Biochem. J. (1984) **218**, 147–155
Printed in Great Britain

γ-Glutamyltransferase is not involved in the bulk uptake of amino acids, peptides or γ-glutamyl-amino acids in yeast (*Saccharomyces cerevisiae*)

Gillian M. PAYNE and John W. PAYNE
Department of Botany, University of Durham, South Road, Durham DH1 3LE, U.K.

(*Received 2 August 1983/Accepted 19 October 1983*)

γ-Glutamyltransferase activity has been measured in yeast (*Saccharomyces cerevisiae*) and shown to be associated mainly with the membrane fraction. A similar level of activity is found in a wild-type strain and in *gap* and *gpp* strains, the latter mutants being defective in the general amino acid and peptide permeases respectively. The activity is inhibited in whole cells by 6-diazo-5-oxo-L-norleucine (N_2O-Nle), azaserine and serine–borate complex; this inactivation seemingly acts from without, for it is similar in (i) control and dicyclohexylcarbodi-imide-treated cells and in (ii) the wild-type and a *gap* mutant, a treatment and a mutation that it has been shown prevents uptake of the inhibitors. Thus a major portion of the γ-glutamyltransferase activity appears to exist in a membrane-bound form that is orientated with its γ-glutamyl-binding site facing the outside. Yeast cells in which γ-glutamyltransferase has been inactivated by N_2O-Nle show no significant change in their rates of uptake of a variety of amino acids, dipeptides and γ-glutamyl-amino acids. The results preclude a major, direct role for γ-glutamyltransferase in the transport of these substrates.

Some years ago, Meister (1973) suggested that transport of amino acids and peptides might occur by a group-translocation mechanism mediated through the concerted action of a set of enzymes constituting the γ-glutamyl cycle. One of these enzymes, γ-glutamyltransferase [(5-glutamyl)-peptide:amino-acid 5-glutamyltransferase; EC 2.3.2.2], located in the membrane, was pictured as translocating extracellular substrates (amino acids or dipeptides) by a vectorial transpeptidation reaction involving intracellular glutathione, the products being a γ-glutamyl derivative of the substrate and the cleaved cysteinylglycine moiety. In subsequent steps of the cycle the free substrate is released by peptidase activity and glutathione is resynthesized. In short, 1 mol of amino acid (or dipeptide) could be transported per mol of glutathione degraded, one turn of the cycle consuming 3 mol of ATP. Subsequently, considerable information on the activities of the γ-glutamyl-cycle enzymes in mammalian tissues *in vivo* and *in vitro* has been obtained (Meister & Tate, 1976; Meister, 1981, 1983), and from this, Meister (1980) has concluded that 'Although a direct and unequivocal demonstration that the γ-glutamyl cycle functions in amino acid transport is still required, the data that are available seem strongly to support the transport hypothesis'.

In micro-organisms the distribution of γ-glutamyltransferase activity is widespread (Meister, 1980; Milbauer & Grossowicz, 1965), although only in the yeast *Saccharomyces cerevisiae* have all the enzymes of the cycle been demonstrated (Mooz & Wigglesworth, 1976). Evidence to support the view that the γ-glutamyl cycle functions in the absorption of amino acids in yeast has been presented by Osuji (1979*a*,*b*, 1980), but other reports have indicated the methodological problems in these studies and invalidated the conclusions (Penninckx *et al.*, 1980; Jaspers & Penninckx, 1981; Robins & Davies, 1980, 1981).

To test directly for the functioning of the γ-glutamyl cycle in transport it seems necessary to establish a relationship between the rate of substrate transport and either the turnover rate of glutathione or the activity of the key enzyme γ-glutamyltransferase. On the basis of the first test, Robins & Davies (1981) concluded that, in the yeast *Candida albicans*, '...the γ-glutamyl cycle plays no role in the absorption of L-amino acids...'. In the present paper we have applied the second test. We provide evidence that, in *Saccharomyces cerevisiae*, γ-glutamyltransferase activity is located mainly in the cell membrane and that it can be inactivated from

Abbreviations used: N_2O-Nle, 6-diazo-5-oxo-L-norleucine; DCCD, NN'-dicyclohexylcarbodi-imide; CCCP, carbonyl cyanide chlorophenylhydrazone.

the outside by covalent modification with glutamine analogues. This inhibition of its activity *in vivo* is without significant effect on the transport of L, or D-amino acids, dipeptides or γ-glutamyl-amino acids. We conclude that γ-glutamyltransferase, and hence the γ-glutamyl cycle, does not function directly in the bulk movement of any of these substrates, but speculate that it could play a co-ordinating role in transmembrane regulation by monitoring extracellular nutrient sources.

Experimental

Materials

L- and D-amino acids, γ-glutamyl-amino acids, L-γ-glutamic acid *p*-nitroanilide, N_2O-Nle, L-azaserine, fluorescamine, DCCD and CCCP were purchased from Sigma, Poole, Dorset, U.K. Peptides were from Sigma and from UniScience, Cambridge, U.K.

