The Diurnal Response of Muscle and Liver Protein Synthesis *in vivo* in Meal-Fed Rats

By P. J. GARLICK,* D. J. MILLWARD and W. P. T. JAMES

Department of Human Nutrition (Clinical Nutrition and Metabolism Unit),
London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, U.K.

(Received 11 July 1973)

1. The rate of protein synthesis in rat tissues was measured by constant intravenous infusion of [14 C]tyrosine. A modification has been developed for the method of calculating the rate of protein synthesis in individual tissues from the specific radioactivity of the free and protein-bound amino acid in tissue at the end of the infusion. This technique gives greater accuracy and allows a greater choice of labelled amino acids. The specific radioactivity of free tyrosine in plasma was used to calculate the plasma tyrosine flux, an index of the rate of protein synthesis in the whole body. 2. Young male Wistar rats were allowed access to food for only 4 h in every 24 h. The tyrosine flux and the rate of protein synthesis in liver and muscle at different periods of time after a single feed were estimated. 3. The tyrosine flux did not alter after feeding nor even after starvation for 48 h. 4. The average fractional rate of protein synthesis in muscle was 7.2%/day, i.e. the proportion of the protein mass which is replaced each day. The rate rose after eating and declined during starvation for 48 h. In addition the rate of muscle protein synthesis correlated with the growth rate of the rat. 5. In liver the average fractional rate of protein synthesis was 50%/day. There was no change in the rate after eating nor after starvation for 48 h. In contrast with muscle this suggests that the changes in protein mass were accompanied by changes in the rate of protein breakdown rather than synthesis.

In mammals the absorption of food protein is not continuous, yet after meals there is little accumulation of free amino acids in the tissues (Munro, 1970). Rapid utilization of the incoming amino acids must therefore occur. Elwyn (1970) has suggested that only a part of the food protein is immediately oxidized and that much of the remainder is incorporated into tissue protein. The present work was designed to show how the rate of synthesis of total (mixed) protein in two different tissues, liver and muscle, responds to a cyclic pattern of food intake.

The liver is usually considered to modulate the inflow of amino acids from the intestine and the concentrations of amino acids in the systemic blood fluctuate much less after a meal than the concentrations in the portal vein (Elwyn, 1970). Muscle tissues are therefore exposed to more constant concentrations of plasma amino acids than the liver and the synthesis of muscle proteins may not respond to sudden dietary changes to the same extent. However, experiments *in vitro* have shown that muscle protein synthesis is increased by insulin (Manchester, 1970). Changes in the metabolism of muscle protein could therefore occur after a feed, despite the small

alterations in plasma amino acid concentrations, as a result of changes in plasma insulin concentrations.

In the present experiments the rate of protein synthesis in the tissues was measured by constant intravenous infusion of [14 C]tyrosine. During the infusion the specific radioactivity of the free amino acid in the blood and tissues reaches a constant (plateau) value and, with certain assumptions, the rate of protein synthesis can be calculated from the specific radioactivity of the free and protein-bound amino acid at the end of the infusion in each individual animal (Waterlow & Stephen, 1968). Groups of animals were infused at intervals after a single feed and the response of muscle and liver protein synthesis was assessed.

Methods

Male Wistar rats (A. Tuck and Sons Ltd., Rayleigh, Essex, U.K.) weighing 80 g were individually caged and fed for 4 h each day. A cubed diet (M.R.C. diet 41B, Bruce & Parkes, 1949) was given at 09:00 h and withdrawn at 13:00 h each day, with access to water at all times. Initially some weight loss occurred but after 2-3 days most animals adapted and recommenced growth. No animal was used for the experiments unless it had a steady growth rate over a period of at least 10 days. At this time venous cannulation

* Present address: Department of Nutritional Sciences, University of Wisconsin, College of Agricultural and Life Sciences, Madison, Wis. 53706, U.S.A.

was performed. After the usual daily meal the rat was anaesthetized with diethyl ether and a cannula was inserted into the left jugular vein, the other end being threaded subcutaneously to emerge behind the ears. The cannula was filled with dilute heparin solution (200 units/ml) and plugged externally with a pin. Rats were returned to their cages and continued feeding normally for 3 days before infusion. Each day the cannula was flushed through with 0.1 ml of heparin solution to prevent blood clotting within the tubing.

Groups of animals were infused for periods of 6 h at different time-intervals after the start of their meal. Infusions for the groups were begun either with the start of the last meal (time 0) or at 6, 12, 18, 24 and 48 h later. Thus the first group was infused while feeding and the last two groups in a starved state. During the infusion the animals sat in a cage which allowed movement and feeding where necessary. Infusions of L-[U- 14 C]tyrosine (The Radiochemical Centre, Amersham, Bucks., U.K., 12.9 μ Ci/ μ mol) were given at a rate of 0.5 ml/h. Physiological saline was added so that the final radioactivity was 2.5 μ Ci/ml. At the end of the 6 h infusion the animals were anaesthetized with diethyl ether while the infusion was continued. Blood (1 ml) was withdrawn from the inferior vena cava; liver and gastrocnemius muscle were then rapidly removed, weighed and homogenized in ice-cold 10% (w/v) trichloroacetic acid. The stomach was removed and weighed as an estimate of residual unabsorbed food. Serum was separated from the blood and serum proteins were precipitated with 10% trichloroacetic acid. Protein precipitates of serum, liver and muscle were washed twice with 10% trichloroacetic acid, the washes being combined with the original supernatants. Further washes resulted in no further extraction of radioactivity. Trichloroacetic acid was extracted with three washes of ether and the residual solution made up to a standard volume. Proteins were hydrolysed in 6M-HCl for 6 h, dried to remove the HCl and then taken up in water.

