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Relationship between Intracellular Amino Acids and Protein Synthesis in the Extensor Digitorum Longus Muscle of Rats

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1. The incorporation into protein, and the accumulation into the free amino acid pools, of radioactive L-leucine and glycine was studied in rat extensor digitorum longus muscle. 2. The tissue was incubated first with ^{14}C-labelled and then with ^{3}H-labelled amino acid. 3. The experimental results were consistent with a model based on the premise that the amino acids in protein were incorporated directly from the extracellular pool.

Kipnis and his colleagues have suggested that there is a functional heterogeneity of the intracellular amino acid pool, only a fraction of which is utilized in protein synthesis. This theory is based on the effect of growth hormone on protein synthesis (Reiss & Kipnis, 1959; Kipnis & Reiss, 1960) and on the kinetics of incorporation of tracer into the protein of lymph-node cells and diaphragm (Kipnis, Reiss & Helmreich, 1961). The conclusions arising from this work were later supported by Kostyo (1964), who carried out experiments on the stimulation of protein synthesis by growth hormone, and by Rosenberg, Berman & Segal (1963) from a mathematical analysis of results obtained with kidney-cortex slices.

The inferences drawn by Kipnis *et al.* (1961) were that there was either a small protein-synthetic pool of amino acids distinct from the main intracellular pool, or that amino acids entering the cell become chemically compartmentalized before entering the intracellular water.

Although this concept has been discussed in subsequent publications (Krahl, 1964; Segal, 1964; Manchester, 1965; Harris & Manchester, 1966; Roscoe, Eaton & Chin Choy, 1968), there are no reports on the relative size of the postulated protein-synthetic pool. This point has been investigated in the present study.

Further, the previous work did not distinguish between amino acid incorporated from the intracellular pool and amino acid effluxed from the tissue during the incubation and subsequently utilized directly from the extracellular fluid for protein synthesis. In the present work these alternatives were investigated by incubating the tissue first in medium containing ^{14}C-labelled amino acid and then in medium containing ^{3}H-labelled amino acid. In this way it was possible to test whether the newly synthesized protein was further enriched by ^{14}C during the second part of the incubation and if it was, whether this could be explained by incorporation directly from the intracellular fluid or by prior passage through the incubation medium.

MATERIALS

[U-^{14}C]Leucine (10mc/m-mole), [G-^{3}H]leucine (236mc/m-mole), [U-^{14}C]glycine (5·0mc/m-mole) and [G-^{3}H]glycine (250mc/m-mole) were obtained from The Radiochemical Centre, Amersham, Bucks. [*carboxyl*-^{14}C]Inulin (164mc/g.) was obtained from New England Nuclear Corp., Boston, Mass., U.S.A.

METHODS

Fed male Sprague–Dawley-derived white rats (Animal Supplies Ltd., Knebworth, Herts.) weighing 60–80g. were killed by decapitation. The muscles were excised by cutting the tendons, care being taken not to damage the fibres. Each muscle was stretched on a stainless-steel frame by the aid of treble knots tied on the two tendons. The frames were made from paper clips. By stretching the muscle, the usual weight increase for this preparation during incubation, 15% in 60min., was decreased to less than 5%. One muscle was placed in a conical flask containing Krebs–Ringer bicarbonate buffer (Umbreit, Burris & Stauffer, 1949) that contained no glucose (2ml.) (pH 7·4 unless otherwise stated), and gassed with O_2+CO_2 (95:5). The medium contained either leucine (0·05mM) (^{14}C, 2·1mc/m-mole; ^{3}H, 28mc/m-mole) or glycine (0·3mM) (^{14}C, 0·45mc/m-mole; ^{3}H, 1·3mc/m-mole). The amino acid concentrations chosen were such as to reflect the concentrations in the plasma of the Sprague–Dawley rat.

