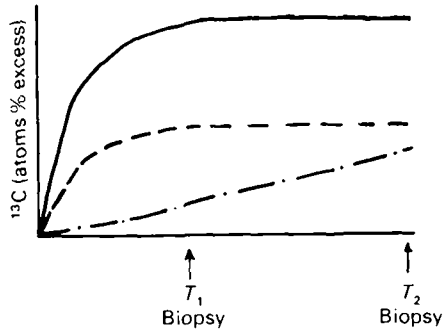
The use of stable isotope tracers as metabolic probes of whole-body and limb metabolism

By M. J. RENNIE, *Department of Physiology, University of Dundee, Dundee DD1 4HN*, and D. HALLIDAY, *Division of Clinical Sciences, MRC Clinical Research Centre, Northwick Park, Harrow, Middlesex HA1 3UJ*

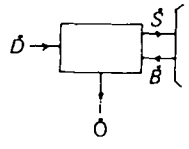
We have been drawn to the use of stable isotopes rather than radioisotope tracers (Halliday & Rennie, 1982) as probes of human metabolism for a number of reasons. The most important of these are the lack of radioactive hazard, the higher sensitivity in some techniques such as measurement of tissue protein synthesis, and the ease of measurement of fractional enrichment in one instrument leading to much greater precision than can be obtained by separate measurements of radioactivity and concentration.

We have for the most part used very simple models for our studies following on in the footsteps of Professor John Waterlow (Waterlow *et al.* 1978), and we routinely use a primed constant-infusion method (Matthews *et al.* 1980; Rennie *et al.* 1982*a*,*b*) (Fig. 1). Since a major aim of our studies is to investigate the nature of protein metabolism, we use an essential amino acid, leucine, as our probe. The aim of the infusion is to achieve plateau labelling of leucine in the free amino acid pool: this facilitates the calculation of the metabolic flux of leucine through that pool and ensures that processes of protein synthesis or amino acid oxidation draw on a precursor pool which is in a steady-state with regard to labelling. By sampling blood we can get some estimate of the labelling of the free pool, although this is inevitably an overestimate because of the problems of partition of amino acids within tissues. However, for accessible tissues this is not a major problem and the same techniques (gas chromatography–mass spectrometry) can be applied to both plasma and intracellular water to measure leucine enrichment. Leucine oxidation may be measured in the whole body by means of collection of expired carbon dioxide with subsequent cryogenic purification and measurement of $^{13}CO_2$ enrichment by isotope-ratio mass spectrometry (Halliday & Read, 1981). Protein synthesis in tissues is measured by the removal of tissue samples either by needle biopsy under local anaesthetic or by open biopsy during surgical procedures, followed by the chromatographic purification of amino acid from hydrolysed protein and the measurement of enrichment of protein-bound amino acid. Although we originally used ion-exchange methods (Rennie *et al.* 1982*a*,*b*) to purify the amino acid, a preparative gas–liquid chromatographic method used to fractionate *N*-propyl amino acid esters has superseded this (W. W. Read, M. J. Rennie and D. Halliday, unpublished results).

How confident can we be of the answers we obtain by these kinds of methods? We have examined the effects of biological variability and the precision and accuracy of the methods. In addition, since there are a number of assumptions



(*a*) Whole body turnover, $\dot{Q}$



$$\dot{Q} = \frac{^{13}C \text{ input}}{\text{Plateau } ^{13}C} = \dot{S} + \dot{O} = \dot{D} + \dot{B}$$

(*b*) Muscle protein synthesis

$$S_m \quad \frac{\Delta \text{ Protein } [1\text{-}^{13}C]\text{leucine}}{\text{Free } [1\text{-}^{13}C]\text{leucine}} \times \frac{1}{T_1 - T_2}$$

