Short Communications

An Investigation of the Location and Possible Compartmentation of the Precursor Pool of Phenylalanine for Protein Synthesis in Human Granulocytes

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Evidence is presented that in human granulocytes the immediate precursor pool of phenylalanine for protein synthesis *in vitro* is intracellular without significant compartmentation.

It has been suggested that the amino acids used for protein synthesis by tissues *in vitro* are drawn selectively from the incubation medium via a special pool compartment, or micro-environment, which is not in free equilibrium with the main intracellular pool of the amino acids. This conclusion has been based on the study of whole skeletal muscle (Kipnis *et al.*, 1961; Hider *et al.*, 1971), fragments of rat pancreas (Van Venrooij *et al.*, 1972), isolated lymph-node cells (Kipnis *et al.*, 1961) and mixed human leucocytes (Winkler, 1972), by using the following isotopically labelled amino acids to mark protein synthesis: L-leucine (Hider *et al.*, 1971; Van Venrooij *et al.*, 1972; Winkler, 1972), DL-leucine (Kipnis *et al.*, 1961), L-lysine (Van Venrooij *et al.*, 1972), glycine (Kipnis *et al.*, 1961; Hider *et al.*, 1971), L-proline (Kipnis *et al.*, 1961) and a mixture of 20 naturally occurring amino acids (Winkler, 1972).

The aim of the present work was to determine if the phenylalanine which is used directly for protein synthesis by human granulocytes *in vitro* is intracellular or extracellular.

Materials and methods

Analytical-grade reagents were used throughout, and solutions were prepared from glass-distilled water. Cycloheximide was purchased from Koch-Light Laboratories (Colnbrook, Bucks., U.K.), and NCS tissue solubilizer from Nuclear Chicago Corp. (G. D. Searle and Co., High Wycombe, Bucks., U.K.), and L-3-[U-¹⁴C]phenylalanine (10 or 513 mCi/mmol) from The Radiochemical Centre, Amersham, Bucks., U.K. The composition of the iso-osmotic buffer used for the granulocyte incubation was: Tris-HCl (20 mM, pH 7.4 at 37°C), KCl (4.5 mM), MgCl₂ (0.8 mM), NaCl (125 mM), Na₂HPO₄ (1 mM), D-glucose (5.5 mM). Granulocytes were separated from human blood as

described previously (Blackburn *et al.*, 1973), and phenylalanine was determined spectrophotofluorimetrically (Andrews *et al.*, 1973).

Studies of incorporation of [U-¹⁴C]phenylalanine into protein. Samples (100 µl) of the granulocyte or L-cell suspension (1 × 10⁷ cells/ml in iso-osmotic buffer) were preincubated at 37°C for 10 min together with 0.45 ml of iso-osmotic buffer. The reaction was started by adding L-3-[U-¹⁴C]phenylalanine [50 µl (2 µCi/ml; 513 mCi/mmol) or 50 µl (6 µCi/ml; 10 mCi/mmol) for the experiments in which extracellular phenylalanine concentrations of 0.3 or 50 µM respectively were required]. The samples were incubated at 37°C for predetermined time-intervals and the reaction was stopped by exposing the cells to ultrasonic vibrations (8 µm amplitude and 20 kHz) for 30 s, immediately followed by the addition of trichloroacetic acid [5 ml; 5% (w/v)]. The resulting precipitates were washed once with trichloroacetic acid at 75–80°C for 20 min, once with trichloroacetic acid at room temperature (~16°C), twice with ethanol-ether (3:1, v/v), dried *in vacuo* over P₂O₅ and the radioactivity was measured. Blank values were obtained by adding cycloheximide (final concentration 10 mM) to the incubation mixture, measuring the radioactivity of the protein precipitate and subtracting this value from the sample value. As a routine the value of the blank was 5% or less of the sample value.

Studies of intracellular phenylalanine specific radioactivity. The intracellular phenylalanine content of granulocytes could not be measured in samples containing as few as 1 × 10⁶ cells, so, whenever experiments were carried out to measure the intracellular phenylalanine specific radioactivity as well as the incorporation of [U-¹⁴C]phenylalanine into the trichloroacetic acid-insoluble material, parallel incubations were performed by using 5 × 10⁶ cells and increasing the volumes of the reagents used proportionately. Samples (0.5 ml) of the granulocyte suspension (1 × 10⁷ cells/ml in iso-osmotic buffer)

