Biochem. J. (1980) **192**, 719–723
Printed in Great Britain

A rapid and convenient technique for measuring the rate of protein synthesis in tissues by injection of [^{3}H]phenylalanine

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(*Received 27 May 1980/Accepted 10 July 1980*)

A rapid procedure for measuring the specific radioactivity of phenylalanine in tissues was developed. This facilitates the accurate determination of rates of protein synthesis in a wide range of tissues by injection of 150 μmol of L-[4-^{3}H]phenylalanine/100g body wt. The large dose of amino acid results in a rapid rise in specific radioactivity of free phenylalanine in tissues to values close to that in plasma, followed by a slow but linear fall. This enables the rate of protein synthesis to be calculated from measurements of the specific radioactivity of free and protein-bound phenylalanine in tissues during a 10min period after injection of radioisotope.

Studies of the rate of protein synthesis and its regulation in tissues of the whole animal require accurate, reliable techniques for measurement. Whereas a number of methods involving administration of labelled amino acids with subsequent measurement of the incorporation of label into protein have been described, the main difficulty is the assessment of the specific radioactivity of the precursor amino acid at the site of protein synthesis. Amino acids from the more readily accessible compartments, such as the total intracellular pool or the plasma, cannot be used reliably for this purpose, because their specific radioactivities may differ from each other and from that of aminoacyl-tRNA (for review see Waterlow *et al.*, 1978). An additional difficulty is that, unless preventive measures are taken, the specific radioactivity of the free amino acid changes rapidly, and in order to measure the time course, large numbers of animals must be killed. A successful way of avoiding both these problems has been to give the labelled amino acid as a very large dose. This is sufficient to flood all possible precursor pools so that they reach nearly the same specific radioactivity, which is then maintained almost constant during the period of incorporation into protein (Henshaw *et al.*, 1971; Dunlop *et al.*, 1975). With one such method we injected 100 μmol of [^{14}C]leucine/100g body wt. into young rats (McNurlan *et al.*, 1979). The specific radioactivity of leucine in liver and small intestine rose rapidly to a value close to that in plasma and then fell slowly, but linearly. This enabled the average specific radioactivity of free leucine during a 10min labelling period to be estimated from measurements on tissues from rats killed at 2min and 10min. This technique has many advantages for measurement of the rate of protein synthesis in tissues with very rapid protein turnover (e.g. liver and gut; McNurlan *et al.*, 1979), but has two drawbacks. The specific radioactivity of leucine was measured with an amino acid analyser fitted with a split-stream attachment for counting of radioactivity. This technique was found to be very slow and expensive. In addition, leucine has been reported to have a role in the control of protein synthesis in muscle (Buse & Reid, 1975; Fulks *et al.*, 1975) and can cause changes in nitrogen balance in starving man (Sherwin, 1978). Although we were not able to detect any change in protein synthesis in liver and gut caused by the large dose of leucine, an alternative amino acid was sought that could be used to make measurements in all tissues, including muscle. Here we describe an improved technique for measuring rates of protein synthesis in animal tissues by injecting a flooding dose (150 μmol/100g body wt.) of L-[4-^{3}H]phenylalanine coupled with a sensitive and selective method for estimating the specific radioactivity of phenylalanine.

Experimental

Labelled compounds were purchased from The Radiochemical Centre, Amersham, Bucks., U.K., and were evaporated to dryness before being dissolved in the appropriate injection solution. L-Tyrosine decarboxylase, β-phenethylamine and leucylalanine were purchased from Sigma (London)

0306-3283/80/110719-05$01.50/1

Chemical Co., Poole, Dorset, U.K., and ninhydrin was from BDH, Poole, Dorset, U.K.

