The effect of azaserine on glutamine uptake by rat renal brush-border membranes

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Azaserine added directly to isolated rat renal brush-border membrane vesicles inhibits uptake of L-glutamine. Azaserine acts as a non-competitive inhibitor of the low- K_m system for glutamine transport, but has no effect on the high- K_m system. Preincubation of the vesicles with azaserine at 37°C for 30 min is not required for transport inhibition to occur, although it is a requirement for γ -glutamyl transpeptidase inhibition. Removal of azaserine from the vesicle preparation by repeated resuspensions in buffer results in a reversal of the transport inhibition but not in reversal of enzyme inhibition. Azaserine also inhibits vesicle uptake of L-proline and α -methyl D-glucoside, indicating a generalized effect on membrane transport systems. The data cast doubt on the postulate that γ -glutamyl transpeptidase might act as the carrier mechanism for glutamine reabsorption by renal cortical cells.

It has long been recognized that glutamine has a significant role in acid–base metabolism in the kidney (Van Slyke *et al.*, 1943; Pitts, 1973). Studies on the therapeutic use of glutamine in cystinuria (Miyagi & Nakada, 1978; Miyagi *et al.*, 1979) have introduced a possible role for glutamine in transport regulation. Uptake of glutamine by isolated rat renal brush-border membrane vesicles has been examined and the conversion of glutamine to glutamic acid in such membranes has been documented (Weiss *et al.*, 1978).

Azaserine (*O*-diazooacetyl-L-serine) bears an obvious structural similarity to glutamine and has been shown to inhibit competitively some glutamine-requiring reactions (Abrams & Bentley, 1959). Novogrodsky *et al.* (1977) reported that the rate of glutamine uptake by a human lymphoid cell line is related to the amount of γ -glutamyl transpeptidase (γ -glutamyltransferase; EC 2.3.2.2) activity on the cell surface. Azaserine is also known to inhibit γ -glutamyl transpeptidase, a membrane-bound enzyme of the renal brush border in the rat (Tate & Meister, 1977). We have utilized azaserine as a membrane probe to study its effect on glutamine uptake into rat renal brush-border vesicles and to study the relationship between γ -glutamyl transpeptidase and glutamine transport. The results form the basis of this report.

Abbreviation used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

Materials and methods

Preparation of vesicles

Adult male Sprague–Dawley rats weighing 250–300 g and fed *ad libitum* on Purina rat chow were killed by decapitation. Kidneys were removed, decapsulated and placed in 0.9% NaCl solution at 4°C. Rat cortical tissue was removed by using a Stadie–Riggs microtome. Brush-border membranes were isolated by using the method of Booth & Kenny (1974). Vesicles were prepared by resuspension of the isolated brush-border membranes in hypo-osmotic 2 mM-Tris/Hepes buffer, pH 7.4, containing 100 mM-mannitol.

Incubation with azaserine

The following protocols were utilized to determine the effects of azaserine on transport of glutamine.

Preincubation effect. About 25 mg of isolated vesicles were incubated at 37°C with 8 ml of buffer alone or with buffer containing 50 mM-azaserine for 30 min. The vesicles were diluted with 6 vol. of cold (0°C) buffer and equilibrated to 22°C before the measurement of uptake. In a parallel group of studies, membranes were suspended in buffer alone or in buffer containing 50 mM-azaserine, immediately diluted with 6 vol. of buffer at 22°C and incubated for 30 min at that temperature before glutamine uptake was measured. The final azaserine

concentration after these manipulations was 7.2 mM.

Direct effect. Vesicles at the same concentration as stated above were incubated at 22°C and then diluted with 6 vol. of buffer. Uptake of glutamine by these vesicles was determined in the absence and presence of 7.2 mM-azaserine.

Residual effect of preincubation. Vesicles that had been preincubated with 50 mM-azaserine for 30 min at 37°C were diluted with 6 vol. of cold buffer and centrifuged at 35 000 *g* for 20 min at 4°C. The pellet was washed twice with 24 vol. of buffer, after which the membranes were suspended in 56 ml of buffer and utilized for measurement of glutamine uptake. Membranes that had been preincubated with 7.2 mM-azaserine at 22°C were treated similarly. The final vesicle-protein concentration after these manipulations was 0.4–0.5 mg/ml as determined by the method of Lowry *et al.* (1951).

