The role of glutathione in amino-acid absorption

Lack of correlation between glutathione turnover and amino-acid absorption by the yeast *Candida utilis*

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The rate of degradation of glutathione has been determined in the yeast *Candida utilis* by using a method that minimizes the effect of amino-acid recycling. When yeast are grown in amino-acid-free medium, the half-life of glutathione was found to be 230 min. *C. utilis* was also found to absorb various L-amino acids rapidly without producing any significant decrease in the half-life of glutathione. While the γ -glutamyl cycle is thus operating in *C. utilis*, the rate of degradation of glutathione is found to be 100 times too slow for the cycle to be mediating the transport of these amino acids.

Glutathione apparently occurs ubiquitously in cells, yet its metabolic activity remains obscure. A role in amino-acid absorption has been proposed, involving the metabolism of glutathione by the γ -glutamyl cycle (Meister, 1973). Assuming that the key enzyme, γ -glutamyltransferase (EC 2.3.2.2), is located in the cell membrane, one mole of amino acid could be transported per mole of glutathione degraded. This localization has been confirmed for a number of mammalian tissues (Meister & Tate, 1976). Much of the support for this proposal is, however, indirect and the validity of the methodology employed has been questioned (Griffith *et al.*, 1978).

Mooz & Wigglesworth (1976) have shown that all enzymes of the γ -glutamyl cycle are present in yeast, and Mooz (1979) has presented evidence to support the view that the γ -glutamyl cycle functions in the absorption of amino acids. A study of several mutant strains of yeast defective in amino-acid transport confirms that the problem is of considerable complexity and such conclusions cannot be drawn (Penninckx *et al.*, 1980).

A direct test of a causal relationship between the γ -glutamyl cycle and the uptake of amino acids is to compare the rate of cycling, i.e. glutathione turnover, with the rate of uptake of amino acids. In this paper we report measurements of the turnover of glutathione and show that it is not correlated with the rate of uptake of a number of L-amino acids. These results contradict those reported by Osuji (1979*a,b*, 1980) and this discrepancy will be examined in the discussion.

Experimental

Materials

$^3\text{H}_2\text{O}$ (185 GBq·ml⁻¹), L-[1- ^{14}C]leucine (2.18 GBq·mmol⁻¹), L-[1- ^{14}C]methionine (2.25 GBq·mmol⁻¹), L-[U- ^{14}C]glutamine (1.55 GBq·mmol⁻¹) and [U- ^{14}C]glycine (4.22 GBq·mmol⁻¹) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. All other reagents were of AR standard and obtained from one of the following: Hopkin and Williams, Chadwell Heath, Essex, U.K.; British Drug Houses, Poole, Dorset, U.K.; Fisons Scientific Apparatus, Loughborough, Leics., U.K.; Sigma Chemical Co., Poole, Dorset, U.K.; and Boehringer Corp. (London), London, U.K.

Culture of yeast

Candida utilis (N.C.Y.C. 737) was obtained from Dr. A. P. Sims (School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, U.K.). Cultures were grown at 25°C with vigorous aeration (approx. 41·min⁻¹) and constant mixing in a medium containing: 28 mM-glucose, 26 mM-NaH₂PO₄, 10 mM-KNO₃, 4.3 mM-K₂SO₄, 1.8 mM-Na₂HPO₄, 0.5 mM-MgCl₂, 0.2 mM-CaCl₂, 16.2 μM -H₃BO₃, 7.3 μM -ZnCl₂, 6.2 μM -FeCl₃, 5.1 μM -MnCl₂, 0.7 μM -CuCl, 0.6 μM -KI and 0.4 μM -Na₂MoO₄. Growth was monitored by the absorption at 640 nm (A_{640}) and converted to cell density (cells·ml⁻¹) when required. Exponential growth was maintained to a maximum A_{640} of 1.0–1.2.

Labelling with $^3\text{H}_2\text{O}$

All manipulations were carried out under sterile conditions. For the determination of the effect of

Abbreviation used: PPO, 2,5-diphenyloxazole.

$^3\text{H}_2\text{O}$ on the growth of yeast, equal samples of culture in exponential-phase growth were added to 100ml of medium and to 100ml of medium containing 74, 37, 18.5, 3.7 and 0.2MBq of $^3\text{H}_2\text{O}\cdot\text{ml}^{-1}$. No significant difference (*t* test) was found between the growth rate of the control and that of any culture containing $^3\text{H}_2\text{O}$ in the medium (Table 1). On the basis of this, $^3\text{H}_2\text{O}$ at 37MBq $\cdot\text{ml}^{-1}$ was used routinely.