Yeast strains

Saccharomyces cerevisiae wild-type Σ1278b, and the isogenic amino acid permease mutants, strain 2512c, *gap* (general amino acid permease-deficient), MG276, *met*-p1 (methionine permease-deficient), 5156d, *gap met*-p1, and the peptide permease mutant PA7101 *gpp* (A.T.C.C. 42880) (Nisbet & Payne, 1979*a*) were maintained and grown as described previously (Nisbet & Payne, 1979*b*). Liquid media contained glucose (2%, w/v), yeast nitrogen base lacking amino acids and NH_4^+ (Difco 0335-15), and (unless otherwise stated) proline (4mg·ml^{-1}) as nitrogen source. Cultures were grown at 28°C with shaking to an A_{660} (measured on a Bausch and Lomb Spectronic 20 spectrophotometer) of 0.2–0.5 (approx. 5×10^6–1.2×10^7 organisms·ml^{-1}, equivalent to 0.2–0.5mg dry wt.·ml^{-1}). Organisms were harvested by membrane filtration and washed with 4vol. of either 20mM-phosphate/citrate buffer, pH4.5, or with 50mM-phosphate buffer, pH6.5, as required.

Transport assays

Uptake of L- and D-amino acids, γ-glutamyl-amino acids and peptides was measured by using an automated fluorescence procedure that continuously measures the concentration of NH_2-containing substrate in solution (Payne & Nisbet, 1981). Briefly, cells are equilibrated in appropriate media for 15min at 28°C, with stirring, with or without transport inhibitors (see the Results section). Cells pretreated with inhibitors were washed and resuspended before use. The assay was initiated by addition of the transport substrate, the suspension was sampled continuously and passed through an in-line filter to remove the yeast; the filtrate was allowed to react with fluorescamine and the concentration of the fluorescent product was continuously measured by using a fluorimeter equipped with a flow cell. Output from the fluorimeter is fed directly to a microcomputer that provides interactive monitoring of uptake and transport kinetic data (Payne & Nisbet, 1981; Payne, 1983; J. T. Gleaves & J. W. Payne, unpublished work).

γ-Glutamyltransferase activity

This was measured by using a modification of the procedure described by Tate & Meister (1978). Routinely, organisms were rendered permeable by the freeze–thaw/Triton X-100 procedure of Miozzari *et al.* (1978). 'Permeabilized' cells (about 3mg dry wt.·ml^{-1}) were incubated with 2mM-γ-glutamic acid *p*-nitroanilide and 10mM-methionine as acceptor at 28°C in 50mM-phosphate buffer, pH8.5 (or 6.5); after 1h the cells were sedimented by centrifugation (12000*g*, 4min; Gelman Hawkesley Microfuge) and the *p*-nitroaniline produced in the supernatant solution was measured at 410nm. For comparative assays of activity in membrane preparations and soluble extracts, cells were sonicated by using an MSE Soniprep instrument, centrifuged (12000*g*, 4min) and the pellet and supernatant solution were used as the membrane fraction and soluble extract respectively.

To study the effects of N_2O-Nle (or azaserine) on enzyme activity, cells were pretreated with the inhibitor at 28°C in 50mM-phosphate buffer, pH6.5 or 8.5, containing 1% (w/v) glucose for the appropriate time (see the Results section). Cells were collected by centrifugation, washed once, centrifuged and resuspended in the above buffer. When serine–borate was used as inhibitor, it was either washed off as above or allowed to remain during the enzyme assay (see the Results section). When serine–borate and N_2O-Nle were used together, the cells were first incubated for 5min with serine–borate and then N_2O-Nle was added (see the Results section).

Results

γ-Glutamyltransferase activity

By using the standard procedure to assay γ-glutamyltransferase, we first examined whole cells, but were unable to detect any activity. However, activity was high in a broken-cell preparation obtained by sonication, with most of the activity being associated with the pellet after centrifugation (Table 1). Quantitatively similar activity could be measured in permeabilized whole cells after freeze–thaw treatment (Table 1). We confirmed the report (Penninckx *et al.*, 1980) that the activity is markedly affected by the nitrogen status of the cells during growth; activity was about 10-fold

Table 1. *γ-Glutamyltransferase activity of sonicated and permeabilized cells of wild-type and transport mutants of S. cerevisiae*
Proline-grown yeast cells were pretreated and the enzyme was assayed at the indicated pH as described in the Experimental section. Key to superscripts: [a]cells were sonicated, all others were permeabilized by freeze–thaw treatment with Triton X-100. Citrate reportedly inhibits the enzyme, so several buffers were tested at pH4.5: [b]citrate/phosphate; [c]acetate; [d]3,3-dimethylglutarate, each at 100mM.

Strain	pH	Activity [nmol·h^{-1}·(mg dry wt.)$^{-1}$]		
		Pellet	Soluble	Total
Σ1278b wild-type[a]	8.5	41	21	57
Σ1278b wild-type	8.5	47	11	56
Σ1278b wild-type	6.5	–	–	59
Σ1278b wild-type	4.5	–	–	1.5[b], 11[c], 7[d]
5156d *gap,met*-p1	8.5	–	–	61
5156d *gap,met*-p1	6.5	–	–	54
PA7101 *gpp*	8.5	42	7	52
PA7101 *gpp*	4.5	–	–	1.5[b], 10[c], 3[d]

greater in cells grown with a poor nitrogen source (proline) compared with NH_4^+ (results not shown). Maximum activity was shown at an incubation pH of 6.5–8.5, a similar range to that reported for the soluble form of the yeast enzyme (Penninckx *et al.*, 1980) and for the mammalian enzyme (Meister & Tate, 1976). Little activity was shown at pH4.5, which is the pH at which the yeast is grown and is the optimum for transport of the putative substrates of the enzyme (see below).

