Identification in Skeletal Muscle of a Distinct Extracellular Pool of Amino Acids, and its Role in Protein Synthesis

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1. The kinetics of radioactive labelling of extra- and intra-cellular amino acid pools and protein of the extensor digitorum longus muscle were studied after incubations with radioactive amino acids *in vitro*. 2. The results indicated that an extracellular pool could be defined, the contents of which were different from those of the incubation medium. 3. It was concluded that amino acids from the extracellular pool, as defined in this study, were incorporated directly into protein.

It has been suggested that the protein of rat skeletal muscle is synthesized either directly from extracellular amino acids or from a pool in rapid equilibrium with the extracellular space (Hider, Fern & London, 1969). Since rapid efflux of amino acids from the intracellular pool has been reported during incubations *in vitro* (Hider, *et al.* 1969) it is possible that, in studies involving radioactive tracers, the specific radioactivities of amino acids contained in the extracellular pool may differ from those of the incubation medium. If this hypothesis is correct it is important to sample the extracellular fluid directly, so as to avoid erroneous assumptions that might lead to a wrong interpretation of data obtained in kinetic studies on protein synthesis and amino acid transport.

To study the composition of the extracellular fluid we have employed a technique previously used by Guidotti, Ragotti & Rossi (1964) and Kostyo (1968) who assumed that during low temperature incubations, extracellular amino acids were washed from the tissue preparations while the intracellular amino acids remained unchanged; these workers used this procedure to avoid the necessity of correcting for radioactive amino acid trapped in extracellular fluid during calculations of distribution ratios. We have developed and adapted this concept for the direct measurement of amino acid concentration and specific radioactivity in the extracellular and intracellular pools of muscle. These parameters have been related to the rate of incorporation of radioactive amino acids into protein. In this way we have attempted to define the precursor pool of amino acid utilized for protein synthesis.

The studies were carried out with the extensor

digitorum longus muscle of the rat, which is well suited to this type of experiment since it can be dissected free of all other muscles and yet be preserved with its fibres intact.

MATERIALS

[U-¹⁴C]Leucine (10 mCi/mmol), [4,5-³H]leucine (20 mCi/mmol), [U-¹⁴C]glycine (5 mCi/mmol), [G-³H]glycine (250 mCi/mmol), D-[1-¹⁴C]mannitol (17 mCi/mmol), [U-¹⁴C]sorbitol (6 mCi/mmol), [carboxy-¹⁴C]inulin (14 mCi/mmol) and [³⁵S]sulphate (carrier free) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

METHODS

Male Sprague-Dawley-derived white rats (Animal Supplies Ltd., Knebworth, Herts., U.K.) weighing 50-60 g were starved for 18 h before decapitation. The extensor digitorum longus muscles (25-30 mg) were excised. Care was taken not to damage the fibres during this procedure. After a knot had been tied on the tendons at the distal end, the muscle was secured across a frame by the aid of this knot and the osseous insertion of the proximal end (Fig. 1). The frames were made from paper clips. The angle α was adjusted so as to maintain the natural length of the muscle. Each muscle was then placed in a conical flask containing 2 ml of Krebs-Ringer bicarbonate buffer, pH 7.4 (Umbreit, Burris & Stauffer, 1949) and gassed with O₂ + CO₂ (95:5). During transferring periods (1 min), the muscle and its frame were removed from one medium, washed twice in Krebs-Ringer bicarbonate buffer, pH 7.4, and added to a second medium, which had previously been preincubated for 1 min at 37°C. The muscle was then gassed with O₂ + CO₂ (95:5).

After incubation by shaking in a water bath at 37°C, the muscle was dipped twice in Krebs-Ringer bicarbonate buffer, pH 7.4, at 2°C and second knots were tied on both tendons inside the frame (X, Fig. 1). The frame angle α was decreased and the newly tied knots were removed to the outside of the frame. The connective tissue between

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Fig. 1. Frame used for maintaining the natural length of extensor digitorum longus muscle.

the bone and the new knot at one end and the old and new knots at the other end of the muscle were then cut away. This avoided contamination of the subsequent incubation medium by the appreciable amount of amino acid carried over from the first incubation medium on the bone and the thread. The angle α was readjusted to maintain the natural length of the muscle and the frame was placed in Krebs-Ringer bicarbonate buffer, pH 7.4 (2 ml; 2°C), and incubated at 2°C. This transferring period lasted 1 min. After incubation, the muscle was dipped in Krebs-Ringer bicarbonate buffer, pH 7.4, at 2°C, removed from the frame and blotted with filter paper.

To extract the intracellular solutes, the muscle was either heated in 2 ml of boiling water for 6 min, or, in the studies of protein synthesis, homogenized with 2 ml of 5% (w/w) sulphosalicylic acid in an all-glass Potter-Elvehjem homogenizer. These homogenates were then poured into centrifuge tubes, centrifuged for 1 min at 400g and the supernatants were decanted.

