Measuring in vivo intracellular protein degradation rates in animal systems

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ABSTRACT: Continual synthesis and breakdown or remodeling of proteins (also called protein turnover) is a principal characteristic of protein metabolism. During animal production, the net differences between synthesis and breakdown represent the actual marketable muscle foods. Because protein synthesis is a highly endergonic and protein breakdown is metabolic energy dependent, efficiency of production can be markedly enhanced by lower muscle protein breakdown rates. Herein, various methodological approaches to studying protein breakdown, with particular emphasis toward food-producing animals, are presented. These include whole-animal tracer AA infusions in vivo, quantifying marker AA release from muscle proteins, and in vitro AA release-based methodologies. From such methods, protein synthesis rates and protein breakdown rates (mass units/time) may be obtained. The applications of such methods and innovations based on traditional methods are discussed. Whole-animal in vivo approaches are resource intensive and often not easily applied to high-throughput metabolic screening. Over the last 25 yr, biochemical mechanisms and molecular regulation of protein biosynthesis and protein breakdown have been extensively documented. Proteolysis is dependent in part on the extent of expression of genes for components of cellular proteolytic machinery during skeletal muscle atrophy. It is proposed that high-throughput methods, based on emerging understanding about protein breakdown, may be useful in enhancing production efficiency.

Key words: marker amino acid, proteolysis, protein synthesis, tracer methodology, transcriptional and translational control, ubiquitin-proteasome


INTRODUCTION

The protein component of lean mass (operationally defined as water plus protein) in the whole body, irrespective of tissue or anatomical location, undergoes a process of remodeling characterized by continual protein synthesis, breakdown, and resynthesis (Schoenheimer, 1942). This overall process, often referred to as protein turnover, is driven by 2 separate but concerted bioprocesses, de novo protein synthesis (i.e., translation; Hershey, 1991) and protein breakdown (Schimke, 1970). Growth or protein accretion of an animal depends on the rate of protein synthesis exceeding that of protein breakdown (Zak et al., 1979; Waterlow, 2006). In mature humans and animals, body protein mass is more or less constant but aging is accompanied by a slow loss of protein mass in particular skeletal muscle protein mass (Solomon and Bouloux, 2006). Farm animals in production situations, however, are rarely in a negative N equilibrium except for females in early lactation or all animals during periods of insufficient feed intake usually related to strategies of limit feeding/compensatory growth or lack of feedstuffs related to weather and economics. In general, however, periods of negative N equilibrium result in lowered performance by animals and, as such, are not deemed as contributing to food production and sustainability of animal agriculture. During disease states or in periods when energy and AA are overtly lacking, both protein biosynthesis and protein breakdown are regulated to maintain as much of the cellular and tissue infrastructure as possible and to contribute to critical metabolic needs of the organism (Waterlow, 2006).

ROLE OF PROTEIN TURNOVER IN ANIMAL PRODUCTION

Although early workers recognized that body protein mass in growing animals was a net product of protein

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biosynthesis and breakdown, the process of protein biosynthesis was elucidated in detail earlier than were the processes involved in protein breakdown (Bergen, 2007). The continual process of body protein breakdown and resynthesis requires both metabolic energy and amino acids. Both of these are provided from feed and represent the primary cost input into animal production. The energetic costs of protein biosynthesis include the formation of the initiation ternary complex, activation of all amino acids and ligation to transfer RNA (tRNA), placing the correct tRNA-AA into the ribosomal acceptor site to match the genetic code in the mRNA, and the translocation of the growing peptide from the A (aminoacyl) to the P (peptidyl) site after peptide bond formation, and finally, termination. The energetic costs for initiation and termination of an average-size protein are small compared with the costs of AA activation (i.e., 2 ATP/mole equivalent of an amino acid) and energy costs related to function of each eukaryotic elongation factor (eEF1 and eEF2) at 1 ATP per peptide bond. Total minimal energetic costs of translation are then 4 ATP/mole equivalent peptide bond formed. This minimal value is often not achieved because the eEF-1–mediated placement of tRNA-AA into the A site is not always efficient, and up to 1 extra mole of ATP may be required for the formation of a peptide bond (Rodnina and Wintermeyer, 1995). This is a net increase of 20 to 25% in energy expenditures. The actual energy costs at the molecular level for protein degradation are less well established, but it takes energy to first synthesize the components of intracellular degradation systems and at least 1 of these components needs metabolic energy to function. The net outcome is that whenever breakdown of protein can be avoided there is a net saving in metabolic energy costs (Bergen and Merkel, 1991a).

