Use of the constant infusion technique for measuring rates of protein synthesis in the New Zealand White rabbit

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(*Received* 12 *January* 1977 – *Accepted* 21 *January* 1977)

1. To study the potential of the constant-infusion technique for measuring rates of protein synthesis in New Zealand White rabbits, animals were infused for up to 6 h with radioactively-labelled tyrosine.
2. Labelled tyrosine from plasma and tissues was isolated from labelled metabolites by ion-exchange chromatography.
3. Analysis of serial blood and muscle biopsy samples removed under anaesthesia showed that the specific radioactivity (SR) of the free tyrosine pools reached an approximately constant value within 2 h.
4. Certain commercial preparations of L-[side-chain 2,3-^{3}H]tyrosine were contaminated with 300 mg radioactive D-tyrosine/g. The D-isomer appeared to enter the muscle intracellular pool.
5. In constant-infusion experiments L-[^{3}H]tyrosine could replace the uniformly-^{14}C-labelled L-isomer for the determination of rates of protein synthesis in muscle. L-[side-chain 2,3-^{3}H]tyrosine may not be suitable for use as a precursor for measuring rates of liver protein synthesis.
6. Evidence is presented that the precursor of liver protein synthesis may not be well defined by the SR for free tyrosine of the homogenate.
7. The technique was used to measure the rates of protein synthesis in adult rabbits. The rates of protein synthesis in liver and muscle were measured and from measurements of tyrosine flux the mean rate of whole-body protein synthesis was calculated as 13·8 g/kg per d.

The mechanisms by which the over-all rate of deposition of muscle protein is controlled at the anabolic and catabolic levels are of importance in the improvement of meat production. In this study the New Zealand White rabbit has been used as an experimental model, since it is large enough to permit assessment of rates of incorporation of isotopes in individual tissues and organs to be made and yet allows isotope costs to be kept relatively low. In addition the isolation procedures and properties of the major muscle protein fractions are particularly well documented for the rabbit.

A first requirement for these studies was a reliable method for measuring protein synthesis rates. These are usually obtained by following the rate of uptake of radioactivity into protein after administration of a labelled amino acid. For individual animals calculation of the rate of uptake is simplified if the isotope is administered by continuous infusion rather than as a single dose, since in the former method the specific radioactivity (SR) of the amino acid in the body free pools increases quickly and becomes approximately constant (Gan & Jeffay, 1967, 1971; Waterlow & Stephen, 1967, 1968; Seta, Sansur & Lejtha, 1973).

The selection of a suitable labelled amino acid for measurement of protein synthesis rates by the constant-infusion method is determined by several factors. An amino acid should be chosen for which the value for protein-bound amino acid:free pool amino acid is as high as possible, thus ensuring rapid turnover of the free amino acid pool. In addition, if synthesized protein is determined radiochemically without further modification, i.e. isolation of the relevant amino acid, then the precursor should not be converted to other protein constituents. In the rabbit these requirements are best met by tyrosine, valine and leucine, all of which have high values for protein-bound amino acid:free pool amino acid and are known not to be significantly metabolized to other amino acids. Because its ultraviolet extinction characteristics would facilitate detection in biological extracts tyrosine was selected as the precursor.

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Table 1. *Composition (g/kg) of semi-synthetic diet* for New Zealand White rabbits*

Casein†	190	Maize starch	385
L-arginine‡	5	Cellulose§	200
DL-methionine‡	2	Arachis oil	30
Glycine‡	2	Mineral mix‖	56
Sucrose	110	Vitamin mix¶	20

* Diets were pelleted using a Laboratory Pellet Mill CL3 (California Pellet Mill Co. Europe, Daventry, Northants.).

† Casein D; Glaxo Laboratories Ltd, Middlesex.

‡ Cambrian Chemical Co. Ltd, London.

§ CEPO SS200; Bayer Chemical Co. Ltd, Glasgow.

‖ Contained (g/kg diet): $CaCO_3$ 12·5, $CaHPO_4$ 10·0, K_2HPO_4 10·0, $KHCO_3$ 8, NaCl 5, $MgSO_4$ 10·0; also (mg/kg diet): $FeSO_4$ 248, KIO_3 1·68, $MnSO_4$ 162, $ZnSO_4$ 177, $CuSO_4$ 29·5, $(NH_4)_6Mo_7O_{24}$ 22·7, $KCr(SO_4)_2$ 9·6, $CoCl_2$ 4·0, Na_2SeO_4 0·9.

¶ Contained (mg/kg diet): choline 1000, thiamin 25, riboflavin 25, pantothenic acid 100, nicotinic acid 200, pyridoxal 10, biotin 2, menaphthone 5, cyanocobalamin 0·05, *myo*-inositol 200, *p*-aminobenzoate 50, retinol 3, cholecalciferol 0·025, vitamin E 15, sucrose 730.

With animals as large as adult rabbits the costs of infusing sufficient ^{14}C-labelled amino acid would be prohibitive, particularly if the SR of individual proteins were to be determined. For this reason it was decided to determine whether the cheaper ^{3}H-labelled amino acid would give comparable results to a ^{14}C-labelled amino acid. This paper reports the use of a biopsy technique to demonstrate that an SR plateau is reached for free tyrosine in plasma and muscle tissue during constant infusion of radioactively labelled tyrosine into the rabbit, and details the problems encountered by using a tritiated rather than a uniformly-^{14}C-labelled amino acid. The method has been used to measure the rates of protein synthesis in the whole body, muscle and liver of adult rabbits.

MATERIALS AND METHODS

Animals

New Zealand White rabbits from the Rowett Research Institute colony were individually caged and fed *ad lib.* from weaning on a pelleted semi-synthetic diet (Table 1). For experimental purposes animals of both sexes were used.

Materials

L-[side-chain 2,3-^{3}H] tyrosine (TRK 282, batch nos. 12, 13, 14, 15 and 16), DL-[side-chain 2-^{14}C]tyrosine (CFA 55, batch no. 18) and L-[U-^{14}C]tyrosine (CFB 74, batch nos. 40, 43 and 46) were obtained from the Radiochemical Centre, Amersham, Bucks. L-[ring 2,6-^{3}H]-tyrosine (NET 444, lot no. 806-196) was obtained from NEN Chemicals GmbH, Frankfurt, West Germany.

Amino acids were supplied by Sigma Chemical Co. Ltd, London. L-alanyl-*N*-carboxy-anhydride was obtained through Alan Hepburn Ltd, Edinburgh, and had been prepared by the carboalkoxy procedure (Greenstein & Winitz, 1961). L-alanyl-L-tyrosine was purchased from Cyclo Chemical, Los Angeles, California, USA.

Other materials were Analar-grade chemicals or the purest commercially-available grade.

Choice of labelled tyrosine

L-[ring 3,5-^{3}H]tyrosine was considered unsuitable for this study because under the conditions used for protein hydrolysis the C—^{3}H bonds are labile (Martin & Marlino, 1965). Under similar conditions both L-[side-chain 2,3-^{3}H]tyrosine and the more expensive

L-[ring 2,6-^{3}H]tyrosine were stable, as judged by SR measurements and recoveries. Both ^{3}H-labelled forms of tyrosine were used as infusates as well as L-[U-^{14}C]tyrosine.