During transferring periods (1min.), the tissue and its frame was removed from one medium, washed twice in Krebs–Ringer bicarbonate buffer and added to the second medium, which had previously been preincubated for 1min. at 37°. The muscle was then gassed with O_2+CO_2 (95:5). The use of the frame minimized any mechanical damage to the tissue. The tissue, which weighed 35–50mg., was

incubated by shaking in a water bath at 37°. After the incubation the tissue was ground with 2 ml. of ice-cold 10% (w/w) trichloroacetic acid in an all-glass Potter–Elvehjem homogenizer. The homogenates were then poured into centrifuge tubes and spun for 1 min. at 400*g*, and the supernatant was decanted. The resulting insoluble fraction was extracted for protein as described by Manchester (1961) and dissolved in Hyamine (1 ml.) (Steinberg, Vaughan, Anfinsen, Gorry & Logan, 1958) at 55° for 1–2 hr. To the cooled Hyamine solution was added 15 ml. of scintillator made up of 0·01% 1,4-bis-(5-phenyloxazol-2-yl)benzene and 0·457% 2,5-diphenyloxazole in toluene. The supernatant of the original homogenate was extracted with ether twice, and then samples (0·2 ml.) were added to a mixture containing 2·8 ml. of ethanol and 7 ml. of the scintillator. Efficiency for counting radioactivity in protein was: ^{3}H, 4·5%; ^{14}C, 53%; and for counting that in aqueous extracts: ^{3}H, 3·9%; ^{14}C, 42%. All counts were corrected with internal standards.

After incubation at least 95% of the radioactivity in the tissue and medium was recovered as unmetabolized amino acid, when subjected to paper chromatography in butan-1-ol–acetic acid–water (12:5:3, by vol.).

The inulin space was represented as a percentage of the tissue wet weight at the end of the incubation and taken as 24%. Total water comprised 80% of the tissue weight. The distribution ratios were calculated as described by Kipnis & Cori (1957). The net amount of amino acid influxed was expressed as mM by multiplying medium concentration by the distribution ratio. The specific radioactivity of the amino acid either in the intracellular pool or in the medium was obtained by dividing the appropriate radioactivity by the relevant pool concentration.

Each graphical point is the mean of six experimental points. Experiments involving the determination of the time-course of these parameters were performed on the same batch of rats. Each batch was divided into three lots and used on consecutive days.

Preparation of samples for amino acid analysis. Portions (1 ml.) of supernatant from each homogenization, corresponding to the various incubation conditions, were combined to make a total pool of 6 ml. To this pool was added 0·2 ml. of 0·6 mM-norleucine and 4 ml. of picric acid (12·5 g./l.). After mixing and centrifugation the supernatant fractions were stored at −20°. Samples from the incubation media were treated in an identical manner. Extracts were thawed, and picric acid was removed from the combined extract by passage through a Dowex 2 column (Stein & Moore, 1954). The eluates were concentrated under reduced pressure at 40° in a rotary evaporator and adjusted to pH 2·0. The samples were analysed for amino acid content with a Technicon amino acid autoanalyser.

Control incubations with ^{14}C- and ^{3}H-labelled amino acids were made under the same conditions to determine the relative loss of radioactivity of the ^{3}H-labelled amino acids when being transformed from free amino acid to the amide-linked form in protein. Whereas ^{14}C-labelled amino acids are stable under these conditions, ^{3}H-labelled amino acids are probably not. The extent of this lability depends on the percentage of ^{3}H atoms on the α-carbon atom. Renal D-amino acid oxidase facilitates labilization of the α-hydrogen atom of L-amino acids (Evans, Green, Spanner & Waterfield, 1963), and it is possible that the enzymes involved in the activation of amino acids for protein synthesis could have a similar effect. Further, the method of processing proteins for radioactivity counting necessitates dissolving the protein in 0·4M-NaOH. This would promote exchange reactions (Evans, 1966), particularly at the α-carbon atom, which possesses the most labile proton attached to carbon. In glycine, 100% of the ^{3}H is on the α-carbon atom, whereas in generally labelled L-leucine the fraction is about 50% (Evans *et al.* 1963). Therefore proportionally less glycine-bound ^{3}H would be expected to be incorporated into protein. By correlating the specific radioactivities of the two labelled forms of the amino acids in the medium and the corresponding ratio of the ^{14}C, to ^{3}H radioactivity incorporated under the identical conditions of a 30 min. incubation (indicated by arrows in Fig. 4), a correction was made for this labilization. It was deduced that 22% of the [^{3}H]-leucine radioactivity and 37% of the [^{3}H]glycine radioactivity was lost between incubation and dissolving the processed protein in Hyamine. These values were used for the determination of the theoretical graphs (Figs. 6 and 7).

