MEASUREMENT OF PROTEIN AND AMINO ACID METABOLISM

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I. INTRODUCTION

Amino acid and protein metabolism are inexorably linked. The whole body flux of most individual amino acids is positively correlated with their content in body protein suggesting that the dominant role of amino acids is protein synthesis (Lindsay 1982). However, amino acids are also oxidisable substrates, providing carbon skeletons for a range of energy yielding pathways. Some amino acids are potent secretagogues of metabolic hormones (Floyd et al. 1963), and variation in concentration of specific amino acids in cerebrospinal fluid is associated with appetite regulation (Tackman et al. 1990). Thus the metabolism of protein and amino acids is of significant practical interest.

Protein deposition is the balance between protein synthesis and degradation. Measurement has concentrated predominantly on synthesis; degradation being estimated by difference where concomitant estimates of deposition are available. Measurement of protein synthesis in vivo relies heavily on use of isotopically labelled amino acids as tracers.

This review summarises some of the technical issues associated with the use of specific amino acids as markers of protein metabolism. Discussion of applications will concentrate predominantly on models of protein metabolism in muscle of farm animals, the preceding paper by Young (1991) having addressed those issues specific to man.

II. METHODOLOGICAL CONSIDERATIONS

A simplified diagram of the flow of a specific amino acid between plasma, extracellular and intracellular pools, and protein in a specific tissue is shown in Figure 1a. Even simpler models (Fig 1b,c) have been used to estimate protein synthesis in tissues. Such models ignore the heterogeneity of rates of protein synthesis and breakdown which occur in different organs and tissues, and within cells, and impose limitations as to the specificity with which different aspects of protein metabolism may be studied. Thus, in vivo estimates of protein metabolism represent the flows through all pools in the organism or tissue under study, and are relatively blunt tools with which to study specific aspects of control. Nonetheless they are useful for studies of nutritional, endocrine, genetic and disease effects on protein metabolism.

(a) Choice of amino acid

For estimation of protein synthesis an amino acid tracer should have the following properties:-

1. A high ratio of protein bound to intracellular free amino acid content (R). Underestimation of protein synthesis due to degradation and hence recycling of label increases as R values decrease (Muramatsu et al. 1987).
2. Similar concentration of free amino acid in intracellular, extracellular and blood plasma pools, to reduce errors in estimation of tissue free amino acid specific radioactivity (SRA) from dilution by extracellular free amino acids, and to permit indirect estimation of precursor SRA.
3. Not be metabolised in the tissue under study or if metabolised be so by well defined pathways with quantifiable degradation products.

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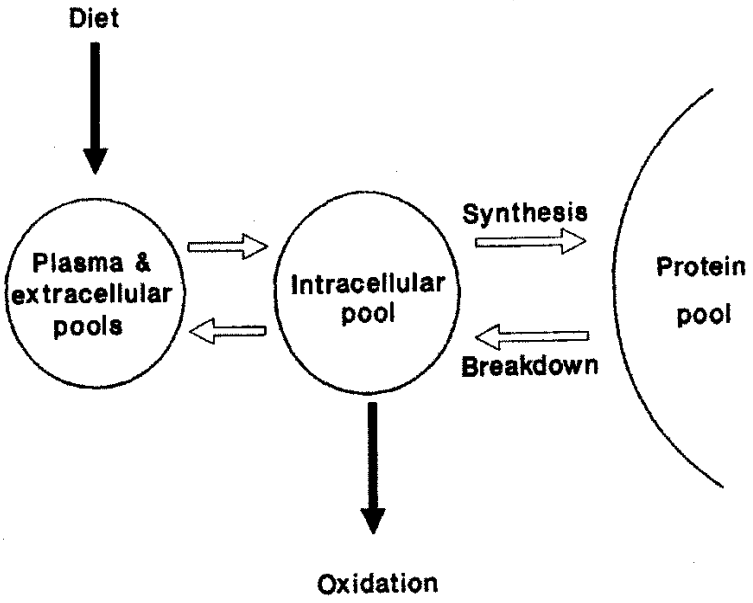
Solubility is important when using flooding dose methodology, to permit rapid injection of a sufficiently large dose to raise SRA in all pools to that of the plasma pool. However, the amino acid should not perturb protein metabolism, at least over the measurement period.

