Protein turnover in pulmonary macrophages

Utilization of amino acids derived from protein degradation

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Conditions were defined under which rates of protein synthesis and degradation could be estimated in alveolar macrophages isolated from rabbits by pulmonary lavage and incubated in the presence of plasma concentrations of amino acids and 5.6 mM-glucose. Phenylalanine was validated as a suitable precursor for use in these studies: it was not metabolized appreciably, except in the pathways of protein synthesis and degradation; it entered the cells rapidly; it maintained a stable intracellular concentration; and it was incorporated into protein at measurable rates. When extracellular phenylalanine was raised to a concentration sufficient to minimize dilution of the specific radioactivity of the precursor for protein synthesis with amino acid derived from protein degradation, the specific radioactivity of phenylalanyl-tRNA was only 60% of that of the extracellular amino acid. This relationship was unchanged in cells where proteolysis increased 2.5-fold after uptake and degradation of exogenous bovine serum albumin. In contrast, albumin prevented the decrease in phenylalanine incorporation observed in macrophages deprived of an exogenous source of amino acids. These observations suggested that macrophages preferentially re-utilized amino acids derived from the degradation of endogenous, but not from exogenous (albumin), protein. However, when the extracellular supply of amino acids was restricted, substrates derived from albumin catabolism could support the protein-synthetic pathway.

Rates at which cellular proteins are synthesized and degraded are influenced by both nutritional and hormonal factors. Among those most extensively investigated is the availability of amino acids [for reviews, see Rannels *et al.* (1977) and Waterlow *et al.* (1978)]. The extracellular supply of amino acids may be influenced by diet and/or by other factors that change the concentration of these compounds in plasma. In addition, degradation of endogenous protein can provide a source of substrate for protein synthesis at the cellular level. In liver, for example, proteolysis contributed amino acids to a compartmented intracellular pool that could supply a significant fraction of the amino acid charged to tRNA (Khairallah & Mortimore, 1976; Ward & Mortimore, 1978). In prolonged amino acid star-

vation, however, this pathway of re-utilization could not provide substrate for net synthesis of protein. In contrast, amino acids derived from the degradation of exogenous proteins would appear to be a potential (net) source of substrate, especially in cells such as macrophages, which demonstrate high rates of endocytic activity and are capable of the uptake and degradation of extracellular proteins to the level of free amino acids (Ehrenreich & Cohn, 1968; Pratten *et al.*, 1977; Silverstein *et al.*, 1977). Although cell nutrition is a function that has been proposed for endocytosis, essentially no information is available regarding the fate of amino acids derived from the degradation of exogenous proteins (Silverstein *et al.*, 1977).

The synthesis of macrophage proteins has been reported to be inhibited by aqueous extracts of cigarette smoke (Low, 1974), trypsin treatment (Ulrich, 1976) and washing by centrifugation (Ulrich, 1977), and to be decreased in suspended cells as compared with those adhered in culture dishes (Leffingwell & Low, 1975). It is not known whether

Abbreviations used: SDS, sodium dodecyl sulphate; dansyl, 5-dimethylaminonaphthalene-1-sulphonyl.

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these effects involve changes in amino acid availability, nor has the effect of amino acid deprivation been explored directly. These factors may, however, be relevant to the role of the macrophage in lung defence, since starvation is known to be associated with impaired macrophage function (Weeks & Kavas, 1979).

Thus the present studies evaluated: (1) conditions under which rates of synthesis and degradation of macrophage proteins could be measured; (2) the extent to which an extracellular protein, bovine serum albumin, was degraded by pulmonary alveolar macrophages; (3) the relative contribution of amino acids derived from exogenous albumin, endogenous protein and the extracellular pool to the charging of aminoacyl-tRNA; and (4) the role of amino acids derived from an exogenous protein source in providing substrates for protein synthesis in cells deprived of free amino acids.

Materials and methods

Cell isolation and incubation

Pulmonary macrophages were isolated (Myrvik *et al.*, 1961) from 2–3 kg male New Zealand White rabbits (Charles River), as detailed elsewhere (Hammer & Rannels, 1981). The yield per rabbit averaged 6×10^7 – 8×10^7 cells, of which $3.2 \pm 0.5\%$ were lymphocytes and polymorphonuclear leukocytes (ten observations).

The cells were washed two to three times by centrifugation (100 g, 5 min), were resuspended in incubation buffers of differing composition (2×10^6 – 3×10^6 cells/ml), and were incubated in suspension (37°C) as described previously (Hammer & Rannels, 1981). Unless otherwise indicated, the incubation buffer was Krebs–Henseleit (1932) bicarbonate buffer (KH buffer) supplemented with 5.6 mM-glucose and equilibrated with $O_2/N_2/CO_2$ (4:15:1). For lung lavage, bivalent cations were omitted from KH buffer and were replaced with NaCl (Brain & Frank, 1973). Other additions to this buffer are detailed below. The concentrations of amino acids added to simulate plasma were (μM): Asp, 14; Asn, 68; Glu, 120; Gln, 652; Ala, 326; Val, 188; Leu, 166; Ile, 108; Ser, 247; Thr, 203; Met, 54; Cys, 100; Pro, 108; Phe, 69; Tyr, 69; Trp, 100; His, 52; Lys, 380; Arg, 119; and Gly, 336 (Watkins & Rannels, 1980), unless indicated otherwise. Fraction V bovine serum albumin (Pentex; Miles Laboratories) and ‘essentially fatty-acid-free’ bovine serum albumin (Sigma) were dialysed before use against 20 vol. of KH buffer for 18 h at 4°C, and were filtered through a 0.45 μm -pore size nitrocellulose filter (Millipore, HAWP).

Cell number was estimated using a haemocytometer; at least 200 cells were counted for each

determination. Viability was assessed by the ability of cells to exclude Trypan Blue (Medzihradsky & Marks, 1975), and from the rate of release of cellular lactate dehydrogenase (Stolzenbach, 1966).

