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Absence of a Role of Gamma-Glutamyl Transpeptidase in the Transport of Amino Acids by Rat Renal Brushborder Membrane Vesicles

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Summary. The role of the enzyme, gamma-glutamyl transpeptidase on the uptake of amino acids by the brushborder membrane of the rat proximal tubule was examined by inhibiting it with AT-125 (L-[αS, 5S]-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid). AT-125 inhibited 98% of the activity of gamma-glutamyl transpeptidase when incubated for 20 min at 37°C with rat brushborder membrane vesicles. AT-125 given to rats *in vivo* inhibited 90% of the activity of gamma-glutamyl transpeptidase in subsequently isolated brushborder membrane vesicles from these animals. AT-125 inhibition of gamma-glutamyl transpeptidase both *in vivo* and *in vitro* had no effect on the brushborder membrane uptake of cystine. Similarly, there was no effect of gamma-glutamyl transpeptidase inhibition by AT-125 on glutamine, proline, glycine, methionine, leucine or lysine uptake by brushborder membrane vesicles. Furthermore, the uptake of cystine by isolated rat renal cortical tubule fragments, in which the complete gamma-glutamyl cycle is present, was unaffected by AT-125 inhibition of gamma-glutamyl transpeptidase. Therefore, in the two model systems studied, gamma-glutamyl transpeptidase did not appear to play a role in the transport of amino acids by the renal brushborder membrane.

Key Words AT-125 · gamma-glutamyl transpeptidase · renal brushborder membrane · amino acids transport

Introduction

Meister and his colleagues have established the cyclical series of reactions known as the gamma-glutamyl cycle and with others have demonstrated the importance of the cycle in the turnover and metabolism of glutathione with its prime locus of operation in the kidney (Orlowski & Meister, 1970, 1971; Van Der Werf, Orlowski & Meister, 1971). Another postulated function of this cycle is the transport of amino acids, especially in the kidney where the reabsorption of amino acids requires a highly efficient process for reclamation of these compounds from tubular fluid (Meister, 1973). The recognition that gamma-glutamyl transpeptidase is membrane-bound and localized primarily in the brushborder of the proximal renal tubule formed the cornerstone of the hypothesis that the enzyme could act as a carrier, translocating amino acids into the cell, where they are subsequently released into the intracellular milieu by the action of gamma-glutamyl cyclotransferase (Orlowski & Meister, 1970).

Support for this postulate has been the report that the purified renal enzyme when incorporated into human erythrocyte membranes, which normally lack the enzyme, enhances their capacity to take up amino acids (Kalra, Sikka & Sethi, 1981) and the observation that purified hog kidney enzyme when inserted into lecithin vesicles mediates the transport of glutamate as a gamma-glutamyl entity when glutathione is present (Sikka & Kalra, 1980). On the other hand the pathway normally present in rabbit erythrocytes does not appear to participate in amino acid transport by those cells (Young, Ellory & Wright, 1975). Patients with an inherited deficiency of gamma-glutamyl transpeptidase reabsorb amino acids by the kidney normally, although they exhibit glutathionuria and increased excretion of gamma-glutamyl cystiene and cystiene mixed disulfides (Schulman et al., 1975; Wright et al., 1979).

Little data is available from experiments employing renal tubule preparations to assess the role of the gamma-glutamyl cycle in amino acid transport. Silbernagl (1981) noted during *in vivo* glutathione microperfusion of rat renal tubules from animals given AT-125 to inhibit gamma-glutamyl transpeptidase that breakdown of the tripeptide was inhibited but the absorption of its constituent amino acids was unimpaired. We have reported that a 60 to 70% inhibition of the enzyme by treatment of brushborder membrane vesicles with azaserine does not impair glutamine uptake (Hsu et al., 1980). In the present studies we have assessed the uptake of amino acids, especially cystine which is an ex-

cellent substrate for gamma-glutamyl transpeptidase, by rat renal brushborder vesicles and tubule fragments where gamma-glutamyl transpeptidase was inhibited 90% by treatment of rats with AT-125 prior to membrane isolation (*in vivo*), and by isolated renal brushborder vesicles where the enzyme was inhibited 98% by their incubation with AT-125 (*in vitro*).