Preparation of samples for scintillation counting. A 5 ml portion of scintillator consisting of 0.2% (w/v) 2,5-bis-5'-tert.-butylbenzoxazol-2-ylthiophen (BBOT) in Triton-toluene-water (5:10:1, by vol.) was added to 0.2 ml of the incubation medium or 0.2 ml of tissue extract. The counts of radioactivity were corrected for quenching by internal standards. Efficiency of radioactivity counting was: ^3H , 41%; ^{14}C , 32%. After a 90 min incubation with [^{14}C]glycine at least 95% of the water-soluble radioactivity in the tissue and medium was recovered as unmetabolized glycine, when subjected to paper chromatography in butan-1-ol-acetic acid-water (12:5:3, by vol.); the corresponding value with [^{14}C]leucine was 80–85%.

Determination of extracellular space. Inulin, mannitol, sorbitol and SO_4^{2-} were used as extracellular markers. The distribution of marker radioactivity between the tissue and the medium reached an equilibrium value, after approx. 120 min for all markers. The inulin space was 17% of the wet weight of the tissue; the mannitol, sorbitol and SO_4^{2-} spaces were all approx. 30% at 37°C and 20% at 2°C. Lower values for inulin spaces have been noted by Ogston & Phelps (1961) and Goodford & Leach (1966). Total water comprised 78% of the tissue weight.

Viability of preparation. After a 90 min incubation in Krebs-Ringer bicarbonate buffer, pH 7.4, the intracellular concentration of potassium for a 25–30 mg muscle fell from 127 to 120 mM. The weight increase after 90 min of incubation was approximately 3%.

Determination of protein. The method of preparation

of radioactively labelled protein for scintillation counting was designed to minimize errors involved in dissolving a known weight of precipitated protein in Hyamine (Steinberg, Vaughan, Anfinsen, Gorry & Logan, 1958).

The insoluble fraction resulting from homogenization of the muscle was resuspended in 2 ml of 5% (w/w) sulphosalicylic acid and heated at 90°C for 15 min. After centrifugation for 1 min at 400g the residue was extracted with acetone (2 ml), acetone-chloroform (1:1, v/v; 2 ml), acetone (2 ml) and water (2 ml). The preparation was centrifuged (1 min at 400g) between each extraction and finally dissolved in 0.1 M-NaOH (1 ml). After centrifugation for 1 min at 400g the residue was discarded. A 0.2 ml sample of the solution was taken for determination of protein content by the method of Lowry, Rosebrough, Farr & Randall (1951), and 0.5 ml was adsorbed on chromatography paper (Whatman 1MM; 10 cm $\times$ 1 cm) that had previously been coiled in a counting vial. The water on the paper was then evaporated at 0.1 mm Hg. When the chromatography paper was dry, 10 ml of scintillator consisting of 0.3% (w/v) 2,5-bis-5'-tert.-butylbenzoxazol-2-ylthiophen in toluene was added. Efficiency of counting radioactivity in protein was: ^3H , 11%; ^{14}C , 40%.

The amount of Lowry-positive material and specific radioactivity (counts/5 min per μg of protein) appearing in each aqueous fraction during the protein preparation are indicated in Table 1. The results show that 5% sulphosalicylic acid is an efficient protein precipitant. The highest specific radioactivity is found in the first two fractions and is largely due to the radioactivity associated with the intracellular amino acids. The specific radioactivity of the protein fraction that was soluble in cold 0.1 M-NaOH was the same as that of the fraction obtained when the residue from the first alkali treatment was dissolved in hot 0.4 M-NaOH. Consequently only the first alkali fraction was used for determination of protein specific radioactivity.

Homogenization in excess of unlabelled glycine (5 mM) or leucine (5 mM) made no difference to the number of counts appearing in protein, when the tissue had been incubated with 500 μM -[^{14}C]glycine or 100 μM -[^{14}C]leucine. Further, in the presence of puromycin (100 $\mu\text{g}/\text{ml}$) and cycloheximide (100 $\mu\text{g}/\text{ml}$), the incorporation of both [^{14}C]glycine or [^{14}C]leucine into the first alkali fraction was decreased to 2–3% of that normally observed. Thus the radioactivity measured by this method was not artificially high due to non-specific binding of ^{14}C -labelled amino acids to protein.

Hydrolysis of ^{14}C -labelled protein. Protein obtained from muscles previously incubated in [^{14}C]glycine was extracted as described above. Portions (0.15 ml) of the resulting protein solution (0.1 M-NaOH) were added to 0.5 ml of redistilled 6 M-HCl and sealed in tubes under N_2 . The samples were hydrolysed for 15, 30 and 45 h at 105°C. Samples (0.2 ml) of the hydrolysates were analysed by automated amino acid chromatography. Fractions of the effluent were collected for scintillation counting. Over 90% of the ^{14}C radioactivity was associated with glycine. Samples (0.2 ml) of the hydrolysates were also counted for ^{14}C radioactivity. Corrections (Tristram, 1967) were made for the comparatively slow rate of hydrolysis of leucine-containing peptides and for the slight decomposition of glycine under these conditions.

