Use of ^{18}O -labelled leucine and phenylalanine to measure protein turnover in muscle cell cultures and possible futile cycling during aminoacylation

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Amino acids labelled with ^{18}O on both carboxy oxygen atoms have the potential for use as non-recyclable tracers to measure protein turnover. During protein synthesis one of the labelled oxygen atoms is removed, and thus release of the mono-labelled amino acid could be used to determine proteolysis. Primary cultures of embryonic-chick skeletal-muscle cells were used to test the use of $^{18}\text{O}_2$ -labelled Leu to measure proteolysis. For 9-day cultures, prelabelled on days 2–8 with medium containing one-half the Leu as $^{18}\text{O}_2$ Leu and one-half as $^2\text{H}_3$ Leu, release of ^{18}O Leu was less than 50 % that of $^2\text{H}_3$ Leu over 24 h, suggesting a loss of the ^{18}O label by a mechanism other than protein synthesis. Medium containing $^{18}\text{O}_2$ Leu, $^2\text{H}_3$ Leu, $^{18}\text{O}_2$ Phe and ^{13}C Phe was then incubated with 9-day cultures to compare the rate of loss of the ^{18}O -label from Leu and Phe with the rate of

uptake of the non-carboxy-oxygen-labelled amino acids. Results for Leu demonstrated an 81 % loss of the ^{18}O label compared with a 33 % decrease in $^2\text{H}_3$ Leu over 12 h. Loss of the ^{18}O label was four times as great for Leu as for Phe. Loss of the ^{18}O label was not decreased by addition of cycloheximide or by addition of a 3-fold excess of Ile, Val and Tyr; thus the loss of label was not due to protein synthesis alone or to misbinding to incorrect tRNAs. Infusion of the isotopes into pigs showed that the ^{18}O label of Leu was not lost during transamination to α -ketoisocaproate (α -oxoisohexanoate). The most probable explanation is that the ^{18}O label is lost as a result of the enzymic deacylation of tRNA, that this process is substantially faster for Leu than for Phe, and that this represents a potentially costly futile cycle for Leu.

INTRODUCTION

The availability of stable isotopes of nitrogen and oxygen to label amino acids offers an advantage in studying protein metabolism over the typically used radioisotopes ^3H and ^{14}C [1]. The development of g.c.–m.s. equipment has permitted analysis and quantification of specific labelled compounds. Although many methods may be used to measure protein degradation, an absolute rate of protein degradation is difficult to determine because of the difficulty of measuring the amount of tracer recycling through rapidly synthesized and degraded proteins. Re-utilization of tracer for protein synthesis during the course of an experiment gives rise to overestimation of the half-life if specific proteins are analysed for tracer, and to underestimation of protein degradation if release of tracer from cells or tissues is measured [2]. The procedures and underlying assumptions for measuring whole-body protein turnover have been reviewed by Waterlow et al. [3]. Errors in typically used methods also arise because of the inability to completely remove the original isotope from the tissue before measurements are made [4].

One method of avoiding re-utilization is to use a natural non-recyclable tracer such as 3-methylhistidine. But 3-methylhistidine indicates degradation of only the major myofibrillar proteins actin and myosin, and myofibrillar proteins make up only about 52–56 % of the proteins in the muscle cell [5]. Alternatively, blocking protein synthesis can be used to prevent re-utilization of labelled amino acids [6]. Cycloheximide has been used to block protein synthesis in many tissue [7] and cell culture systems [8,9]. The use of cycloheximide to resolve absolute rates of protein degradation assumes that cycloheximide itself does not alter protein degradation, at least during the experiment. This as-

sumption may be erroneous, inasmuch as some investigators have reported that cycloheximide can affect the rate of protein degradation [10–12].

In theory, replacing both of the oxygen atoms of the carboxy group of an amino acid with the stable isotope ^{18}O should result in a tracer amino acid that could not recycle through protein because one of the carboxy oxygen atoms is removed during formation of the peptide bond according to the pathway shown in Scheme 1. The ^{18}O -labelled amino acid would start out dilabelled [$+4$ a.m.u. (atomic mass units)], be incorporated into protein as a monolabelled amino acid ($+2$ a.m.u.), and be released from protein as the monolabelled ($+2$ a.m.u.) amino acid. Thus comparing the kinetics of a recyclable amino acid, e.g. ^{13}C -labelled, with the non-recyclable amino acid should indicate the magnitude of recycling of the amino acid and allow calculation of an absolute rate of protein degradation. Central to the use of ^{18}O -labelled amino acids is the assumption that ^{18}O is lost only during peptide synthesis. The amino acid must bind to the aminoacyl-tRNA synthetase enzyme and be quantitatively transferred to the tRNA with the accompanying loss of one ^{18}O . This would provide for a proportional loss of ^{18}O corresponding to the amino acid incorporation into the peptide being synthesized.

