

## Measurement of Protein Synthesis in Rat Lungs Perfused *in situ*

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(Received 5 September 1979)

Compartmentalization of amino acid was investigated to define conditions required for accurate measurements of rates of protein synthesis in rat lungs perfused *in situ*. Lungs were perfused with Krebs–Henseleit bicarbonate buffer containing 4.5% (w/v) bovine serum albumin, 5.6 mM-glucose, normal plasma concentrations of 19 amino acids, and 8.6–690  $\mu$ M-[U-<sup>14</sup>C]phenylalanine. The perfusate was equilibrated with the same humidified gas mixture used to ventilate the lungs [O<sub>2</sub>/CO<sub>2</sub> (19:1) or O<sub>2</sub>/N<sub>2</sub>/CO<sub>2</sub> (4:15:1)]. [U-<sup>14</sup>C]Phenylalanine was shown to be a suitable precursor for studies of protein synthesis in perfused lungs: it entered the tissue rapidly ( $t_{1/2}$ , 81 s) and was not converted to other compounds. As perfusate phenylalanine was decreased below 5 times the normal plasma concentration, the specific radioactivity of the pool of phenylalanine serving as precursor for protein synthesis, and thus [<sup>14</sup>C]phenylalanine incorporation into protein, declined. In contrast, incorporation of [<sup>14</sup>C]histidine into lung protein was unaffected. At low perfusate phenylalanine concentrations, rates of protein synthesis that were based on the specific radioactivity of phenylalanyl-tRNA were between rates calculated from the specific radioactivity of phenylalanine in the extracellular or intracellular pools. Rates based on the specific radioactivities of these three pools of phenylalanine were the same when extracellular phenylalanine was increased. These observations suggested that: (1) phenylalanine was compartmentalized in lung tissue; (2) neither the extracellular nor the total intracellular pool of phenylalanine served as the sole source of precursor for protein; (3) at low extracellular phenylalanine concentrations, rates of protein synthesis were in error if calculated from the specific radioactivity of the free amino acid; (4) at high extracellular phenylalanine concentrations, the effects of compartmentalization were negligible and protein synthesis could be calculated accurately from the specific radioactivity of the free or tRNA-bound phenylalanine pool.

Accurate measurements of protein synthesis *in vitro* require that a suitable radioactive amino acid be identified for use as a precursor and that the specific radioactivity of the amino acid in the pool providing substrate for the synthetic pathway be known. Under ideal conditions, the radioactive amino acid used *in vitro* would equilibrate rapidly with an identifiable pool of precursor amino acids and would not be significantly metabolized by the tissue under study, except in the pathway of protein turnover. Identification of the pool of amino acid providing precursors for protein synthesis is complicated by functional compartmentalization of amino acids within the tissue. Previous studies (Khairallah

& Mortimore, 1976; Vidrich *et al.*, 1977; Martin *et al.*, 1977; McKee *et al.*, 1978) have emphasized that, in the steady state, the specific radioactivity of amino acid bound in the form of an immediate precursor of protein, aminoacyl-tRNA, may not be equal to that of the same amino acid in the extracellular or intracellular pools. These differences in specific radioactivity may be accounted for by compartmentalization of intracellular amino acids (Mortimore *et al.*, 1972; Khairallah & Mortimore, 1976) or by charging of aminoacyl-tRNA with amino acids originating in both the intracellular and extracellular compartments (Airhart *et al.*, 1974; Hod & Hershko, 1976; Vidrich *et al.*, 1977). Since these possibilities have not been systematically investigated in lung tissue, experimental conditions under which rates of synthesis of

Abbreviation used: dansyl, 5-dimethylaminonaphthalene-1-sulphonyl.

lung proteins can be measured accurately are unknown.

The present experiments were carried out in order to define conditions allowing accurate measurements of the rate of protein synthesis in rat lungs perfused *in situ*. Phenylalanine was identified as an amino acid suitable for use in these studies. Compartmentalization of phenylalanine in lung tissue was investigated by comparing the specific radioactivity of the amino acid in the extracellular, intracellular and aminoacyl-tRNA pools as a function of the concentration of extracellular phenylalanine. These studies defined a protocol by which the effects of substrates, hormones and other factors on the synthesis of lung proteins could be investigated.

## Materials and Methods

### *Perfusion of rat lungs in situ*

Male Sprague-Dawley rats (175–200 g), obtained from Charles River Laboratories, were provided with Wayne LabBlox and water *ad libitum* and maintained on a 12h light/dark cycle. After intraperitoneal injection of sodium pentobarbital (50 mg/kg body wt.), the animals were weighed and prepared for lung perfusion as described previously (Rannels *et al.*, 1979b; Watkins & Rannels, 1979). Ventilation was accomplished through a tracheostomy by using a Harvard small-animal respirator (model 680). The ventilatory gas was warmed (37°C), humidified O<sub>2</sub>/CO<sub>2</sub> (19:1) or O<sub>2</sub>/N<sub>2</sub>/CO<sub>2</sub> (4:15:1), as indicated. Tidal volume was 10.0 ml/kg body wt. (72 cycles/min). After thoracotomy and injection of sodium heparin (10 mg/kg body wt.) into the right ventricle, the pulmonary circulation was cannulated by inserting a grooved stainless steel cannula through the left ventricle and mitral valve and tying it at the base of the left atrium. An arterial cannula, filled with perfusate, was inserted through the right ventricle and secured in the pulmonary artery. Perfusion was begun immediately at a pressure of 10–15 cmH<sub>2</sub>O. The animal was transected below the level of the diaphragm, and the lungs were rinsed with warm 0.15 M-NaCl. Perfusion pressure was adjusted to 20 cmH<sub>2</sub>O and a positive end expiratory pressure of 2 cmH<sub>2</sub>O was applied. The preparation was covered with plastic film and placed within a temperature-controlled (37°C) Plexiglas box, constructed as detailed earlier (Exton, 1975; Watkins & Rannels, 1979).

