Measurement of protein turnover in normal man using the end-product method with oral [^{15}N]glycine: comparison of single-dose and intermittent-dose regimens

BY G. GROVE AND A. A. JACKSON

Department of Human Nutrition, University of Southampton, Bassett Crescent East, Southampton SO16 7PX

(*Received 20 June 1994 – Revised 26 January 1995 – Accepted 7 February 1995*)

The 'single-dose end-product' approach for the measurement of protein turnover is the only method suited for application to free-living individuals and for field studies. However, the method has never been widely accepted because the results obtained appear to vary from one situation to another. There is the need for a formal comparison of the different approaches that have been used and the assumptions on which they are based in order to be able to understand the extent to which the variation in the results obtained is real or might be attributed to methodological differences. The present study used the 'prime/intermittent-dose end-product' approach over 18 h as a reference with which to compare the 'single-dose end-product' approach, with urine being collected for periods of 9 to 48 h. N flux was derived in a total of thirteen normal men using oral [^{15}N]glycine and measurement of ^{15}N enrichment in urinary NH_3 or urea, with isotope being given either as prime/intermittent doses or a single dose in separate studies. The pattern of results was similar to those reported in the literature. In all studies the rates of N flux derived from urea were higher than those derived from NH_3, under equivalent conditions, by about 25–30%. The single-dose approach, with urine collection over 9 h, gave results which were consistently higher than the prime/intermittent-dose approach. The extent of the difference was influenced by the duration of time over which the cumulative excretion of isotope in urine was determined, and with NH_3 as the end-product the most consistent estimates of N flux could be obtained with a urine collection of at least 12 h and no greater than 24 h. With urea as the end-product, correction for the label retained in the body pool at 9 h gave similar results for N flux to those derived from the total excretion of label in urea over 24 h. The derivation of values for protein synthesis and protein degradation from measures of flux requires care to ensure that the time periods over which N intake and excretion are measured accord with those for which the measurement of flux apply. It is concluded that measurements of protein turnover similar to those obtained with the prime/intermittent-dose approach can be obtained with the single-dose approach in the fed state during the daytime, either from the excretion of label in urinary NH_3 over a period of 12 h or with the excretion of label in urinary urea over a period of 24 h. The suitability of the method for use in the fasted state or at night time remains to be determined.

Urea: Ammonia: Nitrogen flux

The measurement of protein turnover has provided invaluable information on the dynamic changes in the relative rates of protein synthesis and degradation which take place in a range of physiological or pathological states (Waterlow *et al.* 1978*a*; Waterlow, 1984). For studies in adults there has been a tendency to use the relatively invasive precursor methods with the intravenous administration of isotope, either as a prime/continuous infusion or as a flooding dose of isotope, with measurement of enrichment in the blood and breath (Waterlow, 1967; Garlick *et al.* 1989; Young *et al.* 1989). The invasive nature of these approaches makes them unsuitable for routine use in children or in free-living adults outside the confines of a metabolic ward, although oral [^{13}C]leucine with measurement of

enrichment in urine has been used on one occasion in preterm infants (deBenoist *et al.* 1984). Thus, there has been a continued interest in the development and application of methods which are non-invasive and which might be of practical value in assessing protein kinetics under normal living conditions. The so-called end-product methods are ideally suited to this purpose in that the dose of isotope, most usually [^{15}N]glycine, is taken orally and the enrichment is measured in an end-product of protein metabolism which is excreted in the urine, usually urea or NH_3 (Picou & Taylor-Roberts, 1969; Fern *et al.* 1981; Waterlow, 1984; Jackson *et al.* 1987).

Although the end-product methods have found practical application in a wide range of circumstances, such as malnourished children (Picou & Taylor-Roberts, 1969; Waterlow *et al.* 1978*b*; Jackson *et al.* 1983), preterm infants (Pencharz *et al.* 1977; Jackson *et al.* 1981), pregnant women (deBenoist *et al.* 1985; Willommet *et al.* 1992), malnourished adults (Soares *et al.* 1994) and the elderly (Golden & Waterlow, 1977), because they use simplifying assumptions about amino acid and protein metabolism in the body the methods are open to criticism. The two most important assumptions of the original model which are untenable are that the amino acid pool becomes uniformly labelled following the administration of a single labelled amino acid and the assumption of a single amino acid pool as the precursor pool from which proteins and the end-products of metabolism are formed (Jackson & Golden, 1980; Fern *et al.* 1981). It is clear that there is limited dispersion of label from [^{15}N]glycine to other amino acids (Aqvist, 1951; Vitti & Gaebler, 1963). As there is almost invariably a difference in the enrichment in urinary urea and NH_3 following the administration of either [^{15}N]glycine or other ^{15}N-labelled amino acids, a model which presumes a single precursor pool cannot be sustained (Fern *et al.* 1981, 1985*a*; Fern & Garlick, 1983). However, Fern and colleagues have derived a theoretical solution to the problem and shown that many of these apparent problems can be overcome, in a practical sense, by the use of the 'end-point average', an approach which allows for metabolic and functional compartmentation between two precursor pools (Fern *et al.* 1985*b*). However, when this approach has been used, the labelled urea retained within the body at 9 or 12 h has had to be measured, which has required a blood sample, thereby losing one major advantage of a non-invasive method.

In their exploration of the important factors on which the single-dose end-product method is based, Fern and colleagues conclude that [^{15}N]glycine represents the most suitable choice for a single labelled amino acid in the fed state, and with the end-point average the method provides results which may be repeatable with a variability of less than 5%, and compares well with the invasive approaches using C-labelled amino acids (Fern *et al.* 1981, 1984, 1985*a*, *b*; Fern & Garlick, 1983). In all these studies Fern *et al.* gave the isotope as a single dose, either orally or intravenously. There has never been a direct comparison of this approach with the use of either continuous administration of label, either orally or intravenously, or the approach of prime/intermittent oral doses of isotope (Sim *et al.* 1980; Jeevenandam *et al.* 1985; Jackson *et al.* 1987). The purpose of the present study was twofold: first to compare the results obtained with the end-product method, using [^{15}N]glycine, when the isotope was given either as a single oral dose, or intermittent oral doses, and enrichment measured in either urinary urea or NH_3; second, to determine whether the time course over which urine was collected in the single-dose approach made any difference to the comparison.