The extent of continual protein remodeling appears to vary among farm animal species; this situation has direct consequences on the efficiency of protein deposition. The theoretical energetic efficiency of protein synthesis is about 80% (Pullar and Webster, 1977), but efficiencies may as low as 35% (Bergen and Merkel, 1991a). Work over the years has shown that the ratio of protein synthesis cycles to protein accretion (PS/PA) can range from a low of 2 to 4 to up to almost 10 in food-producing animals [from data summarized by Golián (1984) and Bergen and Merkel (1991a)]. As depicted in Figure 1, chickens and pigs exhibit a PS/PA of 2 to 4, whereas in sheep and cattle this ratio ranges from 7 to 9. Thus, costs of muscle deposition from a net energy cost perspective seem to be greater in ruminants than in nonruminants. This may be related to the principal types of muscle fibers present between these species; however this is not a well established concept. From the data in Figure 1 it may be reasoned that in species with generally higher proportion of type 2 intermediate and 2B fibers (white-glycolytic fibers; i.e., in chicken and pigs), the extent of protein remodeling during the growth period may be lower than in animals with a preponderance of type 1 and 2A muscle fibers (oxidative fibers; i.e., ruminants). Although there are no unequivocal data on the role of muscle fiber types on protein remodeling in growing food animals, during experimental fast-twitch (2X, 2B) fiber to slow-twitch (1A, 2A) fiber transitions in humans and rabbits, a putative increase in proteolytic capacity (based on increased abundance of mRNA for proteolytic genes) was noted (Sultan et al., 2001; Yang et al., 2006). Work is clearly needed to rigorously test this hypothesis supported by the data in Figure 1. Work over the last 25 yr with cattle and swine has, however, clearly shown that a diminution of protein breakdown (as a result of β-agonist use in pigs and use of hormonal implants, such as trenbolone acetate and estrogen implant combinations) can greatly improve production efficiency by affecting protein turnover (Buttery, 1983; Bergen and Merkel, 1991a,b; Hayden et al., 1992).

A remaining frontier of animal production research relates to delineating molecular mechanisms that may affect metabolic efficiencies. Clearly, some biological processes have a fixed thermodynamic efficiency, but many such processes in combination may exhibit variations across animals. If this is true, overall metabolic efficiencies may be amenable to genetic selection (Nkrumah et al., 2007).

TRACER KINETICS IN MEASURING PROTEIN SYNTHESIS AND BREAKDOWN

Overall quantitative aspects of protein breakdown and synthesis can be determined with isotope-tracer...
Protein degradation methodology

Table 1. Glossary of symbols and terms

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
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<tbody>
<tr>
<td>B</td>
<td>Protein mass or pool</td>
</tr>
<tr>
<td>C</td>
<td>Total tracer activity/enrichment in skeletal muscle (B times SA_B)</td>
</tr>
<tr>
<td>SA_i</td>
<td>Specific activity/enrichment of free amino acid</td>
</tr>
<tr>
<td>SA_B</td>
<td>Specific activity/enrichment of incorporated amino acid</td>
</tr>
<tr>
<td>RS</td>
<td>Rate of protein synthesis (mass per unit time) in B</td>
</tr>
<tr>
<td>RB</td>
<td>Rate of breakdown of protein (mass per unit time)</td>
</tr>
<tr>
<td>T</td>
<td>Total radioactivity or enrichment within a defined B</td>
</tr>
<tr>
<td>kS</td>
<td>Fractional protein synthesis rate (FSR)</td>
</tr>
<tr>
<td>kD</td>
<td>Fractional protein breakdown rate (FBR)</td>
</tr>
<tr>
<td>kA</td>
<td>Fractional protein accretion (net) rate (FAR)</td>
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kinetics methodologies. Net gain or accretion of body protein may be determined with tissue protein analyses from serial slaughter samples or with serial measures, regression-based approaches, and dye or deuterium dilutions (Byers, 1979; Anderson et al., 1988; Castro-Bulle et al., 2007). For work in human nutrition, whole-body protein turnover has been of interest to relate to AA needs, whereas in animal agriculture applications, the focus is more often on synthesis and degradation in tissues, particularly in skeletal muscles containing desirable cuts of meat (Bergen, 2007). Additionally, in human exercise and skeletal muscle atrophy-related research, protein synthesis and degradation are being studied intensely at the tissue level.

**Basic Principles of Tracer Kinetics for Protein Synthesis and Breakdown**

It is most convenient to define components and symbols and terms used in describing tracer kinetics; therefore, an appropriate glossary is provided in Table 1. Total protein mass in the whole body, tissues, or cells is a consequence of 2 independent bioprocesses, protein synthesis and protein breakdown such that change in protein mass = synthesis – breakdown (Waterlow et al., 1978; Zak et al., 1979).