Counting of radioactivity of ^3H - and ^{14}C -labelled compounds

^3H and ^{14}C were measured by liquid-scintillation counting in an Intertechnique SL30 liquid-scintillation spectrometer. Samples were prepared either in 10ml of PPO scintillant [7g of PPO/litre of toluene/2-methoxyethanol (6:4, v/v)] or in 5ml of Pico-Fluor 15 (Packard Instrument Co., Downers Grove, IL 60515, U.S.A.) and counted for a suitable period to obtain a background below 5%. Counting efficiency was determined by the addition of an internal standard.

Isolation of glutathione

Glutathione was isolated by the method of Bloch & Snoko (1957) with 5mg of glutathione added to each sample as carrier. The recovery was determined on a small aliquot by the method of Tietze (1969).

Purity of glutathione

The purity of glutathione prepared as above was assessed by the method of Hanes *et al.* (1952). The preparation was evaporated to dryness and the residue was taken into 1.0ml of water. Aliquots (100 μl) were treated for 60min with 5 μl of 300mM-2-mercaptoethanol, sealed under N_2 . *N*-Ethylmaleimide was added to 40mM final concentration and the tubes were resealed under N_2 for a further 60min. Standards of oxidized and reduced glu-

tathione, L-glutamate, L-leucine, γ -L-glutamyl-L-glutamate and γ -L-glutamyl-L-leucine were also prepared. Chromatography was conducted with Whatman no. 1 paper developed by downward elution with butan-1-ol/water (4:1, v/v). The chromatogram was dried and treated in one of three ways: (a) sprayed with ninhydrin (2.0g $\cdot\text{l}^{-1}$) in acetone and incubated for 30min at 100°C; (b) sprayed with *o*-phthalaldehyde (1.0g $\cdot\text{l}^{-1}$) in 0.2M-NaOH and examined under a u.v. lamp; (c) divided into 15mm sections and the ^3H determined by liquid-scintillation counting in 10ml of PPO scintillant.

Fig. 1 shows that the radioactivity co-chromatographs with standard glutathione; only traces are associated with other standards. The fluorescence obtained with *o*-phthalaldehyde shows that the glutathione preparation runs here and there is no other ninhydrin-positive substance present. The preparation is at least 95% radiochemically pure.

Determination of ^3H at C-2 of the γ -glutamyl group of glutathione

Glutathione, prepared as above, was evaporated to dryness by rotary evaporation and the residue was taken into 50 μl water. The ^3H at C-2 of the γ -glutamyl group was released by racemization with 1.0ml of acetic anhydride as described by Humphrey & Davies (1976).

Fig. 1. Purity of glutathione prepared from *C. utilis*. Aliquots of glutathione, prepared and treated as described in the text, were chromatographed by downward elution with butan-1-ol/water (4:1, v/v). The solid blocks show the position of staining the chromatogram with ninhydrin or *o*-phthalaldehyde and also of a glutathione standard similarly stained. The reduced glutathione peak is at R_F 0.27. Standards ran as follows: glutathione, R_F 0.27; γ -L-glutamyl-L-glutamate, R_F 0.18; γ -L-glutamyl-L-leucine, R_F 0.56; L-glutamine, R_F 0.22; L-leucine, R_F 0.62.

Table 1. Effect of $^3\text{H}_2\text{O}$ on the growth of *C. utilis*. Yeast were grown in medium to which $^3\text{H}_2\text{O}$ was added as shown. No significant difference (*t* test) was found between the rate of growth in control medium and in those to which $^3\text{H}_2\text{O}$ had been added. Doubling times are shown as means $\pm$ S.E.M for six observations.