Fig. 1. Diagrammatic representation of enrichment of blood leucine (———), breath carbon dioxide (– – –) and leucine in muscle protein (– · – · –) during a primed constant infusion of [1-^{13}C]leucine. (*a*) Diagrammatic representation of a simple two-pool model for leucine turnover in the whole body where $\dot{Q}$ is total flux, $\dot{D}$ is rate of dietary input, $\dot{B}$ is rate of input from protein breakdown, $\dot{S}$ is rate of removal via protein synthesis and $\dot{O}$ is rate of removal via amino acid catabolism. (*b*) Method of calculating muscle protein synthesis (S_m) from enrichment of biopsies obtained at times T_1 and T_2.

involved in the final calculations of protein turnover from amino acid flux (such as the labelling of precursor pools within tissues and a knowledge of the composition of protein in the tissues with respect to leucine) we have attempted to validate them.

So far as instrumentation is concerned, the isotope-ratio mass spectrometer is capable of detecting one part per 10^6 with a precision of 0·1%, and gas–liquid chromatography plus mass spectrometry of detecting five parts in 10^3 with a precision of 2·0%. These are adequate since the errors do not approach those introduced by biological variation and sample handling before the final measurement. Furthermore, the latter errors are sufficiently small to allow the detection of physiologically- and pathologically-meaningful alterations in protein turnover (see Table 1).

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Table 1. *Variability of analyses of enrichment of biological samples*

(a) Replicate analysis of enrichment of human albumin with [^{13}C]leucine

n 9 Mean 0·0600 SD 0·0038

(b) Reproducibility of enrichment of leucine

$\frac{L}{R} \times 100 \pm$ CV: n 17

Enrichment of free leucine	91 ± 11
Enrichment of protein leucine	99 ± 8

(c) Errors of defining the steady-state during constant infusion of [1-^{13}C]leucine*

	Mean	SD
Plasma leucine enrichment	2·2	1·5
Concentration	2·2	0·6
Expired air ^{13}C	2·9	1·7
Carbon dioxide production	2·9	1·3

L, left; R, right; CV, coefficient of variation

*Expressed as CV of nine individual samples taken at plateau about a mean value.

So far as precursor labelling is concerned we are limited in the extent to which we can approach this problem by the size of tissue samples obtainable. Nevertheless, we have been able to measure enrichment of leucine in the intracellular pool in a variety of muscles, including heart muscle, and also in skin and bowel serosa (Table 2). One useful finding has been that the enrichment of leucine in the free pool in muscle and in plasma is close, in both the fed and fasted conditions, to the enrichment of the transamination product of leucine (α-ketoisocaproate). We have therefore felt justified in making calculations of synthesis rate and whole-body protein turnover on the basis of α-ketoisocaproate labelling.

The concentration of leucine in the tissues compared with that of the whole body is of importance in calculating the scaling factor between leucine turnover and whole-body protein turnover. Although for many years values of 8% leucine in whole-body protein and 10% in muscle protein have been used, these values were based on interpolation rather than on an analysis of whole human carcass (for discussion, see Reeds & Harris, 1981). Reeds & Harris (1981), by analysis of whole sheep and steer carcasses, found 6·6% leucine in protein. We have recently analysed leucine in samples of all the major human tissues taken during autopsy of individuals who suffered sudden accidental death. It is difficult to believe from these results (Table 3) that the currently-accepted values of leucine as 8% of the whole-body protein should be revised downwards by one-quarter unless our understanding of the size of the tissue compartments in man is incorrect.

Whatever our methodological problems, we feel sure that we can produce biologically-meaningful information. Among a number of pieces of evidence that this is so is the finding that in man, as in animals, a linear relationship exists between protein synthetic rate and muscle RNA concentration in samples taken from limb, trunk and heart muscle in patients during operation (Fig. 2).