Table 1. Amount and specific radioactivity of Lowry-positive material of the fractions obtained during the extraction of protein

After incubation for 60 min of an extensor digitorum longus muscle (30 mg) in Krebs–Ringer bicarbonate buffer containing $100\text{ }\mu\text{M}$ - ^{14}C leucine, the muscle was homogenized in 5% (w/w) sulphosalicylic acid. The results are derived from several muscles. Sulphosalicylic acid was removed from the first two fractions for the Lowry test by treatment with Dowex 2 resin. 500 units of Lowry-positive material = 1 mg of extensor digitorum longus muscle protein.

Fractions obtained from muscle	Amount of Lowry-positive material (units)	Sp. radioactivity (counts/5 min per μg of protein)
Supernatant from original homogenization	14	92
Hot 5% sulphosalicylic acid extract	70	2.2
Water extract	80	0.9
Cold 0.1 M-NaOH extract (this fraction is used for protein determination)	2050	17.6
Hot 0.4 M-NaOH extract	480	16.8

Preparation of samples for amino acid analysis. Samples of the media corresponding to each incubation condition were combined and after the addition of norleucine ($0.12\text{ }\mu\text{mol}$), the solutions were stored at -20°C . Tissue extracts obtained by boiling in water were pooled and stored in an identical manner. Before chromatography these samples were acidified with constant-boiling HCl (0.5 ml), concentrated by freeze-drying and adjusted to pH 2.8. The supernatants that were obtained after homogenization in sulphosalicylic acid were also pooled and after the addition of norleucine were passed through a Dowex 2 (Cl^- form) column to remove the sulphosalicylic acid. The eluates were concentrated by freeze-drying and adjusted to pH 2.8.

The samples so obtained were analysed for amino acid content with a Technicon Amino Acid Autoanalyzer, by using a flow rate of 1 ml/min and the following autograd system: chamber 1, pH 2.87 (10%, v/v, methanol); chamber 2, pH 2.87; chambers 3, 4 and 5, pH 3.85 (100 ml of pH 2.87 buffer and 80 ml of pH 5.00 buffer). Each autograd chamber contained 60 ml of buffer. The buffers were prepared as described by Kacser (1966). As reported by Block, Markous & Steele (1966) higher values for the intracellular amino acid concentrations were obtained when sulphosalicylic acid was used as a precipitant.

The specific radioactivities of amino acids in the extracellular, intracellular and medium pools were obtained by dividing the radioactivity by the appropriate pool amino acid concentration.

Experiments designed to validate the determination of extracellular amino acids. By following the examples of Guidotti *et al.* (1964) and Kostyo (1968), incubations at 2°C were used to minimize the transmembrane flux of amino acids.

To find the most suitable period of incubation at 2°C for the removal of as much of the extracellular amino acid from the tissue with minimal contamination by intracellular amino acid, the ^{14}C glycine ($500\text{ }\mu\text{M}$)/ ^{14}C leucine ($100\text{ }\mu\text{M}$) distribution ratio between the intracellular pool and medium was measured by two methods. Two muscles from one animal were incubated at 37°C under identical conditions. One muscle was subsequently incubated at 2°C in a second medium of Krebs–Ringer bicarbonate

buffer, pH 7.4, and the other was used as a control and had no second incubation. The distribution ratios were calculated for the controls, by using an extracellular space correction of 30% but the distribution ratios of muscles that were incubated for a second time were calculated on the basis that there was no radioactivity in the extracellular pool after incubation at 2°C , so that no extracellular space correction need be applied. (Guidotti *et al.* 1964; Kostyo, 1968).

In further experiments, muscles were preincubated with $500\text{ }\mu\text{M}$ - ^3H glycine at 37°C , followed by a 15 min incubation with $500\text{ }\mu\text{M}$ - ^{14}C glycine at 2°C . The contents of the extracellular spaces were then studied by placing the muscles in a third Krebs–Ringer bicarbonate medium, pH 7.4, at 2°C , from which amino acid was absent. If transmembrane amino acid flux is low at 2°C , then, after the first two incubations the intracellular pool would be predominantly labelled with ^3H radioactivity and the extracellular pool with ^{14}C radioactivity. In the third incubation, if the above assumption holds, then ^{14}C radioactivity should appear in the medium rapidly and the tissue ^{14}C space should drop to a low value, whereas no ^3H radioactivity should appear in the medium and the tissue ^3H space should remain constant.

So as to establish that inhibition of efflux from the intracellular pool is rapid when the tissue is cooled to 2°C and thus not yielding an artificially high value for the amount of extracellular amino acid, muscles were preincubated in $500\text{ }\mu\text{M}$ - ^{12}C glycine at 37°C for 60 min and then incubated in either 5 or $500\text{ }\mu\text{M}$ - ^3H glycine at 2°C for various time intervals. The muscle ^3H spaces were determined after the second incubation.

RESULTS

Determination of extracellular amino acids. The results shown in Table 2 indicate that for incubation periods less than 15 min at 2°C , no appreciable loss to the medium of intracellular glycine and leucine occurred, when the two methods for calculating the distribution ratio were compared.

