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## ACTIVE TRANSPORT OF GLUTAMATE IN *STREPTOMYCES HYDROGENANS*

### I. STUDIES ON UPTAKE AND POOL SIZE, AND THEIR INTERRELATIONSHIP

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#### SUMMARY

In *Streptomyces hydrogenans* an active transport of glutamate takes place. The accumulation is highly sensitive to metabolic inhibition. The distribution ratio varies with the extracellular substrate concentration. This ratio was found to be 10 at an extracellular concentration of 3 mM. It is concluded that glutamate is transported *via* two systems which differ widely in their kinetic parameters. Our results indicate that most of the intracellular glutamate is in the free state and not bound to distinct sites of the cell. The amount of intracellular glutamate is about 160  $\mu$ moles per g dry weight, and this pool can be drastically reduced by cold shock treatment. The replenishment of the pool is complete over a wide range of extracellular glutamate concentrations, and it seems that the capacity of the specific system is effective enough to build up a nearly normal glutamate pool. The influx of glutamate is influenced by the composition of the endogenous pool.

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#### INTRODUCTION

Glutamate is actively transported by various cell species such as Ehrlich ascites tumor cells<sup>1-3</sup>, bacteria<sup>4-7</sup> and yeast<sup>8</sup>. Mutants of microorganisms have been cultured showing a reduced rate of glutamate uptake<sup>9,10</sup>. In mycobacteria the transport of glutamate is inducible<sup>11</sup>. We have presented evidence<sup>12,13</sup> that glutamate is actively transported by *Streptomyces hydrogenans* and that the uptake of this amino acid as well as the uptake of all other amino acids so far tested is controlled by feedback mechanisms in this strain.

The present paper describes some kinetic properties of the glutamate uptake, which is mediated by two different active transport systems. These systems differ widely with respect to their affinities towards glutamate and aspartate, respectively.

A special problem arose from an unusual time dependence of glutamate uptake, which is biphasic. This atypical time-course has been interpreted, according to earlier investigations in this laboratory as the result of a positive feedback regulation: the acceleration of active glutamate uptake by a metabolic product of this amino

acid. Such an acceleration of transport has been shown to be independent on protein synthesis<sup>14</sup>.

A second problem concerns the interrelationship between glutamate uptake and the size of the internal glutamate pool. The internal glutamate pool is dependent on the transport activity of glutamate across the cellular membrane, the efflux of glutamate and its metabolic utilization. Accordingly, the low accumulation ratios of glutamate in Ehrlich cells are partly due to its rapid catabolism. Earlier experiments have shown that in *S. hydrogenans* the efflux is negligibly small as compared to its metabolic degradation<sup>14</sup>. A second communication will deal with the ion and pH dependence of active glutamate transport<sup>15</sup>.

#### METHODS

*S. hydrogenans* cells were harvested in the midlog phase and prepared as previously described<sup>16</sup>. The average density of the cell suspension used for transport experiments was 1.5 mg cell dry weight per ml suspension. In this range the amount of label is directly proportional to the amount of cells (Fig. 1). Prior to the uptake experiments, the cells were given a cold shock to remove the amino acids from the endogenous pool. Incubations were carried out at 30° in 0.05 M sodium potassium phosphate buffer (pH 7.1), under aerobic conditions. At various time intervals 2-ml aliquots of the suspensions were withdrawn and cells were separated from the medium by a filter technique<sup>16</sup> as described earlier. Slight corrections had to be made for adsorption of radioactive glutamate to the filter matrix. Dry weights of cells were determined after filtration of a 4-ml aliquot of the suspension through membrane filters (Millipore), washing the cell film with an equal volume of distilled water and drying the cells overnight in a desiccator containing silica gel.

The relative uptake ( $Ru'$ ) of the labeled amino acids was determined from the radioactivity per g cell dry weight ( $u'_c$ ) and the radioactivity per ml medium ( $a'_f$ ).

$$Ru' = \frac{u'_c}{a'_f} \quad (1)$$

For the determination of the trichloroacetic acid-precipitable fraction, 4 ml of the cell suspension were pipetted into an equal volume of 10 % trichloroacetic acid, filtered and subsequently washed with 5 % trichloroacetic acid directly on the filter. Using [<sup>14</sup>C]aminoisobutyrate, which is not incorporated into protein within 1 h, no appreciable amount of label was found in the precipitate. Glutamate in the supernatant was estimated as described by BERNT AND BERGMEYER<sup>17</sup>; the cells were extracted with 0.4 M perchloric acid at 0° for 10 min and subsequently neutralized with potassium phosphate buffer. The cell extracts were chromatographed on paper strips in *n*-butanol-acetic acid-water (4:1:1, by vol.) and scanned by a radiopaper-chromatograph (FH 452 and FHK 1/542, Frieske and Hoepfner, Erlangen, Germany).