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Table 2. *^{13}C enrichment of free leucine or α-ketoisocaproate (α-KIC) measured in tissue water by gas–liquid chromatography and mass spectrometry relative to blood* (100) (*M. J. Rennie and D. Halliday, unpublished results*)

(Mean values and standard deviations; no. of determinations in parentheses)

	Blood α-KIC		Heart (atrium)		Skeletal muscle: Leg (quadriceps)		Skeletal muscle: Leg adductor	Skeletal muscle: Rectus abdominus		Skin		Bowel serosa	
	Mean	SD	Mean	SD	Mean	SD		Mean	SD	Mean	SD	Mean	SD
Fed	82	2	—		85	7	—	87	8	78		—	
	(15)				(7)			(2)		(1)			
Fasted	84	4	86	14	80	4	76	73	12	58	10	55	16
	(17)		(5)		(7)		(1)	(10)		(3)		(4)	

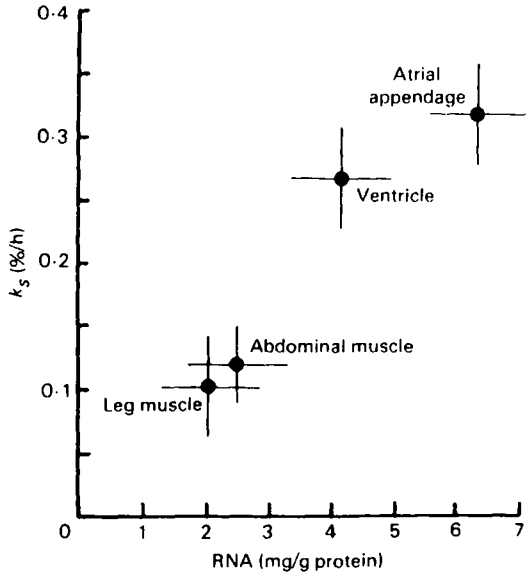

Fig. 2. Relationship between fractional synthesis rate (k_S; %/h) and RNA concentration (mg/g protein) in samples obtained after 6–9 h of constant infusion of [1–^{13}C]leucine in normal man and surgical patients (M. J. Rennie, J. M. M. Vaughan, P. H. Sugden, G. Bennett, W. W. Read, C. Ford and D. Halliday, unpublished results). Points are mean values and standard deviations for seven to ten tissue samples.

Table 3. *Leucine content of human tissues*

(No. of determinations given in parentheses)

Tissue	Tissue wt (% body-wt)	Protein content* (mg/g)	Leucine content (mg/g tissue protein)†	
			Mean	SD
Muscle	37	160	80·3(6)	8·6
Gut (colon, small intestine, oesophagus stomach)	2·1	130	83·5 (6)	7·7
Uterus	—	—	127 (1)	
Liver	3·4	160	86·6 (5)	15·8
Heart (ventricle or atrium)	0·7	160	86·9 (7)	12·3
Skin	7·8	220	56·4 (5)	10·5
Bone (rib)	14·9	190	60·8 (4)	5·7
Lung	4·5	130	88·0 (3)	13·5
Kidney	0·5	150	88·0 (3)	13·5
Testis	—	—	83 (1)	
Spleen	0·2	180	103 (1)	
Aorta and vein	1·0	200	111 (3)	11
Cartilage	—	—	84 (1)	
Adrenal	—	—	89 (1)	
Brain and cord	2·5	120	75 (5)	7·6
Whole blood	8	180	120 (6)	5
Total	82·6			

*Values from Mitchell *et al.* (1945).

†Unpublished values from P. W. Emery and M. J. Rennie.

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Furthermore, in response to a given stimulus, we see changes in whole-body turnover and in muscle protein synthesis in man which are consonant with the changes observed in animals. Although results of other studies concerning the relative proportions in man of protein turnover in muscle and in the whole body (1:2) or the absolute value of protein synthesis in these compartments are not in agreement with our results, there seems little doubt that the twofold changes we found in protein synthetic activity of muscle and the whole body in response to feeding and fasting (Rennie *et al.* 1982*b*) are real biological phenomena. Furthermore, we have been able to observe large differences between normal subjects and those suffering from a number of pathological conditions which cause wasting of skeletal muscle: the differences are so great that it is difficult to believe that they arise from methodological problems (Rennie *et al.* 1983). Generally we observe that in muscle wasting there is a depression in the rate of muscle protein synthesis. We do not see a rapid change in the muscle mass and conclude that, at least in the chronic stages of many myopathies and in wasting due to cancer and surgical operation, the loss of tissue protein is due to a fall in the replacement of protein by synthetic processes.