RESULTS

The distribution ratio of labelled glycine in the intracellular pool to that in the medium increased linearly throughout a 60 min. incubation. The uptake of leucine, unlike that of glycine, was very low and after 60 min. the distribution ratio between the intracellular pool and the medium was only 0·3, in contrast with 3·2 for glycine. The intracellular concentration of the two amino acids remained relatively constant throughout the 60 min. incubation (Fig. 1), glycine at 6·4 mM and leucine at 0·3 mM. To attain a steady state of amino acid flux in and out of the intracellular pool it would have been necessary to extend the incubation period. This was not attempted because after the first hour of incubation extensor digitorum longus muscle rapidly lost intracellular amino acids, especially those initially present in high concentrations (Fig. 1), indicating that a marked change was occurring in the tissue during the prolonged incubation periods.

The appearance of labelled leucine in extensor digitorum longus protein proceeded as a linear function. The rate of incorporation of [^{14}C]glycine was much lower than that of [^{14}C]leucine (Fig. 4).

The effect of sequential incubation of the muscle in ^{14}C- and ^{3}H-labelled amino acids was also studied. After an initial incubation of 30 min. in ^{14}C-labelled amino acid the tissue was transferred to a ^{3}H-labelled amino acid medium. The distribution ratios and specific radioactivities of the ^{14}C- and ^{3}H-labelled amino acids obtained from these studies are depicted for glycine and leucine in Figs. 2 and 3. The shaded areas in Fig. 2 represent the uptake of the ^{3}H-labelled amino acid, and the unshaded that of the ^{14}C-labelled amino acid.

After the transfer of the preincubated tissue to the second medium there was a relatively low efflux of ^{14}C-labelled amino acid (Fig. 2).

The relative incorporations of [^{14}C]- and [^{3}H]-leucine and -glycine into the protein of extensor

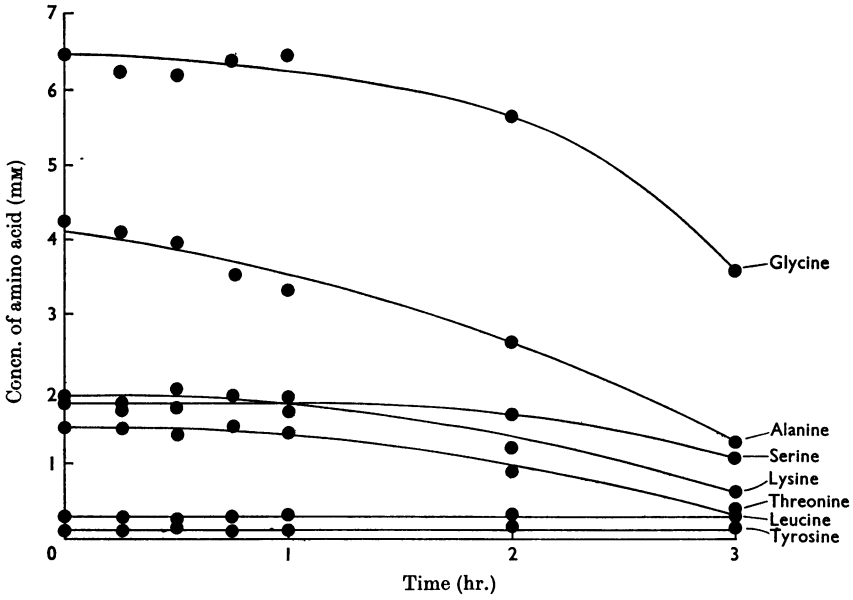


Fig. 1. Variation of intracellular concentration of various amino acids in extensor digitorum longus muscle with time. The graph illustrates glycine, alanine, serine, lysine, threonine, leucine and tyrosine. The tissue was incubated in Krebs–Ringer bicarbonate buffer (2ml.).