#### MATERIALS

[<sup>14</sup>C]Glutamic acid (uniformly labeled, spec. act. of about 200 mC/mmole) was obtained from New England Nuclear Corp., Dreieichenhain, Germany; [<sup>14</sup>C]aspartic acid (uniformly labeled, spec. act. 6.1 mC/mmole) and 2-[1-<sup>14</sup>C]aminoisobutyric

acid (spec. act. 44 mC/mmol) were purchased from the Radiochemical Centre, Amersham, England; glutamate dehydrogenase (EC 1.4.1.3) and NAD<sup>+</sup> (oxidized form) were obtained from Boehringer, Mannheim, Germany; other reagents were obtained from E. Merck, Darmstadt, Germany; Hoffmann La-Roche, Basel, Switzerland; and Serva, Heidelberg, Germany. If not stated otherwise, the amino acids used were L isomers.

*Uptake of radioactivity during incubation with [<sup>14</sup>C]glutamate*

Previously, it has been shown by enzymatic determinations that *S. hydrogenans* strongly accumulates glutamate<sup>14</sup>. If cells are incubated in buffer containing [<sup>14</sup>C]-glutamate the time-course of uptake of label is unusual (Fig. 2) as compared with the uptake of other amino acids in this microorganism, *e.g.* the uptake of the nonmetaboli-

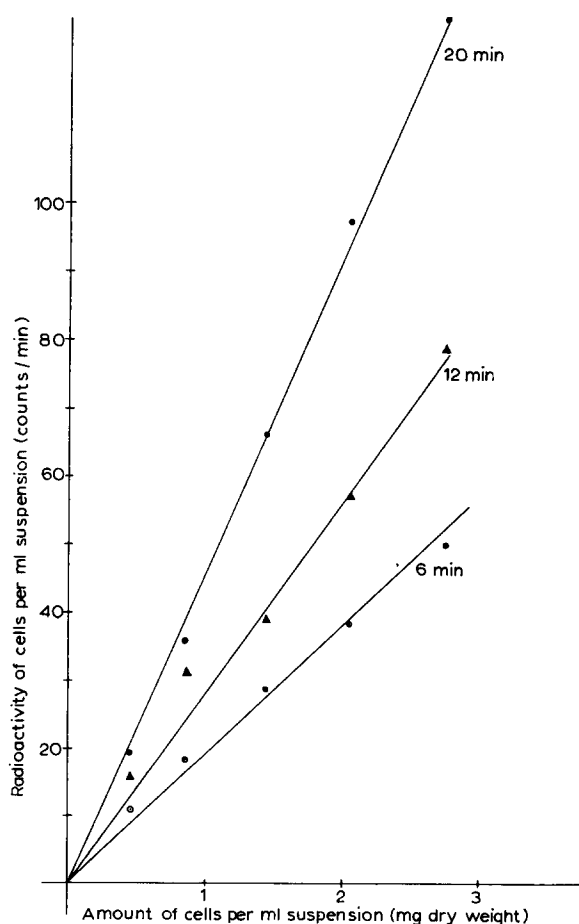


Fig. 1. Relationship between cell dry weight and the uptake of labeled glutamate. The extracellular concentration of glutamate was 0.9  $\mu$ mole/ml. The cellular radioactivity was measured after 6 (○), 12 (▲), and 20 (●) min of incubation in 0.05 M phosphate buffer (pH 7.1) at 30°. A direct proportionality holds over the range tested.

zable 2-aminoisobutyrate, which has been shown to follow a unique function as is expressed by Eqn. (2)

$$\log \frac{Ru'_{\infty} - Ru'_t}{Ru'_{\infty}} = -K \cdot t \quad (2)$$

$Ru'_t$  is the relative uptake at time  $t$ ;  $Ru'_{\infty}$ , the extrapolated steady state value; and  $K$ , a constant.

The uptake values obtained within the first 10–15 min if plotted according to Eqn. 2 give a fairly straight line (inset of Fig. 2). During this period the incorporation of labeled glutamate into protein is slow, probably due to its dilution by endogenous glutamate. Before a steady state of radioactivity is attained the course of the uptake curve changes. This may be due to the increasing incorporation of label into protein, or the transstimulation of the influx by a metabolic product of glutamate described earlier<sup>13</sup>.

