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Whole-body protein and amino acid turnover in man: what can we measure with confidence?

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Measurements of whole-body protein turnover are made either to assess the actual rate of one or more of the components of protein and amino acid turnover in a particular set of circumstances quantitatively, or to evaluate the qualitative responses to some administered stimulus (nutrient, hormone etc.). The achievement of either of these objectives still remains problematic despite considerable efforts to investigate and improve the methodology. Many of these problems are well known, having been reviewed on several occasions (Waterlow *et al.* 1978; Garlick & Clugston, 1981; Bier *et al.* 1985; Millward & Rivers, 1988). Since we are currently engaged in studies in which we have deployed several stable isotopic methods simultaneously, the results of these studies enable us to illustrate most of the major problems which are still outstanding and to address the question: What can we measure with confidence?

THE REGULATION OF WHOLE-BODY PROTEIN HOMEOSTASIS IN NORMAL
HUMAN ADULTS

We are currently investigating the model for protein homeostasis and amino acid requirements proposed by Millward & Rivers (1988). In this model dietary amino acids are shown as serving two different functions, a substrate role for protein synthesis and all other metabolic transformations, and a regulatory influence, which we call the anabolic drive, on many of the individual pathways involved in their utilization (Millward & Rivers, 1989). We have argued that even though the organism does not tolerate sustained increases in tissue concentrations of most of the indispensable amino acids, oxidizing them rapidly, amino acids may exert transient regulatory influences after feeding, before their oxidation. The intake needed to exert this influence should be considered to be part of the requirement for amino acids even though it is oxidized.

The model also takes account of diurnal patterns of feeding with gains of body protein on feeding and post-absorptive losses. We predicted that with increasing habitual protein intake, the diurnal cycling of body protein would involve increasing fasted losses necessitating increasing protein intakes to allow sufficient fed state gains to balance such losses: i.e. the amplitude of diurnal cycling would increase. Thus, protein requirements for overall balance would be determined largely by the level of habitual intake (Millward, 1989, 1990; Millward & Rivers, 1989; Millward *et al.* 1989, 1990).

Nitrogen balance studies in subjects fed at three levels of dietary protein (0.35, 0.75 and 1.5 g protein/kg) for 2 weeks have confirmed the nutritional sensitivity of this diurnal cycling (Price *et al.* 1990). Our current objectives are to confirm and explore the mechanisms of this diurnal cycling in terms of the extent and relative importance of the changes in protein synthesis, degradation and amino acid oxidation. We have used several currently used isotopic approaches simultaneously, i.e. primed constant intravenous infusions of [^{13}C -1]leucine, [^2H (D5)]phenylalanine (D5 phe) and [^2H (D2)]tyrosine (D2 tyr) as well as [^{15}N] glycine, with measurements of carbon dioxide production rates, the extent of $^{13}\text{CO}_2$ enrichment in expired CO_2 , and the enrichment of ^{15}N in urinary urea and ammonia as well as the isotopic abundance in the plasma amino acids and their derivatives, i.e. α -ketoisocaproate (KIC) the transamination product of leucine. The combination of measurement of amino acid oxidation rates and N excretion allows calculation of whole-body protein turnover by end-product methods as well as precursor methods. In the present context this experimental protocol allows a considerable degree of internal methodological validation and it is this aspect which will be emphasized in the present paper. In what follows the results of these measurements will be examined, namely amino acid oxidation to assess whole-body balance, whole-body protein turnover and the mechanisms of diurnal changes in body protein. Since the problems which will become apparent are common to all three questions, their explanations and implications will be discussed at the end.

MEASUREMENT OF AMINO ACID AND N BALANCE

The first question we have examined relates to the nutritional sensitivity of post-absorptive losses. A key feature of the Millward & Rivers (1988) model is that the influence of protein intake on rates of amino acid oxidation persists beyond the immediate feeding period into the post-absorptive state and this results in nutritionally sensitive diurnal cycling. This does appear to occur in terms of N balance (Price *et al.* 1990).

The limitations of the N-balance method have been reviewed on several occasions (Hegsted, 1976; Young, 1986). The major problems include accounting for all the different routes of N loss, persistent overestimation of N balance with unrealistically high apparent positive balances in adults and a lack of sensitivity and precision. One biological problem which can be accounted for is the changing size of the body urea pool. In our subjects fed on a high-protein diet, the losses from the body urea-N pool in a 12 h post-absorptive period accounted for on average 24% of the observed total N loss with individual values as high as 46%, with gains of similar magnitude in the post-prandial state. Without correcting for these changes the distribution of N losses between the post-absorptive and post-prandial phase of the diurnal cycle would be markedly in error, with an underestimate of post-prandial and an overestimate of post-absorptive losses.

Table 1. *Potential problems relating to ^{13}C isotopic measurements of amino acid oxidation rates*

A. Practical problems (i.e. soluble through appropriate additional studies)
1. The extent of isotope retention as bicarbonate
2. Measurement of carbon dioxide production rates
3. The amount of tracer needed which may negate the assumption of a true tracer
4. Variation in baseline tracer enrichment, requiring 'isotopically cold' runs in feeding experiments
5. Liberation and quantitative excretion of labelled carbon as CO_2
6. Oxidation rates as a tracer for overall N excretion influenced by mismatch of food and body tissue amino acid composition
B. Model problems (less tractable and possibly insoluble)
7. The relationship between measured and true precursor amino acid enrichment

Isotopic studies of amino acid oxidation particularly based on ^{13}C have been proposed as an alternative to N balance (Young, 1986). For an indispensable amino acid with a small highly-regulated free pool, if the intake and oxidative loss are measured accurately then the balance of that amino acid is assumed to be protein. If the body protein content of that amino acid is known, the change in balance of the amino acid can be converted to a change in protein or N balance, or both, assuming that the liberated N is excreted and not sequestered in some form. In the case of leucine and phenylalanine these assumptions can be examined to some extent. Many of the kinetic assumptions and isotope-related problems associated with this approach are well known (Bier *et al.* 1985) and were reviewed by Millward & Rivers (1988). The main problems are listed in Table 1.

This list of problems is by no means exhaustive, but has been separated into those problems which are ultimately soluble and those which are currently less tractable. Problem 1 can be avoided by measurement in each study and the importance of this is discussed on p. 209. Problems 2–4 have been discussed previously (Millward & Rivers, 1988). Problem 5 can probably be ignored for leucine and most carboxyl-labelled amino acids but possibly not for threonine. This exhibits unrealistic positive balances (Zhao *et al.* 1986) because of substantial fixation as glycine (Balleve *et al.* 1990). Problem 6 is of central importance when the oxidation rates are measured as a tracer for overall amino acid oxidation and N excretion is discussed on p. 200. Problem 7 relates to the 'model' problems resulting from an inadequate understanding of the kinetic model under analysis and consequent uncertainty of the relationship between the isotopic abundance in the measured species (e.g. plasma amino acid) and the actual precursor for the process under study (protein synthesis or oxidation, or both). In fact it is unlikely that for humans this problem can ever be solved. However, there are several ways in which model assumptions can be empirically tested.