Fig. 2 shows that there was an initially rapid

Table 2. Relationship of intracellular ^{14}C -labelled amino acid content with the length of incubations at 2°C

Amount of intracellular [^{14}C]glycine and [^{14}C]leucine (shown as the ratio of distribution of ^{14}C radioactivity between tissue and medium) was measured either after an incubation at 2°C , or without an incubation at 2°C , but corrected for the extracellular space (30% of tissue wet weight). The extracellular correction assumed that the extracellular concentration of radioactivity was the same as that of the medium. The tissue was preloaded with [^{14}C]glycine ($500\mu\text{M}$) or [^{14}C]leucine ($100\mu\text{M}$) by incubating in 2 ml of Krebs-Ringer bicarbonate buffer for 60 min at 37°C . The values are means of at least three determinations.

Incubation time at 2°C (min)	Distribution ratio of ^{14}C radioactivity corrected for a 30% extracellular space (control, no incubation at 2°C)		Distribution ratio of ^{14}C radioactivity after efflux at 2°C	
	[^{14}C]Glycine	[^{14}C]Leucine	[^{14}C]Glycine	[^{14}C]Leucine
15	2.2	1.1	2.3	1.0
30	2.3	1.2	2.1	0.9
60	2.2	1.1	1.9	0.7
90	2.1	1.1	1.8	0.6

Fig. 2. Efflux of [^{14}C]- and [^3H]-glycine from extensor digitorum longus muscle, and the variation of tissue spaces of both isotopes with time, represented as a percentage of the tissue wet weight. Each muscle was preloaded at 37°C for 30 min with $500\mu\text{M}$ -[^3H]glycine in Krebs-Ringer bicarbonate buffer followed by an incubation in $500\mu\text{M}$ -[^{14}C]glycine at 2°C for 15 min and then incubated at 2°C in Krebs-Ringer bicarbonate buffer (2 ml) containing no glycine. The specific radioactivities of the medium [^3H]- and [^{14}C]-glycine were adjusted to give the same c.p.s./ml. ●, Effluxed [^{14}C]glycine; ○, effluxed [^3H]glycine; ■, tissue [^{14}C]glycine; □, tissue [^3H]glycine.

rate of efflux of [^{14}C]glycine at 2°C from muscles preloaded at 37°C for 30 min first with [^3H]glycine and then subsequently at 2°C for 51 min with

[^{14}C]glycine. In contrast [^3H]glycine appeared only slowly in the third incubation medium. The tissue spaces of the two radioactive labels were followed in the same experiment. The ^3H space remained nearly constant whereas the ^{14}C space fell rapidly during the first 15 min of the third incubation and then remained constant.

The rate of accumulation of [^3H]glycine at 2°C after preloading the muscle with [^{14}C]glycine at 37°C was independent of glycine concentration in the second medium (Fig. 3).

Thus, it would appear that transmembrane amino acid flux is decreased to a minimum at 2°C (Table 2 and Fig. 2) and that this inhibition is induced rapidly (Fig. 3).

The change in concentration and specific radioactivity of glycine accumulating in Krebs-Ringer bicarbonate medium, pH 7.4, at 2°C was followed for 60 min after preincubation in [^{14}C]glycine ($500\mu\text{M}$; $0.6\text{mCi}/\text{mmol}$) at 37°C (Fig. 4). The appearance of [^{14}C]glycine into the medium was rapid over the first 15 min, it then slowed and continued at a constant rate for the remainder of the experiment. The specific radioactivity of the effluxed [^{14}C]glycine fell slowly during the incubation. Similar curves were obtained for leucine after preincubation with $100\mu\text{M}$ -[^{14}C]leucine at 37°C .

As the transmembrane amino acid flux is minimal at 2°C , it is valid to extrapolate to zero time both the linear rate of appearance of total glycine and the linear rate of change of the specific radioactivity of the glycine present in the 2°C incubation medium (Fig. 4). The values so obtained for a 30 mg muscle (0.011mmol and $0.2\text{mCi}/\text{mmol}$; Fig. 4) were attributed to the amount and specific radioactivity of glycine in the extracellular muscle spaces at the end of the incubations at 37°C . The corresponding values for extracellular [^{14}C]leucine after an initial

Fig. 3. Rate of accumulation of [^3H]glycine by extensor digitorum longus muscle at 2°C after preloading the muscle with $500\mu\text{M}$ -[^{12}C]glycine for 60 min in Krebs-Ringer bicarbonate buffer at 37°C . ●, $500\mu\text{M}$ -[^3H]glycine, ○, $5\mu\text{M}$ -[^3H]glycine.

Fig. 4. Efflux of glycine from extensor digitorum longus muscle and change of specific radioactivity of effluxed [^{14}C]glycine with time. Each muscle, initially preloaded at 37°C for 60 min with $500\mu\text{M}$ -[^{14}C]glycine in Krebs-Ringer bicarbonate buffer, was subsequently incubated in Krebs-Ringer bicarbonate buffer (2 ml) at 2°C . ○, Glycine specific radioactivity; □, amount of glycine in incubation medium.

incubation at 37°C with [^{14}C]leucine ($100\mu\text{M}$; 1.7mCi/mmol) for 60 min were 0.0012mmol and 0.53mCi/mmol . If a value of 30% of the wet tissue weight is adopted for the extracellular pool, the concentration of glycine and leucine in this pool, after a 60 min incubation in either $500\mu\text{M}$ -glycine or $100\mu\text{M}$ -leucine, would be 1.2 and 0.13mM respectively. Thus the distribution ratio of glycine between a homogeneous extracellular space and the medium would be 2.4 and for leucine 1.3.