Measurement of phenylalanine uptake and metabolism

To determine the extent to which macrophages metabolized phenylalanine, cells incubated 180 min in the presence of [U - ^{14}C]phenylalanine (Amersham/Searle) were collected by centrifugation and precipitated with 5% (v/v) $HClO_4$ (4°C). The neutralized supernatant was freeze-dried and the residue was subjected to ion-exchange chromatography (Beckman, model 119CL) (Watkins & Rannels, 1980). Typically, 97–99% of the radioactivity applied to the column was recovered. The precipitated cell protein was washed, hydrolysed in 6 M-HCl and the resulting free amino acids were analysed as described above. The production of $^{14}CO_2$ from [U - ^{14}C]phenylalanine (3.6 μM ; 520 $\mu Ci/\mu mol$) was measured by using cells incubated for 90 min in flasks capped tightly with serum stoppers. The generation of 3H_2O from phenyl[2,3- 3H]alanine (0.47 μM ; 16.4 mCi/ μmol ; Amersham/Searle) was measured as described by Williams *et al.* (1981). The time course of [3H]phenylalanine entry into pulmonary macrophages was measured by using [^{14}C]sucrose as a marker of the extracellular space. Rapid separation of cells from medium was accomplished by centrifugation of the cells through silicone oil (Strauss *et al.*, 1976); phenylalanine space was calculated as described previously (Watkins & Rannels, 1980).

Estimation of intracellular phenylalanine concentration

Cells were rapidly chilled to 4°C and [3H]-sorbitol (New England Nuclear) was added; samples were centrifuged and the wet weight of the pellet was determined gravimetrically. Cell-pellet protein and buffer samples were precipitated with 5% (w/v) $HClO_4$. Intracellular phenylalanine concentration was calculated as described previously (Watkins & Rannels, 1980).

[^{14}C]Phenylalanine incorporation into macrophage proteins

For determination of the specific radioactivity of macrophage proteins, precipitated cell samples were washed and the rate of radioactive amino acid incorporation (d.p.m./h per mg of protein) was calculated (Hammer & Rannels, 1981). Data were normalized to reflect an extracellular phenylalanine specific radioactivity of 1.0 $\mu Ci/\mu mol$. The apparent rate of protein synthesis (nmol of phenylalanine incorporated/h per mg of protein), as well as

fractional rates of synthesis were calculated as described previously (Watkins & Rannels, 1980).

Determination of the specific radioactivity of phenylalanyl-tRNA and of extracellular phenylalanine

Aminoacyl-tRNA was extracted by a method modified from that described by Airhart *et al.* (1979) (Method 1). Approx. 6×10^7 cells were incubated in 6 ml of KH buffer for 30 min (37°C) in the presence of [^3H]phenylalanine. Rapidly chilled (4°C) cells were pelleted by centrifugation (100g, 2 min) and were homogenized with a Kinematica Polytron (top speed, 20 s) in 10 ml of a solution containing 5 ml of 50 mM-sodium cacodylate/0.1 M-NaCl, pH 6.0, and 5 ml of freshly distilled phenol (88% in the aforementioned buffer). Bentonite (2.2 mg), sized and deionized as described by Wallyn *et al.* (1974), was added and the suspension was agitated for 30 min at 4°C . The aqueous phase was removed after centrifugation (10000g, 10 min) and re-extracted with an equal volume of phenol for 30 min (4°C). After a second centrifugation, nucleic acids were precipitated from the aqueous phase by addition of 0.1 vol. of 20% (w/v) potassium acetate, pH 5.0, and 2.5 vol. of 95% (v/v) ethanol (-20°C). After at least 18 h at -20°C , the precipitated nucleic acids were recovered by centrifugation (10000g, 5 min), washed five times in 70% ethanol, dried under a stream of N_2 gas, and dissolved in 0.2 ml of water. The pH was adjusted to 9.5–10.0 with 0.1 M-NaOH and the sample was incubated (37°C , 60 min) to deacylate tRNA. Nucleic acids were precipitated by lowering the pH to 3.0 with 0.2 M-HCl. The supernatant was evaporated to dryness in an evacuated centrifuge (Savant Instruments) and stored desiccated (-20°C) until analysed.

In other experiments, cells were sampled by the methods of Ogilvie *et al.* (1979) (Method 2) after precipitation with trichloroacetic acid (final concn. 2%). After agitation of the flask for 1 min at 37°C , 0.1% SDS was added to a final concentration of 0.01%. After it had been shaken for an additional 30 s, the precipitated material was recovered by centrifugation (10000g, 20 min) and resuspended in 4 ml of an ice-cold solution containing 0.25 M-acetic acid, 0.005% SDS and 1 mM-EDTA, pH 4.6. To this was added an equal volume of ice-cold freshly distilled 88% (v/v) phenol (in the aforementioned buffer). The suspension was sonicated on ice for 60 s (Branson sonicator, 70–80 W), homogenized (Polytron, top speed, 20 s), then treated as described above.

In control experiments, when $30 \mu\text{Ci}$ of [^3H]phenylalanine were added to cells that had been incubated in KH buffer containing $345 \mu\text{M}$ -phenylalanine, less than 0.00004% of the radioactive amino acid remained after the subsequent washing pro-

cedures. Moreover, the contaminant represented less than 1% of the average amount of radioactive phenylalanine present in the deacylated tRNA samples.

The specific radioactivity of [^3H]phenylalanine was determined by t.l.c. of [^{14}C]dansyl[^3H]phenylalanine as described previously (Watkins & Rannels, 1980), except that dried amino acid residues were dissolved in $20 \mu\text{l}$ of 0.1 M- NaHCO_3 and were made to react with $20 \mu\text{l}$ of [^{14}C]dansyl chloride in acetone ($2\text{--}4 \mu\text{mol/ml}$, $90\text{--}120 \mu\text{Ci}/\mu\text{mol}$; New England Nuclear). Buffer samples were dansylated similarly, except that $5 \mu\text{l}$ of a 1:5 dilution of buffer was added to $15 \mu\text{l}$ of 0.1 M- NaHCO_3 .