Materials and Methods

Sprague-Dawley rats weighing 250 g were utilized as the source of renal brushborder vesicles which were prepared by the method of Booth and Kenny (1974). For *in vivo* inactivation of gamma-glutamyl transpeptidase, the rats were given intraperitoneally 5 mg of AT-125 (L-(αS, 5S)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid), a gift from Dr. L.J. Hanka of Upjohn, Kalamazoo, Mich. Membrane vesicles were prepared 1 hr after the intraperitoneal injection. For *in vitro* inactivation of the enzyme, the membranes prepared from normal rats were divided and a portion incubated for 20 min at 37°C with 0.25 mM AT-125 in buffer containing 0.25 M sucrose, 0.01 M triethanolamine hydrochloride, pH 7.6. Uptake studies using these treated membranes were compared to those obtained using another portion of the prepared membranes incubated in buffer alone. The uptake of radioactive amino acids, which were obtained from Amersham Co., was determined by rapid filtration procedures published previously for these substrates (McNamara et al., 1976; Hsu et al., 1980; McNamara, Pepe & Segal, 1981). Analyses of gamma-glutamyl transpeptidase and gamma-glutamyl hydrolase (E.C. 2.3.2.2) activities were performed as described by Glossman and Neville (1972).

Isolated renal cortical tubule fragments from adult male Sprague-Dawley rats given 5 mg AT-125 i.p. 1 hr prior to sacrifice were prepared by a modification (Foreman, Hwang & Segal, 1980) of the method of Burg and Orloff (1962). Cystine uptake studies with these tubules were performed as previously described (Foreman et al., 1980) and compared to similar studies using tubules from adult rats not treated with AT-125.

Results

Inactivation of Gamma-Glutamyl Transpeptidase by AT-125

The effect of concentration of AT-125 on brushborder membrane vesicle gamma-glutamyl transpepti-

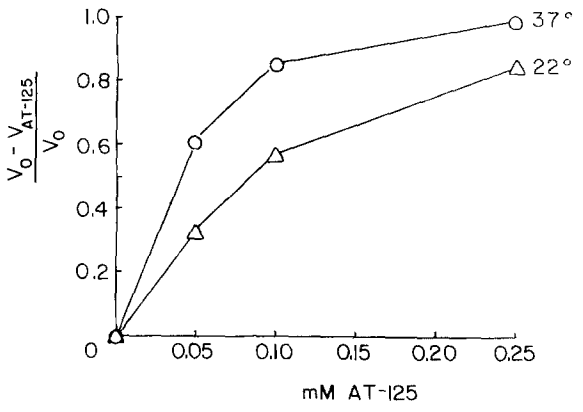

Fig. 1. Effect of AT-125 dose and temperature of incubation on gamma-glutamyl transpeptidase activity of rat renal brushborder vesicles. Brushborder vesicles were incubated at 37°C (○) and 22°C (△) for 30 min in the absence or presence of various concentrations of AT-125, after which an aliquot of the preparation was assayed for gamma-glutamyl transpeptidase activity with gamma glutamyl-*p*-nitroanilide as substrate. Activities were calculated in nmol of *p*-nitroaniline released per min per mg of brushborder protein. V_o represents activity measured in the absence of AT-125, $V_{AT\text{-}125}$ represents activity determined in the presence of AT-125 of amount designated. The ratio of $\frac{V_o - V_{AT\text{-}125}}{V_o}$ indicates the rate of inhibition. Each data point is the mean ± SEM for four determinations. The errors of all the data points are included within the size of each point.

