Source of amino acids for tRNA acylation

Implications for measurement of protein synthesis

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Estimates of protein-synthesis rates using radioisotopes require accurate measurement of the specific radioactivity of the label in protein and in the precursor pool over time. Although the extracellular and intracellular pools of amino acids are easiest to sample, the tRNA pool is the direct precursor and is the appropriate pool for sampling. To test if the intracellular or extracellular pools reflect the tRNA specific radioactivity, a chicken macrophage cell line was incubated in medium containing either 0.23 mM-leucine and 14.5 μCi of [^3H]leucine (tracer dose) or 2.3 μM -leucine plus 145.0 μCi of [^3H]leucine (flooding dose). At both leucine levels, the tRNA specific radioactivity reached a plateau quickly, but did not equilibrate with either the extracellular or intracellular specific radioactivity within 30 min, and remained closer to that of protein. In a second experiment, proteins in chicken macrophages were labelled with [^3H]leucine for 2 days. Labelling medium was removed, and the cells were washed free of residual free [^3H]leucine and incubated with medium containing either 0.23 mM- or 2.3 mM-leucine (unlabelled). The specific radioactivity of leucyl-tRNA leucine reached a plateau within 2 min and remained considerably closer to that in the protein than that in intracellular or extracellular pools for at least 60 min. These results suggest that amino acids from protein degradation are a primary source for charging tRNA. When protein-synthesis rates are estimated by label incorporation, use of extracellular or intracellular specific-radioactivity values result in a marked underestimation.

INTRODUCTION

Measurements of protein-synthesis rates are based on the precursor-product relationship of amino acid incorporation into protein. Accurate assessment of this relationship requires determination of the amino acid precursor and product specific radioactivity. The immediate precursors for protein synthesis are the amino acids acylated to tRNA. The pool of tRNA-bound amino acids is seldom examined, owing to its small size and the technical difficulty involved in isolating aminoacylated tRNA (Zak *et al.*, 1979). Determination of protein-synthesis rates typically uses intracellular or extracellular amino acid specific radioactivity (SA_i and SA_e respectively) and assumes that these pools are representative of aminoacylated-tRNA specific radioactivity (SA_t), or that any difference can be described mathematically (Zak *et al.*, 1979; Waterlow *et al.*, 1978).

Aminoacyl-tRNA specific radioactivity has been shown to be intermediate between those of the intracellular and extracellular amino acid pools in perfused liver (Khairallah & Mortimore, 1976) and heart (McKee *et al.*, 1978). In an effort to eliminate differences between these pools, McNurlan *et al.* (1979) and Garlick *et al.* (1980) used the so-called flooding-dose method originally proposed by Henshaw *et al.* (1971). This method involves addition of a large dose of unlabelled amino acid with the radiolabelled amino acid, which in theory expands the intracellular and extracellular amino acid pools and decreases differences in amino acid specific radioactivities between intracellular, extracellular and tRNA amino acid pools. In some systems, however, the flooding-dose technique fails to force the specific radioactivity of the extracellular, intracellular and tRNA amino acid pools to unity (Airhart *et al.*, 1981; Schneible & Young, 1984; Opsahl & Ehrhart, 1987). These experiments show that failure to measure specific radioactivity of aminoacyl-tRNA results in misrepresentation of protein-synthesis rates.

We describe here a method which allows the accurate measurement of aminoacyl-tRNA specific radioactivity without the time and complexity involved with current techniques, and utilize this method for the characterization of protein metabolism in the avian HD-11 macrophage *in vitro*.

MATERIALS AND METHODS

Cell culture

Chicken HD11 macrophages, a *myc*-transformed avian cell line (Beug *et al.*, 1979), were maintained in 75 cm^2 tissue-culture flasks (Corning Inc., Corning, NY, U.S.A.) with RPMI-1640 + 25 mM-Hepes (Sigma Chemical Co., St. Louis, MO, U.S.A.) + 5% (v/v) newborn-calf serum (Irvine Scientific, Irvine, CA, U.S.A.) + 100 units of penicillin/ml and 100 μg of streptomycin/ml (Sigma). The cells were incubated at 41 °C in a humidified 5% CO_2 atmosphere. Before the start of an experiment, the cells were scraped from the flasks and seeded in 6-well plates at an approximate density of 1×10^6 cells per 9.6 cm^2 well. Upon formation of a confluent monolayer, experiments were initiated.

tRNA isolation

Cells were scraped in ice-cold 0.8% phosphate-buffered saline (PBS; 0.8% NaCl, 2.7 mM-KCl, 8.1 mM- Na_2HPO_4 , 1.5 mM- KH_2PO_4) and spun for 30 seconds at 10000 g . The PBS was aspirated and 0.7 ml of extraction buffer (100 mM-sodium acetate, pH 5.5, 1% SDS and 0.001 M-EDTA) was added to the cell pellet. Cells were lysed by passage through a 23-gauge needle 7 times. An equal volume of extraction-buffer-equilibrated phenol/chloroform/3-methylbutan-1-ol (25:24:1, by vol.) was mixed with the lysate, followed by centrifugation at 14000 g for 10 min. The aqueous phase was removed and re-extracted with an equal volume of phenol/chloroform (1:1, v/v) and pre-

Abbreviations used: SA_i , SA_e , SA_t , intracellular, extracellular and aminoacylated-tRNA specific radioactivity; PBS, phosphate-buffered saline; FSR, fractional synthesis rate; FAR, fractional accretion rate.