Although total protein mass in an animal can be determined analytically, and daily changes in nitrogen status can be determined via an N balance study, such approaches do not suffice to determine protein synthesis and breakdown rates in tissues. Thus, Zak et al. (1979) define the relationship that (using designations from the glossary, Table 1) dB/dt = RS – RB, where dB/dt represents the net change in B, either as accretion when RS is greater than RB or loss when RB is greater than RS. In steady state, when dB/dt = 0, RS = RB. The rate of change in B can be determined with tracer kinetics (i.e., precursor-product relationship; Zilversmit, 1960) using 14C, 13C, 15N, 2H, or even 35S amino acid tracers with single dose (pulse) or continuous infusion experiments. For discussion purposes, the single-dose tracer administration technique can be used to demonstrate the principles of tracer AA kinetics in protein synthesis studies. Upon a single tracer dose, specific activity of the tracee will increase very rapidly in plasma and tissue free AA pools, and then also decline rapidly (Zilversmit, 1960; Zak et al., 1979; Figure 2). At the same time, some tracer is incorporated into the protein and the specific activity of that tracee AA will increase, and once peak labeling has been achieved, will decline. Then, $\frac{dS_{AB}}{dt} = k \left( \int_0^t S_{AI} \, dt - \int_0^t S_{AB} \, dt \right)$. Upon integration, this yields $dS_{AB}/dt = k (S_{AI} - S_{AB})$ or a standard precursor-product relationship (Zilversmit, 1960). These results also indicate that an increase in

![Figure 2. Changes in specific activity (or enrichment), after a single (pulse-dose) tracer amino acid injection, in the plasma or tissue free amino acid pools and the protein pool. The specific activity in plasma or tissue free amino acid pools is $S_{AI}$ whereas $S_{AB}$ is the specific activity of amino acid in the bound (B) or protein pool (Bergen et al., 1987). The arrow on the x-axis denotes start of injection/infusion.](image-url)
protein-specific activity is a consequence of de novo synthesis. In steady state, then $k = k_s$ or $k_D$.

Assuming near steady state early in a tracer isotope experiment, $SA_B$ is much less than $SA_I$ and $k_D = 0$, then $dS/dt = k_SSA_I$. Solving this equation for fractional protein synthesis rate ($k_S$, Table 1), $k_S$ equals $SA_B/SA_I \times t$ (Waterlow et al., 1978; Zak et al., 1979; Garlick et al., 1980; Reeds, 1992). The standard precursor-product relationship implies 2 ways of calculating $k$. During a period when the $SA_I$ is much greater than $SA_B$, $k_S$ can be calculated from the $SA_B$, provided that precursor pool $SA_I$ is also measured. This is precisely the approach of the flooding and continuous infusion methods (Garlick et al., 1973, 1980; Waterlow et al., 1978; Zhang et al., 2002). Further, in a single-pulse setup, when $SA_I$ is much less than $SA_B$ and $dB/dt = 0$, in theory $k_D$ may be determined from the decline in natural log of $SA_B$ after isotope administration has ceased.

Use of Rate Constant Designations in Protein Tracer Kinetics

It has been well documented that the rate of protein synthesis ($R_S$) is dependent on protein mass or product, nor kinetically influenced by substrate concentrations (AA); thus, protein synthesis has been likened to a constant amount process (an adaptation of a zero-order rate constant; Waterlow et al., 1978). The rate constant designation $k_S$ has been assigned, which is expressed as a decimal or a fraction of units/t or the fractional synthesis rate (FSR).

In contrast, protein breakdown is dependent on the protein mass (Schimke, 1970) and is, thus, a constant rate process similar to a first-order rate process or $k_D$. This constant may also be expressed as a decimal or fraction called the fractional breakdown rate (FBR). Similarly, net protein deposition rate (net deposited per unit time) can be expressed as a percentage of the whole protein mass and, therefore, as a fractional constant $k_A$ or fractional accretion rate (FAR). In combination, these rate designations can be combined into the relationship $k_A = k_S - k_D$ or FAR = FSR - FBR.

