined using a protein assay with the protocol optimized for this concentration.

²⁷All the washes are very important to ensure that the free phenylalanine tracer is completely removed. The specific radioactivity of the precursor pool can be several hundred times higher than that of the protein. Thus, even minute levels of contamination can lead to overestimation of the final FSR values.

²⁸The isolation of the MPs is not quantitative in this procedure. Thus, only FSR of the MPs will be obtained. An independent quantitative measurement of the MP concentration in the muscle is needed to estimate ASR.

²⁹At this point an aliquot of the MP fraction can be reserved and used to isolate individual MP proteins, e.g., by SDS/PAGE electrophoresis with or without prior immunoprecipitation of the protein of interest. The purified protein is hydrolyzed as described in

bath at 37°C for exactly 60 min, vortexing every 15 min to assist solubilization of MPs. This step separates the alkali-soluble MPs from the alkali-insoluble collagen. Repeat the centrifugation and then transfer the supernatant to another hydrolysis tube, and chill on ice. To the hydroxide MP solution, add 2/3 of its volume of chilled 1.2M PCA and incubate on ice for 30 min. Centrifuge at 1,500 × *g* at 4°C for 30 min and discard the supernatant. Wash the pellet with 1 mL chilled 0.2M PCA; vortex hard to disperse and centrifuge as before. Pour off the supernatant and leave tubes inverted at room temperature to drain; cap (with Teflon® liners) and store at –20°C.

6. Acid hydrolysis of protein precipitates: The TP and MP acid precipitates should be in KIMAX® tubes at –20°C. Allow samples to thaw at room temperature; working in a fume hood, add 6M HCl to each tube (see Note ³⁰). Use an acid-washed glass rod or glass Pasteur-pipette to completely disperse the pellet in the acid. Place the tubes in the evaporator heating blocks set at 110°C. While the samples are warming up, very gently blow nitrogen gas into each tube taking extreme care not to splash the acid and to avoid cross-contamination. The purpose of this is to displace as much oxygen as possible from the tubes and minimize sample oxidation. After each tube is gassed, loosely screw on the cap. When the samples start to bubble, repeat the nitrogen gassing and this time screw the cap on very tightly. When all samples are done, transfer the tubes to a metal rack and place them in an oven at 110°C to hydrolyze (see Note ³¹). After *exactly* 24 h remove the samples from the oven, cool, and place them in the Savant Speedvac to dry. Once dried, add 4 mL mQ water to each tube and leave to soften at room temperature for 30 min. Vortex at length to redissolve the pellet and then dry the sample down again. Repeat previous wash step two more times, for a total of three washes, and then resuspend the final pellet in 1 mL mQ water. Use a 3 mL syringe to filter the hydrolysate through a 13 mm, 0.4 µm syringe filter into a 1.5 mL microfuge tube. Dry the sample and resuspend it in a volume of water or other diluent as required by the technique that will be used to isolate the phenylalanine and to determine the radioactivity or stable isotope enrichment associated with it (see Note ³²).
7. The specific radioactivity of the phenylalanine in the blood and tissue free amino acid pools and in the protein hydrolysates is measured by HPLC (see Note ³³)

Subheading 3.2, step 6, and its phenylalanine specific radioactivity is then determined. The FSR of that protein is estimated using the same precursor pool values as for the TP and MP. If these analyses are to be performed, it may be necessary to begin with a larger sample size.

³⁰The volume of HCl added depends on how much protein is to be hydrolyzed; this should be at least 1 mL/2 mg of protein. For example, the TP pellet from a 400 µL aliquot of the 1:20 muscle homogenate will contain roughly 4 mg of protein (assumes a muscle protein concentration of 200 mg/g muscle) and the corresponding MP pellet will be 3 mg (assumes MPs to be 50% of TP). Thus, 2 and 1.5 mL of 6M HCl will be added to the TP and MP pellets, respectively. As the exact amount of sample used is known and the protein content of the muscle is measured, the actual amount of protein can be estimated more accurately; however, this is not really necessary unless the muscle protein concentration is expected to be very different, e.g., in immature muscles.

³¹The temperature is critical. At lower temperatures, the hydrolysis of the proteins may not be complete in 24 h. At higher temperatures, the H isotope atom label on the C-4 position of the phenylalanine ring can exchange with H or OH and must be avoided. It is recommended that the heating block and oven are monitored to ensure that they do not go above 110°C before the samples are placed in them.