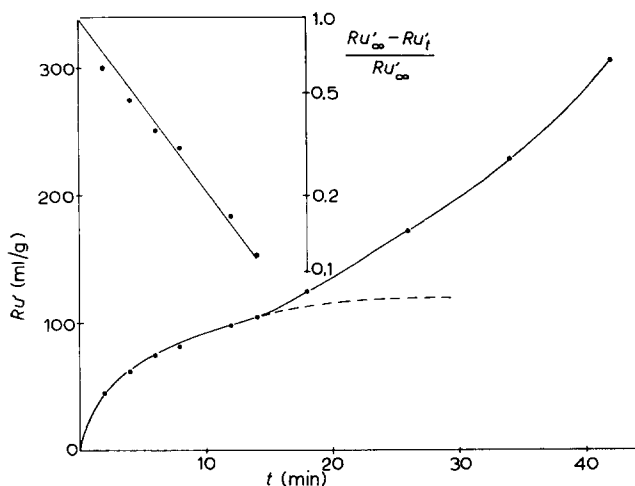


Fig. 2. Uptake of radioactivity during incubation with glutamate ( $0.67 \mu\text{mole/ml}$  medium) in  $0.05 \text{ M}$  phosphate buffer ( $\text{pH } 7.1$ ) at  $30^\circ$ . In the first 15 min of incubation the uptake follows a typical logarithmic function (Eqn. 2) as clearly shown in the inset. The abscissa applies to both graphs. For calculation of the logarithmic plot the value for  $Ru'_{\infty}$  was extrapolated.

In Fig. 3 a similar uptake curve, which represents the total radioactivity inside the cell (Curve A) is corrected for the label incorporated into protein (Curve C). The rate of incorporation into protein increases exponentially with time. This may be due to a decreased isotopic dilution of the transported solute by endogenous substrate and the conversion of glutamate to the other amino acids, which are then incorporated into cellular proteins. According to BRITTEN AND MCCLURE<sup>18</sup>, such a time-course of protein labeling argues against incorporation immediately following the transport step. Curve B of Fig. 3 represents radioactivity in the trichloroacetic acid-soluble fraction, *i.e.* the difference between total radioactivity and the amount of activity precipitated by trichloroacetic acid.

This curve, after reaching temporarily a first plateau, rises towards a second one. That this rise is due to an enhancement of the influx and not a decrease of the efflux has previously been shown<sup>13</sup>. Furthermore, evidence has been presented that

after loading the cells with glutamate for more than 20 min at 30° the influx of amino acids other than glutamate is enhanced. RING<sup>19</sup> has shown that rapid cooling of the cells to -2° opens the cell membrane allowing the small molecules to penetrate, and that rewarming to 30° restores the original permeability. This method can be used to test the effect of glutamate preloading on the uptake of 2-aminoisobutyrate. Alteration of the net uptake curve of 2-aminoisobutyrate after preloading the cells

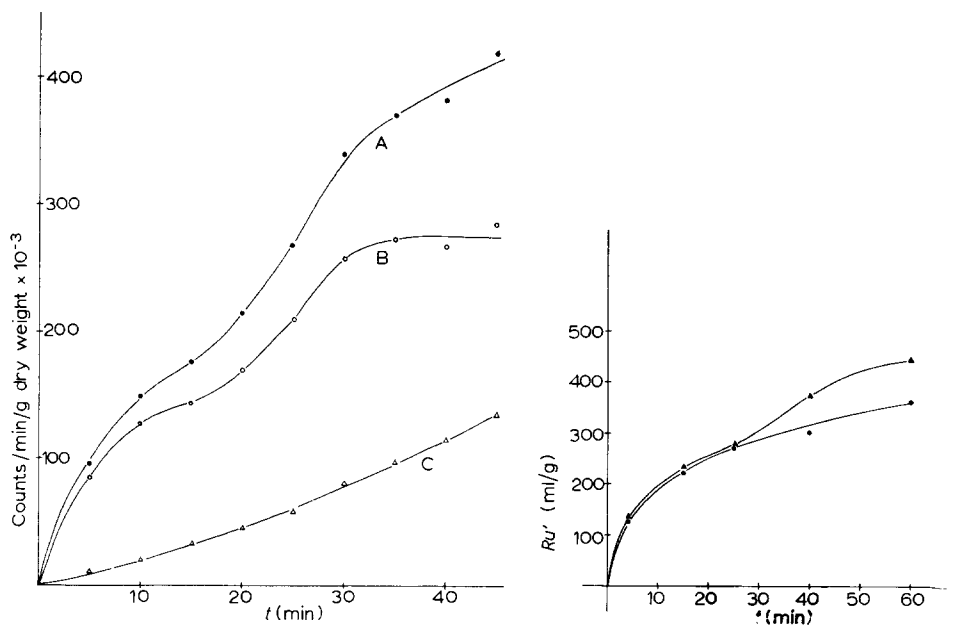


Fig. 3. Uptake of radioactivity and its incorporation into protein. Cells were incubated in 0.05 M phosphate buffer (pH 7.1), containing 4.3  $\mu$ moles uniformly labeled glutamate per ml at 30°. Curve A represents the uptake of radioactivity; Curve C, its incorporation into the trichloroacetic acid-precipitable fraction; Curve B, the difference between (A) and (C), represents the free intracellular radioactivity composed of glutamate *plus* labeled metabolites (*cf.* Fig. 5).