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precipitated overnight by addition of 2.5 vol. of cold (-20°C) ethanol. Nucleic acids were isolated by centrifugation at 14000 g for 30 min. The RNA pellet was washed twice with 1.0 ml of 70 % (v/v) ethanol and resuspended in 0.3 ml of 50 mM-sodium acetate, pH 5.5, for analysis. The method was evaluated for yield, recovery and purity of tRNA. Recovery of a standard and yield were determined by h.p.l.c. separation of nucleic acids on a Waters Gen-Pak Fax column ($4.6\text{ cm} \times 10\text{ cm}$) (Waters, Milford, MA, U.S.A.) with a linear NaCl gradient (25 mM-sodium phosphate/0.3–1.0 M-NaCl) and a flow rate of 0.8 ml/min for 40 min. Purity was evaluated by the A_{260}/A_{280} ratio. All reagents were of molecular-biology grade or the highest quality available.

The isolation procedure was also evaluated for potential contamination by free amino acids. Unlabelled aminoacyl-tRNA samples were spiked with $3.0\text{ }\mu\text{Ci}$ of $[^3\text{H}]$ leucine (153 Ci/mmol) and passed through the isolation procedure. Residual free leucine after the extraction and precipitation was found to be 1–5 % of the original label. Because contamination of isolated tRNA with free amino acids arising from the tissue would result in the underestimation of differences in the SA_i and SA_t pools (which was not seen), a much greater concern is the contamination of isolated tRNA with unlabelled amino acids arising from non-tissue sources. To minimize this source of error, all glassware was washed with acid and 50 % methanol and dried for 24 h at 100°C . Gloves were worn at all times. Reagent blanks were analysed for amino HNO_3 content on a regular basis, and were not found to contain detectable levels. In addition, all samples were run in duplicate, and assays were repeated when the inter-sample difference exceeded 25 %.

Protein synthesis

Two experiments were conducted to examine the effect of amino acid pool expansion on the specific radioactivity of three amino acid pools. The first experiment measured the leucine specific radioactivity in the intracellular, protein and tRNA amino acid pools and the effect on these pools of raising the extracellular leucine concentration to levels 10 times that found in avian plasma. Fractional rates of protein synthesis were calculated from these measurements. Although the first experiment followed the tracer from the extracellular compartment to cell protein, a second experiment was conducted to evaluate the flow of labelled amino acid from protein into the extracellular compartment.

Expt. 1. The rate of protein accretion was determined by measuring the protein content of cells at 12, 24, 36, 48 and 60 h after plating. Cells were grown in media containing either 0.23 mM- or 2.3 mM-leucine. Cells were scraped in 1.0 ml of cold 0.8 % PBS and proteins were precipitated by addition of 0.4 ml of 50 % (w/v) trichloroacetic acid. The pellet was washed twice with 1.0 ml of 8 % trichloroacetic acid. Proteins were solubilized with 0.2 ml of 0.5 M-NaOH, and protein concentration was measured by a micro-Lowry method (Lowry *et al.*, 1951).

Protein synthesis was measured 48 h after plating by changing the medium to one containing either 0.23 mM-leucine and $14.5\text{ }\mu\text{Ci}$ of $[^3\text{H}]$ leucine (153 Ci/mmol ; New England Nuclear) (tracer dose), or 2.3 mM-leucine plus $145.0\text{ }\mu\text{Ci}$ of $[^3\text{H}]$ leucine (flooding dose). Labelling media also contained $3.0\text{ }\mu\text{Ci}$ of $[^3\text{H}]$ phenylalanine/ml (110.4 Ci/mmol ; New England Nuclear). Cells were incubated with labelling media for 2, 5, 10 or 30 min. Labelling medium was removed from adherent cells and the plates were rapidly rinsed by passage through five 1-litre beakers of ice-cold PBS. Cells were scraped in 1.0 ml of PBS. Portions (0.4 ml) of the media and cell suspension were precipitated by addition of 0.08 ml of 50 % trichloroacetic acid; the supernatant was designated as the free pool and the precipitate as the protein-bound pool. The precipitate was washed with $4 \times 1.0\text{ ml}$ of 8 %