This view is for the moment largely hypothetical since we do not have good methods for the measurement of protein breakdown. We have attempted to develop such methods in two ways. One approach has been to measure the efflux of 3-methylhistidine, an amino acid which is released from myofibrillar proteins on degradation and not re-utilized: this is achieved by measuring the concentration of the amino acid in arterial blood and venous blood draining the muscles of the leg (Lundholm *et al.* 1982). We have been able to show that, contrary to general expectations, the increased total α-amino-nitrogen efflux associated with muscle wasting is not found in certain chronic conditions such as cancer cachexia and malnutrition. Furthermore, following mild to moderate trauma accompanying elective gastrointestinal surgery, there is no indication of an increase in 3-methylhistidine efflux from leg tissues at the time when total N excretion is markedly increased (Rennie *et al.* 1984). At present these techniques simply measure the amounts of 3-methylhistidine, and of tyrosine, as indicators of total α-NH_2-N balance. The usefulness of the technique is to some extent limited by the difficulty of measuring very small concentrations of 3-methylhistidine. The measurement of 3-methylhistidine may be made more sensitive and precise by the use of mass-spectrometric techniques (Matthews *et al.* 1981*a*,*b*) and even more information may be obtained in the future by measuring the dilution between arterial and venous blood of exogenously-labelled 3-methylhistidine.

Another approach is to gain some insight into the processes of muscle synthesis and degradation by means of monitoring the uptake and release of leucine in muscle tissues (or indeed any other tissues accessible to arterial and venous blood measurements) and leucine oxidation. We have applied these techniques in studies of normal subjects and in those with wasting diseases of muscle. For example, by measuring the dilution of label between arterial and venous circulations in leucine and its keto acid α-ketoisocaproate and by measuring the production of labelled

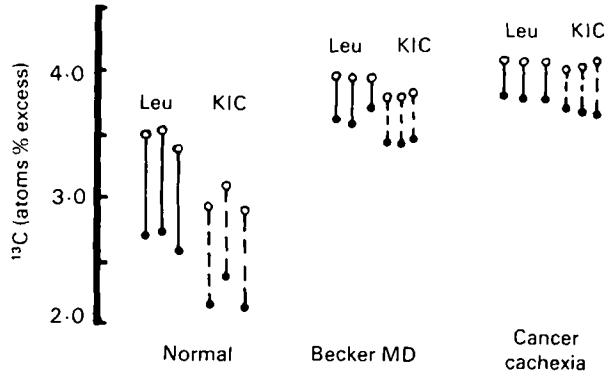

Fig. 3. Labelling of leucine (leu) and α-ketoisocaproate (KIC) obtained during constant infusion of [1-^{13}C]leucine from arterialized and deep forearm venous blood samples in normal subjects and in patients suffering from muscle wasting as a result of Becker muscular dystrophy and cancer cachexia (M. J. Rennie, D. Halliday, R. C. Griggs, R. H. T. Edwards and S. M. Matthews, unpublished results).