Concentration of $^3\text{H}_2\text{O}$ (MBq $\cdot\text{ml}^{-1}$)	Doubling time (h)
None (control)	2.3 ± 0.07
74	2.5 ± 0.04
37	2.3 ± 0.14
18.5	2.0 ± 0.05
3.7	2.4 ± 0.07
0.2	2.2 ± 0.09

Preparation of [γ -glutamyl-2- ^3H]glutathione

(Carbonato)bis(ethylenediamine) cobalt (III) chloride, prepared by the method of Springbørg & Schäffer (1973), was complexed with glutathione (Legg & Steele, 1971). Treatment of an aliquot of the preparation with NaBH_4 released free glutathione with a recovery of 88%. Further aliquots of the complex were sealed with $^3\text{H}_2\text{O}$ at $37\text{MBq}\cdot\text{ml}^{-1}$ and incubated at 40°C for 7 days. On opening, samples were treated with NaBH_4 and the free glutathione was separated from contaminants and unreacted $^3\text{H}_2\text{O}$ on Dowex 50 (Keyes & Legg, 1976).

Test of the validity of the acetic anhydride method when applied to glutathione

A sample of [γ -glutamyl-2- ^3H]glutathione, prepared as described above, was divided gravimetrically into equal portions and evaporated to dryness. One portion was racemized with acetic anhydride; the other was incubated with water. The ^3H released as $^3\text{H}_2\text{O}$ was determined for the treated portion; the total ^3H present was determined in the untreated portion. The values obtained differ by less than 2% from the mean, indicating that acetic anhydride effectively removes all the ^3H at C-2 of the γ -glutamyl moiety of glutathione.

Determination of the degradation constant of glutathione

The yeast from 400ml of culture, growing in medium containing $^3\text{H}_2\text{O}$ were harvested by vacuum filtration onto Whatman GF/A paper, briefly washed, and resuspended in 400ml of unlabelled medium. For the next 3 or 4 h samples of approx. 50ml were withdrawn at various intervals into pre-weighed flasks containing 5 ml of cold trichloroacetic acid ($500\text{g}\cdot\text{l}^{-1}$). Each flask was reweighed, to obtain the sample size. Sampling was performed by temporarily blocking the air outlet vent, allowing the ensuing pressure to force culture through an outlet tube dipping into the medium.

Glutathione was isolated from each sample and the ^3H content at C-2 of the γ -glutamyl group of glutathione was determined as described above.

The degradation constant (k_d) of glutathione was also determined by using [$\text{U}-^{14}\text{C}$]glycine to label the yeast. Culture (400ml) was grown in medium containing [$\text{U}-^{14}\text{C}$]glycine at $1.85\text{kBq}\cdot\text{ml}^{-1}$ and treated exactly as described above for the $^3\text{H}_2\text{O}$ -grown culture.

Determination of the ^3H at C-2 in free glutamic acid

Yeast were grown on medium containing $^3\text{H}_2\text{O}$ ($37\text{MBq}\cdot\text{ml}^{-1}$) for 2 h, harvested, briefly washed, and resuspended in unlabelled medium. Samples (50ml) were withdrawn, the yeast were harvested onto Whatman GF/A paper by vacuum filtration

and killed by plunging the filter into ethanol/water (3:2, v/v) at room temperature. L-Glutamate (1mg) was added as carrier, the debris was removed by centrifugation, and L-glutamate was isolated by ion-exchange chromatography on Dowex resins (Humphrey, 1975). The recovery of L-glutamate was measured with glutamic dehydrogenase (Bernt & Bergmeyer, 1965) and the ^3H present at C-2 was determined by racemization with acetic anhydride (Humphrey & Davies, 1976).

Purity was confirmed by chromatography on Whatman no. 1 paper with pyridine/acetic acid/water (10:7:3, by vol.). Standards of L-glutamate and L-aspartate were co-chromatographed. Chromatograms were either developed with the ninhydrin method or sliced and prepared for liquid-scintillation counting as described above. L-Glutamate prepared this way ran with R_F 0.39 and was 98% radiochemically pure. Standard L-glutamate ran with R_F 0.39 and L-aspartate with R_F 0.25.

Uptake of amino acids by Candida utilis

Yeast were cultured with 1.0mM-L-amino acid added to the medium. An aliquot was removed, filtered on Whatman GF/A paper, briefly washed and resuspended to give $(10\text{--}15)\times 10^6\text{cells}\cdot\text{ml}^{-1}$. Approx. 10ml of this suspension was added to a pre-warmed flask containing 185–18.5kBq of ^{14}C -labelled L-amino acid and various amounts of concentrated stock L-amino acid to give a final concentration in the range 0.001–1.0mM. Six 1ml samples were taken over 3–5min and the yeast was extracted by filtration under vacuum onto Sartorius cellulose nitrate filters (1.2 or $0.45\mu\text{m}$ pore diameter). After washing with 10ml of ice-cold water, the ^{14}C present was determined by liquid-scintillation counting in 10ml of Pico-Fluor 15 scintillant. The cell density in the culture remaining was determined by measuring A_{640} and was converted to $\text{cells}\cdot\text{ml}^{-1}$.