Chromatography of dansylated amino acids was performed in two dimensions by the methods of Hartley (1970) and Peterson *et al.* (1973). Samples were prepared, chromatographs were developed and the specific radioactivity of phenylalanine was calculated as described by Watkins & Rannels (1980). Before calculation of phenylalanine specific radioactivity, dansylphenylalanine radioactivity was corrected for radioactivity that chromatographed in the position of dansylphenylalanine in samples lacking phenylalanine (McKee *et al.*, 1978). For buffer samples, KH buffer containing plasma concentrations of amino acids (except phenylalanine) was diluted and analysed as described above (blank: 252 d.p.m.). For tissue samples, $20 \mu\text{l}$ of 0.1 M-sodium bicarbonate was made to react with [^{14}C]dansyl chloride (blank: 49 d.p.m.).

Degradation of endogenous macrophage proteins

Cells were incubated in buffer devoid of phenylalanine and bovine serum albumin, but containing $20 \mu\text{M}$ -cycloheximide (Sigma). Samples (0.5 ml) were chilled to 4°C and the cells were pelleted by centrifugation. The cell pellet was solubilized in NaOH for determination of protein content (Lowry *et al.*, 1951), bovine serum albumin being used as standard. Buffer samples were assayed in triplicate for phenylalanine concentration by using the tRNA-binding assay of Rubin & Goldstein (1970), as modified previously (Chua *et al.*, 1978). For measurements of the net uptake or release of phenylalanine in the absence of cycloheximide, cells were incubated in KH buffer containing $2.1 \mu\text{M}$ -phenylalanine in order to provide sufficient substrate for protein synthesis (Hammer & Rannels, 1981).

The rate of degradation of endogenous macrophage proteins was determined in the presence of albumin by measuring the generation of trichloroacetic acid-soluble radioactivity from cells in which proteins had been prelabelled with radioactive phenylalanine as described above. To investigate the effect of albumin on the degradation of macrophage proteins of longer half-life, the rate of proteolysis in

cell monolayers was determined by a modification of the protocol of Dean (1979).

Degradation of bovine serum albumin

In order to determine the rate at which macrophages degraded serum albumin, cells were incubated in buffer devoid of phenylalanine, but containing 2% of fraction V bovine serum albumin (BSA in the equation below) and 20 μ M-cycloheximide. The phenylalanine content of HClO₄ extracts was measured by ion-exchange chromatography (Watkins & Rannels, 1980), with leucylalanine as an internal standard. Cell protein concentration per ml of buffer was determined on separate samples (0.5 ml) of cells that were washed twice with 4 ml of saline (0.9% NaCl) (4°C). The rate of phenylalanine release was calculated as described above. The rate of albumin clearance was calculated by using the formula:

$$\begin{aligned} \text{Albumin clearance} &= \text{phenylalanine release due to albumin (nmol/h per mg of protein)} \times \\ (\mu\text{l/h per } 10^6 \text{ cells}) & \times \frac{0.151 \text{ mg of protein}}{10^6 \text{ cells}} \times \frac{\text{nmol of BSA}}{27 \text{ nmol of phenylalanine}} \times \frac{66.2 \mu\text{g of BSA}}{\text{nmol of BSA}} \times \frac{\mu\text{l of medium}}{\mu\text{g of BSA}} \end{aligned}$$

Other methods

Unless stated otherwise, radioactivities of samples were counted in 10 ml of Formula 947 scintillation fluid (New England Nuclear) in a liquid-scintillation counter (Beckman, model 250). Data were corrected for spillover and for counting efficiency by using an external-standard-ratio method. Protein was assayed by the method of Lowry *et al.* (1951). Dry weight (0.224 mg/10⁶ cells) was obtained by weighing pellets containing known numbers of cells after drying overnight. The intracellular water volume (2.8 μ l/mg dry wt.) was obtained by subtracting the sorbitol space and the dry weight from the wet weight of pellets containing known numbers of cells.

Except where indicated, data in the Figures and Tables are presented as means $\pm$ S.E.M. Significance was determined with Student's *t* test by using a two-tailed analysis; values with *P* < 0.05 were considered to be significantly different.

Results

Estimation of protein synthesis in rabbit pulmonary macrophages

Phenylalanine was chosen for use in studies of protein synthesis in rabbit pulmonary macrophages because lung tissue lacks phenylalanine hydroxylase activity (Udenfriend & Cooper, 1952) and because these cells appeared not to metabolize the amino acid (Woodside & Massaro, 1979). When macrophages were incubated for 3 h under conditions used

to measure protein synthesis, amino acid analysis of HClO₄ extracts of cell pellets and cell protein hydrolysates revealed that 97 and 99% of the radioactivity recovered in the respective samples was in the phenylalanine peak. Similar observations were made when the cells were incubated under the conditions of protein-degradation experiments (see below). These results suggested that the conversion of phenylalanine into other amino acids or acid-soluble metabolites was not significant. Additional studies indicated that oxidation of [¹⁴C]phenylalanine to ¹⁴CO₂ was negligible. Finally, cells incubated in buffer containing phenyl[2,3-³H]alanine, plasma concentrations of other amino acids and 20 μ M-cycloheximide produced negligible amounts of ³H₂O. In the absence of other amino acids, ³H₂O production increased somewhat, but represented degradation of less than 0.25% of the added radioactive phenylalanine/h. When macrophages

were incubated under conditions used in subsequent estimates of protein synthesis, the [³H]phenylalanine space reached a value of approx. 2 ml/g in less than 60 s, then remained constant for at least 120 min. Taken together, these data indicated that radioactive phenylalanine was suitable for use in measurements of protein turnover in rabbit pulmonary macrophages.