γ-Glutamyltransferase activity in amino-acid- and peptide-transport mutants of S. cerevisiae

On the basis of the proposed role of the γ-glutamyl cycle in transporting a range of amino acids, it has been speculated that *S. cerevisiae* mutants defective in the general amino acid permease (*gap*) might be deficient in enzymes of the γ-glutamyl cycle (Mooz, 1979; Penninckx *et al.*, 1980). However, Penninckx *et al.* (1980) reported a similar activity for the soluble form of γ-glutamyltransferase in the wild-type, in *gap* strains and in mutants defective in certain specific amino acid permeases. We have confirmed these conclusions for the γ-glutamyltransferase activity measured in permeabilized cells of the transport mutant strain 5156d (Table 1).

Furthermore, a feature of the γ-glutamyl-cycle model is that γ-glutamyltransferase could function to transport small peptides as well as amino acids (Meister & Tate, 1976; Meister *et al.*, 1977). Indeed, certain dipeptides are excellent acceptors in the assay of the enzyme *in vitro* (Mooz & Wigglesworth, 1976; Meister & Tate, 1976). However, to date there has been no reported test of its possible involvement in peptide transport. We have demonstrated the presence in *S. cerevisiae* of a single permease for di- and oligo-peptides (general peptide permease, *gpp*) (Nisbet & Payne, 1979*a*) and isolated a specific *gpp* mutant (Nisbet & Payne, 1979*b*). When we tested this *gpp* strain we found no significant difference between the level of its γ-glutamyltransferase activity and that of the parent strain (Table 1).

Inhibition of γ-glutamyltransferase activity

In mammalian tissues, γ-glutamyltransferase activity can be inhibited by the glutamine analogues N_2O-Nle and L-azaserine (Meister, 1981). We found they could also inhibit this enzymic activity in sonicated cell preparations and in permeabilized cells of *S. cerevisiae* (Table 2). More importantly, whole cells were also inhibited (Table 1, Fig. 1). Inhibition with N_2O-Nle (or azaserine) was rapid and irreversible, in accord with other findings that these reagents form covalent active-site derivatives (Inoue *et al.*, 1977; Tate & Meister, 1977). This is in contrast with serine–borate, which it has been suggested forms an analogue of the normal transition-state intermediate (Tate & Meister, 1978), for which inhibition was reversible and activity could be restored by washing (Table 3). Furthermore, serine–borate could protect against the irreversible inhibition caused by N_2O-Nle (Table 3). The mixture of amino acids present in casein hydrolysate protected against N_2O-Nle inhibition in whole cells, whereas glutathione, the presumed natural substrate of γ-glutamyltransferase, competed with the artificial substrate γ-glutamic acid *p*-nitroanilide in the standard assay for the enzyme (Table 2).

Inhibition of membrane-bound γ-glutamyltransferase is mediated extracellularly

When whole cells of *S. cerevisiae* were incubated with the inhibitors N_2O-Nle, azaserine or serine–borate and their presence in the medium was monitored continuously by using the automated fluorescamine procedure, it was clear that each of these amino acids was rapidly taken up by the cells. This observation raised the possibility that the inhibition of γ-glutamyltransferase might actually

Table 2. *Inhibition of γ-glutamyltransferase activity in whole and permeabilized cells*

Yeast cells were prepared and treated with inhibitors as described for Fig. 1. After inhibition treatments the cells were washed and residual activity assayed as in Table 3. Key to superscripts: [a]cell types: WC, whole cells; PC, permeabilized cells prepared by Triton X-100 treatment. [b]Values in parentheses are pH of inhibition treatment when this differed from assay pH; for pH 4.5, 200 mM-phosphate/citrate buffer was used. [c]Activities in pellet; [d]activity in soluble extract. [e]Reduced glutathione (GSH) was added only to the final enzyme assay incubation as a competitor for the artificial substrate γ-glutamic acid *p*-nitroanilide. CH is casein acid hydrolysate.

	Inhibition treatment		Residual activity (%) measured at:	
Strain	Cell type[a]	Additions	pH 8.5	pH 6.5
Σ1278b	WC	N_2O-Nle (0.1 mM)	50 (4.5)[b]	–
Σ1278b	WC	N_2O-Nle (0.1 mM)	55 (6.5)[b]	–
Σ1278b	WC	N_2O-Nle (0.1 mM)	49	–
5156d	WC	N_2O-Nle (0.1 mM)	47 (4.5)[b]	–
5156d	WC	N_2O-Nle (0.1 mM)	50 (6.5)[b]	–
5156d	WC	N_2O-Nle (0.1 mM)	96	–
Σ1278b	WC	N_2O-Nle (1 mM)	30	23
Σ1278b	WC	N_2O-Nle (1 mM) + CH (0.5%, w/v)	67	54
Σ1278b	WC	N_2O-Nle (1 mM) + GSH (2 mM)	30	25
Σ1278b	WC	CH (0.5%, w/v) or GSH (2 mM)	100	100
Σ1278b	PC	N_2O-Nle (1 mM)	0	28
Σ1278b	PC	N_2O-Nle (1 mM) + GSH (2 mM)	3	28
Σ1278b	PC	N_2O-Nle (1 mM) + CH (0.5%, w/v)	6	50
Σ1278b	PC	CH (0.5%, w/v) or GSH (2 mM)	100	100
Σ1278b	WC	N_2O-Nle (0.1 mM)	–	45 (45[c], 47[d])
Σ1278b	PC	N_2O-Nle (0.1 mM)	3 (1[c], 0[d])	–
Σ1278b	WC	Azaserine (0.1 mM)	76	–
Σ1278b	WC	Azaserine (0.2 mM)	59	–
Σ1278b	PC	Azaserine (0.2 mM)	4	–
Σ1278b	PC	GSH (2 mM)[e]	75	–
Σ1278b	PC	GSH (10 mM)[e]	29	55

be mediated intracellularly after uptake of the inhibitors. That this is not the case is clear from the following observations.