METHODS

Subjects

The studies were carried out in normal male adults aged 22–48 years, who were in good health at the times of investigation (Table 1). The subjects agreed to participate in the study

Table 1. *The height, weight and age of male subjects in whom whole-body nitrogen flux was determined, with derived values for total body water (Watson* et al. *1980) and body mass index*

Subject	Age (years)	Weight (kg)	Height (m)	Total body water (litres)	Body mass index (kg/m^2)
1	22	70	1·65	42	25·7
2	27	59	1·51	36	25·9
3	28	55	1·63	36	20·7
4	29	81	1·83	47	22·8
5	26	100	1·77	53	31·9
6	24	76	1·79	45	23·8
7	22	74	1·71	43	25·5
8	22	76	1·80	45	23·5
9	26	75	1·86	45	21·6
10	31	77	1·85	45	22·4
11	48	80	1·87	45	23·1
12	23	85	1·84	48	25·0
13	22	76	1·55	44	31·6
Mean	27	76	1·74	44	24·9
95% CI	23–31	69–82	1·67–1·82	41–47	23–27

when the nature of the investigations had been explained to them. The studies had the approval of the joint ethical committee of the Southampton Hospitals and South West Hampshire Health Authority. Nine subjects underwent both the single-dose and intermittent-dose protocols, and two subjects each undertook either the single-dose or the intermittent-dose protocol. The study was divided into two parts. In the first part of the study protein turnover was measured either by the prime/intermittent-dose approach over 18 h or by the single-dose approach over 9 h. In the second part of the study five of the subjects in whom protein turnover had been measured by the prime/intermittent-dose approach over 18 h and the single-dose approach, initially for 9 h, had the study extended with urine collections being continued for a total period of 48 h.

Diets

The habitual dietary intake of each subject was assessed from a 3 d dietary recall. The intake was standardized on study days to provide 1·1–1·2 g protein/kg per d and to approximate the habitual intake of energy (about 11 MJ/d). Food was provided for the subjects in the form of sandwiches which contained standardized amounts of energy and protein. On the day of the study the sandwiches were eaten every 3 h from 06.00 to 18.00 hours.

Protocols

Single-dose approach. A urine collection was made between 06.00 and 09.00 hours for the measurement of baseline enrichment. At 09.00 hours a single dose of 200 mg [^{15}N]glycine (99 atoms percent excess, Cambridge Isotope Laboratories, Massachusetts, USA) made up in water was taken orally. For the subjects in the first part of the study, urine was collected between 09.00 and 18.00 hours. For the subjects in the second part of the study, urine was collected every 3 h from 09.00 hours for 24 h and then as two samples of 12 h each for a further 24 h, to bring the total collection period to 48 h. For each subject 20 ml venous

blood was drawn from an antecubital vein into a heparinized container, 9 h after the ingestion of the isotope. The blood sample was used to determine the amount of labelled urea retained in the urea pool at 9 h.

Intermittent-dose approach. A urine collection was made between 21.00 hours and midnight for the measurement of baseline enrichment. At midnight a priming dose of [^{15}N]glycine dissolved in water was taken orally. The amount of glycine was equivalent to 18 h of infusion. From 06.00 hours, intermittent doses of [^{15}N]glycine were taken every 3 h (equivalent to 0·005 mg N/kg per h) until 18.00 hours. Urine was collected at intervals of 3 h until 21.00 hours.

Analyses

The urine was collected into acidified containers and stored at −4° until analysis. The contents of urea-N and NH_3-N in urine were assayed by the Berthelot reaction (Kaplan, 1965). NH_3 was isolated from urine by alkaline aeration. The NH_3-free urine was then reacted with urease (*EC* 3.5.1.5; Jack Bean Type III) to liberate urea-N as NH_3 which was collected by alkaline aeration (Jackson *et al.* 1980). Alkaline hypobromite was used to liberate N_2 gas for entry into the mass spectrometer and enrichment was measured with a triple collector isotope-ratio mass spectrometer (Sira 10, VG Isogas, Middlewich, Ches.).

The blood was centrifuged at 2500 *g* for 20 min and the plasma removed. The concentration of urea in plasma was determined by an automatic method (Technicon/Bayer Smac 2 Analyzer, using the diacetyl monoxime method). The total body urea pool was derived on the assumption that urea was distributed throughout total body water (TBW). TBW was estimated using the equation of Watson *et al.* (1980) for males:

$$\text{TBW} = 2{\cdot}447 - (0{\cdot}095\,16 \times \text{age}) + (0{\cdot}1074 \times \text{height}) + (0{\cdot}3362 \times \text{mass}),$$

where age is in years, height is in cm and mass is in kg.

Plasma samples were deproteinized with perchloric acid (100 ml/l) and the urea-N was extracted and isolated from the deproteinized plasma for the measurement of enrichment using the same approach as for urine.

Calculations

For the intermittent-dose studies plateau enrichment in urinary NH_3 and urea was determined by visual inspection. The level of enrichment at plateau was used to calculate N flux (Picou & Taylor-Roberts, 1969):

$$Q = d/En,$$

where Q is flux, d is the rate of administration of [^{15}N]glycine and En is the plateau enrichment in the end-product.

For the single-dose studies, flux was derived from the amount of isotope excreted in the end-product over a specified period of time. In principle the method requires that the period of time over which excretion is measured is that required to ensure that the isotope has been completely cleared from the body pool of the end-product. There is no *a priori* method of knowing the most appropriate time over which urine should be collected to ensure that the body pool has been completely cleared. Previous experience has suggested that a period of between 9 and 12 h might be suitable for NH_3, with a longer period being required for urea. (Prolonging the period of urine collection will lead to errors as the method assumes that all label which passes into protein will not return to the amino acid pool over the duration of measurement. With time this assumption becomes progressively less tenable as label returns to the amino acid pool from protein degradation.) Flux was derived from the amount of label excreted in the urine over 9 h in all eleven subjects. In the five subjects in whom urine

was collected for 48 h, values for flux were derived at each time period over which the urine had been collected, i.e. 9, 12, 15, 18, 24, 36 and 48 h, based on the cumulative excretion of label in the two end-products with time.

In addition, the amount of label retained in the urea pool at 9 h was used to adjust the result of flux based upon the label excreted in urinary urea at 9 h. Flux was calculated by the method of Waterlow *et al.* (1978*b*) and Fern *et al.* (1981):

$$Q = d \times E_x / e_x,$$

where Q is flux, d is the amount of isotope administered, E_x is the amount of NH_3-N or urea-N excreted and e_x is the amount of isotope excreted as NH_3-N or urea-N over the period of study. Where urea is used as the end-product to calculate flux at 9 h, e_x includes the amount of isotope retained in the urea pool at 9 h.

Differences between sets of data were sought by the use of the Wilcoxon Rank sum test for paired data.

RESULTS

All the studies were completed satisfactorily, although two of the five subjects involved in the extended study with the single-dose approach slept through the urine collection at 03.00 hours. The age, weight and height of the subjects and the calculated values for TBW and BMI are shown in Table 1.