The data in Table 1 shows that as extracellular phenylalanine concentration was varied from 4.3 μ M to 690 μ M, with phenylalanine specific radioactivity held constant, incorporation of [¹⁴C]phenylalanine into protein increased 3-fold. Extracellular phenylalanine concentrations in this experiment ranged between one-sixteenth and ten times the plasma concentration (69 μ M). No effect of extracellular phenylalanine concentration on histidine incorporation was observed. These results showed that changing extracellular phenylalanine over this range of concentrations did not alter the rate of protein synthesis, and indicated that decreased incorporation at low extracellular phenylalanine concentrations was due to an effect of extracellular phenylalanine concentration on the specific radioactivity of the phenylalanine pool serving as precursor for the synthetic pathway. Over longer periods of incubation, an extracellular phenylalanine concentration of at least 345 μ M was required to maintain optimal rates of incorporation. When the concentration of extracellular phenylalanine was increased from 345 μ M to 5 mM, [¹⁴C]phenylalanine incorporation

Table 1. *Effect of extracellular phenylalanine on incorporation of ^{14}C -labelled amino acids into macrophage proteins*
Cells were incubated for 40 min in KH buffer equilibrated with O_2/CO_2 (19:1) and containing 2% fraction V bovine serum albumin, plasma concentrations of 19 amino acids and [^{14}C]phenylalanine (sp. radioactivity $1.0\ \mu\text{Ci}/\mu\text{mol}$) at concentrations ranging from 4.3 to $690\ \mu\text{M}$. In a second experiment, cells were incubated as above, except that $520\ \mu\text{M}$ -[^{14}C]histidine was added (sp. radioactivity $1.0\ \mu\text{Ci}/\mu\text{mol}$). Additional studies showed that lowering oxygen in the gas phase to 20% ($\text{O}_2/\text{N}_2/\text{CO}_2$; 4:14:1) had no effect on the incorporation of [^{14}C]phenylalanine ($345\ \mu\text{M}$) into the macrophage proteins (95% O_2 , $13\,266 \pm 1100$; 20% O_2 , $14\,248 \pm 235$ d.p.m./h per mg of protein); thus subsequent experiments were performed at the lower, more physiological, oxygen concentration. The amino acid composition of macrophage proteins, determined after hydrolysis of a purified protein sample, was as follows (nmol/mg of protein): aspartic acid, 638 ± 5 ; threonine, 376 ± 18 ; serine 352 ± 27 ; proline, 345 ± 5 ; glutamic acid, 780 ± 15 ; glycine, 510 ± 7 ; alanine, 557 ± 2 ; valine, 421 ± 15 ; half-cystine, 94 ± 17 ; methionine, 132 ± 1 ; isoleucine, 352 ± 19 ; leucine, 655 ± 8 ; tyrosine, 205 ± 12 ; phenylalanine, 280 ± 5 ; lysine, 433 ± 8 ; histidine, 150 ± 2 ; arginine, 380 ± 12 . These data account for lower rates of histidine incorporation as compared with phenylalanine. All results are means $\pm$ S.E.M. for three to five separate determinations. * $P < 0.05$ versus $345\ \mu\text{M}$ -phenylalanine. Abbreviation used: N.D., not determined.

[Extracellular phenylalanine] (μM)	Radioactive amino acid	Incorporation (d.p.m./40 min per mg of protein)	
		[^{14}C]Phenylalanine	[^{14}C]Histidine
4.3		$3388 \pm 321^*$	5118 ± 71
8.6		$5424 \pm 415^*$	N.D.
34.5		$7911 \pm 375^*$	N.D.
69		8472 ± 265	N.D.
345		9481 ± 405	N.D.
690		9886 ± 532	4811 ± 131

did not increase further ($345\ \mu\text{M}$: $32\,286 \pm 735$; 5 mM: $34\,178 \pm 1320$ d.p.m./2 h mg of protein; $n = 3$). Thus, given the duration of incubation and the average cell concentration used in these experiments, an extracellular phenylalanine concentration of $345\ \mu\text{M}$ or higher was required to attain maximal suppression of the utilization of proteolysis-derived phenylalanine in the pathway of protein synthesis.

Other studies showed that the intracellular concentration of phenylalanine (y) increased linearly as extracellular phenylalanine (x) was raised from $4\ \mu\text{M}$ to $345\ \mu\text{M}$ (results not shown). The equation of the line relating these parameters ($y = 1.71x + 46.5$) indicated that phenylalanine was concentrated intracellularly. These results, in common with those above, suggested that proteolysis-derived phenylalanine could dilute the specific radioactivity of an intracellular precursor pool more effectively as the extracellular phenylalanine concentration was lowered.

Dilution of the specific radioactivity of the pool of phenylalanine serving as precursor for protein synthesis was less evident when bovine serum albumin was absent from the medium (Fig. 1). The effect of albumin was more apparent as the time of incubation was increased (a) and as the extracellular phenylalanine concentration was decreased (b). For example, as compared with $69\ \mu\text{M}$ -phenylalanine, where no effect of albumin was observed; at $4.3\ \mu\text{M}$, [^{14}C]phenylalanine incorporation declined by 70% when albumin was present, but only by 42%

when the protein was absent from the medium (Fig. 1b). Taken together with the fact that macrophages would be expected to take up and degrade exogenous protein, these results suggested a significant contribution of albumin-derived amino acid to the pool of precursors for protein synthesis.

Role of albumin in providing amino acids for the pathway of protein synthesis.

To investigate further the possibility that albumin-derived amino acid was used for protein synthesis, changes in the extracellular concentration of phenylalanine were measured as a function of time, with or without bovine serum albumin (Fig. 2). Because phenylalanine was not metabolized except in protein synthesis and degradation, net changes in the free amino acid reflected the balance between these pathways. Whereas net release of phenylalanine was observed when macrophages were incubated with 2% albumin, net uptake of the amino acid was evident when albumin was absent (Fig. 2a). These results suggested that the cells did degrade significant amounts of exogenous albumin to free amino acid. Additional experiments were designed to quantify the rate of albumin degradation in cells in which protein synthesis was inhibited. Fig. 2(b) shows that when macrophages were incubated without albumin in the presence of cycloheximide ($20\ \mu\text{M}$), release of phenylalanine into the medium was linear over 3 h (6.03 nmol of phenylalanine released/h per mg of cell protein). Because the intracellular concentration of phenylalanine, which

Fig. 1. Effect of albumin on the incorporation of $[^{14}\text{C}]$ phenylalanine at low extracellular phenylalanine concentrations. Macrophages were incubated in KH buffer containing plasma concentrations of 19 amino acids and 4.3, 8.6, 34.5 or 69 μM -phenylalanine, with (closed symbols) or without (open symbols) 2% fraction V bovine serum albumin. $[^{14}\text{C}]$ Phenylalanine was added at a final specific radioactivity of $5.0 \mu\text{Ci}/\mu\text{mol}$ in all cases. (a) Incorporation of $[^{14}\text{C}]$ phenylalanine as a function of time. Incorporation in the presence of 34.5 μM -phenylalanine was omitted from this part of the Figure. (b) Incorporation values at 120 min were expressed as a percentage of the incorporation measured in the presence of 69 μM -phenylalanine with 2% (■) and without (□) albumin. The results are from a single experiment.