Fig. 4. Alteration in 2-aminoisobutyrate uptake after preloading the cells with glutamate. During the first incubation the cells were loaded with glutamate (30  $\mu$ moles per ml phosphate buffer) for 40 min at -2°. In the second incubation after removal of glutamate the 2-aminoisobutyrate uptake (extracellular concentration 0.45 mM) was followed at 30°.  $\blacktriangle$ , preloaded;  $\bullet$ , control.

with glutamate at -2° is shown in Fig. 4. Two batches of a cell suspension were given a cold shock and during this cold shock one of them was preloaded with glutamate. In a second incubation at 30°, after removal of extracellular glutamate, the uptake of 2-aminoisobutyrate was measured in both batches. Whereas the uptake curve of the preloaded cells is similar in shape to the uptake curve of glutamate (*cf.* Curve B of Fig. 3), the uptake by the unloaded control follows a uniform logarithmic time-course (*cf.* Eqn. 2).

Since we used uniformly labeled glutamate it is not surprising that the radioactivity of trichloroacetic acid-soluble fraction represents other compounds too. Within the first 20 min of incubation at 30°, less than 10% of the glutamate taken up are metabolically converted. However, after 60 min of incubation the enzymatic

assay shows about 50 % of the radioactivity belonging to compounds other than glutamate. This was confirmed by chromatography of the perchloric acid-soluble fraction of cells pretreated with chloramphenicol and loaded with [ $^{14}\text{C}$ ]glutamate (Fig. 5). Planimetric analysis of the three ninhydrin positive peaks shows that only 50 % of the total label appears in the peak of glutamate. This concludes that all amino acids derived from glutamate can be incorporated into protein. After 60 min of incubation 40 % of the total cellular radioactivity can be detected in the protein fraction.

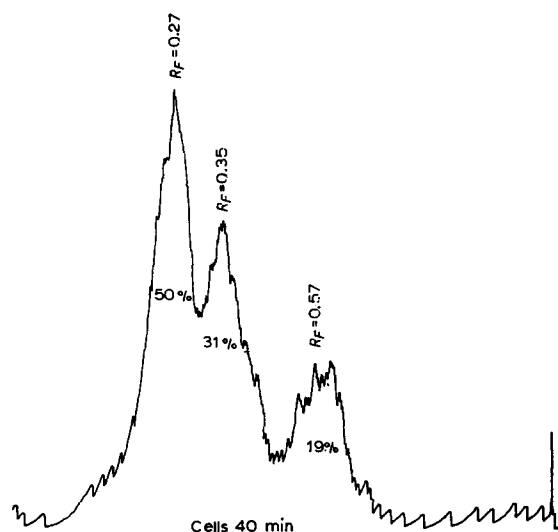


Fig. 5. Radiochromatogram of an extract of *Streptomyces* cells incubated with 2 mM uniformly labeled glutamate. Cells were incubated in 0.05 M phosphate buffer (pH 7.1) containing 50  $\mu\text{g}$  chloramphenicol per ml for 40 min at 30°. Cells were extracted as described in METHODS. The chromatogram was registered by an automatic recorder. The arrows indicate the start and the front of the solvent. Percent values indicate the areas determined planimetrically for the three peaks.

#### *The intracellular pool of glutamate*

The intracellular pool of glutamate was analysed enzymatically using glutamate dehydrogenase. Cells harvested in the intermediate phase of exponential growth from complete medium (Table I) contain about 160  $\mu\text{moles}$  glutamate per g dry weight.

TABLE I

#### COMPOSITION OF THE CULTURE MEDIUM

For details see ref. 16.

Components	g/l
Bacto beef extract	4.0
Bacto yeast extract	1.0
Casein-peptone, tryptic digest	4.0
Glucose	10.0
NaCl	2.5

The pool is osmotically sensitive. If cells are washed three times with distilled water, they contain 100  $\mu$ moles glutamate per g dry weight (Table II). Further washings with phosphate buffer reduce the endogenous pool, which after 10 washing procedures may be as low as 35 % of the initial one (Fig. 6). Cold shock given twice for 20 min without any intermediate rewarming causes more than 70 % loss of the initial glutamate. Besides rendering the cellular membranes more permeable, the endogenous synthesis of glutamate may be completely blocked at this low temperature.

*Dependence of the distribution ratio on the extracellular concentration of glutamate*

As indicated in Table III, the steady state distribution ratio of glutamate increases considerably with decreasing concentrations of glutamate in the medium; the intracellular concentration of glutamate varies only slightly. Even at low concentrations (0.04  $\mu$ mole glutamate per ml medium) a nearly normal glutamate pool is built up.