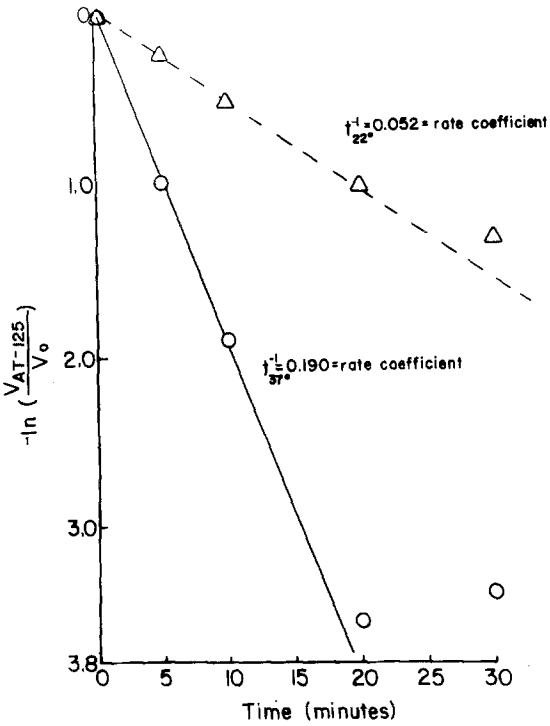

Fig. 2. Effect of incubation time and temperature upon the initial rate of inhibition of gamma glutamyl transpeptidase of rat renal brushborder vesicles in the presence of 0.25 mM AT-125. Brushborder vesicles was incubated at 37°C (○) and 22°C (△) for the designated time period in the absence or presence of 0.25 mM AT-125, after which an aliquot of the preparation was assayed for the gamma-glutamyl transpeptidase activity. For details of assay and calculation *see* legend of Fig. 1

dase incubated for 30 min at 22 and 37°C is shown in Fig. 1. At 37°C 98% inhibition of the enzyme occurred with 0.25 mM AT-125. The inactivation with the same inhibitor concentration was less at 22°C (84% inhibition). The inactivation with time of incubation in 0.25 mM AT-125 is shown in Fig. 2. The inactivation is logarithmic with a rate coefficient (t^{-1}) at 37°C equal to 0.190 and at 22°C, $t^{-1} = 0.052$. The inhibition at 37°C appeared to be essentially complete after 20 min. On the basis of these findings incubation of membranes to provide optimal inhibition of enzyme at 98% was performed routinely at 37°C for 20 min in 0.25 mM AT-125. The inhibition resulting from incubating the membranes under the above conditions was not reversed by repeated washing (Table 1), which is consistent with the known actions of AT-125 as an affinity site inhibitor. Thus, the activity of the enzyme during incubation of membranes for time-dependence determinations was unaltered.

For *in vivo* inactivation of gamma-glutamyl transpeptidase the rats were injected intraperitoneally with various amounts of AT-125 dissolved in 0.9% saline. The enzyme activity in kidney membranes isolated from kidneys removed 1 hr after injection of the drug was dose-dependent as shown in Table 2. Administration of 5 mg of AT-125 caused a 90% inhibition of the membrane gamma-glutamyl transpeptidase while causing no alteration in the activity of another brushborder marker enzyme, alkaline phosphatase.

Vesicle Response to Osmolarity Change

Rat renal brushborder vesicles prepared by the method of Booth and Kenny (1974) behave as osmometers with a linear decrease in uptake of most substrates with increasing osmolarity (McNamara et al., 1976; Hsu et al., 1980). It has been shown by McNamara et al. (1981) that total cystine uptake by renal brushborder membrane vesicles includes a significant capacity for vesicular binding of the substrate. In the present study the effect of osmolarity on cystine uptake by vesicles isolated from rats treated with AT-125 was compared to vesicles from control animals. The uptake of cystine was unchanged by inhibition of gamma-glutamyl transpeptidase at all osmolarities employed indicating that gamma-glutamyl transpeptidase inhibition had no effect on binding of cystine to the brushborder membrane.

Effect of Gamma-Glutamyl Transpeptidase Inhibition of Membrane Vesicle Amino Acid Uptake

The time-dependent uptake of glutamine, cystine and proline by vesicles whose gamma-glutamyl transpeptidase is inhibited is shown in Fig. 3. Glu-

Table 1. Reversibility of AT-125 effect upon membrane gamma-glutamyl transpeptidase (*in vitro*)

BK prep[a]	S.A. μmol PNA/mg/min	% of activity remaining	% inhibition
1. Control BK (37°C × 30 min)	17.15	100	0
2. BK + 0.25 mM AT-125 (37°C × 30 min)	0.241	1.41	98.59
3. Washed control	16.05	93.6	6.40
4. Washed AT-125-treated BK	0.108	0.63	99.37

[a] Data obtained were the results of two different membrane vesicle preparations. BK is brushborder membrane preparation isolated by the $MgCl_2$ method of Booth and Kenny.