Results*Release of ^3H at C-2 from free glutamate during the 'chase' period*

The validity of the proposed method of measuring glutathione turnover requires glutamate that becomes labelled during the exposure to $^3\text{H}_2\text{O}$ rapidly to lose ^3H from C-2 during the 'chase' period. To examine this point, *C. utilis* was grown on medium containing $^3\text{H}_2\text{O}$ ($37\text{MBq}\cdot\text{ml}^{-1}$), glutamate was isolated, and the ^3H at C-2 was estimated as described above. Fig. 2 shows that the loss of ^3H from C-2 of glutamate is not monophasic. Initially there is a rapid loss of label with a $t_{\frac{1}{2}}$ of 1.9min, followed by a slower loss of $t_{\frac{1}{2}}$ approx. 25min. The rapidly transaminated pool represents about 80% of the total. The turnover in both pools is much faster

than the turnover of the ^3H at C-2 of glutamate in glutathione.

Measurement of glutathione degradation

An aliquot of yeast, brought into exponential-phase growth, was added to 400 ml of medium containing $^3\text{H}_2\text{O}$ at $37\text{ MBq}\cdot\text{ml}^{-1}$ and cultured for 8–10 generations to a final A_{640} of 0.4–0.8. Cells were harvested, washed and transferred to ^3H -free medium. Samples were taken at intervals and the ^3H content at C-2 of the glutamate of glutathione was determined. There was a steady exponential decline in the ^3H content, from which the degradation constant of glutathione under standard growth conditions was estimated to be $3.0 \pm 0.3\text{ min}^{-1}$ (Fig. 3), giving a $t_{1/2}$ of $230 \pm 35\text{ min}$.

The decay curve is fitted by a single exponential ($P < 0.001$). Glutathione-decay curves obtained after

labelling with L-[^{35}S]cysteine have been interpreted (Tateishi & Higashi, 1978) to mean the existence of two glutathione pools. It is, therefore, necessary to check for the existence of a rapidly-turning-over pool of glutathione which disappears in the time taken to wash and resuspend the yeast. Experiments to measure the loss of ^3H at C-2 from the glutamate of glutathione during this period (2–3 min) indicate a maximum loss of 7%. This suggests either that there is a single pool of glutathione or that, if there is a second pool, it is very small.

When glutathione was labelled with [^{14}C]glycine the degradation constant was estimated to be $1.4 \pm 0.4\text{ min}^{-1}$, giving a $t_{1/2}$ of $490 \pm 102\text{ min}$.

Fig. 2. Decay curve for the loss of ^3H from C-2 of glutamate in *C. utilis*

Yeast was grown for 2 h on medium containing $^3\text{H}_2\text{O}$ at $37\text{ MBq}\cdot\text{ml}^{-1}$ and glutamate was prepared from samples taken at the times shown during a subsequent unlabelled 'chase' on ^3H -free medium. The content of ^3H at C-2 was determined as described in the text.

Fig. 3. Decay curve for the loss of ^3H from C-2 of the γ -glutamyl group of glutathione in the absence of exogenous amino acid

C. utilis was cultured on medium containing $^3\text{H}_2\text{O}$ at $37\text{ MBq}\cdot\text{ml}^{-1}$, harvested and resuspended on ^3H -free medium. Samples were taken at the times shown, glutathione was prepared and the content of ^3H at C-2 of the γ -glutamyl group was determined as described in the text. The Figure shows results compiled from four separate experiments. The graph was constructed by linear regression analysis and the fit is better than 0.1% ($P < 0.001$).

Table 2. Effect of an exogenous supply of L-amino acid on the turnover of glutathione

Yeast were grown without added amino acid (control) or with L-amino acid added at the initial concentration shown and in the presence of $^3\text{H}_2\text{O}$ at $37\text{ MBq}\cdot\text{ml}^{-1}$. After 8–10 generations they were harvested and transferred to ^3H -free medium with L-amino acid added as before. At suitable times samples were taken, from which glutathione was extracted and the ^3H at C-2 of the γ -glutamyl group was determined as described in the text. The level of significance of the difference in the rate of loss of ^3H in the presence of L-amino acid was determined by using a t test. The number of experiments (n) and total number of samples taken (N) are given. Values are given as means $\pm$ S.E.M. N.S., not significant.