digitorum longus muscle are shown in Fig. 4. The unshaded areas represent ^{14}C-labelled protein and the shaded areas ^{3}H-labelled protein. The initial specific radioactivities of both [^{3}H]- and [^{14}C]-leucine in the incubation medium were adjusted to give approximately the same number of c.p.m./mg. of protein in a fixed 30min. incubation (arrows, Fig. 4). The sum of the rates of incorporation of the two labelled leucines into protein proceeded as a linear function. There was an appreciable fall in the rate of incorporation of ^{14}C radioactivity appearing in protein during the second incubation, and after 10min. there was no measurable incorporation of additional [^{14}C]leucine. Essentially the same results were obtained with glycine (Fig. 4); the incorporation of [^{14}C]glycine ceased immediately the second incubation was started.

The specific radioactivities of [^{14}C]- and [^{3}H]-leucine and -glycine in the incubation media remained at constant values, apart from a small initial decrease of less than 5%. Efflux of ^{14}C-labelled amino acid into the second medium, containing either glycine (0·3mM) or L-leucine (0·05mM), was negligible; less than 5% of the radioactivity appeared in the medium after 30min.

If the tissue was incubated in an amino acid-free medium, there was a rapid efflux of endogenous amino acids (Fig. 5).

Theoretical estimation of the relative incorporation of ^{14}C- *and* ^{3}H-*labelled amino acids in protein.* A theoretical graph, based on the assumption that the intracellular pool was an obligatory intermediate in protein synthesis, is shown in Fig. 6. The curve was calculated by assuming that the rate of incorporation of tracer into protein would be proportional to the specific radioactivity of the intracellular pool at time t. This assumption is valid since during the incubation the intracellular concentration of amino acid did not vary significantly (Fig. 1). The relationship may be represented as:

$$dC/dt = ka \quad (1)$$

where C corresponds to the radioactivity in protein, k is a proportionality constant and a is the specific radioactivity of the intracellular pool at time t. It follows that:

$$C_t = k' \int_0^t a\,dt \quad (2)$$

Therefore the radioactivity incorporated into protein at any time t is proportional to the area under the specific radioactivity–time graph (Fig. 3) at time t. This relationship assumes that the labelled amino acid is stable and undergoes no loss of radioactivity when incorporated into protein. However, this is not so for ^{3}H-labelled amino acids and the percentage losses of radioactivity from [^{3}H]glycine and [^{3}H]leucine have been estimated (see the Methods section). By using this correction, a curved

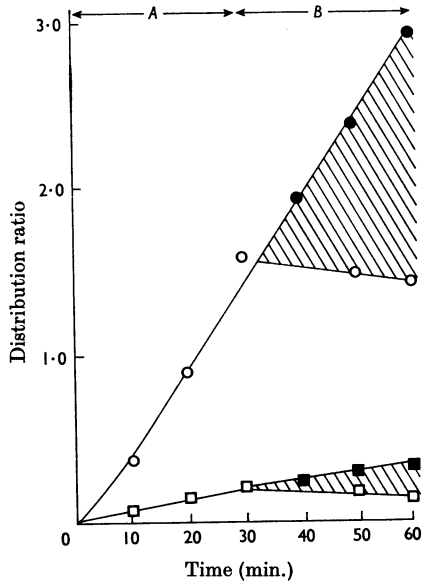


Fig. 2. Variation, with time, of the uptake of [^{14}C]-glycine and -leucine and [^{3}H]-glycine and -leucine during a sequential incubation of tissue, initially in Krebs–Ringer bicarbonate buffer (2ml.) containing [^{14}C]glycine (0·3mM) or [^{14}C]leucine (0·05mM) for 30min. (*A*), and then in Krebs–Ringer bicarbonate buffer (2ml.) containing [^{3}H]glycine (0·3mM) or [^{3}H]leucine (0·05mM) for 30min. (*B*). The shaded area represents the accumulation of ^{3}H-labelled amino acid. The ordinate shows the distribution ratio between the intracellular pool and the medium. ○, [^{14}C]-Glycine contained in Krebs–Ringer bicarbonate buffer; ●, [^{3}H]glycine contained in Krebs–Ringer bicarbonate buffer; □, [^{14}C]leucine contained in Krebs–Ringer bicarbonate buffer; ■, [^{3}H]leucine contained in Krebs–Ringer bicarbonate buffer.

line representing total incorporation into protein was calculated from the relationship given in eqn. (2) (Fig. 6). The shaded area represents ^{3}H-labelled protein and the unshaded represents ^{14}C-labelled protein.