The relationship between the distribution ratio and the extracellular concentration has been derived by HEINZ AND MARIANI<sup>20</sup> for glycine transport in Ehrlich cells on the basis of a pump-leak model. A similar relationship can be derived if the exit of a solute is carrier mediated and if the  $K_m$  value for this exit is higher than the intracellular concentration or, if additional metabolic processes consuming this solute are

TABLE II

ESTIMATION OF INTRACELLULAR GLUTAMATE USING GLUTAMATE DEHYDROGENASE<sup>17</sup>

Cells were removed from complete medium by centrifugation at  $3000 \times g$ . Each value indicates a mean of six determinations.

Conditions	Intracellular glutamate ( $\mu$ moles/g dry wt.)
A without further treatment	166.3
B 3 times washed with distilled water	99.5
C as B and subjected to cold shock	48.4
D as B and twice subjected to cold shock	29.1
E as B and 10 times washed with phosphate buffer	45.0

TABLE III

DEPENDENCE OF THE DISTRIBUTION RATIO OF GLUTAMATE ON ITS EXTRACELLULAR CONCENTRATION

The cells were incubated in 0.05 M phosphate buffer (pH 7.1), for 60 min at 30°.

Concentration (mM)		Distribution ratio
extracellular	intracellular	
5.5	33.5	6.1
4.6	37.5	8.2
4.4	35.0	7.9
0.4	37.3	93.3
0.04	30.1	805.0

far from being saturated. This is expressed by a generalized pump and leak model as is given by Eqn. 3

$$\frac{v_{\max} [S]_o}{K_m + [S]_o} = k_1[S]_i + k_2[S]_i + \dots k_n[S]_i \quad (3)$$

where the saturable influx (left term) is equal to the sum of non-saturable processes, one term of the sum being equal to the exit, the other ones to metabolic processes.

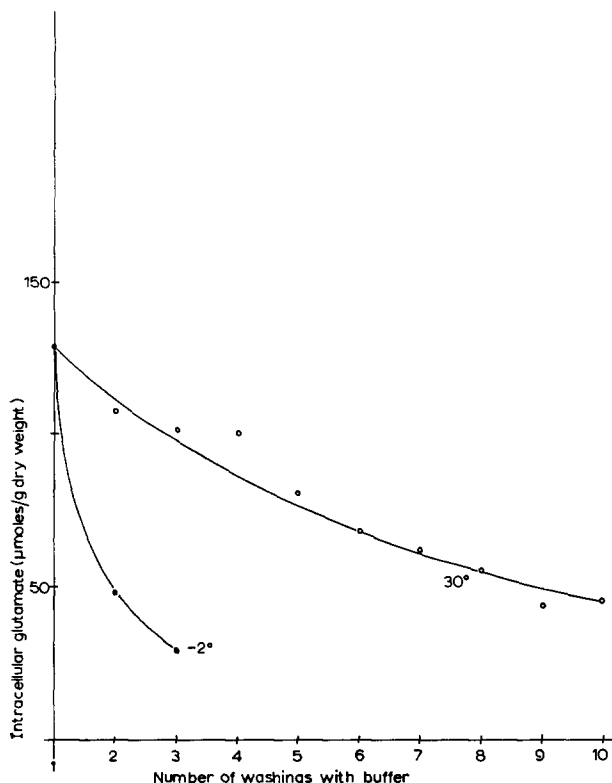


Fig. 6. Effect of repeated washings on the glutamate pool of *S. hydrogenans*. The cells were washed with phosphate buffer at two different temperatures (30° and -2°) and the intracellular glutamate was determined using an optical test<sup>17</sup>.

$k_1$ ,  $k_2$  and  $k_n$  are velocity constants;  $[S]_o$  and  $[S]_i$  are the concentrations of the solute outside and inside the cell, respectively.

The function describing the dependence of the distribution ratio on high extracellular concentrations ( $[S] \gg K_m$ ) is hyperbolic as is shown by a rearrangement of Eqn. 3 under this condition.

$$\frac{v_{\max}}{k_1 + k_2 + \dots k_n} = Ra[S]_o ; Ra = \frac{[S]_i}{[S]_o} \quad (4)$$

The dependence of  $[S]_i$  upon  $[S]_o$  is formulated in Eqns. 5a and 5b for two extremely different cases



$$[S]_i = \frac{v_{\max}}{\Sigma k} \quad ([S]_o \gg K_m) \quad (5a)$$

$$[S]_i = [S]_o \cdot \frac{v_{\max}}{K_m \cdot \Sigma k} \quad ([S]_o \ll K_m) \quad (5b)$$

Thus, the intracellular concentration of glutamate will only be constant and independent upon  $[S]_o$  if  $[S]_o \gg K_m$ . However, since the values for  $[S]_o$  listed in Table III are in the range of or lower than  $K_{mII}$  (see below), the constancy of the internal glutamate pool over a wide range of extracellular glutamate can only be understood if the sum of the different velocity constants,  $\Sigma k$ , is variable, *e.g.* as a result of regulatory events. Further experiments are in progress to clarify this.