After incubation of an extensor digitorum longus muscle for 60 min in Krebs-Ringer bicarbonate solution containing $500\mu\text{M}$ -glycine at 37°C , the

Fig. 5. Time-course of the specific radioactivity of [^{14}C]glycine in protein and the intracellular and extracellular pools and the total incorporation of [^{14}C]glycine into protein of extensor digitorum longus muscle. The muscle was incubated in Krebs-Ringer bicarbonate solution (2 ml) containing glycine ($500\mu\text{M}$). □, Extracellular [^{14}C]glycine specific radioactivity; △, intracellular [^{14}C]glycine specific radioactivity; ○, protein [^{14}C]glycine specific radioactivity; ○, total quantity of [^{14}C]glycine incorporated into protein.

total amount of glycine present in the intracellular pool of a 30 mg muscle was $0.14\mu\text{mol}$. From Fig. 4 it can be extrapolated that the glycine (approx. 2nmol) that appeared in the medium after 15 min originated from the intracellular pool; this represents approx. 1.5% of the total free intracellular glycine. For leucine the corresponding value is 2.5%.

Incorporation of amino acids into protein. By maintaining the natural length of the muscle, the coefficient of variation of the rate of protein synthesis as measured by incorporation of [^{14}C]glycine was 5.5%. This is similar to that reported by Buresova, Gutmann & Klicpera (1969), when the muscles were stretched under a known controlled tension.

The time-course for both the incorporation of [^{14}C]glycine and [^{14}C]leucine into protein and the variations of specific radioactivity of the intra- and extra-cellular pools of the respective amino acids are shown in Figs. 5 and 6. The rate of incorporation of [^{14}C]glycine becomes constant between 45 and 60 min (Fig. 5); this corresponds to the time at which the specific radioactivity of the glycine extracellular pool approaches a constant value. The specific radioactivity of the intracellular pool

Fig. 6. Time-course of the specific radioactivity of [¹⁴C]leucine in the intracellular and the extracellular pools and the total incorporation of [¹⁴C]leucine into protein of extensor digitorum longus muscle. The muscle was incubated in Krebs-Ringer bicarbonate solution (2ml) containing leucine (100 μM). ■, Extracellular [¹⁴C]leucine specific radioactivity; ▲, intracellular [¹⁴C]leucine specific radioactivity; ●, total quantity of [¹⁴C]leucine incorporated into protein.

continues to increase throughout the 90min incubation. The specific radioactivity of glycine in the protein increases in a similar manner to the time-course of the incorporation of [¹⁴C]glycine into protein.

By contrast, the rate of incorporation of [¹⁴C]leucine into protein occurs at a linear rate almost from the start of the experiment (Fig. 6). The specific radioactivity of extracellular leucine attains in less than 15min an equilibrium value that is maintained during the 90min incubation. The intracellular leucine specific radioactivity increases at a slower rate than that of the extracellular leucine. The distribution ratio of ¹⁴C radioactivity at 90min between the medium and intracellular pool was 2.5 for glycine and 1.1 for leucine.

The rates of incorporation of [¹⁴C]glycine into protein after 60min incubation in Krebs-Ringer bicarbonate solution were determined by measuring the amount of [¹⁴C]glycine incorporated into protein after 50, 60 and 70min and related to the specific radioactivities of the extracellular and intracellular

Fig. 7. Relationship between the rate of incorporation of [¹⁴C]glycine into protein and the specific radioactivity of extracellular and intracellular [¹⁴C]glycine, obtained at various medium concentrations of glycine (0.02–10 mM) after a 60min incubation. The tissues were incubated in Krebs-Ringer bicarbonate solution at 37°C, for 50, 60 and 70 min. The rate of incorporation of [¹⁴C]glycine into protein at 60 min was extrapolated from the values of the total [¹⁴C]glycine incorporated into protein after 50, 60 and 70 min. ○, Intracellular glycine specific radioactivity; ■, extracellular glycine specific radioactivity.

[¹⁴C]glycine after 60min (Fig. 7). Although there was a linear relationship between the rate of incorporation of [¹⁴C]glycine into protein and the specific radioactivity of this amino acid in the extracellular pool, no such relationship was observed with the intracellular pool (Fig. 7). To establish that the rate of protein synthesis was not altered by a change in the medium glycine concentration, muscles were incubated in various concentrations of [¹⁴C]glycine together with a single concentration of [³H]leucine. The results shown in Table 3 indicate that the amount of ³H radioactivity incorporated into protein remains constant irrespective of the glycine concentration.