The objective of the present study was to use skeletal-muscle cell cultures to test assumptions for the use of ^{18}O -labelled amino acids in the study of protein turnover *in vivo*. Leu and Phe were used to test the ^{18}O -labelling model because of their extensive use in protein-turnover studies. Results demonstrated a substantial loss of ^{18}O label from Leu not due to incorporation into protein and therefore suggested futile cycling of Leu during aminoacylation.

Abbreviations used: a.m.u., atomic mass units; DMEM, Dulbecco's modified Eagle's medium; KIC, α -ketoisocaproic acid (α -oxoisohexanoic acid); APE, atoms percent excess.

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Scheme 1 Model of ^{18}O -carboxy-labelled amino acid use in measuring protein degradation

Proposed model showing how a di-oxygen-labelled amino acid would be incorporated into protein as the mono-oxygen-labelled amino acid. The mono-oxygen-labelled amino acid would be released through protein degradation and measured.

MATERIALS AND METHODS

Muscle cell cultures

Primary cultures of chick skeletal-muscle cells were isolated from 12-day-old embryos as described by Ridpath et al. [13]. Incubated (12 days), specific-pathogen-free eggs from a layer strain of chicken (Single Comb White Leghorn) were obtained from Hy-Vac Laboratory Eggs (Gowrie, IA, U.S.A.). Cells from the leg muscles of the embryos were separated by mechanical dissociation and plated at a density of 5.5×10^6 cells in 3 ml of medium per 60 mm-diameter collagen-coated culture plate. Medium consisted of 500 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented to contain all 20 amino acids (DMEM deficient, low glucose, powdered form; Sigma Chemical Co., St. Louis, MO, U.S.A.), 60 ml of horse serum (Gibco, Grand Island, NY, U.S.A.), 15 ml of embryo extract, 6 ml of penicillin G solution (resulting in 50 i.u./ml of media; Gibco) and 3.25 ml of Fungizone (resulting in 1.39 μg of amphotericin B/ml in the media; Sigma). Cultures were grown for 8 days under a humidified air/ CO_2 (19:1) atmosphere at 37 °C and the culture medium was replaced with 3 ml of fresh medium every 24 h. On days 5 and 6, 5×10^{-5} M cytosine β -D-arabinofuranoside was added to kill any dividing cells and to leave a relatively pure culture of fused, non-dividing myotubes.

For expt. I, which was conducted to measure protein degradation, medium fed on days 2–8 contained stable isotopes of Leu to label cell proteins. On day 9, cultures were rinsed once with PBS (3.3 mM NaH_2PO_4 /6.7 mM Na_2HPO_4 /150 mM NaCl, pH 7.0), and fresh medium containing all natural Leu was added. Release into the medium of the isotopes previously used to label cell proteins was then measured for 24 h and corrected for uptake of a radioisotope tracer from the release medium.

Expts. II and III were done to examine the loss of the ^{18}O label from the medium of 9-day cultures. Medium fed on day 9 contained labelled Leu and Phe as described in the Results section. During the next 24 h, measurement of the various

isotopes of Leu and Phe in the medium were compared. Treatments applied to the cultures included addition of 0.375 mM cycloheximide (Sigma), use of medium with and without embryo extract and addition of a 3-fold excess of Ile, Val and Tyr. Addition of 0.375 mM cycloheximide blocked 95 % of the uptake of [^{14}C]Leu into the trichloroacetic acid-precipitable fraction of muscle cell protein (results not shown). As judged by phase-contrast microscopy, this concentration of cycloheximide did not visibly affect the cells' ability to contract and to remain adhered to the culture plates. Control plates received the same medium without the treatments. In addition, labels were measured in incubated medium alone for 24 h, both with and without embryo extract and with and without a 3-fold excess of Ile, Val and Tyr.

Stable isotope labels

L-[1,1- ^{18}O]Leu and L-[1,1- ^{18}O]Phe were prepared from the corresponding amino acids by exchange of the carboxy oxygen atoms in H_2^{18}O at low pH. Dry crystalline amino acid (200 mg) was added to 3 ml of H_2^{18}O (96 % ^{18}O ; Cambridge Isotopes, Cambridge, MA, U.S.A.), and the preparation was gassed with HCl gas for 2–3 min until the pH was < 1.0 . The mixture was capped and incubated at 100 °C for 30 min, and the H_2^{18}O was removed under vacuum. Fresh H_2^{18}O was added to the vacuum-dried amino acid, and the process repeated twice more, after which the amino acid was neutralized with 3 M NaOH. The amino acid was then dissolved in 95 % ethanol to make a 10 % amino acid solution, which was placed in a -20 °C freezer until amino acid crystals had formed (2–3 days). The crystals were separated by centrifugation, and the crystallization process was repeated twice more. After final crystallization the amino acid was freeze-dried. A yield of 70 % was obtained with an approximate analysis of 93 % dilabelled, 4 % monolabelled and the remainder unlabelled.