Nitrogen flux

The derived values for N flux based on urinary NH_3 and urinary urea for both the intermittent-dose and single-dose approaches are shown in Table 2 and Fig. 1. With the prime/intermittent approach a satisfactory plateau was achieved in all the studies from the sample taken at 09.00 hours, with the variation in enrichment at plateau being 10% for NH_3 and 5% for urea. In the single-dose approach, flux based on the excretion of label in urea includes the label retained in the body urea pool at 9 h. For the intermittent dose, flux derived from NH_3 enrichment (26 mg N/kg per h) was significantly lower than that from urea enrichment (32 mg N/kg per h, $P < 0{\cdot}002$) and the same was true for the single-dose approach (NH_3, 37 mg N/kg per h; urea 42 mg N/kg per h, $P < 0{\cdot}05$). The intermittent-dose approach gave results for flux which were consistently less than the single-dose approach, with either NH_3 ($P < 0{\cdot}002$) or urea ($P < 0{\cdot}002$) as the end-product. Hence flux determined from the end-product average was higher in the single-dose approach (40 mg N/kg per h) than with the intermittent dose (29 mg N/kg per h, $P < 0{\cdot}002$).

One reason for the consistent difference between the two approaches might have been that the period over which urine had been collected in the single-dose approach was not sufficiently long to allow the label to be adequately cleared from the body pool. For this reason in five subjects the period of collection was increased to 48 h and a value for flux was derived from the cumulative excretion of label after progressively longer periods of time.

In Fig. 2 the pattern of enrichment in urinary NH_3 and urinary urea is shown in the five subjects for the period up to 48 h. The maximum amount of label excreted in urinary NH_3 was passed over 3 h, in the first urine, with a rapid fall to low levels within 12 h. Small amounts of label continued to be excreted in the urine up to 48 h. The pattern of excretion in urinary urea was more variable, with the maximum amount excreted over any time period being only one fifth that in NH_3. The excretion of relatively large amounts of label in urea was prolonged over 9 h. Measurable amounts of label were excreted over the entire period, although by 48 h the low levels reached were not different from those in NH_3.

Fig. 3 shows the values derived for flux in these five subjects for each successive period of collection. With the prime/intermittent approach, flux derived from the plateau in NH_3

Table 2. *Whole-body nitrogen flux in male subjects determined with oral [^{15}N]glycine, given either as intermittent doses or a single dose, and enrichment measured in either urinary ammonia or urinary urea**

	Intermittent dose (mg N/kg per h)			Single dose (9 h) (mg N/kg per h)		
Subject	Ammonia	Urea	Average†	Ammonia	Urea	Average†
1	22	30	26	—	—	—
2	26	34	31	41	27	34
3	36	35	36	—	—	—
4	28	36	32	45	48	47
5	26	32	29	40	44	42
6	32	34	33	34	51	43
7	—	—	—	40	52	46
8	—	—	—	29	33	31
9	24	32	28	27	39	33
10	31	26	29	44	47	46
11	17	34	26	46	45	46
12	27	35	31	29	47	38
13	19	26	23	32	30	31
Mean	26	32	29	37	42	40
95% CI	22–30	30–35	27–32	32–42	36–48	35–44

* For details of procedures, see pp. 492–495.
† Arithmetic average of flux derived from urea and ammonia.

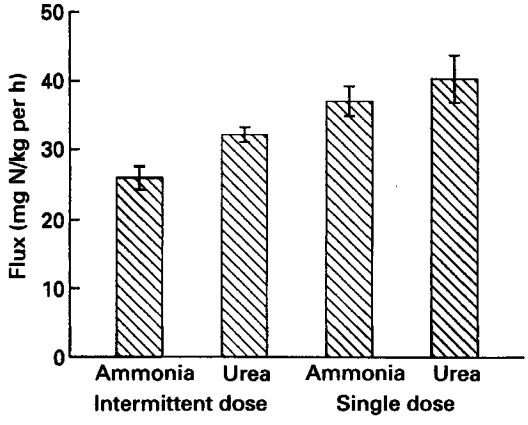

Fig. 1. A comparison of the values derived for whole-body nitrogen flux in eleven normal adult men following either a single oral dose or prime/intermittent oral doses of [^{15}N]glycine and measurement of enrichment of either ammonia or urea collected over 9 h with the single dose and 18 h with the prime/intermittent dose. For details of procedures, see pp. 492–495. Values are means with their standard errors indicated by vertical bars.

enrichment (23 (SD 6) mg N/kg per h) was significantly greater than that derived from the plateau in urea enrichment (31 (SD 5) mg N/kg per h; $P < 0{\cdot}05$). With the single-dose approach, flux derived from excretion of label in NH_3 at 9 h (35·4 mg N/kg per h) was about 10% greater than at 15 h (31·5 mg N/kg per h), but there was little further change from 15 to 24 h. For flux derived from the excretion of label in urea, the value at 9 h corrected for label retained in the urea pool (40 mg N/kg per h) was similar to that

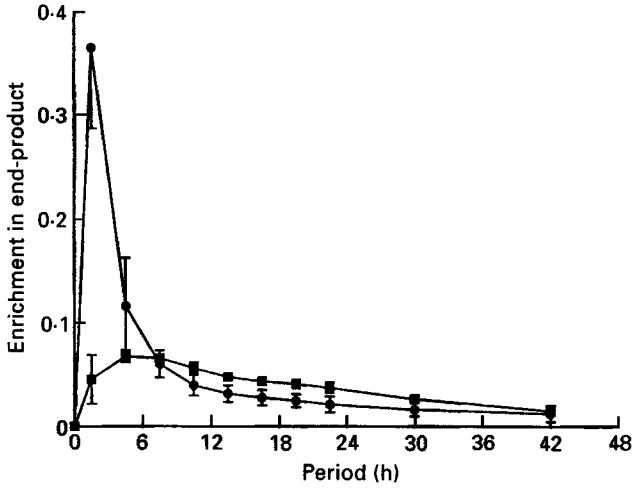

Fig. 2. The pattern of enrichment over 48 h in ammonia (●) and urea (■) isolated from urine of five normal adult men, following a single oral dose of [^{15}N]glycine. For details of procedures, see pp. 492–495. Values are means with their standard errors indicated by vertical bars.

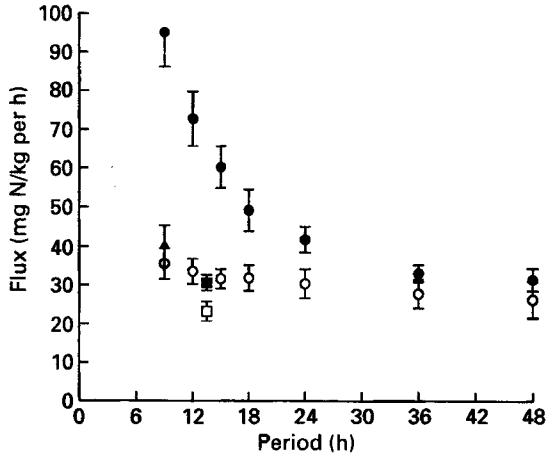

Fig. 3. Derived values for whole-body nitrogen flux in five subjects, either based on the amount of label excreted as ammonia (○) or urea (●) in urine following a single oral dose of [^{15}N]glycine, or based on the enrichment of ammonia (□) or urea (■) in urine over 18 h, following prime/intermittent oral doses of [^{15}N]glycine. In the single-dose method, the derived value for whole-body nitrogen flux when the labelled urea retained within the urea pool of the body is allowed for (▲). Values are means with their standard errors indicated by vertical bars.

obtained from urinary excretion alone at 24 h (42 mg N/kg per h). Label continued to be excreted in urea beyond 24 h, however there was little further change from 36 h (33 mg N/kg per h) to 48 h (31 mg N/kg per h) and at this time the value for flux was similar to that obtained with NH_3 between 15 and 24 h, and with urea in the prime/intermittent approach. The continued loss of label in urinary NH_3 beyond 24 h is likely to represent the return of label to the amino acid pool as a consequence of protein degradation. It is more difficult to identify the extent to which this factor contributes to the continued excretion of label in urea between 24 and 48 h.