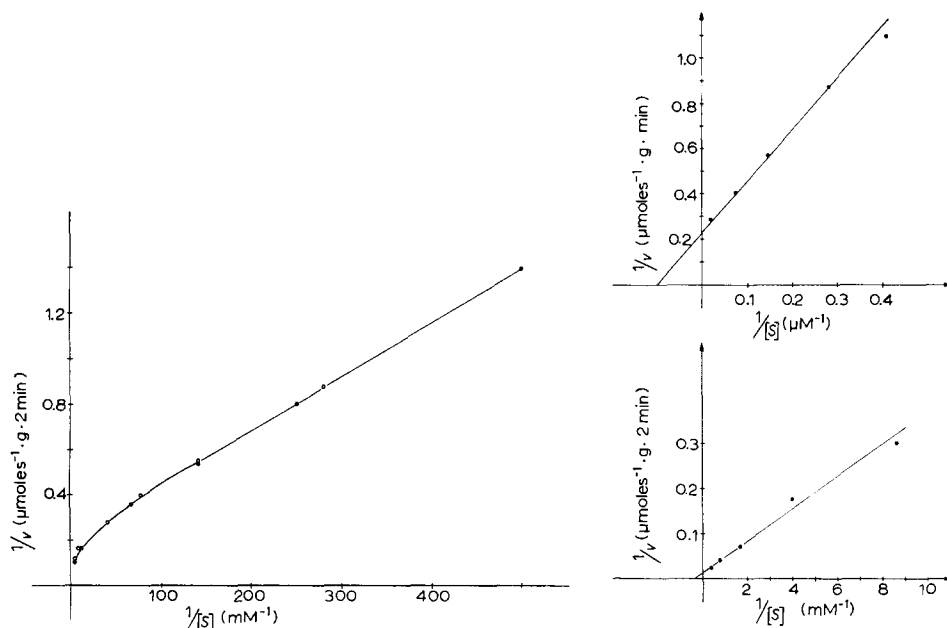


Fig. 7. Lineweaver-Burk plot of glutamate uptake. Cells were incubated in 0.05 M phosphate buffer (pH 7.1) for 2 min at 30°. The curve drawn was calculated from the kinetic parameters as shown in Fig. 8. (○) experimental points; (●) calculated points.

Fig. 8. Double-reciprocal plots of glutamate uptake in the  $\mu\text{M}$  range and in the mM range. Cells were incubated in 0.05 M phosphate buffer (pH 7.1), for 2 min at 30°. The upper curve shows the dependence of the glutamate influx on its extracellular concentration in the  $\mu\text{M}$  range. The lower curve shows the dependence in the mM range after correction of the influx as described in the text.

#### *Dependence of the influx of glutamate on its extracellular concentration*

The dependence of the influx on the extracellular glutamate concentration is shown in Fig. 7. The double reciprocal plot of Lineweaver and Burk does not give a straight line: at high concentrations the curve seems to deviate towards the zero point. This could indicate an additional non-saturable pathway of glutamate entry. Free diffusion obeying Fick's law, however, seems unlikely in view of the following observation: the influx of glutamate even at high extracellular concentrations remains highly sensitive to 2,4-dinitrophenol (Table IV). However, the results obtained with

2,4-dinitrophenol are not conclusive as long as intracellular endergonic binding of glutamate cannot be ruled out. As will be shown later, available evidence favours the assumption that at least a major part of the intracellular glutamate is in the free state. Lineweaver-Burk plots in Fig. 8 exhibit two straight lines, one corresponding to the  $\mu$ mole range, the other to the mmole range. Therefore, we assume that there are two transporting systems with different affinities involved in the transport of glutamate and aspartate. Similar systems have been described in other microorganisms,

TABLE IV

## INHIBITION OF THE GLUTAMATE INFLUX BY 2,4-DINITROPHENOL

The cells were incubated in phosphate buffer (pH 7.1) at 30°. Pretreatment with 1 mM 2,4-dinitrophenol for 10 min. The concentrations used are in the range, in which the uptake velocities in a double-reciprocal plot deviate from the linear course.

Pretreatment	Extracellular glutamate, $a_f$ (mM)	$Ru'$ (4 min)	$v (= Ru' \cdot a_f)$ ( $\mu$ moles/g per 4 min)	Transport activity (%)
Buffer	0.225	230.0	51.8	100
2,4-Dinitrophenol	0.225	6.3 *	1.4	2.7
Buffer	1.35	57.0	77.0	148.6
2,4-Dinitrophenol	1.35	4.1 *	5.5	10.6

\* No significant accumulation of glutamate related to cell water (4–5 ml/g).