Uptake of amino acids

The uptake of amino acids at 22°C was determined in the presence of a 100 mM inward sodium gradient by using filtration techniques as described by McNamara *et al.* (1976). Results of amino acid-uptake studies are expressed as uptake in excess of that of 3-*O*-[methyl-³H]methyl-D-glucose or L-[1-³H(N)]glucose, sugars that are thought to enter the brush-border by diffusion (McNamara *et al.*, 1976; Weiss *et al.*, 1978).

To determine the effect of azaserine at 22°C on the uptake of amino acids by brush-border vesicles, 0.4 ml of freshly prepared membrane vesicles in 2 mM-Tris/Hepes/100 mM-mannitol buffer, pH 7.4, at 22°C was added at the starting time to a disposable 10 mm × 75 mm test tube containing 0.1 μCi of ¹⁴C-labelled amino acid, 0.1 μCi of 3-*O*-[methyl-³H]methyl-D-glucose or 0.4 μCi of L-[1-³H(N)]glucose, 50 μmol of NaCl, 4.04 μmol of azaserine and unlabelled amino acid to bring the incubation mixture to the desired final concentration of amino acid. The final volume of the mixture was 0.565 ml. The samples were vortex-mixed during the first 6 s of incubation. After a total incubation time ranging from 15 s to 20 min, the incubation mixture was transferred by Pasteur pipette to a Millipore filter apparatus. Uptake was stopped by rapid filtration of the mixture through a Millipore filter (HAWP, 0.45 μm) which was washed once with 5 ml of solution containing 2 mM-Tris/Hepes/0.9% NaCl, pH 7.4. The filter was air dried and counted in a Packard Tricarb scintillation spectrometer in 10 ml of diluted Concentrol (Yorktown Research, Miami, FL, U.S.A.).

The azaserine treatment at 22 and 37°C had no effect on the ability of brush-border membranes to form sealed vesicles as measured by uptake of L-glucose and 3-*O*-methyl-D-glucose which decreased as the osmolarity of the medium was

increased by the addition of sucrose. Both the azaserine-treated vesicles and control vesicles were osmotically active with no apparent binding component for the freely-diffusible 3-*O*-methyl-D-glucose or L-glucose.

Enzyme assay

γ-Glutamyl transpeptidase activity was examined by using the procedure of Glossmann & Neville (1972). To determine the effect of azaserine on the activities of membrane-bound γ-glutamyl transpeptidase, membrane vesicles (3 mg/ml) in 2 mM-Tris/Hepes buffer (pH 7.4) containing 100 mM-mannitol were preincubated at 22 or 37°C in the presence or absence of 7, 25, or 50 mM-L-azaserine for the designated time (0–60 min). Aliquots of the preincubated samples were then diluted 1:7 with buffer and assayed in quadruplicate as follows. Vesicle suspension (20 μg/50 μl) in 2 mM-Tris/Hepes buffer containing 100 mM-mannitol, with or without azaserine, was preincubated at 37°C for 1.8 min with 100 mM-Tris/HCl, pH 8.2, 2 mM-glycylglycine and 20 mM-MgCl₂ in a final volume of 0.8 ml. The assay was initiated by the addition of 200 μl of 10 mM-γ-glutamyl-*p*-nitroanilide at 37°C. After 1 min, the assay was terminated by the addition of 0.5 ml of 1.5 M-acetic acid at 0°C. The final azaserine concentrations in the enzyme assay for vesicles preincubated with 7, 25, and 50 mM-azaserine were 0.05, 0.18 and 0.36 mM, respectively. Assay incubations in which final azaserine concentrations were as high as 7 mM were also performed.

Materials

L-[U-¹⁴C]Glutamine, L-[U-¹⁴C]proline, L-[1-³H-(N)]glucose and 3-*O*-[methyl-³H]methyl-D-glucose were purchased from New England Nuclear Corp. [U-¹⁴C]α-Methyl D-glucoside was obtained from Calatomic. L-Azaserine (*O*-diazooacetyl-L-serine), γ-glutamyl-*p*-nitroanilide and Hepes were purchased from Calbiochem. All other chemicals were of the highest purity available.