Table 3. *Protein turnover in normal adult men, measured with intermittent doses of [^{15}N]glycine from the average enrichment in urinary ammonia and urea over 18 h with derived values for protein synthesis and protein degradation**

(The dietary intake of protein is expressed as g/kg per d and also as mg N/kg per h as an average over a 24 h period, or mg N/kg per h as an average over a 12 h period and degradation has been calculated both as an average for the 24 h period and for the 12 h period during which the food is ingested)

	Intake						Degradation	
Subject	(g protein/kg per d)	(mg N/kg per h) (24 h)	(mg N/kg per h) (12 h)	Excretion (mg N/kg per h)	Flux (mg N/kg per h)	Synthesis (mg N/kg per h)	(mg N/kg per h) (24 h)	(mg N/kg per h) (12 h)
1	1·19	7·9	15·8	4·6	26	21	15	10
2	1·22	8·1	16·2	3·6	31	26	20	14
3	1·30	8·7	17·4	3·9	36	32	25	18
4	1·22	8·1	16·2	7·6	32	24	20	16
5	1·22	8·1	16·2	7·3	29	22	18	13
6	1·20	8·0	16·0	9·1	33	24	21	17
9	1·17	7·8	15·6	7·1	28	21	20	12
10	1·16	7·7	15·4	7·5	29	22	21	14
11	1·20	8·0	16·0	5·0	26	21	18	10
12	1·18	7·9	15·8	7·1	31	24	23	15
13	1·17	7·8	15·6	4·4	23	19	15	7
Mean	1·20	8·0	16·0	6·1	29	23	20	13
95% CI	1·18–1·23	7·8–8·2	15·7–16·4	4·9–7·4	27–32	21–26	18–22	11–16

* For details of procedures, see pp. 492–495.

Protein turnover

The results for N flux are used to derive values for protein synthesis and protein degradation on the assumption that the flow of N into the pool is derived from the dietary intake and protein degradation, while flows from the pool are towards excretion and protein synthesis. In Table 3 the value for flux is given as the arithmetic average of results based on the excretion of label in urinary urea and urinary NH_3 (Fern *et al.* 1985*b*). This has been used to derive estimates of protein synthesis and degradation. There has not been any consistency in the way authors have chosen to approach these derivations. For example intake, degradation, excretion and synthesis have been variously expressed and derived as per hour, per 9 h, per 12 h or per 24 h depending on either the duration of the study or the overall interest of the investigator. In Table 3 the values for protein synthesis and degradation are shown, with degradation being derived in two different ways. In the first, protein intake is taken to be 1·2 g/kg per d and degradation derived as mg N/kg per h for a 24 h period. In the second, intake has been taken to be 16 mg N/kg per h over 12 h and degradation derived as mg N/kg per h over a 12 h period. Intake can be ascertained with reliability and the excretion of urinary NH_3 and urea-N has been used to approximate N excretion. A protein intake of 1·2 g/kg per d provides 192 mg N/kg per d or 8 mg N/kg per h over a 24 h period, but in reality this is ingested over 12 h. Therefore, for the duration of the fed state during which the measurement of N flux is derived the intake would be 16 mg N/kg per h. With the intermittent-dose method protein synthesis was 23 mg N/kg per h and degradation 13 mg N/kg per h over the 12 h period. When degradation was derived on the assumption that the intake was that for a 24 h period the value for degradation was 20 mg N/kg per h.

DISCUSSION

General considerations for the measurement of nitrogen flux and protein turnover

It is clear that proteins are synthesized and degraded on a continuous basis and that the ability to measure the rate at which these processes take place provides information of practical value. Available methods are based on the measurement of the flow of an amino acid or N through its respective pool and the derivation of the major components which contribute to that flow. However, given the complexity of the processes involved, any attempt to measure whole-body protein turnover can only provide an approximation, not least because the time taken to make the measurement is longer than the time taken for some proteins to be synthesized and degraded. This does not make the measurement of no value, but in order to interpret the results sensibly it is important to understand the limitations of the approaches used. As all the approaches are based on assumptions of one form or another it is desirable that, at the same time as the methods are applied in practice to enrich understanding, the assumptions on which the methods are based are continuously explored. It is only in this way that the methods themselves can be used more critically to clarify the nature of the complex processes they seek to describe.

The end-product methods have the great advantage that they are non-invasive and potentially simple to use in practice. The use of a N label means that the fate of label within the N pool is followed directly. The primary assumption of the original single-pool model of protein turnover required that the tracer amino acid should donate N to protein synthesis and to the end-product in the same proportions as total N moves to protein synthesis and the end-product (Picou & Taylor-Roberts, 1969; Jackson & Golden, 1980). Protein turnover measured with uniformly labelled protein has been used as the reference against which to assess the usefulness of individual amino acids as tracers and it is clear that few amino acids when used alone as the tracer give similar results (Picou & Taylor-Roberts, 1969; Fern & Garlick, 1983). It is coincidental that the most widely used tracer, glycine,

appears to be the most suitable in this respect (Taruvinga *et al.* 1979; Jahoor, 1982; Fern *et al.* 1985*a*, *b*), however the use of the end-product average for deriving flux (Fern *et al.* 1981) serves to minimize the differences and potentially useful information can be derived by assuming that the different results obtained with the different end-products enables understanding of changes in different precursor pools (Fern *et al.* 1985*b*). On first principles it is to be expected that an amino acid which donates N disproportionately to either protein synthesis or to end-product is less likely to be an acceptable tracer, as has been found (Taruvinga *et al.* 1979; Jahoor, 1982; Fern *et al.* 1985*a*). The transaminating non-essential amino acids which are actively involved in moving N to the end-products of metabolism (alanine, glutamate, aspartate) are likely to give estimates of flux which are lower than the reference, while essential amino acids (lysine, leucine, valine) will tend to give overestimates of flux. The fact that glycine, a conditionally essential amino acid, gives results most similar to uniformly labelled protein would suggest that on the one hand glycine does not carry N to the end-product disproportionately, and on the other that the rate of glycine formation is closely matched to the metabolic requirement for glycine in synthetic pathways, including protein synthesis. The high quantitative demand for the *de novo* synthesis of glycine to satisfy a range of metabolic pathways probably indicates that as a matter of course *de novo* synthesis of this amino acid is operating close to capacity (Jackson, 1991).