The perfusate was Krebs-Henseleit bicarbonate buffer (Krebs & Henseleit, 1932) containing 4.5% (w/v) bovine serum albumin (fraction V, Pentex; Miles Laboratories), 5.6 mM-glucose and 19 amino acids at the concentrations found in rat plasma, as follows ( $\mu$ M): aspartic acid, 14; asparagine, 68; glutamic acid, 120; glutamine, 652; serine, 247;

threonine, 203; proline, 108; glycine, 336; alanine, 326; valine, 188; isoleucine, 108; leucine, 166; tyrosine, 69; lysine, 380; histidine, 52; arginine, 119; methionine, 54; half-cystine, 10; tryptophan, 10 (Morgan *et al.*, 1971). Perfusate phenylalanine was present at the concentration indicated. The mixture of amino acids was adjusted to pH 7.4 before addition to the perfusate. Fraction V albumin was dialysed overnight against Krebs-Henseleit buffer and filtered (Millipore, 0.8  $\mu$ m) before use. The perfusate was equilibrated with the same humidified gas mixture used to ventilate the lungs. The first 30–50 ml of perfusate to pass through the preparation was discarded; 100 ml of perfusate was recirculated. Radioactive isotopes were added to the perfusate reservoir after recirculation was begun.

### *Measurement of [<sup>14</sup>C]phenylalanine and [<sup>3</sup>H]sorbitol space and incorporation of <sup>14</sup>C-labelled amino acid into protein*

For determination of [<sup>14</sup>C]phenylalanine and [<sup>3</sup>H]sorbitol space, lungs were rinsed in a beaker containing 0.15 M-NaCl (2°C), trimmed free of large airways and blotted on filter paper. A weighed portion of the tissue was homogenized in 2 ml of 0.5 M-HClO<sub>4</sub> and centrifuged (10 000 g, 10 min). Radioactivity in the tissue extract and perfusate was determined in a liquid scintillation counter (Beckman LS-250), and spaces were calculated as outlined previously (Rannels *et al.*, 1974). Incorporation of <sup>14</sup>C-labelled amino acid into lung protein was measured as described earlier (Rannels *et al.*, 1979a). Duplicate samples of dried protein (4–10 mg) were weighed into scintillation vials, dissolved in 1.0 ml of NCS tissue solubilizer (Amersham/Searle), and counted in 10 ml of Formula 949 scintillator (New England Nuclear).

### *Analysis of phenylalanine content and specific radioactivity in lung tissue and perfusate*

For amino acid analysis, aliquots of perfusate were precipitated with an equal volume of 1.0 M-HClO<sub>4</sub>. Lungs, trimmed and blotted as described above, were homogenized in 0.5 M-HClO<sub>4</sub> using a Polytron PT10 (Brinkman) homogenizer. Extracts of perfusate or lung tissue were neutralized with KOH and freeze-dried, the residue was taken up in 0.05 M-lithium citrate buffer, pH 2.20, and applied to the column (type W-3P spherical resin; Beckman) of a Beckman 119CL amino acid analyser. Phenylalanine was separated by using a rapid elution protocol as described earlier (Rannels *et al.*, 1975), using leucyl-alanine as internal standard. Initial experiments, performed as described below, indicated that radioactivity added to the perfusate as [U-<sup>14</sup>C]phenylalanine (New England Nuclear) remained as phenylalanine throughout the course of perfusion. Thus phenylalanine specific radioactivity

was estimated by dividing the d.p.m./ml of sample applied to the column of the amino acid analyzer by the amount (nmol) of phenylalanine/ml of this sample. Intracellular phenylalanine concentrations were calculated as follows:

$$\text{Concentration } (\mu\text{M}) = \frac{[\text{lung phenylalanine content (nmol/g)}] - \{[\text{perfusate phenylalanine concentration (nmol/ml)}] \times [\text{sorbitol space (ml/g)}]\}}{\{[\text{total lung water (ml/g)}] - [\text{sorbitol space (ml/g)}]\}}$$

The specific radioactivity of intracellular phenylalanine was calculated as follows:

$$\text{Specific radioactivity (d.p.m./nmol)} = \frac{\{[\text{lung phenylalanine content (nmol/g)}] \times [\text{lung phenylalanine specific radioactivity (d.p.m./nmol)}]\} - \{[\text{perfusate phenylalanine concentration (nmol/ml)}] \times [\text{perfusate phenylalanine specific activity (d.p.m./nmol)}] \times [\text{sorbitol space (ml/g)}]\}}{[\text{lung phenylalanine content (nmol/g)}] - \{[\text{perfusate phenylalanine concentration (nmol/ml)}] \times [\text{sorbitol space (ml/g)}]\}}$$

In some experiments amino acids obtained from perfusate or from hydrolysed lung protein (Watkins & Morgan, 1979) were resolved more completely (Rannels *et al.*, 1974). In order to determine the distribution of radioactivity in each amino acid, fractions of the effluent from the column of the amino acid analyser were collected and radioactivity in each fraction was determined in a liquid-scintillation counter. The amino acid composition of lung proteins was determined after hydrolysis of a purified protein sample obtained from lungs washed free of blood with 0.15 M-NaCl. Amino acids were present as follows (nmol/mg of protein; mean  $\pm$  s.e.m. for four observations): aspartic acid, 647  $\pm$  6; threonine, 408  $\pm$  6; serine, 414  $\pm$  5; proline, 358  $\pm$  8; glutamic acid, 835  $\pm$  9; glycine, 597  $\pm$  12; alanine, 639  $\pm$  8; valine, 523  $\pm$  7; cysteine, 70  $\pm$  14; methionine, 115  $\pm$  7; isoleucine, 370  $\pm$  4; leucine 716  $\pm$  10; tyrosine, 222  $\pm$  4; phenylalanine, 308  $\pm$  5; lysine, 459  $\pm$  8; histidine, 157  $\pm$  3; arginine, 378  $\pm$  5. The phenylalanine content thus obtained was used for calculations of the fractional rate of protein synthesis by the following formula:

$$\text{Fractional rate (\%/day)} = \frac{\text{phenylalanine incorporation (nmol/h per mg of protein)}}{\text{phenylalanine content of lung protein (nmol/mg of protein)}} \times 24 \times 100$$