Addition to medium	$10^3 \times k_d$ (min^{-1})	$t_{1/2}$ (min)	Significance	$n(N)$
None (control)	3.02 ± 0.26	230 ± 35	—	4 (30)
L-Glutamine (1.0 mM)	3.45 ± 0.31	201 ± 16	N.S.	2 (15)
L-Methionine (0.2 mM)	3.35 ± 0.78	207 ± 39	N.S.	3 (19)
L-Leucine (0.2 mM)	1.83 ± 0.41	378 ± 69	$P < 0.02$	2 (13)

Effect of exogenous amino acids on the degradation of glutathione

An amino acid was added to the growth medium throughout the experimental period. The degradation constants obtained in the presence of three amino acids and the control are given in Table 2. No significant change in the rate of degradation of glutathione was found in the presence of 1.0 mM-L-glutamine or 0.2 mM-L-methionine, but 0.2 mM-L-leucine caused a significant decrease (*t* test: $P < 0.02$) in the rate of degradation of glutathione. The decay curves are fitted by single exponentials (correlation coefficients: L-glutamine, $P < 0.001$; L-methionine, $P < 0.001$; L-leucine, $P < 0.01$).

Effect of growth on the glutathione content of the cells

As the cell density increases, the glutathione content per cell rises, reaching a plateau at about 10^7 cells·ml⁻¹ (Fig. 4). Mooz (1979) also reported that the glutathione content of *Saccharomyces cerevisiae* changed with cell density. All estimates of k_d reported here were performed on cells at a density of $(7-40) \times 10^6$ ml⁻¹. All measurements of the initial velocity of uptake of amino acids used cells at a density of $(10-20) \times 10^6$ ml⁻¹.

Influx of L-amino acids into *Candida utilis*

The rate of influx of L-glutamine, L-methionine and L-leucine was found to be constant during the first 5 min. The uptake of L-glutamine appears, on a double reciprocal plot, to be dependent on a single function of K_t 410 μ M and V_{max} 104 pmol·min⁻¹·(10⁶ cells)⁻¹. The uptake of L-methionine and L-leucine both appear dependent on

Fig. 4. Effect of growth on the glutathione content of *C. utilis*

Yeast were grown on medium and the glutathione content of cells at various cell densities was determined by the method of Tietze (1969). Cell density was determined by A_{640} and was converted to cells·ml⁻¹. The graph is compiled from a number of separate experiments.

two functions (Fig. 5). For L-methionine there is a high affinity system of K_t 23 μ M and V_{max} 31 pmol·min⁻¹·(10⁶ cells)⁻¹ with a second system of K_t 495 μ M and V_{max} 96 pmol·min⁻¹·(10⁶ cells)⁻¹. For L-leucine there is a high affinity system of K_t 5.6 μ M and V_{max} 22 pmol·min⁻¹·(10⁶ cells)⁻¹ and a second system of K_t 530 μ M and V_{max} 99 pmol·min⁻¹·(10⁶ cells)⁻¹.

Discussion

Methodology

Previous determinations of the half-life of glutathione in mammalian tissues have yielded a wide range of values (Sekura & Meister, 1974). In all

Fig. 5. Effect of concentration on the rate of influx of L-amino acids into *C. utilis*

An aliquot of yeast growing on medium containing in (a) 0.2 mM-L-methionine and in (b) 1.0 mM-L-leucine was harvested, washed and resuspended in medium at $(1-2) \times 10^7$ cells·ml⁻¹. Approx. 10 ml of this suspension was added to a pre-warmed vessel containing (a) L-[1-¹⁴C]methionine at 18.5–185 kBq·ml⁻¹ and unlabelled L-methionine to give final concentrations of 0.01–1.0 mM or (b) L-[1-¹⁴C]leucine at 18.5–185 kBq·ml⁻¹ and unlabelled L-leucine to give final concentrations of 0.001–1.0 mM. Samples were taken for 5 min, during which time the rate of influx remained linear.

these cases the use of ^{14}C -, ^{15}N - or ^{35}S -labelled amino acids is likely to have caused problems with metabolic recycling of label (Davies & Humphrey, 1978) and the persistence of the primary source of radiolabel in the system. Sekura & Meister (1974) demonstrate the difficulties of trying to correct for these. Other results have been interpreted as a biphasic decay (Tateishi & Higashi, 1978) but the elevated initial values they report are equally likely to be due to these problems. We find that in *Candida utilis* the use of $[^{14}\text{C}]$ glycine leads to a 2-fold overestimate of the $t_{\frac{1}{2}}$ of glutathione.