Although the simplest model for whole-body protein turnover presumes a single pool of amino acids, this is clearly a simplification of the real situation. Of practical relevance for the end-product method is the demonstration that different results are obtained depending on whether urea or NH_3 is used as the end-product (Jackson & Golden, 1980; Fern *et al.* 1981). Fern *et al.* (1985*b*) have shown that the arithmetic average of the values for flux derived from urea and NH_3 should give an appropriate estimate on the assumption that there is equal partitioning of the tracer dose between the two precursor compartments, whereas the harmonic average would be more appropriate if both the precursor compartments had equal rates of flux. Without evidence one way or another we are not able to say which alternative is most likely to reflect reality. Nevertheless, the solution to the problem has been to take the average of the two values obtained with the two end-products (Fern *et al.* 1981, 1985*a*, *b*). The enrichment in the two end-products will be determined by the relative enrichments of the precursor pools for each end-product, and the suggestion that the urea pool tends to reflect precursors derived from a splanchnic pool, with NH_3 reflecting precursors derived from a somatic pool, especially muscle (Fern *et al.* 1985*b*), has found some support in practice (Soares *et al.* 1991, 1994). As the implications of these assumptions may have considerable practical importance, there is the need for formal exploration in detail.

With uniformly labelled protein, urinary urea tends to be more highly enriched than urinary NH_3, whereas the converse is found for glycine (Fern & Garlick, 1983). It has been suggested that this can be accounted for by the pattern of amino acids in the protein and the differential fate of their label (Fern & Garlick, 1983). The amino acids glutamine, glutamate, aspartate and arginine may be of particular importance in this respect. They comprise a significant proportion of the amino acid residues in protein, and tend to be deaminated in the gastrointestinal tract (Windmueller & Spaeth, 1980) with a substantial proportion of the N passing to the liver as NH_3, where it will be preferentially incorporated into urea, with the amino acids never becoming a functional component of the general amino acid pool. This is probably an important factor in accounting for the relatively higher enrichment in urinary urea than NH_3. These observations underlie the very great importance of the metabolic channelling of amino-N in intermediary metabolism (Jahoor, 1982). This area has not been explored in great detail, but is of undoubted importance both for amino acid metabolism and the interpretation of tracer studies (Jahoor, 1982; Jahoor

Table 4. *Derived values for whole-body nitrogen flux, determined following oral doses of [^{15}N]glycine and measurement of enrichment in urinary ammonia or urea*

Study	Duration (h)	Flux from ammonia (mg N/kg per h)	Flux from urea (mg N/kg per h)
Intermittent dose			
Jackson *et al.* (1987)	3	40	—
Jackson *et al.* (1987)	18	19·8	28·8
Badaloo *et al.* (1989)	18	20·8	33·3
Present study	18	23·1	30·5
Single dose			
Fern *et al.* (1981)	9	30·7	41·8
Present study	9	35	40
Fern & Garlick (1983)	12	29·1	35·4
Soares *et al.* (1991)	12	30·9	39·5
Present study	12	33	—
	15	32	—
	36	—	33

et al. 1988). The implications for the interpretation of studies of whole-body protein turnover are seen in the marked difference in the pattern of enrichment of urinary urea and NH_3, depending on whether the label is given by the oral or the intravenous route (Fern *et al.* 1981, 1984, 1985*a*).

Therefore, in the present study it has been assumed that glycine given by the oral route is, at the present time, the tracer and approach of choice for end-product studies. We have been interested to explore the limitations of its use in practice. This is a complex area for investigation and the present study has shown that even with a single amino acid there is the need to define with some care the conditions under which experimental studies are conducted if comparisons are to be drawn between the results from different groups and for different experimental conditions in the same group.

Nitrogen flux measured by the different approaches

For any one method the variability in the measurement of N flux between individuals is in the region of 25%, which is in the same order as the variation between different methods. In Table 4 a comparison is drawn of the results obtained in the present study with other studies from the literature in which the same methods have been used in normal adults. The profiles of enrichment and the values derived for protein turnover in the present study are not different from those reported by other workers using similar methods. Pacy *et al.* (1994) gave [^{15}N]glycine intravenously as a short bolus and derived protein turnover based on the average enrichment in the end-product over a period of 9 h. Their values for N flux, 34–46 mg N/kg per h on intakes of 0·77 and 1·59 g protein/kg per d respectively were similar to those given in Table 4; and the estimates of protein synthesis over a wide range of protein intake were similar to those obtained using the precursor method with [^{13}C]leucine. The methods appear to be reproducible (Fern *et al.* 1984, 1985*a*) and any differences which have been observed are not likely to be due to variability in the selection of the subjects between studies, but rather are intrinsic to the measurements themselves. The basic assumptions underlying the method have been reviewed (Waterlow *et al.* 1978*a*; Fern *et al.* 1981; Golden & Jackson, 1981), and the points of interest for the present work are the duration of study period in the single-dose approach, the comparative results

between the single-dose and intermittent-dose approaches and the use of alternative end-products, and their influence on the estimate of N flux.

Single-dose approach

The pattern of excretion of label in urinary NH_3 and urea following a single dose of [^{15}N]glycine has been clearly characterized (San Pietro & Rittenberg, 1953; Wu & Bishop, 1959; Golden & Jackson, 1981; Fern & Garlick, 1983). Attention has been drawn to the apparent precursor–product relationship between the enrichment in urinary NH_3 and in urinary urea (Golden & Jackson, 1981). In the present study the excretion of label was similar to that obtained by other investigators. N flux is derived from the amount of label excreted in a defined period of time. The choice of time period has to be a compromise between the time taken for the label to clear the pool of either NH_3 or urea, and the time taken for significant amounts of label to return to the end-product from the degradation of protein which has been labelled with the isotope.

In most other studies where the single-dose method has been used to determine protein turnover from the excretion of label in urinary NH_3 the collection period has been either 9 h or 12 h. In the present study 9 h was probably too short a period, with a 12 h period of collection giving results which were similar to those at 24 h. Pragmatically, there is often the need to be able to complete a study in a single day and under these circumstances a 9 h period of collection might be acceptable, but a 12 h period would be preferable. With differing durations of measurement different components of protein turnover are being measured. In the study where the time period of urine collection was extended beyond 24 h to 48 h the results at the later time points appeared to be affected by the return of label, leading to an apparent fall in N flux of about 20% per d.