The specific radioactivity of protein and free tyrosine was assayed as described by Garlick & Marshall (1972). In essence, the tyrosine was converted into tyramine by L-tyrosine decarboxylase; the tyramine was extracted from an alkaline salt solution and then recovered into dilute acid. Finally, the solution was assayed fluorimetrically for tyramine by the nitroso-naphthol technique of Waalkes & Udenfriend (1957). By using a specific decarboxylase, any contamination of L-tyrosine with highly labelled D-tyrosine derived from the infusate is obviated and measurement of radioactivity in other tissue compounds is avoided. Waterlow & Stephen (1968) showed that during 6 h infusions of L-[14 C]lysine appreciable errors in the estimate of specific radioactivity of free lysine in tissue would occur if the infusate had racemized to the extent of only 0.5%; this is well within the stated limits of purity of the compound.

Counting of the extracted aqueous solution for 14 C radioactivity was performed on a Beckman LS 150 liquid-scintillation counter by using 1,4-dioxan with 10% (w/v) naphthalene and 0.5% 2,5-diphenyloxazole as scintillant. A correction was made for the counting efficiency (about 50%) by the external standard channels-ratio method and all samples were counted to a standard error of better than 1.5%.

Free and protein-bound tyrosine concentration in tissues was measured by reaction with nitroso-naphthol (Udenfriend & Cooper, 1952b; Waalkes & Udenfriend, 1957). Total free α -amino nitrogen in muscle was determined on the trichloroacetic acid supernatant by the ninhydrin method (Jagenburg, 1959).

Calculations

The method of calculating rates of protein synthesis from data from infusion of [14 C]lysine has been given by Waterlow & Stephen (1968). The present approach is a modification of this method which is applicable to studies on a wider range of tissues. The present calculations also permit the use of any amino acid for the measurement of protein synthesis rates by constant infusion.

Amino acid flux

When a labelled amino acid is infused intravenously its specific radioactivity in the blood plasma and free amino acid pool of the tissues soon reaches a constant or plateau value that is maintained for many hours (Loftfield & Harris, 1955; Waterlow & Stephen, 1967; Gan & Jeffay, 1967). Then according to Waterlow & Stephen (1968):

$$I = F \cdot S_{\text{pmax}} \quad (1)$$

where I = rate of infusion of isotope (μ Ci/h), F = flux of amino acid (μ mol/h) and S_{pmax} = specific radioactivity of free amino acid at plateau value (μ Ci/ μ mol).

The flux represents the rate of exit of amino acid from the pool by synthesis into both protein and other compounds as well as by catabolic pathways.

As demonstrated previously in a number of laboratories (e.g. Hider *et al.*, 1971) the free amino acid pool of the body cannot be considered as a single homogeneous compartment. Intracellular amino acid specific radioactivities during infusion are lower than those in the blood (Waterlow & Stephen, 1968; Gan & Jeffay, 1967). Thus the flux of the body's free amino acids may be underestimated when measurements are made solely on the plasma.

Specific radioactivity of free amino acids in tissue

These differences between plasma and intracellular specific radioactivities also affect calculations of the

rate of synthesis of proteins in individual tissues. The calculations require measurement of the specific radioactivity of the amino acid pool at the site of protein synthesis. In the absence of measurements of the specific radioactivity of the amino acid bound to tRNA it is conventional to consider the precursor specific radioactivity to be equal to that of the total intracellular free amino acid. The intracellular tyrosine specific radioactivity has therefore been used in the calculations shown here. In addition, a measure of the minimum possible value for the rate of protein synthesis has been calculated from the specific radioactivity of tyrosine in blood, as during an infusion this specific radioactivity must be greater than, or equal to, that of the precursor.

To calculate the rate of protein synthesis in a tissue from the specific radioactivity of the intracellular free amino acid at the end of the infusion, it is also important to have some information about the time-course by which the specific radioactivity reaches a plateau. Some knowledge of this can be obtained from measurements on plasma. Waterlow & Stephen (1967) took serial samples of blood from rats during infusion of [^{14}C]lysine and showed that the plasma lysine specific radioactivity rose to a plateau by a pathway closely approximating to a simple exponential of the type:

$$S_p = S_{p\max} \cdot (1 - e^{-\lambda_p t}) \quad (2)$$

where S_p = specific radioactivity of plasma amino acid, $S_{p\max}$ = value of S_p when the plateau value has been reached, λ_p = rate constant and t = time. The value of λ_p for lysine was shown to be between 12 and 24 days $^{-1}$ (Waterlow & Stephen, 1967). With infusions of [^{14}C]tyrosine we have shown that the plateau value is reached more rapidly ($\lambda_p = 80$ days $^{-1}$) and is maintained for at least 6h.