trichloroacetic acid and solubilized in 0.2 ml of 0.5 M-NaOH. A sample of this protein solution was hydrolysed with 6 M-HCl at 115°C for 22 h for determination of protein-bound leucine specific radioactivity. The remaining cell suspension was used to isolate tRNA as described above. Amino acids were removed from the tRNA by adjusting the solution pH to 10 by addition of 0.5 M-NaOH, followed by incubation at 41°C for 1 h. After amino acid hydrolysis, nucleic acids were precipitated with 2.5 vol. of cold ethanol (-20°C). The supernatant was designated as the tRNA-bound amino acid pool. Amino acid concentrations were determined by phenyl isothiocyanate derivative formation and reverse-phase h.p.l.c. separation of the phenylthiocarbamoyl-amino acids, with u.v. detection of the conjugated amino acids at 254 nm (Bidlemyer *et al.*, 1984). Specific radioactivities for all pools were determined by collection of the leucine and phenylalanine peaks, with radioactivity (d.p.m.) measured by liquid-scintillation counting using a Packard LS1600 liquid-scintillation spectrophotometer. Quench correction was achieved by use of an external standard. Fractional synthetic rates (FSR) were calculated from the following equation:

$$\text{FSR} = (\text{SB}/\text{SA}_x) \times t \times 100 \quad (1)$$

where SB is the specific radioactivity of the amino acid in the protein pool, SA_x is the specific radioactivity of the amino acid in the precursor pool, where x could be the intracellular, extracellular or tRNA pool, t is time expressed in days and FSR has the units %/day.

Protein accretion was best described by the quadratic equation:

$$\text{Protein } (\mu\text{g}) = 624.28 + 17.22t + 0.265t^2 \quad (r^2 = 0.992) \quad (2)$$

and the first derivative (eqn. 3) represented the rate of protein accretion as a function of time in days:

$$\frac{d(\mu\text{g of protein})}{dt} = 17.22 + 0.53t \quad (3)$$

Fractional accretion rates (FAR) were calculated by dividing the solution to the first derivative (eqn. 3) at 48 h, the time of the synthesis experiment, by the total protein present at 48 h (eqn. 2) and expressing the result on a per-day basis:

$$\text{FAR} = \frac{\text{Total protein accreted (Eqn. 3)}}{\text{total protein present (Eqn. 2)}} \times \text{time} \times 100 \quad (4)$$

Expt. 2. Cell culture was carried out as previously described except that, after confluency, cell proteins were labelled for 2 days by incubation in medium containing $3.0\text{ }\mu\text{Ci}$ of $[^3\text{H}]$ leucine/ml. Immediately before the experiment, the labelling medium was removed and the cells were washed with 5.0 ml of non-labelled medium. After the wash, cells were incubated with RPMI 1640 containing either 0.23 mM or 2.3 mM-leucine (unlabelled). Samples were collected at 5, 10, 30 and 60 min. Sample processing, amino acid analysis and determination of amino acid specific radioactivity were as above.

Statistical analysis

Data from Expt. 1 were analysed by the General Linear Model (GLM) procedure of SAS version 6.03 (SAS Institute, Cary, NC, U.S.A.). As there were different numbers of observations for the individual cell means, least-squares means and the least-squares means standard error for each cell were generated on the basis of the two-way analysis of variance model:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + e_{ijk} \quad (5)$$

where Y_{ijk} is the k th observation (e.g. specific radioactivity, FSR) at the j th time of incorporation ($j = 1, \dots, 4$) of the i th amino acid pool ($i = 1, 2, 3$), μ is a constant common to all observations, α_i

is a fixed effect common to all amino acid pools, β_j is a fixed effect common to all time points, $\alpha\beta_{ij}$ is the fixed interaction between time of incorporation and amino acid pool, and e_{ijk} is the residual associated with each observation with null mean and variance σ^2 . This model was used to analyse data at each of the two extracellular leucine concentrations (0.23 mM and 2.30 mM). Differences between means within a given extracellular leucine concentration were detected by using the PDIF option with least-squares means generated by the GLM procedure of SAS.

Data from Expt. 2 contained equal numbers of observations in all cells, thus eliminating the need for least-squares means. In this case arithmetic means and the associated standard error were generated from the model

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + e_{ijk} \quad (6)$$

where Y_{ijk} is the k th observation (e.g. specific radioactivity) at the j th time of incorporation ($j = 1, \dots, 4$) of the i th amino acid pool ($i = 1, \dots, 4$), and μ , α_i , β_j , $\alpha\beta_{ij}$ and e_{ijk} are as described for eqn. (5). This model was used to analyse data at each of the two extracellular leucine concentrations (0.23 mM and 2.30 mM). Differences between means within a given extracellular leucine concentration were determined by the Student-Newman-Kuels multiple-range test.