Constant-infusion technique

Radioactively-labelled tyrosine for infusion was diluted in sterile saline solution (9 g sodium chloride/l) without the addition of carrier amino acid. Total dosages for each experiment were measured accurately and approximated to 0·3–1·0 mCi/kg body-weight for [^{3}H]tyrosine and 20-100 μCi/kg body-weight for [^{14}C]tyrosine. Infusions were performed using a Slow Infusion Apparatus (Scientific and Research Instruments Ltd, Edenbridge, Kent) connected to a 10 ml plastic syringe via coiled flexible tubing (made from PP25; Portex Ltd, Hythe, Kent) to a Butterfly-25 Infusion Set (Abbot Laboratories Ltd, Queenborough, Kent) inserted in the peripheral ear vein. This allowed animals normal movement within the cage, but to minimize interference with the catheter animals were lightly harnessed with a length of Tubigrip G4 or G5 (Seton Products Ltd, Oldham, Lancs) over the front legs and thorax. The normal infusion rate was 1·16 ml/h for a period of 6 h. Animals were allowed access to water and food throughout this time, except that food was withheld for the final hour of the infusion.

Immediately infusions were terminated the animals were killed by cervical dislocation, the throat slit and blood collected in a heparinized container. Main organs and individual muscles were quickly removed and frozen in liquid nitrogen. The blood was centrifuged and the plasma removed, frozen and stored with the tissue samples at $-20°$ until further use.

Rabbits for biopsy experiments were anaesthetized using Saffan (Glaxo Laboratories Ltd, Greenford, Middlesex) administered through a Butterfly-25 Infusion Set placed in the peripheral ear vein and then a Teflon catheter (medical grade, 0·69 mm i.d., 1·22 mm o.d.; Becton Dickinson & Co. Ltd, Rutherford, New Jersey, USA) was inserted in a carotid artery. This catheter was fitted to a three-way tap allowing blood sample removal and infusion of sterile saline solution containing heparin (25 U/ml) to help maintain constant blood volume. Biopsy samples (0·3–0·6 g) were excised from the longissimus muscles at specified intervals. Usually four biopsies were removed from each longissimus, all incisions being packed with sponge rubber and then closed with Michel clips (Downs Bros and Mayer & Phelps Ltd, Mitcham, Surrey). Blood and tissue samples were treated as described previously.

Preparation of samples for chromatography

Before treatment, plasma and tissue samples were weighed and a measured volume of 1 mM-norleucine added to a final level of approximately 0·1 μmol/g tissue.

Plasma samples (1 ml) were treated with 2·5 ml 0·5 M perchloric acid (PCA) at 4°, and after standing for 10 min they were centrifuged at 1000 *g*. The protein pellets were washed twice with 0·5 M-PCA (1·25 ml) at 4° and the washings combined with the original supernatant fraction. Tissue samples (1–3 g) were homogenized (ILA Homogenizer Model X 10; Internationale Laboratoriums-Apparate GmbH, Dottingen, West Germany) in water (5 ml) at 4°, 2·5 M-PCA (1·5 ml) was added and PCA-soluble material extracted as described previously. All protein precipitates were retained.

Plasma and tissue extracts were adjusted to approximately pH 4 using 2 M-potassium hydroxide. After standing on ice for 20 min, precipitated potassium perchlorate was removed by centrifugation at 1000 *g*, and the supernatant fractions were freeze-dried. The residue was dissolved in 0·1 M-hydrochloric acid for chromatography.

Protein precipitates were washed twice with 5 ml dilute trichloroacetic acid (TCA) (50 g/l) and dissolved in 0·5 M-sodium hydroxide (2–4 ml) by stirring for 1 h at 20°. First experiments included an initial treatment with hot (90°) dilute TCA (50 g/l) containing

unlabelled tyrosine to remove any tyrosyl-tRNA of high SR, but later studies showed this refinement to be unnecessary. The protein was then reprecipitated by addition of TCA to a final concentration of 150 g/l and washed twice with 5 ml dilute TCA (50 g/l). Samples were further washed with 5 ml ethanol–water (95:5, v/v) containing potassium acetate (10 g/l) and then with two 5 ml treatments of warm chloroform–ethanol–diethyl ether (1:2:2, by vol.). Finally the samples were washed at least six times with diethyl ether and allowed to air-dry.

For protein analysis, 20 mg dried powder was placed in Pyrex tubes (medium wall), 2 ml 0·1 M-NaOH was added and the sample was left to stand for 1 h at 21°. Three drops of antifrothing reagent, capryl alcohol, were added, then 2 ml conc. HCl (Aristar; British Drug Houses, Poole, Dorset). The solution was frozen in liquid N_2 and de-aerated under slow thawing using a rotary vacuum pump (Model ED100; Edwards Vacuum Components, Crawley, Sussex). Tubes were sealed under reduced pressure and heated at 110° for 24 h. Samples were then dried under reduced pressure and dissolved in 2 ml 0·2 M-sodium citrate, pH 2·2, for chromatography.

Ion-exchange chromatography and determination of tyrosine SR

Chromatography was performed using an amino acid analyser (The Locarte Co., London W14) in the preparative, split-stream or analytical mode. When appropriate, a timed-flow valve device was used to divert only that part of the eluate containing tyrosine to a fraction collector, the remainder going to waste.

PCA-soluble extracts equivalent to 0·5–0·8 g original sample were chromatographed on a resin (The Locarte Co.) with 'bed' dimensions 270 × 9 mm at 50°. The buffer was 0·2 M-sodium citrate, pH 4·25, and its flow-rate and that of the ninhydrin reagent was 30 ml/h. A peristaltic pump (Gilson Minipuls; Gilford Instruments Ltd, Teddington, Middlesex) was used to transfer approximately one-third of the column effluent to a fraction collector for determination of radioactivity and to replace this effluent with buffer. The remainder of the effluent passed through the colorimeter for tyrosine and norleucine estimation. By chromatographing radioactively-labelled tyrosine standards the SR of tyrosine in extracts could be calculated. The tyrosine content (/g tissue wet weight) and the proportion of the total radioactivity present in tyrosine were also determined. The results were not corrected for extracellular space.

Hydrolysates equivalent to 5–15 mg protein were chromatographed preparatively on a resin (The Locarte Co.) with 'bed' dimensions 500 × 9 mm at 50°. The buffer was 0·2 M-sodium citrate, pH 4·25, flow-rate 30 ml/h. The eluate was collected by a fraction collector and the tyrosine located by its extinction at 274·5 nm. Pooled fractions were assayed for tyrosine content using the amino acid analyser and were assayed also for radioactivity.