In the tissues also the free amino acid specific radioactivity reaches a plateau; the value is lower than

that in the blood because of dilution by unlabelled amino acid from protein breakdown (Gan & Jeffay, 1967; Waterlow & Stephen, 1968; Aub & Waterlow, 1970). This plateau for the intracellular amino acid is approached by a more complex pathway than that in the plasma. We show below that, provided the infusion is long enough, the specific radioactivity of the free amino acid in the tissue (S_i) can also be expressed as a single exponential of exactly the same type as eqn. (2) without sacrificing accuracy in the estimate of protein synthesis rate, i.e.:

$$S_i = S_{i\max} \cdot (1 - e^{-\lambda_i t}) \quad (3)$$

This equation requires the choice of values for the rate constant λ_i . If the intracellular pool is assumed for the purposes of the present study to be homogeneous, constant in size and to receive free amino acids by exchange with the plasma pool, by synthesis *de novo* and by protein breakdown, it may be represented by the model shown in Scheme 1. The change in specific radioactivity (S_i) in the pool is given by:

$$\frac{dS_i}{dt} = \frac{V_x}{q} \cdot S_p - \frac{V_z}{q} \cdot S_i$$

where q is the amount of amino acid in the pool, V_x is the rate of entry of labelled amino acid from the blood and V_z is the rate of exit of amino acid into protein, into other chemical reactions or back into the plasma. The time-course of S_p has been measured and approximated to a single exponential function (eqn. 2) which can be substituted in the differential equation. Solving with the initial conditions $S_i = 0$ when $t = 0$ gives an equation with two exponentials:

$$S_i = S_{i\max} \cdot \left[\frac{\lambda_p (1 - e^{-\frac{V_z}{q} t}) - V_z/q (1 - e^{-\lambda_p t})}{\left(\lambda_p - \frac{V_z}{q} \right)} \right] \quad (4)$$

where $S_{i\max} = V_x/V_z \cdot S_{p\max}$.

Scheme 1. The movement of amino acid into and out of the free amino acid pool in tissue

The model is used to determine the time-course of specific radioactivity of the free amino acids in tissue during a constant infusion. V_x is the rate of entry of amino acid from the plasma with specific radioactivity S_p . V_y is the total rate of entry of unlabelled amino acid from all other sources including intracellular protein breakdown and synthesis *de novo*. V_z is the total rate of exit of the amino acid from the pool with specific radioactivity S_i . This includes both exit into the plasma and the synthesis of new protein as well as oxidation and other chemical reactions.

The value of λ_p is known but not that of V_z/q which represents the fractional rate of exit of amino acid from the intracellular pool by all possible routes. Since this value cannot be measured it is necessary to substitute parameters that can be measured more easily. In the steady state it can be shown that $V_z = PV_y$, where $P = S_{pmax.}/(S_{pmax.} - S_{imax.})$ and V_y is the rate of entry of unlabelled amino acids. For essential amino acids V_y represents only amino acids derived from protein breakdown. Hence $V_y = Q \cdot k_d$, where Q is the amount of the amino acid in protein and k_d is the fractional rate of protein degradation. Tyrosine is not essential, but is only synthesized to an appreciable extent in the liver (Udenfriend & Cooper, 1952a and see below). In the steady state therefore, $V_y = Qk_s$, where k_s is the fractional rate of protein synthesis.

If Q/q is denoted by R , eqn. (4) becomes:

$$S_i = S_{imax.} \cdot \left[\frac{\lambda_p(1 - e^{-PRk_s t}) - PRk_s(1 - e^{-\lambda_p t})}{(\lambda_p - PRk_s)} \right] \quad (5)$$

This expression for S_i could be used for the precursor specific radioactivity in the calculation of the rate of protein synthesis. It is, however, rather cumbersome and the single exponential shown in eqn. (3) is an adequate approximation provided that a suitable value for λ_i , the rate constant, is chosen and that the period of infusion is long enough. Eqn. (5) contains two exponentials, $\exp(-\lambda_p t)$ and $\exp(-PRk_s t)$. Since $P > 1$ then $PRk_s > Rk_s$. Three conditions are therefore possible: (1) $PRk_s > Rk_s > \lambda_p$: in this condition the term $\exp(-PRk_s t)$ of eqn. (5) rapidly becomes negligible and the appropriate value for λ_i in eqn. (3) is λ_p . The value of λ_p can be measured by blood sampling in a different group of animals. With infusions of tyrosine this condition would occur with rapidly turning over proteins such as in the liver. The synthesis of tyrosine from phenylalanine in liver would tend to improve the validity of the approximation.

(2) $\lambda_p > PRk_s > Rk_s$: here the other exponential, $\exp(-\lambda_p t)$ rapidly disappears and the value of λ_i is PRk_s .

(3) $PRk_s > \lambda_p > Rk_s$: in this case it has been shown that the curve derived with these conditions approximates to a single exponential with a rate constant λ_i equal to Rk_s .

When tyrosine is infused the third condition is applicable to muscle, which has a slower turnover rate than liver.

Accumulation of isotope in protein

If the specific radioactivity of the precursor of protein synthesis is assumed to be equal to that of the free amino acid in tissue as derived above (eqn. 3) an expression for the accumulation of isotope in protein can be derived as described by Swick (1958), i.e.:

$$\frac{S_B}{S_i} = \frac{\lambda_i}{(\lambda_i - k_s)} \cdot \frac{(1 - e^{-k_s t})}{(1 - e^{-\lambda_i t})} - \frac{k_s}{(\lambda_i - k_s)} \quad (6)$$

where S_i and S_B are the specific radioactivities of the free and protein-bound amino acid at the end of the infusion and k_s is the fractional rate of protein synthesis. Accurate determination of the value of λ_i is unnecessary and the approximate values derived above are adequate since quite large variations in the value of λ_i result in small variations in the value of k_s (Waterlow & Stephen, 1968).