#### Isolation of tRNA from perfused lung tissue

For determination of the specific radioactivity of tRNA-bound phenylalanine, perfused lungs were frozen rapidly between blocks of aluminium cooled to the temperature of liquid N<sub>2</sub> (Wollenberger *et al.*, 1960). The frozen tissue was pulverized at the same temperature in a porcelain mortar and pestle. For extraction and purification of aminoacyl-tRNA, the lung powder was homogenized in 10 vol. of a solution containing 0.05 M-sodium cacodylate,

pH 6.0, and 0.17 M-NaCl. The homogenate was centrifuged (10000g, 10 min) and supernatant deproteinized with an equal volume of freshly distilled phenol [88% (w/v) in the same buffer] by mixing continuously for 60 min (4°C). The aqueous phase

was removed after centrifugation and re-extracted with additional phenol. Ethanol (2.5 vol.) containing

potassium acetate (20 g/l) was added to the pooled aqueous phases to precipitate the nucleic acids (-20°C). The precipitate was recovered by centrifugation (10000g, 10 min), washed three times with cold 95% ethanol, and resuspended in a small volume of distilled water. The pH was adjusted to 9-10 with NaOH; the sample was incubated at 37°C for 60 min, then readjusted to pH 4.0 with HCl. After centrifugation (10000g, 10 min), the supernatant was evaporated to dryness in an evacuated centrifuge (Savant Instruments) (McKee *et al.*, 1978; Airhart *et al.*, 1979).

#### Analysis of the specific radioactivity of [<sup>3</sup>H]phenylalanine by using [<sup>14</sup>C]dansyl-chloride

Amino acid mixtures, containing [2,6(n)-<sup>3</sup>H]-phenylalanine (Amersham/Searle) from perfusate or aminoacyl-tRNA (above), were taken up in 100  $\mu$ l of 0.1 M-NaHCO<sub>3</sub>, pH 9.8 (Airhart *et al.*, 1979). An equal volume of acetone containing 2 mM-[<sup>14</sup>C]dansyl chloride (Amersham/Searle) of known specific radioactivity was added and the sample incubated at 30°C for 30 min in the dark. Addi-

tional acetone (3.6 ml) was added to precipitate the salt; the supernatant was dried and taken up in 20  $\mu$ l of 50% (v/v) pyridine; 5  $\mu$ l was applied to a 7.5 cm  $\times$  15 cm micro-polyamide sheet (Schleicher and Schuell, Keene, NH, U.S.A.). Chromatography was as described previously (McKee *et al.*, 1978): in the first dimension (15 cm) with water/formic acid, 100:3 (v/v); in the second dimension with benzene/acetic acid, 9:1 (v/v); again in the second dimension with ethylacetate/meth-

anol/acetic acid (20:1:1, by vol.). Radioactivity was recovered from fluorescent spots identified under u.v. light by elution with 2 ml of water-saturated ethyl acetate. To determine the  $^3\text{H}/^{14}\text{C}$  ratio, samples were counted in New England Nuclear Formula 947 and corrected for efficiency of counting and spillover with a channels-ratio technique using an external standard. The specific radioactivity of [ $^3\text{H}$ ]phenylalanine was calculated as outlined earlier (McKee *et al.*, 1978).

Measurements of the specific radioactivity of phenylalanyl-tRNA were done by t.l.c. of [ $^{14}\text{C}$ ]dansyl-[ $^3\text{H}$ ]phenylalanine because this approach offered the advantages of easy application and high sensitivity. Determination of the specific radioactivity of 50–100 pmol of phenylalanine derived from tRNA required that the specific radioactivity of phenylalanine in the perfusate be increased 300- and 1200-fold at high (690  $\mu\text{M}$ ; 17.5  $\mu\text{Ci/ml}$ ) and low (8.6  $\mu\text{M}$ ; 2  $\mu\text{Ci/ml}$ ) concentrations of perfusate phenylalanine respectively. Determinations of the specific radioactivity of [ $^3\text{H}$ ]phenylalanine required that blanks derived from amino acid mixtures devoid of phenylalanine be run in parallel with each set of samples. In these blanks, radioactivity from [ $^{14}\text{C}$ ]dansyl-chloride chromatographed in the position of dansyl-phenylalanine. The amount of radioactivity detected was in direct proportion to the specific radioactivity of the [ $^{14}\text{C}$ ]dansyl-chloride employed. After subtraction of values determined by this procedure from those observed in phenylalanine-containing mixtures, chromatographic measurements were in excellent agreement with determinations made by using a Beckman amino acid analyzer. Similar observations were reported previously in experiments to determine the specific radioactivity of [ $^{14}\text{C}$ ]phenylalanine using [ $^3\text{H}$ ]dansyl-chloride (McKee *et al.*, 1978). Other control experiments showed that addition of [ $^3\text{H}$ ]phenylalanine of high specific radioactivity to extracts of unperfused lungs did not result in detectable radioactivity due to  $^3\text{H}$  in the position of the dansyl-phenylalanine spot. No  $^3\text{H}$  was detected in this position when the deacylation step was omitted from the tRNA-extraction protocol.

#### Statistics

Significance of differences between means was determined by Student's *t*-test.

