Organ-specific measurements of protein turnover in man

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Over the last few years there has been increasing interest in protein synthesis and degradation in individual organs in man. As outlined in the previous paper (Millward *et al.* 1991), there are difficulties with the interpretation of measurements of the whole-body turnover. Moreover, in many conditions (e.g. cancer) the response differs in individual tissues and is not adequately reflected by measurements on the whole body (e.g. Pain *et al.* 1984). The aim of this presentation, therefore, was to review the available methods for assessing rates of protein turnover in individual tissues of human subjects. Only the isotopic methods will be discussed here, specifically those using stable isotopes, as these are more suitable for use in man. The non-isotopic methods, 3-methyl histidine excretion for muscle protein degradation (Long *et al.* 1988) and polyribosome analysis for protein synthesis (Wernerman *et al.* 1985), will not be considered here.

CONSTANT INTRAVENOUS INFUSION

The principle behind administering a labelled amino acid and measuring the incorporation of label into protein is simple enough. However, in practice it is complicated by the need to measure the time-course of isotopic enrichment of the free amino acid precursor. The constant-infusion method was developed to simplify this measurement, because the enrichment of the plasma amino acid rapidly rises to a plateau, enabling the entire time-course to be defined by a measurement at a single point in time (Waterlow *et al.* 1978). The technique was originally developed for use in animals with radioactive labels (Waterlow & Stephen, 1968; Garlick *et al.* 1973), but has been successfully adapted for measurements in man with stable isotopes.

The first uses of this approach in man were with [15]N. Halliday & McKeran (1975) infused [^{15}N]lysine into volunteers for 21–30 h and measured the incorporation of label into samples of muscle taken by percutaneous needle biopsy. Similarly Stein *et al.* (1978) gave 12–19 h infusions of [^{15}N]glycine to patients and measured the incorporation of ^{15}N into protein of various tissues taken during surgery. The rates of protein synthesis obtained by Stein *et al.* (1978; Table 1) showed that visceral tissues turned over two- to fourfold less rapidly than the corresponding tissues in young rats (Waterlow *et al.* 1978). Furthermore, there was a relationship between rates in malignant tumours and those in their tissues of origin. More recently measurements in skeletal muscle have been made by Halliday *et al.* (1988) with infusions of [1-^{13}C]leucine. The time-period required for measurement was reduced to 8 h by the use of a priming dose of isotope to achieve plateau labelling more rapidly, and technical improvements in mass spectrometry enabled the enrichment of free and protein-bound amino acid to be measured in very small samples of plasma or muscle. Some results obtained with this technique, showing

Table 1. *Rates of protein synthesis in human visceral tissues measured by [¹⁵N]glycine infusion (Stein et al. 1978)*

(Mean values and standard deviations)

	Protein synthesis (%/d)	
	Mean	SD
Liver	15	7
Stomach	22	10
Colon	9	6
Tumours	18	8

Table 2. *Muscle protein synthesis in post-absorptive and fed volunteers measured by constant infusion of [1-¹³C]leucine and arterio–venous (a–v) difference with [1-¹³C,¹⁵N]leucine (from Halliday et al. (1988))*

(Mean values and standard deviations)

	Protein synthesis (%/d)			
	Infusion		a–v difference	
	Mean	SD	Mean	SD
Post-absorptive	1·10	0·29	1·22	0·19
Fed	1·80	0·34	1·87	0·48

that rates of protein synthesis in quadriceps muscle are higher in fed volunteers than in post-absorptive volunteers, are given in Table 2.

The attainment of plateau labelling requires that the subject should be in a steady metabolic state throughout infusion, which might be difficult to achieve in some hospital patients. However, a more serious limitation of the method involves the measurement of the enrichment of the precursor for protein synthesis. During infusion the enrichment of the free amino acid in the tissue reaches a plateau which is lower than that in the plasma, typically by 20% in muscle and possibly more in some other tissues. Since it is not possible in man to measure directly the time-course of intracellular enrichment, it has become common practice to use instead the enrichment of α-ketoisocaproate (KIC), which is thought to be produced from intracellular leucine in muscle tissues (Matthews *et al.* 1982). However, this does not necessarily overcome the problem, since there is ample evidence from work both in vitro and in animals that the enrichment at the site of protein synthesis can be different from either the intracellular or plasma amino acid (Waterlow *et al.* 1978). Moreover, it has been shown by Bennet *et al.* (1989) that the enrichment of plasma KIC can differ from that of muscle free leucine. In their study the rate of protein synthesis in tibialis muscle of post-absorptive volunteers was 1·10%/d when calculated using the plasma free leucine, 1·32%/d from the plasma KIC and 2·26%/d from the muscle free leucine. With infusion of an amino acid mixture the rate of