One way of testing model assumptions is to compare rates of amino acid oxidation with measured N excretion.

[^{13}C]leucine oxidation studies. The kinetic model assumed for the first tracer we have used, leucine, is shown in Fig. 1. During a primed constant infusion the enrichment of the leucine in the free pool achieves a constant plateau value which can be measured in sampled plasma (or urine when plasma sampling is difficult as with preterm infants (De Benoist *et al.* 1984; Kandil *et al.* 1991)). The advantage of leucine is that the enrichment of its α -keto acid (KIC) can be measured in the plasma, and since this must be derived

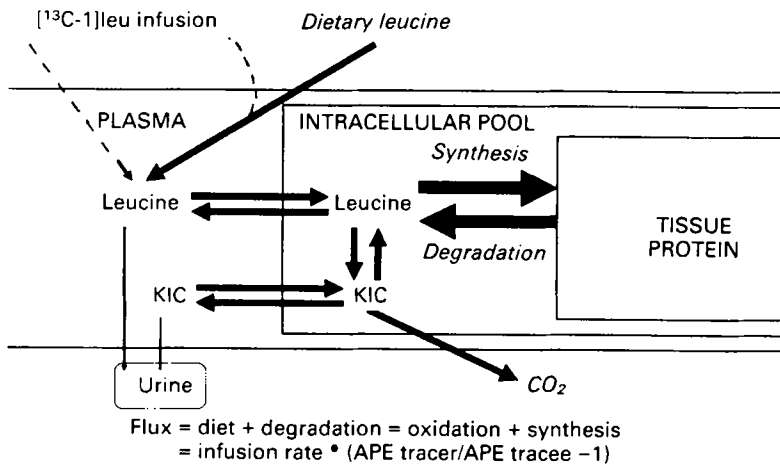


Fig. 1. The kinetic model assumed for leucine. KIC, α -ketoisocaproate; APE, atoms % excess.

from transamination of intracellular leucine it can be assumed to give a better estimate of the true precursor enrichment for both flux and oxidation. The leucine oxidation rate in the fasted state is assumed to reflect leucine liberated from protein degradation. Its loss should be accompanied by an equivalent amount of oxidation of the other amino acids liberated from protein resulting in N excretion. The amount of this can be calculated from the leucine:N value for whole-body and tissue protein. The values used by us are the mean values for muscle and liver given in the food tables (Paul & Southgate, 1978).

During feeding the situation is different and more complicated. Some of the food protein is deposited, the rest oxidized. To predict N excretion the leucine:N value for the oxidized food fraction must be known. This can differ substantially from that of body protein. When the leucine:N value in food is higher than that in tissues, then, following any net deposition which has occurred, the amino acid mixture left to be oxidized will be even more enriched with leucine. In this case the leucine oxidation could markedly overestimate the N excretion. Fortunately the ratio in the food oxidized can be calculated from the measured rate of leucine oxidation. Thus, the leucine balance (intake-oxidation) is converted into an associated N balance and this N is subtracted from the food N to determine the excreted N predicted from the leucine oxidation. As an example of this in our studies the leucine:N value for food ranged from 4.13 to 4.75 $\mu\text{mol}/\text{mg}$ N compared with an estimated 3.92 $\mu\text{mol}/\text{mg}$ N for tissue protein. In the oxidized fraction the calculated ratio increased to values as high as 5.57 which would have meant a 42% overestimation of the N excretion were it not recognized. In fact for the complete data-set of fed state leucine oxidation rates, N excretion would have been overestimated by on average 20% without correction for the food leucine content. In the past this problem has not been recognized (Golden & Waterlow, 1977).

Fig. 2 shows the relationship between measured N excretion in the fed and fasted state and the ratio, N excretion predicted from leucine oxidation:the measured N excretion. A value of 1 would imply perfect agreement. After the correction for food leucine content

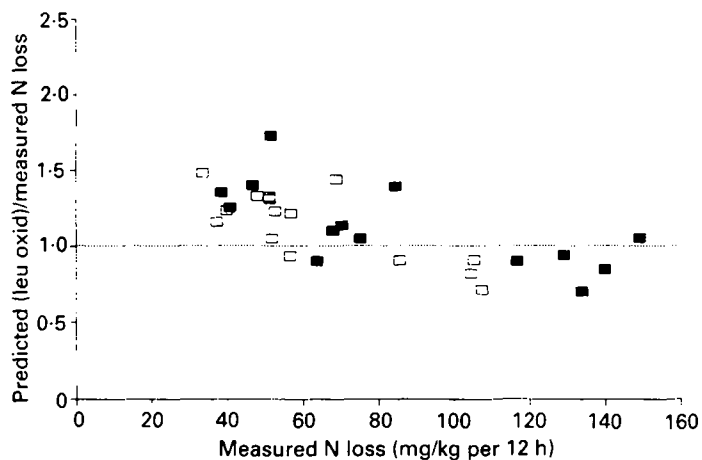


Fig. 2. The relationship between measured nitrogen excretion in the fed (■) and fasted (□) state and the ratio, N excretion predicted from leucine oxidation:the measured N excretion. A value of 1 would imply perfect agreement. Leucine oxidation and N balance were measured in normal adults after a 2-week period of adaptation to daily intakes of 0.35, 0.75 and 1.5 g protein/kg. The 12 h N loss was calculated from N excretion corrected for changes in the body urea pool calculated from plasma urea concentration and estimates of body water.

any residual difference between predicted and actual N excretion must reflect other errors. If average values are examined the agreement is not too bad (overall ratio 1.13 (SD 0.25)) but there is an obvious intake-related trend from underestimation at high intake (0.92 (SD 0.2)) to a marked overestimation at low intakes (1.34 (SD 0.19)). This is the first and most important indication of a major problem which as we shall see has considerable ramifications.

[²H (D5)]phenylalanine hydroxylation (phe(OH)) studies. Phenylalanine is an alternative to leucine, with the advantage that its flux and oxidation can all be measured by plasma sampling, with no need for CO₂ measurement or isotope-ratio mass spectrometry (Clarke & Bier, 1982; Thompson *et al.* 1989). However, the model assumptions in this case are particularly problematic. The oxidation component of the phenylalanine flux (Fig. 3) is phe(OH) to tyrosine and can be measured from the appearance of isotope in tyrosine during the infusion. With ring-deuterated phenylalanine (D5), D4 tyrosine (D4 tyr) will accumulate. However, the rate of that accumulation will also depend on the tyrosine flux (Q_t) which must also be measured. This can be calculated from the phenylalanine flux assuming a phenylalanine:tyrosine value for tissue protein, but the rate of phe(OH) must be known. For these reasons it is better to measure it by simultaneous infusion of D2 tyr. If the D4 tyr pool is primed whilst priming the D5 phe and D2 tyr then the phenylalanine flux can be measured and resolved and the Q_t also measured. Tyrosine oxidation cannot be assessed in this model.