Figure 1
(a) 3 pool model of amino acid transfer to tissue protein (from Golden and Waterlow 1977).



(b) Simplified 2 pool model used to calculate non steady state changes in protein synthesis from measurements solely of plasma pool. This assumes no change in size of protein pool (Wootton 1985).

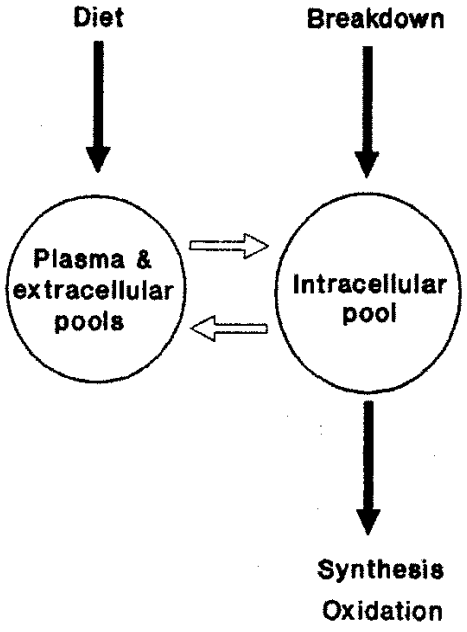
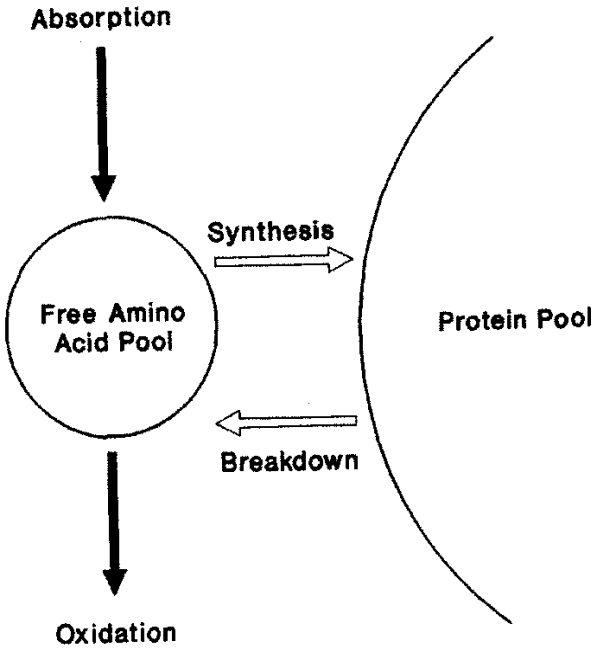


Figure 1
(c) Simplified 2 pool model used to calculate protein synthesis by measuring extent of labelling of tissue protein relative to an estimate of precursor labelling (Muramatsu et al. 1987).



Characteristics of some amino acids likely to be most useful for muscle are shown in Table 1. Phenylalanine (Phe) is not metabolised in muscle (Harris et al. 1991) and there appears to be no net transfer to muscle from the red cell (Jefferson et al. 1977). Leucine (Leu), like the other branched chain amino acids isoleucine (Ile) and valine (Val) is transaminated in muscle to its corresponding oxo-acid. The oxo acids may be subsequently oxidised in muscle, although the major sites of oxidation of these acids are the liver and kidney. Leucine uptake by muscle may involve transfer from the red cell, for Barrett et al. (1987) reported higher net Leu uptakes across the muscle of dogs when calculated from whole blood compared with plasma. The neutral essential amino acids referred to above are carried by the Na^+ independent L-transporter system (Christensen 1990) and are not concentrated within the cell. Although the branched chain amino acids are metabolised in muscle the degradation products can be measured and accounted for in calculation of protein metabolism (Nissen and Haymond 1981).