TABLE V

## INHIBITION OF THE INFLUX (1 min) OF ASPARTATE AND GLUTAMATE BY AMINO ACIDS

The rest activity =  $v_i/v$ , where  $v$  is the influx without inhibitor and  $v_i$  the influx in the presence of inhibitor. The concentrations of the added amino acids at pH 6.0 and at pH 7.1 were 0.6 mM and 0.133 mM, respectively. In the glutamate influx experiments the concentrations of the added amino acids were 2.3 mM.

1st amino acid	2nd amino acid	$\alpha$ (pH 6.0)	$\alpha$ (pH 7.1)
L-[ <sup>14</sup> C]Asp (0.041 mM)	None	1	1
	L-Asp	0.20	0.54
	L-Glu	0.20	0.56
	2-aminoisobutyrate	0.92	0.84
	CycloLeu	0.92	0.85
	L-Leu	1	0.91
	L-Glu(NH <sub>2</sub> )	0.87	1
	L-Asp(NH <sub>2</sub> )	0.87	1
	D-Glu	1	—
	L-Lys	1	1
	L-Arg	1	1
	L-Met	1	1
	L-Phe	0.97	1
	L-Tyr	—	0.96
	L-His	—	1
L-[ <sup>14</sup> C]Glu (0.034 mM)	None	—	1
	L-Glu	—	0.31
	D-Glu	—	0.96

e.g. by FERROLUZZI-AMES<sup>21</sup> for the transport of histidine, tyrosine, tryptophan and phenylalanine in *Salmonella typhimurium* and by GRENSON<sup>22</sup> for the lysine transport in *Saccharomyces cerevisiae*.

If there are two systems, the intercept on the ordinate of a Lineweaver-Burk plot is the reciprocal value of  $v_{\max I} + v_{\max II}$ , and in determining  $K_{mII}$ , the measured velocities have to be corrected by subtracting the velocities of System I. In this way the following parameters are obtained

System I:  $K_{mI} = 1 \cdot 10^{-5}$  M;  $v_{\max I} = 4$   $\mu$ moles/g per 2 min

System II:  $K_{mII} = 2 \cdot 10^{-3}$  M;  $v_{\max II} = 50$   $\mu$ moles/g per 2 min.

### *Specificity of the transport systems*

The specificity of the glutamate or aspartate transport is dependent on their extracellular concentrations. Competition studies at low concentrations as indicated in Table V show that the transport system is specific for dicarboxylic amino acids. The affinity of aspartate for the transport site is similar to that of glutamate. The  $\omega$ -amides of aspartic acid and glutamic acid do not appreciably compete for aspartate influx. Hence, the  $\omega$ -carboxyl group appears to be necessary for specific binding. At pH 7.1, neutral amino acids compete only slightly, if at all, and this slight effect is almost completely abolished on shifting the pH to 6.0, even at concentrations 3.7 times higher than at pH 7.1. The dependence of uptake of dicarboxylic amino acids on protons has already been described<sup>14</sup>. In Table V the rest activities of various amino acids for aspartate uptake are listed. The system is highly stereospecific, because D-glutamate, even at high concentrations (Table V), does not show any significant inhibition of L-aspartate or L-glutamate influx. In this respect the system differs from those transporting neutral or basic amino acids in this microorganism, which do not exhibit such a high degree of stereospecificity<sup>23</sup>.

The competition pattern changes with increasing extracellular glutamate concentration: at high concentrations the neutral amino acids indicated in Table VI are inhibitory.

### *Regulation of glutamate uptake*

Previously it has been described that the active uptake of amino acids into *S. hydrogenans* is controlled by positive and negative feedback mechanisms<sup>12, 13, 24</sup>.

TABLE VI

INHIBITION OF THE GLUTAMATE INFLUX BY NEUTRAL AMINO ACIDS AT HIGH EXTRACELLULAR GLUTAMATE CONCENTRATIONS

The 2-min influx was measured in 0.05 M sodium-potassium phosphate buffer (pH 7.1) at 30°. The concentrations of glutamate and of the inhibitors were 4.08 mM and 40 mM, respectively. Rest activities are mean values of three experiments.

<i>Inhibitor</i>	<i>Rest activity</i>
2-Aminoisobutyrate	0.50
Leucine	0.34
Glycine	0.34
Methionine	0.31
Glutamate	0.44
Aspartate	0.44

Accordingly the influx of glutamate is influenced by intracellular amino acids or by some of their derivatives: *e.g.* preincubation of the cells in a medium containing 0.9 mM glutamate for 60 min enhances the subsequently measured 1-min influx of labeled glutamate by about 70 %. Similar results are obtained if glutamate in the medium is replaced by aspartate. This enhancement of influx, which becomes apparent after about 20 min of incubation with glutamate<sup>13</sup> (*cf.* Fig. 4) influences the shape of the net uptake curve. Thus, the time-course of glutamate uptake becomes S-shaped and may formally be compared with an auto-activative process.