L-[5,5,5- ^3H]Leu and L-[1- ^{13}C]Phe (Tracer Technologies Inc., Sommerville, MA, U.S.A.) were utilized as non-carboxy oxygen labels. The calculated Leu concentration in normal medium was 84 $\mu\text{g}/\text{ml}$. [5,5,5- ^3H]Leu and [1,1- ^{18}O]Leu were each added as approximately one-half the normal Leu amount in expt. I in which cell proteins were prelabelled with stable isotopes of Leu. In expts. II and III, all stable isotope labels of Leu and Phe were added to the medium placed on 9-day-old cell cultures. Additional measurements were made to determine if any loss of label used occurred in medium alone. Leu in the medium was the same as that used in expt. I, and the calculated Phe concentration in the initial medium consisted of 53 μg of natural Phe/ml, 26 μg of [1- ^{13}C]Phe/ml and 26 μg of [1,1- ^{18}O]Phe/ml.

Radioisotope labels

In expt. I, in which skeletal-muscle cell cultures were prelabelled with [^3H]Leu and [^{18}O]Leu and release of these labels into the medium was measured, L-[^3H]Leu was used to determine uptake of Leu from the medium used to measure label release. For this, 5000 μCi of [^3H]Leu (approx. 50 mCi/mmol; Amersham, Arlington Heights, IL, U.S.A.) was dried under N_2 , diluted to a volume of 2.5 ml with water, and sterilized by filtration. The [^3H]Leu was added directly to the release medium at the start of the measurement period at the rate of 20 μl (approx. 2.7 μCi) per 10 ml of release medium, and each plate received 3.2 ml of release medium.

In vivo measurement of [1,1- ^{18}O]Leu conversion into [1,1- ^{18}O] α -ketoisocaproic acid (KIC; α -oxoisohexanoic acid)

In vivo conversion of [1,1- ^{18}O]Leu into [1,1- ^{18}O]KIC was studied

using two 30 kg pigs. The pigs were surgically implanted with two silicon catheters (Silastic; Dow Corning, Midland, MI, U.S.A.) in the vena cava 7 days before study. On the day of study a continuous infusion of [5,5,5- ^3H]Leu and [1,1- ^{18}O]Leu was begun with a syringe infusion pump (Harvard Apparatus). Over a 4 h period, 100 mg of each isotope had been infused and the plasma levels of each had reached a plateau. Blood was sampled every 20 min from 2 to 4 h after the infusion period. Enrichment of ^3H and ^{18}O isotopes of both Leu and KIC were determined by g.c.-m.s. To avoid loss of ^{18}O from KIC, plasma was first treated with 4 vol. of ice-cold acetone to precipitate proteins. After centrifugation, the supernatant was dried at 50 °C under N_2 and resuspended in 2 ml of 0.05 M phosphate buffer, pH 8.0. A small disposable column, containing 5 ml of a 30 % slurry of Whatman-RPL-2 C18 resin (Sigma) in methanol, was first conditioned with buffer. The sample was then applied to the column, and the column was eluted with 1 ml aliquots of buffer. Leu was eluted in 3–7 ml, whereas KIC was eluted in 8–12 ml. These fractions were then dried at 50 °C under N_2 . The dried fraction was then derivatized as described below. Leu was monitored by g.c.-m.s. as described above and [5,5,5- ^3H]KIC and [1,1- ^{18}O]KIC were monitored at m/z 304 and 305 respectively.

Collection and processing of samples

Samples of media (20 μl) were taken for g.c.-m.s. and scintillation counting (LS1700 liquid scintillation counter; Beckman, Fullerton, CA, U.S.A.) at selected times during the release period as described in the Results section. For scintillation counting, the 20 μl media samples were taken at 2, 8 and 24 h and deposited directly into scintillation vials, and 2 ml of cocktail (Scintiverse BD; Fisher, Pittsburg, PA, U.S.A.) was immediately added. Samples for g.c.-m.s. analysis were simultaneously taken, immediately frozen at -20 °C, and dried as soon as possible at 70 °C under N_2 . Immediately before drying, 100 μl of a Nle standard solution (21 μg of Nle/ml) was added to each sample. Samples were then derivatized with 100 μl of acetonitrile plus 100 μl of *N*-methyl-*N*-(*t*-butylchlorosilane)trifluoroacetamide containing 1 % *t*-butyldimethylchlorosilane (Regis Chemical Company, Morton Grove, IL, U.S.A.) [14]. A system (Hewlett-Packard 5970A) was used to measure [^{18}O]Leu and [$^{18}\text{O}_2$]Leu and Phe at m/z 304 and 306 respectively, and [^{13}C]Phe and [$^2\text{H}_3$]Leu at m/z 303 and 305 respectively. Peak heights for Leu and Phe were compared with the peak height for Nle to calculate the quantity of each.