The time-courses for all the parameters shown in Figs. 5 and 6 were also followed for glycine and leucine during a 45min incubation in either 500 μM-¹²C]glycine or 100 μM-¹²C]leucine after an initial 45min incubation in the corresponding ¹⁴C-labelled amino acids. The rate of incorporation of ¹⁴C

Table 3. *Effect of variation of the medium glycine concentration on the rate of incorporation of [³H]leucine into protein*

Extensor digitorum longus muscles were incubated for 60 min in Krebs–Ringer bicarbonate solution (2 ml) containing [¹⁴C]glycine (5.00–0.02 mM) and [³H]leucine (100 μM). The total incorporations of [¹⁴C]glycine and [³H]leucine into protein are shown. The values are means of six determinations.

Concn. of glycine in the medium (mM)	10 ^{−4} × Incorporation of [¹⁴ C]glycine (μCi/mg of protein)	10 ^{−2} × Incorporation of [³ H]leucine (μCi/mg of protein)
5	1.5	1.2
2	2.2	1.25
1	2.5	1.05
0.1	4.4	1.1
0.01	5.4	1.15

Fig. 8. Time-course of the incorporation of [¹⁴C]glycine and [¹⁴C]leucine during a sequential incubation of tissue in Krebs–Ringer bicarbonate buffer (2 ml). The initial incubation (A) was for 45 min in medium containing [¹⁴C]glycine (500 μM) or [¹⁴C]leucine (100 μM) and the second (B) was for 45 min in medium containing [¹²C]glycine (500 μM) or [¹²C]leucine (100 μM). ■ and ○, Control incubations, when the second incubation (B) contained either [¹⁴C]glycine (500 μM) or [¹⁴C]leucine (100 μM). ■, Incorporation of leucine; ○, incorporation of glycine.

radioactivity into protein is shown in Fig. 8. After the initial 45 min, there was a sharp decrease in the rate of incorporation of radioactivity into protein; this was more marked with leucine than with glycine. Fig. 8 also shows that virtually all the [¹⁴C]leucine that appeared in protein during the second incubation was incorporated in the first 15 min of this incubation. The apparent decrease in the amount of [¹⁴C]leucine in protein during the

Fig. 9. Time-course of the specific radioactivities of the intracellular and extracellular [¹⁴C]leucine and [¹⁴C]glycine during the second half of a sequential incubation of tissue in Krebs–Ringer bicarbonate solution (2 ml). This time-course corresponds to that of (B) in Fig. 8. The muscle was incubated in ¹²C-labelled amino acid after having been previously incubated for 45 min in ¹⁴C-labelled amino acid. ■, Extracellular leucine specific radioactivity; □, extracellular glycine specific radioactivity; ▲, intracellular leucine specific radioactivity; △, intracellular glycine specific radioactivity.

second incubation was not statistically significant. For glycine, the rate of protein synthesis after the first 15 min in the second incubation was 18% of that when the incubation was continued in the presence of [¹⁴C]glycine (Fig. 8). The rate of decrease of the specific radioactivity of the intra- and extra-cellular ¹⁴C-labelled amino acids during these

Table 4. *Rate of incorporation of ^{14}C - and ($^{14}\text{C} + ^{12}\text{C}$)-labelled extensor digitorum longus protein*

The estimations are based on the assumption that protein is synthesized from the extracellular amino acid pool, and utilize the results given in Figs. 5 and 6. When muscles were incubated with [^{14}C]leucine (100 μM , 1.7 mCi/mmol) at 37°C, the steady-state specific radioactivity of [^{14}C]leucine in the extracellular pool was 0.53 mCi/mmol, and the rate of incorporation of [^{14}C]leucine into protein was 2×10^{-5} μCi per mg of protein/min. Thus the velocity of leucine entering protein

$$= \frac{\text{rate of incorporation of } [^{14}\text{C}]\text{leucine into protein}}{\text{sp. radioactivity of } [^{14}\text{C}]\text{leucine in precursor pool}}$$

$$= 2.0 \times \frac{10^{-5}}{0.53} = 3.8 \times 10^{-5} \mu\text{mol/min per mg of protein}$$

For [^{14}C]glycine an assumed value of 0.19 mCi/mmol (Fig. 1) was used for the calculation.

	Rate of incorporation of [^{14}C]amino acids at 60 min ($\mu\text{Ci}/\text{min}$ per mg of protein)	Rate of amino acid incorporation ($\mu\text{mol}/\text{min}$ per mg of protein)	Amount of amino acid ($\mu\text{mol}/\text{mg}$ of protein)
Glycine	2.9×10^{-6}	1.55×10^{-5}	0.3
Leucine	2.0×10^{-5}	3.80×10^{-5}	0.42

Table 5. *Estimated $t_{\frac{1}{2}}$ for extensor digitorum longus protein*

The half-life for protein synthesis was calculated from the rate of incorporation at 60 min and amount of the amino acid contained in 1 mg of protein. Both these quantities are given in Table 4.

$$\text{Protein } t_{\frac{1}{2}} = \frac{\text{amount of amino acid contained in 1.0 mg of protein}}{\text{rate of incorporation of amino acid into protein}} \times 0.5$$

$$\text{For leucine } t_{\frac{1}{2}} = \frac{0.21}{3.8 \times 10^{-5}} \text{ min} = \frac{21}{3.8 \times 1.44} = 3.9 \text{ days}$$