#### Results

##### *Selection of a $^{14}\text{C}$ -labelled amino acid precursor for studies of protein synthesis in perfused rat lung*

An initial series of experiments was designed to select an appropriate radioactive amino acid for use as a precursor to investigate the synthesis of lung proteins. Earlier studies indicated that lung tissue lacked phenylalanine hydroxylase (Udenfriend &

Cooper, 1952; Thet *et al.*, 1977) and that this amino acid was incorporated into proteins of the perfused lung at a rate sufficient to allow quantification of radioactivity in protein (Rannels *et al.*, 1979a,b). Since an ideal precursor amino acid would not be converted to other compounds by the tissue under study (Rannels *et al.*, 1977; Waterlow *et al.*, 1978), metabolism of [ $^{14}\text{C}$ ]phenylalanine was investigated in the present lung preparation. Lungs were perfused with buffer containing normal plasma concentrations of amino acids and 690  $\mu\text{M}$ -[ $^{14}\text{C}$ ]phenylalanine. After 180 min, the distribution of radioactivity in each amino acid was determined by ion exchange chromatography, as outlined in the Materials and Methods section. More than 96% of the radioactivity present in the acid-soluble fraction of lung tissue co-eluted with the phenylalanine peak. Furthermore, the phenylalanine peak contained 95% of the radioactivity present in hydrolysates of lung proteins. No radioactivity was associated with the tyrosine peak. These observations indicated that [ $^{14}\text{C}$ ]phenylalanine was not converted to other amino acids or acid-soluble products by perfused lungs.

Additional experiments showed that [ $^{14}\text{C}$ ]phenylalanine in the perfusate equilibrated rapidly with the tissue pool of the amino acid. The [ $^{14}\text{C}$ ]phenylalanine space reached an equilibrium value of  $0.74 \pm 0.01$  ml/g within 10 min and remained stable for up to 3 h thereafter (Fig. 1a). The tissue space available to the extracellular marker [ $^3\text{H}$ ]sorbitol, remained well below values observed for [ $^{14}\text{C}$ ]phenylalanine, suggesting that rapid equilibration of the amino acid did not reflect damage to the membranes of cells in the preparation (results not shown). Additional calculations indicated that phenylalanine equilibration was a first-order process, with a half-time of 81 s (Fig. 1b).

The rate of incorporation of [ $^{14}\text{C}$ ]phenylalanine into lung protein was linear for at least 3 h of perfusion (Fig. 2, closed symbols). In order to determine how rapidly this linear rate of incorporation was attained, lungs were exposed for short periods of time (3–30 min) to perfusate containing [ $^3\text{H}$ ]phenylalanine at high specific radioactivity. Incorporation values from these experiments (Fig. 2, open symbols) fell on the line derived from the 3 h time course and displayed no lag in incorporation at early time points. These experiments complemented those shown in Fig. 1, and suggested that the pool of phenylalanine providing precursors for charging of tRNA and, thus, for the protein synthetic pathway, rapidly reached a steady-state specific radioactivity.

##### *Conditions required for estimates of the rate of protein synthesis*

The data in Fig. 3 suggested that a more detailed investigation of the nature of the pool providing

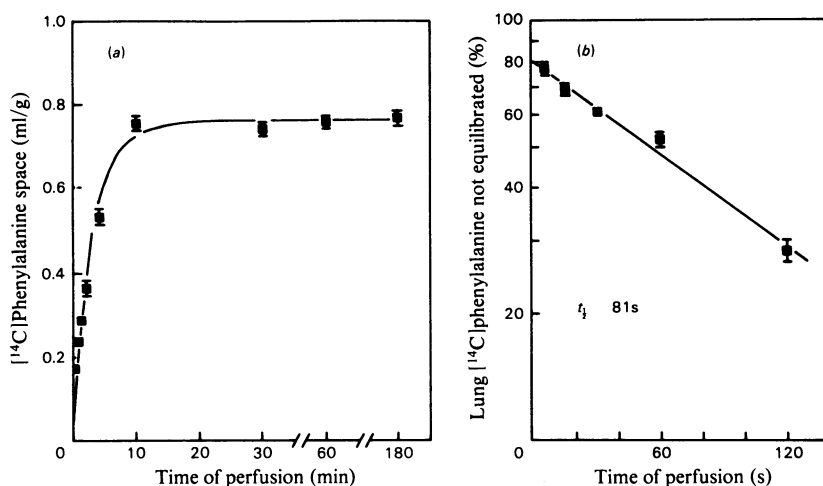


Fig. 1. Phenylalanine entry into perfused lungs

Lungs were perfused for the period indicated as described in the Materials and Methods section. The perfusate contained [ $^{14}\text{C}$ ]phenylalanine ( $690\mu\text{M}$ ) and normal plasma concentrations of other amino acids. The perfusion apparatus was modified by inserting a Y-tube just in front of the pulmonary artery. This allowed perfusate containing radioactive phenylalanine to be introduced rapidly into the pulmonary circulation and avoided the mixing time required when radioactivity was added directly to the perfusate reservoir. Phenylalanine space was calculated as described earlier (Rannels *et al.*, 1974). Values obtained after 10 min were taken to represent equilibrium (100%; b). In (b), the  $y$ -intercept was accounted for by rapid equilibration of phenylalanine in the extracellular space. Equilibration of the remaining lung phenylalanine with phenylalanine in the perfusate was described by the equation  $y = 79.9e^{0.0086t}$ , where  $y$  represents the percentage of lung phenylalanine not equilibrated with the perfusate at time  $t$  (s). Each data point represents the mean  $\pm$  s.e.m. for five or six observations. Where the s.e.m. is not shown, it did not extend beyond the symbol. Results obtained for lungs perfused with buffer equilibrated with 95% or 20%  $\text{O}_2$  were similar and were combined in the Figure.