Statistics and calculations

Results from prelabelling cell cultures with the stable isotopes are expressed as μg of isotope/ml of medium. Results from experiments in which the stable isotopes are placed in culture medium and measured are expressed as a percentage of initial isotope enrichment. Initial isotope enrichment [(peak height of isotope)/(peak height of natural + peak heights of all other isotopes present)] was considered as 100 %. At each time point the value for each isotope was then calculated and expressed as a percentage of the initial enrichment. Plasma Leu and Phe values are expressed in atoms percent excess (APE). This was calculated as the ratio of the amount of isotope in the sample to the amount of the naturally occurring isotope that was present and expressed as a percentage.

Differences between treatments were assessed by using analysis of variance with the General Linear Models procedure of the Statistical Analysis System (SAS Institute Inc., Cary, NC,

U.S.A.). Individual treatment means were compared by using the least-squares means calculations in the case of unequal replication and Duncan's multiple range test for means where cell sizes were equal.

RESULTS

Leucine release from prelabelled muscle cell cultures

Expt. I examined the use of $^{18}\text{O}_2$ -labelled Leu to measure protein turnover in muscle cell cultures. Cultures were grown for 8 days in medium with one half of the Leu as [5,5,5- ^3H]Leu and the other half as [1,1- ^{18}O]Leu. Labels were added to the medium beginning at 24 h, before most myoblast fusion and the accompanying increase in protein synthesis had begun. Thus essentially all cell proteins in 8-day cultures should have been synthesized from labelled Leu, and proteins in the cells should have contained one half the Leu as the 5,5,5- ^3H isotope and the other half as the 1- ^{18}O isotope of Leu, according to the model presented in Scheme 1. Medium containing unlabelled Leu was placed on the cultures on day 9 and release of [5,5,5- ^3H]Leu and [1- ^{18}O]Leu into the medium was measured for 24 h (Figure 1). Values obtained for appearance of the isotopes used to prelabel the cells were corrected for any utilization of Leu for protein synthesis or oxidation by measuring the decrease in the [^3H]Leu tracer added to the release medium. The corrected value for release of either of the two stable isotopes used to prelabel the cell proteins represents protein degradation over the 24 h period. Because each of the isotopes used to prelabel cell proteins, [5,5,5- ^3H]Leu- and [1,1- ^{18}O]Leu, should have labelled the cell proteins nearly equally, the release of each isotope from cell proteins and into the culture medium should have been equal, in the absence of recycling. The release of the monolabelled ^{18}O isotope of Leu, however, was less than half that of the release of the ^3H isotope of Leu. At all times, significantly ($P < 0.01$) less [1- ^{18}O]Leu appeared in the medium than did [5,5,5- ^3H]Leu. The total Leu concentration in the release medium decreased from 24.4 to 21.6 $\mu\text{g}/\text{ml}$ during the 2–24 h measurement period. Percentage enrichments increased from 1.17 ± 0.09 to 3.61 ± 0.24 % for [1- ^{18}O]Leu and 3.37 ± 0.22 to 9.88 ± 0.92 % for [5,5,5- ^3H]Leu over the same period. The difference between the release of [1- ^{18}O]Leu and the release of [5,5,5- ^3H]Leu shown in Figure 1 was too great to be due to the recycling of Leu in protein. This

Figure 1 Release of [5,5,5- ^3H]Leu and of [1- ^{18}O]Leu from prelabelled cells

[5,5,5- ^3H]Leu and [1- ^{18}O]Leu were released into culture medium from cells previously labelled with medium containing half the Leu as each isotope on day 2 and up until day 8 of culture. [1- ^{18}O]Leu release was significantly ($P < 0.01$) less than [5,5,5- ^3H]Leu release. Sampling times were 2, 8 and 24 h. Results are from three plates in each of 2 weeks, and SEM bars are indicated on each data point.

Table 1 Loss of ^{18}O from $[1,1-^{18}\text{O}]\text{Leu}$ and $[1,1-^{18}\text{O}]\text{Phe}$ from culture medium

The stability of $[1,1-^{18}\text{O}]\text{Leu}$ and of $[1,1-^{18}\text{O}]\text{Phe}$ in the cell-culture medium was measured by incubation of medium containing the isotopes as described for expts. II and III in the Materials and methods section. Samples were taken from duplicate sets of plates at 1, 8 and 24 h with the 24 h results shown. Total Leu and Phe concentrations did not change over the 24 h period. The average Leu concentration in all samples was 75.4 ± 2.7 and 75.9 ± 2.3 $\mu\text{g}/\text{ml}$ of medium at 0 and 24 h respectively. The average Phe concentration in all samples was 100.0 ± 4.1 and 96.5 ± 2.5 $\mu\text{g}/\text{ml}$ medium at 0 and 24 h respectively.