The model problem in this case is more severe than for leucine since there is no plasma amino acid derivative which might indicate precursor pool enrichment. In addition the calculation of hydroxylation involves a formula which has two 'correction factors' for

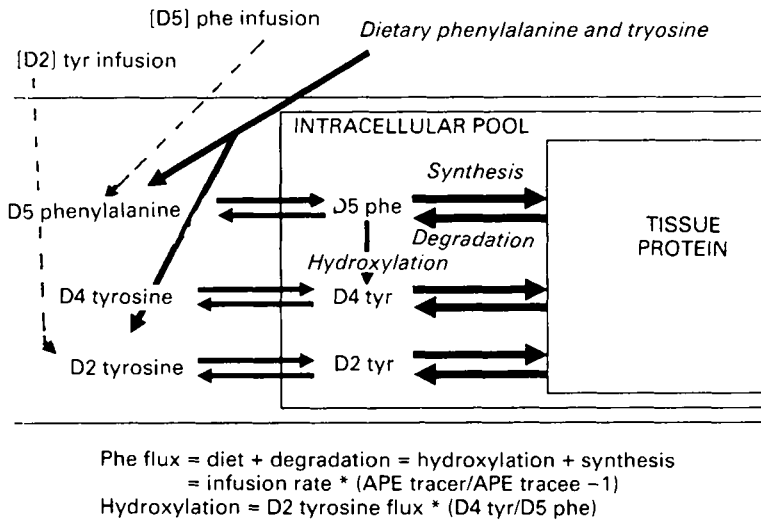


Fig. 3. The kinetic model assumed for phenylalanine and tyrosine. The oxidation component of the phenylalanine flux is phenylalanine hydroxylation to tyrosine and this is measured from the appearance of isotope in tyrosine during the infusion. Phenylalanine and tyrosine kinetics can be evaluated by primed constant intravenous infusion of [^3H](D5) phenylalanine and [^2H](D2) tyrosine with priming of the [^2H](D4) tyrosine pool (Thompson *et al.* 1989). APE, atoms % excess.

precursor compartmentation which are multiplied together so compounding any error. The formula for calculation of pheOH from the Q_t , the atoms % excess (APE) of D5 phe and D4 tyr is

$$\text{phe(OH)} = Q_t \times \frac{\text{D4 tyr}}{\text{D5 phe}} \quad 1$$

It can be assumed that Q_t calculated from plasma D2 tyr will be an underestimate due to the real precursor being lower than the plasma value. For leucine the KIC:plasma leucine APE value is usually about 0.8 implying the 'plasma' flux will underestimate the true flux by 0.8. The magnitude of this factor for tyrosine (which we can call tyr(f)) is unknown. In addition the D5 phe value which is the hepatic phenylalanine pool APE may be lower than the measured plasma value by an unknown amount (which we can call phe(h)). However, the D4 tyr which is measured in the plasma originates from the hepatic D4 tyr pool and should have the same enrichment. Thus, no correction of this value is required. This means that

$$\text{true phe(OH)} = \text{measured phe(OH)} \times \frac{1}{\text{tyr(f)} \times \text{phe(h)}} \quad 2$$

How can these factors be determined? Tyr(f) can to some extent be evaluated by taking advantage of a phenomenon identified by several authors (Waterlow *et al.* 1978; Garlick & Clugston, 1981; Bier *et al.* 1985) which relates to impact of such an error on

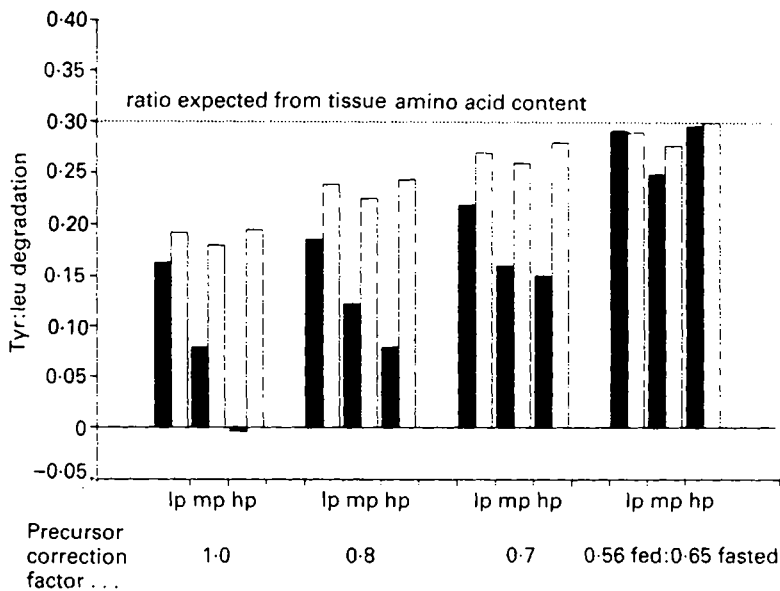


Fig. 4. The degradation rate for tyrosine expressed as a fraction of that of leucine in the fed (■) and fasted (□) state on the three diets: low-protein (lp), moderate-protein (mp), high-protein (hp). The expected value of the tyrosine:leucine degradation ratios is that predicted from the relative composition of body protein.

the calculation of degradation during feeding. During feeding the degradation rate is flux-intake. If the flux was grossly underestimated so that its value was less than that of the intake, degradation could be negative, a theoretical impossibility. When this occurs it must involve gross error and is easily spotted, but such a single observation does not reveal the extent of the error. However, we can make use of our data-set involving different dietary protein intake levels and multiple tracers to examine the impact of correcting the plasma flux on the apparent degradation rate in a way which does allow the extent of the error to be identified. This is illustrated by the values in Fig. 4. This shows the degradation rate for tyrosine expressed relative to that of leucine in the fed and fasted state on the three diets. For tyrosine, degradation is calculated taking into account *de novo* synthesis from phenylalanine as well as any dietary intake. It is assumed that the leucine values, calculated from the KIC APE, are reliable. In this case any underestimation of the error in Q_t should be apparent in two ways: (a) the ratio, tyrosine:leucine degradation rates should be less than the expected value of 0.299 (based on the known amino acid composition of body protein); (b) the fed-state rate of degradation will be progressively more underestimated as the intake increases. On this basis there is obvious error in the uncorrected values. Agreement with the leucine values is apparent when the plasma flux is divided by 0.56 and 0.65 for the fed and fasted states respectively, suggesting that for tyrosine the true precursor enrichment is a lower proportion of the plasma amino acid than with leucine. A similar analysis for phenylalanine, (Fig. 5), indicates that 0.7 is the appropriate correction factor.