For Expt. 2 it was of interest to examine the effect of extracellular leucine concentration on specific radioactivities of the four amino acid pools. In a preliminary analysis the data were fitted by the three-way analysis of variance model:

$$Y_{ijkl} = \mu + \alpha_i + \beta_j + \tau_k + \alpha\beta_{ij} + \alpha\tau_{ik} + \beta\tau_{jk} + e_{ijkl} \quad (7)$$

where Y_{ijkl} is the l th observation (e.g. specific radioactivity) at the k th extracellular leucine concentration ($k = 1, 2$) at the j th time of incorporation ($j = 1, \dots, 4$) of the i th amino acid pool ($i = 1, \dots, 4$), τ_k is a fixed effect common to both levels of extracellular leucine, and μ , α_i , β_j , $\alpha\beta_{ij}$ and e_{ijkl} are as described for eqn. (5). By using this model it was determined that the time of incorporation by level of extracellular leucine interaction ($\beta\tau_{jk}$) was not statistically significant ($P = 0.172$), whereas the level of extracellular leucine by amino acid pool ($\alpha\tau_{ik}$) was statistically significant ($P = 0.0017$). Thus the model was simplified to the two-way model:

$$Y_{jkl} = \mu + \beta_j + \tau_k + \beta\tau_{jk} + e_{jkl} \quad (8)$$

where Y_{jkl} is the l th observation (e.g. specific radioactivity) at the k th extracellular leucine concentration ($k = 1, 2$) at the j th time of incorporation. All other terms are as described for eqn. (7). This model was run separately for each of the four amino acid pools and was used to test the effect of extracellular leucine concentration on each of the four amino acid pools. Statistical significance was accepted at $P < 0.05$ for all analyses conducted.

RESULTS

Effect of leucine on protein metabolism

No differences in the rates of protein accretion owing to leucine concentration were observed (Fig. 1), so the data were pooled to describe protein FAR (eqn. 4). Protein FAR at 48 h was 49.6 %/day.

An important assumption in using the flooding-dose method is that the increased concentration of the amino acid does not alter protein metabolism. It is known that in some cell systems addition of amino acids stimulates protein synthesis (Rannels *et al.*, 1974). The branched-chain amino acids, in particular, have been shown to stimulate protein synthesis in muscle (Fulks *et al.*, 1975; Tischler *et al.*, 1981). The effect of leucine on protein metabolism was examined by measuring incorporation of [3 H]-phenylalanine into protein for each concentration of leucine. No differences were observed in rate or level of phenylalanine

incorporation between the two concentrations of leucine (Fig. 2a), suggesting that leucine did not influence protein synthesis. The rate of leucine incorporation into protein was similarly unaffected by the leucine concentration in the medium (Fig. 2b).

Amino acid pool specific radioactivity

The first experiment examined the effect of the flooding-dose technique on the specific radioactivities of three pools of amino acid. The effect of increased leucine concentration on protein synthesis was also examined. Leucine specific radioactivity for extracellular (SA_e), intracellular (SA_i) and aminoacyl-tRNA (SA_t) amino acid pools was significantly higher ($P < 0.05$) for cells treated with the flooding dose (2.3 mM-leucine) as compared

Fig. 1. Influence of leucine concentration on the rate of protein accretion in macrophages

The rate of protein accretion was determined by measuring the trichloroacetic acid-precipitable protein content of cells at 12, 24, 36, 48 and 60 h after plating. Cells were grown in medium containing either a tracer (0.23 mM; ●, dotted line) or a flooding (2.3 mM; △, dashed line) level of leucine. Values represent means $\pm$ S.E.M. Where error bars are absent, symbol-size is greater than the S.E.M.

Fig. 2. Influence of tracer and flooding leucine concentrations on the accumulation of radio-labelled amino acids into macrophage protein

HD11 chicken macrophages were incubated in medium containing either 0.23 mM-leucine or 2.3 mM-leucine and 3.0 μ Ci of [3 H]-phenylalanine/ml (a) or either 14.5 μ Ci of [3 H]-leucine (tracer dose) or 145.0 μ Ci of [3 H]-leucine (flooding dose) respectively (b). The accumulation of label into cell protein was determined at the indicated times. The level of leucine in the medium did not significantly affect the rate (slope) of label incorporation for either [3 H]-leucine or [3 H]-phenylalanine. Dotted and dashed lines represent regression lines for 0.23 mM- and 2.3 mM-leucine respectively. Values represent means $\pm$ S.E.M.

Fig. 3. Influence of tracer and flooding leucine concentrations on the specific radioactivity of free and tRNA-bound leucine

HD11 chicken macrophages were incubated in medium containing either 0.23 mM-leucine and 14.5 μ Ci of [3 H]leucine (a; tracer dose) or 2.3 mM-leucine plus 145.0 μ Ci of [3 H]leucine (b; flooding dose). At the indicated times, leucine in the following compartments was isolated for determination of specific radioactivity: ●, intracellular; ○, medium (extracellular); △, tRNA. Values represent means $\pm$ S.E.M.

with the tracer dose (0.23 mM) of leucine (Fig. 3). Although the specific radioactivity of leucine in serum-free medium was the same for both the 0.23 mM and 2.3 mM treatments, the relative contribution of leucine from the 5% added serum resulted in a slightly lower specific radioactivity in the final 0.23 mM-leucine medium compared with the 2.3 mM medium.