Conditions	Percentage loss of isotope (mean $\pm$ S.E.M.)	
	$[1,1-^{18}\text{O}]\text{Leu}$	$[1,1-^{18}\text{O}]\text{Phe}$
In complete medium, 3 weeks at 4 °C	No loss	No loss
In complete medium, 24 h at 37 °C	44.3 ± 1.9	3.15 ± 0.35
In complete medium with added Ile, Val and Tyr (3 $\times$ normal), 24 h at 37 °C	54.3 ± 2.0	3.35 ± 3.45
In medium without embryo extract, 24 h at 37 °C	6.2 ± 0.5	-3.2 ± 1.4

result suggests that ^{18}O label was removed from Leu either before or after incorporation into protein.

Stability of $[1,1-^{18}\text{O}]\text{Leu}$

The stability of the ^{18}O labels of $[1,1-^{18}\text{O}]\text{Leu}$ and $[1,1-^{18}\text{O}]\text{Phe}$ incubated in cell-culture medium is shown in Table 1. The ^{18}O labels were stable under the conditions used for the cell-culture studies, except for incubation in complete medium at 37 °C. Then only Leu was significantly ($P < 0.01$) affected, and a 44 % loss of label was seen. The concentration of Leu in the medium remained constant during this period. The average Leu concentration in all samples was 75.4 ± 2.7 and 75.9 ± 2.3 $\mu\text{g}/\text{ml}$ of medium at 0 and 24 h respectively. Incubation of the labels in medium without embryo extract at 37 °C resulted in no loss of either ^{18}O label. These results suggest that embryo extract contains the necessary enzymes capable of exchanging the carboxy oxygen atoms of Leu. The addition of a 3-fold excess of Ile, Val and Tyr did not prevent the significant loss of ^{18}O label from Leu during incubation in complete medium. Amino acid oxidase was also used to convert $[1,1-^{18}\text{O}]\text{Leu}$ into $[1,1-^{18}\text{O}]\text{KIC}$, and no loss of the ^{18}O label was seen (results not shown). In addition, the ^{18}O labels of both leucine and phenylalanine were stable during processing of media or plasma for g.c.-m.s. analysis, as determined by comparison with Nle standards.

Loss of ^{18}O label from Leu and Phe in cell cultures

The relatively limited release of monolabelled $[^{18}\text{O}]\text{Leu}$ from the muscle cell cultures prelabelled in expt. I appeared to be due to loss of the label by a process other than protein synthesis, resulting in lower levels of the label in cell proteins. Thus, in expt. II, we compared losses of ^{18}O label from Leu and from Phe to losses of non-carboxy-labelled amino acids in the cell-culture medium. Medium containing the $^{18}\text{O}_2$ -labelled Leu and Phe was incubated with 9-day muscle cell cultures, and changes in the amounts of dilabelled, monolabelled and unlabelled Leu and Phe in the medium were determined over 12 h. The decrease in $^{18}\text{O}_2$ -labelled Leu and Phe was compared with the decrease in a second label, either ^{13}C for Phe or ^3H for Leu. Results are shown in Figure 2. In complete medium (controls, Figures 2a and 2b), the amount of $[1,1-^{18}\text{O}]\text{Leu}$ decreased 81 % over 12 h (Figure 2a). This decrease was accompanied by a small increase in mono- ^{18}O -labelled Leu and by a large increase in unlabelled Leu (Figure 2b), demonstrating the conversion of $[1,1-^{18}\text{O}]\text{Leu}$ to unlabelled

Leu. Loss of the ^{18}O label was significantly ($P < 0.01$) greater than loss of $[5,5,5-^3\text{H}]\text{Leu}$ from the medium (Figure 2a). The magnitude of the loss of ^{18}O label from Phe was much less than that from Leu (Figure 2a), and little increase in $[1-^{18}\text{O}]\text{Phe}$ or unlabelled Phe was seen (Figure 2b). A smaller but still significant ($P < 0.01$) difference is seen between loss of $[1,1-^{18}\text{O}]\text{Phe}$ and $[1-^{13}\text{C}]\text{Phe}$ than between the Leu isotopes, $[1,1-^{18}\text{O}]\text{Leu}$ and $[5,5,5-^3\text{H}]\text{Leu}$. In control plates the total Leu concentration decreased by approx. 9 % over 12 h.

To determine whether loss of ^{18}O label was due to protein synthesis, a simultaneous experiment was conducted in the presence of 0.375 mM cycloheximide (Figures 2c and 2d). Addition of cycloheximide to the medium did not decrease loss of the ^{18}O label from Leu in the medium (Figure 2c). In fact, the loss was significantly ($P < 0.05$) increased from 81 % without to 89 % with cycloheximide treatment. Cycloheximide did significantly ($P < 0.05$), lessen the loss of ^{18}O from Phe, but only from 25 % loss in controls to 20 % loss with cycloheximide (Figure 2c). Utilization of $[5,5,5-^3\text{H}]\text{Leu}$ was significantly ($P < 0.05$) reduced with cycloheximide treatment. The utilization of $[1-^{13}\text{C}]\text{Phe}$ from the medium was so little to begin with that the reduction was not significant. Total Leu in the medium did not change significantly over the 12 h period in the cycloheximide-treated plates.