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were preincubated for 10 min at 37°C, after the addition of a further 2.25 ml of iso-osmotic buffer. The reaction was started by adding L-3-[U-¹⁴C]-phenylalanine [250 μ l (2 μ Ci/ml; 513 mCi/mmol) or 250 μ l (6 μ Ci/ml; 10 mCi/mmol) for the experiments in which extracellular phenylalanine concentrations of 0.3 μ M or 50 μ M respectively were required]. The samples were incubated at 37°C for predetermined time-intervals and the reaction was stopped by cooling on ice. The samples were centrifuged at 75g_{av.} for 5 min at 4°C and the supernatant was discarded. The pellets of cells were resuspended in iso-osmotic buffer (1 ml) and centrifuged at 75g_{av.} for 5 min at 4°C, the supernatant was discarded and the pellets of the cells were dried *in vacuo* over P₂O₅. Water (100 μ l) was added to the desiccated cell preparations, which were then left in ice for 10–15 min. The cells were then disrupted by ultrasonic vibrations (6 μ m, 20 kHz) for 30 s while the sample was cooled in ice. Trichloroacetic acid (100 μ l of 0.6 M solution) was added to each sample and the precipitate allowed to form for 15 min before the samples were centrifuged at 1000g_{av.} for 10 min at 4°C. Portions (20 μ l) of the supernatant were either assayed for phenylalanine or added to glass counting vials and dried *in vacuo* over P₂O₅, and the radioactivity was measured. The radioactivity (d.p.m./20 μ l) of the trichloroacetic acid-soluble material divided by the phenylalanine content (nmol/20 μ l) gives the intracellular free phenylalanine specific radioactivity.

Measurement of radioactivity. Samples, dissolved in NCS tissue solubilizer (0.5 ml, 0.3 M in toluene), were mixed with 10 ml of scintillator solution [0.05 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene and 4 g of 2,5-diphenyloxazole/litre of toluene] in glass counting vials, and the radioactivity was measured in a Nuclear Chicago mark I liquid-scintillation spectrometer, cooled to 4°C. The efficiency of counting was 85%.

Culturing and harvesting of L-cells. L-cells (a continuous line of normal C3H mouse subcutaneous fibroblasts) were grown as a monolayer culture in Gibco L-15 tissue-culture medium (Gibco-Biocult, Paisley, U.K.) containing 10% (v/v) foetal bovine serum (Flow Laboratories, Ayr, U.K.). The cells were harvested by washing the cell cultures twice with buffer solution (pH 7.3) containing NaCl (8.0 g/l), KCl (0.2 g/l), Na₂HPO₄ (1.15 g/l) and KH₂PO₄ (0.2 g/l) and then adding trypsin [5.0 ml, 0.25% (w/v) in the buffer solution described above, but with the pH adjusted with 1 M-NaOH to pH 7.8] to each culture. After 10 min the cell suspensions were pooled, 2 ml of foetal bovine serum was added to inactivate the trypsin, and the cells were separated by centrifugation at 75g_{av.} for 5 min at 4°C. The pellets of cells were resuspended in iso-osmotic buffer to a concentration of 1×10^7 cells/ml and were kept on ice until used.

Fig. 1. Time-course for the incorporation of [U-¹⁴C]phenylalanine into protein by human granulocytes *in vitro*

The concentration of phenylalanine in the incubation medium was (a) 0.3 μ M (the mean $\pm$ 1 s.d. of duplicate values obtained in four separate experiments are shown, expressed as a percentage of the maximum value; the range of maximum values observed is 800–1400 d.p.m.) or (b) 50 μ M (the means of duplicate values obtained in two separate experiments are shown, expressed as a percentage of the maximum value; the range of maximum values observed is 300–500 d.p.m.).

Results

Incorporation of [U-¹⁴C]phenylalanine into protein. Fig. 1(a) shows the time-course of the incorporation of [U-¹⁴C]phenylalanine into protein by isolated human granulocytes *in vitro* incubated with 0.3 μ M-[U-¹⁴C]phenylalanine. There was a time-lag of about 30 min before a linear rate of incorporation with time was attained. Preincubating these cells for 40 min instead of 10 min before adding the [U-¹⁴C]phenylalanine did not increase the rate of labelled amino acid incorporation into the cell protein. However, increasing the concentration of phenylalanine in the incubation medium from 0.3 to 50 μ M abolished the initial non-linear part of the curve (Fig. 1b).

Supplementing the incubation medium with a mixture of the following amino acids: L-alanine, L-arginine, L-asparagine, L-aspartate, L-cysteine, L-

Fig. 2. Time-course of the intracellular phenylalanine specific radioactivity

Human granulocytes were incubated with (a) 0.3 μM-[U-¹⁴C]phenylalanine (the means of duplicate values from three separate experiments are shown, expressed as a percentage of the mean plateau value; the range of mean plateau values observed is 2600–4500 d.p.m./nmol) or (b) 50 μM-[U-¹⁴C]phenylalanine (the means of duplicate values from two separate experiments are shown, expressed as a percentage of the mean plateau value; the range of mean plateau values observed is 1500–2600 d.p.m./nmol).

cystine, L-glutamate, L-glutamine, glycine, L-histidine, hydroxy-L-proline, L-isoleucine, L-leucine, L-lysine, L-methionine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine and L-valine, to give concentrations in the incubation medium which were the same as the physiological concentrations in plasma (the concentration of phenylalanine being 50 μM), did not increase the incorporation of radioactivity into the cell protein.