The SA_e of cells incubated with 0.23 mM-leucine did not change over the course of the experiment. SA_i increased quickly, and by 5 min was not significantly different from SA_e (Fig. 3). SA_t tended to increase and reach an apparent plateau by 10 min, at approx. 20% of SA_e or SA_i .

The SA_e for 2.3 mM-leucine tended to decline over time; however, only the 30 min time point was significantly lower than the previous time points (Fig. 3). As with 0.23 mM-leucine, leucine SA_e and SA_i were equal by 5 min. SA_t was approx. 25%

of the extracellular or intracellular specific radioactivity over the course of the experiment, and was not affected by time.

The second experiment was designed to examine the effect of a flooding dose of amino acid on specific radioactivity in the extracellular, intracellular and aminoacyl-tRNA pools of amino acids when proteins were prelabelled with [3 H]leucine. The experiment was designed to examine the same parameters as the initial experiment, with the exception that label would originate from the intracellular protein-bound pool rather than the extracellular free amino acid pool. Specific radioactivity of protein-bound amino acids (SB) remained constant throughout the experiment for both levels of leucine (Table 1). SA_e was constant for the first 30 min, and increased by 58% and 32% for 0.23 mM- and 2.3 mM-leucine respectively during the second 30 min. Specific radioactivity of the intracellular amino acids did not change with time for the tracer level of leucine; however, for the flooding dose, SA_i decreased by 40% during the course of the experiment. SA_t was not affected by increasing the extracellular leucine concentration. However, specific radioactivity of this pool declined during the first 30 min and then increased over the next 30 min. It is not known why SA_t was equivalent to that in the protein at 60 min in the presence of a flooding level of leucine, but not when a tracer level was used. Specific radioactivity in the tRNA pool was typically 60% of the protein specific radioactivity for either level of external leucine. Leucine SA_t was 10 and 2% of SB for 0.23 mM- and 2.3 mM-leucine respectively.

In Expt. 1, FSR values calculated from either SA_e or SA_i were slightly higher for the tracer level of leucine, owing to the small difference in specific radioactivity in the medium (Table 2); however, these differences were not significant ($P < 0.05$). No statistical differences were observed between SA_i and SA_e ; however, SA_e tended to be greater than SA_i , hence FSRs calculated from SA_i were greater than rates calculated from SA_e . The main differences observed were those between the synthetic rates calculated by using either the SA_e or SA_i and those by using SA_t . FSRs for cells incubated in 0.23 mM-leucine were 3–6-fold higher, when means pooled across time were compared, when calculated from SA_t as compared with values calculated from SA_i and SA_e .

FSRs for cells incubated with 2.3 mM-leucine exhibited the same response observed with the tracer level of amino acid. FSR calculated from SA_e or SA_i was highest at 2 min and reached a plateau between 5 and 30 min (Table 2). FSR calculated from

Table 1. Influence of medium leucine concentration on the specific radioactivity of tRNA-bound leucine when label originates from protein degradation

Proteins in chicken HD11 macrophages were labelled with [3 H]leucine for 2 h. Immediately before the experiment, the labelling medium was removed and the cells washed free of residual free [3 H]leucine. After the wash, cells were incubated with medium containing either 0.23 mM- or 2.3 mM-leucine (unlabelled). At the indicated times, leucine in the following compartments was isolated for determination of specific radioactivity: intracellular (IC); medium (EC); tRNA; protein. Values represents means $\pm$ S.E.M. ($n = 4$). Means in a row with different letters are significantly different ($P < 0.01$).

[Leucine]	Time (min)	Sp. radioactivity (d.p.m./pmol of leucine)			
		EC	IC	tRNA	Protein
0.23 mM	5	0.248 $\pm$ 0.016 ^a	1.62 $\pm$ 0.20 ^b	10.80 $\pm$ 1.88 ^c	17.66 $\pm$ 0.13 ^d
	10	0.248 $\pm$ 0.028 ^a	2.04 $\pm$ 0.11 ^b	10.82 $\pm$ 1.56 ^c	18.69 $\pm$ 0.12 ^d
	30	0.234 $\pm$ 0.024 ^a	2.00 $\pm$ 0.02 ^b	9.70 $\pm$ 1.61 ^c	17.50 $\pm$ 0.19 ^d
	60	0.384 $\pm$ 0.018 ^a	2.02 $\pm$ 0.05 ^b	12.15 $\pm$ 1.67 ^c	19.13 $\pm$ 0.49 ^d
	Mean	0.278 $\pm$ 0.019 ^a	1.92 $\pm$ 0.07 ^b	10.87 $\pm$ 0.79 ^c	18.24 $\pm$ 0.21 ^d
2.3 mM	5	0.038 $\pm$ 0.003 ^a	0.491 $\pm$ 0.023 ^b	13.69 $\pm$ 1.77 ^c	19.15 $\pm$ 0.06 ^d
	10	0.037 $\pm$ 0.005 ^a	0.382 $\pm$ 0.039 ^b	10.78 $\pm$ 2.01 ^c	17.06 $\pm$ 0.11 ^d
	30	0.034 $\pm$ 0.004 ^a	0.340 $\pm$ 0.016 ^b	8.87 $\pm$ 0.62 ^c	18.92 $\pm$ 0.26 ^d
	60	0.048 $\pm$ 0.004 ^a	0.292 $\pm$ 0.010 ^b	19.00 $\pm$ 2.48 ^c	18.09 $\pm$ 0.15 ^d
	Mean	0.039 $\pm$ 0.002 ^a	0.376 $\pm$ 0.022 ^b	13.08 $\pm$ 1.29 ^c	18.31 $\pm$ 0.22 ^d