Male Wistar rats (Charles River, Margate, Kent, U.K.) weighing approx. 75g were maintained on a 12h-light/12h-dark cycle and were fed on a synthetic diet prepared in the laboratory containing 20% (w/w) casein. When they had reached the appropriate body weight, each rat was restrained in a cloth and injected with radioisotope via a lateral tail vein. For the flooding dose, L-[4-^{3}H]phenylalanine was combined with unlabelled phenylalanine (150mM in water) to give approx. 50μCi/ml. In the experiments with tracer doses of amino acids the solutions contained 7.7μCi of L-[U-^{14}C]threonine (10mCi/mmol)/ml plus 38μCi of L-[4,5-^{3}H]lysine (15Ci/mmol)/ml in either 0.9% (w/v) NaCl or 150mM unlabelled phenylalanine. In all experiments rats were injected with 1.0ml/100g body wt. At various times after injection rats were decapitated, blood was collected in heparinized tubes, and tissues were rapidly excised and frozen in liquid N_2. Tissues that required careful dissection, such as individual muscles, were crudely removed and chilled in ice before dissection. The mucosa and serosa of the jejunum were separated as described previously (McNurlan *et al.*, 1979).

Frozen tissues were pulverized between two aluminium blocks precooled in solid CO_2. The powder from 0.1–0.5g of tissue was then precipitated in 3ml of cold 2% (w/v) $HClO_4$ and centrifuged at 2800*g* for 15min. To the supernatant was added 1.5ml of saturated tripotassium citrate, resulting in precipitation of $KClO_4$ and a pH close to 6.0. This sample was centrifuged at 2800*g* for 15min, and the supernatant was used for measurement of the specific radioactivity of free phenylalanine. The precipitate containing protein was washed three times with 10ml of 2% $HClO_4$, resuspended in 10ml of 0.3M-NaOH and incubated at 37°C for 1h. In the experiments with tracer doses of [^{3}H]lysine and [^{14}C]threonine, this solution was used for radioactivity counting with a Beckman LS 150 instrument adjusted for ^{3}H/^{14}C separation and a xylene-based scintillant as described by Fricke (1975), and for measurement of RNA and protein as described previously (McNurlan *et al.*, 1979).

Protein-bound phenylalanine was obtained by re-precipitating the protein from the NaOH solution with 2ml of 20% (w/v) $HClO_4$, washing the pellet with 5ml of 2% (w/v) $HClO_4$ twice and then hydrolysing the protein in 5ml of 6M-HCl for 24h at 110°C. HCl was removed by evaporation to dryness and the amino acids were resuspended in 3ml of 0.5M-sodium citrate, pH6.3.

Determination of the specific radioactivity of [^{3}H]phenylalanine involved its enzymic conversion into β-phenethylamine. A 1ml portion of the supernatant or hydrolysate was incubated with 0.5ml of a suspension of L-tyrosine decarboxylase (acetone-dried powder from *Streptococcus faecalis* type I), overnight at 50°C. The enzyme was suspended in 0.5M-sodium citrate, pH6.3 (0.7 unit/ml for supernatants, 1.4 unit/ml for hydrolysates; one unit gives 1μmol/min at 37°C and pH5.5) containing 0.5mg of pyridoxal phosphate/ml. β-Phenethylamine was extracted by adding 1ml of 3M-NaOH and shaking with 10ml of chloroform/n-heptane (1:3, v/v). The organic layer was removed, added to 5ml of chloroform plus 4.0ml of 0.01M-H_2SO_4 and shaken. A 1.0ml (2.0ml for hydrolysates) sample of the upper (aqueous) phase was removed for liquid-scintillation counting in a xylene-based scintillant (Fricke, 1975) with 20–30% efficiency. Count rates varied depending on the size and type of tissue, but with 200mg of tissue were typically 1000–2000c.p.m. for supernatants, 100–200c.p.m. for muscle protein hydrolysates and >1000c.p.m. for liver and gut hydrolysates. A further 1ml (of supernatants) or 0.02ml (of hydrolysates) was assayed for phenethylamine by a modification of the method of Suzuki & Yagi (1976), by using 0.1–1.0ml of 20mM-β-phenethylamine in 0.01M-H_2SO_4 as a standard. To 1.0ml of the solution of β-phenylethylamine in 0.01M-H_2SO_4 was added 0.5ml of 2mM-leucylalanine, 1.0ml of 50mM-ninhydrin and 2.5ml of 1.0M-potassium phosphate, pH8.0. Samples were incubated at 60°C for 1h, then cooled in ice for 15min. The fluorescence at 495nm (excitation 390nm) was measured in a Locarte (London W.12, U.K.) fluorimeter by drawing the sample into a flow cell and waiting a fixed period (usually 5s) before taking readings. Incubation with peptide and ninhydrin and all subsequent steps were performed in the dark.