³²Again one can make some rough calculations based on the initial amount of protein and assuming that protein is 6% phenylalanine. Thus, a 4 mg protein pellet will contain approximately 0.24 mg or 1.4 µmol of phenylalanine.

and scintillation counting (for radioactive tracers or mass spectrometry for stable isotope tracers). Values (dpm/nmol) are calculated for the specific radioactivity (or MPE if a stable isotope tracer was used) of the blood phenylalanine (SA_{BLD}) at T_1 and T_2 , the tissue free pool (SA_{FP}), and the protein (TP and MP) bound tracer (SA_{TP} or SA_{MP}).

3.3. Calculations for Estimating Protein Synthesis Rates

Before performing the final calculations, determine the ratio of tissue SA_{FP} at T_3 to the SA_{BLD} at T_2 for each animal. If values are <0.95 or >1 , it indicates that there likely was a problem (several possible causes), and it is unlikely that accurate, valid FSR values will be obtained.

To determine the *average* SA_{FP} ($\overline{SA_{FP}}$) for the entire labeling period (see Notes ³⁴ and ³⁵), calculate the rate of change of SA_{BLD} over the labeling time:

$$\Delta_{BLD} [(dpm/nmol) / min] = [(SA_{BLD} \text{ at } T_1) - (SA_{BLD} \text{ at } T_2)] / (T_2 - T_1).$$

Then use this value, to calculate $\overline{SA_{FP}}$:

$$\overline{SA_{FP}} = SA_{FP} \text{ at } T_3 + [\Delta_{BLD} \times (T_3/2)].$$

Calculate the FSR (in %/day) values for TP and MP:

$$FSR_{TP} = \left(SA_{TP} / \overline{SA_{FP}} \right) \times (1,440/T_3) \times 100,$$

$$FSR_{MP} = \left(SA_{MP} / \overline{SA_{FP}} \right) \times (1,440/T_3) \times 100.$$

The ASR_{TP} (mg protein/day) is estimated as:

$$ASR_{TP} = (FSR_{TP}/100) \times TP \text{ mass of the muscle.}$$

³³There are some reports where the radioactivity of the samples (blood and tissue free pools, and protein bound) are counted directly, and the concentration of the phenylalanine is measured on a separate aliquot of the sample rather than by isolating the phenylalanine and determining its radioactivity. The former approach has the advantage that it is faster and less tracer can be used for labeling the animal in vivo because larger amounts of sample can be counted. However, it has the major drawback that it assumes that all the activity in the sample is associated with the phenylalanine; this probably is not the case and would introduce an unknown amount of error.

³⁴A number of assumptions go into the estimation of the average specific radioactivity or enrichment of the precursor pool. The first is that the specific radioactivity of the aminoacyl-tRNA, the true precursor, and muscle free amino acid pools (a surrogate that is measured in this protocol) are equilibrated throughout the labeling period. There has been extensive debate in the literature, because the routine measurement of phenylalanine-tRNA specific radioactivity or enrichment is not as practical as measuring that of the tissue free pool. Under the conditions described in this protocol, we have verified that the assumption is valid (16). If the isotope solution has been administered intraperitoneally, a correction to the precursor pool value is recommended to account for the slower equilibration of the injected tracer (25, 26). In the present calculations, we also assume that any decrease in specific radioactivity over the 30 min labeling period is linear. It is more likely to be an exponential decrease, but the error introduced by assuming it is linear is negligible.

³⁵If a number of muscles from the *same* animal are analyzed separately, it is not necessary to measure the SA_{FP} for each muscle. We have evaluated this numerous times, and determined that the variation in SA_{FP} among muscles is not greater than the analytical error for any one muscle. This assumption is not necessarily valid for different tissues; e.g., it is not advisable to use a muscle SA_{FP} value for calculating liver FSR.

The TP mass of a muscle is the product of the total wet weight of that muscle and the protein concentration determined from the sample of homogenate taken in Subheading 3.2, step 4.

3.4. Approach for Estimating Rates of Protein Degradation

Estimates of protein degradation averaged over relatively chronic periods (days) during which there are measureable changes in protein mass can be calculated from the protein synthesis and protein accretion rates (for example see refs. (13, 14)). To derive these data, the change in the protein mass (as mg/day) of the muscle of interest over an interval is measured by collecting and quantifying protein mass at the beginning and the end of the interval. The average protein synthesis rate over this time is then determined; a single measurement can be made at the midpoint of the interval, or the weighted average of a number of measurements is taken. Alternatively, if several values are obtained so that the change in muscle mass over time can be described with an equation, the velocity at the time points when protein synthesis is measured can be estimated by differentiation. The difference between protein synthesis and accretion rates will represent the average degradation rate.

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