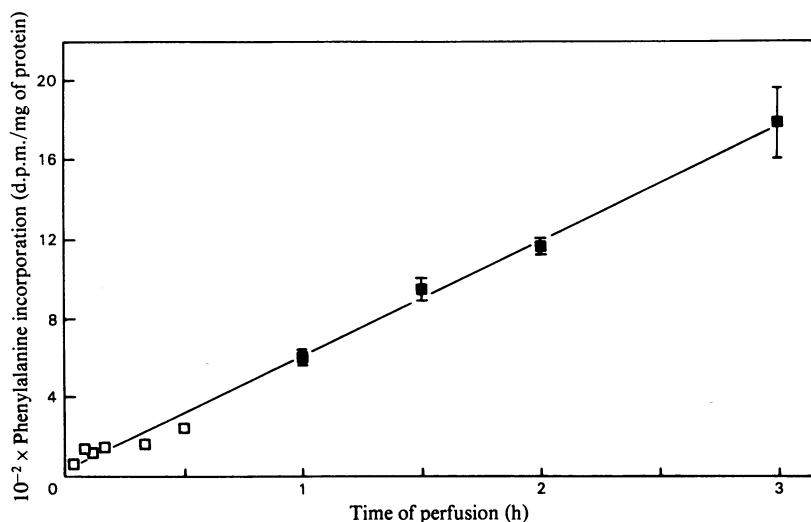


Fig. 2. Incorporation of radioactive phenylalanine into lung protein

Incorporation of radioactive phenylalanine into lung protein was determined after the period of perfusion indicated, as described in the Materials and Methods section. In experiments depicted by the closed symbols, the perfusate contained  $690\mu\text{M}$ -[ $^{14}\text{C}$ ]phenylalanine (specific radioactivity 320 d.p.m./nmol). The line was derived by least-squares linear regression from experiments of 60 min duration or longer; the equation of the line was  $y = 581x + 35$ . Data shown by the open symbols were obtained in experiments where the perfusate contained [2,6(n)- $^3\text{H}$ ]phenylalanine (specific radioactivity, 3200 d.p.m./nmol). These incorporation values were normalized to constant phenylalanine specific radioactivity. Each point represents the mean  $\pm$  s.e.m. for 7 to 18 observations. Where s.e.m. is not shown, it did not extend beyond the symbol.

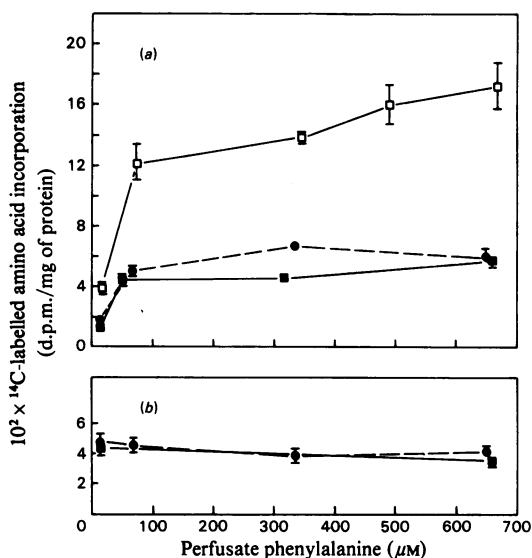


Fig. 3. Effect of perfusate phenylalanine on incorporation of radioactive amino acids into lung protein. Lungs were perfused for 60 or 180 min, as indicated, as described in the Materials and Methods section. The perfusate contained 10–690  $\mu\text{M}$ -phenylalanine. Either [ $^{14}\text{C}$ ]phenylalanine (specific radioactivity, 320 d.p.m./nmol) or [ $^{14}\text{C}$ ]histidine (specific radioactivity, 850 d.p.m./nmol) was present in experiments depicted in (a) or (b), respectively. The perfusate was equilibrated with  $\text{O}_2/\text{CO}_2$  (19:1) (circles) or  $\text{O}_2/\text{N}_2/\text{CO}_2$  (4:15:1) (squares). Closed and open symbols represent data from lungs perfused for 60 and 180 min respectively. Data represent the mean  $\pm$  S.E.M. for four to seven observations.

precursors for protein synthesis was required. As perfusate phenylalanine was increased over a 50-fold range, at constant specific radioactivity, the rate of incorporation of [ $^{14}\text{C}$ ]phenylalanine into lung protein increased 4-fold (Fig. 3a). Perfusate phenylalanine concentrations in this experiment represented the range between 0.125 and 10 times the normal plasma concentration of 69  $\mu\text{M}$ , and were plotted as measured at the end of the perfusion period. Similar effects of perfusate phenylalanine concentrations on phenylalanine incorporation were evident in 60 min experiments, whether lungs were equilibrated with a gas mixture containing 20 or 95%  $\text{O}_2$ , or when perfusion was continued for 180 min (20%  $\text{O}_2$ ). In contrast, incorporation of [ $^{14}\text{C}$ ]histidine did not decrease in the presence of low perfusate phenylalanine concentrations (Fig. 3b). Similar rates of histidine incorporation were observed in lungs exposed to 20 or 95%  $\text{O}_2$ . These experiments indicated that reduced rates of [ $^{14}\text{C}$ ]phenylalanine incorporation at low extracellular

phenylalanine concentrations did not represent inhibition of protein synthesis, but, rather, reflected an effect of extracellular phenylalanine on the specific radioactivity of phenylalanine serving as precursor for the synthetic pathway.

Fig. 4(a) shows that intracellular phenylalanine increased linearly as perfusate phenylalanine was raised over the range shown earlier. A similar relationship between extracellular and intracellular phenylalanine was maintained during 180 min of perfusion (20%  $\text{O}_2$ ) or in the presence of 95%  $\text{O}_2$  (60 min; results not shown). Extrapolation of this curve to zero extracellular phenylalanine suggested that the concentration of intracellular phenylalanine would be maintained at about 31  $\mu\text{M}$  if no phenylalanine were present in the perfusate. This amounted to nearly 50% of the intracellular phenylalanine normally present in the tissue and presumably represented phenylalanine derived from protein degradation. At low perfusate phenylalanine concentration, the specific radioactivity of intracellular phenylalanine was 20% of that of the amino acid in the perfusate after 60 min *in vitro* (Fig. 4b). After 180 min, the ratio of these specific radioactivities had nearly doubled, primarily as a result of decreased specific radioactivity of the perfusate (Table 1). As the concentration of the amino acid was increased in the perfusate, the specific radioactivity of the intracellular pool reached that of extracellular phenylalanine (Fig. 4 and Table 1). Similar relationships were apparent after 60 and 180 min of perfusion (20%  $\text{O}_2$ ) or upon exposure of the preparation to higher  $\text{O}_2$  concentrations.