With infusion of tyrosine the value of λ_i varies from tissue to tissue (see above). In liver $\lambda_i = \lambda_p$, therefore:

$$\frac{S_B}{S_i} = \frac{\lambda_p}{(\lambda_p - k_s)} \cdot \frac{(1 - e^{-k_s t})}{(1 - e^{-\lambda_p t})} - \frac{k_s}{(\lambda_p - k_s)} \quad (7)$$

In muscle $\lambda_i = Rk_s$, therefore:

$$\frac{S_B}{S_i} = \frac{R}{(R - 1)} \cdot \frac{(1 - e^{-k_s t})}{(1 - e^{-Rk_s t})} - \frac{1}{(R - 1)} \quad (8)$$

These equations enable the rate of protein synthesis in liver and muscle to be calculated from the experimentally derived specific radioactivities of tyrosine in the protein and free amino acid pool of the tissue at the end of the infusion. Because of the doubt about the site of the precursor pool, values of k_s have also been calculated from the specific radioactivity of free tyrosine in the blood. This is done by a method analogous to that shown above since the time-course of specific radioactivity is similar in blood and tissues (eqns. 2 and 3). The formula for calculating the rate of protein synthesis from the specific radioactivity of the tissue protein and of the amino acid in blood is the same in all tissues:

$$\frac{S_B}{S_p} = \frac{\lambda_p}{(\lambda_p - k_s)} \cdot \frac{(1 - e^{-k_s t})}{(1 - e^{-\lambda_p t})} - \frac{k_s}{(\lambda_p - k_s)} \quad (9)$$

Eqns. (7), (8) and (9) were solved graphically for k_s values by plotting curves of k_s versus S_B/S_i or S_B/S_p at fixed values of t , λ_p and R . The values of k_s corresponding to experimental values of S_B/S_i or S_B/S_p were then read off from these curves. Results for the fractional rate of protein synthesis (k_s) are given with the units %/day, i.e. the proportion of the protein mass which is replaced each day.

Results

Growth and food intake of rats

The rats adjusted within a few days to their feeding schedule and then began to grow steadily. Table 1 shows that there were some differences in the growth rate of the groups of animals as well as the expected individual variability between rats in each group. These growth differences proved to be of importance when assessing the individual values for protein syn-

thesis in the groups of animals (see below). Food intake measurements showed that on average the rats consumed 11 g of food during the 4h feeding period but the actual amount was related both to the weight of the animal and to their growth rate. Analysis of stomach weight, used as an index of food retained within the stomach, showed that some food remained within the stomach for more than 12h after a meal. Only after 24h was the stomach completely empty. The animals thus adapted to the feeding regime by eating rapidly and then retaining food within the stomach well after the eating period. Absorption of

amino acids was thus continuing during the second, and to a more limited extent during the third, infusion period.

Tyrosine flux

Measurements of plasma tyrosine flux showed that despite the variability in food absorbed during the different infusion periods the flux rates were surprisingly constant. Even when food was withheld for 2 days there was no appreciable decrease in the tyrosine flux.

Table 1. *Average weight, growth rate and tyrosine flux of each group of rats used for the infusions*

Rats, accustomed to a cyclic feeding regime, were fed between 0 and 4h and were infused with L-[^{14}C]tyrosine at the times indicated after the start of this meal. No further food was given during the experiment. Body weight was measured before the feed and growth rates were calculated for the 10-day period preceding the infusion. The stomach weight was measured at the end of the infusion as an index of gastric emptying and absorption. The tyrosine flux was calculated from the specific radioactivity of free tyrosine in the plasma at the end of the infusion as described in the 'Calculations' section. Each result is the mean $\pm$ s.d. for between five to seven rats. None of the differences in tyrosine flux is significant (analysis of variance, $P > 0.05$).

Infusion time after start of last meal (h)	No. of rats	Body weight (g)	Stomach weight (g)	Growth rate (% of body wt./day)	Tyrosine flux ($\mu\text{mol/h per 100 g}$)
0-6	5	126 $\pm$ 10	14.4 $\pm$ 3.3	2.21 $\pm$ 0.77	25.9 $\pm$ 2.9
6-12	6	114 $\pm$ 13	7.4 $\pm$ 1.9	2.81 $\pm$ 0.55	23.1 $\pm$ 1.8
12-18	6	104 $\pm$ 8	2.9 $\pm$ 0.7	2.19 $\pm$ 0.40	28.0 $\pm$ 4.1
18-24	5	110 $\pm$ 13	1.1 $\pm$ 0.1	2.80 $\pm$ 0.30	30.0 $\pm$ 6.9
24-30	7	107 $\pm$ 12	1.0 $\pm$ 0.1	2.12 $\pm$ 0.56	27.4 $\pm$ 7.3
48-52	5	96 $\pm$ 14	1.0 $\pm$ 0.2	2.62 $\pm$ 0.83	24.8 $\pm$ 1.1

Table 2. *Rate of protein synthesis and free amino acid content of gastrocnemius muscle of meal-fed rats*

Rats were infused with L-[^{14}C]tyrosine for periods of 6h after which they were killed and gastrocnemius muscles removed and treated as described in the text. The estimate of the fractional rate of protein synthesis was calculated from the specific radioactivity of tyrosine in protein and in the free amino acid pool of the tissue by using eqn. (8). R is the ratio of protein-bound/free tyrosine in the muscle used in this calculation. The minimum value for the rate of protein synthesis was calculated by using eqn. 9, from the specific radioactivity of tyrosine in protein and free tyrosine in blood. The value of λ_p (eqn. 8) used in this calculation was 80 days $^{-1}$ for all groups (see the text). S_i/S_p is the ratio of free tyrosine specific radioactivity in the tissue to that in blood at the end of the infusion. The concentration of α -amino N in the muscle was determined with ninhydrin and standardized against alanine. Each value is the mean $\pm$ s.d. for the group. Analysis of variance shows that the observed differences in the estimate of the protein synthetic rate are highly significant ($P < 0.001$) but that differences in S_i/S_p and α -amino N concentration are not significant ($P > 0.05$).