Fig. 7 shows a theoretical curve based on an alternative hypothesis that protein was synthesized from amino acid in the total extracellular pool. A straight line representing total incorporation into protein (Fig. 7) results, which is to be expected as the precursor specific radioactivity is constant all the time. The shaded area represents ^{3}H-labelled protein, and the unshaded represents ^{14}C-labelled protein.

DISCUSSION

Glycine and leucine were chosen for this study because the uptake patterns of these two amino acids in a variety of tissues are different: the behaviour of glycine is characteristic of the A system, and leucine of the L system (Oxender & Christensen, 1963; Manchester, 1966; Riggs, Pan & Feng, 1968). The differences observed in this work (Figs. 1, 2 and 4) and a marked inhibition of glycine uptake in Na^{+}-free medium (R. C. Hider, E. B. Fern & D. R. London, unpublished work) accentuate the dissimilarities in the mode of accumulation of the two amino acids in extensor digitorum longus muscle.

Although [^{14}C]glycine and [^{14}C]leucine had intracellular specific radioactivities of the same order (Fig. 3), the rate of incorporation of [^{14}C]leucine was ten times that of [^{14}C]glycine in extensor digitorum longus (Fig. 4). As the total proteins isolated from extensor digitorum longus (R. C. Hider, E. B. Fern & D. R. London, unpublished work) and soluble mammalian enzymes (Dixon &

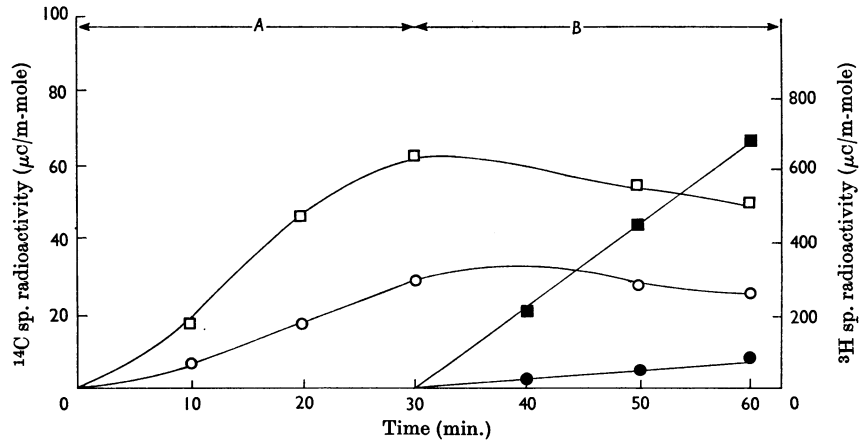


Fig. 3. Time-course of specific radioactivities of intracellular leucine and glycine during a sequential incubation of tissue in Krebs–Ringer bicarbonate buffer (2ml.). The initial incubation (*A*) was for 30min. in medium containing ^{14}C-labelled amino acid, and the second (*B*) was for 30min. in medium with ^{3}H-labelled amino acid. The right-hand ordinate gives ^{3}H specific radioactivity and the left-hand ordinate gives ^{14}C specific radioactivity. □, [^{14}C]Leucine (0·05mM); ■, [^{3}H]leucine (0·05mM); ○, [^{14}C]glycine (0·3mM); ●; [^{3}H]glycine (0·3mM).