The apparent rate of protein synthesis was calculated on the basis of the specific radioactivity of the extracellular or intracellular phenylalanine pool (Table 1). When perfusate phenylalanine was at the normal plasma concentration (69  $\mu\text{M}$ ), apparent rates of synthesis differed by nearly a factor of two. At lower concentrations of extracellular phenylalanine, the discrepancy became larger. In contrast, as perfusate phenylalanine was increased above the normal plasma concentration, apparent rates of protein synthesis calculated on the basis of the specific radioactivity of either pool of precursor were the same. These experiments indicated that neither the extracellular nor the total intracellular phenylalanine pool served as the sole source of amino acid for the synthetic pathway.

#### Protein synthesis determined on the basis of the specific radioactivity of phenylalanyl-tRNA

Additional experiments were carried out to define the relationship between the specific radioactivities of perfusate and tRNA-bound phenylalanine. [ $^3\text{H}$ ]phenylalanine was employed in order to achieve a 1000-fold increase in the specific radioactivity of perfusate phenylalanine. T.l.c. of [ $^{14}\text{C}$ ]dansyl-[ $^3\text{H}$ ]-

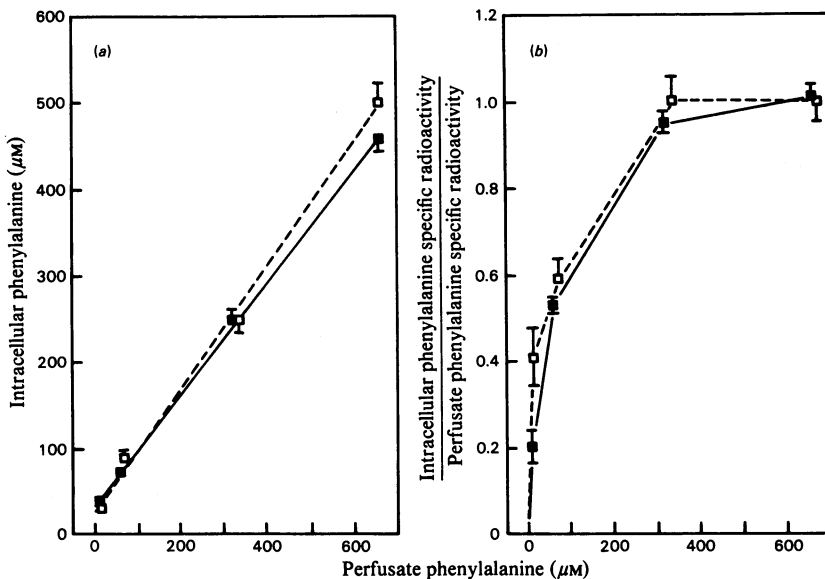


Fig. 4. Effect of perfusate phenylalanine on the concentration and specific radioactivity of intracellular phenylalanine in perfused lungs

Lungs were perfused for 60 (closed symbols) or 180 (open symbols) min with buffer equilibrated with a gas mixture containing  $O_2/N_2/CO_2$  (4:15:1). Perfusate phenylalanine was varied over the range indicated. Data were plotted as a function of the perfusate phenylalanine concentration measured at the end of the perfusion period (Table 1). The concentration and specific radioactivity of intracellular phenylalanine were calculated from determinations made on lung tissue samples using values for the sorbitol space published previously (Watkins & Rannels, 1979). These values were 0.440 and 0.520 ml/g after 60 and 180 min of perfusion respectively. In (a) equations of the lines are  $y = 0.64x + 35.40$  (60 min, closed symbols) and  $y = 0.71x + 27.40$  (180 min, open symbols).

phenylalanine was used to determine phenylalanine specific radioactivities. This approach increased the sensitivity of the determinations sufficiently to allow measurements of phenylalanine derived from the tRNA extracted from the lungs of one rat (about 1.3 g of lung). At a low perfusate phenylalanine concentration, the specific radioactivity of phenylalanyl-tRNA reached 66% of the specific radioactivity of perfusate phenylalanine after 60 min (Table 2). Thus the rate of protein synthesis calculated on the basis of the specific radioactivity of phenylalanyl-tRNA was intermediate to that calculated on the basis of specific radioactivity of phenylalanine in the perfusate (Table 2) and intracellular pools (compare Tables 1 and 2). When extracellular phenylalanine was raised to 10 times the normal concentration, the specific radioactivity of tRNA-bound phenylalanine reached that of phenylalanine in the perfusate. Under these conditions, the synthetic rate calculated from phenylalanyl-tRNA was unchanged compared with that observed at low extracellular amino acid concentration and was equal to that calculated from the specific radioactivity of phenylalanine in the perfusate.

## Discussion

Accurate estimates of the rate of protein synthesis from incorporation of radioactive amino acids into protein require that the specific radioactivity of the pool of amino acids providing precursors for the synthetic pathway be taken into account. This approach is complicated when the amino acid of interest is metabolized in pathways other than those of protein turnover or is compartmentalized within the tissue. The difficulty presented by either of these factors depends upon the amino acid chosen, as well as the tissue under study. These considerations have been reviewed in detail (Rannels *et al.*, 1977; Waterlow *et al.*, 1978; Zak *et al.*, 1979). For example, although phenylalanine served as a suitable precursor for studies of protein synthesis in hearts (Morgan *et al.*, 1971), skeletal muscle (Jefferson *et al.*, 1977) and lung (present studies; Chiang *et al.*, 1979), it was readily metabolized to tyrosine by perfused kidneys (J. A. Engle & D. E. Rannels, unpublished observations). In perfused lungs, phenylalanine offered the additional advantage that it entered the tissue rapidly (Fig. 1) and apparently reached the sites of charging of aminoacyl-tRNA without appreciable delay (Fig. 2).