muscle synthesis rose significantly to 1·51%/d calculated from plasma leucine and 1·78%/d from the KIC, but the rate calculated from muscle leucine was not significantly altered at 2·57%/d. Thus, not only were the rates calculated from the various available precursors different, but also the conclusion about the response to treatment differed. In work in vitro and in animals this ambiguity has been avoided by some workers by measurement of the direct precursor of protein synthesis, amino acyl tRNA (e.g. Airhart *et al.* 1974). This may be difficult for routine use in human tissues, since its half-life with respect to amino acylation is of the order of 1 s (Airhart *et al.* 1974) and rapid freezing to prevent changes in enrichment after tissue sampling would be required.

ARTERIO–VENOUS DIFFERENCE

Determination of the net uptake or outflow of an amino acid from an organ or limb, by measuring the arterial and venous concentrations and the blood flow-rate, has been a valuable tool in metabolic research for many years (e.g. Pozefsky *et al.* 1969). This technique gives only the net balance of amino acid, but if a constant infusion of labelled amino acid is given simultaneously it is possible to estimate the rates of synthesis and degradation of protein also (Cheng *et al.* 1985). With this approach, therefore, the incorporation of label into protein is not measured directly, but instead it is assumed that any label that disappears from the circulation as it passes through the tissue must be entering protein. The relevant measurements are (1) blood flow-rate, (2) the arterio–venous difference in concentration, which gives the net balance of amino acid, (3) the arterio–venous difference in isotopic enrichment, which gives the rate of protein degradation, and (4) the arterio–venous difference in the concentration of isotope in the blood, which gives the rate of protein synthesis (2, 3 and 4 are not independent, so if two are measured, the third can be calculated).

The major advantage of this approach is that it enables both the synthesis and degradation of protein to be determined simultaneously, which cannot be achieved by any other method. Thus, for example, Gelfand & Barrett (1987) were able to show that infusion of insulin into post-absorptive subjects caused the balance of phenylalanine across the forearm to change from negative to positive. This was accompanied by a decrease in forearm protein degradation, but no change in protein synthesis. Cheng *et al.* (1987) used labelled leucine to study the response to feeding. On the assumption that protein metabolism in the forearm represents mainly muscle, they have demonstrated an increase in protein synthesis with feeding, which was the same as that obtained previously with the constant-infusion method (Table 2).

There are a number of limitations of the arterio–venous balance method, some of which have been discussed previously (Wernerman & Vinnars, 1987). Although the technique avoids the need to take tissue biopsies, it is necessary to sample arterial blood and to measure blood flow-rate. The organ or limb studied must have a well-defined circulation, such that all the blood from a defined volume of tissue can be sampled: the forearm or leg are regarded as suitable, as are the splanchnic tissues. It is an advantage if the amino acid chosen is not metabolized in the tissue. Thus, phenylalanine is better than leucine, since with the latter it is necessary to measure also the arterio–venous differences of the oxidation products, KIC and carbon dioxide. A steady metabolic state of the free amino acid in the tissue is essential. For example, the rate of output of phenylalanine from the forearm in the post-absorptive state of about 10 μmol/h per l

Table 3. *Muscle protein synthesis in post-absorptive volunteers measured by flooding dose or constant infusion with [1-^{13}C]leucine, calculated using either the plasma leucine or α-ketoisocaproate (KIC) as precursor (values from Garlick* et al. *(1989 and unpublished results))*

(Mean values and standard deviations)

| | Protein synthesis (%/d) | | | |
| | Plasma KIC | | Plasma leucine | |
	Mean	SD	Mean	SD
Flooding dose (*n* 10)	1·95	0·37	1·86	0·37
Constant infusion (*n* 3)	1·04	0·20	0·78	0·19

tissue (Gelfand & Barrett, 1987) compares with a free phenylalanine pool size in the tissue of about 50 μmol/l tissue: thus, a small change in the free amino acid pool size would appreciably alter the inflow or outflow from the tissue, and hence apparently alter the balance of protein. Finally, this technique requires assumptions to be made about the enrichment of the precursor for protein synthesis in exactly the same way as the constant-infusion method, with the added limitation that intracellular enrichments cannot be measured. This should be borne in mind when making the comparison between these two methods shown in Table 2, since any error resulting from the precursor pool measurement will appear equally with the two techniques.