The second requirement to calculate the phe(OH) is knowledge of the relative enrichment of the APE of the hepatic D5 phe pool. In fact this can be measured. For those export proteins synthesized by the liver which occur in small rapidly-turning-over

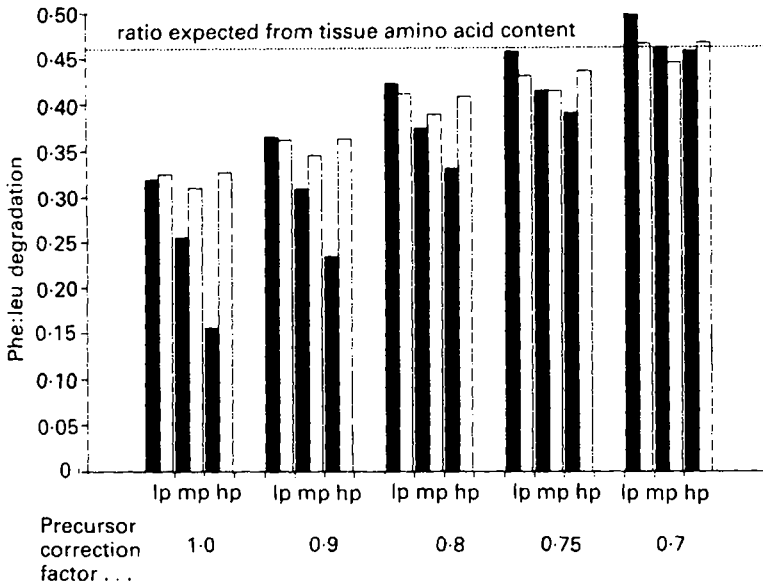


Fig. 5. The degradation rate for phenylalanine expressed as a fraction of that of leucine in the fed (■) and fasted (□) state on the three diets: low-protein (lp), moderate-protein (mp), high-protein (hp). The expected value of the phenylalanine:leucine degradation ratios is that predicted from the relative composition of body protein.

plasma pools, their isotope enrichment should rapidly achieve the same value as that of their hepatic precursor pool. One such protein is the B100 apolipoprotein which can be easily isolated. P. J. Reeds (personal communication) has measured the relative isotope enrichment of leucine and phenylalanine in this protein compared with that in the plasma amino acid pool in adult women during a constant infusion in the fed and fasted state. P. J. Reeds' (personal communication) findings indicate values for phenylalanine of 0.81 fed and 1 fasted, whilst for leucine values are different at 0.62 fed and 0.73 fasted. Whilst the leucine values are what might be expected, a little lower than the KIC values (generally 0.8 that of leucine), reflecting faster turnover and more isotope dilution in liver, the phenylalanine values are surprising. Why they should be higher than leucine is not known. Furthermore the equality with plasma in the fasted but not fed state is puzzling. Fasting would be expected to induce accelerated proteolysis in liver lowering rather than raising the enrichment.

These values for the hepatic-plasma phenylalanine enrichment can be combined with the Q_p correction factors to give correction factors to adjust the phe(OH) 0.454 (0.56×0.81), and 0.65 (0.65×1.0) for the fed and fasted state. In Fig. 6 the impact of these corrections on the prediction of N excretion from phe(OH) is shown. Without correction the predicted:actual N excretion value falls from an overestimation of 1.55 (SD 0.66) on the low-protein diet to an underestimation of 0.65 (SD 0.19) on the high-protein diet, values broadly similar to the leucine values discussed previously. However, the application of these correction factors which are based on the best estimates and predictions we can identify result in excessive losses at all intakes; the apparent overestimation of N excretion is worsened, the ratio being 3.00 (SD 1.00) and 1.13 (SD

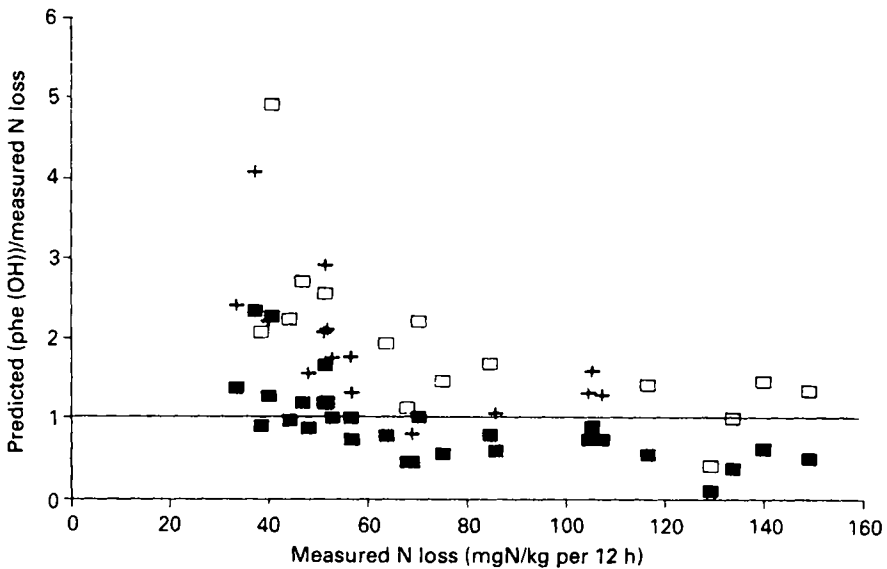


Fig. 6. The relationship between measured nitrogen excretion and the ratio, N excretion predicted from phenylalanine oxidation:the measured N excretion. Phenylalanine hydroxylation (phe(OH)) in the fed and fasted state is shown calculated from the uncorrected plasma values for [^2H (D5)]phenylalanine, [^2H (D2)]tyrosine and [^2H (D4)]tyrosine, and from these values corrected by the factors (0.45 fed and 0.65 fasted; see p. 204). (□), Corrected; (+), uncorrected (fed); (■), uncorrected (fasted).

0.25) on the low- and high-protein diets respectively. This would suggest that the actual correction factors are intermediate between 1 and those we have applied, although there is no logical way in which we can derive them. At present we must conclude that further work on phe(OH) is needed. What is most important, however, is that the pattern of the mismatch between predicted and measured N excretion is the same as for leucine and the biological significance of this is discussed on p. 212.

Diurnal cycling from amino acid oxidation measurements. These results indicate that caution must be used when applying amino acid oxidation studies to verify whole-body N balance. Leucine oxidation appears to be reasonably satisfactory at high protein intakes but less so at low levels, whilst phenylalanine does not currently allow accurate assessment. The fed-state gains and fasted losses indicated by N balance and leucine oxidation on the three diets are shown in Fig. 7. The tendency for leucine oxidation to overestimate N excretion on the low protein intakes results in an underestimate of gain and overestimate of loss. Nevertheless if the moderate- and high-protein diets are compared the nutritionally sensitive diurnal cycling of body protein identified from the N-balance measurements is confirmed by the leucine oxidation studies.