Table 1. *Effect of perfusate phenylalanine on the apparent rate of protein synthesis in perfused lungs*

Lungs were perfused for 60 or 180 min with buffer equilibrated with a gas mixture containing 95% or 20% O<sub>2</sub>, as outlined in Fig. 3. The concentration and specific radioactivity of perfusate and lung phenylalanine were determined, and the specific radioactivity of intracellular phenylalanine was calculated as described in the Materials and Methods section. The apparent rate of protein synthesis was calculated from incorporation data (Fig. 3) by using the specific radioactivity of perfusate or intracellular phenylalanine measured at the end of the perfusion period. Data represent the mean  $\pm$  s.e.m. of four to eight observations. Significance of differences between means was determined only for apparent rates of protein synthesis. \*,  $P < 0.01$  versus perfusate, same experimental conditions; †,  $P < 0.05$  versus perfusate, same experimental conditions; ‡,  $P < 0.05$  versus 690  $\mu$ M-phenylalanine.

Parameter	O <sub>2</sub> in gas mixture (%)	Time of perfusion (min)	Initial perfusate [phenylalanine] ( $\mu$ M)			
			... 8.6	69	345	690
Phenylalanine specific radioactivity (d.p.m./nmol)						
Perfusate	20	60	182 $\pm$ 6	358 $\pm$ 13	287 $\pm$ 1	303 $\pm$ 14
		180	130 $\pm$ 17	290 $\pm$ 15	312 $\pm$ 9	315 $\pm$ 5
	95	60	246 $\pm$ 4	375 $\pm$ 23	344 $\pm$ 7	332 $\pm$ 3
Intracellular	20	60	36 $\pm$ 7	189 $\pm$ 4	274 $\pm$ 8	291 $\pm$ 7
		180	50 $\pm$ 7	169 $\pm$ 13	311 $\pm$ 12	333 $\pm$ 17
	95	60	48 $\pm$ 8	207 $\pm$ 24	303 $\pm$ 10	345 $\pm$ 13
Apparent rate of protein synthesis (nmol of Phe/h per mg of protein) based on the specific activity of:						
Perfusate phenylalanine	20	60	0.81 $\pm$ 0.04‡	1.25 $\pm$ 0.09‡	1.61 $\pm$ 0.07‡	1.94 $\pm$ 0.12
		180	1.00 $\pm$ 0.10‡	1.41 $\pm$ 0.14	1.54 $\pm$ 0.02	1.86 $\pm$ 0.18
	95	60	0.75 $\pm$ 0.05‡	1.37 $\pm$ 0.07‡	1.95 $\pm$ 0.04	1.85 $\pm$ 0.11
Intracellular phenylalanine	20	60	4.58 $\pm$ 0.81*‡	2.36 $\pm$ 0.10*‡	1.70 $\pm$ 0.10	1.85 $\pm$ 0.16
		180	3.64 $\pm$ 0.42*‡	2.54 $\pm$ 0.36*	1.40 $\pm$ 0.04	1.74 $\pm$ 0.17
	95	60	4.19 $\pm$ 0.37*‡	2.63 $\pm$ 0.32*‡	2.18 $\pm$ 0.07†	1.81 $\pm$ 0.16

Table 2. *Estimates of protein synthesis based on the specific radioactivity of phenylalanyl-tRNA*

Lungs were perfused for 60 min with buffer equilibrated with O<sub>2</sub>/N<sub>2</sub>/CO<sub>2</sub> (4:15:1). The specific radioactivity of perfusate and tRNA-bound phenylalanine from the same preparation were determined by using t.l.c. of [<sup>14</sup>C]dansyl-[<sup>3</sup>H]phenylalanine as described in the Materials and Methods section. Rates of phenylalanine incorporation were determined from separate lungs perfused under the same conditions. The rate of protein synthesis was calculated as outlined in Table 1. Data represent the means  $\pm$  s.e.m. for four determinations. \*,  $P < 0.05$  versus perfusate; †,  $P < 0.01$  versus perfusate.

Parameter	Perfusate [phenylalanine] ( $\mu$ M)	... 8.6	690
Phenylalanine specific radioactivity (d.p.m./pmol)			
Perfusate		197 $\pm$ 21	51 $\pm$ 3
tRNA		130 $\pm$ 12*	53 $\pm$ 4
10 <sup>-3</sup> $\times$ Phenylalanine incorporation, (d.p.m./h per mg of protein)		230 $\pm$ 10	93 $\pm$ 8
Protein synthesis (nmol/h per mg of protein)			
Perfusate		1.17 $\pm$ 0.05	1.84 $\pm$ 0.16
tRNA		1.78 $\pm$ 0.08†	1.76 $\pm$ 0.15

Finally, phenylalanine was easily and rapidly separated from other amino acids by ion exchange chromatography or by t.l.c. of the dansyl-phenylalanine derivative.

The present experiments suggested that phenylalanine was compartmentalized within the perfused

lung. Furthermore, the specific radioactivity of phenylalanine serving as precursor for protein synthesis was subject to dilution by non-radioactive phenylalanine derived from protein degradation. The latter event was most clearly reflected by the decrease in [U-<sup>14</sup>C]phenylalanine incorporation at



low, as compared with high, extracellular concentrations of the amino acid. Two lines of evidence showed that the altered incorporation did not reflect reduced rates of protein synthesis: (1) incorporation of [ $^{14}\text{C}$ ]histidine was not affected as extracellular phenylalanine was decreased and (2) rates of protein synthesis calculated on the basis of specific radioactivity of phenylalanyl-tRNA were the same when the perfusate contained high or low concentrations of phenylalanine.