#### DISCUSSION

The uptake of glutamate into *S. hydrogenans* is an energy-dependent and carrier-mediated process. Its dependence on metabolic energy is visualized by an almost complete inhibition of uptake by 2,4-dinitrophenol. The uptake of glutamate occurs against its concentration gradient. The relationship between influx and extracellular concentration of glutamate suggests that uptake occurs by at least two pathways. From a mere kinetic analysis it is difficult to decide whether the second pathway is by diffusion or by an additional transport system with a low affinity for the substrate. However, free diffusion should not be inhibited by dinitrophenol, which blocks the energy supply but does not presumably influence the membrane permeability. However, the influx is markedly inhibited by dinitrophenol even at high extracellular glutamate concentrations. Furthermore, if the major part of the entry at high concentration range were attributed to free diffusion, this influx component of [<sup>14</sup>C]glutamate should not be inhibited by [<sup>12</sup>C]glutamate because free diffusion of the labeled species is by definition independent of the movement of other solutes. In all experiments in which the radioactivity in the medium is kept constant, the addition of the unlabeled species causes an appreciable decrease in the uptake of radioactivity.

Our data lead to the assumption of two binding sites with different affinities for glutamate. The location of these sites remains unknown; the existence of two pathways for lysine entry into yeast cells has been postulated by GRENSON<sup>22</sup> on the basis of two mutants, *arg-p<sub>1</sub>* and *lys-p<sub>1</sub>*, the first one being able to take up lysine only *via* the specific system, the second mutant only *via* the unspecific one. At present it seems appropriate to interpret our results in a similar manner. The affinities of glutamate to its transport sites, insofar as they are adequately represented by the *K<sub>m</sub>* values, differ widely from each other. With respect to its entry, aspartate behaves in essentially the same manner, and the measurable parameters of its transport are nearly equal to those of glutamate transport.

The specificity of System I seems to be restricted to dicarboxylic amino acids. The competition studies with D-glutamate conclude that this system is highly stereospecific. The lack of inhibition of glutamate uptake by its  $\omega$ -amide suggests that the  $\omega$ -carboxylic group or its negative charge is a prerequisite for specific binding. From these results it appears that substrate binding occurs with its two carboxylic groups and the amino group in L position. System II, on the contrary, is non-specific.

Another important question concerns the state of glutamate inside the cell. It might be that there is a large number of intracellular binding sites, and that the glutamate extracted from the cell does not represent the free amino acid but, rather the amount of previously bound molecules being released during the extraction pro-

cedure. There are two observations which disagree with this assumption: (a) the rise to a second steady state (*cf.* Fig. 3) observed for uptake of glutamate. Since this transstimulation of the glutamate influx is also observed during incubation in a medium containing 50  $\mu\text{g}$  chloramphenicol per ml, the rise cannot have been caused by an increase of binding sites, provided the acceptors are protein molecules. But if the intracellular binding sites are located on compounds other than proteins, it is hardly understandable how preloading with glutamate should increase these sites when the cells have energy enough from a high extracellular glucose concentration. (b) Cells harvested in the middle phase of exponential growth have a glutamate pool of about 160  $\mu\text{moles/g}$  dry wt. By applying cold shock this pool can be drastically reduced to less than 30  $\mu\text{moles/g}$  dry wt. Therefore, one may assume that glutamate inside the cell is to a major part in the free state.

The glutamate taken up is rapidly metabolized, so that after 60 min of incubation at 30° only 30 % of the total cellular radioactivity represents glutamate. 40 % of the intracellular label was found in the trichloroacetic acid-precipitable fraction. There is evidence from radiochromatograms that this percentage is not only due to incorporation of glutamate into proteins but also of other amino acids derived from the carbon chain of glutamate. In the absence of net protein synthesis the pool size remains unchanged because for each molecule incorporated into protein another is released by breakdown. Without net synthesis and after saturation of the intracellular pool the net flux of glutamate into the cell corresponds to the conversion and breakdown of this amino acid. The influx of glutamate is influenced by amino acids present at the *trans* side of the membrane<sup>12, 13, 24</sup>. Preloading the cells with L-glutamate enhances the uptake of labeled glutamate. It was previously pointed out<sup>13</sup> that this transstimulation is not identical with the preloading effect described by HEINZ<sup>25</sup> and that it is not caused by glutamate itself, but, rather, by one of its derivatives. From these and previous findings it is concluded that the uptake of glutamate is regulated by the intracellular pool of free amino acids and some derivatives.

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