MEASUREMENT OF WHOLE-BODY PROTEIN TURNOVER

The second question we have examined is to what extent is whole-body protein turnover sensitive to dietary protein intake? Millward & Rivers (1988) suggested that the nutritionally variable diurnal cycling might be caused in part through increased protein

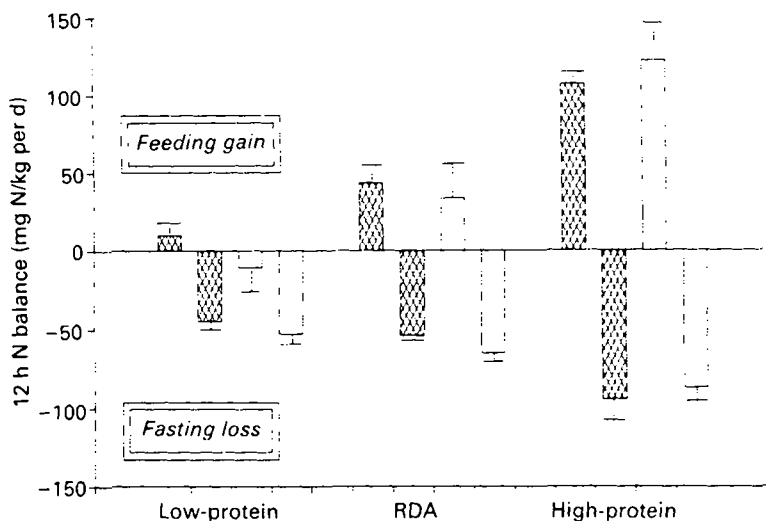


Fig. 7. Fed-state gains and fasted losses indicated by nitrogen balance (\square) and leucine oxidation (\square) on the three diets: low-protein, RDA (recommended dietary allowance), high-protein. Values are means with one standard deviation represented by vertical bars.

turnover with increased protein intake. In our studies we can calculate the daily turnover rate from the leucine and phenylalanine values, and the fed-state rate from the [^{15}N]glycine values.

Daily turnover rate: precursor and end-product methods. The amino acid flux can be calculated from the leucine and phenylalanine isotopic values in two different ways. In the first method the flux calculated from the plateau enrichment of the amino acid (e.g. phenylalanine or tyrosine) or a derivative (e.g. KIC as an indicator of the intracellular leucine pool), can be resolved into its components on the basis of the relationship:

$$\begin{aligned} \text{flux} &= \text{input (diet + de novo synthesis + protein degradation)}, & 3 \\ &= \text{output (oxidation + protein synthesis)}. & 4 \end{aligned}$$

For leucine and phenylalanine it is usually assumed that there is no *de novo* synthesis. For tyrosine since we cannot currently measure its oxidation, the Q_t cannot be resolved into protein synthesis. *De novo* synthesis does occur, however, (the phe(OH) rate) so that degradation can only be calculated when this factor is also measured. From the previous discussion about the extent of any underestimation of the flux based on plasma sampling, it is clear that potential 'model' or precursor problems loom large in any calculations based on phenylalanine and tyrosine. Given the additional difficulty of estimating phe(OH), estimation of protein synthesis rates from the phenylalanine flux and degradation rates from the Q_t (also dependent on phe(OH)) is probably unwise. Thus, the phenylalanine degradation rate (flux - intake) is the most secure derived value for phenylalanine. In comparing whole-body turnover rates with different amino acids by precursor methods, the assumption must be made that the distribution of the amino acids

throughout the individual tissue proteins, which might differ in terms of their turnover rates and response to dietary insults, is the same. There is no real way of assessing this. In the rat, measurements with the large-dose method do indicate that the fractional whole-body synthesis rates measured with leucine and phenylalanine are the same, albeit faster than with lysine and slower than with threonine (Obled *et al.* 1989). This would support the use of these two amino acids to make the comparisons reported here.

The second method of calculation is the end-product method. It has been suggested that end-product methods may be a better alternative in that the 'model' assumptions can be different and less dependent on absolute assessment of precursor labelling (Golden & Waterlow, 1977; Imura & Walser, 1988). These methods rely on the following principle. During a continuous or single dose of an amino acid tracer:

$$\frac{\text{tracer excreted in end-product}}{\text{isotope administration rate or dose}} = \frac{\text{amount of end-product}}{\text{flux}}, \quad 5$$

$$\text{thus, flux} = \frac{\text{amount of end-product}}{\text{tracer excretion/dose}}. \quad 6$$

Thus, in principle only isotope excretion rates have to be measured with no plasma sampling required. This approach has been applied to [¹⁴C]leucine oxidation studies (Golden & Waterlow, 1977; Imura & Walser, 1988) as well as to ¹⁵N studies (Waterlow *et al.* 1978; Fern *et al.* 1985), although there are considerable differences in the model assumptions for these two tracers.

The influence of dietary protein intake on whole-body protein turnover expressed as the daily rate of whole-body protein degradation, is shown in Fig. 8, calculated by precursor methods for leucine (from plasma KIC), and phenylalanine (assuming the plasma: true flux value is 0.7) and from leucine oxidation by the end-product method. (An end-product calculation for phenylalanine based on hydroxylation has not been attempted because of the precursor problems discussed previously.) For both leucine and phenylalanine precursor methods, there is no measurable influence of these three intakes of dietary protein on turnover. The lower content of phenylalanine in body protein accounts for its lower overall flux. In contrast the leucine-oxidation end-product method indicates a marked increase in turnover (70% from low to high) in response to the increased protein intake. Clearly the end-product method indicates a quite different response, although as will become apparent if the method assumptions are looked at closely then it is not surprising in the present circumstances.

Fed-state turnover rate: ¹⁵N end-product methods. The ¹⁵N end-product method relies on model assumptions about the transfer of the ¹⁵N in the administered amino acid into the end-product (urea or ammonia) and into protein synthesis. Equation 6 is valid if the same proportions are transferred through these two pathways. In fact there is no independent way in which this can be assessed. What has been shown is that for various ¹⁵N-labelled amino acids, [¹⁵N]glycine appears to be intermediate between the two extremes illustrated by alanine (or aspartate) and lysine (Fern *et al.* 1985) and that the calculated flux with [¹⁵N]glycine is of the same order as that measured with [¹³C]leucine. Also the partition of the isotope between the two end-products urea and ammonia is different and influenced by the route of isotope administration (oral as opposed to

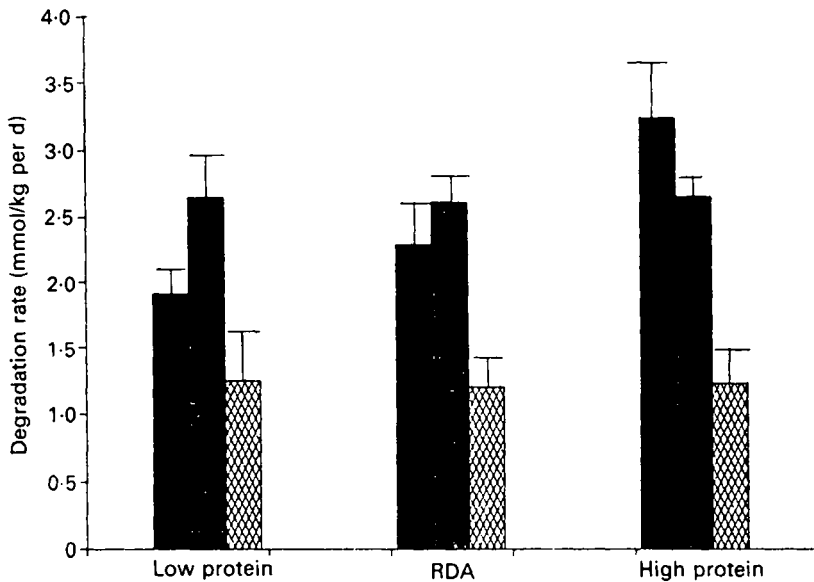


Fig. 8. Influence of dietary protein intake on daily rate of whole-body protein degradation. Values calculated by precursor methods for leucine (from plasma α -ketoisocaproate), and for phenylalanine (assuming the plasma: true flux ratio is 0.7) and from leucine oxidation and nitrogen excretion by the end-product method. RDA, recommended dietary allowance. (■), Leucine (end-product method); (■ with dots), leucine (precursor method); (■ with cross-hatch), phenylalanine (precursor method). Values are means with one standard deviation represented by vertical bars.

intravenous). Fern *et al.* (1985) argued that calculation of an end-product average flux value from isotope excretion in urea and ammonia would to some extent allow for amino acid and N compartmentation and provide a better estimate of the true flux. The average could be calculated as an arithmetic mean assuming an equal partition of isotope between the two metabolic pools or a harmonic mean, i.e.

$$Q_{\text{harmonic}} = \frac{2}{(1/Q_{\text{urea}} + 1/Q_{\text{ammonia}})},$$

which assumes an equal turnover of the two pools.