Finally, loss of labels was determined in the presence of a 3-fold excess of the other branched-chain amino acids and Tyr. Results were similar to those for controls in that there was a greater loss of $[1,1-^{18}\text{O}]\text{Leu}$ than of $[5,5,5-^3\text{H}]\text{Leu}$ ($P < 0.01$), and a much smaller, but significant ($P < 0.01$), loss of $[1,1-^{18}\text{O}]\text{Phe}$ than of $[1-^{13}\text{C}]\text{Phe}$. As in the controls, loss of the ^{18}O label from Leu was about four times as great as loss of ^{18}O from Phe.

Loss of ^{18}O label from Leu and Phe with cell cultures in medium without embryo extract

Complete cell-culture medium caused a loss of the ^{18}O label from Leu, but not from Phe (Table 1). This loss seemed to be due to embryo extract, for the loss only occurred in complete medium and not in medium without embryo extract. Because the embryo extract in the culture medium exhibited activity capable of removing ^{18}O label from Leu, in expt. III culture medium containing the labels as in expt. II but not the embryo extract was placed on 9-day old cell cultures. In Table 2, incubation of the labels in culture medium without embryo extract and containing either a 3-fold excess of Ile, Val and Tyr or 0.375 mM cyclo-

Figure 2 Incubation of [5,5,5- ^2H]Leu, [1,1- ^{18}O]Leu, [1- ^{13}C]Phe, and [1,1- ^{18}O]Phe in complete medium with 9-day-old cell cultures

[5,5,5- ^2H]Leu, [1,1- ^{18}O]Leu, [1- ^{13}C]Phe and [1,1- ^{18}O]Phe were added to cell culture medium of 9-day-old cells. Results are shown as percentages of initial enrichment. (a) and (b) (controls) are with complete medium. (c) and (d) are with 0.375 mM cycloheximide, and (e) and (f) are with a 3-fold excess of Ile, Val and Tyr added to the medium. In all instances loss of [1,1- ^{18}O]Leu from the medium was significantly ($P < 0.01$) greater than that for any other label. The loss of ^{18}O from Leu is also seen in a significant ($P < 0.01$) increase in unlabelled leucine in the medium. Cycloheximide resulted in less [5,5,5- ^2H]Leu loss ($P < 0.05$) and an increase ($P < 0.05$) in loss of [1,1- ^{18}O]Leu when compared with control counterparts. Addition of the 3-fold excess of Ile, Val and Tyr had no effect on loss of labels from the medium. Sampling times were 0, 1, 4, 8, and 12 h. Results are from three plates, and SEM bars are indicated on each data point (where not seen, error bars are within the symbols).

Table 2 Effect of treatments on the loss of ^{18}O from [1,1- ^{18}O]Leu and from [1,1- ^{18}O]Phe in medium without embryo extract of 9-day old cell cultures

Medium without embryo extract containing labelled Leu and Phe was placed on 9-day-old skeletal-muscle cell cultures. Abundance of each label in the medium was measured for 12 h. Different superscripts indicate significant differences ($P < 0.05$) between the ^{18}O -labelled Leu and Phe, between ^{18}O -labelled amino acids and the corresponding ^2H or ^{13}C -labelled amino acids and among the treatments.

Treatment	Label remaining after 12 h in medium with embryo extract (% of initial enrichment $\pm$ S.E.M.)			
	Leu		Phe	
	1,1- ^{18}O	5,5,5- ^2H	1,1- ^{18}O	[1- ^{13}C]
Control	0.167 ^a $\pm$ 0.020	0.642 ^c $\pm$ 0.025	0.734 ^e $\pm$ 0.017	0.970 ^f $\pm$ 0.036
Cycloheximide	0.082 ^b $\pm$ 0.019	0.752 ^a $\pm$ 0.005	0.765 ^d $\pm$ 0.015	1.036 ^f $\pm$ 0.040
Added amino acids	0.207 ^a $\pm$ 0.006	0.664 ^c $\pm$ 0.031	0.745 ^e $\pm$ 0.005	0.946 ^f $\pm$ 0.064

heximide is shown. Results were similar to those obtained with complete medium, as shown in Figure 2. The decrease in total Leu in control and added-amino-acid-treated plates was 12%, with no loss of total Leu seen in cycloheximide-treated plates.

Again, a significant ($P < 0.01$) loss of both Leu and Phe ^{18}O labels occurred compared with the non-carboxy-oxygen labels used. This loss was, however, four times greater with Leu than with Phe.