Although protein synthesis rate is not a function of protein mass or substrate concentration, the use of $k_S$ as a rate constant for mathematical purposes is valid in protein synthesis studies (Waterlow et al., 1978; Zak et al., 1979). During steady state, if C (or $B \times SA_B$) equals amount of isotope in the total protein pool B, then $dC/dt$ is the rate of incorporation of isotope from the precursor pool or $R_c \times SA_I$ (protein synthesis, zero order, constant amount process) minus loss by breakdown or $k_D$ $\times$ total isotope activity in protein pool B (here designated as C). Early in a tracer infusion experiment, tracer incorporation into protein will be negligible whereas $R_c$ is not affected by tracer infusions. Then to express $R_c$ as a fractional rate constant ($k_S$), both sides of the equation $dC/dt = (R_c \times SA_I - k_D C)$ may be divided by protein mass or B, resulting in $dSA_B/dt = (\text{synthesis rate} [R_c/B] - \text{breakdown rate} [k_D C])$ (Waterlow et al., 1978). But $R_c/B$ is the fractional rate of synthesis and can be likened to $k_S$. Because in steady state $k_S = k_D$, this equation can be rewritten as $dSA_B/dt = k_S (SA_I - SA_B)$, the standard precursor-product relationship (Zilversmit, 1960). For a more in-depth exploration of these kinetics, especially for application to growing (non-steady-state) animals, readers are encouraged to consult Atkins (1969), Steele (1971), Shipley and Clark (1972), Garlick et al. (1973, 1980), Waterlow et al. (1978), Zak et al. (1979), Bergen et al. (1987), Nissen (1992), Wolfe (1992), and Waterlow (2006).

Determination of FSR, FAR, and FBR in Muscle Foods Species: General Approach and Utility

In research with animals, it is often desirable to determine FBR, FSR, and FAR after a specific treatment or during differing physiological and dietary states. Generally speaking, FSR of all or individual proteins within a tissue can be determined using infusions of AA isotopes. To this end, a continuous infusion approach (Garlick et al., 1973), a flooding dose approach (excess tracee with tracer; Garlick et al., 1980), and a pulse dose approach (Zhang et al., 2002) to determine protein synthesis have been developed and are widely utilized. In many respects, the latter 2 methods are similar except that excess tracee AA are not used in the pulse-dose system of Zhang et al. (2002). In the continuous infusion approach, because $SA_I$ will take some time to plateau (or reach $SA_I$ max), such infusions may require greater time intervals and thus, the specific activity of the initial labeled tracee will be much lower before $SA_I$ max has been achieved. This may result in a significant error in the obtained value for $k_S$ for short-time tracer infusion studies. Garlick et al. (1973) developed appropriate mathematical formulas and procedures to overcome this deficiency. If continuous infusions are continued sufficiently long then a final tissue protein $SA_B$ can be divided by the $SA_{I \text{max}} \times t$ to obtain $k_S$ or FSR as customary for the flooding dose approach (Garlick et al., 1980). Long-term continuous infusion experiments require significant resources when applied to food animal studies; therefore, this procedure has been applied infrequently in food animal studies particularly with large, near-harvest animals (Garlick et al., 1976; Mulvaney et al., 1985; Bergen et al., 1987, 1989; Lobley et al., 1990; Rivera-Ferre et al., 2005). To obtain reliable FSR values, it is critical to accurately measure the applicable $SA_I$ or devise an experimental strategy such that the $SA_I$ remains nearly the same over the period of tracer incorporation into proteins. Again, this concept forms the basis of the flooding dose technique and this procedure has been applied by numerous workers across many laboratories. In most cases FSR values obtained by continuous infusion vs. a flooding dose are essentially identical (Cas o et al., 2006).

The FAR of total protein in a given tissue or specific proteins within tissues may be determined by dye or deuterium dilutions in vivo to measure total body water.
and derived body protein content, by serial animal harvests, muscle sampling and analysis or with a regression approach (Byers, 1979; McCarthy et al., 1983; Reeds et al., 1986; Anderson et al., 1988; Castro Bulle et al., 2007). As microanalytical techniques and new in vivo methods (such as imaging or dual-energy x-ray absorptiometry) to measure body composition become more available (Ellis, 1992, 2007; Mitchell, 2007), serial biopsies and direct muscle protein estimates may replace serial slaughter approaches to determine FAR.