L-cells incorporated [U-¹⁴C]phenylalanine into the cell protein in a linear manner for the first 40 min of the incubation period, when the phenylalanine concentration in the medium was the same (0.3 μM) as that which gave non-linear incorporation of radio-

activity for the first 30 min of incubation with human granulocytes.

Intracellular phenylalanine specific radioactivity. The specific radioactivity of the intracellular free phenylalanine of granulocytes incubated with 0.3 μM-[U-¹⁴C]phenylalanine increased during the first 30 min of incubation, and then remained steady until the experiments were stopped after 60 min (Fig. 2a). The specific radioactivity of the intracellular phenylalanine reached its steady maximum value more rapidly (within 5–10 min) when the concentration of phenylalanine in the incubation medium was increased from 0.3 to 50 μM (Fig. 2b). Chromatographic separation of the intracellular amino acids after incubation showed that 98% of the ¹⁴C was in phenylalanine, with no measurable labelling of tyrosine or other amino acids.

Discussion

A linear increase of incorporation of labelled amino acid into protein with respect to time had previously been observed during studies *in vitro* using fragmented rat pancreas (Van Venrooij *et al.*, 1972), intact rat diaphragm and isolated guinea-pig lymph-node cells (Kipnis *et al.*, 1961) and isolated human leucocytes (Winkler, 1972), although a lag phase occurred before the specific radioactivity of the intracellular free amino acid reached a constant value. Using the isolated rat extensor digitorum longus preparation, Hider *et al.* (1971) observed a time-lag before the rate of radioactive amino acid incorporation into protein became constant which was significantly shorter than the time-interval required for the intracellular amino acid specific radioactivity to become constant. The above-mentioned workers therefore suggested that in these tissues the amino acids used in protein synthesis were taken directly from an extracellular pool and not from the intracellular pool.

In our studies the time-course of [U-¹⁴C]phenylalanine incorporation into protein became linear only after 30 min of incubation when a phenylalanine concentration of 0.3 μM was used (Fig. 1a). Similar results were obtained by Regier & Kafatos (1971) in their studies of protein synthesis in the galea of the giant silkworm (*Antheraea polyphemus*). This observation suggests that the specific radioactivity of the precursor phenylalanine pool did not become constant until 30 min after the start of incubation, a time-course identical with that leading to constant specific radioactivity of the intracellular free amino acid pool (Fig. 2a), suggesting identity of the two pools. The observation that preincubating granulocytes for 40 min instead of 10 min before adding [U-¹⁴C]phenylalanine did not increase the initial rate of labelled amino acid incorporation into cell protein suggests that the true rate of protein synthesis was not increasing during incubation. Supplementing

the incubation medium with the full range of amino acids which occur naturally in protein did not accelerate the incorporation of [$U-^{14}C$]phenylalanine into protein during the first 30 min of incubation, indicating that the apparently non-linear rate of protein synthesis was not due to a shortage of other amino acids needed to complete polypeptide chains.

Linear time-courses of radioactive amino acid incorporation into protein are obtained when amino acid concentrations greater than $0.3 \mu M$, in some cases as much as $400 \mu M$ (Kipnis *et al.*, 1961), are used in the incubation medium. A higher concentration of amino acid would be expected to saturate the influx carrier of the amino acids more rapidly and therefore accelerate the attainment of uniform specific radioactivity in the intracellular pool, as shown in Fig. 2(b). In addition the time-course of incorporation into protein became linear throughout the experiment when the concentration of phenylalanine in the incubation medium was increased from 0.3 to $50 \mu M$ (Fig. 1b).

The observation that L-cells, treated experimentally like the granulocytes, incorporated [$U-^{14}C$]phenylalanine into protein with a linear time-course during the first 40 min of incubation in $0.3 \mu M$ -phenylalanine indicated that genuine differences between tissues do occur, and might account for the difference between our and earlier findings. The time-scales of the two sets of experiments with granulocytes (Figs. 1 and 2) suggest that phenylalanine used directly for protein synthesis is derived from the intracellular pool. When $0.3 \mu M$ -phenylalanine was used, a steady maximum specific radioactivity of intracellular free phenylalanine was reached after 30 min incubation, coinciding with the attainment of the linear increase in protein radioactivity. Further, when the maximum specific radioactivity of free amino acid was reached

more quickly (i.e. in 5–10 min), with $50 \mu M$ -phenylalanine, linear incorporation into protein was attained earlier.

It is noteworthy that the pattern of labelling in the protein of heart, liver and skeletal muscle (Fern & Garlick, 1973) observed after continuous infusion of rats with [$U-^{14}C$]glycine resembled that of the tissue free amino acids rather than that of plasma, in accord with our present findings in granulocytes.

We conclude that, in our experimental system, granulocytes draw on a homogeneous intracellular pool of free phenylalanine for protein synthesis, and not on an extracellular pool or a minor subcompartment of the intracellular pool.

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