Firstly, in a *gap* mutant at pH 6.5 the uptake of N_2O-Nle is barely detectable (Table 4), but the kinetics of γ-glutamyltransferase inhibition resembles that in the wild-type strain in which N_2O-Nle is rapidly transported. Secondly, comparable inhibition of γ-glutamyltransferase activity occurred in the wild-type strain whether the cells were incubated with the inhibitor at pH 4.5, 6.5 or 8.5, even though at pH 8.5 uptake of the inhibitors (and most other transport substrates) was not detectable. Finally, we tested the point specifically by incubating *S. cerevisiae* with DCCD, which inactivates the plasma-membrane-bound ATPase responsible for generating a protonmotive force that is coupled to the transport of amino acids and peptides via the *gap* and *gpp* systems respectively (Eddy, 1980; Serrano, 1980; Payne & Nisbet, 1981). Treatment with DCCD at pH 6.5 inhibits uptake of N_2O-Nle, but the kinetics of γ-glutamyltransferase inhibition by N_2O-Nle remained similar to that with untreated cells (Fig. 2). Similar observations were made when the cells were pretreated at pH 4.5 (results not shown). At 1 mM, DCCD was without noticeable effect on γ-glutamyltransferase activity, although at higher concentrations (5 mM) it did cause some inhibition (results not shown). Treatment with 0.1 mM-CCCP, which dissipates the proton gradient and inhibits transport (Payne & Nisbet, 1981), similarly did not prevent inactivation of γ-glutamyltransferase by N_2O-Nle (results not shown).

The above results lead us to conclude that, in *S. cerevisiae*, extensive (but clearly not all) γ-glutamyltransferase activity occurs at the plasmalemma, and it is orientated so that a binding site is accessible to exogenous inhibitor analogues.

Relationship of amino acid transport to γ-glutamyltransferase activity

The ability to inactivate γ-glutamyltransferase in whole cells makes it feasible to assess the involvement of the enzyme in transport. Although most of the substrates of interest are unavailable in radioactively labelled form, precluding use of a conventional transport assay, with the continuous

fluorescence procedure we can measure uptake of all classes of substrate (Payne & Nisbet, 1981; Payne, 1983). Furthermore, we showed previously that use of radioactively labelled substrates in fact gives marked underestimates of true uptake rates (Payne & Nisbet, 1980).

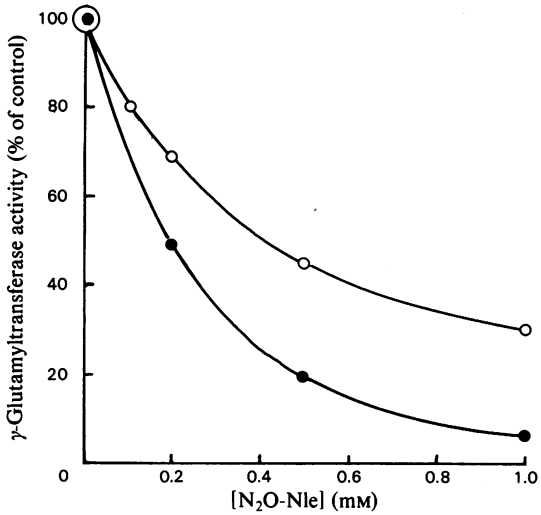

Fig. 1. *Inhibition of γ-glutamyltransferase activity by treatment of whole cells with N_2O-Nle*

Cells of strain Σ1278b were suspended at 10mg dry wt.·ml^{-1} in 200mM-potassium phosphate buffer, pH6.5 (●) or pH8.5 (○), containing 1% (w/v) glucose and the indicated concentrations of N_2O-Nle, and incubated at 28°C for 15min. The samples were immediately centrifuged (3min) and the cells washed in their respective buffer before being resuspended in the same buffer for permeabilization and enzyme assay.

The amino acids studied were selcted on the basis of their good acceptor activity for γ-glutamyltransferase (Meister & Tate, 1976). In addition, citrulline and D-amino acids were studied, because they are specific substrates for the general amino acid permease (Rytka, 1975; Eddy, 1980). In all cases it was found that prior inhibition of γ-glutamyltransferase by incubation of whole cells with N_2O-Nle was without significant inhibitory effect on the transport of any tested amino acid (Table 4). In addition, we studied the pH optimum for transport so that we could compare it with the pH optimum of γ-glutamyltransferase. For this study (Fig. 3) methionine was chosen, as this has been reported to be a good substrate for γ-glutamyltransferase (Meister & Tate, 1976); in addition, uptake of γ-glutamylglycine was studied. This study confirmed previous reports (Eddy, 1980) that a pH of about 4.5 is optimal for transport, and this should be contrasted with the optimum for γ-glutamyltransferase at pH6.5–8.5.