Results

Effect on enzyme activity

The presence of up to 7 mM-azaserine in a suspension of brush-border membrane vesicles in 2 mM-Tris/Hepes/100 mM-mannitol buffer, pH 7.4 at 22°C, for as long as 30 min resulted in no inhibition of γ-glutamyl transpeptidase activity. Indeed, the presence of 7 mM-azaserine in the enzyme assay did not inhibit γ-glutamyl transpeptidase activity. Only after incubation of the vesicles with azaserine at 37°C was an inhibitory effect seen. Fig. 1 shows the effect on γ-glutamyl transpeptidase activity of incubating rat renal brush-border membrane vesicles

in 2 mM-Tris/Hepes/100 mM-mannitol buffer with 7, 25 or 50 mM-azaserine for up to 30 min at 37°C. No inhibition of enzyme activity occurred after 3 min of incubation, and only 15–20% inhibition occurred after 10 min of incubation at 37°C. No significant difference in inhibition after 10 min of incubation with the three azaserine concentrations occurred.

Fig. 1. Effect of azaserine doses and incubation time on γ -glutamyl transpeptidase activity of rat kidney brush-border vesicles

Brush-border vesicles were incubated at 37°C in the absence (○) or presence of 7 mM- (●), 25 mM- (■), or 50 mM- (▲) L-azaserine as described in the text for the times indicated, after which an aliquot of the preparation was assayed for γ -glutamyl transpeptidase activity with γ -glutamyl-*p*-nitroanilide as substrate. Activities were calculated in mmol of *p*-nitroaniline released/min per mg of brushborder protein and are expressed as a percentage of the activity of the control vesicles. Each data point is the mean $\pm$ S.E.M. for four determinations. The errors of all the data points are included within the size of each point.

After 20 min of incubation, the rate of inhibition by 50 mM-azaserine was appreciably greater than that at 7 or 25 mM-azaserine. The inhibition by 50 mM-azaserine was maximal after 30 min of incubation since incubation for 60 min (result not shown) revealed no further inhibition. γ -Glutamyl transpeptidase inhibition was correlated with glutaminase inhibition in the vesicles, since we were unable to observe hydrolysis of glutamine to glutamate when the vesicle contents were examined by paper chromatography (Weiss *et al.*, 1978) during transport studies in which the enzyme was maximally inhibited.

The residual effect of azaserine treatment on the γ -glutamyl transpeptidase activity of membrane vesicles preincubated at 22 or 37°C in the presence or absence of azaserine is shown in Table 1. There was no significant difference between γ -glutamyl transpeptidase activity of membrane vesicles that were preincubated at 22 or 37°C in the absence of azaserine either before or after additional washings (Table 1, conditions 1, 3, 5 and 7). There was also no effect on the enzyme activity when 7.2 mM-azaserine was added to, or washed out from, vesicle suspensions at 22°C (Table 1, conditions 2 and 4). However, the γ -glutamyl transpeptidase activity was inhibited by 60% when membrane vesicles were preincubated with 50 mM-azaserine for 30 min at 37°C (Table 1, condition 6). Removal of azaserine by washing did not restore the lost activity (Table 1, condition 8).

Effects of transport

To parallel the studies of enzyme activity, the dose-response to azaserine concentration of [14 C]-glutamine uptake for 30 s by brush-border vesicles was examined under conditions where γ -glutamyl transpeptidase is uninhibited. The results are shown in Fig. 2. It may be seen that adding as little as

Table 1. Effect of azaserine treatment on γ -glutamyl transpeptidase activity of brush-border membrane vesicles
Washing indicates vesicles have undergone the 'wash-out' procedure explained in the text consisting of repeated resuspensions of the vesicles in 2 mM-Tris/Hepes/100 mM-mannitol buffer, pH 7.4. * $P < 0.001$; the values shown are means $\pm$ S.E.M. from three different brush-border preparations where assays were done in quadruplicate in each preparation.

Condition	Preincubation for 30 min at:		Washing	Activity of γ -glutamyl transpeptidase	
	Temperature (°C)	[Azaserine] (mM)		Specific (mmol of <i>p</i> -nitroaniline/min per mg of protein)	Relative (% of control)
1	22	0	—	3.57	100 $\pm$ 3.15
2	22	7.2	—	3.67	102.7 $\pm$ 5.21
3	22	0	+	3.62	101.2 $\pm$ 1.86
4	22	7.2	+	3.68	103.0 $\pm$ 3.61
5	37	0	—	3.61	101.0 $\pm$ 3.13
6	37	50	—	1.42	39.6 $\pm$ 1.88*
7	37	0	+	4.08	104.0 $\pm$ 0.52
8	37	50	+	1.48	41.4 $\pm$ 0.79*

Fig. 2. Azaserine dose-response curve of glutamine uptake by rat kidney brush-border vesicles at 22°C in the presence of an Na^+ gradient

Uptake after 30 s of incubation with an Na^+ gradient was determined for 0.02 mM-glutamine ($\blacktriangle$), 0.06 mM-glutamine ($\triangle$), 1.5 mM-glutamine ($\bullet$), and 2.1 mM-glutamine ($\circ$), by the methods described in the text. All points are expressed relative to uptake in the absence of azaserine, which was taken to be 100%. Values given are the means for eight determinations for each substrate concentration. The errors of all the data points are included within the size of each point.