Liquid-scintillation counting

Samples were assayed for radioactivity in standard vials or minitubes containing 10 ml and 3 ml respectively of liquid scintillator (NE260; Nuclear Enterprises (GB) Ltd, Edinburgh) using a liquid-scintillation spectrometer (Packard Tri-Carb Model 3315; Packard Instrument Co., Illinois, USA). Two standards were prepared of L-[side-chain 2,3-^{3}H]-tyrosine and L-[U-^{14}C]tyrosine at 5 μCi/ml, and the radioactivity in 5 μl of each of these standards in 10 ml NE260 was measured. Variations in counting efficiency associated with different buffers and different volumes were determined relative to the standard conditions and appropriate corrections applied to values obtained for experimental samples.

Enantiomeric composition of tyrosine

The method was based on that of Manning & Moore (1968). To radioactively-labelled tyrosine samples (freeze-dried if necessary) was added 1 ml 0·45 M-sodium borate buffer, pH 10·2, containing DL-tyrosine (1 mg/ml). L-alanyl-*N*-carboxyanhydride (1·5 mg) was added, with vigorous mixing, at 4° and the reaction allowed to proceed for 2 min. After adjustment to pH 12·5 using 1 M-NaOH the solution was incubated at 37° for 4 h. Finally the solution was adjusted to pH 2 with 2 M-HCl.

Ion-exchange chromatography was performed with the 270 mm column described previously, using 0·2 M-sodium citrate buffer, pH 4·25, at 68°. The eluate was collected and the fractions assayed for radioactivity.

Muscles

The nomenclature of the individual muscles was that of Bensley (1948), as modified by Kerr (1955).

Calculation of protein synthesis rates

The methods of calculation were based on those described by Garlick, Millward & James (1973).

Tyrosine plasma flux and whole-body protein synthesis rate. The plasma flux (F; μmol/h) was calculated from the relationship:

$$F = \frac{I}{S_{p(\max)}}, \tag{1}$$

where I is the rate of infusion of isotope (μCi/h), $S_{p(\max)}$ is the plateau value of free plasma tyrosine SR (μCi/μmol).

Whole-body protein synthesis rate (P; g/d) was calculated from F using the equation:

$$P = (2{\cdot}4 \times 10^{-3}) \times F \times \frac{M}{C}, \tag{2}$$

where M is the molecular weight of tyrosine, C is the tyrosine content of body protein (%).

Tissue protein fractional-synthesis rates. The rate equation describing the protein-bound tyrosine SR (S_B) is:

$$\frac{d}{dt} S_B = k_s S_x - k_d S_B \text{ (Zilversmit, 1960)}, \tag{3}$$

where k_s and k_d are the fractional rates of protein synthesis and degradation respectively (assumed constant for the infusion period), S_x and S_B are the time-dependent SR of the precursor and protein-bound amino acid respectively.

For infusions of 6 h, the value for $k_d S_B$ only becomes significant compared with that for $k_s S_x$ in those tissues with large fractional-synthesis and degradation rates. In this latter instance, since the tissue k_d value must be close to that for k_s, the approximation can be made:

$$k_d S_B = k_s S_B.$$

Also if it is assumed that the 'time-course' of S_x can be described approximately by the expression:

$$S_x = S_{x(\max)} (1 - e^{-\phi t}), \tag{4}$$

where ϕ is a rate-constant $\left(\frac{1}{\text{time}}\right)$ then equation 3 can be rewritten as:

$$\frac{d}{dt} S_B = k_s S_{x(\max)} (1 - e^{-\phi t}) - k_s S_B. \tag{5}$$

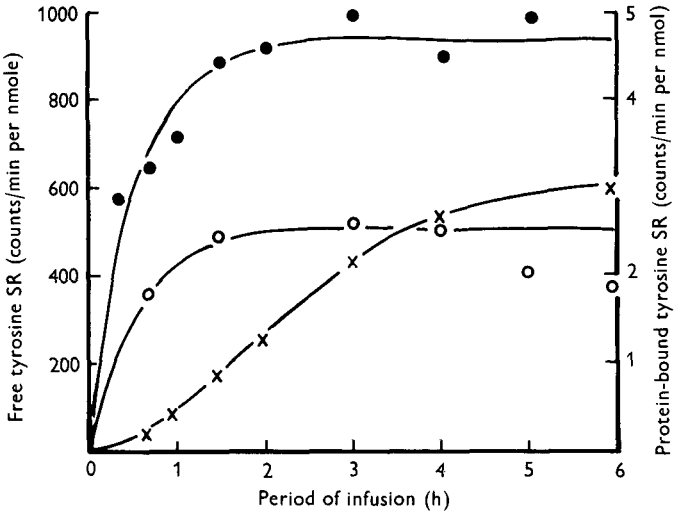

Fig. 1. Changes in tyrosine specific radioactivity (SR) in New Zealand White rabbit no. 87 (female, body-weight 2·54 kg) during constant infusion of L-[ring 2,6-^{3}H]tyrosine at 400 μCi/h for a 6 h period. (●), Plasma free tyrosine; (○), longissimus muscle free tyrosine; (×), longissimus muscle protein-bound tyrosine. Plots of the type $S = S_{max}(1\text{-}e^{-\phi t/24})$ were fitted to the free tyrosine values obtained for the first 4 h, where t is the period of infusion (h), ϕ is a rate constant (/d), S_{max} is the asymptotic value of S) SR; counts/min per nmol):

$$\text{Plasma } S = 940\ (1\text{-}e^{-45\cdot8t/24}),$$
$$\text{Muscle } S = 503\ (1\text{-}e^{-43\cdot9t/24}).$$

Integrating equation 5 with respect to time, and using the condition that $S_B = 0$ when $t = 0$ to evaluate the integration constant, and finally eliminating $S_{x(max)}$ using equation 4, gives the equation:

$$\frac{S_B}{S_x} = \frac{\phi}{\phi - k_s} \times \frac{1 - e^{-k_s t}}{1 - e^{-\phi t}} - \frac{k_s}{\phi - k_s}. \qquad (6)$$

Equation 6 is the equation used by Garlick *et al.* (1973) to calculate protein fractional-synthesis rates. Using this equation, plots of S_B/S_x *v.* k_s were made for defined values of ϕ and t. From these, k_s could be calculated for experimentally-determined estimates of S_B/S_x. The assigned value of ϕ need only be approximate for values greater than 20/d since it can be shown that the derived estimate of k_s is relatively insensitive to changes in ϕ exceeding this level.

Depending on whether the SR of the free tyrosine pool of plasma (S_p) or the relevant tissue (S_i) was used to define the precursor, then the fractional rates of protein synthesis were defined as $k_{s(p)}$ and $k_{s(i)}$ respectively.