Relationship of peptide transport to γ-glutamyltransferase activity

Peptide transport can also be measured by using the fluorescence assay (Payne & Nisbet, 1981). When this was used with a variety of peptides, uptake was not noticeably affected by pretreatment of wild-type cells with N_2O-Nle (Table 4). However, uptake of these and all other tested peptides is not detectable in the *gpp* mutant, although it is the same in *gap* mutants as in the wild-type strain (Table 4; Payne & Nisbet, 1979*a*; J. W. Payne & T. M. Nisbet, unpublished work).

Table 3. *Effect of serine–borate on γ-glutamyltransferase activity*

Exponential-phase cells of strain Σ1278b were suspended at approx. 1.5×10^8 cells·ml^{-1} in 0.25ml of 50mM-potassium phosphate buffer, pH6.5 or 8.5, containing 1% (w/v) glucose, plus serine and borate as shown and incubated at 28°C for 5min. N_2O-Nle was added as shown and incubation continued for 15min. For washing, samples were centrifuged, washed with 1.0ml of 50mM-potassium phosphate buffer, pH6.5 or 8.5, centrifuged, and resuspended in 0.25ml of 200mM-potassium phosphate buffer, pH6.5 or 8.5. A 5μl portion of 2.5% Triton X-100 was added and the cells permeabilized by freezing in liquid air and then thawing. Non-washed cells were permeabilized immediately after incubation. After dilution to 1.0ml by addition of methionine (10mM) and γ-glutamic acid *p*-nitroanilide (2mM) residual γ-glutamyltransferase activity was measured.

Additions	Sample washed	Residual activity (%) measured at: pH6.5	pH8.5
None	Yes	100	100
Borate (10mM)	Yes	–	89
Serine (2mM)+borate (10mM)	No	55	34
Serine (2mM)+borate (10mM)	Yes	81	92
Serine (5mM)+borate (10mM)	No	–	23
Serine (5mM)+borate (10mM)	Yes	–	89
Serine (2mM)+borate (10mM)+N_2O-Nle (1mM)	Yes	50	–
Serine (5mM)+borate (10mM)+N_2O-Nle (1mM)	Yes	–	58
N_2O-Nle (1mM)	Yes	15	36

Table 4. *Uptake of amino acids, peptides and γ-glutamyl-amino acids in S. cerevisiae with and without N_2O-Nle treatment*
Exponential-phase cells were washed and resuspended in phosphate/citrate buffer, pH 4.5 or 6.5, to give 7–10 mg dry wt·ml^{-1} and equilibrated for 10 min at 28°C. 1 mM-N_2O-Nle was added and the cells incubated for 15 min. After washing and resuspension at 1 mg dry wt·ml^{-1} cells were equilibrated for 15 min at 28°C in buffer containing 1% (w/v) glucose. Substrate (0.1 mM) was added and uptake measured continuously for up to 15 min using the fluorescamine procedure.

		Uptake rates [nmol·min^{-1}·(mg dry wt.)$^{-1}$]			
		pH 6.5		pH 4.5	
Strain	Substrate	Untreated	N_2O-Nle-treated	Untreated	N_2O-Nle-treated
Σ1278b	Arg	34	32	–	–
	Gln	29	25	40	40
	Leu	33	32	–	–
	Met	38	37	–	–
	D-Ala	–	–	17	18
	D-Leu	0	–	1	1
	Citrulline	17	20	35	33
	N_2O-Nle	25	27	–	–
	Ala-Ala	2	2	3	3
	Ala-Met	2	2	3	3
	Leu-Leu	1	1	1	1
	γ-Glu-Ala	0	0	3	2
	γ-Glu-Gly	–	–	6	6
	γ-Glu-Glu	–	–	<1	–
	γ-Glu-Leu	–	–	0	–
5156d	Arg	18	17	–	–
	Gln	4	4	–	–
	Met	3	4	–	–
	Citrulline	0	0	–	–
	Ala-Ala	–	–	3	3
	Ala-Met	–	–	3	3
	γ-Glu-Gly	0	–	0	–
	N_2O-Nle	<1	–	–	–

Transport of γ-glutamyl-amino acids

Meister and colleagues (Griffith *et al.*, 1979; Meister, 1983) have presented evidence for the uptake of γ-glutamyl-amino acids by mammalian tissues through a mechanism that is distinct from that used by typical α-amino acids and have suggested that it may involve γ-glutamyltransferase. The fluorescence assay can also be used with γ-glutamyl-amino acids, and so we studied their uptake by *S. cerevisiae*. Some were transported quite rapidly, whereas uptake of others was undetectable (Table 4). Those showing most uptake were studied further, and it was found that their transport was not impaired in cells in which γ-glutamyltransferase activity had been inhibited by N_2O-Nle (Table 4). Furthermore, their rate of uptake was not diminished in the peptide-transport mutant *gpp*, in which uptake of normal α-linked peptides, e.g. α-glutamylalanine, is not detectable. In contrast, uptake of all tested γ-glutamyl-amino acids was lost in a *gap* mutant studied over a range of pH (Fig. 3 and Table 4). We conclude that, in *S. cerevisiae*, γ-glutamyl-amino acids are recognized as amino acid (glutamine) analogues by the general amino acid permease and their bulk uptake does not involve γ-glutamyltransferase.

