Measurement of the rates of protein synthesis in rabbits

A method for the estimation of rates of change in the specific radioactivities of free amino acids during continuous infusions

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1. A method is described by which, from analysis of terminal samples, the rate constants that define the changes in specific radioactivity of free amino acids during continuous infusions can be estimated. The method involves the infusion of ^3H -labelled and ^{14}C -labelled forms of an amino acid for different, but overlapping, periods. 2. The procedure was developed for infusions of proline and tyrosine into New Zealand White rabbits and the rate constants were determined for blood and muscle. 3. The rate constant for equilibrium of radioactive free proline was much lower in muscle than in blood, and indicated that a plateau condition may not be attained in muscle by the end of a 6 h infusion. 4. Comparison of the ratio of areas under the curves of estimated specific radioactivity plotted versus time with the $^3\text{H}/^{14}\text{C}$ ratio of bound amino acid in muscle protein suggested that radioactive proline and tyrosine may be incorporated preferentially from an extracellular rather than an intracellular source.

The continuous infusion of labelled amino acids has become a widely used technique for the measurement of rates of protein synthesis in body tissues (Waterlow & Stephen, 1967; Garlick *et al.*, 1973; Nicholas *et al.*, 1977; Waterlow *et al.*, 1978). During an infusion, the specific radioactivities of the free amino acid in the various pools of the body rise to approximately constant plateau values. For free amino acids in blood it has been found empirically that the change in specific radioactivity with time could be described satisfactorily by an exponential equation with a single rate constant. Another form of equation, including a second rate constant, can be derived to describe the change in specific radioactivity in the intracellular free pools. The areas under these curves, over the period of the infusion, can be calculated if the rate constants are known. The total flows of radioactive free amino acid from the blood or intracellular pools to any other pool, e.g. the protein-bound amino acid, are proportional to these areas, provided that total flows remain constant. The calculation of the areas under the curves is therefore an important step in the estimation of fractional rates of protein synthesis (Waterlow & Stephen, 1967; Garlick *et al.*, 1973).

At present, however, no practical method exists whereby the rate constants for the changes in specific radioactivity of free amino acids in blood and tissues can be easily determined for individual

animals. The present paper details a dual-label method in which analysis of terminal samples from the infusion permits estimates to be made of the rate constants. The technique has been used primarily with labelled proline in rabbit muscle (Palmer *et al.*, 1980), but is applicable to many other situations.

Experimental

Materials

L-[5- ^3H]Proline (12 Ci/mmol), L-[U- ^{14}C]proline (300 mCi/mmol), L-[side-chain-2,3- ^3H]tyrosine (20 Ci/mmol), L-[ring-2,6- ^3H]tyrosine 40 Ci/mmol and L-[U- ^{14}C]tyrosine (400 mCi/mmol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K.

Methods

New Zealand White rabbits (0.9–2.5 kg) were infused as described by Nicholas *et al.* (1977). Two infusion solutions, which contained either the ^3H -labelled amino acid alone or the ^3H - and ^{14}C -labelled amino acids together, were prepared such that the ^3H -labelled amino acid was at the same concentration ($\mu\text{Ci/ml}$) in both. Infusions were started with the ^3H -labelled amino acid alone, and, at known times after the start of the experiment, the mixture of ^3H - and ^{14}C -labelled amino acid was substituted for the remainder of the infusion; corrections were made