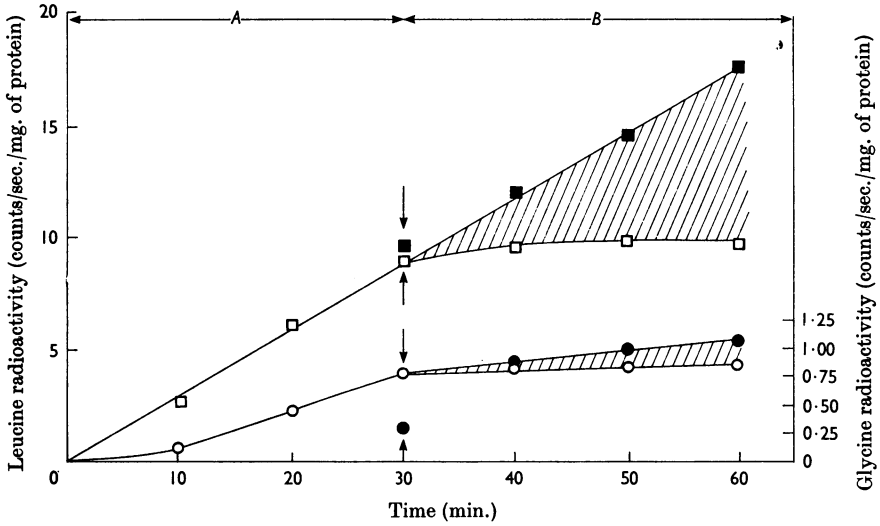


Fig. 4. Time-course of the incorporation of [^{14}C]-leucine and -glycine and [^{3}H]-leucine and -glycine during a sequential incubation of tissue in Krebs–Ringer bicarbonate buffer (2ml.). The initial incubation (*A*) was for 30min. in medium containing [^{14}C]leucine (0·05mM) or [^{14}C]glycine (0·3mM) and the second (*B*) was for 30min. in medium containing [^{3}H]leucine (0·05mM) or [^{3}H]glycine (0·3mM). The shaded area represents incorporation of ^{3}H-labelled amino acid. The values of incorporation during standard 30min. incubation periods for ^{14}C- and ^{3}H-labelled amino acids (see the text) are indicated by the arrows. □, [^{14}C]Leucine; ■, [^{3}H]leucine; ○, [^{14}C]glycine; ●, [^{3}H]glycine. The incorporations of glycine and leucine are drawn on different scales: the right-hand ordinate gives glycine radioactivity and the left-hand ordinate gives leucine radioactivity.

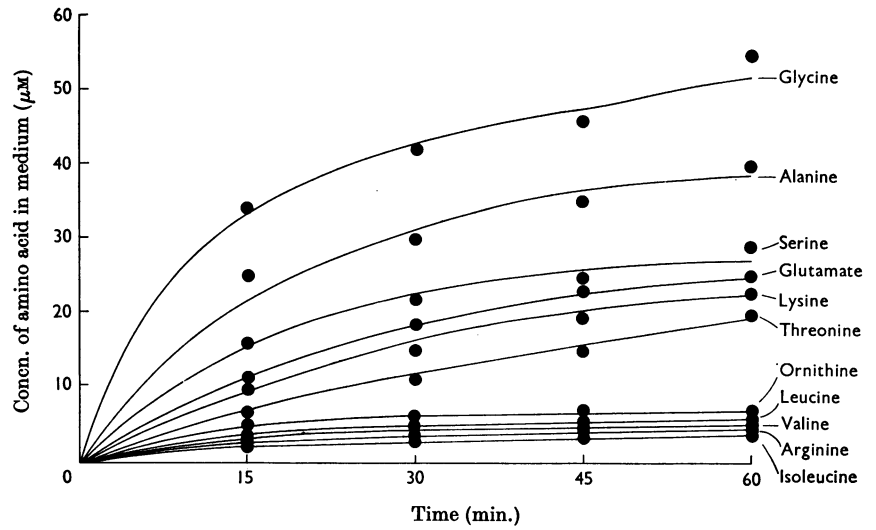


Fig. 5. Appearance of free amino acids in Krebs–Ringer bicarbonate solution (1ml.) containing one extensor digitorum longus muscle.