The use of $^3\text{H}_2\text{O}$ to label amino acids coupled with racemization to examine the loss of ^3H from C-2 has been established as a satisfactory technique to examine the degradation of proteins in *Lemna minor* (Humphrey & Davies, 1976). This technique is now shown to be equally applicable to the amino-terminal residue of glutathione.

Half-life of glutathione

We find that in healthy yeast growing aerobically with a doubling time of 120–160 min the $t_{\frac{1}{2}}$ is 230 min. Osuji (1979a) claims a $t_{\frac{1}{2}}$ of 39.2 h (2352 min), 10 times our value. To measure a $t_{\frac{1}{2}}$ of this duration would necessitate observations over at least 20 h. Osuji started with 5 g of yeast in 200 ml of medium, claimed to be growing with a doubling time of 4 h. If these conditions were sustained, the medium would contain 160 g of yeast after 20 h, or 800 mg · ml $^{-1}$. At this concentration the yeast can no longer be aerobic and steady-state conditions have thus not been maintained, making Osuji's results difficult to interpret in any consideration of the aerobic physiology of this organism.

There is no evidence for a small pool of glutathione turning over extremely rapidly in *C. utilis*, as the difference in specific activity of glutathione extracted before and after resuspension is only a few per cent. Two pools, of $t_{\frac{1}{2}}$ 1.7 and 28.5 h, have been reported for rat liver (Tateishi & Higashi, 1978) but the interpretation of the data in this way is questionable and the standard error of these results also permits the data to be fitted by a single exponential.

We conclude therefore that in *C. utilis* there is essentially a single pool of glutathione, turning over with a $t_{\frac{1}{2}}$ of 230 min.

Effect of exogenous amino acids on glutathione turnover

When L-amino acids are added to the growth medium, the rate of loss of ^3H from the C-2 of the γ -glutamyl moiety of glutathione is not stimulated, suggesting no increase in the turnover of this molecule. Indeed, with L-leucine there appears to be a slight, but significant, decrease in glutathione degradation. The reason for this is not clear but it

may be caused by an inhibitory action on γ -glutamyltransferase, as reported for rat liver (Thompson & Meister, 1977), although L-leucine does not appreciably inhibit the enzyme from *S. cerevisiae* (Penninckx *et al.*, 1980). The effect may, however, have a less direct cause, as leucine has been found to affect protein synthesis and breakdown in isolated muscle (Goldberg & Chang, 1978).

If glutathione turnover were associated with the uptake of amino acid, then, as the medium becomes depleted of amino acid and the uptake rate decreases, so the turnover of glutathione should decrease, leading to a rate of loss of ^3H from C-2 of the γ -glutamyl moiety that cannot be fitted by a single exponential. For the amino acids tested the data is satisfactorily fitted by a single exponential, showing that this is not the case, as, under the conditions studied, the absorption of amino acid was not saturated.

These findings are in direct contrast with those of Osuji (1979a), which we have already questioned (Robins & Davies, 1980). Osuji claims that four amino acids, exhibited over the range 2.5–250 μM , all cause a decrease in the $t_{\frac{1}{2}}$ of glutathione as measured by the 2- ^3H method. With the exception of L-glutamate (300 min) all values were considerably greater than those found here. With L-arginine and L-leucine, Osuji claims further that the effect is concentration-dependent yet, despite all four amino acids being at sub-saturating concentrations, no such effect occurs with L-histidine and L-glutamate. None of Osuji's curves extrapolate to a common value at zero concentration, which is incompatible with the model under consideration.