Table 2. I.P. dose response of BK marker enzyme[a]

I.P. Dose (mg AT-125/250 g rat)	APase (% of control)				γ-GTPase (% of control)			
	Control BK	AT-125 BK	*t* value	% inhibition	Control BK	AT-125 BK	*t* value	% inhibition
2 mg	100 ± 22.30 (6)	81.75 ± 9.15(6)	$t = 0.7571(10)$ $P > 0.5$	18.25% (NS)	100 ± 0.15(6)	39.28 ± 5.54(6)	$t = 10.96(10)$ $P < 0.01$	60.72%
4 mg	ND	ND	—	ND	100	28.19	—	71.81%
5 mg	100 ± 12.55 (6)	98.97 ± 13.42	$t = 0.0561(10)$ $P > 0.9$	1.03% (NS)	100 ± 16.75 (5)	9.08 ± 1.39(5)	$t = 5.4095(8)$ $P < 0.001$	90.92%

[a] The number of determinations is indicated in the parentheses behind the mean value ± SE. *t* and *P* values indicate the difference between control and AT-125. Abbreviations: NS, not significant; ND, not determined.

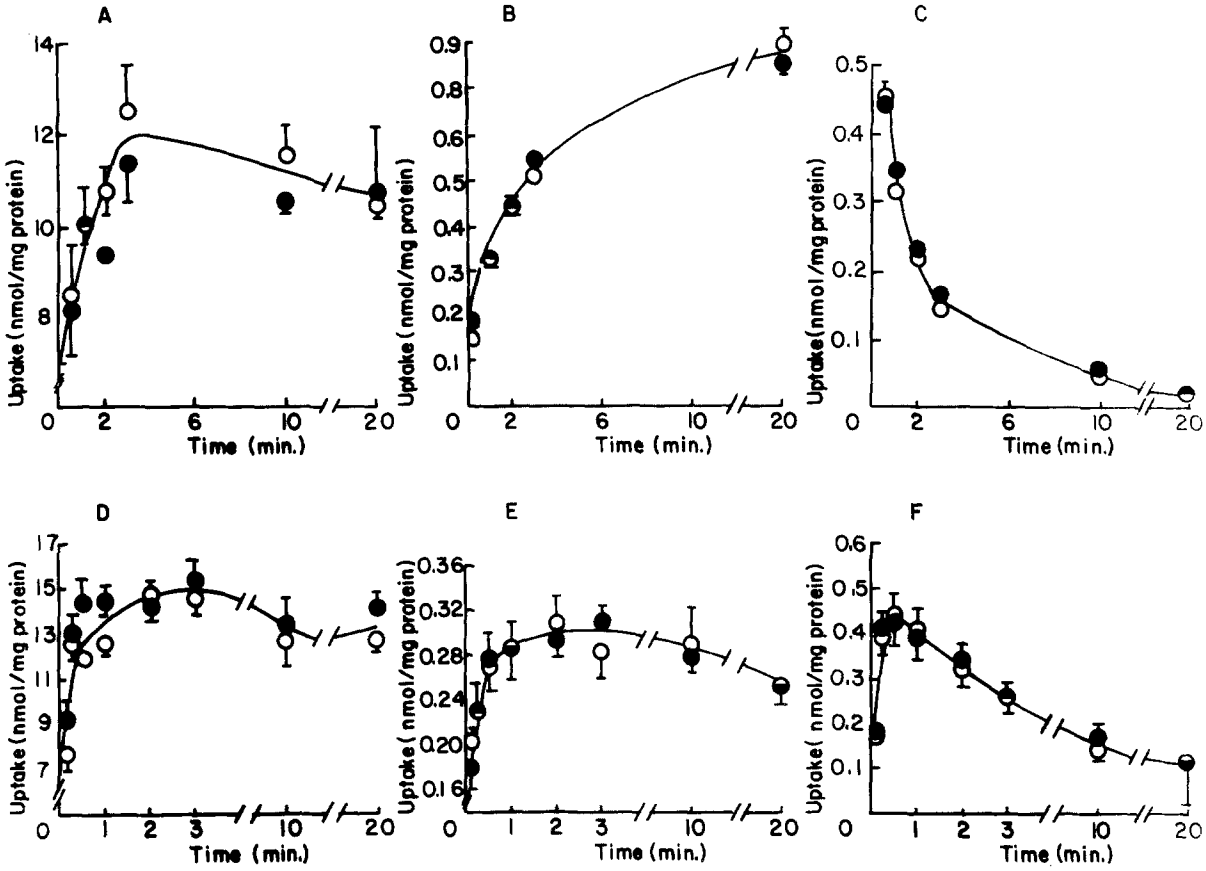

Fig. 3. *In vivo* and *in vitro* effect of AT-125 on the time course of uptake of glutamine, cystine and proline by control and AT-125-treated membrane vesicles. Brushborder vesicles were obtained as described in the text. The time course of uptake by membrane vesicles in THM buffer incubated with designated concentrations of L-[^{14}C]-glutamine, L-[^{14}C]-proline and L-[^{35}S]-cystine was measured under the conditions of 100 mM Na^+ gradient at 22°C. (*A*) represents the time course of 2 mM L[^{14}C]-glutamine uptake by control (○) and *in vivo* AT-125-treated (●) membrane vesicles. Values shown are the mean ± SEM for 8 determinations. (*B*) presents the time course of 0.02 mM L-[^{35}S]-cystine uptake by control (○) and *in vivo* AT-125-treated (●) membrane vesicles. Values shown are the mean ± SEM for 16 determinations. (*C*) shows the time course of 0.02 mM L-[^{14}C]-proline uptake by control (○) and *in vivo* AT-125-treated (●) membrane vesicles. Values shown are the mean ± SEM for 4 determinations. (*D*) represents the time course of 2 mM L-[^{14}C]-glutamine uptake by control (○) and *in vitro* AT-125-treated (●) membrane vesicles. Values shown are the means ± SEM for 12 to 15 determinations. (*E*) illustrates the time