Fig. 2. Effect of albumin on the release of phenylalanine from pulmonary macrophages. (a) Cells were incubated in KH buffer containing plasma concentrations of 19 amino acids and 2.1 μM -phenylalanine, with (■) or without (□) 2% fraction V bovine serum albumin. The buffer phenylalanine concentration was measured at the times indicated; the results were normalized to 2×10^6 cells/ml. Values represent the mean $\pm$ S.E.M. of three to four separate determinations. (b) Macrophages were incubated as above, except that 20 μM -cycloheximide was present and phenylalanine was omitted from the original buffer. Results are means $\pm$ S.E.M. for seven to eight separate observations.

Fig. 3. *Degradation of macrophage proteins prelabelled with $[^3\text{H}]$ phenylalanine*

Pulmonary macrophage proteins were prelabelled during 3 h incubation with KH buffer containing plasma concentrations of 19 amino acids and $4.3 \mu\text{M}$ $[^3\text{H}]$ phenylalanine ($60 \mu\text{Ci}/\mu\text{mol}$). (a) After prelabelling, the cells were washed three times and incubated in KH buffer as described above, but without $[^3\text{H}]$ phenylalanine, and containing the concentration of phenylalanine indicated. (b) Prelabelled cells were incubated in the presence of 5 mM non-radioactive phenylalanine, with (■) and without (□) 2% fraction V bovine serum albumin, and degradation was measured as described in the Materials and Methods section.

represented less than 2% of the total phenylalanine released by 10^6 cells, did not change significantly during the experiment (0 min, $39 \pm 14 \mu\text{M}$; 180 min, $56 \pm 20 \mu\text{M}$; four observations each), the rate of appearance of phenylalanine in the medium provided a direct index of the rate of protein degradation. Addition of 2% bovine serum albumin to the medium increased phenylalanine release approx. 2.5-fold (Fig. 2b). When the cells were removed after 60 or 120 min and incubation of the medium was continued, no further increase in phenylalanine concentration was observed (180 min phenylalanine concentration = $100.6 \pm 2.1\%$ of that measured when the cells were removed; four observations). The degradation of exogenous albumin appeared therefore to be a cell-mediated and presumably intracellular event. If the addition of albumin did not affect the degradation of endogenous macrophage proteins, the increment of phenylalanine release observed in the presence of the protein (Fig. 2b) would represent albumin degradation.

The experiments illustrated by Fig. 3 examined the effect of albumin on the rate of degradation of endogenous macrophage proteins. The generation of acid-soluble radioactivity from macrophage proteins prelabelled with $[^3\text{H}]$ phenylalanine was maximal when 5 mM non-radioactive phenylalanine was present to suppress re-utilization (Fig. 3a). The rate

of breakdown of endogenous proteins labelled in this experiment, primarily those with short half-lives, was not affected when 2% albumin was present (Fig. 3b).

Additional experiments confirmed and extended these results by assessing the degradation of proteins with longer half-lives. Adherent macrophage monolayers were prelabelled with $[^3\text{H}]$ phenylalanine for 18 h in medium without albumin. The degradation of cellular proteins was measured over 180 min with and without 2% albumin present, either immediately, or 8 h after prelabelling. The fraction of the initial radioactive protein degraded per h differed nearly 2-fold for the two intervals of measurement (2.16 ± 0.04 versus $1.27 \pm 0.04\%/h$); however, the rate of degradation of both classes of proteins was unaffected by albumin (2.08 ± 0.02 and $1.24 \pm 0.02\%/h$). Finally, the rate of phenylalanine release was directly proportional to albumin concentration over a 300-fold range (Fig. 4). Approx. 12.6 nmol of phenylalanine were released during 180 min per mg of macrophage protein for each 1% of albumin in the medium. Taken together, these experiments indicated that significant amounts of free amino acid were generated by pulmonary macrophages from the hydrolysis of exogenous fraction V bovine serum albumin in a dose-dependent fashion and that these amino acids could be utilized for synthesis of cellular protein.

Fig. 4. Effect of extracellular albumin concentration on the rate of albumin degradation

Cells were incubated as described in Fig. 2(b) in the presence of 0.02–6.0% fraction V bovine serum albumin. Phenylalanine release after 180 min of incubation was corrected for that due to the degradation of endogenous proteins. Values are the means for two separate experiments, except at 2.0% albumin, which represents the mean $\pm$ S.E.M. of seven separate observations.