for the volume of the infusion lines. In some experiments blood samples were obtained throughout the infusion from small longitudinal cuts made in a peripheral vein in the opposite ear to that in which the infusion was performed. With care, six or seven samples (0.5–1.0 ml) could be obtained through a single incision throughout the course of the infusion. At the end of the infusion, blood and tissue samples were collected in liquid N₂ before treatment and analysed by methods similar to those described by Nicholas *et al.* (1977) and Lobley *et al.* (1980). Samples were chromatographed preparatively on a Locarte amino acid analyser, and fractions containing the relevant amino acids were pooled, cognizance being taken of the fact that some separation of ³H-, ¹²C- and ¹⁴C-containing forms of amino acids occurs on ion-exchange resins (Nicholas *et al.*, 1977). The pooled samples were then analysed for amino acid content and radioactivity. The latter was determined in either NE 260 (Nuclear Enterprises, Edinburgh, Scotland, U.K.) or Picofluor TM30 (Packard Instruments, Reading, Berks., U.K.) scintillation fluid by using a two-channel Beckman LS-345 scintillation counter. Each channel was adjusted to give minimal overlap of the spectra of ³H and ¹⁴C; typical conditions were ¹⁴C counted with an efficiency of 67.3% and a ³H spill-over of 0.37%, and ³H measured at 32.5% efficiency with a spill-over of 22.5% of the ¹⁴C. The radioactivity of each sample was counted for at least 10 min to a maximum counting error of 1% (2 × s.d.) for the samples of free amino acid and 1.5% (2 × s.d.) for the protein-bound amino acid.

Biopsy experiments

The first experiment, under Saffan anaesthesia,

was essentially similar to those described by Nicholas *et al.* (1977). In the second experiment a catheter was inserted in the femoral artery and sodium pentobarbitone (15 mg/ml) was administered in doses of 0.1–0.2 ml (approx. 0.75 ml/h was required to maintain anaesthesia in a 1.4 kg animal).

Results and mathematical analysis

Proline infusions

The ³H/¹⁴C ratios of free proline isolated from various tissues at the end of the infusions are shown in Table 1. In all cases, at the time intervals selected, the ratio for free proline from blood (*r_b*) was higher than that of the infusate (*r_i*). If the plateau conditions had been attained within the period of the infusion of amino acid containing the second isotopic label, then *r_b*/*r_i* would equal unity. During infusions of rabbits 1 and 4 serial blood samples were taken, and the measured changes in specific radioactivity of free proline in blood from rabbit 1 are shown in Fig. 1(a). The experimental curve approximates to an equation of the form:

$$S_b = S_{b, \max.} (1 - e^{-\phi t}) \quad (1)$$

where *S_b* and *S_{b, max.}* are the specific radioactivities of free proline in the blood at time *t* (days) and at plateau respectively, and *φ* is a rate constant (days⁻¹). These results are in agreement with other studies that have shown that an equation of this form describes the change in specific radioactivity of blood free proline in chickens and rabbits during a continuous infusion (Laurent *et al.*, 1978; Robins, 1979). From eqn. (1) the rate constants (*φ*) for the changes in specific radioactivity of free proline in

Table 1. ³H/¹⁴C ratios of free proline in blood and tissue homogenates
For details see the text. Values in parentheses are (ratio in infusate)/(ratio in tissue).

Rabbit no.	Infusion periods (h)		³ H/ ¹⁴ C ratio of free proline					
	[³ H]Proline	[¹⁴ C]Proline	Infusate	Blood	Muscle	Intestine	Liver	Skin
1	0–6	5–6	3.01	3.45 (0.87)	10.50 (0.29)			
2	2–10	8–10	3.05	3.62 (0.84)	6.31 (0.48)	4.70 (0.65)	4.58 (0.67)	4.36* (0.70)
3	0–6	3–6	4.19	4.35 (0.96)	5.34 (0.66)	4.66 (0.90)		4.71 (0.89)
4	0–3.8	3–3.8	3.01	3.75 (0.80)				
	0–4.9	3–4.9	3.01	3.07 (0.98)				
	0–6	3–6	3.01	3.02 (1.00)	3.77 (0.80)			
5	0–6	3–6	2.65	2.91 (0.91)	4.69 (0.57)			

* From Robins (1979).