CO_2, it is possible to calculate the components of protein synthesis and breakdown within the tissue. Unfortunately leucine is not an ideal tracer in this respect, since it undergoes transamination within muscle. The absolute values of some of the components of leucine metabolism, such as delivery of unlabelled leucine via protein breakdown, are likely to be unreliable since their calculation is only possible by reference to assumed values for the equilibrium constant of transamination. At present we are only able to make approximate estimates of the constant by reference to measurements made in animal muscle or by kinetic measurements in the whole body of man (Matthews *et al.* 1980; Livesey & Lund, 1980). Nevertheless, while recognizing the constraints involved, the values produced by these methods for tissue synthesis and breakdown are of the same order as those arrived at by other techniques. Certainly this technique is able to highlight differences in metabolism in different physiological conditions such as exercise and hypoxia and in different clinical conditions such as the wasting associated with cancer or muscle disease (Fig. 3).

It may be that for the purposes of probing intracellular metabolism, leucine is not the best tracer to use for muscle because of transaminating reactions; phenylalanine and tyrosine, which are not metabolized in muscle, may give clearer results. Nevertheless, whatever the tracer to be used, gas chromatography–mass spectrometry appears to be the method of choice since the precision of estimation of fractional labelling is so much better than with alternative methods based on radiochemical techniques.

In summary, the use of stable isotope-labelled tracers in metabolism has increased greatly in recent times (Halliday & Rennie, 1982; Matthews & Bier, 1983) due to the ease of application of techniques which are effectively hazard-free and potentially much more powerful and precise than previous techniques. We have concentrated here on measurements using amino acids as tracers but of

course the same techniques could be used with fat and carbohydrate tracers. It is our expectation that the application of such techniques in metabolic and nutritional research will considerably extend our knowledge in this area.

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REFERENCES

Halliday, D. & Read, W. W. (1981). *Proceedings of the Nutrition Society* **40**, 321–334.
Halliday, D. & Rennie, M. J. (1982). *Clinical Science* **63**, 485–496.
Livesy, G. & Lund, P. (1980). *Biochemical Journal* **188**, 705–713.
Lundholm, K., Bennegård, K., Edén, E., Svaniger, G., Emery, P. W. & Rennie, M. J. (1982). *Cancer Research* **42**, 4807–4811.
Matthews, D. E. & Bier, D. M. (1983). *Annual Review of Nutrition* **3**, 309.
Matthews, D. E., Bier, D. M., Rennie, M. J., Edwards, R. H. T., Halliday, D., Clugston, G. A. & Millward, D. J. (1981*a*) *Science* **214**, 1129–1131.
Matthews, D. E., Motil, K. J., Rohrbaugh, D. K., Burke, J. F., Young, V. R. & Bier, D. M. (1980). *American Journal of Physiology* **238**, E473–E479.
Matthews, D. E., Starren, J. B., Drexler, A. J., Kipnes, D. M. & Bier, D. M. (1981*b*). *Analytical Biochemistry* **110**, 308–317.
Mitchell, H. H., Hamilton, T. S., Steggerda, F. R. & Bean, H. W. (1945). *Journal of Biological Chemistry* **158**, 625–637.
Reeds, P. J. & Harris, C. I. (1981). In *Nitrogen Metabolism in Man*, pp. 391–408. [J. C. Waterlow and J. M. L. Stephen, editors]. London & New Jersey: Applied Science Publishers.
Rennie, M. J., Bennegard, K., Eden, E., Emery, P. W. & Lundholm, K. (1984). *Metabolism* **33** 250–256.
Rennie, M. J., Edwards, R. H. T., Halliday, D., Matthews, D. E., Wolman, S. L. & Millward, D. J. (1982*a*). *Nature* **296** 165–167.
Rennie, M. J., Edwards, R. H. T., Halliday, D., Matthews, D. E., Wolman, S. L. & Millward, D. J. (1982*b*). *Clinical Science* **63**, 519–523.
Rennie, M. J., Emery, P. W., Edwards, R. H. T., Halliday, D., Lundholm, K. & Millward, D. J. (1983). *Clinical Physiology* **3**, 387–398.
Waterlow, J. C., Garlick, P. J. & Millward, D. J. (1978). *Protein Turnover in Mammalian Tissues and in the Whole Body*. Amsterdam: North-Holland.

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