At extracellular concentrations of phenylalanine below those in the plasma, a significant contribution of albumin-derived amino acids to the charging of phenylalanyl-tRNA was observed by comparing rates of [14 C]phenylalanine incorporation in cells incubated with or without the exogenous protein (Fig. 1). At higher concentrations of phenylalanine, no decrease in the rate of incorporation was observed when albumin was present (results not shown). These observations suggested that albumin-derived amino acids may not be utilized for protein synthesis in preference to those present in the medium. Direct investigation of this point required that the steady-state specific radioactivity of phenylalanyl-tRNA be measured. The experiments illustrated by Fig. 5 showed that when the extracellular [14 C]phenylalanine concentration was $345 \mu\text{M}$, the specific radioactivity of macrophage proteins increased linearly during 180 min of incubation. Incorporation of radioactive phenylalanine was linear from zero time (Fig. 5, inset), indicating that the specific radioactivity of phenylalanyl-tRNA rapidly reached a steady-state value. Thus subsequent determinations of the specific radioactivity

Fig. 5. Time course of [14 C]phenylalanine incorporation into macrophage proteins

Cells were incubated as described in Fig. 1 in the presence of $345 \mu\text{M}$ -[14 C]phenylalanine (sp. radioactivity $1.0 \mu\text{Ci}/\mu\text{mol}$). Results are means $\pm$ S.E.M. for the number of separate determinations shown in parentheses. The equation of the line was $y = 207x + 686$, where y (incorporation) is in d.p.m./mg of protein and x (incubation period) is in min. Inset: cells were treated similarly, except that [^3H]phenylalanine was added at a specific radioactivity of $25 \mu\text{Ci}/\mu\text{mol}$. The results from a single experiment were adjusted to reflect a phenylalanine specific radioactivity of $1.0 \mu\text{Ci}/\mu\text{mol}$. The units of the ordinate and abscissa are the same as those shown on the main panel.

of phenylalanyl-tRNA were made after 30 min of incubation.

Results of these measurements are shown in Table 2. When cells were incubated in buffer containing $345 \mu\text{M}$ -[^3H]phenylalanine ($500 \mu\text{Ci}/\mu\text{mol}$) and 2% fraction V bovine serum albumin, and the tRNA was extracted by Method 1, the specific radioactivity of tRNA-bound phenylalanine was 64% of that of extracellular phenylalanine. A similar relationship between these values was observed when aminoacyl-tRNA was extracted from cells rapidly precipitated with trichloroacetic acid (Method 2). The second protocol was employed to avoid changes in aminoacyl-tRNA specific radioactivity that might result if sampling methods were not sufficiently rapid (Ogilvie *et al.*, 1979). The apparent rate of protein synthesis (nmol of phenylalanine incorporated/h per mg of cell protein) calculated on the basis of the

Table 2. Relationship between the specific radioactivity of extracellular and tRNA-bound phenylalanine

Cells were incubated for 30 min in KH buffer (10^7 cells/ml) containing plasma concentrations of 19 amino acids and $345 \mu\text{M}$ -phenylalanine. Fraction V bovine serum albumin (2%) was present as indicated. $[2,6\text{-}^3\text{H}]\text{Phenylalanine}$ (Amersham/Searle) was added to a final specific radioactivity of approx. $500 \mu\text{Ci}/\mu\text{mol}$. The specific radioactivity of extracellular phenylalanine and phenylalanyl-tRNA was determined by measurement of $[^{14}\text{C}]\text{dansyl}[^3\text{H}]\text{phenylalanine}$ radioactivity. The average radioactivity in dansylphenylalanine spots derived from tRNA was: Method 1 2785 ± 641 ^3H d.p.m.; 1257 ± 329 ^{14}C d.p.m.; Method 2: 1843 ± 659 ^3H d.p.m.; 710 ± 177 ^{14}C d.p.m. These results corresponded to an average of approx. 5 or 3 pmol of phenylalanine derived from tRNA per sample for Methods 1 or 2 respectively. Results are means $\pm$ s.e.m. for the numbers of separate determinations shown in parentheses.

* $P < 0.01$ versus extracellular phenylalanine under the same conditions.

Parameter	Bovine serum albumin	Cell extraction protocol ...	Method 1 (cell centrifugation)	Method 2 (precipitation with trichloroacetic acid)
$[^3\text{H}]\text{Phenylalanine}$ specific radioactivity (d.p.m./pmol)				
Extracellular phenylalanine	+		1043 ± 90 (5)	1098 ± 22 (4)
	—		1074 ± 119 (4)	1100 ± 29 (3)
tRNA-derived phenylalanine	+		675 ± 79 (5)*	585 ± 92 (4)*
	—		616 ± 114 (4)*	590 ± 141 (3)*
$[^3\text{H}]\text{Phenylalanine}$ specific radioactivity ratio ($100 \times \text{tRNA/extracellular}$)				
	+		64.4 ± 4.3 (5)	53.4 ± 8.4 (4)
	—		56.3 ± 5.8 (4)	53.1 ± 11.9 (3)

specific radioactivity of extracellular phenylalanine was more than 40% below that calculated on the basis of phenylalanyl-tRNA specific radioactivity.

In similar studies of intact lung tissue (Watkins & Rannels, 1980), an extracellular phenylalanine concentration sufficient to obtain maximal rates of phenylalanine incorporation led to equal specific radioactivities of the amino acid in the extracellular and aminoacyl-tRNA pools. Under those conditions, rates of protein synthesis could be calculated accurately on the basis of the specific radioactivity of extracellular phenylalanine; in the present studies, this was clearly not the case (Table 2). The observed decrease in the specific radioactivity of tRNA-derived phenylalanine, as compared with the extracellular amino acid, might have reflected preferential re-utilization of non-radioactive phenylalanine derived from the degradation of exogenous albumin. When 2% albumin was omitted from the medium, however, no changes in the specific radioactivities of extracellular or tRNA-bound phenylalanine or in the apparent rate of protein synthesis were observed, with either method of extraction of aminoacyl-tRNA (Table 2). Thus, in contrast with phenylalanine from the breakdown of endogenous protein, albumin-derived phenylalanine did not appear to be preferentially charged to tRNA under these conditions of incubation.

Effect of albumin in amino acid-deprived macrophages

A final series of experiments sought to define a role for amino acids derived from the degradation of bovine serum albumin in the support of protein synthesis in cells deprived of exogenous amino acids.