RESULTS

Changes in free tyrosine SR during constant infusion

The removal of muscle and blood samples under anaesthesia avoided the expense and biological variation involved in the slaughtering of animals in series, at differing intervals following similar treatments. Using L-[ring 2,6-^{3}H]tyrosine under these conditions the SR of protein-bound tyrosine increased linearly with the period of infusion, after a short lag period, and the free tyrosine SR in plasma and muscle approached a plateau within 2 h (Fig. 1). In contrast, this 'plateau' condition was not reached for plasma or muscle free

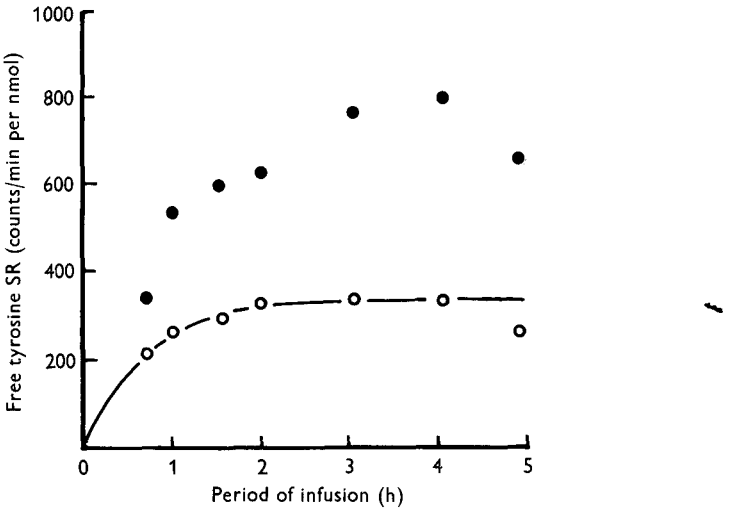

Fig. 2. Changes in longissimus muscle free tyrosine specific radioactivity (SR) in New Zealand White rabbit no. 46 (male, body-weight 3·08 kg) during constant infusion of L-[side-chain 2,3-^{3}H]-tyrosine (batch no. 13) at 250 μCi/h for a 6 h period. (●), Uncorrected values; (○), values corrected for D-tyrosine contamination; fitted curve shown $S = 339\ (1\text{-}e^{-34 \cdot 3t/24})$, where S is the SR (counts/min per nmol), t is the period of infusion (h).

tyrosine when L-[side-chain 2,3-^{3}H]tyrosine (batch no. 13) was infused (Fig. 2). During the latter part of these biopsy experiments, bleeding from the excision areas became a problem and the general trauma caused probably accounted for the observed decrease in the SR of free tyrosine and the reduction in the rate of uptake of radioactivity into protein.

An indication of the reason for the differing behaviour of the two labelled compounds came from the finding that approximately 30 % of the radioactivity in what was nominally L-[side-chain 2,3-^{3}H]tyrosine (batch nos. 12 and 13) was resistant to L-tyrosine carboxy-lyase (*EC* 4.1.1.25) (*Streptococcus faecalis*) and yet co-chromatographed with tyrosine. This suggested that the commercial preparation might contain an unacceptable level of radioactively-labelled D-tyrosine.

Enantiomeric purity of commercial radioactively-labelled L-*tyrosine preparations*

The method of Manning & Moore (1968) was adapted to determine quantitatively the amount of D-isomer radioactivity present in labelled tyrosine samples (see p. 5). Test material was diluted with non-radioactively-labelled DL-tyrosine and after reaction with L-alanyl-*N*-carboxyanhydride the resulting diastereoisomeric dipeptides were separated by ion-exchange chromatography. The conversion of tyrosine to L-alanyl dipeptide was approximately 80 %. Most of the remaining radioactivity was unreacted tyrosine but two minor radioactively-labelled compounds which eluted before the main dipeptides were not identified (Fig. 3).

The enantiomeric composition of the radioactivity in tyrosine samples was determined from the distribution of radioactivity between the dipeptides, L-alanyl-D-tyrosine and L-alanyl-L-tyrosine. The profiles obtained with L-[side-chain 2,3-^{3}H]tyrosine (batch No. 13) and DL-[side-chain 2-^{14}C]tyrosine (batch no. 18) are shown in Fig. 3. Table 2 summarizes the calculated D-isomer content of these and other commercial preparations. The sensitivity of the method was such that 2 % of the radioactivity as D-isomer would have been detected. The only materials which failed to meet specification were three successive batches (nos. 12,

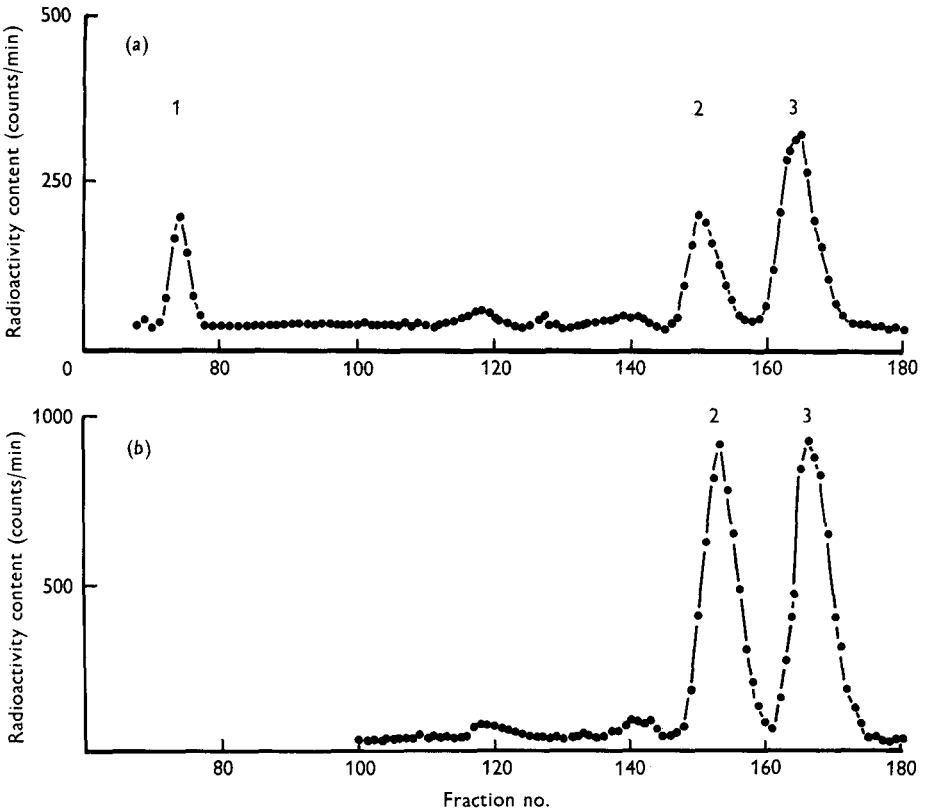

Fig. 3. Separation of L-alanyl-D-tyrosine and L-alanyl-L-tyrosine by ion-exchange chromatography (see p. 5). Sixty fractions/h were collected. (a) L-[side-chain 2,3-^{3}H]tyrosine (batch no. 13); (b) DL-[2-^{14}C]tyrosine (batch no. 18). (1) Tyrosine, (2) L-alanyl-D-tyrosine, (3) L-alanyl-L-tyrosine.