Results

Because other amino acids (principally tyrosine) became labelled by injection of [^{3}H]phenylalanine, the decarboxylation step was adopted as a means of isolating labelled phenylalanine (as β-phenethylamine) from these contaminants. Phenylalanine decarboxylase activity is present in preparations of tyrosine decarboxylase which was slightly cheaper than the same supplier's preparation of phenylalanine decarboxylase. The tyramine produced by the tyrosine decarboxylase is not extracted into the heptane/chloroform. Although the activity of the enzyme was found to be very low, adequate yields of β-phenethylamine (40–75%) were obtained by changing the incubation conditions from those recommended by the supplier, from pH5.5 to 6.3 and from 37 to 50°C. The yield was also improved by keeping the total incubation volume to a minimum (1–1.5ml), by extending the incubation

time and by providing the cofactor, pyridoxal phosphate.

The fluorimetric procedure for assaying β-phenethylamine gave a linear response up to 25 nmol per tube. However, in preliminary experiments we found the fluorescent product to be highly unstable. Loss of fluorescence was greatly accelerated by light and heat, both during incubation and by the excitation beam of the fluorimeter. Consequently, the incubation and subsequent steps were carried out in the dark, and the fluorescence was read in a flow cell by a standardized procedure (see the Experimental section).

Repeated measurements of the specific radioactivity of a standard [^{3}H]phenylalanine solution over a period of 4 months (19 determinations) gave a coefficient of variation of 3.2%. Measurements on tissue supernatants and protein hydrolysates from rats injected with [^{3}H]phenylalanine did not reveal the presence of any interfering substances. Tissue blanks (sample without enzyme) and enzyme blanks (enzyme without sample) contained no phenethylamine or radioactivity. Also, incubation with enzyme and subsequent solvent-extraction steps did not extract any substances which inhibited or enhanced the fluorescence produced by a standard solution of β-phenethylamine.

Fig. 1 shows the specific radioactivity of free phenylalanine in plasma and in tissues at different times after injection of 150 μmol of L-[4-^{3}H]phenylalanine/100 g body wt. Maximum values were obtained within 2 min, and thereafter the fall in specific radioactivity was slow but linear. This enabled the fractional rate of protein synthesis (k_s) to be calculated from the specific radioactivity of phenylalanine in protein (S_B) at 10 min and the mean specific radioactivity of free phenylalanine in the tissue ($\bar{S}_A$) between 0 and 10 min. The value of $\bar{S}_A$ was obtained by killing animals at 2 and 10 min, and the formula for calculating k_s has been given by McNurlan *et al.* (1979), i.e.

$$k_s = \frac{S_B \times 100}{\bar{S}_A \times t}$$

where t is the incorporation time in days and the units of k_s are % per day. In order to determine the value of k_s for each individual rat in the 10 min group it was necessary to calculate its own value for $\bar{S}_A$. This was done by multiplying each free amino acid specific radioactivity at 10 min ($S_{A(10)}$) by the value of $\bar{S}_A/S_{A(10)}$ obtained from the group means. It should be noted that 2 and 10 min were nominal times, in that these were the times at which rats were killed. The actual times used in the calculations were adjusted to take account of the additional period between killing the rat and cooling the tissue. Fractional synthesis rates (per cent of protein pool renewed per day) obtained with this technique in a number of tissues with a wide range of turnover rates are shown in Table 1.

Table 2 shows the effect of injecting rats with 150 μmol of unlabelled phenylalanine/100 g body wt.