By using the assumption that protein forms 16% of the wet tissue wt. (Mitchell, Hamilton, Steggerda & Bean, 1945), the rate of amino acid incorporation is also represented as a percentage of the intracellular amino acid pool. For leucine $3.8 \times 10^{-5} \times 60 \mu\text{mol}$ are incorporated/h per mg of protein (Table 4), therefore for a 30 mg tissue $3.8 \times 6 \times 10^{-4} \times 4.8 = 1.1 \times 10^{-2} \mu\text{mol}/\text{tissue}$ are incorporated into protein every hour. As the intracellular leucine content of a 30 mg muscle, after incubation in 100 μM -leucine for 1 h is $0.6 \times 10^{-2} \mu\text{mol}$, the percentage of total intracellular leucine utilized per 60 min =

$$\frac{1.1 \times 10^{-2}}{0.6 \times 10^{-2}} \times 100 = 180\%$$

For glycine the intracellular content of a 30 mg muscle after incubation in 500 μM -glycine for 1 h is 0.14 μmol ; this value was used in the corresponding calculation involving glycine.

	Estimated $t_{\frac{1}{2}}$ of protein (days)			% of total intracellular pool utilized/60 min assuming the extracellular pool to be precursor
	Assuming the extra- cellular pool to be precursor	Assuming the intra- cellular pool to be precursor	Assuming the incubation medium to be precursor	
Glycine	6.7	2.1	21	3.5
Leucine	3.9	3.7	14.5	180

double-incubation experiments was also followed (Fig. 9). There was an extremely rapid decrease in the specific radioactivity of the extracellular pool but a slower decrease of the specific radioactivity of the intracellular pool, particularly for glycine. After 45 min of incubation in unlabelled amino acid,

the specific radioactivity of the intracellular leucine pool had decreased to 15% of the value observed at the beginning of the second incubation. The specific radioactivity of the intracellular glycine pool fell to only 70% of its initial value. For the extracellular pools the corresponding values were

7% for leucine and 16% for glycine (Fig. 9). The main bulk of [^{14}C]glycine and [^{14}C]leucine that was effluxed during the second incubation appeared in the medium during the first 15 min.

Table 4 shows the rate of incorporation of radioactivity into protein after 60 min of incubation in Krebs-Ringer bicarbonate buffer containing either [^{14}C]leucine (100 μM) or [^{14}C]glycine (500 μM). These values were derived from Figs. 5 and 6. Table 4 also shows estimates of the amounts of these two amino acids present in the protein. The rates of incorporation of glycine and leucine have been based on the assumption that protein is synthesized from the extracellular amino acid pool.

The theoretical half-lives as shown in Table 5 were obtained by assuming that protein was synthesized from one of three amino acid pools: the medium, a homogeneous extracellular pool, or a homogeneous intracellular pool. The amount of amino acid incorporated into protein is represented in Table 5 as a percentage of the intracellular amino acid pool. Clearly there is a very rapid turnover of muscle leucine, when compared with that of glycine.

DISCUSSION

The results shown in Table 2 confirm the earlier assumptions of Guidotti *et al.* (1964) and Kostyo (1968) that incubations at 2°C were effective in removing amino acids from the extracellular fluid without contamination by intracellular amino acid. The results displayed in Fig. 2 indicate that at 2°C only a minimal amount of amino acid was lost from the intracellular compartment into the incubation medium. This being so, these conditions were utilized for the determination of the concentration and specific radioactivity of glycine and leucine in the extracellular space of muscle. Thus it was possible, in these experiments, to study the concentrations and specific radioactivities of amino acid in the intra- and extra-cellular compartments and to relate them to the rate of protein synthesis. Indeed, it has proved important to make a separate determination of the contents of the extracellular space for, as Kostyo (1968) pointed out, there is no proof of the generally held assumption that exchange between the constituents of the extracellular pool and of the incubation medium is much more rapid than exchange between intra- and extracellular pools. In fact a 60 min incubation in 500 μM -glycine resulted in an extracellular glycine concentration of 1.2 mM when the extracellular space was taken to be 30% of the wet tissue weight. This gave a glycine distribution ratio of 2.4 between the extracellular fluid and the medium. After incubation in 100 μM -leucine the extracellular concentration was 130 μM and the distribution ratio 1.3. Thus there were clear differences between both the amino acid concentra-

tions and, as evidenced from Figs. 4, 5 and 6, the amino acid specific radioactivities of the extracellular fluid and the incubation medium.

These observations are analogous to other studies showing that, under a variety of experimental conditions, there is an extracellular microenvironment, or 'unstirred layer', that has a different composition from the bulk phase in which the tissue is bathed (Hogben, Tocco, Brodie, & Schanker, 1959; Dainty & House, 1966). Viewed in this light it is now possible to visualize why, when muscle preparations are incubated with a radioactive amino acid, the distribution ratio of amino acid concentrations between the intracellular pool and the medium is greater than that of the radioactivity between these two pools (Guroff & Udenfriend, 1960; Pain & Manchester, 1970). The difference in amino acid specific radioactivity across the plasma membrane would be much less than that existing between the incubation medium and the intracellular pool.