When urea is used as the end-product the main difficulty is the large size of the urea pool and the extended time taken for label to be cleared completely. In studies in which the clearance of label from the urea pool has been measured directly it has taken between 24 and 36 h for labelled urea to clear the pool, virtually completely (Fern & Garlick, 1983; Moran & Jackson, 1990; Jackson *et al.* 1993). The label probably moves to the formation of urea within a short period of the administration of the single dose, therefore to get around the problem of extended periods of measurement the amount of urea retained within the urea pool of the body has been estimated (Fern *et al.* 1981). This estimate is based on the measurement of the concentration of urea in plasma, the assumption that urea distributes throughout TBW and the derivation of TBW either by direct measurement, but more usually by an indirect approach based upon anthropometry. The value derived for N flux based on the excretion of label in urea at 9 h with a correction for retained label is similar to that based on excretion of label at 24 h. This means that the amount of label in the urea pool at 9 h is similar to the amount of label excreted in urinary urea from 9 h to 24 h. Therefore in order to determine the amount of label which has passed to urea in the 9 h period, either the label retained within the pool can be determined from a blood sample, or the label lost from the pool up to 24 h can be measured directly. In a non-invasive study the extension of the period of urine collection to 24 h may be preferred to a blood sample at 9 h (note this does not give a value for flux over a 24 h period). The collection of urine also requires fewer assumptions to be made about the size of the urea space, based on an anthropometric derivation of TBW. Values based on the excretion of label in urea at 36 to 48 h are significantly less than at 24 h. It is likely that this can be attributed to the return of label from protein degradation rather than more complete clearance of the urea pool, but it is not possible to know with certainty. However, as the relative difference for both NH_3 and urea is similar from 24 to 48 h, it appears more likely that the change should be attributed to return of label rather than clearance of the pool.

Comparison of single dose with intermittent dose

The underlying principle of the single-dose approach is that label introduced to the pool reliably traces the fate of material present in the pool, and in essence therefore traces the fate of label as it passes from the pool. In contrast, the intermittent-dose approach is based on the dilution of label within the pool, determined by the rate of entry of unlabelled material into the pool, thereby determining the movement of material into the pool. Hence, if each of the two approaches gives a reliable measurement they are only likely to give similar results when the rate of movement of material into the pool and the rate of movement of material out of the pool are similar, i.e. where a steady metabolic state exists. The derivation of flux in the single-dose approach is based on the determination of amounts of material, whereas the derivation of flux in the intermittent-dose approach is based on the measurement of the proportions of labelled and unlabelled species of the material. In the present study there were consistent differences in the results obtained with the two different approaches for each of the two end-products. Given that the two approaches were conducted in the same individuals and that the experimental conditions were sufficiently similar, the consistent differences are most likely to be attributable to real differences between the methods rather than other confounding variables. At the present time we have no adequate explanation for the differences, but there are two important possibilities which are presently being explored. First, the priming dose cannot be assumed to be without any metabolic effect, although it has been shown that relatively large single doses of labelled glycine do not exert any obvious influence on the determination of flux (Fern *et al.* 1981). Second, in the intermittent-dose approach the priming dose has been given at midnight and the measurement of flux carried out the following day. There is evidence for diurnal differences in aspects of protein kinetics (Garlick *et al.* 1980; Millward *et al.* 1991; El-Khoury *et al.* 1994) and it cannot be assumed that label given overnight is handled in the same way as label given during the daytime (Fern *et al.* 1981), nor that there is necessarily negligible return of label from a prime dose of label given the night before.

End-product

For both the single-dose and the intermittent-dose approaches, a consistent finding is that for comparative measurements flux derived from measurements of enrichment in urinary NH_3 are lower than for urinary urea. In the intermittent-dose approach the prime is given to reduce the time taken for plateau enrichment to be achieved in urinary urea. Any prime which is suitable for the large urea pool will invariably be inappropriately large for the smaller, more rapidly turning over NH_3 pool (Jackson *et al.* 1987). Hence, there will be an overshoot in the enrichment in urinary NH_3 and a tendency towards the plateau value being falsely elevated during the period of study (Shipley & Clarke, 1972). However, it is likely that this effect is small and not sufficient alone to account for the differences in the final levels of enrichment in urea and NH_3 as similar differences persist when no priming dose is given (Sim *et al.* 1980; Jeevenandam *et al.* 1985; Jackson *et al.* 1987). In the single-dose method the assumption is made that all label moving to the end-product is either excreted in the urine within the time course of the experiment or can be accounted for as being retained within the pool. However, urea is not an end-product of metabolism and on an intake of 1·2 g protein/kg per d about 25% of the urea-N produced will be salvaged through the metabolic activity of the colonic microflora (Hibbert & Jackson, 1991). At least half of the salvaged N will return to the amino acid pool and from the point of view of the model will be seen as having moved into protein synthesis. This could theoretically account for an apparent difference in flux derived from urea and NH_3. However, this would only be applicable in the single-dose approach which follows the movement of label from the

pool, and not in the intermittent-dose approach which determines the movement of material into the pool (*vide supra*).

Neither of the above explanations is sufficient to explain the consistent difference between urea and NH_3, and the suggestion has been made that the difference is more likely to be a reflection of the differing patterns of enrichment in the precursors for urinary urea and NH_3. It has been suggested that as urinary NH_3 derives predominantly from glutamine that enrichment in NH_3 will reflect the enrichment in glutamine derived from a muscle pool of amino acids (Fern & Garlick, 1983; Fern *et al.* 1985*b*). In contrast, the hepatic production of urea would tend to reflect the pattern of enrichment in hepatic amino acids, representative of a visceral pool (Fern *et al.* 1985*b*). This idea has derived some support from the evidence which shows that there may be substantial differences in the patterns of labelling in the end-products depending on whether the amino acid tracers are given by the intravenous or the oral route (Fern *et al.* 1981, 1985*a*; Jackson *et al.* 1987). This explanation has been found attractive and has been used as an explanation to allow for a possible mechanism through which changes in protein turnover and energy expenditure take place in adaptive response to chronic energy deficiency (Soares *et al.* 1991, 1994). In the absence of a more formal exploration in which the enrichment of precursors and products are measured in the same study protocol we are not able to know how justified these propositions might be. However, given the considerable importance which attaches to a clear understanding of the mechanisms which underlie successful adaptation to changes in dietary intake there is the need to clarify this point.

Protein turnover

As the derivations of protein synthesis and degradation are based on the value obtained for N flux, any errors in the derivation of flux will be reflected in the results for synthesis and degradation. However, even when an acceptable value for flux is obtained there are still other important considerations in the derivation of synthesis and degradation. In the present study N flux has been derived in the fed state during the daytime period. Protein synthesis is calculated as flux minus N excretion. Two important sources of potential error are that not all N excreted during the period was included in the calculation, and that not all 'potential' losses of N are recorded during the period of the study. It is possible to make estimates for the first category, which includes other urinary losses of N and miscellaneous losses. The second category would include urea-N retained within the urea pool of the body and N which has moved to faecal losses, but has not been passed in the study period. Failure to account for these losses would mean that the tendency is for protein synthesis to represent an overestimate. A similar problem applies to the derivation of protein degradation. In nutrition studies protein intake is expressed on a daily basis, but in fact intake is discontinuous throughout the day. The problem this presents in calculating degradation is shown in Table 3. A protein intake of 1·2 g/kg per d represents a N intake of 192 mg N/kg per d or 8 mg N/kg per h. In reality, as the food was only taken over a period of 12 h, the actual intake for this period was 16 mg N/kg per h. Therefore, strictly it is only justifiable to derive a value for degradation over the period of study itself. As shown in Table 3, degradation derived over the period of study was only 13 mg N/kg per h, compared with 20 mg N/kg per h when intake was taken as the average intake over the 24 h.