1.5 mM-azaserine directly to the uptake assay would result in 50% inhibition of uptake for both 0.02 and 0.06 mM-L-glutamine, whereas the addition of 7 mM-azaserine is necessary for about 40% inhibition of uptake of 1.5 and 2.1 mM-glutamine.

The time course of uptake of 0.02 and 2.1 mM-L-glutamine by brush-border membrane vesicles in the absence and presence of 7.2 mM-azaserine under the condition of an Na^+ gradient at 22°C is shown in Fig. 3. The typical 'overshoot' phenomenon of stimulated uptake was observed for 2.1 mM-L-glutamine (Fig. 3b) but not for 0.02 mM-L-glutamine (Fig. 3a). Azaserine lowered the uptake values for glutamine over the time course studied. For 0.02 mM-glutamine, the uptake values in the presence of 7.2 mM-azaserine never exceeded 27% of the values for glutamine uptake in the absence of azaserine over the entire 20 min studied. For 2.1 mM-glutamine, the maximal uptake at 1 min in the presence of azaserine was 64% of that in the absence of azaserine, whereas after 20 min of incubation in the presence of azaserine, uptake was 86% of the control. Thus the presence of azaserine seemed to exert a stronger inhibitory effect on transport at lower glutamine concentrations.

Fig. 3. Effect of azaserine on the time course of uptake of glutamine by isolated rat renal brush-border membrane vesicles at 22°C in the presence of an Na^+ gradient

(a) Shows the uptake of 0.02 mM-glutamine in the absence ($\triangle$) or presence ($\blacktriangle$) of 7 mM-azaserine. (b) Shows the uptake of 2.1 mM-glutamine in the absence ($\triangle$) or presence ($\blacktriangle$) of 7 mM-azaserine. Uptake was assayed by the methods described in the text. Values shown are the means ($\pm$ S.E.M.; bars) of eight to twelve determinations.

Effects on kinetic parameters

We have previously reported that the kinetics of glutamine transport by brush-border membrane vesicles indicate the presence of two saturable systems for uptake (Weiss *et al.*, 1978). The uptake of glutamine by brush-border membrane vesicles at 22°C in the absence and presence of 7.2 mM-azaserine was concentration-dependent and saturable, as shown in Fig. 4. The Hofstee plot for the concentration dependence of initial (15 s) glutamine uptake shows a two-limbed curve that indicates the presence of multiple transport systems (Neame & Richards, 1972) for the vesicles in the absence of azaserine. The transport parameters determined from the Hofstee plot were K_{m1} , 0.63 mM; $V_{max,1}$,

Fig. 4. Hofstee plots for the concentration-dependence of glutamine uptake by brush-border vesicles

Uptake after 15 s of incubation at 22°C with an Na⁺ gradient was measured over a range of 0.0184–3.913 mM-glutamine in the absence (△) and presence (▲) of 7.2 mM-azaserine. Glutamine uptake by vesicles that were preincubated at 37°C for 30 min without azaserine (○) or with 50 mM-azaserine followed by dilution to 7.2 mM-azaserine (●) was also measured. The velocity of uptake, v , is measured in nmol/15 s per mg of protein and $[S]$ is the glutamine concentration (mM). Values given are the means of 8 to 24 determinations for each substrate concentration. The lines represent the best fit for the data with or without the addition of azaserine.