Table 2. D-*isomer content of various commercial radioactively-labelled tyrosine preparations*

Compound	Manufacturer	Batch no.	D-isomer content (mg/g)
L-[U-^{14}C]tyrosine	RC	43, 46	ND
DL-[2-^{14}C]tyrosine	RC	18	479
L-[side-chain 2,3-^{3}H]tyrosine	RC	12	328
L-[side-chain 2,3-^{3}H]tyrosine	RC	13	308
L-[side-chain 2,3-^{3}H]tyrosine	RC	14	350
L-[side-chain 2,3-^{3}H]tyrosine	RC	15	ND
L-[side-chain 2,3-^{3}H]tyrosine	RC	16	ND
L-[ring 2,6-^{3}H]tyrosine	NEN	806-196	ND

ND, not detected; RC, The Radiochemical Centre, Amersham, Bucks.; NEN, NEN Chemicals GmbH, Frankfurt, West Germany.

Table 3. *Contribution (%) of radioactivity in the* D*-isomer to radioactivity in total free tyrosine of blood and muscle of a New Zealand White rabbit during constant infusion of* L*-[side-chain 2,3-*^{3}*H]tyrosine*

(A 3·08 kg rabbit was infused with L-[side-chain 2,3-^{3}H]tyrosine (batch no. 13)* at 250 μCi/h for 5 h)

Period of infusion (min)	Contribution of D-isomer (%)	
	Blood	Longissimus muscle
0 (Infusate)	30·5	30·5
20	49·7	—
43	56·3	37·6
60	—	43·9
93	—	47·4
120	—	50·8
184	62·0	55·8
254	67·4	58·0
295	—	60·0

* For details, see p. 2 and Table 2.

13 and 14) of L-[side-chain 2,3-^{3}H]tyrosine. The level of D-isomer contamination in all of these batches was greater than 300 mg/g, which confirmed the earlier observations using L-tyrosine carboxy-lyase.

Accumulation of radioactively-labelled D*-tyrosine in blood and muscle*

After infusion with the radioactively-labelled tyrosine contaminated with D-isomer, analysis by the dipeptide method of free tyrosine from muscle biopsy samples revealed a steady increase in the contribution (%) of the D-isomer to the total tyrosine radioactivity. The contribution of D-tyrosine reached 60 % after 5 h infusion and there was a similar and slightly faster increase in the blood (Table 3).

Correction for D-isomer radioactivity of the free muscle tyrosine SR values in Fig. 2 showed that a plateau had indeed been reached within 2 h, which agreed with the behaviour of L-[ring 2,6-^{3}H]tyrosine.

As a result of this work the manufacturers have modified the preparations of the L-[side-chain 2,3-^{3}H]tyrosine and a more recent batch of this compound has shown a D-isomer content of < 20 mg/g (batch no. 15). Only with the seriously contaminated batches of this material was there a problem with D-isomer accumulation in the animal. With all other ^{3}H- and ^{14}C-labelled tyrosine preparations infused, radioactively-labelled D-isomer was not detected in plasma and tissues using the dipeptide method.

Metabolic conversion of tyrosine

Although the procedure of separating tyrosine from the biological fluids was time-consuming, it was necessary as a considerable proportion of the radioactivity present in tissue extracts was not in tyrosine (Table 4). This proportion varied from tissue to tissue, and was much higher in 'red' muscles than in 'white' when L-[U-^{14}C]tyrosine was infused. After ion-exchange chromatography, the distribution of the non-tyrosine radioactivity into acidic and basic components also showed marked differences between tissues (Nicholas, Lobley & Harris, unpublished results). As would be expected the pattern of labelling differed between ^{14}C- and ^{3}H-labelled infusates. In all instances, however, > 99 % of the radioactivity in protein was in tyrosine.

Table 4. *Total perchloric acid (PCA)-soluble radioactivity (%) present as tyrosine in body tissue fluids of a New Zealand White rabbit after infusion of [^3H]- and [^{14}C]tyrosine*

(75 μCi L-[U-^{14}C]tyrosine (batch no. 43)* and 1 mCi L-[side-chain 2,3-^{3}H]tyrosine (batch no. 15)* were infused into a female rabbit of body-wt 660 g for a 6 h period. The PCA-soluble extract was prepared as described on p. 3 and the tyrosine separated by ion-exchange chromatography)

	PCA-soluble radioactivity present as tyrosine (%)	
Radioactive label in tyrosine ...	^{14}C	^{3}H
Tissue		
Plasma	55·3	68·0
Brain	52·0	75·6
Liver	16·9	65·5
Kidney	32·9	37·8
Heart	35·9	41·0
Muscles:		
'White'		
Extensor digitorum longus	63·3	87·0
Tibialis anterior	61·4	75·3
Longissimus	61·1	72·5
Gluteus longus	61·1	78·4
'Red'		
Soleus	35·8	61·3
Semimembranosus proprius	37·1	73·7

* For details, see p. 2 and Table 2.

Validity of using [^3H]tyrosine for measuring protein synthesis rates

To determine whether L-[^{3}H]tyrosine and L-[U-^{14}C]tyrosine gave comparable results when used to determine rates of protein synthesis, dual-label experiments were performed in which a mixture of L-[side-chain 2,3-^{3}H]tyrosine (batch no. 15) and L-[U-^{14}C]tyrosine (batch nos. 43 and 46) was constantly infused. During isolation of the tyrosine by ion-exchange chromatography, partial resolution of the ^{3}H- and ^{14}C-labelled amino acids was observed. [^{3}H]-tyrosine was eluted before, and [^{14}C]tyrosine after, the detection of ultraviolet extinction (Fig. 4). Gaitonde & Nixey (1972) have shown a similar resolution of ^{12}C- and ^{14}C-labelled amino acids by ion-exchange chromatography. These results stress the importance of using the whole elution peak when making SR measurements.

The values for ^{3}H:^{14}C for tyrosine from the free amino acid pools of most muscles and organs were similar to that of the infusate except for liver (Table 5). Free tyrosine from this latter organ had a reduced ^{3}H:^{14}C value although the values for the protein-bound tyrosine of liver and plasma, in common with those from the other tissues studied, approached that of the infusate. The net effect of the reduced ^{3}H:^{14}C value was that $k_{s(i)}$ values calculated for the liver with L-[side-chain 2,3-^{3}H]tyrosine as the infusate were considerably higher than those calculated for L-[U-^{14}C]tyrosine, and are consequently unreliable.