Conclusion

One of the important objectives of being able to measure whole-body protein turnover is to be able to understand the mechanisms through which the body responds to differences in intake, functional state, or diseased state. It is of importance to be able to do this in free-

living individuals and therefore there is the need for methods which are minimally invasive and relatively low cost. The end-product method is ideally suited for this purpose, but there are a number of assumptions of the method which need to be explored. (1) The tracer of choice deserves further exploration, and ultimately the preferred choice will be determined by a more detailed understanding of the metabolism of the N of individual amino acids. For the time being glycine is still the preferred option. (2) The design of individual studies has been the subject of the present report. We conclude that the preferred approach is a single-dose study with oral label over a 12 h period. The derivation of flux should be based on the loss of label to urea and NH_3 over 12 h. The loss of label can be measured as the amount of label in urinary NH_3 over 12 h and the amount of label in urea over 12 h (either as label in urinary urea plus label retained in urea in the urea pool or label recovered in urinary urea over 24 h). Results should be expressed as mg N/kg per h, and protein degradation should be calculated from the intake over the 12 h period when the fate of label is being determined. The most suitable way to calculate protein synthesis is less certain and awaits understanding from further experimentation. However, in the interim it should be based on the loss of N in urine with suitable adjustments for miscellaneous losses, faecal losses, and urea-N retained within the N pool. (3) There is the need to determine how fasting as distinct from daytime/night time (Fern *et al.* 1981) modifies the pattern of isotope movement and the derivations of protein turnover with the single dose. (4) There is the need for a more formal exploration of the extent to which isotope partitioning between the two end-products is influenced by either the relative mass, or the metabolic activity, of the visceral and somatic tissues. (5) There is the need to determine the extent to which the assumptions can be found to hold for different metabolic or pathological states.

It is acknowledged that proteins which turnover with a very short half-life are not included in the measurements of flux carried out in the present work, and the turnover of these proteins requires different approaches for their quantification (Jackson *et al.* 1987; Garlick *et al.* 1989). Our present results would suggest that the single-dose, end-product method for the measurement of protein turnover can provide useful information, but should be carried out under conditions where appropriate account can be taken of the assumptions which have been made.

During the conduct of this work, G.G. was an MRC scholar.

REFERENCES

Aqvist, S. E. G. (1951). Metabolic interrelationships among amino acids studied with isotopic nitrogen. *Acta Medica Scandinavica* **5**, 1046–1064.

Badaloo, A., Jackson, A. A. & Jahoor, F. (1989). Whole body protein turnover and resting metabolic rate in homozygous sickle cell disease. *Clinical Science* **77**, 93–97.

deBenoist, B., Abdulrazzak, Y., Brooke, O. G., Halliday, D. & Millward, D. J. (1984). Measurement of whole body protein turnover in the preterm infant with intragastric infusion of 1-[1-^{13}C]leucine and sampling of the urinary leucine pool. *Clinical Science* **66**, 155–164.

deBenoist, B., Jackson, A. A., Hall, J. StE. & Persaud, C. (1985). Whole body protein turnover in Jamaican women during normal pregnancy. *Human Nutrition: Clinical Nutrition* **39C**, 167–180.

El-Khoury, A. E., Fukagawa, N. K., Sanchez, M., Tsay, R. H., Gleason, R. E., Chapman, T. E. & Young, V. R. (1994). Validation of the tracer-balance concept with reference to leucine: 24-h intravenous tracer studies with L-[1-^{13}C]leucine and [^{15}N-^{15}N]urea. *American Journal of Clinical Nutrition* **59**, 1000–1011.

Fern, E. B. & Garlick, P. J. (1983). The rate of whole body metabolism in the whole body of man measured with ^{15}N glycine and uniformly labelled ^{15}N wheat. *Human Nutrition: Clinical Nutrition* **37C**, 91–107.

Fern, E. B., Garlick, P. J., McNurlan, M. A. & Waterlow, J. C. (1981). The excretion of isotope in urea and ammonia for estimating protein turnover in man with [^{15}N]glycine. *Clinical Science* **61**, 217–228.

Fern, E. B., Garlick, P. J., Sheppard, H. G. & Fern, M. (1984). The precision of measuring the rate of whole-body nitrogen flux and protein synthesis in man with a single dose of [^{15}N]-glycine. *Human Nutrition: Clinical Nutrition* **38C**, 63–73.

Fern, E. B., Garlick, P. J. & Waterlow, J. C. (1985*a*). Apparent compartmentation of body nitrogen in one human

subject: its consequences in measuring the rate of whole-body protein synthesis with ^{15}N. *Clinical Science* **68**, 271–282.

Fern, E. B., Garlick, P. J. & Waterlow, J. C. (1985*b*). The concept of the single body pool of metabolic nitrogen in determining the rate of whole-body nitrogen turnover. *Human Nutrition: Clinical Nutrition* **39C**, 85–99.

Garlick, P. J., Clugston, G. A., Swick, R. W. & Waterlow, J. C. (1980). Diurnal pattern of protein and energy metabolism in man. *American Journal of Clinical Nutrition* **33**, 1983–1986.

Garlick, P. J., Wernerman, J., McNurlan, M. A., Essen, P., Lobley, G. E., Milne, E., Calder, G. A. & Vinnars, E. (1989). Measurement of the rate of protein synthesis in muscle of postabsorptive young men by injection of a 'flooding dose' of [1-^{13}C]leucine. *Clinical Science* **77**, 329–336.

Golden, M. H. N. & Jackson, A. A. (1981). Assumptions and errors in the use of ^{15}N-excretion data to estimate whole body protein turnover. In *Nitrogen Metabolism in Man*, pp. 323–344 [J. C. Waterlow and J. M. L. Stephen, editors]. London: Applied Science Publishers.

Golden, M. H. N. & Waterlow, J. C. (1977). Total protein synthesis in elderly people: a comparison of results with [^{15}N]glycine and [^{14}C]leucine. *Clinical Science* **53**, 277–288.

Hibbert, J. M. & Jackson, A. A. (1991). The intra-individual variation in urea kinetics measured in a single individual over a period of four years. *European Journal of Clinical Nutrition* **45**, 347–351.

Jackson, A. A. (1991). The glycine story. *European Journal of Clinical Nutrition* **45**, 59–65.