Fig. 9 shows the mean values for fed-state protein synthesis on the three protein diets calculated as both arithmetic and harmonic means and this has been compared with the leucine-KIC values calculated for the fed state. The most obvious feature of the findings is the considerable variability in the ^{15}N values compared with the leucine values. The coefficient of variation ranges up to 36% and 48% for the arithmetic and harmonic means respectively, values which though high are still less than the variance in the urea and ammonia values from which these values were calculated. With this variability no influence of diet can be identified with statistical certainty from the ^{15}N values, although the mean values clearly increase with increasing dietary protein. In contrast the leucine values have a variance of only 7–8% so that the 37% increase in the fed-state synthesis from the low- to the high-protein diet is highly significant.

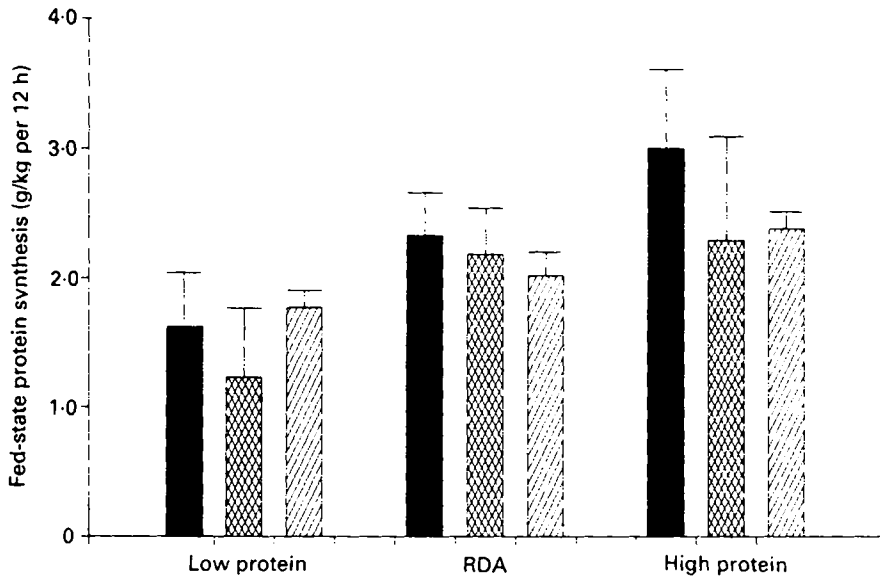


Fig. 9. Fed-state protein synthesis on the three protein diets: low-protein, RDA (recommended dietary allowance), high-protein. Values determined by ^{15}N glycine, (calculated as both arithmetic (■) and harmonic means ▨ of the flux values determined from ^{15}N excretion in ammonia and urea) and by ^{13}C leucine (α -ketoisocaproate-precursor method) (▧). Values are means with one standard deviation represented by vertical bars.

THE MECHANISMS OF DIURNAL CHANGES IN BODY PROTEIN

The final question involves the identification of the targets for the anabolic drive, protein synthesis, degradation and amino acid oxidation, which mediate diurnal cycling. In fact there has been considerable recent debate as to the relative importance of changes in protein synthesis as opposed to degradation on feeding. In studies we carried out some years ago (Rennie *et al.* 1982), we showed that the low rate of protein synthesis in fasting was stimulated by feeding. Whilst these studies have been subsequently confirmed for muscle with further ^{13}C -1]leucine-biopsy studies (Halliday *et al.* 1988) and forearm studies (Cheng *et al.* 1987), others have argued that in the whole body, no influence of feeding on protein synthesis can be identified and proteolysis is the regulatory target (McNurlan & Garlick, 1989).

The resolution of the flux into synthesis and degradation, requires intake and oxidation to be known accurately. Since the rate of oxidation may change markedly with feeding, errors in measurement of this response could influence the ability to detect changes in protein synthesis. As indicated in Table 1 natural ^{13}C in food can contribute to expired $^{13}\text{CO}_2$ and this requires separate assessment of food contribution. The extent of H^{13}CO_3 retention in the body must also be known since, if it is ignored, this would result in underestimation of the oxidation rate. In most studies in the literature the fractional H^{13}CO_3 recovery is assumed to be fixed at 0.80. However, several authors have pointed to the need for its measurement and showed it may vary between feeding and fasting. We have measured H^{13}CO_3 recovery in breath during an 8 h infusion (4 h fasting, 4 h

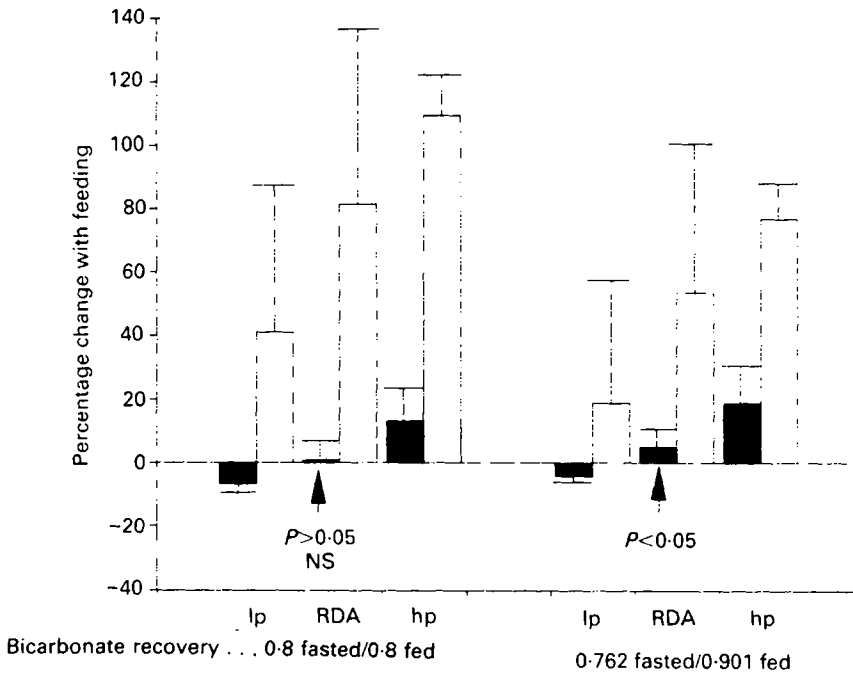


Fig. 10. Influence of H^{13}CO_3 recovery on the apparent response of leucine oxidation (\square) and protein synthesis (\blacksquare) to feeding. lp, low-protein; RDA, recommended dietary allowance; hp, high-protein. Values are means with one standard deviation represented by vertical bars. NS, not significant.

feeding), showing that the recovery was lower than this commonly assumed value in the fasted state (0.762) rising to 0.901 with feeding (Wenhum *et al.* 1991). The influence of these H^{13}CO_3 recovery values on the response of oxidation and protein synthesis to feeding is shown in Fig. 10. Clearly if the usual values of 0.80 recovery had been used, the increase in the oxidation rate is markedly overestimated and any stimulation of protein synthesis is underestimated.