Fig. 1. Relative specific radioactivities of free proline and tyrosine in blood and muscle of anaesthetized and non-anaesthetized rabbits during continuous infusions

The results are expressed as fractions of the specific radioactivity of the blood sample taken at 6 h. (a) Rabbit 1: O, free ^3H proline in blood. (b) Rabbit 8 anaesthetized with Saffan: O, free ^3H proline in blood; ●, free ^3H proline in muscle. (c) Rabbit 9 anaesthetized with sodium pentobarbitone: O, free ^3H proline in blood; ●, free ^3H proline in muscle. (d) Rabbit 8 anaesthetized with Saffan: O, free ^3H tyrosine in blood; ●, free ^3H tyrosine in muscle.

blood for each animal can be calculated from r_b and r_i by an iterative solution of:

$$r_b = r_i \left(\frac{1 - e^{-\phi t_H}}{1 - e^{-\phi t_C}} \right) \quad (2)$$

where t_H and t_C represent the times (days) of infusion for ^3H proline and ^{14}C proline respectively. This and all subsequent analysis assumes that the animal is in a steady-state condition with respect to the size and entry and exit rates of the various pools of proline in the body throughout the period of the infusion, and that transfer between pools or compartments is a first-order process.

The calculated values of ϕ are shown in Table 2. The ratios $S_b/S_{b,\max}$ predicted from the rate constants obtained for rabbits 1 and 4 were within 5% of those measured directly from the serial samples of blood.

The $^3\text{H}/^{14}\text{C}$ ratio of free proline in muscle was consistently higher than that in blood or in the other tissues examined (Table 1), indicating that progression towards plateau conditions was much slower in muscle. By an analysis similar to that described by Garlick *et al.* (1973), the derived equation that describes this progression was:

$$S_m = S_{m,\max} \left(\frac{\nu(1 - e^{-\phi t}) - \phi(1 - e^{-\nu t})}{(\nu - \phi)} \right) \quad (3)$$

where S_m and $S_{m,\max}$ are the specific radioactivities of free proline in muscle at time t and at plateau respectively, and ν is a rate constant (days^{-1}) that describes the total fractional rate of exit from the pool of free proline in muscle (first-order process). By a reasoning similar to that used to calculate ϕ , values for ν were derived from the $^3\text{H}/^{14}\text{C}$ ratio of

free proline in the muscle homogenate (r_m) by the numerical solution of:

$$r_m = r_1 \left(\frac{v(1 - e^{-\phi t}) - \phi(1 - e^{-vt})}{v(1 - e^{-\phi t}) - \phi(1 - e^{-vt})} \right) \quad (4)$$

by using the values of ϕ obtained from solution of eqn. (2).

The calculated values of u are given in Table 2. The fractional rate constants for proline in muscle were always lower than those for blood. Substitution of known values of S_m and t together with the estimates of ϕ and v into eqn. (3) allowed $S_{m, \max.}$ to be calculated. From this it was possible to estimate how close to plateau the [^3H]proline in muscle was at the end of infusions. Terminal values that exceeded 95% of the expected plateau value were obtained, except for rabbit 5, for which only 78% of the predicted value was achieved. Fig. 2 shows the predicted change in S_m with time both for rabbits 5 and 1.

The areas under the specific-radioactivity-time curves over the entire infusion periods were calculated for the free pools of blood and muscle by integration of eqns. (1) and (3) respectively:

$$\int_0^t S_b \cdot dt = \frac{S_{b, \max.}}{\phi} (\phi t + e^{-\phi t} - 1) \quad (5)$$

$$\int_0^t S_m \cdot dt = t \cdot S_{m, \max.} \left(1 - \frac{v^2(1 - e^{-\phi t}) - \phi^2(1 - e^{-vt})}{\phi v(v - \phi)t} \right) \quad (6)$$

Table 2. Calculated rate constants, ϕ and v , for the change in specific radioactivity of free proline in blood and muscle homogenate, and relative areas of $^3\text{H}/^{14}\text{C}$ for free proline in blood and muscle estimated from ϕ and v compared with measured $^3\text{H}/^{14}\text{C}$ in muscle total protein.

Rabbit no.	ϕ^*	$v^\dagger$	Relative areas of $^3\text{H}/^{14}\text{C}$ for free proline		$^3\text{H}/^{14}\text{C}$ ratio in muscle total protein
			Blood‡	Muscle§	
1	49	14	28.8	103.9	47.1
2	22	17	19.4	40.9	24.9
3	26	19	10.1	13.9	10.0
4	47				
	49				
	46	15	6.7	9.5	8.9
5	19	8	6.8	11.4	7.7

* From eqn. (2).

† From eqn. (4).

‡ From eqn. (5).