Incorporation of $[^{14}\text{C}]\text{phenylalanine}$ was measured in the presence of zero ($0\times$), one ($1\times$) and five ($5\times$) times plasma concentrations of amino acids and high of phenylalanine (Fig. 6). Rates of phenylalanine incorporation in the presence of $1\times$ and $5\times$ plasma concentrations of amino acids did not differ significantly, either in the presence or absence of 2% albumin. When amino acids were omitted from the medium, phenylalanine incorporation decreased 30% in the absence of albumin, but when albumin was present, the rate remained within 10% of the control. Thus albumin prevented the decrease in $[^{14}\text{C}]\text{phenylalanine}$ incorporation observed when the extracellular supply of amino acids was restricted. Decreased rates of incorporation observed in amino acid-deprived cells were not associated with increased release of the cytosolic enzyme lactate dehydrogenase ($1\times$, 1.6 ± 0.3 ; $0\times$, $1.9 \pm 0.2\%$ released/h; three observations each), suggesting no change in cell viability.

Further studies revealed that the submaximal rate of phenylalanine incorporation observed in amino acid-deprived cells was restored to the control rate in the presence of 2% bovine serum albumin (essentially free of fatty acids) ($0\times$ plus defatted albumin, $99.0 \pm 2.6\%$ of $1\times$ control) or by addition of $1\times$ amino acids after the inhibition had been allowed to develop (reversed, $101.3 \pm 3.7\%$ of $1\times$ control). The submaximal rate of incorporation was not prevented by the presence of plasma concentrations of (1) the nine essential amino acids arginine, histidine, isoleucine, leucine, lysine, methionine, threonine, tryptophan and valine ($72.1 \pm 4.2\%$ of control); (2) cysteine, tryptophan and the branched-chain amino acids leucine, isoleucine, and valine ($72.9 \pm 1.3\%$ of

Fig. 6. Effect of extracellular amino acid concentrations on the synthesis of macrophage proteins

Cells were incubated for 120min in KH buffer containing $690\mu\text{M}$ -phenylalanine and either zero (0x), one (1x), or five (5x) times plasma concentrations of 19 amino acids, with (■) or without (□) 2% fraction V bovine serum albumin. [^{14}C]-Phenylalanine was added at a specific radioactivity of $1.0\mu\text{Ci}/\mu\text{mol}$. Values are means $\pm$ S.E.M. for four to five separate determinations. Similar observations were made after 40 and 80min of incubation.

* $P < 0.05$ versus 0x plus bovine serum albumin and 1x minus bovine serum albumin; ** $P < 0.05$ versus 1x plus bovine serum albumin.

control); or (3) glutamine, glutamate, asparagine, aspartate, serine and alanine ($80.5 \pm 2.7\%$ of control). The last group of amino acids represented those compounds with intracellular concentrations that were less than 25% of control (1x) when no exogenous amino acids (0x) were provided (results not shown).

The intracellular pool of phenylalanine in macrophages incubated without exogenous amino acids would support a maximal rate of protein synthesis for less than 3 min. Therefore continued synthesis of proteins at 70% of the maximal rate by amino acid-deprived cells (minus albumin) presumably involved re-utilization of amino acids derived from degradation of endogenous proteins. In cells incubated with $20\mu\text{M}$ -cycloheximide in the absence of albumin, no difference in the rate of proteolysis was observed when buffer amino acids were zero, one or

five times plasma concentrations (0x, 11.83 ± 0.47 ; 1x, 11.22 ± 0.54 ; 5x, 12.16 ± 0.48 nmol of phenylalanine released/3h per mg of protein; four observations each). With 2% albumin present, phenylalanine release (plus cycloheximide) was also unaffected when amino acids were eliminated from the medium (1x, 14.79 ± 0.45 ; 0x: 14.57 ± 0.85 nmol of phenylalanine/h per mg of protein; three observations each). These results, combined with the fact that albumin did not affect the degradation of endogenous macrophage proteins, suggested that when the extracellular supply of amino acids was restricted, amino acids derived from exogenous albumin were used to support protein synthesis in pulmonary alveolar macrophages.

Discussion

Investigations of rates of protein synthesis and degradation are simplified when a radioactive precursor amino acid not otherwise metabolized by the cell under study is employed. Rapid equilibrations of the extracellular amino acid with the intracellular pool and with aminoacyl-tRNA are additional properties of a desirable precursor. The present studies confirmed and extended previous observations (Woodside & Massaro, 1979) that suggested that phenylalanine was suitable for use in studies of protein turnover in pulmonary alveolar macrophages. Recent observations showed that phenylalanine is metabolized in perfused rat heart by a mitochondrial aminotransferase (Williams *et al.*, 1981). Heart rapidly produced $^3\text{H}_2\text{O}$ from phenyl-[2,3- ^3H]alanine, but net production of phenylpyruvate or other metabolites of phenylalanine was negligible. This pathway has not been examined in other tissues, but could result in a significant underestimation of the rate of protein degradation as calculated on the basis of phenylalanine release. The present studies showed that macrophages did not generate significant amounts of $^3\text{H}_2\text{O}$ from phenyl-[2,3- ^3H]alanine. The maximum rate of $^3\text{H}_2\text{O}$ production observed amounted to metabolism of about 2pmol of phenylalanine/h per 2×10^6 cells, which represented, at most, 0.14% of the minimal rate of phenylalanine release and thus introduced insignificant error into these determinations.

Few quantitative comparisons of the specific radioactivity of extracellular and tRNA-bound amino acids have been published. Studies in several perfused tissues (Khairallah & Mortimore, 1976; McKee *et al.*, 1978; Watkins & Rannels, 1980) suggested that little if any amino acid derived from protein breakdown was re-utilized in protein synthesis when the extracellular precursor concentration was high. Similar conclusions were reached by Baker & Shiman (1979), who compared the

specific radioactivity of leucine in the precursor pool with that in acid hydrolysates of newly synthesized albumin secreted by cultured hepatoma cells. Furthermore, Stanchfield & Yager (1979) reported equal specific radioactivities of extracellular and tRNA-bound leucine from cultured hepatocytes incubated in the presence of 500 μ M-leucine. In contrast, the specific radioactivity of phenylalanyl-tRNA in macrophages reached an average of only 57% of that of extracellular phenylalanine during incubation under comparable conditions (Table 2). This finding suggested that intracellular compartmentation resulted in charging of a significant portion of tRNA from a pool of amino acids that did not readily equilibrate with an extracellular source of radioactivity, and was consistent with preferential re-utilization of amino acids derived from protein degradation. Righetti *et al.* (1971) made similar observations regarding amino acid re-utilization by examining the specific radioactivities of several amino acids in immunoprecipitates of ferritin newly synthesized by HeLa cells. In addition, aminoacyl-tRNA specific radioactivities below those of the extracellular free amino acid were observed in sea-urchin gastrulae (Regier & Kafatos, 1977) and in cultured human lung fibroblasts (Hildebran *et al.*, 1980). Taken together, the latter studies emphasize that raising extracellular concentrations of precursor cannot be used as a general approach to simplify determinations of the absolute rate of protein synthesis, but that this protocol must be validated in each system studied.