Discussion

Current views on role of the γ-glutamyl cycle in transport

Debate continues over the proposal, made originally by Meister (1973), that the γ-glutamyl cycle may function in amino acid and dipeptide transport (Meister, 1973, 1980; Meister & Tate, 1976; Meister *et al.*, 1977). Circumstantial evidence for this view is the observation that the key enzyme, γ-glutamyltransferase, is frequently found in tissues associated with high levels of amino acid absorption (Meister, 1981). Most attempts to test the hypothesis have been carried out with mammalian cells and limited to amino acid absorption, and for technical reasons have generally been indirect in nature. No definitive answer has been

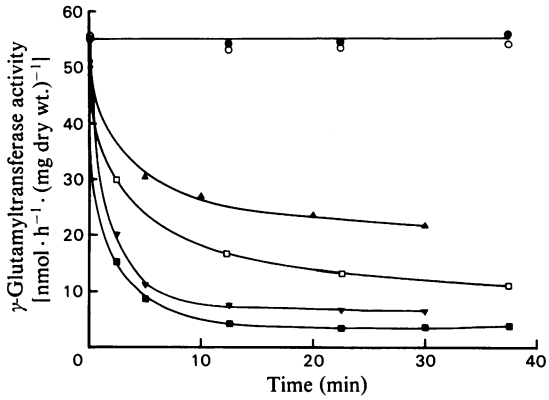

Fig. 2. *Kinetics of inhibition of γ-glutamyltransferase activity by treatment of whole cells with N_2O-Nle and effect of pretreatment with DCCD*

Cells of strain Σ1278b were suspended at 10mg dry wt.·ml^{-1} in 100mM-potassium phosphate buffer, pH6.5, containing 1% (w/v) glucose and incubated with (open symbols) or without (closed symbols) 1mM-DCCD for 15min at 28°C. Incubations were continued for the indicated times after addition of N_2O-Nle as follows: 0.2mM (▲), 0.5mM (▼), 1mM (■ and □), controls with no addition (● and ○). After centrifugation and washing, cells were permeabilized and assayed for γ-glutamyltransferase in the standard way.

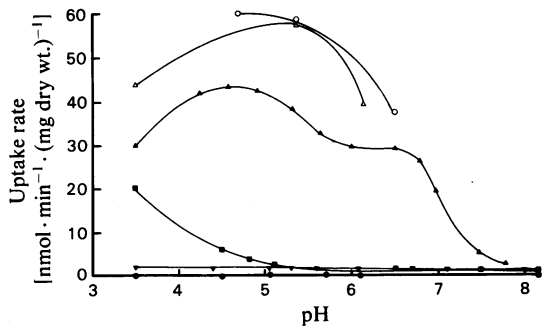

Fig. 3. *Influence of pH on the uptake of methionine and γ-Glu-Gly in S. cerevisiae*

Yeast cells were suspended at 1mg dry wt.·ml^{-1} in 20mM-phosphate/citrate buffer, pH3.5–7.0, or 50mM-phosphate buffer, pH7.5–8.5, containing 1% (w/v) glucose. Uptake rates were measured by using the fluorescamine assay. Uptake of methionine (0.025mM) is shown for wild-type strain Σ1278b (○), strain MG276 *met*-p1 (▲), strain 5156d *gap,met*-p1 (▼), and at 0.25mM for strain MG276 *met*-p1 (△). Uptake of γ-Glu-Gly (0.1mM) is shown for strain Σ1278b (■) and for strain 5156d *gap, met*-p1 (●).

reached, and evidence for and against the proposal has been forthcoming (see Meister & Anderson, 1983, for latest review).

In micro-organisms, studies aimed at testing the proposed transport function have been limited to amino acid uptake and confined to yeast, in which all enzymes of the cycle have been demonstrated (Mooz & Wigglesworth, 1976). The balance of evidence from these results is against the cycle playing a role in amino acid uptake. Thus, Osuji's (1979*a*,*b*, 1980) claims to the contrary have been rightly criticized (Robins & Davies, 1980; Meister, 1980) and the turnover of glutathione shown to be too slow for its stoichiometric involvement in the amino acid uptake observed in the yeast *Candida utilis* (Robins & Davies, 1981). In addition, a finding (Mooz, 1979) that a mutant with a deficiency in the general amino acid permease (*gap*) showed an associated decrease in levels of glutathione and glutathione synthesis activity has been questioned (Jaspers & Penninckx, 1981).

A direct test of the hypothesis is to investigate the relationship between the activity *in vivo* of the key enzyme, γ-glutamyltransferase, and the transport capability of the cells (Novogrodsky *et al.*, 1977). Our development of a procedure to measure uptake of any peptide or amino acid without the need for these to be radioactively labelled (Payne & Nisbet, 1981; Payne, 1983) offered an opportunity to explore this possibility in *S. cerevisiae*.

γ-Glutamyltransferase activity in S. cerevisiae

We started by examining γ-glutamyltransferase activity in *S. cerevisiae*. In previous studies a soluble form of the enzyme from yeast was described (Mooz & Wigglesworth, 1976; Penninckx *et al.*, 1980). It seems probable that, in broken cells, several enzymes may be able to show activity towards the artificial substrate γ-glutamic acid *p*-nitroanilide. However, the enzyme needs to be membrane-bound to function in transport according to the γ-glutamyl-cycle model (Meister, 1973) and so we sought evidence for its association with the yeast plasmalemma. In whole cells, at pH8.5, we could detect no activity, perhaps because the substrate γ-glutamic acid *p*-nitroanilide may be unable to penetrate the cell envelope. However, after subjecting cells to a simple freeze–thaw procedure, considerable activity was found. By using this technique we confirmed a previous report (Penninckx *et al.*, 1980) that activity is increased about 10-fold when *S. cerevisiae* is grown on a poor nitrogen source in place of NH_4^+; accordingly, we used proline-grown cells in our studies. Cells grown on proline also show a similar increase in the transport of peptides (Nisbet & Payne, 1979*b*) and of amino acids via the *gap* permease (Eddy, 1980). In broken cell preparations, activities were comparable with those in permeabilized cells, and the bulk of the activity was associated with the pellet, with only about 20% in the supernatant extract. We

could not distinguish between these activities by using various criteria, e.g. pH optimum, effects of inhibitors, although others have suggested that two forms of the activity may exist (Penninckx *et al.*, 1980).