(b) Precursor pool problem

Protein synthesis is calculated from the ratio of protein bound label to that of the precursor amino acid following administration of tracer (e.g. Fig 1c). The precursor SRA, or in the case of stable isotopic tracer, enrichment, is that of amino acyl-tRNA bound amino acid at the time of recruitment to the ribosomal complex. However, accurate measurement is difficult. The extremely low concentration of the specific amino acid-tRNA in a tissue (eg 0.5-1 nmol valyl-tRNA/g rat liver) and the subsequent difficulty of extraction, are less severe constraints than the rapid turnover of the amino acyl-tRNA complex (half life ~0.3-2 secs, Airhart et al. 1974). Thus it is impossible to obtain a direct measure of the SRA of amino acids bound to tRNA without exchange with other free amino acids in the cell. In addition, there is evidence, albeit conflicting, of compartmentalisation of amino acid pools, and differential charging of tRNA from pools of differing SRA at least within liver cells (Mortimore et al. 1972; Airhart et al. 1974). Several

approaches have been developed to overcome the problem of identity and measurement of the precursor pool SRA.

Table 1.

The ratio of blood to plasma concentration (B/P) of the neutral amino acids valine (Val), isoleucine (Ile), leucine (Leu), tyrosine (Tyr) and phenylalanine (Phe) in adult sheep. Also shown is the ratio of concentration in intracellular water of ovine hind limb muscles to plasma concentration (I/P), and the ratio of content of amino acid bound to protein to the amount of amino acid in intracellular water (R). Values shown are means ±SEM, together with solubility (sol) of the same amino acids (g/L) at 25°C.

	B/P	I/P	R	sol *
Val	0.94 ± 0.04	1.41 ± 0.12	521 ± 54	88.5
Ile	0.93 ± 0.05	1.44 ± 0.16	878 ± 80	41.2
Leu	1.09 ± 0.07	1.31 ± 0.07	1047 ± 105	24.3
Tyr	0.90 ± 0.08	2.15 ± 0.25	527 ± 30	0.45
Phe	0.99 ± 0.04	1.53 ± 0.09	738 ± 68	29.6

unpublished data of Oddy, V.H., Warren, H.M. and Jones, A.W.
* from Merck Index

III. ATTEMPTS TO OVERCOME THE PRECURSOR SPECIFIC ACTIVITY PROBLEM

(a) Flooding dose method

Henshaw et al. (1971) suggested the use of a large, or flooding, unlabelled dose of amino acid in conjunction with label, to increase specific activity in all pools of the body to that of the plasma pool. Leucine (McNurlan et al. 1979), and Phe (Garlick et al. 1980) were initially used to overcome the precursor pool identity crisis. The procedure involves injection of ~20 times the body pool of free amino acid, and sampling within 10-20 mins of injection. Large quantities of tracer are needed to incorporate measureable quantities in tissue protein within the time span of the measurement. In addition, one complication is that large doses of amino acids may themselves perturb protein metabolism and thus alter the measurements being made. Leucine, for example, is a potent secretagogue of insulin (Floyd et al. 1963), and can both increase protein synthesis and decrease degradation in rat muscle (Buse and Reid 1975). However, McNurlan et al. (1982) failed to detect direct effects of Leu on protein synthesis in rats whilst measuring rate of protein synthesis by flooding dose. Phe was thought not to directly alter protein synthesis, and became the preferred amino acid for flooding dose estimation of protein synthesis, although recent studies in lambs indicate a transient increase in plasma insulin following a large dose of Phe (Lobley et al. 1990). In small animals this methodolgy has provided considerable progress, but the relatively low solubility of Phe, and the high cost of label has until recently precluded the use of this methodology with farm animals.