FLOODING-DOSE TECHNIQUE

The difficulty of measuring the true precursor enrichment when labelled amino acids are given in trace amounts has prompted the development of the flooding-dose approach in man. This technique has become the method of choice for measuring protein synthesis rates in isolated tissues and cell cultures in vitro and in animal tissues in vivo, because when the concentration of the labelled amino acids is raised, the difference between the intra- and extracellular enrichment and that of amino acyl tRNA is minimized (Henshaw *et al.* 1971; Mortimore *et al.* 1972; Ballard, 1982). This method has been widely used for measuring protein synthesis rates in animal tissues by injection of a flooding dose of radioactively-labelled leucine or phenylalanine (McNurlan *et al.* 1979; Garlick *et al.* 1980). We have recently been able to show that this approach can be used equally well for measuring muscle protein synthesis in man with stable isotopes. When volunteers were injected intravenously with 0·05 g [1-^{13}C]leucine/kg the enrichments of the plasma and muscle free leucine and of plasma KIC became almost equal over a period of 90 min (Garlick *et al.* 1989). It was, therefore, possible to estimate the rate of protein synthesis from the increase in enrichment of protein-bound leucine in muscle (taken by needle biopsy) over the 90 min period. The rates of synthesis calculated from either the plasma leucine or plasma KIC were almost equal (Table 3), illustrating the effectiveness of the flooding procedure in minimizing the uncertainty arising from differences in labelling of potential precursors.

The rate of muscle protein synthesis of almost 2%/d given by flooding with leucine is higher than that obtained by constant infusion (Halliday *et al.* 1988; Table 2). This does
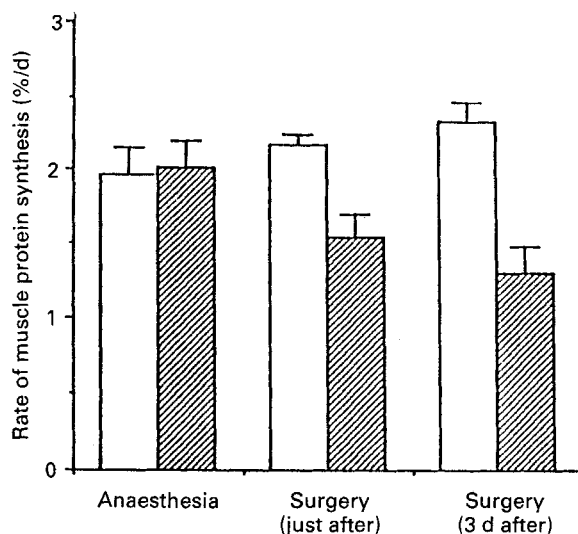
Fig. 1. Rates of muscle protein synthesis measured by flooding dose of [1-^{13}C]leucine in patients undergoing abdominal surgery. Measurements were made in all subjects before anaesthesia (control; □) and during anaesthesia, immediately after surgery was complete or 3 d after surgery (during which time patients were not fed). Values are means with their standard errors represented by vertical bars. Values from Essen *et al.* 1990*a,b*.

not result from analytical differences, since our own measurements with constant infusion show the same discrepancy (Table 3). The difference probably reflects the better estimate of precursor enrichment with flooding dose. However, it is important to rule out the possibility that the large dose of leucine itself had stimulated muscle protein synthesis, since stimulation of protein synthesis by leucine is known to occur in isolated rat muscles in vitro (e.g. Fulks *et al.* 1975). This possibility seems unlikely in human muscle in vivo, however, since the rate given by flooding with phenylalanine (1·89%/d, McNurlan *et al.* 1989) is similar to that given by leucine (Table 3) and phenylalanine has not been shown to influence muscle protein synthesis. Furthermore, the distribution of ribosomes in muscle of volunteers was not altered by injection of a large dose of leucine (M. A. McNurlan, P. Essen, P. J. Garlick and J. Wernerman, unpublished results).

There are several advantages to the flooding-dose procedure, in addition to the better estimate of precursor labelling. In particular, there is no requirement for a metabolic steady-state and the measurement period is relatively short. The technique is, therefore, particularly suited to measurements of acute changes in patients. For example, sequential measurements in surgical patients have shown that the anaesthetic has no effect on muscle protein synthesis, but immediately after surgery was completed there was a significant decrease (Essen *et al.* 1990*b*, Fig. 1). At 3 d after surgery there was an even more marked decrease in protein synthesis (Essen *et al.* 1990*a*, Fig. 1).