The effect of liver homogenate on the ^{3}H:^{14}C value was studied to determine whether the decrease in ^{3}H:^{14}C was occurring in the preparation of the tissue. An animal was selected that had been previously infused with L-[side-chain 2,3-^{3}H]tyrosine and for which the liver S_i/S_p value was only 0·195, less than half the value found normally with L-[U-^{14}C]tyrosine infusions. To portions of liver (approximately 1 g) from this rabbit was added an excess of radioactivity as L-[side-chain 2,3-^{3}H]tyrosine and L-[U-^{14}C]tyrosine in proportion to that used in dual-label infusates. The increase in tyrosine concentration caused by this addition

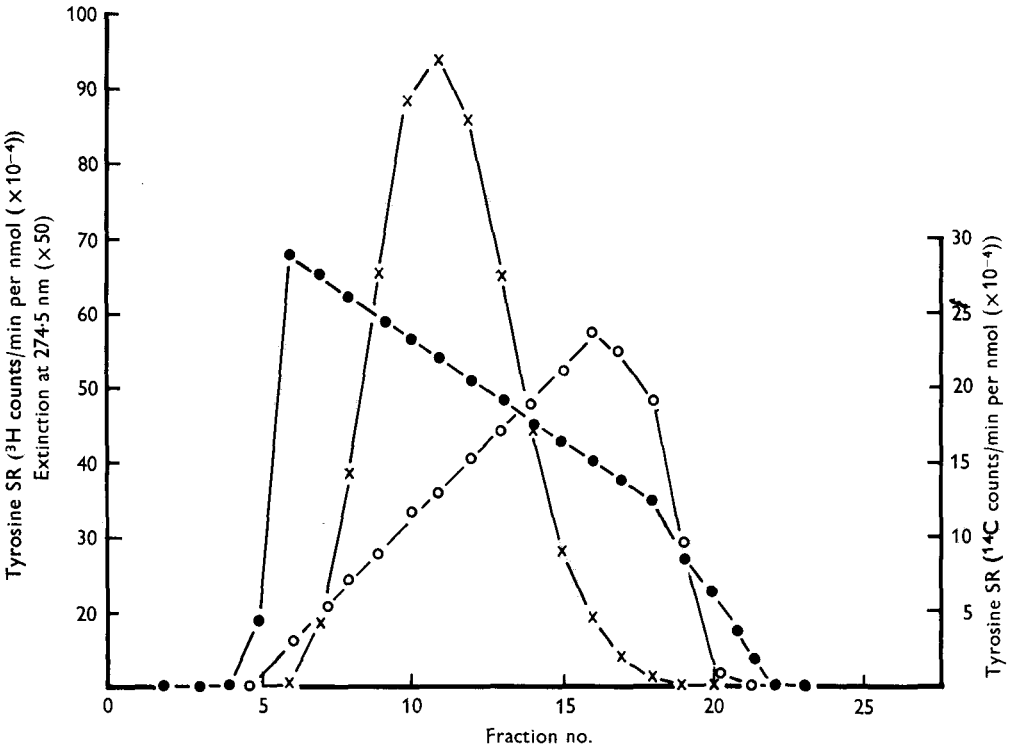

Fig. 4. Partial separation of L-[side-chain 2,3-^{3}H]tyrosine (batch no. 15) and L-[U-^{14}C]tyrosine (batch no. 46) by ion-exchange chromatography under conditions used to analyse protein hydrolysates (see p. 4). Forty fractions/h were collected. (●), ^{3}H specific radioactivity (SR); (○), ^{14}C SR; (×), extinction at 274·5 nm.

Table 5. *Values for ^3H:^{14}C for free and protein-bound tyrosine in tissues of New Zealand White rabbits after 6 h infusions of radioactively-labelled amino acid*

(Values in parentheses are the body-wt (kg) of the rabbits. The following infusates were administered in a 6 h period: for rabbit no. 126, 0·77 mCi L-[side-chain 2,3-^{3}H]tyrosine (batch no. 15)* and 0·077 mCi L-[U-^{14}C]tyrosine (batch no. 46)*; for rabbit no. 115, 1·0 mCi L-[side-chain 2,3-^{3}H]tyrosine (batch no. 15)* and 0·075 mCi L-[U-^{14}C]tyrosine (batch no. 43)*; for rabbit no. 110, 1·74 mCi L-[side-chain 2,3-^{3}H]tyrosine (batch no. 15)* and 0·105 mCi L-[U-^{14}C]tyrosine (batch no. 46)*)

Rabbit no.	126 (0·49)		115 (0·66)		110 (3·63)	
Form of tyrosine ...	Free	Bound	Free	Bound	Free	Bound
Tissue (Infusate)	(2·43)	—	(3·56)	—	(3·80)	—
Plasma	2·51	2·64	3·61	3·98	3·85	3·85
Liver	2·04	2·65	2·52	3·64	3·02	3·91
Other sites†	2·37–2·56	2·39–2·64	3·49–3·74	3·62–3·70	3·64–3·90	3·53–3·78

* For details, see p. 2 and Table 3.

† The range of values includes those for kidney, brain, heart, diaphragm, muscles (soleus, semimembranosus propius, extensor digitorum longus, tibialis anterior, gluteus longus, longissimus).

Table 6. *Effect of processing of perchloric acid (PCA)-soluble material from liver of a New Zealand White rabbit on ^3H:^{14}C of added radioactively-labelled tyrosine*

(To liver samples from a 1·78 kg rabbit was added 10 μl solution containing 0·3 μCi L-[side-chain 2,3-^{3}H]tyrosine (batch no. 15)* and 0·033 μCi L-[U-^{14}C]tyrosine. The samples were then homogenized directly in 0·5 M-PCA or in water and then treated with 2·5 M-PCA as described on p. 3. The PCA-soluble material was then prepared for isolation of tyrosine by ion-exchange chromatography (see p. 4))

Sample wt (g)	Homogenizing medium	^3H:^{14}C of isolated tyrosine
(Added tyrosine)	—	(2·46)
0·818	Water	2·49
0·699	Water	2·52
0·958	0·5 M-PCA	2·49
0·804	0·5 M-PCA	2·45

* For details, see p. 2 and Table 2.

Table 7. *Rates of protein synthesis in adult New Zealand White rabbits infused with radioactively-labelled tyrosine*

(Animals were infused with 1·2–1·8 mCi L-[side-chain 2,3-^{3}H]tyrosine (batch nos. 15 and 16)* for a 6 h period. Rabbit nos. 83 and 110 also received 88 μCi L-[U-^{14}C]tyrosine (batch nos. 43 and 46)*. Protein fractional synthesis rates ($k_{s(i)}$, $k_{s(p)}$) were calculated using the free tyrosine specific radioactivity of tissue and plasma respectively. Whole-body protein synthesis rate was calculated assuming that rabbit protein contains 40 mg tyrosine/g)

Sex ...	Male				Female					
Rabbit no. ...	83	143	108	94	263	142	110	265	Mean	SD
Body-wt (g)	3020	3605	3615	4260	3315	3360	3630	4135	3618	413
Tyrosine plasma flux (μmol/h)	405	405	584	380	523	514	406	378	449	79
Whole-body protein synthesis rate: g/d	44·0	44·0	63·5	41·3	56·9	55·9	44·2	41·1	48·9	8·6
g/kg body-wt per d	14·6	12·2	17·6	9·7	17·2	16·6	12·2	9·9	13·8	3·2
Protein fractional synthesis rate (/d)										
Muscle										
$k_{s(i)}$	0·0204	0·0169	0·0177	0·0134	0·0173	0·0208	0·0220	0·0216	0·0188	0·0029
$k_{s(p)}$	0·0141	0·0133	0·0144	0·0090	0·0122	0·0160	0·0147	0·0173	0·0139	0·0025
Liver										
$k_{s(i)}$	0·285	—	—	—	—	—	0·397	—	(0·317)	
$k_{s(p)}$	0·133	0·164	0·194	0·147	0·083	0·163	0·200	0·148	0·154	0·037

There was no significant difference between the male and female groups (t test, $P > 0·1$) for any measurement.