Table 2. Least-squares means of protein fractional synthesis rates in macrophages calculated from three possible precursor pools

HD11 chicken macrophages were incubated in medium containing either 0.23 mM-leucine and 14.5 μ Ci of [3 H]leucine (tracer dose), or 2.3 mM-leucine plus 145.0 μ Ci of [3 H]leucine (flooding dose). At the indicated times, FSRs (%/day) were calculated from the specific radioactivity of [3 H]leucine in protein and in three potential precursor pools: extracellular (EC), intracellular (IC) and aminoacylated tRNA (T). Least-square means $\pm$ standard error of the least-square means in columns with different superscripts are significantly different ($P < 0.05$). The numbers in parentheses indicate the sample size for that mean.

[Leucine]	Pool	Time (min)...	FSR (%/day)				Mean
			2	5	10	30	
0.23 mM	EC		182 $\pm$ 198 ^a (3)	165 $\pm$ 198 ^a (3)	129 $\pm$ 109 ^a (10)	114 $\pm$ 99 ^a (12)	148 $\pm$ 79 ^a (28)
	IC		565 $\pm$ 198 ^a (3)	223 $\pm$ 198 ^a (3)	177 $\pm$ 109 ^a (10)	128 $\pm$ 99 ^a (12)	273 $\pm$ 79 ^a (28)
	T		1252 $\pm$ 198 ^b (3)	868 $\pm$ 243 ^b (2)	1057 $\pm$ 109 ^b (10)	631 $\pm$ 130 ^b (7)	952 $\pm$ 89 ^b (22)
2.3 mM	EC		159 $\pm$ 69 ^a (3)	87 $\pm$ 69 ^a (3)	97 $\pm$ 38 ^a (10)	93 $\pm$ 36 ^a (11)	109 $\pm$ 28 ^a (27)
	IC		272 $\pm$ 69 ^a (3)	99 $\pm$ 84 ^a (2)	113 $\pm$ 38 ^a (10)	101 $\pm$ 34 ^a (12)	146 $\pm$ 30 ^a (27)
	T		550 $\pm$ 119 ^b (2)	424 $\pm$ 69 ^b (3)	340 $\pm$ 38 ^b (9)	525 $\pm$ 49 ^b (6)	460 $\pm$ 38 ^b (19)

SA_i averaged 3–4-fold greater ($P < 0.05$) than the rates derived by using SA_e or SA_i.

DISCUSSION

tRNA isolation

Isolation of tRNA and measurement of specific radioactivity of the associated amino acids are difficult and time-consuming, a principal reason why the process is seldom performed for the determination of protein-synthesis rates. The main goal of tRNA isolation for measurement of specific radioactivity is rapid removal of protein and DNA while maximizing recovery of aminoacyl-tRNA. The technique described above accomplishes this goal with two phenol/chloroform extractions. Further extractions were unnecessary, as judged by the A_{260}/A_{280} ratio and ion-exchange chromatography.

RNA typically gives an A_{260}/A_{280} ratio of 2.06, depending on nucleotide composition. The sodium acetate/chloroform/phenol extraction described here resulted in a ratio of 2.13, indicating little contamination from protein or DNA. Ion-exchange chromatography confirmed the lack of any interfering contaminants. Composition of samples prepared as described was typically 50% tRNA and 50% 5 S RNA and other small RNA species. Yield was approx. 13.5 μ g of tRNA/ 2×10^6 cells. Contamination of isolated RNA by free leucine after the isolation procedure was found to be less than 5%, indicating that the primary source of label when RNA samples are subjected to alkali hydrolysis was the tRNA fraction. Recovery of a tRNA standard, processed as described, was 47%.

This procedure provided sufficient amino acid from tRNA to determine accurately the specific radioactivity in cell-culture samples. Amino acid levels released from tRNA varied from barely detectable (histidine) to 600 pmol (cysteine), with leucine at approx. 100 pmol/6-well plate. Phenyl isothiocyanate derivative formation of amino acids allowed accurate separation and quantification for concentrations as low as 25 pmol.