Table 3 *In vivo* conversion of [5,5-²H]Leu and [1,1-¹⁸O]Leu into [5,5-²H]KIC and [1,1-¹⁸O]KIC

Experiments were conducted using two 30 kg pigs in which labelled Leu was infused until a plateau of plasma enrichment was reached. Plasma Leu and KIC were then measured every 20 min from 2 to 4 h post infusion and an average APE was then calculated for each. Ratios of Leu to KIC were also calculated for both of the isotopes used.

Isotope	Plasma enrichment (APE)	
	Pig 1	Pig 2
[5,5- ² H]Leu	1.32	1.61
[5,5- ² H]KIC	1.10	1.04
Ratio ...	0.83	0.64
[1,1- ¹⁸ O]Leu	0.575	0.546
[1,1- ¹⁸ O]KIC	0.481	0.461
Ratio ...	0.83	0.89

In vivo conversion of [1,1-¹⁸O]Leu to [1,1-¹⁸O]KIC

To examine possible loss of ¹⁸O by the conversion of Leu into KIC, Leu, labelled with either ²H or ¹⁸O, was infused into two pigs and the enrichments of both the labelled Leu and the resulting labelled KIC were measured (Table 3). The ratio of [¹⁸O]Leu to [¹⁸O]KIC was the same or higher than the ratio of [²H]Leu to [²H]KIC. These results indicate that ¹⁸O is not lost from Leu upon *in vivo* transamination to KIC.

DISCUSSION

The goal of this study was to determine if ¹⁸O labelling could be used to estimate tracer re-utilization in protein-turnover studies, according to the model shown in Scheme 1 and described in the Introduction. For this model to be valid, label loss must occur only during protein synthesis. For both ¹⁸O-labelled Leu and Phe, loss of label in the medium was greater than loss of a non-carboxy-oxygen-labelled counterpart (i.e. the ¹⁸O label was lost at a rate greater than utilization of Leu for protein synthesis or other processes, such as oxidation). This loss of the ¹⁸O label from

Leu was four times as great as from Phe. Addition of cycloheximide to block protein synthesis failed to prevent the loss of ¹⁸O label. This loss of label, therefore, was not due to protein synthesis alone or to cycling through rapidly degraded proteins. We also showed that the loss was not due to simple exchange, because ¹⁸O-labelled Leu and Phe are stable under all conditions encountered in the experiments, except for Leu in complete medium. This loss of ¹⁸O from Leu in complete medium without cells was due to the embryo extract in the complete medium. However, the rate of ¹⁸O loss from Leu was faster in medium with cells than in complete medium alone, the loss of ¹⁸O also occurred with cells without embryo extract in the medium, and the loss of ¹⁸O did not occur in medium without embryo extract in the absence of cells. Therefore this process occurs in cells and cell extracts only.

In Figure 2(c) a rise in the initial [1-¹³C]Phe is seen. The reason for this rise in the amount of this isotope is unknown, and this rise was not seen in other experiments in this laboratory. This should not change the interpretation of the results demonstrating that there is a difference in the rate of ¹⁸O loss from Leu versus that from Phe.

Carboxy oxygen atoms are not involved in the transaminase reaction of amino acids [15]. For Leu we have shown that, *in vivo*, the ¹⁸O label is quantitatively transferred to KIC by transamination (Table 3). A 20% decrement in the enrichment of KIC occurred with both ¹⁸O and ²H labels and is thought to be due to the lower enrichment of intracellular Leu compared to the enrichment of plasma Leu. Thus intracellularly derived KIC enrichment is lower than the correspondingly labelled Leu enrichment in plasma. This *in vivo* experiment was done in pigs because only very low concentrations of KIC were found in medium from muscle cell cultures.

The most plausible remaining explanation for the loss of ¹⁸O label is that this loss occurs during the aminoacylation process. Aminoacylation of tRNA is a two-step process [16], as shown in Scheme 2. In step 1, the amino acid is activated with the liberation of PP_i [17]. In step 2, the amino acid is transferred to the tRNA, liberating AMP, which contains the ¹⁸O lost from the amino acid [18]. The amino acid-tRNA-enzyme complex dissociates to yield the aminoacylated tRNA, which is then used for protein synthesis, as shown in pathway 3A in Scheme 2. That step 1 is reversible *in vitro* has been known for some time, and has been shown by the addition of ³²PP_i to the reaction mixture [19]. Because cells contain very low levels of PP_i and because energy from hydrolysis of PP_i is a stabilizing factor, reversal of this step is probably not a major reaction *in vivo* and is unlikely to account for the loss of the ¹⁸O label. Another reaction, enzymic deacylation, which is the hydrolysis of the amino-acid-tRNA complex (pathway 3B in Scheme 2), has also been shown to occur [20]. Enzymic deacylation does not require either PP_i or AMP, as is required for the reversal of either steps 1 or 2 respectively of the aminoacylation process [20]. Enzymic deacylation occurs at various rates depending upon the synthetase enzyme [20]. There is no relationship between rate of deacylation and non-cognate-amino-acid or tRNA binding [21–23]. And, because the rate of deacylation for misbinding and correct binding are similar, the deacylation reaction probably plays a small part in regulating the specificity of aminoacylation *in vivo* [22]. Enzymic deacylation is probably significant as a mechanism for the loss of ¹⁸O from our ¹⁸O-labelled amino acids. Because two high-energy phosphate bonds are utilized in step 1, and because this energy is lost by deacylation, this process represents a futile cycle that could occur as part of charging tRNA for protein synthesis.