* For details, see p. 2 and Table 2.

was less than 4%. Samples were then immediately homogenized either in water or 0·5 M-PCA and the free amino acid extract prepared by the usual procedure. As can be seen in Table 6 there was no significant difference in $^3H:^{14}C$ of the isolated free tyrosine compared with the added material.

Rates of protein synthesis in adult rabbits

A group of eight adult rabbits (four male and four female) of body-weight greater than 3 kg was infused with [^{3}H]tyrosine, and the fractional-synthesis rates of muscle and liver protein determined from the SR of protein-bound and free tyrosine at the end of the infusion. Fractional rates were calculated from a standard plot based on equation 6, in which ϕ, which defines the kinetics of the free tyrosine SR, was assigned the value of $40/\bar{d}$ from consideration of the results of the biopsy experiments (Figs. 1, 2). For each tissue Table 7 shows $k_{s(i)}$ and $k_{(s)p}$ values, these being the fractional-synthesis rates derived from the free tyrosine SR of the tissue and plasma respectively. The values shown are for mixed muscle from the back and hind limbs and will be representative of skeletal muscle in general.

Liver protein $k_{s(i)}$ values are shown only for two animals which received [^{14}C]tyrosine as well as [^{3}H]tyrosine, and these values were calculated from the corresponding values for [^{14}C]tyrosine. As already discussed the use of L-[side-chain 2,3-^{3}H]tyrosine alone would result in erroneously high estimates for liver protein $k_{s(i)}$. The mean value shown for $k_{s(i)}$ has been calculated, therefore, by multiplying the mean value for $k_{s(p)}$ of the group of rabbits by the average value for $k_{s(i)}:k_{s(p)}$ for the two rabbits receiving [^{14}C]tyrosine. These animals had values for $k_{s(i)}:k_{s(p)}$ of liver protein of 0·47 and 0·51. It should be emphasized, however, that the rate of protein synthesis in liver will be underestimated since much of the plasma protein synthesized by the liver during the infusion will have been exported.

Also shown in Table 7 is the tyrosine plasma flux derived from the plasma free tyrosine SR and the rate of infusion of isotope. The rate of whole-body protein synthesis was calculated from the tyrosine plasma flux (Garlick *et al.* 1973) assuming that rabbit whole-body protein contains 40 mg tyrosine/g (Nicholas, Lobley & Harris, unpublished results).

DISCUSSION

This study was intended to determine whether by infusing [^{3}H]tyrosine at a constant rate into the rabbit for several hours the SR of the free tyrosine pools could be maintained approximately constant, facilitating the calculation of protein synthesis rates.

In rabbits under anaesthesia it has been shown, using the blood-sampling and biopsy techniques, that within 2 h the SR of free tyrosine in plasma and muscle reached plateau values. Owing to technical difficulties it has not yet proved possible to maintain a long-term catheter in the vascular system of the rabbit to determine the interval required to reach plateau in the non-anaesthetized animal.

Perry (1975) did not observe a plateau in the pig after an 8 h infusion of nominally L-[side-chain 2,3-^{3}H]tyrosine, although there was linear incorporation of radioactivity into protein. The commercial preparation used was TRK 282, batch no. 13, which here was shown to contain approximately 300 mg radioactively-labelled D-isomer/g. It is probable that the apparent failure to reach a plateau in the pig was due to accumulation of the D-tyrosine in the free pools of the body fluids, as was found for the rabbit. In support of this, other workers using a constant infusion of L-[U-^{14}C]tyrosine in pigs have been able to demonstrate a plateau for plasma tyrosine SR within 2 h (Garlick, Burk & Swick, 1976).

The term 'muscle free tyrosine' used in this paper is in fact a composite of the free tyrosine in the intracellular and extracellular fluids including residual blood. If it is assumed that

the D-tyrosine is distributed uniformly in blood and extracellular fluid and that these comprise 20–30 % of total muscle volume (Goldspink, 1966) then the difference in SR of muscle free 'total' and 'true L-' tyrosine (Fig. 2) can only be explained by the D-isomer entering the intracellular space. This may provide a novel method of measuring the transport rates for amino acids relative to their rates of catabolism and uptake into protein. Failure to recognize the presence of this contaminant would have resulted not only in the apparent inability to observe plateaux but also would have given gross underestimations of the rates of protein synthesis. For example, Fig. 2 shows that the uncorrected values include a D-isomer contribution of 60 % to the S_i value and this would result in a similar percentage decrease in the calculated $k_{s(i)}$ value.

D-isomer contamination is more likely to be encountered with labelled amino acids prepared chemically as racemic mixtures and then resolved enzymically (perhaps incompletely) than with uniformly ^{14}C-labelled L-amino acids obtained from Chlorella protein hydrolysates. The dipeptide chemical resolution method used in this work is a very simple and convenient method for determining the enantiomeric composition of commercial preparations and biological samples.

The problem of D-isomer contamination was raised by Waterlow & Stephen (1967, 1968) in studies which involved the constant infusion of rats with L-[U-^{14}C]lysine. However, direct evidence for the presence of D-isomer was not presented and it is possible that the substantial levels of non-L-lysine radioactivity in acid-soluble extracts of liver, muscle and plasma represented mainly metabolites of L-lysine.

Since after infusion experiments a considerable fraction of the PCA-soluble radioactivity in the plasma and tissues was present in products of tyrosine catabolism, it was necessary to isolate the amino acid and determine its true SR. The distribution of radioactivity between L-tyrosine and its metabolic products is not uniform throughout the tissues of the body, there being particularly interesting differences between 'red' and 'white' muscles in the labelling of metabolites from L-[side-chain 2,3-^{3}H]tyrosine and L-[U-^{14}C] tyrosine. The nature and site of production of these metabolites has not been investigated. Use of total radioactivity rather than the true tyrosine SR would also give an underestimate of protein synthesis rates. The extent of underestimation (%) would be approximately the same as the contribution (%) of metabolites to the radioactivity of the PCA-soluble material. For completeness the protein-bound tyrosine was also isolated on the amino acid analyser and this had the added advantage of allowing larger amounts of protein (i.e. the tyrosine equivalent) to be measured for radioactivity.