Glutathione turnover and amino-acid absorption rates

In its original form it is an intrinsic property of the γ -glutamyl cycle that, for every molecule of amino acid absorbed, one molecule of glutathione must be degraded (Orlowski & Meister, 1970; Meister, 1973). Subsequently, Sekura & Meister (1974) proposed that a series of near-equilibrium transpeptidation reactions involving the γ -glutamyl moiety may permit the absorption of several molecules per molecule of glutathione degraded, thus forming a facilitated diffusion mechanism.

The proposed cycle could function in amino-acid transport by turning over relatively slowly, responding to the presence of amino acid by a rapid increase in cyclic flux, leading to an increased k_d . It is shown that this does not occur in *C. utilis* (Table 2). Alternatively, the cycle could 'free-wheel' at maximal or near-maximal capacity, since Revel & Ball (1959) have shown that the γ -glutamyl moiety may be donated to water in the absence of amino acid. Although this model is energetically highly

Table 3. Relationship between the observed velocity of influx of L-amino acids and the turnover of glutathione

The molar ratio, that is the ratio of moles of L-amino acid absorbed to moles of glutathione turned over per unit time and per unit cell density, is given for different conditions.

Amino acid added to medium	Concentration (mm)	Velocity of influx [pmol·min ⁻¹ ·(10 ⁶ cells) ⁻¹]	Glutathione degraded [pmol·min ⁻¹ ·(10 ⁶ cells) ⁻¹]	Molar ratio
L-Glutamine	1.0	99.5	0.97	103:1
	'Infinite'	103.6 ($V_{\max}$)	0.97	107:1
L-Methionine	0.2	27.8	0.94	30:1
	'Infinite' (high affinity)	30.8 ($V_{\max}$)	0.94	33:1
	'Infinite' (low affinity)	96.4 ($V_{\max}$)	0.94	103:1
L-Leucine	0.2	27.2	0.51	53:1
	'Infinite' (high affinity)	22.0 ($V_{\max}$)	0.51	43:1
	'Infinite' (low affinity)	99.2 ($V_{\max}$)	0.51	195:1

unfavourable, it cannot be disregarded. Under these conditions no stimulation by exogenous amino acid will be observed, but k_d must still be sufficiently high to account for the rate of amino acid absorption.

An examination of the uptake kinetics of the L-amino acids used to test the hypothesis show that this is not the case. From the measured quantity of glutathione present and the degradation constant, it may be calculated that 30–200 molecules of L-amino acid are absorbed for every molecule of glutathione degraded (Table 3). This ratio is incompatible with the model, even if the idea of facilitated diffusion is invoked. To obtain a 1:1 ratio, the $t_{1/2}$ of glutathione must be 1.5–6 min if the whole pool is involved.

It may be argued that all or a portion of the glutathione pool is turning over extremely rapidly. We have shown that, at the most, 7% of the glutathione present may represent a rapidly-turned-over pool. If this were responsible for the absorption of L-amino acids, it needs to turn over with a $t_{1/2}$ of less than 100s depending on the L-amino acid used. While not impossible, this seems highly improbable.

Osuji (1979b) has attempted to relate the rate of absorption of L-amino acids to the turnover of glutathione. Osuji did not, however, measure the initial rate of absorption but incubated large quantities of yeast with ¹⁴C-labelled amino acid for up to 10h before measuring the remaining external concentration. Subsequently Osuji allocated portions of the influx to a γ -glutamyl pathway and to a facilitated-diffusion pathway on the basis of uptake over the first 30min (Osuji, 1980). As it is doubtful that the yeast used by Osuji were growing aerobically, and as he has not measured the initial rate of uptake of amino acids, we consider that his conclusions must be open to question.

Mooz (1979) found that the *gap* mutant of *S. cerevisiae* (2512c) is deficient in glutathione synthetase (EC 6.3.2.3) activity and suggested that this supports a role of glutathione turnover in the uptake of some amino acids by this yeast. Penninckx *et al.*

(1980), however, find γ -glutamyltransferase activity in *S. cerevisiae* not to differ from the wild type in a number of amino-acid-permease defective mutants, including *gap*. They thus fail to support Mooz (1979) but rather find the enzyme subject to repression. As the turnover of glutathione in this organism has not been estimated, any suggested correlation remains circumstantial.

We are thus led to conclude that the γ -glutamyl cycle plays no role in the absorption of L-amino acids by yeast. Those tested are, on kinetic evidence, the most likely candidates but the influx of L-glutamine, L-methionine and L-leucine in *C. utilis* is considerably greater than the turnover of glutathione.

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