§ From eqn. (6).

These areas can then be used to calculate the fractional rates of protein synthesis (k_s) from:

$$k_s (\text{days}^{-1}) = \frac{S_b}{\int_0^t S_b \cdot dt \text{ (or } \int_0^t S_m \cdot dt)} \quad (7)$$

where S_b is the specific radioactivity of the protein-bound amino acid.

The ratio of the areas for the ^3H -labelled and ^{14}C -labelled free proline of blood and muscle provides an estimate of the relative amounts of the two isotopic labels that outflow from each pool. These ratios were compared with the relative amounts of [^3H]- and [^{14}C]-proline that appeared in the synthesized protein (Table 2) in an attempt to determine the source of radioactive amino acids used for protein synthesis.

Proof that the predicted changes in muscle free proline radioactivity (eqns. 4 and 6) represent physiological conditions can only be obtained by direct measurement, by either a serial slaughter or a biopsy technique. The latter method was selected, as

Fig. 2. Predicted changes in the specific radioactivity of muscle free proline

Values of ϕ and v from Table 2 were substituted in eqn. (3):

$$S_m = S_{m, \max.} \left(\frac{v(1 - e^{-\phi t}) - \phi(1 - e^{-vt})}{(v - \phi)} \right)$$

The ratio $S_m/S_{m, \max.}$ is expressed for rabbit 1 (—), $\phi = 49$, $v = 14$, and rabbit 5 (---), $\phi = 19$, $v = 8$. The predicted times at which $0.95 S_{m, \max.}$ would be attained were 5.5 and 10h respectively for rabbits 1 and 5. The relative areas under the curves for the 6h infusion were 1:0.53 respectively.

Table 3. ³H/¹⁴C ratios of free tyrosine in blood and tissue homogenates
For details see the text. Values in parentheses are (ratio in infusate)/(ratio in tissue).

Rabbit no.	Infusion periods (h)		³ H/ ¹⁴ C ratio of free tyrosine					
	[³ H]Tyrosine	[¹⁴ C]Tyrosine	Infusate	Blood	Muscle	Intestine	Liver	Skin
2	0–10*	6–10	5.60	5.70 (0.98)	6.05 (0.93)	5.91 (0.95)	5.18 (1.08)	5.96‡ (0.94)
3	0–6*	3–6	3.57	3.65 (0.98)	3.77 (0.95)	4.21 (0.85)	3.39 (1.05)	4.06 (0.88)
6	0–4†	3–4	3.65	5.15 (0.71)				
	0–6	3–6	3.65	3.70 (0.99)	4.40 (0.83)	4.47 (0.82)	4.20 (0.87)	4.40 (0.83)
7	0–4†	3–4	3.65	4.70 (0.78)				
	0–6	3–6	3.65	3.80 (0.96)	4.10 (0.89)	4.47 (0.82)	3.80 (0.96)	4.47 (0.82)

* L-[side-chain-2,3-³H]Tyrosine.
† L-[ring-2,6-³H]Tyrosine.
‡ From Robins (1979).

it avoids inter-animal variation and is less expensive in materials. Furthermore, it has been used successfully under both general (Nicholas *et al.*, 1977) and local (Simon *et al.*, 1978a) anaesthesia. In biopsy experiments performed by methods similar to those described by Nicholas *et al.* (1977), however, plateau conditions were not achieved: the specific radioactivities of free proline in both the blood and the muscle homogenate continued to rise throughout the 6 h infusion whether Saffan or sodium pentobarbitone was used as anaesthetic (Figs. 1b and 1c). In none of the samples did the S_m/S_b ratio exceed 0.31, a value considerably below that found for non-anaesthetized rabbits (Palmer *et al.*, 1980). Proline metabolism under anaesthesia is clearly abnormal, for several reports have shown that $S_{b,max.}$ is rapidly attained during infusions of proline in conscious animals (Laurent *et al.*, 1978; Robins, 1979; the present study, Fig. 1a). Moreover, in these experiments, simultaneous infusion of tyrosine gave rise to plateau conditions in muscle (Fig. 1d), with results similar to those previously reported (Nicholas *et al.*, 1977). This latter observation suggests that possible effects of anaesthesia on amino acid supply from food absorption were not responsible for the lack of attainment of plateau conditions for proline.