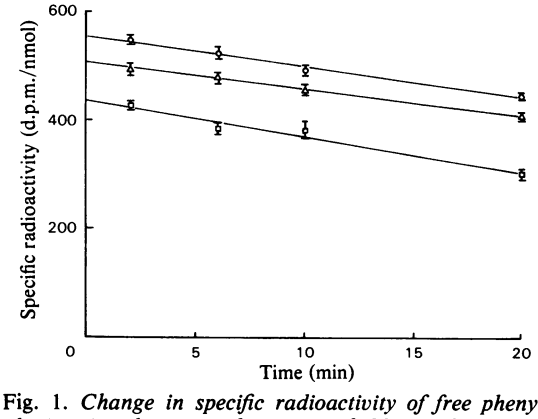

Fig. 1. *Change in specific radioactivity of free phenylalanine in plasma and tissues of 90 g male rats at different times after injection of 150 μmol of* L-[*4-*^{3}*H*]*-phenylalanine/100 g body wt.*

Each point represents the mean ± S.E.M. from four animals: ○, plasma; △, gastrocnemius muscle; □, jejunal mucosa. The points for liver were indistinguishable from those for gastrocnemius muscle in this experiment.

Table 1. *Fractional rates of protein synthesis in tissues of young male rats*

Rats (130 g) were injected with 150 μmol of [4-^{3}H]-phenylalanine/100 g body wt. and tissues were taken from four killed after 2 min and six killed after 10 min. Spleen and lung were taken from a different set of rats, from which four were killed at each of 2 min and 10 min. Specific radioactivities of free and protein-bound phenylalanine in tissues were measured and rates of protein synthesis were calculated as described in text. Results are means ± S.D.

Tissue	Fractional synthesis rate, k_s (%/day)
Jejunum:	
mucosa	119.2 ± 8.6
serosa	51.4 ± 5.1
Liver	83.3 ± 8.0
Spleen	68.3 ± 7.9
Lung	32.7 ± 2.6
Heart	20.0 ± 2.0
Muscle:	
Gastrocnemius*	16.9 ± 1.6
Soleus	21.3 ± 4.0
Extensor digitorum longus	18.0 ± 2.8

* Includes plantaris.

Table 2. *Incorporation of [^{3}H]lysine and [^{14}C]threonine injected in tracer amounts in the presence and absence of a large dose of unlabelled phenylalanine*

Rats (90g) were injected with a 1.0ml/100g body wt. dose of a solution of L-[U-^{14}C]threonine plus L-[4,5-^{3}H]lysine in either 0.9% NaCl (six rats) or 150mM unlabelled phenylalanine (six rats). They were killed after 10min and tissues were removed as described in the Experimental section. The incorporation of each tracer into protein was measured and expressed as d.p.m./mg of RNA (±S.E.M.) ($\times 10^{-2}$) to minimize scatter resulting from variations in RNA concentration. None of the differences between results with the tracer alone and with tracer plus phenylalanine is significant.

	[^{3}H]Lysine		[^{14}C]Threonine	
	Tracer alone	Tracer + phenylalanine	Tracer alone	Tracer + phenylalanine
Jejunal mucosa	570 ± 42	594 ± 29	252 ± 29	288 ± 20
Liver	700 ± 57	707 ± 32	183 ± 24	238 ± 25
Gastrocnemius	231 ± 16	200 ± 21	114 ± 16	108 ± 11
Heart	353 ± 17	320 ± 22	102 ± 13	100 ± 11

on incorporation into tissue protein of tracer doses of [^{3}H]lysine and [^{14}C]threonine. The incorporation of neither tracer was significantly altered in any tissue, suggesting that the large dose of phenylalanine did not in itself influence protein synthesis.

Discussion

The advantages of injecting a flooding dose of labelled amino acid for measuring rates of protein synthesis in tissues have been discussed previously (McNurlan *et al.*, 1979; Garlick, 1980). When a tracer dose of labelled amino acid is given, the specific radioactivities of the free amino acid in different compartments (e.g. plasma and tissue) may differ widely, thus aggravating the problem of defining the specific radioactivity at the site of protein synthesis (for review see Waterlow *et al.*, 1978). With a flooding dose this problem is minimized, since the specific radioactivity in tissues approaches that in the plasma (>90% in liver and muscle, Fig. 1). In addition, the time course of specific radioactivity of the free amino acid is linear, enabling it to be defined by measurement at two time points only. This contrasts with single injection of a tracer dose, when the complex time course obtained requires many time points for accurate definition (see, e.g., Peters & Peters, 1972). The short time period for incorporation eliminates the need to account for label which is incorporated and then lost again through breakdown of very rapidly turning-over proteins. The short duration also allows more accurate definition of the metabolic state of the animal (e.g. with regard to food absorption) than is possible with other techniques such as constant infusion of [^{14}C]tyrosine for 6h (Garlick *et al.*, 1973).