Infusion time after last meal (h)	Estimate of protein synthetic rate (%/day)	Minimum value for protein synthetic rate (%/day)	S_i/S_p	α -Amino N ($\mu\text{mol/g wet wt.}$)	R
0-6	6.1 $\pm$ 1.1	4.6 $\pm$ 1.7	0.698 $\pm$ 0.174	22.7 $\pm$ 5.0	445 $\pm$ 39
6-12	7.7 $\pm$ 1.3	5.0 $\pm$ 1.2	0.650 $\pm$ 0.100	22.4 $\pm$ 7.9	420 $\pm$ 27
12-18	7.4 $\pm$ 0.9	5.7 $\pm$ 0.6	0.746 $\pm$ 0.133	22.5 $\pm$ 8.2	467 $\pm$ 28
18-24	7.5 $\pm$ 1.2	6.1 $\pm$ 1.3	0.747 $\pm$ 0.076	13.3 $\pm$ 6.4	581 $\pm$ 39
24-30	4.8 $\pm$ 0.7	3.8 $\pm$ 0.7	0.693 $\pm$ 0.130	24.4 $\pm$ 5.6	399 $\pm$ 32
48-54	3.8 $\pm$ 0.5	2.8 $\pm$ 0.4	0.699 $\pm$ 0.059	20.5 $\pm$ 4.7	392 $\pm$ 20

Free amino acids in muscle

Measurements of the α -amino N content and the ratio of protein-bound/free tyrosine concentrations in muscle (Table 2) suggested that the ingested food had little influence on the amino acid concentrations in muscle, although a decrease was seen in the animals killed immediately before the next meal was due.

Fig. 1. Correlation of growth rate with the rate of protein synthesis in muscle

The growth rate of meal-fed rats was measured over 10 days before infusion (Table 1). Groups of rats were infused with [^{14}C]tyrosine for 6h periods at different times after their last meal and the fractional rate of protein synthesis in gastrocnemius muscle was calculated from the specific radioactivity of the free and bound tyrosine in muscle at the end of the infusion. The growth rate for the individual rat was subtracted from the mean growth rate for its group. The result was plotted against the difference between the rate of protein synthesis in muscle of the individual rat and the mean for the group. The scatter diagram therefore demonstrates the correlation of protein synthesis rate in muscle with growth rate, independent of variations in protein synthesis rate owing to feeding. The correlation coefficient r is 0.577 which is significant at $P < 0.001$. The line is the regression of protein synthesis rate (y) on growth rate (x) where $y = 0.962x$.

During starvation, however, the total free amino acid concentrations returned to normal.

Protein synthesis in muscle

Despite the small changes in tissue concentration of amino acids the synthesis rate of muscle protein altered in response to the diet. The average rate of synthesis of proteins in the gastrocnemius muscle during the days' infusions (Table 2) was 7.2%/day, confirming observations (Waterlow & Stephen, 1968; Garlick, 1969; Millward, 1970a) which suggested that muscle protein had a moderately rapid turnover rate. The present studies, with measurements made at different times after a meal, also show that muscle is a dynamic organ capable of responding to both feeding and starvation by alterations in its rate of protein

Table 3. Effect of feeding on the rate of protein synthesis in muscle, corrected for differences in the mean growth rates for the groups

The fractional rate of protein synthesis in gastrocnemius muscle at different times after a feed was estimated from the specific radioactivity of free and protein-bound tyrosine in muscle after 6h infusion of [^{14}C]tyrosine. The differences in synthesis rate between the groups shown in Table 2 were corrected for the observed differences in growth rate for the groups (Table 1) by reference to the equation for the correlation of growth rate and protein synthesis rate (Fig. 1). The table shows the rates of synthesis appropriate to a growth rate of 2.12% of body wt./day (the mean growth rate of the 24–30h group). The correction was made from the gradient of the regression shown in Fig. 1 by the formula $c = y - 0.962(x - 2.12)$, where c = corrected mean rate of protein synthesis for the group, y = observed mean rate of protein synthesis for the group (Table 2) and x = mean growth rate for the group. The statistical significance of the differences between the groups was tested by analysis of covariance (Armitage, 1970). The protein synthesis rate between 0–6h was significantly different from that between 12–18h ($P < 0.02$), 24–30h ($P < 0.001$) and 48–54h ($P < 0.001$).

Infusion time after last meal (h)	Corrected rate of protein synthesis (%/day)
0–6	6.03
6–12	7.06
12–18	7.36
18–24	6.86
24–30	4.78
48–54	3.31

synthesis. The synthesis rate of 6.1%/day during feeding rose by a fifth during the subsequent periods as absorption was completed.