It is not clear from such studies with broken cells whether the observed distribution of enzyme activity reflects the situation in the original cell, but supporting evidence for a membrane location of γ-glutamyltransferase came from the effects *in vivo* of inhibitors that covalently modify the enzyme (Inoue *et al.*, 1977; Tate & Meister, 1977). Thus N_2O-Nle, azaserine, and serine–borate inactivate the enzyme under conditions in which these amino acids are not taken up. For example, the kinetics of enzyme inactivation by N_2O-Nle was similar for DCCD-treated cells, in which uptake of N_2O-Nle was inhibited, and for normal whole cells. In addition, γ-glutamyltransferase was inactivated by N_2O-Nle or azaserine in whole cells of a wild-type strain and of a *gap* mutant, although the *gap* mutant was unable to transport the inhibitors. Finally, in whole cells the transferase is most effectively inhibited by N_2O-Nle at a high pH (8.5), although its uptake is barely detectable (pH optimum for uptake is about 4.5), although anomalously we did find that the *gap* mutant was only marginally inhibited at pH 8.5.

We conclude that, in *S. cerevisiae*, a major proportion of γ-glutamyltransferase activity exists in a membrane-bound form that is orientated so that its γ-glutamyl-binding site is accessible to inhibitory analogues acting at the outside surface. This conclusion on the location and orientation of the enzyme parallels results with mammalian cells (Kuhlenschmidt & Curthoys, 1975; Horiuchi *et al.*, 1978; Meister *et al.*, 1980; Inoue & Morino, 1981).

γ-Glutamyltransferase activity in transport mutants of S. cerevisiae

Penninckx *et al.* (1980) reported a similar activity for the soluble form of γ-glutamyltransferase in a wild-type strain of *S. cerevisiae* and a *gap* mutant. We confirmed this conclusion here for the combined activity of membrane and soluble forms of the enzyme in strains carrying mutations in *gap* and in the peptide permease, *gpp*. Clearly, the transport defects in these mutants cannot be related to their levels of γ-glutamyltransferase. If γ-glutamyltransferase is a component of the *gap* permease, as has been speculated (see Penninckx *et al.*, 1980), then it would be expected that N_2O-Nle should inhibit its own uptake, for we have shown that N_2O-Nle enters via the *gap* permease (no uptake in *gap* mutant); in fact, when N_2O-Nle is incubated with whole cells, γ-glutamyltransferase is rapidly inhibited, but uptake of N_2O-Nle continues at a steady rate.

Role of γ-glutamyltransferase in transport in S. cerevisiae

The essence of the present findings is that, when γ-glutamyltransferase is largely inactivated in *S. cerevisiae*, there is no significant change in the rates of uptake of amino acids or dipeptides. We conclude that, in contrast with the role envisaged for it in the original form of the γ-glutamyl cycle (Meister, 1973; Meister & Tate, 1976), γ-glutamyltransferase is not *directly* involved in mediating uptake of amino acids or dipeptides.

Two points need to be made concerning the conclusion as stated above. Firstly, in all discussions of the γ-glutamyl cycle, Meister has emphasized his belief that its transport role represents but *one* mode of (say) amino acid uptake. Significantly, no speculation has been made about the proportion of total uptake that might be mediated via the cycle. Thus we conclude that, in *S. cerevisiae* grown as described, *bulk* uptake of amino acids, dipeptides and γ-glutamyl-amino acids does not involve γ-glutamyltransferase directly. Our procedures do not permit us to exclude the possibility that (say) a few per cent of the measured uptake might be mediated directly by γ-glutamyltransferase (see below). Secondly, the γ-glutamyl cycle has been modified from its original form (Meister, 1973). Thus, primarily to accommodate evidence indicating the 'outward' orientation of γ-glutamyltransferase, the cycle has recently been depicted (Meister *et al.*, 1980; Meister, 1983) as having a discrete step for outward translocation of intracellular glutathione that does not involve γ-glutamyltransferase. This glutathione is shown as reacting with acceptors to form γ-glutamyl adducts through the action of γ-glutamyltransferase. These adducts are pictured as being absorbed subsequently in separate steps. It is possible for our results to be accommodated by the current model.