In the relationship \( \text{FAR} = \text{FSR} - \text{FBR} \), determining 2 of the rates enables calculation of the third rate. This “2-out-of-3” approach has been applied differently to swine vs. cattle. Urinary excretion of a skeletal muscle protein marker amino acid, N-\(\gamma\)-methyl-histidine (NMH), may be used to determine FBR (Young and Munro, 1978). This approach (the urinary excretion of NMH) cannot be applied to pigs and some other species (Harris, 1981; Bergen et al., 1987), but can be used with cattle (Bergen et al., 1987), as well as humans and rodents (Young and Munro, 1978), and yields FBR values for individual animals (McCarthy et al., 1983; Hayden et al., 1992; Castro-Bulle et al., 2007). When studying how hormones and exogenous agents may influence protein turnover, researchers have often implemented a 2-out-of-3 approach in animal production-related research (Mulvaney et al., 1985; Reeds et al., 1986; Hayden et al., 1992; Castro-Bulle et al., 2007). For example, in our work with pigs we determined FSR and FAR and estimated FBR for skeletal muscle (Mulvaney et al., 1985; Bergen et al., 1989); whereas, with cattle we determined FBR and FAR and estimated FSR for skeletal muscle (McCarthy et al., 1983; Hayden et al., 1992). Any problems in obtaining the 2 experimentally determined values will be reflected in the calculated FBR or FSR. When using the FAR-FSR-FBR approach, such values reflect an overall composite rate for synthesis, breakdown, and accretion of all the mixed proteins in the applicable tissue and not of any individual classes of proteins (except for the NMH-based direct technique, which relates mostly to myofibrillar proteins in skeletal muscle). In this author’s view, these fractional rate values derived by difference need to be critically scrutinized as to whether excessive variations in FAR and FSR or FBR values have occurred. This may invalidate values (FBR or FSR) obtained by difference calculations. Other potential problems affecting fractional rate estimates may include estimation of tissue protein content and total body protein mass. Moreover, experimental approaches to obtain FAR, FBR, and FSR values really differ and while combined into a useful mathematical relationship, it should be realized that each of the fractional rates measured, as discussed previously, are not based on the same underlying assumptions. In addition, these constants should not be construed as reaction velocity constants (Waterlow, 2006). For example, FAR values are based on serial assessment of changes in whole body or individual tissue protein mass over days or months, FBR (in cattle) are based on NMH excretion for 1 or more days, whereas FSR values are based on tracer infusions of individual animals with short intervals, in which a “quasi” steady state (with respect to muscle protein mass) can be assumed. Additional isotopic tracer infusion approaches to directly measure FBR have been developed more recently where a quasi steady state may also be assumed (Rathmacher and Nissen, 1998; Zhang et al., 2002).

Whole-Body Amino Acid Kinetics

Infusions of isotopes can also be used to estimate an overall entry rate or flux (\(Q\)) of an amino acid in the whole animal from which protein synthesis and breakdown can be estimated. Such a strategy will reflect all tissues and protein pools, but skeletal muscle contains most of the protein mass in large animals. Liver and the gastrointestinal tract could also be major contributors to the data obtained. A 2-pool stochastic model of protein metabolism was initially proposed (for example, by Picou and Taylor-Roberts, 1969) and later, multiple pools and compartmental analyses were applied to whole human or large tissue (such as arm or leg muscle) pool AA kinetics (Wolfe, 1992). A 2-pool model is presented here to illustrate basic principles (Figure 3). After a tracer essential amino acid has been continuously infused and its plasma specific activity plateaued (frequent blood sampling required; Figure 3), then Flux (\(Q\)) is as follows: \(Q\) (moles AA/unit time) = \([\text{Infusion rate (mole/t)}]/[\text{Plaque specific activity (dpm/mole)}]\). Then, from the metabolic pool (Figure 3a), \(Q\) (flux) = all out (synthesis, \(S\) + excretion, \(E\)) = all in (breakdown, \(D\) + intake, \(I\)).

In the postabsorptive state, \(Q\) equals breakdown of the whole-body protein pool. Instead of sampling the circulation, \(Q\) may also be based on specific activity analysis of urinary end products of an infused tracer AA (Duggleby and Waterlow, 2005).

DIRECT MEASURES OF TISSUE PROTEIN BREAKDOWN

Tischler (1992) noted that tissue proteolysis may be measured directly in vitro with nonisotopic AA release in studies using incubated tissue preparations, by pulse-chase labeling in studies using isolated cells in culture, and by dilution of perfusate isotopic AA specific activities in perfused organs. Experiments with incubated tissues have been applied frequently to study comparative rates of protein breakdown in vitro with isolated strips of muscle. This in vitro strategy was extensively applied to study the effect of hormones and many other variables on tissue protein breakdown using phenylalanine or NMH release into the incubation medium (Goldberg et al., 1980; Skjaerlund et al., 1988, Tischler, 1992). Muscle strip incubations may also be used to measure protein synthesis; but irrespective of whether such strips arise from animals with a high FSR, \(R_9\) would almost always be less than the observed
Figure 3. A 2-pool model of whole-body protein metabolism (panel a) and plasma specific activity, SAi, upon continuous injection of a tracer AA vs. time (panel b). In panel a, the model includes a metabolic (entry or exit) pool, A, and a whole-body protein pool, B. Components entering the pool are AA arising from protein breakdown, D, and dietary intake, I. Components leaving the metabolic pool are AA incorporated into the protein pool, B (synthesis, S) and catabolism of AA and urinary excretion (irreversible loss, E). The rate of entry and exit of a given AA, or flux, Q, into the metabolic pool, can be assessed from a continuous infusion of tracer (panel b) when SAi reaches a plateau. Then Q (in moles of AA/time) equals moles of AA that enters (I, D) and amounts of AA that leaves (S, E) the metabolic pool, such that: Q = S + E = D + I. Because I and E of an AA can be measured, both S and D can be calculated (Bergen et al., 1987). The arrow on the x-axis denotes start of injection/infusion.