Although these reactions have been well-characterized in cell homogenates, few studies have been conducted with whole living

Scheme 2 The aminoacylation reactions

Pathways 1 and 2 depict the two steps in aminoacylation of tRNA. Pathway 3A depicts utilization of aminoacylated-tRNA for protein synthesis. Pathway 3B depicts the enzymic deacylation reaction in which water hydrolyses the amino acid from the 'proper' tRNA while this is still complexed with the synthase enzyme. AA, E and tRNA represent an amino acid, the corresponding aminoacyl synthase and the corresponding tRNA respectively.

cells. Our results demonstrate that muscle cells in culture, as well as embryo extract, are capable of exchanging the carboxy oxygen atoms of Leu at an extremely high rate, and of Phe at a lower rate. By blocking protein synthesis with cycloheximide, we have demonstrated that ^{18}O label loss is not due to cycling of the label in a fast-turnover pool of proteins and, thus, is probably related to tRNA charging. In fact, the loss of ^{18}O label from Leu was increased with cycloheximide treatment, which would be expected if of the two possible pathways, utilization for protein synthesis or an alternative pathway after aminoacylation, one was blocked by cycloheximide. Furthermore, by adding an excess of branched-chain amino acids other than Leu we have shown that the loss of ^{18}O label is probably not due to error correction due to Leu or Phe misbinding to other tRNAs for structurally similar amino acids. Phe has been shown to bind to tRNA^{Val} and *vice versa* [21], and Ile has been shown to bind to tRNA^{Phe} [24]. However, adding an excess of Val, Ile and Tyr did not alter loss of ^{18}O from Leu or Phe. Thus it is probably safe to say that most of the label loss in the case of Leu is caused by enzymic deacylation of the proper combination, i.e. Leu-tRNA^{Leu}. The loss of ^{18}O is substantially less for Phe, either with or without added amino acids.

Phe may be suitable for use as a tracer in *in vivo* studies because the magnitude of the ^{18}O loss is much smaller than with Leu. This difference in the rate of ^{18}O loss may be due to the difference in V_{max} for each synthase enzyme. The V_{max} of acylation for tRNA^{Leu} synthase is more than twice that of tRNA^{Phe} synthase [25]. The reported K_m values for Phe and Leu are 2 μM and 6 μM respectively [25]. Because intracellular concentrations of both amino acids are much higher than the K_m for each [25], each enzyme should be operating at V_{max} . However, these studies used tissue homogenates to measure activity, and the authors admit that such a procedure may not be an accurate representation of the *in vivo* concentrations at the site of the enzyme.

Leu has been shown to increase protein synthesis *in vitro* [26,27]. The mechanism of this response is unknown. Our results suggest that enzymatic deacylation of tRNA^{Leu} may contribute to instability of the acylated tRNA and that this process may limit Leu-tRNA^{Leu} formation *in vitro*, which may in turn slow the rate of protein synthesis. If however, the Leu-tRNA complex is unstable and hydrolysis via enzymic deacylation occurs at a high frequency, then increasing the concentration of Leu in the cell should shift the equilibrium of the deacylation reaction and act to stabilize the Leu-tRNA^{Leu} complex. Earlier work has suggested that no change in the concentration of Leu-tRNA^{Leu} occurred in response to increasing concentrations of Leu that resulted in increased protein synthesis [25,28]. Morgan et al. [28] reported that increasing the Leu concentration to five times normal in rat heart increased the tRNA-bound Leu from 2.65 ± 0.28 to 3.21 ± 0.26 nmol/g. Although this was a 21% increase, it was not significant ($P > 0.05$), possibly due to a small sample size. This result alone may not rule out the possibility that the additional Leu was acting to stabilize the acylated tRNA and increase protein synthesis by increasing Leu-tRNA^{Leu} concentration.

In conclusion the high rate of apparent futile cycling during the charging of tRNA^{Leu} precludes its use as a non-recyclable tracer

using the ^{18}O labelling method. While some loss of ^{18}O from Phe seems to occur in the same manner, the magnitude of the loss may not preclude it from being used in this method. This futile cycling has important implications on the amount of energy needed for protein synthesis. In addition, the fact that the rate of futile cycling for Leu is so high may be related to the unique effect that Leu has on protein metabolism. Although, in theory, the ^{18}O labelling of protein should provide an alternative to other labelling methods, certain aminoacyl-tRNA synthase enzymes seem to be capable of catalysing deacylation to the point that label integrity is lost too quickly for use.

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