The specific radioactivity of the free amino acids in tissue was lower than that in plasma because of the continued supply of unlabelled tyrosine from muscle protein breakdown. During the course of the experiment, from the onset of feeding until 54 h later, there was an approximately constant relationship between the plasma and tissue specific radioactivities (Table 2). If plasma values are taken as equal to the precursor specific radioactivity then the values for synthesis are 20–30% lower than those calculated from the tissue pool and represent minimum values for the synthesis rate of the tissue's protein. When these lower values are chosen for assessing the effects of feeding the same response in synthetic rate is observed, in keeping with the constant ratio of plasma to tissue specific radioactivities.

Starving the animals for 48 h led to a profound fall in muscle protein synthesis rates to half the values found during a normal day's feeding cycle. The response was rapid, since the delay of a feed for 6 h, i.e. in those infused between 24 and 30 h after the last meal, led to a large decrease in synthesis: a further fall occurred with continued starvation but the main effect was shown early.

Since some of the variations in synthetic rates of muscle proteins in individual rats within a group seemed to be related to their previous growth rate it was necessary to establish whether growth rates as well as the period of feeding could influence the values obtained for muscle protein synthesis. This was

initially analysed by correlating the growth with the individual animal's protein synthesis rate, once the effect of feeding had been eliminated. The difference between the individual rat's growth rate and that of its own group was plotted against the deviation of the rat's synthesis rate from the mean synthetic rate of its own group. Thus, only variations from the mean group value were correlated with growth rates and no account was taken of the differences owing to feeding. The result, shown in Fig. 1, indicates that there was a significant correlation between growth and synthesis of muscle protein. An increase in muscle weight was therefore accomplished, at least in part, by an increase in the synthesis rate. The contribution of the rate of muscle protein breakdown in determining the accumulation of muscle protein could not be evaluated from these observations.

This demonstrable relationship between growth and muscle synthesis rates then allowed a re-evaluation of the effects of feeding on rates of muscle synthesis. Each animal's synthetic rate was corrected for the growth component by applying a correction factor from the slope of the graph in Fig. 1. The effect of feeding on the corrected synthesis rate is shown in Table 3. Thus true increases were seen in synthetic rates after feeding in groups of animals with equivalent growth rates. Similarly, the effects of starvation are as striking as when first calculated.

Gan & Jeffay (1967) and Waterlow & Stephen (1968) showed that the ratio of tissue to plasma free amino acid specific radioactivity is a measure of the proportion of amino acids that enter the tissue from plasma and from breakdown of unlabelled proteins.

Table 4. *Rate of synthesis of fixed liver proteins and concentration of free tyrosine in liver of meal-fed rats*

Rats were infused with L-[¹⁴C]tyrosine for 6 h after which 1 g of liver was taken from each and treated as described in the text. The estimate of the rate of protein synthesis was calculated from the specific radioactivity of free and protein-bound tyrosine in the tissue by using eqn. (7). The minimum value for the rate of protein synthesis was calculated from the specific radioactivity of tyrosine in liver protein and free tyrosine in the blood by using eqn. (9). The value of λ_p used in these calculations (eqns. 7 and 9) was the same for all groups (80 days⁻¹, see the text). The free tyrosine concentration was measured on the trichloroacetic acid supernatant of liver by reaction with nitrosonaphthol (see the text). S_i/S_p is the ratio of free tyrosine specific radioactivity in the liver to that in systemic blood at the end of the infusion. Each value is the mean $\pm$ S.D. for the group. Analysis of variance shows that there is a significant increase in the estimate of the rate of protein synthesis between 18 and 24 h ($P < 0.001$) but differences in S_i/S_p and the free tyrosine concentration are not significant ($P > 0.05$).

Infusion time after last meal (h)	Estimate of protein synthetic rate (%/day)	Minimum value for protein synthetic rate (%/day)	S_i/S_p	Free tyrosine concentration (nmol/g wet wt.)
0–6	44.4 $\pm$ 8.3	20.4 $\pm$ 7.3	0.426 $\pm$ 0.123	164 $\pm$ 89
6–12	42.0 $\pm$ 7.0	20.2 $\pm$ 2.4	0.506 $\pm$ 0.111	83 $\pm$ 25
12–18	47.5 $\pm$ 6.7	22.9 $\pm$ 3.7	0.501 $\pm$ 0.088	89 $\pm$ 16
18–24	63.8 $\pm$ 5.6	29.0 $\pm$ 2.6	0.470 $\pm$ 0.022	94 $\pm$ 6
24–30	46.1 $\pm$ 8.8	20.6 $\pm$ 5.0	0.440 $\pm$ 0.103	102 $\pm$ 31
48–54	47.1 $\pm$ 5.3	27.2 $\pm$ 3.3	0.590 $\pm$ 0.010	89 $\pm$ 12

In muscle there was no significant alteration in this proportion at any time during the day (Table 2).

Synthesis of fixed liver protein

The rapid rate of protein synthesis in the liver (Table 4) corresponds to an average half-life of 1.4 days compared with an average half-life for muscle protein of 9.6 days. In contrast with muscle, liver protein synthesis did not respond to a meal nor to 48 h of starvation (Table 4). The only animals with a significantly different rate were those fed 18 h before and these had little food remaining in their stomachs. When the minimum values for the rate of protein synthesis were calculated from the blood free tyrosine specific radioactivity value as precursor, the pattern during the day was the same.