Protein breakdown rate may be estimated in vivo directly from the rate of loss of total isotopic label from equilibrium prelabeled protein or a mixture of proteins in an animal by measuring the rate of isotope release from the tissue proteins (Schimke, 1970; Reeds, 1992). For application in vivo, serial biopsies of desired muscle targets may be obtained after stopping isotope administration (or sampling of muscles after serial slaughter of animals), protein fractions may be isolated, isotope enrichment or specific activity quantified, and $k_D$ determined according to the formula $T_t / T_0 = e^{-k_D t}$ (Koch, 1962; Waterlow et al., 1978; Bergen et al., 1987), where $T$ is the total protein tracer-label content of the whole applicable tissue. There are 2 major limitations of this approach. First, the inability to counteract the effect of recycling of the tracer isotope in animal systems (even when so-called nonrecycled labels are used) leads to significant overestimates of $t_{1/2}$ and underestimates of the rate of breakdown. Second, it cannot be assumed that the kinetics of label decay for mixed tissue proteins are described by a single rate constant. If only short experimental periods are used, then decay of short $t_{1/2}$ proteins in mixed proteins may disproportionately influence the results even if they only represent a small fraction of total mixed proteins. Thus decay measures need to be continued for a sufficiently long timeframe to also include the slowest-decaying components. Other issues that make the procedure impractical include the number of animals required during the decay period, isotope expenses, and difficulties in measuring protein pool sizes over the experimental period. All these variables, which may be well controlled or measured in a muscle cell tissue culture system (Clark and Zak, 1981), severely curtail a general application of this approach to directly determine skeletal muscle protein breakdown rates in food-animal species.

Wolfe’s laboratory (Zhang et al., 2002) has recently proposed an improved isotope tracer infusion method to determine FBR. This procedure is based on pulse injection of 3 stable isotopes of a nonmetabolizable indispensible AA for muscle (usually phenylalanine) at 0, 30, and 55 min, respectively, in rabbits. Multiple arterial blood samples were obtained at intervals from 5 to 115 min after the injection of the first tracer and muscle biopsies were obtained at 5, 30, and 60 min (Zhang et al., 2002). The physiological basis of this approach will be described here, but detailed mathematical relationships and proofs are presented in Zhang et al. (2002). These researchers reasoned that the precursor-product relationship is usually only applied to movement of tracer from the tissue (muscle) free AA pool to the bound (protein) pool for determinations of FSR. However, the movement of tracee from the bound to the intracellular pool, in principle, is a precursor-product relationship (Zilversmit, 1960) useful to determine FBR. These workers further reasoned that in a physiological steady state, intracellular protein breakdown and movement of the tracee AA will result in an
enrichment gradient (during tracer infusion) between arterial blood and the intracellular pool. In a tracer study, it can be shown that in the absence of intracellular protein breakdown, enrichment in the intracellular free AA pool will be equal to the tracer enrichment in the arterial blood when an AA that is not metabolized in skeletal muscle (such as L-phenylalanine) is used as tracer. When the transport tracer/tracee from the arterial blood into the intracellular free AA pool is constant, dilution of enrichment of the tracer AA in the intracellular pool is strictly a function of intracellular protein breakdown (i.e., FBR). From here, Zhang et al. (2002) developed a set of equations to determine FBR and FSR. Importantly, this procedure does not require a tracer enrichment plateau nor precursor pool enrichments (the latter a requirement for the flooding dose procedure), which are more or less stable during the period of tracer incorporation. Additionally, blood flow measures often needed for compartmental kinetic approaches are not required. Zhang et al. (2002) measured arterial and intracellular enrichments in arterial blood and muscle biopsy samples, which sufficed for FBR calculations when the ratio of intracellular free tracee content to protein bound tracee content is known. For FSR, the enrichment of the bound pool was also required. Results for FBR and FSR were similar to estimates based on a previously validated 3-pool compartmental AA kinetics model (Zhang et al., 2002) and the FSR is comparable to FSR obtained from the flooding dose procedure. At this time, the Zhang et al. (2002) procedure has not been used in food animal research (Chinkes, 2005).