This limitation has in part been overcome by Attaix and colleagues (Attaix et al. 1986; 1988) who have used Val as a label in pre ruminant lambs. In larger animals, other amino acids, Leu (Harris et al. 1986), proline (Pell and Bates 1987) and Phe (Lobley et al. 1991) have been

used. Another modification of flooding dose methodology for use in large animals has been to increase the time between injection and sampling. Typically the period between injection of label and sampling is increased from 10 to 60-100 mins thus decreasing the requirement for label by 5-10 fold. However, equilibration of intracellular and blood pools may not be attained by this modification. Moreover, elevated intracellular concentration reduces the R value, which in turn increases errors due to recycling of label which increase with time (Muramatsu et al. 1987). The recent studies of Lobley et al. (1991) suggest that optimisation of the Phe flooding dose methodology for muscle (a tissue of slow turnover) by use of 15 times the estimated free Phe pool in the body is insufficient to provide accurate estimation of precursor pool SRA, and thus protein synthesis in tissues of higher turnover (skin, liver, rumen wall and small intestine).

The flooding dose procedure has recently been developed for serial measurement of protein synthesis, through use of stable isotopes and serial biopsy in man (Garlick et al. 1989) and sheep (Lobley et al. 1990).

(b) Reciprocal labelling

An alternate approach to overcoming measurement of precursor pool SRA takes advantage of labelled branched chain amino acids (BCAA), which are transaminated in a range of tissues, and measurement of the SRA of the corresponding oxo acid (reciprocal labelling). This may or may not be combined with measurement of arteriovenous difference of labelled amino acid across a tissue bed to provide simultaneous estimates of protein synthesis, gain and degradation.

Reciprocal labelling capitalises on the intracellular localisation of the branched chain amino transferase and oxidase enzymes and assumes that intracellular amino acids exchange freely with oxo acids. The SRA of the oxo acid of leucine 4-methyl-2-oxopentanoic acid (MOP) has been used to estimate the intracellular SRA of Leu (Rennie et al. 1982; Schwenk et al. 1985).

Although this technique is commonly used in man, it is unlikely to be valid in ruminants, and sheep in particular. Ruminants have 5-10 fold lower rates of transamination of BCAA than do monogastric species (Lindsay 1980; Busboom et al. 1984; Nissen and Ostaszewski 1985). This species difference is not simply a function of ruminant digestion, as inter-conversion of Leu and MOP in muscle and whole body of pre ruminant (milk fed) lambs is less than 25% that of non ruminant species (Oddy and Lindsay 1986; Oddy et al. 1987). In muscle of pre ruminant lambs the SRA of ^{14}C-Leu in tissue protein after infusion of 1-^{14}C Leu, is approximately double that of SRA of ^{3}H-Leu in tissue protein incorporated from (4,5-^{3}H) Leu derived from intracellular conversion of (4,5-^{3}H) MOP to (4,5-^{3}H) Leu. Variation in label exchange between Leu and MOP indicate that Leu and MOP pools mix less at high rates of net Leu uptake by muscle, than at low rates (Table 2). Clearly, reciprocal labelling of MOP from Leu is not a suitable means to estimate the SRA of the precursor pool, and thus provide an accurate measurement of protein synthesis, at least in ruminant species.

(c) Arteriovenous difference

Protein metabolism can be estimated from exchange of labelled amino acids across a defined tissue bed. This method offers several advantages over other procedures. Protein degradation and gain may be estimated at the same time as protein synthesis, and serial measurements of protein metabolism are possible. Hence the method can be used for measurement of short term effectors of protein gain and degradation not possible with tissue biopsy procedures. The procedure however is technically demanding. Ideally the arteriovenous difference (AV) method requires a labelled amino acid which is not metabolised, except for incorporation into protein in the tissue under study. Parameters of protein metabolism by the AV method are calculated from mass (gain) and isotopic transfer (synthesis and degradation), although an estimate of precursor pool SRA is still required. Amino acids which are metabolised in the tissue can be used provided that end products of metabolism can be measured. Thus Leu, which is transaminated and decarboxylated in muscle can be used as a marker provided that alteration in

SRA of CO_2 and MOP across the tissue are measured and accounted for in the calculations (Nissen and Haymond 1983; Oddy and Lindsay 1986).