In Fig. 11 the percentage changes in protein synthesis and degradation during the fast-fed transition on the three diets are shown as indicated by the leucine-precursor method, and by the end-product method. The leucine-precursor method indicates that degradation is depressed by feeding on all three protein intakes whilst the response of protein synthesis to feeding depends on the level of protein intake, falling slightly on a low-protein diet but increasing on the moderate and especially the high protein intake. This overall pattern is also observed with the end-product method, although there is considerable variability with the data, (so much so that most of the responses are not significantly different from zero!). Clearly the end-product method on its own would not allow a satisfactory answer to this particular question, but when considered in conjunction with the precursor method does support the fall in degradation and the variable response of protein synthesis.

METHODOLOGICAL DISCREPANCIES

The results which most obviously raise questions about the methodologies employed here are the mismatch between the leucine and phenylalanine oxidation rates and N

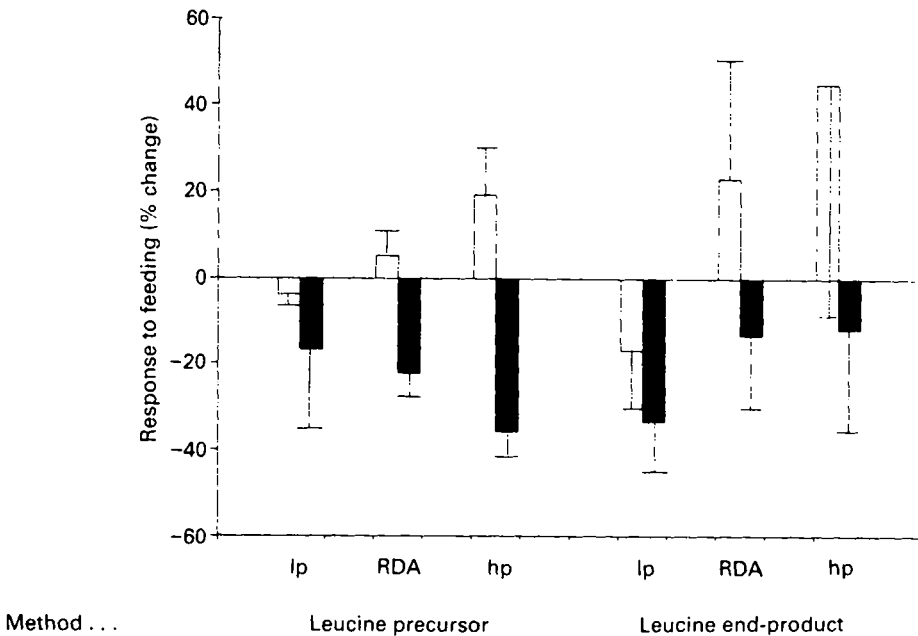


Fig. 11. Influence of feeding on whole-body protein synthesis (\square) and degradation (\blacksquare) in subjects habituated to three levels of dietary protein: lp, low-protein; RDA, recommended dietary allowance; hp, high-protein. Values are shown as the percentage changes from the fasted state as measured by the leucine precursor and end-product methods, and are means with their standard errors represented by vertical bars.

excretion (Figs 1 and 6), and the difference between the precursor and end-product methods in assessing the response of whole-body protein turnover to dietary protein (Fig. 8). In fact these discrepancies are connected and reflect the mismatch between the oxidation rates and N excretion.

The end-product method based on leucine oxidation requires (1) the leucine-precursor pool for protein synthesis is equally isotopically enriched as the oxidation precursor pool, i.e. KIC, and (2) leucine oxidation always quantitatively traces N excretion.

Assumption 1 is difficult to evaluate. In theory compartmentation could result in different enrichments of the leucyl tRNA and KIC. Furthermore differences between tissues in both plateau precursor enrichments and the relative extent of the two processes of protein synthesis and oxidation also could result in potential errors. However, since the same assumption is made for the precursor method, even if it was incorrect it could not account for the difference between the two methods.

Assumption 2 is much more problematic in the present circumstances for two reasons.

First, as discussed previously, in calculations based on the fed-state leucine oxidation rate as a tracer for N excretion, an allowance must be made for the different leucine content of the food and tissue. In fact such an allowance is only possible when the absolute leucine oxidation rate is known from the plasma KIC enrichment, ruling out the basic method for studies in the fed state unless an absolute match between the leucine content of food and tissue can be arranged.

Second, the assumption that leucine oxidation traces N excretion does not hold in the present circumstances given the increasing mismatch between N excretion predicted from leucine oxidation and the actual N excretion (Fig. 2). The influence of this is marked. On the low-protein diet the leucine overestimated N excretion by 34 (SD 19)% compared with 16% on the moderate-protein diet and an 8 (SD 20)% underestimation on the high-protein diet. Whatever the biological explanation of this mismatch, it is apparent from eqn 6 that its consequence will be a marked underestimation of the flux on the low- and moderate-protein diets and an overestimation on the high-protein diet compared with the values measured by the precursor method.

In fact the mismatch between the leucine oxidation and N excretion is entirely responsible for the difference between the precursor and end-product methods. When the precursor for oxidation and protein synthesis is assumed to be the same (e.g. KIC), oxidation/flux calculated from the KIC enrichment is the same as ^{13}C excretion/infusion rate (as long as the correction for the infusion rate as a contribution to the flux is made). Agreement between leucine oxidation (expressed as N) and measured N excretion will automatically mean that the two methods will give the same answer. Recalculation of the end-product protein turnover values with correction factors based on the predicted:observed excretion ratios shown in Fig. 2, will result in values exactly equal to the leucine-precursor values.

The mismatch between leucine oxidation and N excretion will also influence the flux resolution into synthesis and degradation when the flux is calculated by the two methods (Fig. 11). As with the 24 h degradation rates (Fig. 8) the recalculations of the data after applying a correction factor based on the value of the leucine oxidation–N excretion mismatch would result in an exact reproduction of the leucine precursor results. Clearly the apparent overestimation of leucine oxidation or underestimation of N excretion must be explained if we are to answer the questions we posed at the outset.