Muscle protein metabolism has been studied extensively because of its large mass, its sensitivity to nutrition and trauma and its accessibility for study. However, measurements of protein metabolism in other tissues can lead to important information regarding their growth or function. The flooding method is easily adapted for measuring protein

Table 4. *Rates of protein synthesis in healthy liver and colonic mucosa in comparison with rates obtained in a variety of pathological conditions*

(Measurements were by flooding dose of [1-^{13}C]leucine and tissues were taken at surgery (Heys *et al.* 1989, 1990*a*). Values under 'colon' were for the mucosa, except those marked * which were the tumours themselves)

| | Protein synthesis (%/d) | | | |
| | Liver | | Colon | |
Pathology	Mean	SE	Mean	SE
Healthy	23·5	1·4	9·5	1·5
Ulcerative colitis	36·8	1·0	26·8	3·2
Colon cancer	23·5	1·0	22·6	1·9*
Villous adenoma	—		38·1	2·6*

synthesis in tissues taken at surgery. Values for liver and colonic mucosa are shown in Table 4 (Heys *et al.* 1989, 1990*a*). The rate of synthesis in healthy colonic mucosa is about fivefold higher than that in skeletal muscle (see Table 3), whilst that in liver is about tenfold higher. A similar relationship between these three tissues has been observed in fasting young rats (McNurlan *et al.* 1982), although the absolute values were three- to fourfold higher in the rat. A substantial fraction of proteins synthesized in the liver is subsequently exported into the circulation, and their rate of synthesis can also be evaluated by this technique, provided allowance is made for the temporal delay arising from processing of the protein in the liver before detection of labelled protein in the plasma. The rate of albumin synthesis measured by flooding with [1-^{13}C]leucine in normal volunteers was 7·9 (SE 0·4)% intravascular albumin mass/d, and this rose to 20·4 (SE 2·0)%/d in nephrotic patients, who excrete albumin in the urine (Ballmer *et al.* 1990).

Table 4 also shows rates in liver and colonic mucosa in a variety of pathological conditions. In patients with ulcerative colitis both liver and colon protein synthesis were elevated compared with normal: an increase in whole-body protein synthesis has previously been demonstrated in this type of patient (Powell-Tuck *et al.* 1984). In colorectal cancer, however, the rate of liver protein synthesis was normal. This differs from findings obtained in tumour-bearing mice in which liver protein synthesis was elevated (Pain *et al.* 1984). This illustrates the difference between experimental tumours in animals, which are usually large, and the non-metastatic colon tumours in man studied here, and emphasizes the value of making direct measurements of the human condition. By contrast, the malignant tumour itself had a very much higher rate of protein synthesis than the healthy mucosa. The highest rate of synthesis, however, was seen in the benign villous adenoma. This might be a reflection not only of its rate of growth, but also of a high rate of mucus production.

The rate of protein synthesis in tumours in vivo has previously been used as an index of its rate of growth (Mullen *et al.* 1980; Emery *et al.* 1989). This is of value because rates of tumour growth in human patients are difficult to assess by conventional means, particularly over short periods of time. We have used this approach to assess the response of tumours to the provision of nutrients. Since cancer patients frequently lose weight, it may be considered advisable to provide nutritional support in order to improve their nutritional status and their ability to withstand anti-tumour therapy or surgery. It is

important to know, however, whether the nutrients might also stimulate the growth of the tumour. Patients with colorectal cancer were, therefore, either fasted during the day preceding surgery or were given total parenteral nutrition, continuing until surgery had commenced. At surgery rates of tumour protein synthesis were then measured (Heys *et al*. 1990*b*). The rate of tumour protein synthesis in the fed patients (44 (SE 3)%/d) was almost twice as high as that in the fasted patients (23 (SE 2)%/d), suggesting that the growth of the tumour is responsive to the exogenous supply of nutrients. Whereas it is not feasible to starve cancer patients, assessment of tumour protein synthesis might provide a method for selecting a diet with a specific composition which will not stimulate the tumour, whilst maintaining the normal host tissues.

SUMMARY AND CONCLUSIONS

Methods that were originally developed for animal studies have now been modified for measuring tissue protein turnover in man with stable isotopes. Of the three techniques which have been discussed, only the arterio–venous difference method has the capacity to measure both the synthesis and degradation. This technique is, however, difficult to perform and has a number of potential sources of error, one of the most important being the assessment of precursor labelling. Constant infusion of a labelled amino acid with measurement of the incorporation into protein of a biopsy is a simpler and more precise technique for measuring synthesis in patients as well as volunteers, but the most appropriate means of assessing the precursor labelling still remains to be worked out. The flooding-dose procedure aims to minimize the difficulty of assessing precursor labelling and there is no evidence that the unphysiological dose of labelled amino acid given influences the synthesis rate which is measured. It is rapid to perform and is very well suited to measurements in patients, in whom a wide range of tissues can be studied.

These advances in techniques have been facilitated particularly by improvements in mass spectrometry, which have allowed the use of stable in place of radioactive isotopes, and have enabled measurements of isotopic enrichments to be made in small samples of tissue. These techniques for assessing the dynamics of protein metabolism in individual tissues are now being used to answer nutritionally and clinically important questions in human volunteers and patients.

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