4.34 nmol/15 s per mg; K_{m_2} , 2.56 mM; $V_{max,2}$, 12.77 nmol/15 s per mg. With the Hofstee parameters as initial estimates, we calculated kinetic parameters from eqn. (1) to give the best fit to the observed total uptake by using a Digital PDP 12 computer:

$$v_{total} = \frac{V_{max,1}[S]}{[S] + K_{m_1}} + \frac{V_{max,2}[S]}{[S] + K_{m_2}} \quad (1)$$

The calculated values were: K_{m_1} , 0.45 mM; $V_{max,1}$, 2.00 nmol/15 s per mg; K_{m_2} , 7.50 mM; $V_{max,2}$, 18.00 nmol/15 s per mg. The Hofstee plot for glutamine uptake by vesicles in the presence of 7.2 mM-azaserine at 22°C indicated a single uptake system with an apparent K_m and V_{max} for glutamine of 6.64 mM and 14.08 nmol/15 s per mg. It should be emphasized that there was no inhibition of γ -glutamyl transpeptidase activity by azaserine during these kinetic studies.

Included in Fig. 4 are data obtained from vesicles that had been incubated at 37°C for 30 min in buffer alone or with 50 mM-azaserine before dilution to 7.2 mM-azaserine. Preincubation of membrane vesicles at 37°C for 30 min in buffer alone did not

Fig. 5. Effect of removal of azaserine on the time course of uptake of glutamine at 22°C in the presence of an Na⁺ gradient by brush-border membrane vesicles that had been preincubated at 37°C for 30 min in the absence (○) or presence (●) of 50 mM-azaserine

The preincubated vesicles were diluted and washed by the methods described in the text. (a) Shows the uptake of 0.02 mM-glutamine; (b) shows the uptake of 2.1 mM-glutamine. Values shown are the means $\pm$ S.E.M. for four determinations.

affect the kinetic parameters of glutamine uptake, which were calculated to be: K_{m_1} , 0.44 mM; $V_{max,1}$, 1.68 nmol/15 s per mg; K_{m_2} , 7.90 mM; $V_{max,2}$, 17.95 nmol/15 s per mg. A single system with K_m 7.45 mM and V_{max} 18.60 nmol/15 s per mg was evident after preincubation with 50 mM-azaserine at 37°C. The same inhibition of the low- K_m system was observed when 7.2 mM-azaserine was added directly, when vesicles were preincubated with 7.2 mM-azaserine at 22°C for 30 min, and when 7.2 mM-azaserine was present after preincubation with 50 mM-azaserine at 37°C and subsequent dilution at 22°C (Fig. 4). Thus, the observed inhibition of transport by 7.2 mM-azaserine does not have as a prerequisite a parallel inhibition of γ -glutamyl transpeptidase resulting from the prior incubation with azaserine at 37°C.

To determine any residual transport inhibition by

azaserine, experiments were performed in which membranes were washed to remove unbound azaserine and these results are shown in Figs. 5 and 6. Fig. 5 shows the time course of uptake of 0.02 and 2.1 mM-L-glutamine by brush-border membrane vesicles that had been washed after preincubation at 37°C for 30 min in buffer alone or in the presence of 50 mM-azaserine. The inhibitory effect of azaserine upon the time course of uptake (shown in Fig. 3) was completely removed after the washing procedure. γ -Glutamyl transpeptidase activity remained inhibited by 60–70%.

The Hofstee plot for concentration-dependent glutamine uptake by vesicles that had been preincubated with 7.2 mM-azaserine at 22°C and then washed was a two-limbed curve (Fig. 6a) with kinetic parameters similar to those of the control preparation (see Fig. 4). The washing procedure had no apparent effect on the K_m values for the two systems in either the control or the azaserine-exposed vesicles and resulted in only slight variations in V_{max} values. Computer calculated parameters for the initial uptake of glutamine from washed control vesicles were: K_{m1} , 0.46 mM; V_{max1} , 2.37 nmol/15 s per mg; K_{m2} , 7.80 mM; V_{max2} , 12.40 nmol/15 s per mg. It is important to note the reappearance of two saturable systems for glutamine uptake after removal of the 7.2 mM-azaserine by repeated resuspension of the vesicles. These parameters were calculated to be: K_{m1} , 0.44 mM; V_{max1} , 2.65 nmol/15 s per mg; K_{m2} , 7.80 mM; V_{max2} , 16.80 nmol/15 s per mg. Parallel determinations of

the enzyme activity of the vesicles indicated that γ -glutamyl transpeptidase was not inhibited during these kinetic studies.