A significant contribution by protein degradation to the total intracellular pool of free phenylalanine was suggested by the intercept of the line relating intracellular to extracellular phenylalanine concentrations (Fig. 4). Participation of this protein-derived amino acid in the synthetic pathway was indicated by comparison of the specific radioactivity of tRNA-bound phenylalanine with that of the amino acid in the intracellular and extracellular pools. At low extracellular phenylalanine concentration, the intermediate specific radioactivity of phenylalanyl-tRNA suggested mixing of amino acids derived from the perfusate and from protein degradation. Similarly, aminoacyl-tRNA specific radioactivities between those of the intracellular and extracellular pools were reported in liver (Khairallah & Mortimore, 1976; Vidrich *et al.*, 1977) and in heart (Martin *et al.*, 1977; McKee *et al.*, 1978), where models for compartmentalization of intracellular amino acids were proposed. In the latter system, a detailed kinetic analysis could not resolve whether mixing of amino acids from the extracellular pool and from proteolysis occurred at the level of free amino acids or subsequent to charging of aminoacyl-tRNA at several sites. At high perfusate phenylalanine concentration, equal specific radioactivities of extracellular, intracellular and tRNA-bound phenylalanine probably reflected an increase in the relative contribution of perfusate-derived amino acid to the synthetic pathway rather than elimination of compartmentalization. The practical advantage offered by these observations is that synthetic rates can be calculated accurately on the basis of specific radioactivity of perfusate phenylalanine.

In addition to complicating estimates of the specific radioactivity of amino acid precursors for protein synthesis, low extracellular phenylalanine concentrations provided the disadvantage that the specific radioactivity of the amino acid was diluted with time, in spite of a relatively large recirculating perfusate volume. This was reflected in decreasing perfusate phenylalanine specific radioactivity between 60 and 180 min *in vitro* (Table 1) and in the time-dependent changes in the intracellular/extracellular specific radioactivity ratio (Fig. 4). Progressive dilution of the radioactive amino acid pool could lead to an overestimate of the average rate of

protein synthesis when amino acid specific radioactivities were measured only at the end of the experimental period. This error is exaggerated at very low precursor concentrations, as when tracer amounts of radioactive amino acids are added to the extracellular medium at high specific radioactivity, and may be compounded under conditions where rates of protein degradation are changed. If protein-derived amino acid mixes rapidly with the free amino acid pool and does not accumulate intracellularly, the rate of dilution of the specific radioactivity of the free amino acid can provide an estimate of the rate of protein degradation (Rannels *et al.*, 1975; Chua *et al.*, 1979). In the experiments shown in Table 1, dilution of the specific radioactivity of perfusate phenylalanine between 60 and 180 min could be accounted for by release of 196 nmol of phenylalanine/h per g of lung, representing the degradation of about 0.64 mg of protein/h per g of lung. If the lungs were assumed to contain 118 mg of protein/g (Rannels *et al.*, 1979a), phenylalanine release amounted to 1.66 nmol/h per mg of protein, a rate in good agreement with the rate of synthesis (Table 2). Rates of protein synthesis measured in these experiments were equal to turnover rates of 13.8%/day. As would be expected, this value was considerably below that reported by Chiang *et al.*, (1979), calculated on the basis of release of radioactivity from lung proteins prelabelled for 5 h with [ $^{14}\text{C}$ ]phenylalanine. In contrast, Garlick *et al.* (1976), using a 6 h constant infusion of [ $^{14}\text{C}$ ]tyrosine, reported the turnover of pig lung proteins *in vivo* to be 18.3%/day. These rates were equivalent to incorporation of 2.35 nmol of phenylalanine/h per mg of protein. However, the RNA content of pig lung exceeded that of rat lung by 42%; thus, when rates were expressed on the basis of the protein synthetic capacity of the tissue (per mg of RNA), the efficiency of protein synthesis observed in the lungs of the two species was similar (rat lung perfused *in vitro*, 69.2 and pig lung *in vivo*, 64.5 nmol of phenylalanine incorporated/h per mg of RNA). The similarity between these rates may, however, be fortuitous. Millward has reported *in vivo* rates of protein synthesis per mg RNA in rat liver, kidney, heart and skeletal muscle to be higher than the 5.4 mg of protein/day per mg of RNA observed in the present experiments (Waterlow *et al.*, 1978, p. 462). Although those studies were carried out in younger animals, they raise the possibility that conditions required to support protein synthesis at rates *in vivo* may not have been optimized in the present studies. Resolution of these discrepancies requires more specific information regarding hormones and other factors which regulate the synthesis of lung proteins.

Comparisons of lungs exposed to gas mixtures containing 95% or 20%  $\text{O}_2$  showed little or no

difference, supporting earlier observations of similar protein metabolism in lungs perfused under these conditions (Watkins & Rannels, 1979). In contrast, Bassett & Fisher (1979) observed increased  $^{14}\text{CO}_2$  production from glucose as early as the first hour of exposure to hyperbaric  $\text{O}_2$ , although several other metabolic parameters were unchanged. Prolonged exposure to high  $\text{O}_2$  tensions affected the metabolism of intact lungs (Block & Fisher, 1977; Bassett & Fisher, 1979) and of lung-derived cells in culture (Simon *et al.*, 1979).

A variety of models have been proposed to account for the functional compartmentalization of amino acids in tissue and cell preparations *in vitro* (for review, see Rannels *et al.*, 1977; Waterlow *et al.*, 1978). Although the observations reported here were qualitatively similar to those made earlier in other tissues, application of specific models of amino acid compartmentalization to lung is greatly complicated by the cellular heterogeneity of the tissue (Kuhn, 1976). Five major cell populations (type I and type II pneumocytes, interstitial cells, endothelial cells and pulmonary macrophages) comprise relative volumes ranging from 15 to 26% of lung parenchymal mass (Barry *et al.*, 1979). Whether the compartmentalization observed in the present studies reflected functional barriers within or among cell types is presently unknown. Related experiments suggested that phenylalanine was compartmentalized in pulmonary macrophages in a fashion qualitatively similar to that observed in intact lungs (J. A. Hammer & D. E. Rannels, unpublished work). Information on the other major cell types present in lung tissue is not available.

This work was supported by grant HL-20344 from the National Heart, Lung and Blood Institute of the National Institutes of Health. D. E. R. is the recipient of Research Career Development Award HL-00294 from the NHLBI. We thank Dr. R. B. Low for providing access to an unpublished manuscript, Ms. K. E. Giger for excellent technical assistance, and Ms. P. A. Gering for typing this manuscript.

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