Amino acid specific radioactivity

For purposes of estimating rates of protein synthesis, the vast majority of the published literature is based on the assumption that SA_i is similar to SA_e or SA_i. Only rarely have investigators made measurements of SA_i or attempted to eliminate or quantify the differences between the amino acid pools for use in the calculation of synthesis rates. In previous studies, SA_i was observed to be intermediate between intracellular and extracellular specific radioactivities (Airhart *et al.*, 1974, 1981; McKee

et al., 1978; Hammer & Rannels, 1981). Airhart *et al.* (1974) suggest that, owing to the higher specific radioactivity observed in the aminoacyl-tRNA as compared with the intracellular specific radioactivity, a portion of the tRNA is charged directly from an extracellular source, e.g. linked to transport of amino acid into the cell. Hod & Hershko (1976) used the time course of label incorporation into protein to describe a similar model where the precursor of protein synthesis, aminoacyl-tRNA, is charged from both the intracellular and extracellular pools of amino acid. Examination of the precursor-product relationship has shown that the pattern of amino acid incorporation into protein can be described by tRNA charging from various amino acid sources (Hod & Hershko, 1976; Airhart *et al.*, 1974; Li *et al.*, 1973). Rather than conducting the complex experiments necessary to describe and quantify such a system, the flooding dose is often used in an attempt to eliminate the differences between amino acid pools and avoid the necessity of measuring aminoacyl-tRNA specific radioactivity.

The flooding-dose method assumes that, by exposing the cell to a high concentration of amino acid, the specific radioactivities in all pools (extracellular, intracellular, tRNA) become equivalent and relatively constant. If amino acids acylated to tRNA are derived from the intracellular or extracellular amino acid pools, or both, the specific radioactivity of these pools would be representative of the precursor specific radioactivity. This method has proved successful in the perfused rat liver (Khairallah & Mortimore, 1976), heart (McKee *et al.*, 1978) and lung (Watkins & Rannels, 1980). In other systems, primarily cell or tissue culture, the aminoacyl-tRNA specific radioactivity was observed to be below the intracellular and extracellular specific radioactivities, despite equilibration of the intracellular amino acid pool with the extracellular amino acids (Airhart *et al.*, 1981; Hildebran *et al.*, 1981; Schneible & Young, 1984; Opsahl & Ehrhart, 1987; Hall & Yee, 1989).

Our first experiment examined the sensitivity of intracellular and aminoacyl-tRNA specific radioactivities to extracellular amino acid concentration. The underlying assumptions surrounding the behaviour of amino acid pools during expansion of these two compartments were examined. Fig. 3 shows the specific radioactivities in the three potential precursor pools of amino acid for two extracellular concentrations of amino acid, 0.23 mM- and 2.3 mM-leucine, representing a tracer and a flooding dose of amino acid respectively. Specific radioactivities in the extracellular and intracellular pools for both leucine concentrations reached equilibrium and were equivalent after 5 min of incubation. Aminoacyl-tRNA specific radioactivity, however, re-

mained well below that of the extracellular or intracellular pools. This observation suggests that either: (1) a single tRNA pool charges from two discrete sources of intracellular amino acid, one a low-specific-radioactivity pool which equilibrates slowly with other amino acid pools, and the other a rapidly equilibrating high-specific-radioactivity pool; presumably the low-specific-radioactivity pool arises from protein as a result of protein degradation and the high-specific-radioactivity pool is intracellular or extracellular amino acids; or (2) tRNA is compartmented into two pools, one which is charged directly from an intracellular amino acid pool primarily originating from protein degradation and is insensitive to the expansion of the intracellular amino acid pool, and the other is a smaller pool which equilibrates with intracellular or extracellular amino acids.

To examine further the possibility that tRNA is charged by amino acids from protein degradation before they mix with the general IC pool, a second experiment was conducted using cells with pre-labelled protein. In this experiment, labelled amino acids in the cell were supplied only by protein degradation. If tRNA is charged from an intracellular pool which equilibrates with the extracellular pool, SA_i would approximate to SA_e and/or SA_i . The data presented in Table 2 demonstrate that tRNA specific radioactivity is more representative of the protein specific radioactivity than is either the extracellular or intracellular specific radioactivity. This supports the data from the previous experiment where specific radioactivity in tRNA remained below extracellular or intracellular specific radioactivities, indicating that tRNA specific radioactivity was diluted by unlabelled amino acids derived from protein degradation. The compartmentalization of multienzyme complexes and the channelling of metabolites is well described for many metabolic pathways (Srere, 1987). Sivaram & Deutscher (1990) have provided evidence for two pools of arginyl-tRNA, suggesting the channelling of aminoacyl-tRNA for protein synthesis. Similar channelling of leucine from protein degradation would explain the observation reported here.