The ratio of liver/plasma free tyrosine specific radioactivity (Table 4) was less than that in muscle. This is consistent with the faster breakdown of liver protein, the synthesis of tyrosine from phenylalanine in liver and the influx of unlabelled amino acids from protein breakdown within the gut. In spite of the rapid absorption of food during the early part of the day there were no significant changes in this ratio during the feeding cycle or even after 48 h of starvation. It is possible, therefore, that during the period of rapid absorption of tyrosine from the gut the ratio of free tyrosine specific radioactivities was only preserved because the influx of unlabelled dietary amino acids was balanced by a decrease in the entry of unlabelled tyrosine from intracellular protein breakdown and tyrosine synthesis within the liver.

Discussion

Measurement of protein synthesis rate

Constant infusion of an isotopically labelled amino acid was chosen because it was well suited to measurement of the rate of protein synthesis during short periods of a few hours only. Because the specific radioactivity of the free amino acid rises rapidly to a plateau value, it is only necessary to make measurements at the end of the infusion in order to determine the rate of protein synthesis in each separate animal (Garlick & Millward, 1972). The results from both liver and muscle obtained by infusion of tyrosine agree quite well with those from infusion of lysine (Waterlow & Stephen, 1968), and glycine (Garlick, 1969, 1972), and by an independent technique with $^{14}\text{CO}_2$ -labelling of amino acids (Millward, 1970a).

The use of tyrosine is preferable to glycine or lysine because the plateau value of specific radioactivity of tyrosine is reached more rapidly and the method for the chemical estimation of tyrosine specific activity is much simpler (Garlick, 1972; Garlick & Marshall, 1972). Work with both glycine and tyrosine has suggested some modifications to the original calculations

of rates of synthesis made by Waterlow & Stephen (1968), and these have been set out in detail under 'Calculations'. The new formulae are more accurate and can be used for calculating synthetic rates in most tissues with infusions of lysine, glycine or tyrosine.

A number of assumptions made in determining the synthetic rates are unlikely to lead to inaccuracy in its measurement. First, the assumption of a constant plateau of specific radioactivity is valid for both blood and tissues when a number of different amino acids are infused (Gan & Jeffay, 1967; Waterlow & Stephen, 1967, 1968; Garlick & Marshall, 1972; Seta *et al.*, 1973). Secondly, a single value for the rate constant for the rise of specific radioactivity in plasma was assumed for all groups when calculating the rate of protein synthesis in liver. It can be shown that any errors involved in this assumption are small, particularly when tyrosine with its small pool size is labelled by infusions lasting 6 h, during most of which the specific radioactivity is at a plateau value. The calculated rate of protein synthesis is independent of the duration of the infusion between 1 and 10 h (Garlick, 1969; Garlick & Marshall, 1972) which means that both the estimate of the rate of rise of precursor specific radioactivity to plateau value and the assumption that the plateau, once reached, is maintained must have been correct. Finally, the assumption was made that both the free and protein-bound amino acid pools would remain constant in size during the experiment. The inflow of dietary tyrosine caused only small changes in the free tyrosine concentrations in muscle (Table 2) and liver (Table 4) and the rate of infusion of carrier tyrosine was kept low so that it made little contribution to the total tyrosine flux.

The exact site of the free amino acid precursor of protein synthesis has not yet been located and this influences the accuracy of all measurements of protein synthesis rate made by incorporation of isotope into protein. A number of observations of the kinetics of incorporation of isotopically labelled amino acids into protein of isolated tissues *in vitro* suggest incorporation from a pool that equilibrates with the extracellular amino acids more rapidly than does the total intracellular free amino acid pool (Kipnis *et al.*, 1961; Rosenberg *et al.*, 1963; Guidotti *et al.*, 1964; Kostyo, 1964; Hider *et al.*, 1969, 1971; Adamson *et al.*, 1972). By contrast, perfusion experiments with heart (Morgan *et al.*, 1971) have shown that the kinetics of incorporation into protein are consistent with the rise in specific radioactivity of the total intracellular free amino acid. It is probable that neither the specific radioactivity of the amino acid in the total intracellular nor that in the extracellular pool is equal to that of the precursor of protein synthesis because of compartmentation of the free amino acid pool such as that demonstrated by Mortimore *et al.* (1972) in perfused liver. However, under the condi-

tions of a constant infusion of a labelled amino acid, it has been shown that a better estimate of the rate of protein synthesis is derived from the specific radioactivity of the free amino acid in the tissue rather than that in the plasma (Fern & Garlick, 1973). We have therefore calculated rates of protein synthesis from the specific radioactivity of free tyrosine in the tissue. Values derived from the specific radioactivity of tyrosine in plasma have also been included for completeness.

With the constant infusion technique the differences between the intracellular and plasma amino acid specific radioactivities are minimized because both follow a similar time-course to plateau value. Yet an error of 50% in absolute synthetic rates is still possible because, in for example the liver, the intracellular specific radioactivity is about half of that in blood. The specific radioactivity of free tyrosine in muscle is closer to that in blood (a ratio of 0.7) so that estimates of protein synthesis in muscle are probably more reliable. Because the ratios of intracellular/plasma specific radioactivities are the same in the different groups of animals, changes in synthetic rate after feeding (Table 2) can be taken as being genuine even though the actual values based on total intracellular pools may be over-estimates of the true rates.

Finally, the rates of protein synthesis are averages for the total protein of the tissue and need not apply to any individual protein whose concentration may vary in a different way from the remainder. The present studies assessed the nutritional impact of diet on the protein mass in muscle and liver and did not assess the effects of diet on quantitatively minor, though metabolically important, proteins.