Skeletal muscle is a major tissue in whole-body protein metabolism. This is especially true in large animals (i.e., livestock) and humans compared with rodents. A novel AA, NMH, was isolated from actin and myosin (reviewed by Bergen et al., 1987) and it was reported subsequently that this AA arises upon posttranslational methylation of specific histidine residues in both major myofibrillar proteins (reviewed by Young and Munro, 1978; Bergen et al., 1987). Early work showed that NMH was released by intracellular proteolysis and excreted mostly unchanged in human urine (Bergen et al., 1987). Young and Munro (1978) recognized the potential of urinary NMH excretion as an index of muscle protein proteolysis, but with humans it is necessary to avoid all dietary sources of NMH (muscle foods) for urinary NMH excretion data to be valid. Furthermore, because creatinine excretion is a function of total muscle activity and mass, a urinary NMH to creatinine ratio could be used to express proteolysis as a fraction of protein mass. Three criteria were developed for the application of this marker AA as an index of protein catabolism: 1) NMH is present exclusively in skeletal muscle at a constant amount; 2) NMH released upon proteolysis is neither recycled into proteins nor metabolized, and 3) NMH is rapidly and quantitatively excreted in the urine (Young and Munro, 1978). Up to 20% of total urinary NMH excretion may be contributed from nonmuscle myofibrillar protein; however, over 90% of all body NMH is found in skeletal muscle (Afting et al., 1981; Harris, 1981; Bergen et al., 1987). In some species (e.g., pigs and sheep), NMH excretion does not follow muscle proteolysis because NMH is sequestered in the nonexcreted NMH-containing dipeptide balenine, whereas in chickens and mice, NMH seems to be metabolized before final excretion (reviewed in Bergen et al., 1987). To be able to obtain FBR, skeletal muscle protein pool size and NMH concentration in protein must also be determined or known (Bergen et al., 1987). Total daily NMH excretion may additionally be corrected by assuming that 20% of urinary NMH is from nonmuscle sources (at least in humans; Afting et al., 1981). Fractional breakdown rate is then calculated as daily NMH excretion × (total skeletal muscle NMH pool)−1 (Bergen et al., 1987).

Because of the chronic balenine problem in pigs (Harris, 1981), Rathmacher et al. (1996) and Rathmacher and Nissen (1998) developed a NMH tracer infusion compartmental analysis approach to determine NMH release from muscle protein that is applicable to humans, pigs, lambs, and cattle. The determined NMH release (typically micromoles/day) is then divided by whole-body skeletal muscle or myofibrillar protein pool for FBR estimates. The Rathmacher et al. (1996) approach does require serial blood samples to develop the exponential curve of NMH enrichment decline vs. time. This procedure has been used in some pig protein breakdown studies in recent years (Jones and Stahly, 1999; Yang et al., 2000). To date, no other molecules suitable as a marker for proteolysis that adhere to the criteria set by Young and Munro (1978) have emerged (Chinkes, 2005).

APPLICATION OF CONTEMPORARY GENOMIC REGULATION STRATEGIES TO STUDYING PROTEIN BREAKDOWN IN SKELETAL MUSCLES OF FOOD ANIMALS

Uses of classical approaches, discussed previously, to assess protein synthesis and breakdown in food animals are expensive (e.g., reagents, isotopes, and animals), and require suitable experimental animal facilities and skilled technical human resources. Thus, any of the classical isotope and marker AA-based approaches to follow protein breakdown do not lend themselves to studies with the very large numbers of animals required to assess a) genotype variations in protein metabolism efficiency for selection purposes and b) genotype by performance enhancer interactions in growth trials.