An alternative explanation for the apparent insensitivity of the aminoacyl-tRNA specific radioactivity to changes in extracellular specific radioactivity suggested by Airhart *et al.* (1974) is the possible existence of a non-labile pool of tRNA. According to this theory, the amino acids acylated to this tRNA pool would turn over very slowly and thus provide a pool that dilutes the specific radioactivity of the tRNA pool which is actually used for protein synthesis. In the first experiment, SA_i at 2 min was not different from that at later time periods, demonstrating very rapid equilibrium with the pools from which it is charged. The existence of a non-labile pool of tRNA should result in a slow equilibration rate, which was not observed. Fast equilibration is supported by calculating the turnover of the tRNA based on the measured synthesis rates and the tRNA pool size. Assuming 1000 μg of protein at day 1 and a 50% fractional accretion rate, 500 μg of protein, containing 267 nmol of leucine, would accumulate in 24 h. Assuming 50% recovery of the original tRNA and that aminoacyl-tRNA samples contained 100 pmol of leucine, the resultant pool size is 200 pmol of leucine acylated to leucyl-tRNA. If FSR is 800%/day (Table 2), 4268 nmol of leucine would be incorporated into protein per day, requiring the tRNA pool to turn over every 1.6 s if it is used exclusively to support protein synthesis. Airhart *et al.* (1974) made a similar calculation indicating that the tRNA pool in liver turns over every 0.3–2.0 s. Certainly, a turnover rate of around 1 s would permit equilibration of a single pool by the time of our first observation at 2 min.

Protein synthesis

In Expt. 1, FSR calculated from tRNA specific radioactivity

was always greater than FSR calculated from the other two pools, indicating that use of intracellular or extracellular specific radioactivity in this system underestimates FSR. The question arises as to the efficacy of the flooding-dose method for the study of protein synthesis in cell culture and other systems where the relationship between amino acid pools has not been determined. The use of SA_i for the calculation of protein-synthesis rate and the flooding-dose method underestimated FSR 7-fold. Although the use of SA_i is inappropriate for quantification of protein synthesis, its use to determine proportional changes due to nutritional, hormonal or other treatments is not compromised as long as the differences are large enough and do not affect charging of tRNA. The possibility that treatments that affect the rate of protein degradation influence the proportion of tRNA charged directly from protein degradation, and consequently the specific radioactivity of tRNA, needs to be examined.

A potential disadvantage of using SA_i for calculating FSR is the higher variability inherent in its measurement. In both Expts. 1 and 2, the coefficient of variation for SA_i averaged about twice as high as that for SA_e . Consequently, FSR values calculated with SA_i have greater coefficients of variation compared with those calculated by using either SA_i or SA_e . The greater variability is due mostly to the extra sample manipulations needed to extract tRNA before amino acid and radioisotope determination.

Estimates of FSR were highly variable at early time points, owing to the difficulty of measuring the very low amounts of radioactivity incorporated into protein. The estimates of the specific radioactivity of tRNA could be made reliably even at 2 min, as indicated by the low standard-error values (Fig. 3). In general, estimates of FSR decreased with time regardless of the precursor pool used for calculation. Presumably this is due to the bias of longer measurement times towards longer-half-life proteins and the inclusion of very-short-half-life proteins and processed fragments in the early measurements.

The observation that tRNA charging is linked to protein degradation is understandable both energetically and nutritionally. By reutilizing a significant proportion of the amino acids released from protein degradation, the cell can prevent the efflux of essential amino acids from the cell and minimize their loss to amino acid catabolism. Efficient reutilization of amino acids could result in decreased energy requirements associated with amino acid transport into the cell. If the acylation of tRNA is tied directly to protein degradation, it would be possible to recover some of the energy in the amino acid gradient across the lysosome, thereby decreasing the energy required for protein turnover.

Rates of protein synthesis observed in transformed macrophages growing at a fast rate reported in these studies is considerably greater than that reported in primary pulmonary macrophages (Airhart *et al.*, 1981). Primary cultures of macrophages had an FSR of 62%/day; however, primary cultures do not actively divide and usually die within 1 week. Interestingly, the FSR calculated from SA_i was almost 3 times greater than FSR calculated from SA_e in primary cultures of macrophages (Airhart *et al.*, 1981). The significant contribution of protein degradation to supplying amino acids for protein synthesis and the apparent compartmentalization of amino acid pools is so far confined to a limited number of cell-culture systems. The active role of the macrophage in the degradation of exogenous proteins and its high content of lysosomes and proteolytic enzymes would suggest that this cell type may be unique in its handling of amino acids. Hammer & Rannels (1981) have demonstrated that macrophages preferentially use amino acids from the degradation of endogenous proteins to charge tRNA, but not those from exogenous protein taken up from the medium. We have found similar evidence for the charging of tRNA from amino acids

released from protein degradation in a variety of tissues *in vivo* (D. M. Barnes, K. C. Klasing & C. C. Calvert, unpublished work). Further work is needed to determine the extent of this amino acid partitioning and the method of its regulation.

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