Tyrosine flux

The tyrosine flux represents amino acid that leaves the free amino acid pool for protein synthesis and by other chemical reactions. Oxidation is probably 10–20% of total flux (Neale, 1971). If these reactions of tyrosine other than incorporation into protein are neglected, an estimate of the rate of protein synthesis occurring in the whole body can be made. By using the value for the tyrosine content of the whole rat (Munro, 1970), a tyrosine flux of $30 \mu\text{mol/h}$ per 100 g body wt. (Table 1) is equivalent to a protein synthesis rate of about 40 g/day per kg body wt. This value is very similar to the whole body protein synthesis rate estimated by infusion of both [^{14}C]lysine and [^{14}C]glycine (James, 1972).

In spite of the cyclic pattern of food intake, significant differences in tyrosine flux during the day were not observed. This may in part be the result of large variations between animals in the groups and also to the retention of food in the stomach during most of the day. After a further 24 h of starvation there was still no significant fall in flux. This is similar to the

observation of Waterlow & Stephen (1967, 1968) that only after 6 weeks of protein deprivation was a fall in lysine flux obtained. However, measurements made on plasma take no account of the distribution of protein synthesis in the whole organism. More precise information on the incorporation of isotope into protein of two tissues, liver and gastrocnemius muscle, was therefore obtained.

Synthesis of muscle protein

The average fractional rate of protein synthesis during the day obtained with infusion of tyrosine (7.2%/day) corresponds to a half-life of 9.6 days. This is slightly lower than that obtained by infusion of lysine (Waterlow & Stephen, 1968) and glycine (Garlick, 1969) or by measurement of the rate of decay of [^{14}C]aspartate and [^{14}C]glutamate in protein (Millward, 1970a) and is probably the result of the slower rate of growth (2.5%/day) obtained with meal-fed rats.

During the day the rate of protein synthesis in muscle clearly follows a cyclic pattern (Table 3). During starvation there is a rapid fall in the rate of protein synthesis, similar to that observed in both starved and protein-depleted rats by Waterlow & Stephen (1968) and Millward (1970b). Young (1970) predicted this type of cyclic pattern of protein synthesis as a response to the secretion of insulin after a meal. The concentration of free amino acids in muscle does not appear to be directly responsible since neither free tyrosine concentration nor total α amino N were seen to alter with feeding or starvation (Table 2).

Because the average rate of protein turnover in muscle is of the same order as the growth rate of the animal it is possible to detect the increment in synthesis rate required for growth to occur. The regression of protein synthesis rate on growth rate (Fig. 1) has a gradient close to unity which shows that all of the growth can be accounted for by a change in the rate of synthesis. A similar relationship between growth rate and protein synthesis rate was observed by Henshaw *et al.* (1971). In the same way Waterlow & Stephen (1968) observed a correlation between the fractional rate of protein synthesis in muscle and the size of the rats, which is equivalent to the relationship between growth and protein synthesis, since small rats grow more rapidly.

Liver

The rate of protein synthesis in the liver was found to be about 50%/day. Only the rate of synthesis of the fixed liver proteins is measured since the plasma proteins such as albumin, which are made within the liver, only remain there for about 30 min (Rothschild *et al.*, 1970). With an infusion lasting 6 h therefore, the component of radioactivity in intracellular plasma proteins will be very small.

Unlike the muscle, in the liver there was no response of protein synthesis to feeding, nor to 24 or 48 h of starvation. Only during the 18–24 h period was there a significant increase in the rate of protein synthesis, which we are unable to explain. The lack of response to starvation is in agreement with measurements made on starved and protein-deprived rats. In these animals there is a rapid loss of liver protein and perhaps a fall in the absolute rate of protein synthesis, but the fractional rate of synthesis is maintained or even elevated (Waterlow & Stephen, 1968; Haider & Tarver, 1969; Gaetani *et al.*, 1961; Wannemacher *et al.*, 1971).

During cyclic feeding (or after feeding) dramatic changes in liver weight and protein content occur (Leveille & Chakrabarty, 1967). We estimate that the liver protein content rises by 20% in the first 8 h after feeding and thereafter falls again (D. J. Millward, P. J. Garlick & W. P. T. James, unpublished work). This would require a very large change in the rate of protein synthesis or that protein breakdown should almost cease. Small increases in synthesis rate might not be detected, particularly after a meal when unlabelled amino acids were entering the liver from the food and when the precursor specific radioactivity may have been altered. The large change in synthesis needed to increase the protein content by 20% in 8 h would have been observed. Since no appreciable change in synthesis occurred we conclude that the change in protein content was primarily the result of changes in protein breakdown. Perfusion experiments with liver confirm this, for high concentrations of amino acids in the perfusate cause only a moderate increase in the rate of synthesis, but protein breakdown almost ceases (Woodside & Mortimore, 1972). Insulin also inhibits protein breakdown in the perfused liver (Mortimore & Mondon, 1970).

In liver, therefore, we have a different mechanism from that in muscle. The major effect of food absorption appears to be a decrease in the rate of protein breakdown, with a subsequent increase in the protein content of the tissue.

Hence it is important when studying the regulation of protein turnover *in vivo* not only to consider the changes in both synthesis and breakdown, but also that the effect in one tissue may be quite different from that in another.

The authors are grateful to Professor J. C. Waterlow for discussion and advice. P. J. G. and D. J. M. thank the Medical Research Council and W. P. T. J. the Wellcome Trust for financial support.

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