Tyrosine infusions

The ³H/¹⁴C ratios of free tyrosine from the various tissue preparations (Table 3) were subjected to analysis similar to that described for proline, and the derived values for ϕ and ν are presented in Table 4. The rate constants for blood were slightly lower than those reported by Nicholas *et al.* (1977), and the calculated time at which 95% of plateau value

Table 4. Calculated rate constants, ϕ and ν , for the change in specific radioactivity of free tyrosine in blood and muscle homogenate, and relative areas of ³H/¹⁴C for free tyrosine in blood and muscle estimated from ϕ and ν compared with measured ³H/¹⁴C in muscle total protein

Rabbit no.	ϕ *	ν †	Relative areas of ³ H/ ¹⁴ C for free tyrosine		³ H/ ¹⁴ C ratio in muscle total protein
			Blood‡	Muscle§	
2	24	27	16.7	20.7	16.8
3	30	50	8.3	9.5	.8.4
6	36				
	34	20	8.3	11.4	9.5
7	29				
	25	36	9.1	10.6	9.3

* From eqn. (2).
† From eqn. (4).
‡ From eqn. (5).
§ From eqn. (6).

would be attained was also longer than observed by those workers. The results from the biopsy experiment (Fig. 1d) gave a lower rate of rise for S_m than that found previously (Nicholas *et al.*, 1977). The relative ³H/¹⁴C areas of free tyrosine in blood and the muscle homogenate are compared with the ³H/¹⁴C ratio of bound tyrosine in muscle total protein (Table 4).

Discussion

The advantages offered by the technique of continuous infusion of labelled amino acids for the measurement of the rates of protein synthesis are

many (Waterlow & Stephen, 1967; Waterlow *et al.*, 1978). The final calculations do require, however, some knowledge of the rates at which the specific radioactivities of the free amino acid change in blood and the tissues. A theoretical approach based on the relative sizes of the free and protein-bound amino acid pools has been described (Garlick *et al.*, 1973; Garlick, 1978). Practical measurements of the rate constants in tissues have, hitherto, come from biopsy (Nicholas *et al.*, 1977; Simon *et al.*, 1978a) or serial-slaughter (Simon *et al.*, 1978b) experiments, but both of these systems have limitations. In particular, interpretation of the results of serial slaughter of a group of animals after infusions of different duration is often complicated by variation between individual animals. In the biopsy method there are only a limited number of tissues from which successive removal of samples is feasible. Furthermore, the effect of anaesthesia is uncertain, as was demonstrated for proline. The values obtained for S_m do not include a correction for free proline in the extracellular fluid, and generally it is assumed in experiments with isotopically labelled amino acids that the concentrations and specific radioactivities of free amino acids in the extracellular space are similar to those in blood. As the ratio by volume of extracellular to intracellular fluid in muscle is probably 0.1–0.3:1 (Goldspink, 1966), it would therefore appear that most of the observed radioactive free proline in muscle homogenate could be confined to the extracellular space and that considerable inhibition of proline transport into muscle cells occurred under anaesthesia.

The dual-label system described in the present study has the particular advantage that it is applicable to single experiments. The only samples required are the terminal ones usually removed at the end of continuous infusions. The theoretical assumptions presented in the analysis are also those in general use for the derivations of equations to calculate rates of protein synthesis by the technique of continuous infusion, i.e. that the pools of amino acid are in a steady state throughout the infusion, that transfer between pools is a first-order process, that a simple exponential equation provides an adequate description of the specific radioactivity of the free pool in blood, and that return of labelled amino acid from protein degradation is insignificant during the experimental period. The infusion period, especially that for the amino acid containing the second isotope, can be varied to permit the greatest sensitivity in the measurement. The times selected in some of these experiments were to enable extra information to be obtained on rates of procollagen synthesis (Robins, 1979; Palmer *et al.*, 1980); to obtain more precise measurements of ϕ the ^{14}C -labelled amino acid would, for preference, have been infused for the last 1–2 h of the experiment for the