Webb, 1964) each have approximately equal glycine and leucine contents, it seemed likely that the amino acid utilized for protein synthesis did not originate from the total intracellular pool. If the amino acids used in protein synthesis did come from this source, the appearance of radioactivity in the protein would be expected to follow a curvilinear plot (Kipnis *et al.* 1961). Kipnis *et al.* (1961) based their theory of functional heterogeneity of the intracellular pool on such evidence with diaphragm

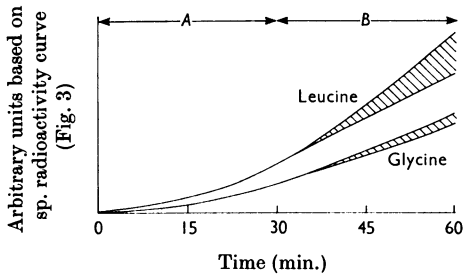


Fig. 6. Prediction of variation of incorporation of [^{14}C]- and [^{3}H]-leucine and -glycine, based on the assumption that intracellular amino acids are precursors of protein. The arbitrary units for protein synthesis are based on the area of the specific-radioactivity curve (Fig. 3) as given by eqn. (2) (see the text). The shaded area represents incorporation of ^{3}H-labelled amino acid and the unshaded area that of ^{14}C-labelled amino acid. *A*, 30min. period of incubation with ^{14}C-labelled amino acid; *B*, 30min. period with ^{3}H-labelled amino acid.

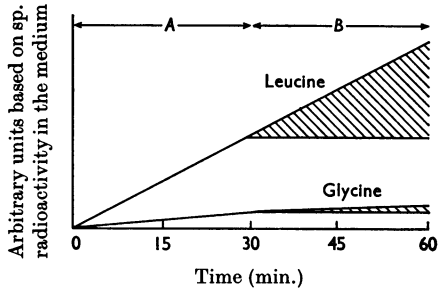


Fig. 7. Prediction of variation of incorporation of [^{14}C]- and [^{3}H]-leucine and -glycine, based on the assumption that extracellular amino acids are precursors of protein. The arbitrary units for protein synthesis are based on the area of a graph of specific radioactivity in the medium against time. The shaded area represents incorporation of ^{3}H-labelled amino acid and the unshaded area that of ^{14}C-labelled amino acid. *A*, 30min. period of incubation with ^{14}C-labelled amino acid; *B*, 30min. period with ^{3}H-labelled amino acid.

and lymph-node cells. However, conflicting findings were reported with lymph-node slices (Askonas & Humphrey, 1958) and diaphragm (Manchester & Wool, 1963). In the present study protein synthesis proceeded in a linear manner for leucine but not for glycine (Fig. 4).

To establish the significance of the apparently different modes of incorporation of glycine and leucine, ^{14}C- and ^{3}H-labelled amino acids were added sequentially to the muscle. During the second incubation period the amino acid intracellular pool is initially labelled with ^{14}C and the extracellular pool with ^{3}H. If protein were synthesized directly from the amino acids in the medium, incorporation of ^{14}C-labelled amino acid would cease and the ribosomes would utilize only ^{3}H-labelled amino acid (Fig. 7). If the intracellular amino acid pool was utilized as a direct precursor then incorporation of ^{14}C-labelled amino acid would continue initially unhindered and at a rate in excess of ^{3}H-labelled amino acid incorporation throughout the remaining incubation (Fig. 6).

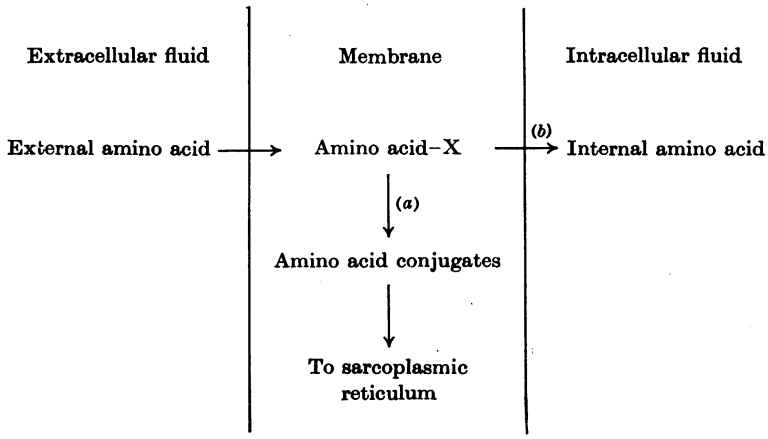
The coincidence of the experimental curves of Fig. 4 with the theoretical curves of Fig. 7 is strong evidence for the theory that amino acids entering protein are in extremely rapid equilibrium with the external amino acid pool. The anomalous pattern of the incorporation of glycine at the earliest times (Fig. 4) remains unexplained.