Jackson, A. A., Danielsen, M. S. & Boyes, S. (1993). A non-invasive method for measuring urea kinetics with a single dose of [$^{15}N^{15}N$]urea in free-living humans. *Journal of Nutrition* **123**, 2129–2136.

Jackson, A. A. & Golden, M. H. N. (1980). [^{15}N]Glycine metabolism in normal man: the metabolic α-amino-nitrogen pool. *Clinical Science* **58**, 517–522.

Jackson, A. A., Golden, M. H. N., Byfield, R., Jahoor, F., Royes, J. & Soutter, L. (1983). Whole body turnover and nitrogen balance in young children at intakes of protein and energy in the region of maintenance. *Human Nutrition: Clinical Nutrition* **37C**, 433–446.

Jackson, A. A., Golden, M. H. N., Jahoor, P. F. & Landman, J. P. (1980). The isolation of urea nitrogen and ammonia nitrogen from biological samples for mass spectrometry. *Analytical Biochemistry* **105**, 14–17.

Jackson, A. A., Persaud, C., Badaloo, V. & deBenoist, B. (1987). Whole-body protein turnover in man determined in three hours with oral or intravenous ^{15}N-glycine and enrichment in urinary ammonia. *Human Nutrition: Clinical Nutrition* **41C**, 263–276.

Jackson, A. A., Shaw, J. C. L., Barber, A. & Golden, M. H. N. (1981). Nitrogen metabolism in preterm infants fed human donor breast milk: the possible essentiality of glycine. *Pediatric Research* **15**, 1454–1461.

Jahoor, F. (1982). In vivo nitrogen metabolism in the rat. PhD thesis, University of the West Indies.

Jahoor, F., Jackson, A. A. & Golden, M. H. N. (1988). In vivo metabolism of nitrogen precursors for urea synthesis in the postprandial rat. *Annals of Nutrition and Metabolism* **32**, 240–244.

Jeevenandam, M., Brennan, M. F., Horowitz, G. D., Rose, D., Mihiranian, M. H., Daly, J. & Lowry, S. F. (1985). Tracer priming in human protein studies with [^{15}N]glycine. *Biochemical Medicine* **34**, 214–225.

Kaplan, A. (1965). Urea nitrogen and ammonia. In *Standard Methods in Clinical Chemistry*, vol. 5, pp. 245–256 [S. Meites, editor]. New York: Academic Press.

Millward, D. J., Price, G. M., Pacy, P. J. H. & Halliday, D. (1991). Whole-body protein and amino acid turnover in man: what can we measure with confidence? *Proceedings of the Nutrition Society* **50**, 197–216.

Moran, B. J. & Jackson, A. A. (1990). Metabolism of ^{15}N-labelled urea in the functioning and defunctioned human colon. *Clinical Science* **79**, 253–258.

Pacy, P. J., Price, G. M., Halliday, D., Quevedo, M. R. & Millward, D. J. (1994). Nitrogen homeostasis in man: the diurnal responses of protein synthesis and degradation and amino acid oxidation to diets with increasing protein intakes. *Clinical Science* **86**, 103–118.

Pencharz, P. B., Steffee, W. P., Cochran, W., Scrimshaw, N. S., Rand, W. M. & Young, V. R. (1977). Protein metabolism in human neonates: nitrogen-balance studies, estimated obligatory losses of nitrogen and whole-body turnover of nitrogen. *Clinical Science and Molecular Medicine* **52**, 485–498.

Picou, D. & Taylor-Roberts, T. (1969). The measurement of total protein synthesis and catabolism and nitrogen turnover in infants in different nutritional states and receiving different amounts of dietary protein. *Clinical Science* **36**, 283–296.

San Pietro, A. & Rittenberg, D. (1953). A study of the rate of protein synthesis in humans. II. Measurements of the metabolic pool and the rate of protein synthesis. *Journal of Biological Chemistry* **201**, 457–473.

Shipley, R. A. & Clarke, R. E. (1972). *Tracer Methods for In Vivo Kinetics*. New York: Academic Press.

Sim, A. J. W., Wolfe, B. M., Sugden, B., Young, V. R. & Moore, F. D. (1980). Nitrogen turnover in man. *Journal of Parenteral and Enteral Nutrition* **4**, 180–183.

Soares, M. J., Piers, L. S., Shetty, P. S., Jackson, A. A. & Waterlow, J. C. (1994). Whole body protein turnover in chronically undernourished individuals. *Clinical Science* **86**, 441–446.

Soares, M. J., Piers, L. S., Shetty, P. S., Robinson, S., Jackson, A. A. & Waterlow, J. C. (1991). Basal metabolic rate, body composition and whole-body protein turnover in Indian men with different nutritional status. *Clinical Science* **81**, 419–425.

Taruvinga, M., Jackson, A. A. & Golden, M. H. N. (1979). Comparison of ^{15}N-labelled glycine, aspartate, valine and leucine for measurement of whole-body protein turnover. *Clinical Science* **57**, 281–283.

Vitti, T. G. & Gaebler, O. H. (1963). Effects of growth hormone on metabolism of nitrogen from several amino acids and ammonia. *Archives of Biochemistry and Biophysics* **101**, 292–298.

Waterlow, J. C. (1967). Lysine turnover in man measured by intravenous infusion of L[U-^{14}C]lysine. *Clinical Science* **33**, 507–515.

Waterlow, J. C. (1984). Protein turnover with special reference to man. *Quarterly Journal of Experimental Physiology* **69**, 409–438.

Waterlow, J. C., Garlick, P. J. & Millward, D. J. (1978*a*). *Protein Turnover in Mammalian Tissues and in the Whole Body*. Amsterdam: North Holland Publishing Company.

Waterlow, J. C., Golden, M. H. N. & Garlick, P. J. (1978*b*). Protein turnover in man measured with ^{15}N: comparison of end products and dose regimes. *American Journal of Physiology* **235**, E165–E174.

Watson, P. E., Watson, T. D. & Ball, R. (1980). Total body water volumes for adult males and females estimated from simple anthropometric measurements. *American Journal of Clinical Nutrition* **33**, 27–39.

Willommet, L., Schutz, Y., Whitehead, R., Jéquier, E. & Fern, E. B. (1992). Whole body protein metabolism and resting energy expenditure in pregnant Gambian women. *American Journal of Physiology* **263**, E624–E631.

Windmueller, H. G. & Spaeth, A. E. (1980). Respiratory fuels and nitrogen metabolism in vivo in small intestine of fed rats. Quantitative importance of glutamine, glutamate and aspartate. *Journal of Biological Chemistry* **255**, 107–112.

Wu, H. & Bishop, C. W. (1959). Pattern of N^{15}-excretion in man following administration of N^{15}-labeled glycine. *Journal of Applied Physiology* **14**, 1–5.

Young, V. R., Bier, D. M. & Pellet, P. L. (1989). A theoretical basis for increasing current estimates of the amino acid requirements in adult man, with experimental support. *American Journal of Clinical Nutrition* **50**, 80–92.

Printed in Great Britain