proline infusions and during the last 1 h for tyrosine. As the value of r_b approaches that of r_i , then small errors in their measurement will have an increasingly large effect on the derived value of ϕ . The effect of such inaccuracies on the final calculations is best illustrated by considering two extreme examples. Rabbit 3 (Table 2) had a derived ϕ of 26 days⁻¹ for proline, considerably less than the maximal value (60 days⁻¹) found in growing rabbits (Robins, 1979). If a value of 60 days⁻¹ is substituted in the subsequent calculations, then v is decreased from 19 to 13 days⁻¹, but the relative areas change much less, for blood from 10.1 to 9.1 and for muscle from 13.9 to 13.2. The muscle on k_s is also small, such that the calculated value based on free proline in blood would decrease by 9.1% and that based on the muscle homogenate by 2.5%. Similarly, for rabbit 2 (Table 4), if the value for ϕ of 24 days⁻¹ for tyrosine is substituted by 40 days⁻¹ (Nicholas *et al.*, 1977) then v decreases from 27 to 20 days⁻¹ and the relative areas from 16.7 to 15.5 for blood and from 20.7 to 20.3 respectively for muscle. The decreases in k_s would be 4.2 and 0.8%, based on the free tyrosine in blood and muscle respectively. The effect of such substitutions for ϕ in the other infusions would be correspondingly less. Thus relatively large changes in calculated values of ϕ , much greater than could have arisen by experimental error, do not significantly alter the values derived subsequently from the results.

For proline good agreement was found when ϕ estimated by the dual-label method was compared with that determined directly. Although it was not possible to obtain direct proof of the changes in S_m predicted from estimates of ϕ and v , these assume only that the transfers follow first-order kinetics, a basis used for the derivation of most calculations of rates of protein synthesis. In the calculations, r_m has been taken to represent the ratio of the intracellular amino acid, but it is in fact that of the homogenate, and this will include free proline in blood and extracellular fluid. The values of v for proline (Table 2) would decrease by 13–30% if non-intracellular proline contributed 20% to the homogenate pool at an $r = r_b$. In consequence, the ratio of the $^3\text{H}/^{14}\text{C}$ areas would increase for muscle (Table 2). The v values for free proline in muscle were lower than those for tyrosine, consistent with the report that, in rat muscle, the fractional entry rate of tyrosine was 5 times that of proline (Banos *et al.*, 1973). The low values of v compared with ϕ mean that it is not always valid to assume that S_m follows a simple exponential function during continuous infusions (Garlick *et al.*, 1973).

This analysis describes the changes in S_b and S_m , but whether either of these reflects the specific radioactivity of the true precursor of protein synthesis, the aminoacyl-tRNA, is the subject of much

controversy (see Waterlow *et al.*, 1978). Although no measure of the specific radioactivity of the precursor can be obtained from this method, some idea of the relative contributions of radioactive amino acids from extracellular and intracellular sources might be obtained from the relative areas of the ^3H -labelled and ^{14}C -labelled amino acid from the free pools as compared with the amount of the two isotopic labels that appears in the protein, provided (1) that the rate of incorporation into protein is constant over the period of the infusion, and without any time lag, and (2) that there is no substantial loss of radioactivity from muscle protein over the period of the infusion. This latter assumption is probably valid for muscle.

In Tables 2 and 4 it can be seen that the $^3\text{H}/^{14}\text{C}$ ratios of both proline and tyrosine in muscle protein are more consistent with the precursor being supplied mainly from an extracellular (blood) source than from the intracellular (homogenate) free amino acid pool. This observation is slightly surprising for proline, as it has been suggested that other non-essential amino acids, notably glycine and serine, are incorporated into muscle protein from the intracellular fluid (Fern & Garlick, 1974). In addition, Robins (1979) has shown that proline from an amino acid pool(s) of average specific radioactivity less even than the intracellular value is used for procollagen synthesis in skin. The present method does not allow any conclusions to be drawn about the specific radioactivity of the precursor, and hence the true rate of protein synthesis, but the results suggest that labelled proline and tyrosine are derived preferentially from extracellular sources for the synthesis of muscle total protein.

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