During the last 25 yr, knowledge about protein synthesis (Hershey, 1991; Proud, 2007) and degradation mechanisms have increased substantially (Goll et al., 2003; Reinstein and Ciechanover, 2006). Based on current understanding, calcium-activated proteases, the ubiquitin-proteasome system (UPS), lysosomes (to a limited extent), and other mechanisms such as autophagy are responsible for intracellular protein breakdown
Genomic tools are becoming more available and specific and is also possibly related to growth rate in poultry. a factor in lactation-related muscle protein breakdown demonstrates that transcription of UPS components is work of Clowes et al. (2005) and Harper et al. (1999) been widely explored and is not well understood. The healthy, fast-growing, food-producing animals has not sis is involved in skeletal muscle protein accretion in transcriptional regulation of muscle proteolysis and during disease-induced muscle proteolysis, the degree to which transcriptional regulation of muscle proteolysis is involved in skeletal muscle protein accretion in healthy, fast-growing, food-producing animals has not been widely explored and is not well understood. The work of Clowes et al. (2005) and Harper et al. (1999) demonstrates that transcription of UPS components is a factor in lactation-related muscle protein breakdown and is also possibly related to growth rate in poultry. Genomic tools are becoming more available and specific in skeletal muscle (Costelli et al., 2005; Finn and Dice, 2006). It has been well recognized that overall capacity of protein synthesis is a function of cellular ribosome content (Proud, 2007). Control of translation, however, appears to be principally accomplished by posttranslational mechanisms via endocrine and signaling cascades that mediate phosphorylation or dephosphorylation of initiation and elongation factors and (or) associated binding proteins of the ribosome-based protein synthesis system (Proud, 2007). The regulation of proteolysis in muscle appears more complex and is both dependent on transcription of genes for the proteolytic mechanism (Reinstein and Ciechanover, 2006) and on the inhibition and activation of cellular calpains and components of UPS (Goll et al., 2003, 2008). It has been suggested that capacity for proteolysis in cells is sufficient to result in rapid, total tissue dissolution unless proteolysis is restrained by inhibitory mechanisms (Goll et al., 2008). Contrariwise, during skeletal muscle atrophy and sepsis, expression of genes for the components of the intracellular protein breakdown mechanism is elevated (Lecker et al., 2004; Sacheck et al., 2007). It is not altogether clear why, during onset of muscle wasting with a putative substantial tissue stored proteolytic capacity (Goll et al., 2008), gene expression for UPS components is rapidly elevated unless the turnover rate of ubiquitin-proteasomal constituents is itself elevated during rapid phases of protein breakdown. Upon induction of sepsis or muscle atrophy, a parallel increase in gene regulation of inflammation-related transcription factors and expression of several genes associated with proteolytic mechanisms has been noted, but a direct relationship between up-regulation of these genes and increased proteolytic activity has not been demonstrated (Hasselgren et al., 2005). Researchers trying to delineate induction mechanisms of disease-induced skeletal muscle protein atrophy have shown a clear up-regulation of gene expression of UPS components with respect to cancer, renal failure, fasting or anorexia, and diabetes (type II)-related muscle protein wasting (Price, 2003; Lecker et al., 2004; Cao et al., 2005; Eley and Tisdale, 2007). From such studies, there is agreement that up-regulated expression and activity of UPS is involved in development of muscle atrophy (Price et al., 1996; Hasselgren et al., 2005; Nakashima et al., 2005; Wang et al., 2006; Wyke and Tisdale 2006; Sadiq et al., 2007). Although there is universal agreement that transcription is a primary regulator of UPS components during disease-induced muscle proteolysis, the degree to which transcriptional regulation of muscle proteolysis is involved in skeletal muscle protein accretion in healthy, fast-growing, food-producing animals has not been widely explored and is not well understood. The work of Clowes et al. (2005) and Harper et al. (1999) demonstrates that transcription of UPS components is a factor in lactation-related muscle protein breakdown and is also possibly related to growth rate in poultry. Genomic tools are becoming more available and specific.

swine and beef cDNA microarrays have been developed and validated (Collier et al., 2006; Tuggle et al., 2007). Although transcriptional profiling is experimentally well defined, changes in mRNA abundance for any gene do not necessarily imply regulation at the level of gene expression but reflect potential capacity for synthesis of a given protein (Carey and Smale, 2000).

Application of proteomics may also be useful in studying proteolytic mechanisms in food animals. One approach would be to study abundance of components of cellular protein breakdown systems using electrophoresis and mass spectrometry (Cummins et al., 2004; Xu and Peng, 2006). Assessing products of protein degradation (i.e., the degradome) is another proteomic approach (Schilling and Overall, 2007) that may be fruitful in quantifying degradation rates in food animals. Proteomic approaches are resource intensive and have not been widely used or validated to evaluate protein turnover in skeletal muscle. Clearly, new data and revelations on regulatory and quantitative aspects of protein breakdown may be expected in the future. The combination of muscle biopsies and laboratory assessment of protein synthesis and breakdown status will be a strategy to explore many unanswered questions in food animal protein metabolism.

**SUMMARY**

Traditional isotopic tracer and marker AA-based methods to quantify protein breakdown, synthesis, and accretion applicable to food-producing animals have been enumerated. Newer, well-developed methods based on modifications of traditional approaches have emerged during the last 10 yr but have not been widely applied to food animal research. Because protein breakdown and synthesis are energy-dependent processes, these processes are directly related to animal production costs. High-throughput methods are needed to study molecular regulation of protein turnover, and such understanding must be applied to regulate protein turnover during animal production. It is proposed that both transcriptional profiling and proteomic assessments should be applied to study molecular regulation of proteolysis to strengthen the livestock industry’s continued quest to enhance efficiency of muscle growth.

**LITERATURE CITED**


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