When vesicles that had been preincubated at 37°C for 30 min with 50 mM-azaserine were washed to remove unbound azaserine, there was the reappearance of two transport systems. This is evidenced by the two-limbed Hofstee plots for initial uptake of glutamine by such vesicles (Fig. 6b). The calculated parameters for these vesicles were: K_{m1} , 0.40 mM; V_{max1} , 0.55 nmol/15 s per mg; K_{m2} , 7.80 mM; V_{max2} , 19.0 nmol/15 s per mg. Calculated parameters [by using eqn. (1)] for best fit of the data for control vesicles preincubated at 37°C were: K_{m1} , 0.35 mM; V_{max1} , 1.00 nmol/15 s per mg; K_{m2} , 7.80 mM; V_{max2} , 13.50 nmol/15 s per mg. It must be emphasized that the reappearance of two saturable systems for glutamine uptake after removal of azaserine in this instance was not accompanied by recovery of γ -glutamyltranspeptidase activity in the brush-border membrane vesicles.

Brush-border vesicle uptake of glutamine on the low- K_m system was completely inhibited by 7.2 mM-azaserine, making it impossible to determine the mode of action of azaserine inhibition. To investigate the kinetics of inhibition, concentration-dependent studies of initial (15 s) glutamine uptake in the presence or absence of 1 mM-azaserine were performed at 22°C. Computer analysis of the data [by using eqn. (1)] indicated the following kinetic parameters: K_{m1} , 0.41 mM; V_{max1} , 0.25 nmol/15 s per mg; K_{m2} , 7.4 mM; V_{max2} , 18.7 nmol/15 s per mg. Thus, azaserine appears to act in a noncompetitive fashion by lowering the V_{max1} of glutamine entry on the low- K_m system approx. 10-fold compared with control vesicles.

Effects on sodium dependence

Sodium dependence of glutamine transport was examined in vesicles incubated for 30 min at 37°C in the absence and presence of azaserine under conditions of maximal inhibition of both transport and γ -glutamyl transpeptidase activity. Azaserine treatment had no effect on the apparent affinity of 13 mM for sodium of the glutamine transport systems in rat renal brush-border membrane vesicles which we have previously reported (Weiss *et al.*, 1978).

Specificity of azaserine inhibition of transport

The specificity of azaserine inhibition of the transport systems of the brush-border membrane was explored by using L-proline and a model sugar, α -methyl D-glucoside, in addition to glutamine. The results are presented in Table 2. Uptake of all the substrates tested was inhibited by azaserine. When membrane vesicles were repeatedly resuspended to remove azaserine, uptake of both low and high

Fig. 6. Hofstee plots showing the effect of azaserine removal on the concentration-dependence of glutamine uptake by brush-border vesicles that had been preincubated with the compound

(a) Shows glutamine uptake by brush-border vesicles that were preincubated at 22°C in the absence (Δ) and presence ($\blacktriangle$) of 7.2 mM-azaserine before removal of the azaserine by repeated resuspensions in buffer. (b) Shows uptake by brush-border vesicles that were preincubated at 37°C for 30 min in buffer alone ($\circ$) or with 50 mM-azaserine ($\bullet$) before dilution and washing. The velocity of uptake, v , is expressed as nmol/15 s per mg and $[S]$ is the glutamine concentration (mM). The means of eight determinations for each substrate concentration are shown.

Table 2. *Effects of azaserine incubation and washings on initial uptake of amino acids and sugar*

* $P < 0.001$ for differences from control; † $P < 0.02$ for differences from control. Values in parentheses represent the number of determinations.

Substrate	Concentration (mM)	Treatment	Uptake (% of control)			
			Control	Azaserine present	Control (after washing)	Azaserine-treated vesicles (after washing)
L-Glutamine	0.02		100 ± 1.18 (16)	39.0 ± 1.31 (15)*	118.3 ± 9.92 (12)	120.1 ± 7.66 (12)†
	2.1		100 ± 1.51 (13)	61.4 ± 2.68 (15)*	95.5 ± 5.58 (15)	114.9 ± 10.26 (16)
L-Proline	0.02		100 ± 2.22 (14)	53.1 ± 1.44 (13)*	104.6 ± 10.46 (16)	100.9 ± 5.79 (14)
	2.1		100 ± 3.87 (13)	65.9 ± 3.69 (12)*	138.7 ± 18.76 (15)	95.6 ± 12.43 (16)
α -Methyl D-glucoside	0.02		100 ± 1.41 (16)	55.3 ± 1.85 (13)*	91.1 ± 11.31 (16)	79.3 ± 7.01 (16)†
	2.1		100 ± 3.99 (10)	72.4 ± 2.11 (10)*	118.3 ± 10.59 (10)	110.7 ± 6.71 (10)

concentrations of glutamine and proline, as well as uptake of high concentrations of α -methyl D-glucoside, was restored to control levels. Uptake of low concentrations of α -methyl D-glucoside was only partially restored by removal of azaserine.