The problem of contamination of L-tyrosine in body fluids by its metabolites and D-tyrosine has also been overcome using a specific enzymic estimation of L-tyrosine SR (Garlick & Marshall, 1972). This method involved the determination of tyramine SR following treatment of acid-soluble extracts with L-tyrosine carboxy-lyase. However, enzymic methods are available for only a few amino acids and their use may preclude the use of amino acids labelled in particular positions. For example, the enzymic method mentioned previously would not be suitable for tyrosine labelled with ^{14}C in the C-1 position, the material of choice for measuring the rate of tyrosine oxidation in vivo (James, Garlick, Sender & Waterlow, 1976). Therefore, whilst enzymic methods may provide a very convenient method of determining SR in certain situations, the methods described in this paper are more generally applicable.

The constant-infusion technique does permit average free amino acid pool SR to be determined reasonably accurately, but calculation of true protein synthesis rates is hampered by the current ambiguity concerning the site of the precursor amino acids. The extent of uncertainty which this ambiguity introduces can be illustrated using the protein fractional-synthesis rates for adult rabbits given in Table 7. Here the values for $k_{s(p)}$;$k_{s(i)}$

Table 8. *Contribution of protein synthesis in muscle and liver to whole-body protein synthesis in adult New Zealand White rabbits*

(Protein synthesis rates ($A_{s(i)}$, $A_{s(p)}$) for a rabbit of body-wt 3·618 kg were calculated from fractional synthesis rates $k_{s(i)}$, $k_{s(p)}$, calculated using the free tyrosine specific radioactivity of tissue and plasma respectively) given in Table 7, using the equation:

$$A_s = k_s \times \text{tissue wt} \times \text{protein content of tissue}^*;$$

the values for $A_{s(i)}$, $A_{s(p)}$ expressed as a percentage of the whole-body protein synthesis rate are shown in parentheses)

Tissue ...	Muscle	Liver
% live wt†	43·3	2·8
Tissue wt:g	1567	101
Protein synthesis rate (g/d)		
$A_{s(i)}$	6·3 (12)	6·8 (13)
$A_{s(p)}$	4·6 (9)	3·3 (7)

* Derived from the estimation of nitrogen content (n 6): 33·98 ± 0·42 mg N/g tissue × 6·25 = 212·4 mg protein/g tissue. The protein content of liver was assumed to be similar.

† Values obtained by carcass dissection (n 3).

are 0·74 and 0·49 for muscle and liver respectively, which indicates a considerable margin of error particularly for liver.

The question of whether, with radioactive incorporation experiments in vivo, the precursor is better represented by the intracellular or extracellular free amino acid SR has received considerable attention in recent years. Hider, Fern & London (1969), van Venrooij, Poort, Kramer & Jansen (1972) and Ilan & Singer (1975) have presented evidence that the labelled amino acids are incorporated into protein without first equilibrating with the intracellular pool, whereas this is contrary to the findings of Fern & Garlick (1974), Mowbray & Last (1974) and Li, Fulks & Goldberg (1973). These diverse results were obtained by different methods and using different amino acids. This latter point may be important as the fractional entry and turnover rates of individual free amino acids of the tissues (see Banos, Daniel, Moorhouse & Pratt, 1973) vary widely.

In dual-label experiments it was found that $^3H:^{14}C$ in liver and plasma protein was the same for the plasma free tyrosine and was higher than that for the free tyrosine of liver. We believe that the decrease in $^3H:^{14}C$ for the free tyrosine of liver was due to loss of the ^3H attached to C-2 as a result of transamination, which is a reversible process. L-tyrosine aminotransferase (*EC* 2.6.1.5) is present in high activity in liver, and for rabbit no. 115 (Table 5) it can be calculated, after correction for residual blood contamination, that nearly 100% of the ^3H atoms at position C-2 had been lost. The anomaly of $^3H:^{14}C$ for liver appears to indicate that the homogenate (mainly intracellular) free amino acid is not representative of the precursor for protein synthesis in this tissue. In addition, as the value for plasma free tyrosine $^3H:^{14}C$ was close to that of the infusate, this suggests that although the transaminated tyrosine dominated the liver homogenate pool, it made little contribution to the circulating plasma amino acid.

Although the values for liver and plasma protein $^3H:^{14}C$ were similar to that for the free tyrosine of plasma it need not necessarily follow that the SR of the precursor is close to that of plasma tyrosine. While the results indicate that the tyrosine precursor pool in liver cells does not exchange rapidly with the main intracellular tyrosine, its SR may become less than that of the plasma amino acid as a result of dilution with unlabelled tyrosine from protein breakdown.

Until the controversy concerning the precursor is settled, probably by comparing the SR

of the free amino acids from the body pools with those of the amino-acyl-tRNA or the nascent polypeptide chains, it can only be postulated that the fractional rates of synthesis of tissue proteins are probably within the range given by $k_{s(t)}$ and $k_{s(p)}$.

The value for $k_{s(t)}$ for mixed skeletal muscle protein from adult rabbits was 0·0188/d, which is lower than the value of 0·049 for adult rats (Millward, Garlick, Stewart, Nnanyelugo & Waterlow, 1975) but comparable to 0·018 for mature wethers (Buttery, Beckerton, Mitchell, Davies & Annison, 1975).

Although in terms of fractional rates muscle is much less active in synthesizing protein than liver, the large mass of muscle relative to liver leads to the conclusion that these tissues make a comparable contribution to the whole-body protein synthesis in the adult rabbit (Table 8). The $A_{s(t)}$ value for muscle in these rabbits was 6·1 g/d or 12 % of the whole-body protein synthesis rate. In the adult rat, muscle accounted for 16 % of the total protein synthesis (calculated from Millward, Garlick, James, Sender & Waterlow, 1975; Millward, Garlick, Stewart *et al.* 1975) but, interestingly, in larger species such as adult man (Halliday & McKeran, 1975) and sheep (Buttery *et al.* 1975) this tissue was found to synthesize more than half the total.

These calculations are dependent clearly on the accuracy of the measurements for individual tissues and the whole body. The assumptions implicit in the calculation of whole-body protein synthesis rates from tyrosine plasma flux measurements have been discussed by James *et al.* (1976). The rabbits in this study were found to have a mean whole-body protein synthesis rate, based on tyrosine plasma flux, of 13·8 g/kg per d. Using the same technique corresponding values (g/kg per d) for other species were 27·7 for adult rats (calculated from Millward, Garlick, James *et al.* 1975) and 5·7 for adult man (Garlick *et al.* 1976).

Currently there is much interest in the energy cost of protein synthesis in animals. The metabolic rate of adult rabbits under similar conditions to those applying during the infusion periods in this work is 385 kJ/kg$^{0.75}$ per d (J. D. Pullar, personal communication). If it is assumed that the synthesis of 1 g protein requires the expenditure of 4·46 kJ (Webster, 1976) then protein synthesis accounts for about 22 % of the total energy requirement of the adult rabbit.

The authors wish to thank Mr G. Wenham for expert advice and supervision during the biopsy experiments, Mr J. Digweed for constructing the timed-flow valve, and Mr W. Boyne and his staff for computer analysis of theoretical and practical information. They also wish to acknowledge the excellent technical expertise of Mr G. Milne, Mrs J. M. Lovie and Mrs V. Milne.

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Printed in Great Britain