The rate of synthesis of macrophage proteins calculated from the average specific radioactivity of phenylalanyl-tRNA was 10.0 nmol of phenylalanine incorporated/h per mg of cell protein, 1.7 times that calculated from the specific radioactivity of extracellular phenylalanine. Synthesis amounted to approx. 36 μ g of protein/h per mg of cell protein and represented a fractional rate of synthesis (86%/day) which was 4–6-fold higher than those reported for the turnover of total proteins in pig lung *in vivo* (Garlick *et al.*, 1976) and in perfused rat lungs (Watkins & Rannels, 1980). These comparisons underscore the importance of studying purified cell preparations isolated from heterogeneous tissues such as the lung.

When the release of phenylalanine more than doubled as a result of albumin catabolism, the ratio of the specific radioactivities of phenylalanine in the tRNA-derived and extracellular pools did not change. The contribution of a significant supply of amino acid derived from the degradation of endocytosed exogenous protein to charging of tRNA has not been investigated previously. The present observations suggested that, with high concentrations of extracellular phenylalanine, phenylalanine derived from the degradation of exogenous protein, in

contrast with endogenous protein, was not preferentially utilized in protein synthesis. This result, which reflected compartmentalization of amino acids from these sources, suggested that endogenous and exogenous proteins were catabolized by different pathways. This hypothesis is consistent with the studies of Poole *et al.* (1980), which showed significant effects of several inhibitors of lysosomal function on the degradation of exogenous, but not endogenous, proteins by cultured macrophages.

Measurement of the degradation of a preparation of bovine serum albumin that was not derivatized or intentionally denatured represented a significant departure from previous studies, where iodine-labelled albumin was used. This could be particularly important in the light of reports that iodination may accelerate protein endocytosis and degradation. Moore *et al.* (1977) showed that the rate at which rat yolk sac endocytosed and degraded iodinated albumin increased progressively as the extent of albumin denaturation increased. Similarly, degradation of proteins taken up by oocytes was three to fifteen times faster when the molecules were iodinated (Chloramine-T method) than when they were labelled under more mild conditions by reductive alkylation (Opresko *et al.*, 1980). In addition, the present approach to measuring albumin degradation greatly increased sensitivity as compared with the degradation of the iodinated protein (Pratten *et al.*, 1977). This difference may account for the inability of Kirsch *et al.* (1972) to detect significant degradation of biologically screened 125 I-labelled rat serum albumin by alveolar or peritoneal macrophages.

On the basis of the amount of albumin degraded over 3 h of incubation, an albumin clearance of 0.15 μ l/h per 10^6 cells was calculated. Clearance rates for the non-digestible fluid-phase pinocytotic substrates 125 I-labelled polyvinylpyrrolidone and [14 C]sucrose by cultured peritoneal macrophages were 0.03 and 0.02 μ l/h per 10^6 cells respectively (Pratten *et al.*, 1977). Estimates of fluid-phase endocytosis based on uptake of [3 H]sucrose by cultured rabbit alveolar macrophages were 0.08–0.13 μ l/h per 10^6 cells (Kaplan & Nielsen, 1978). In contrast, adsorption of formaldehyde-denatured albumin to the cell membrane led to higher rates of clearance (0.4–1.1 μ l/h per 10^6 cells) (Pratten *et al.*, 1977). Furthermore, whereas the degradation of fraction V albumin was directly proportional to protein concentration over a 300-fold range (0.02–6.0%), saturation kinetics were observed well within the present range of substrate concentrations when uptake involved membrane adsorption (Silverstein *et al.*, 1977; Moore *et al.*, 1977). Taken together, these results suggested that the present preparation of albumin was taken up primarily in the liquid phase and that membrane adsorptive processes

played a less significant role than in the uptake of denatured or derivitized protein preparations.

Submaximal rates of amino acid incorporation were observed in response to omission of any one essential amino acid in cultured ascites cells (van Venrooij *et al.*, 1972), fibroblasts (Christman, 1973) and HeLa cells (Vaughan & Hansen, 1973). In agreement with those studies, decreased amino acid incorporation in macrophages incubated without added exogenous amino acids was rapidly reversed upon re-addition of 19 amino acids to the cells. The inhibition was not prevented by the branched-chain compounds, which stimulated protein synthesis in heart and skeletal muscle (Rannels *et al.*, 1974; Chua *et al.*, 1979; Li & Jefferson, 1978), or by essential amino acids. Furthermore, neither amino acids (cysteine, tryptophan) normally present at low concentrations intracellularly nor a group of amino acids most extensively depleted in amino acid-deprived cells prevented the inhibition.

Eagle & Piez (1960) previously reported that HeLa cells cultured in medium containing serum proteins prelabelled with radioactive amino acids utilized less than 5% of those amino acids for synthesis of cellular proteins. Furthermore, a similar protein mixture did not permit cell growth in amino acid-deficient medium. Unfortunately, those authors did not determine the extent to which HeLa cells actually degraded protein from the medium. The fact that amino acid-deprived macrophages degraded albumin to free amino acids suggested that increased phenylalanine incorporation in the presence of the protein could have been mediated by an increase in amino acid availability as a result of albumin catabolism. This consideration is of special interest in light of the studies outlined above, in evaluating the potential physiological importance of exogenous proteins as a source of cell nutrition (Silverstein *et al.*, 1977).

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