Discussion

Azaserine is known to inhibit a number of reactions involving glutamine, such as the amination of xanthylic acid to guanylic acid in a mammalian system (Abrams & Bentley, 1959), the amination of the nicotinic acid moiety of ADP in bacterial enzyme preparations (Pitillo & Hunt, 1967) and the transpeptidase and hydrolase activities of kidney γ -glutamyl transpeptidase (Tate & Meister, 1977). To this list we now add the inhibitory effect of azaserine on glutamine transport in isolated rat renal brush-border membrane vesicles. Transport of glutamine into human lymphocytes has been reported by Willis & Seegmiller (1977), as well as by Novogrodsky *et al.* (1977, 1979), also to be inhibited by 6-diazo-5-oxo-L-norleucine and by the chloroketone L-2-amino-4-oxo-5-chloropentanoate, which are other glutamine analogues related to azaserine.

Glutamine transport in brush-border vesicles is characterized by a dual mechanism with two K_m values. In this report we have shown that azaserine acts as a non-competitive inhibitor of the low- K_m system, such that 1 mM-azaserine results in a 10-fold lower V_{max} for this high-affinity system. It has no effect on the high K_m , low-affinity system. The inhibition is reversible when azaserine is removed from the membrane preparation by repeated centrifugation and resuspension in buffer.

Studies on the modes of action of azaserine on *Escherichia coli* (Williams & Tritz, 1977) and on a number of isolated enzymes (Stadtman & Prusiner, 1973) indicate that, once azaserine binds to an enzyme, the diazo group of azaserine reacts co-

valently with the enzyme, usually through a thio group (French *et al.*, 1963), thus exerting an inhibiting effect. When the interaction of azaserine with purified rat kidney γ -glutamyl transpeptidase was examined, Tate & Meister (1977) suggested that, during preincubation at 37°C for 30 min, azaserine could bind to the enzyme site that normally binds the α -amino and α -carboxy moieties of γ -glutamyl donors. Although preincubation with *p*-chloromercuribenzoate did not affect purified γ -glutamyl transpeptidase (Will & Hopfer, 1979), suggesting that a thiol group might not be involved, a 'buried' thiol group cannot be excluded. We have confirmed the necessity of the preincubation at 37°C for azaserine inhibition of γ -glutamyl transpeptidase in isolated rat renal brush-border membrane vesicles and have demonstrated the irreversibility of this inhibition by repeated washings of the vesicles to remove azaserine.

Data in our studies allow us to analyse the relationship between renal brush-border-membrane glutamine transport and γ -glutamyl transpeptidase, which has often been suggested to be a carrier of glutamine and other amino acids. The facts are as follows.

(1) Azaserine inhibits the low- K_m system for glutamine uptake when it is present during the measurement of entry of glutamine into membrane vesicles. Though azaserine is a structural analogue of glutamine, the inhibition appears to be non-competitive in nature. Under these conditions, where glutamine uptake is inhibited, there is no inhibition of γ -glutamyl transpeptidase.

(2) Since the uptakes of proline and α -methyl D-glucoside, which have no postulated relationship to γ -glutamyl transpeptidase activity, are also inhibited, it appears that the presence of azaserine has a widespread effect on various transport functions of the membrane.

(3) When the membrane vesicles are preincubated at 37°C, presumably to bind azaserine to

γ -glutamyl transpeptidase, producing an irreversible 70% inhibition of enzyme activity, and the unbound azaserine is removed by washing, there is no inhibition of glutamine uptake by vesicles.

These observations cast doubt on the role that γ -glutamyl transpeptidase plays in the low- K_m glutamine transport system. The high- K_m system that we have described in brush-border membrane vesicles, which accounts for the majority of glutamine uptake (Weiss *et al.*, 1978) is not affected under any experimental conditions. We chose to examine glutamine transport because glutamine is an excellent substrate for γ -glutamyl transpeptidase. Our findings do not support the postulated role of this enzyme as a carrier mechanism for glutamine reabsorption by renal cortical tubule cells (Meister, 1973). The enzyme could, however, play a role in transport of other amino acids that we have not yet examined.

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