Table 2.

The ratios of $^{14}C/^{3}H$ leucine in isolated muscle proteins from milk fed lambs, compared to intracellular Leu SRA derived from simultaneous infusion of (1-^{14}C) Leu and (4,5-^{3}H)MOP. Because ^{3}H Leu is derived predominantly from transamination from (4,5-^{3}H)MOP in muscle cells, the ratio of the protein bound to intracellular free Leu derived from each label should indicate the extent of mixing of plasma derived Leu with the intracellular Leu and MOP pools. If the Leu and MOP pools were completely mixed before protein synthesis the ratio would be 1. The table also shows the corresponding net Leu uptake and ratio of MOP/Leu flux in muscle (Oddy 1986).

^{14}CLeu/^{3}HLeu bound/free	Net Leu uptake nmol/g/min	MOP/Leu flux in muscle
3.79	3.2	0.25
2.45	2.7	0.19
1.65	2.65	0.45
1.45	1.6	0.375
1.10	0.8	0.54

However, the complexity of measurements needed (Leu, MOP and CO_2 concentration and SRA) reduces the utility of this approach for large scale studies. Phe is not transaminated or converted to Tyr in muscle (Harris et al. 1991) thus simpler kinetic models may be used for calculation of protein synthesis and degradation (see Barrett et al. 1987). However, the Phe content of muscle protein is less than 50% that of Leu making AV difference of Phe more difficult to quantify.

Sources of error not present in tissue analysis include those introduced by heterogeneity of protein metabolism and blood flow in the tissue under study. Arteriovenous anastomoses lead to arterial blood bypasssing the tissue bed, and increase SRA in venous blood. Consequently, venous SRA may overestimate intracellular SRA. It is also possible that net amino exchange with the tissue under study may not be exclusively via free amino acids. McCormick and Webb (1982) and Jois et al. (1985) indicated significant net difference of peptide amino acids across the hind limb. The methods for measurement of peptide amino acid exchange are crude, and there have been no studies of labelled peptide exchange which would convincingly indicate a role for peptide exchange across muscle, although the possibility cannot be excluded. Nonetheless, estimates of specific muscle protein synthesis rates obtained with flooding dose methods, differ from estimates simultaneously obtained using the arteriovenous difference technique (Lobley et al. 1991; Harris et al. 1991).