Since an earlier study (Hider *et al.* 1969) had demonstrated that a source of amino acids other than the total free intracellular pool was used for protein synthesis in skeletal muscle, further experiments were performed in an attempt to link the extracellular pool of amino acids, as defined in the present investigation, with amino acid incorporation into protein. The results presented in Fig. 7 shows that for glycine whereas the relationship between intracellular specific radioactivity and the rate of incorporation of ^{14}C radioactivity into protein was complex, there was linearity between the extracellular specific radioactivity and incorporation into protein. Providing changes in extracellular glycine concentration do not affect the protein synthetic rate, and this is apparently the case (Table 3 in Jefferson & Korner, 1969), the evidence suggests that the extracellular pool is the direct source of amino acids for protein synthesis. Further evidence that a homogeneous intracellular pool of glycine is not utilized for incorporation into protein can be adduced from Fig. 8. Although the specific radioactivity of the intracellular glycine fell but little in the second half of the sequential incubation (Fig. 9), the rate of protein synthesis rapidly decreased (Fig. 8). This result confirmed that reported in an earlier communication (Hider *et al.* 1969). Although a similar pattern was found for leucine incorporation (Fig. 8), the rapidity with which this amino acid equilibrated between extra- and intracellular fluid compartments (Figs. 6 and 9) precluded any identification of the precursor pool for the incorporation of this amino acid into protein.

When calculations were made on the basis that the extracellular pool was the amino acid source for protein synthesis, the resulting values for protein t_1 were close to those previously reported (Waterlow & Stephen, 1968; Garlick, 1969). The values

in the present study were obtained with growing rats; consequently the protein t_1 will be slightly lower than that of mature rats, where the rates of protein synthesis and degradation are equal. However, this difference would only be small, as indicated by Garlick (1969) who studied rats within a weight range 80–200 g.

Since the intracellular concentrations of glycine and leucine remain relatively constant throughout 90 min incubation in glycine (500 μ M) or leucine (100 μ M) respectively, there must be a very rapid interchange between some free amino acids and protein due to protein synthesis and catabolism. For example the amount of leucine incorporated into protein during the course of 60 min incubation was equivalent to 180% of that contained in the intracellular pool (Table 5) and Gan & Jeffay (1967) calculated that under steady-state conditions, 30% of the lysine incorporated into rat muscle protein was derived directly from protein catabolism. The rapid turnover of amino acids, particularly those with low intracellular concentrations, would contribute further to the differences between the distribution ratio of amino acid concentrations and radioactivity between the intracellular and incubation medium pools (Guroff & Udenfriend, 1960; Pain & Manchester, 1970).

It is inherently unlikely that the t_1 for protein will be different for each amino acid, as indicated in Table 5. Of the values given in Table 5 those for leucine may approach the true value. This amino acid exchanges rapidly across the cell membrane (Oxender & Christiansen, 1963) with the result that the extra- and intra-cellular pools quickly equilibrate (Figs. 6 and 9). Thus a similar protein t_1 is found when the calculations are based on either the extra- or intra-cellular pools. This contrasted with the situation for the slowly exchanging glycine (Oxender & Christiansen, 1963) where extra- and intra-cellular pools gave protein t_1 values differing from each other and from those based on the leucine results. The results with glycine (Fig. 5 and Table 5) could be interpreted to show that the extracellular fluid may itself be compartmentalized with an inner region whose glycine specific radioactivity lies between that of the total extra- and intra-cellular fluid. If this inner region was the site of the protein precursor pool, a calculation using intermediate glycine values would give a t_1 similar to that calculated from the results with leucine.

Measurement of the extracellular space with various markers at 37 and 2°C (see the Methods section) gave further indication of a heterogeneous extracellular space. These results mirror those of a rigorous study with brain slices, where extracellular compartmentation was also invoked (Cohen, Stampleman & Lajtha, 1970). In skeletal muscle it has been suggested that there are two zones in the extra-

cellular space (Cotlove, 1954; Gould & Rawlinson, 1966) and that interstitial concentration gradients exist (Crone & Garlick, 1970). Although the sarcoplasmic reticulum is not extracellular to large molecules (Page, 1964; Eisenberg & Eisenberg, 1968) unlike the transverse-tubular system (Huxley, 1964; Endo, 1966; Kelly, 1969) it may be extracellular for small molecules and ions (Blinks, 1965; Birks & Davey, 1969) forming part of the sucrose space (Dydynska & Wilkie, 1963).

This work with extracellular markers provides a basis for the present finding with amino acids. A labyrinthine extracellular space could explain how amino acids might penetrate to the sites of acylation without prior equilibration with the intracellular pool; further, it is apparent from the present investigation that the extracellular amino acid pool is distinct from that of the incubation medium. Consequently in studies of this nature it is no longer valid to assume values for extracellular amino acids based on incubation medium concentrations.

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