The rapid efflux of amino acids from the tissue preparation (Fig. 5) renders them available for protein synthesis *in vitro*. This observation casts doubt on the conclusions drawn by Wool (1969) that, because protein synthesis can apparently occur in a medium from which amino acid is initially absent, there is thus no amino acid available for incorporation from an extracellular pool.

Consideration of the two general interpretations proposed by Kipnis *et al.* (1961) leads us to favour that of chemical compartmentalization. Fig. 4 clearly shows that, after the tissue has been moved from the ^{14}C- to the ^{3}H-containing medium, there is virtually no increase in ^{14}C-labelled protein content. If a protein-synthetic pool of free amino acids exists (Kipnis *et al.* 1961; Manchester, 1965) it is probably small and undergoes a rapid turnover. Otherwise one might have expected a significant increase in the synthesis of ^{14}C-labelled protein after removal of the muscle from the ^{14}C-containing medium. Thus either the amino acids are activated directly from the extracellular pool, or there is a very small intracellular compartment important in protein synthesis that is more rapidly labelled than the pool as a whole.

Hendler (1962) presented a theoretical model for protein synthesis that postulated direct incorporation via the extracellular pool. Appreciable concentrations of lipid–amino acid complexes have been shown to exist in a number of different tissue preparations (Hendler, 1963; Schwartzman, Crawhall & Segal, 1966; Wilcox, Dishman & Heimberg, 1968) and work with *Escherichia coli* cells has shown that an amino acid-catalysed ATP–pyrophosphate exchange occurs at the cell surface membrane (Roberts, Bensch & Carter, 1964).

A model taking into account all the facts reported in this paper is presented in Scheme 1. It is proposed that amino acids in the extracellular pool are transported into the membrane (possibly the T-tubular membrane) by means of a 'carrier'. This membrane-



Scheme 1.

bound carrier (amino acid–X) then has two possible routes: (*a*) interaction with ATP or another conjugate, with subsequent removal for protein synthesis; (*b*) entry into the intracellular pool and dissociation to the free amino acid and carrier.

This model would also explain several other observations that are superficially contradictory to the concept of protein being synthesized directly from the extracellular amino acids. Riggs & Walker (1963) found that in the ascites-tumour cell the presence of puromycin caused leucine uptake to follow a similar pattern to that of cycloleucine; they also showed that in the absence of the antibiotic, when incorporation of leucine into protein is possible, there was a much lower rate of accumulation into the intracellular pool. Essentially the same observation was made with the complete spectrum of protein containing amino acids, by Castles, Wool & Moyer (1965) in isolated 'intact' diaphragm and by Scharff & Wool (1965) in perfused heart. These findings were interpreted as showing that the intracellular amino acid pool was being depleted as soon as it was formed, as a result of the demand of protein synthesis. However, an alternative explanation using the model proposed in Scheme 1 is also possible. If puromycin blocks protein synthesis there will be a build-up of protein precursors from the site of inhibition to the hypothetical step (*a*), and thus proportionally much more of the carrier complex (amino acid–X) will be dissociated to the free intracellular amino acid pool via step (*b*), giving rise to a greater accumulation of free amino acid.

Further support for the conclusions drawn from this study is given by the observations by Waterlow & Stephen (1968). These workers found, from experiments with the rat *in vivo*, that a calculated rate of protein turnover in liver would be more in accord with that previously reported by others if the source of amino acid utilized for protein synthesis were the extracellular rather than the intracellular pool.

Thus there appears to be no direct relationship between the total intracellular pool and protein synthesis. It may be that the biological role of this amino acid pool is to act as a reservoir. Indeed, Van Slyke & Meyer (1913) and London, Foley & Webb (1965) have showed *in vivo* that amino acids stored in muscle are released into the circulation in the starved state.

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