Phenylalanine is preferable to leucine, used previously by McNurlan *et al.* (1979), in several ways. It is more soluble than leucine, so that a larger dose may be given, and the pool of free phenylalanine in tissues is smaller than that of leucine (Waterlow *et al.*, 1978). The result is that the specific radioactivities in tissues and plasma are closer. The specific assay procedure for phenylalanine is far more convenient and more reliable than the column-chromatographic method used for leucine. The specific radioactivity in 30–40 samples can easily be measured each day by this technique. The sensitivity of this assay is such that measurements may be made on single muscles such as soleus or extensor digitorum longus, which may weigh 50mg or less. For muscle it was important to use an amino acid other than leucine, because studies *in vitro* have indicated a possible role for leucine in the regulation of protein synthesis and breakdown (Buse & Reid, 1975; Fulks *et al.*, 1975).

It is, however, important to ascertain that the large amount of phenylalanine injected does not in itself influence rates of protein synthesis in tissues. This is difficult to prove without having another reliable technique for measuring these rates. We chose the simple procedure of injecting a tracer dose of a labelled amino acid, with or without 150μmol of unlabelled phenylalanine/100g body wt., and measuring the incorporation into protein without any correction for precursor specific radioactivity. Thus, changes in transport to the site of protein synthesis would also influence the incorporation of label. We therefore chose two amino acids, lysine and threonine, whose transport carriers are believed to be different from that for phenylalanine and different from each other (Christensen, 1969). This would be expected to minimize, but not to prevent, interference with their transport by the large dose of phenylalanine. The effect of phenylalanine on incorporation into protein was not always the same with both amino acids, indicating that such interference with transport had probably occurred, similar to the effects of leucine on [^{14}C]lysine

transport reported previously (McNurlan *et al.*, 1979). However, in no tissue, and with neither amino acid, was the effect of phenylalanine statistically significant. Similarly, mixtures of amino acids containing phenylalanine have been shown to have no effect on protein synthesis in incubated muscle (Fulks *et al.*, 1975) or in perfused liver (Woodside & Mortimore, 1972). Indeed, high concentrations of labelled phenylalanine have frequently been used to measure protein-synthesis rates in perfused heart and muscle (Jefferson *et al.*, 1974). It therefore seems unlikely that the injected phenylalanine has any direct effect on protein synthesis.

The rates of protein synthesis shown in Table 1 are within the range of those produced by other methods in rats of the same age. The rates in liver and jejunal mucosa are very similar to those given by injection of a flooding dose of [^{14}C]leucine (McNurlan *et al.*, 1979). These measurements made no allowance in the calculation of k_s for the time between killing the rat and cooling the tissues (approx. 1 min), which accounts for the apparently higher values obtained with [^{14}C]leucine. As described previously (McNurlan *et al.*, 1979), the value for liver obtained during a 10 min incorporation period is the total rate of synthesis of cellular plus secreted (plasma) proteins. The values for heart and gastrocnemius muscle are very similar to those obtained by constant infusion of [^{14}C]tyrosine for 6 h. The difference in rate of synthesis between the various muscle types is striking. Jejunal serosa (smooth muscle) is by far the most rapid, followed by cardiac muscle and the skeletal muscles. The standard deviation in most tissues was about 10% of the mean. This is similar to values obtained by other techniques (McNurlan *et al.*, 1979; Garlick *et al.*, 1975) and must represent the variability in an apparently homogeneous population of rats. In soleus and extensor digitorum longus muscles the standard deviation was nearly 20%. This probably represents additional analytical error resulting from the use of tiny samples of tissue (approx. 15 mg) in the assay.

We conclude that injection of a flooding dose of [^{3}H]phenylalanine is an accurate method for measuring rates of protein synthesis in a wide range of tissues of small animals. When combined with a rapid, sensitive assay for the specific radioactivity of free and protein-bound phenylalanine from tissues, the technique is a significant advance on previously published techniques.

We are grateful to the Medical Research Council and to the Wellcome Trust for financial support.

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