Hypothetical role for γ-glutamyl derivatives in transport regulation

Finally, we propose that the role of the cycle in transport may not be as generally presented, i.e. simply a translocation mechanism for amino acids and dipeptides, but rather it may serve to communicate to the cell interior information on the availability of extracellular nutrients. The information would be contained in the types and amounts of γ-glutamyl derivatives being absorbed, and could be used to co-ordinate the supply of extracellular nutrients with the activities of the primary transport systems involved in their uptake and of the enzymes involved with their intracellular metabolism. This hypothesis is based on a number of premises. Thus, because it is more efficient to transport actively a preformed nutrient

than to synthesize it from simpler precursors, a major portion of cellular energy is devoted to transport, and we assume there will have been great selective pressure to evolve efficient regulation of these transport processes (Payne, 1980). However, active-transport mechanisms are characterized by the fact that their substrates are accumulated in an unmodified form, and after uptake are indistinguishable from endogenously synthesized molecules. This identity of form serves well for regulating the intracellular biosynthetic and catabolic pathways that are controlled by the intracellular concentration of the molecule, but it provides no effective means to regulate transport in response to exogenous supply. We speculate that uptake of γ-glutamyl derivatives, produced through reaction of particular nutrients with glutathione and γ-glutamyltransferase at the cell membrane, could supply specific messengers for use as one means of transmembrane regulation; uptake in the γ-glutamyl form need be only a tiny fraction of that of the unmodified form by active transport. To perform this function, however, it is logical that γ-glutamyltransferase should occur in regions of high transport activity.

It seems significant to us that glutamine and cystine, key substrates in nitrogen and sulphur nutrition respectively, should be particularly good acceptors for γ-glutamyltransferase. It may be that the γ-glutamyl derivatives of glutamine and cystine could play roles in nitrogen and sulphur regulation similar to that played by cyclic AMP in carbon regulation. In general, there appears to be a positive correlation between good acceptor activity (e.g. glutamine, cystine, methionine, serine, glycine and peptides) and nutritional value.

We gratefully acknowledge the financial support of the Science and Engineering Research Council for this work.

References

Eddy, A. A. (1980) in *Microorganisms and Nitrogen Sources* (Payne, J. W., ed.), pp. 35–62, John Wiley, Chichester and New York

Griffith, O. W., Bridges, R. J. & Meister, A. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 6319–6322

Horiuchi, S., Inoue, M. & Morino, Y. (1978) *Eur. J. Biochem.* **87**, 429–437

Inoue, M. & Morino, Y. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 46–49

Inoue, M., Horiuchi, S. & Morino, Y. (1977) *Eur. J. Biochem.* **73**, 335–342

Jaspers, C. & Penninckx, M. (1981) *FEBS Lett.* **132**, 41–44

Kuhlenschmidt, T. & Curthoys, N. P. (1975) *Arch. Biochem. Biophys.* **167**, 519–524

Meister, A. (1973) *Science* **180**, 33–39

Meister, A. (1980) in *Microorganisms and Nitrogen Sources* (Payne, J. W., ed.), pp. 493–509, John Wiley, Chichester and New York

Meister, A. (1981) *Curr. Top. Cell. Regul.* **18**, 21–58

Meister, A. (1983) *Science* **220**, 472–477

Meister, A. & Anderson, M. E. (1983) *Annu. Rev. Biochem.* **52**, 711–760

Meister, A. & Tate, S. S. (1976) *Annu. Rev. Biochem.* **45**, 559–604

Meister, A., Tate, S. S. & Thompson, G. A. (1977) *CIBA Found. Symp.* **50**, 123–144

Meister, A., Griffith, O. W., Novogrodsky, A. & Tate, S. S. (1980) *CIBA Found. Symp.* **72**, 135–161

Milbauer, R. & Grossowicz, N. (1965) *J. Gen. Microbiol.* **41**, 185–194

Miozzari, G. F., Niederberger, P. & Hutler, P. (1978) *Anal. Biochem.* **90**, 220–233

Mooz, E. D. (1979) *Biochem. Biophys. Res. Commun.* **90**, 1221–1228

Mooz, E. D. & Wigglesworth, L. (1976) *Biochem. Biophys. Res. Commun.* **68**, 1066–1072

Nisbet, T. M. & Payne, J. W. (1979*a*) *FEMS Microbiol. Lett.* **6**, 193–196

Nisbet, T. M. & Payne, J. W. (1979*b*) *J. Gen. Microbiol.* **115**, 127–133

Novogrodsky, A., Tate, S. S. & Meister, A. (1977) *Biochem. Biophys. Res. Commun.* **78**, 222–229

Osuji, G. O. (1979*a*) *FEBS Lett.* **105**, 283–285

Osuji, G. O. (1979*b*) *FEBS Lett.* **108**, 240–242

Osuji, G. O. (1980) *FEBS Lett.* **110**, 192–194

Payne, J. W. (1980) in *Microorganisms and Nitrogen Sources* (Payne, J. W., ed.), pp. 335–359, John Wiley, Chichester and New York

Payne, J. W. (1983) *Biochem. Soc. Trans.* **11**, 794–798

Payne, J. W. & Nisbet, T. M. (1980) *FEBS Lett.* **119**, 73–76

Payne, J. W. & Nisbet, T. M. (1981) *J. Appl. Biochem.* **3**, 447–458

Penninckx, M., Jaspers, C. & Wiame, J. M. (1980) *Eur. J. Biochem.* **104**, 119–123

Robins, R. J. & Davies, D. D. (1980) *FEBS Lett.* **111**, 432

Robins, R. J. & Davies, D. D. (1981) *Biochem. J.* **194**, 63–70

Rytka, J. (1975) *J. Bacteriol.* **121**, 562–570

Serrano, R. (1980) *Eur. J. Biochem.* **105**, 419–424

Tate, S. S. & Meister, A. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 931–935

Tate, S. & Meister, A. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 4806–4809