Implications of the amino acid oxidation studies: urea salvage? The overestimate of N excretion by leucine and phenylalanine oxidation on the low-protein diets exhibit the same pattern but for phenylalanine given the precursor problems inherent in the calculations their quantitative importance is more difficult to estimate than for leucine. Is a simple 'Model' problem likely to be responsible? Overestimation of oxidation would result from an underestimation of the precursor enrichment. The maximum possible error would be a 25% overestimate if the tissue KIC enrichment was higher than plasma KIC and equal to the plasma leucine, most unlikely. The mismatch could reflect a temporal problem with leucine oxidation higher during the measurement periods, (end of fasting and beginning of feeding), than the average for the complete 12 h periods over which the N excretion was measured. Again this is the opposite of what would be expected especially for the fasted measurement.

An underestimation of N loss on low-protein diets is also unlikely. If this occurred and body N was lost at the rate predicted by leucine or phenylalanine on the low-protein diet then much more marked weight loss should have occurred. The actual N balance on the low-protein diet was equivalent to a loss of lean tissue of 0.91 kg over 14 d which was within the measured values. In contrast the leucine and phenylalanine oxidation rates predicted N loss equivalent to 1.94 and 5.91 kg lean tissue/14 d, rates which did not occur.

The apparent mismatch between amino acid oxidation and N excretion on low-protein diets can be explained by the selective retention of the N from leucine and phenylalanine

(but not other amino acids) in some form, which appears highly unlikely. The most likely explanation relates to urea salvage in the lower gut. Urea hydrolysis and incorporation into amino acids by microfloral protein which was then degraded and recycled into the body, would account for the better efficiency of utilization of N compared with amino acid-C skeletons. Thus, amino acid oxidation would be proportional to urea synthesis rather than excretion.

The methodological implications of urea recycling. Urea recycling will reduce the fraction of the end-product formed (urea synthesis) which is excreted and, in the case of ^{15}N recycled into body protein, also reduce the fraction of the isotope dose excreted in the end-product. For $^{13}\text{CO}_2$ as indicated previously this will result in an underestimate of the flux. For ^{15}N since the amount of the isotopic dose will also be reduced this would overestimate the flux and these two opposite flux errors would in effect cancel. However, it is not entirely clear how the flux should be calculated from the ^{15}N data under these circumstances so that without further difficult analysis of an already complicated model the values obtained by this method, when urea recycling is occurring, should be treated with even more caution than usual.

Any *de novo* synthesis of amino acids from urea recycling would reduce the calculated degradation rate (eqn 3). However, the extent of this would be small, equal to about 25 and 14% of the oxidation rate on the low- and moderate-protein diets, and lowering the degradation rates by between 3 and 4%. Since this would occur in both fed and fasted state it would not influence the apparent change in the degradation rate with feeding.

The biological implications of urea recycling. Although the *de novo* synthesis of indispensable amino acids from urea which are made available to the organism is judged unlikely by many, there is evidence, (largely derived from the work of Jackson and colleagues; see Millward *et al.* 1989) that it does occur in man. The occurrence of urea recycling is not in question. The contentious issue is its biological importance and the extent of its regulation. According to Jackson *et al.* (1988), it is regulated since the extent of recycling varies in a complex way with protein intake; it is not simply related to the absolute dietary intake, but rather to the intake in relation to the metabolic demand for protein synthesis.

The implications of urea salvage and the *de novo* synthesis of indispensable amino acids are profound. The extent implied by our results with leucine on the low-protein diet is a daily recycling of *de novo* synthesized amino acids equivalent to 25% of the dietary intake and a higher percentage (at least 43) for phenylalanine. This makes the concept of protein quality meaningless with the potential for qualitative modification of the amino acid balance by the lower gut and allows for considerable adaptation as discussed elsewhere (Millward *et al.* 1989). However, in the present context where we are concerned with use of stable isotopes to measure whole-body balance, urea salvage and amino acid recycling will also make it impossible to measure balance precisely.

CONCLUSION

Our intention in the present paper has been to consider the question 'what can we measure with confidence'? In identifying the pitfalls which can arise and the major areas of uncertainty which exist, it seems to us that we can be reasonably sure that [^{13}C]leucine-KIC studies in the whole body do allow us to investigate protein and amino acid metabolism with relative confidence. Our conclusions are shown in Fig. 12 in which

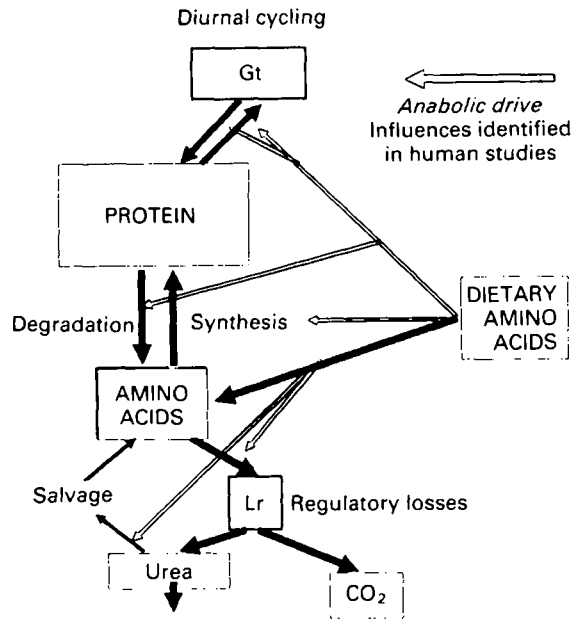


Fig. 12. Components of the anabolic drive of dietary protein on whole-body protein and amino acid metabolism. Dietary protein influences the amplitude of diurnal cycling, (transient gain, Gt), the rates of whole-body protein synthesis and degradation (Lr), the extent of regulatory oxidative losses of amino acids and the extent of urea salvage and *de novo* synthesis of amino acids in the large bowel.

the components of the anabolic drive of dietary protein on whole-body amino acid and protein metabolism identified by our isotopic studies are shown.

Nutritionally sensitive diurnal cycling of body protein is confirmed even though on low-protein diets the agreement of the isotopic studies with N balance is less. The protein gains on feeding are mediated by a fall in the protein degradation rate on all diets coupled with a response in protein synthesis which varies from a small fall on low-protein diet to a substantial increase with the high-protein diet. The consequent increases in net protein synthesis allow some deposition even though feeding markedly stimulates amino acid oxidation even on a low-protein diet. The extent to which the daily rate of whole-body protein turnover is sensitive to the dietary protein level is relatively small and not easy to identify with the currently available reliable methods. Only when the measurements are made during feeding can an influence be identified with protein synthesis on a high-protein diet, about 37% higher than that measured on a low-protein diet. Clearly our interpretation of the leucine oxidation–N excretion mismatch as indicating urea salvage and *de novo* indispensable amino acid synthesis will be contentious and will require further investigation. In the case of phenylalanine we do have some way to go in solving the considerable model problems relating to the precursor enrichment. The measurement of the hepatic precursor enrichment through apolipoprotein studies offers a partial solution, but the problem remains for the rest of the body tissues. [¹⁵N]glycine studies do not appear to offer any advantage over

[¹³C]leucine in the present circumstances, but may well offer advantages in other circumstances where the need to trace N specifically is paramount.

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