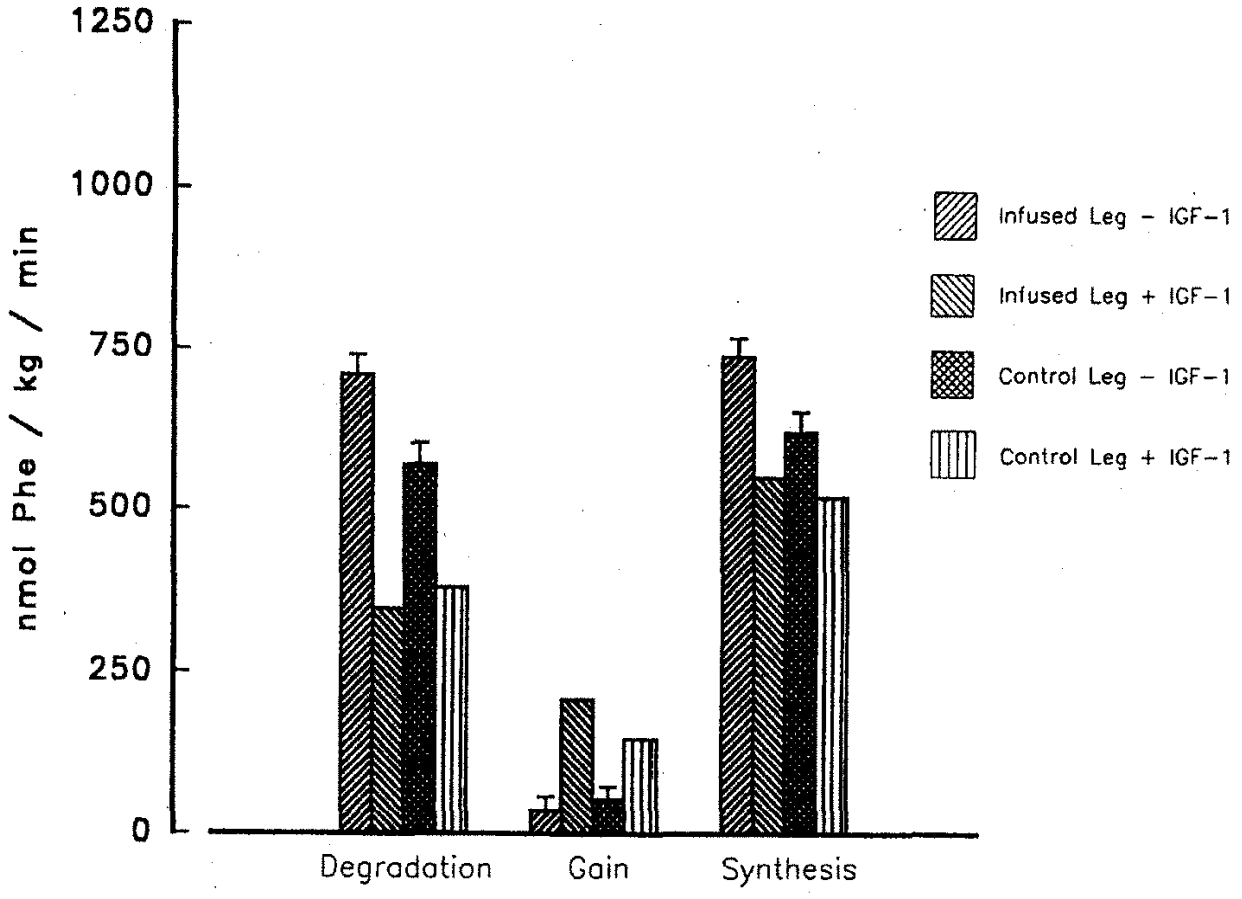
(d) Applications

We have used the AV method in conjunction with the muscle preparation developed by Domanski et al. (1974) to investigate effects of insulin and IGF-1 on muscle protein metabolism in lambs (Oddy et al. 1987; Oddy et al. 1991). These studies have shown the importance of protein degradation in regulating muscle protein gain in young sheep and lambs. This is illustrated in

Figure 2 which shows that infusion of IGF-1 into lambs fed a maintenance diet, increased protein gain through a reduction in protein degradation; protein synthesis tended to decrease.

In addition to measurement of protein metabolism in muscle by the AV method, it is possible to obtain simultaneous estimates of some parameters of protein metabolism in the whole body. In studies of the physiological consequences of selection for and against weaning weight we have shown that sheep selected for weight at weaning have increased body protein gain per unit feed intake, associated with decreased rates of protein degradation in muscle and the whole body (Oddy et al. 1989).

Figure 2
Effect of IGF-1 infusion (15 μg/kg/hr) for 4 hrs into lambs fed a maintenance diet.
The IGF-1 was infused into the femoral artery of one hind leg, the other leg was used as control.
-/+ IGF-1 = measurements made prior to and during infusion respectively
(data from V.H. Oddy and P.C. Owens).



IV. CONCLUSION

Limited specificity of protein turnover studies using amino acids as labels restricts application to study of effects of large scale modifiers of protein metabolism and growth. Identification of the points controlling protein metabolism are closer to resolution, not through studies of protein turnover at the level of aggregation described above (predominantly translational), but by study of gene expression (transcription). The methods described above provide valuable tools to isolate and identify aspects of control of protein and amino acid metabolism, and to study the gross effects of likely controlling agents. They do not provide the resolution necessary to show, by themselves, where such control may lie.

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