course of 0.02 mM L-[^{14}C]-glutamine uptake by control (○) and *in vitro* AT-125-treated (●) vesicles. Values shown are the mean ± SEM for 8 determinations. (*F*) represents the time course of 0.06 mM L-[^{14}C]-proline uptake by control (○) and *in vitro* AT-125-treated (●) vesicles. Values shown are the mean ± SEM for 12 determinations

tamine and cystine are excellent substrates for the transpeptidase while proline is believed not to interact with the enzyme (Karkowsky & Orlowski, 1978). Figures 3*A* and 3*D* reveal the vesicle uptake of 2 mM glutamine where the enzyme was inhibited *in vivo* and *in vitro*, respectively. In neither case was there a significant difference in glutamine uptake between the control and AT-125 exposed membranes. Since glutamine transport also occurs via a high-affinity system (Weiss et al., 1978), the uptake of 0.02 mM glutamine by vesicles treated with AT-125 *in vitro* was also studied. The results shown in Fig. 3*E* reveal an uptake curve unchanged from the normal. Cystine uptake by vesicles whose enzyme was inhibited *in vivo* (Fig. 3*B*) and proline uptake by vesicles whose enzyme was inhibited both *in vivo* and *in vitro* was unaltered (Fig. 3*C* and 3*F*). There was no decrease in the uptake of other amino acids, glycine, methionine, leucine and lysine with gamma-glutamyl transpeptidase inhibition as shown in Table 3.

Cystine Uptake by Isolated Tubule Fragments

Because membrane vesicles are devoid of glutathione and other components of the gamma-gluta-

Table 3. Rate of uptake (0.5 min) of 0.02 and 2.1 mM amino acid and sugar by AT-125-treated BK (*in vivo*, i.p. 5 mg/250 g rat)[a]

Substrate	Concentration (mM)	% of uptake		*t* value	*P*
		Control BK	AT-125 BK		
Glycine	0.02	100 ± 2.17(8)	108.45 ± 6.01(8)	1.322	>0.2 (NS)
	2.1	100 ± 3.64(8)	90.63 ± 5.18(8)	1.480	>0.1 (NS)
L-methionine	0.02	100 ± 4.52(7)	108.11 ± 3.60(7)	1.400	>0.1 (NS)
	2.1	100 ± 4.14(8)	103.55 ± 7.36(8)	0.420	>0.6 (NS)
L-leucine	0.02	100 ± 2.05(8)	111.10 ± 3.91(8)	2.52	<0.05
	2.1	100 ± 3.23(8)	109.81 ± 8.51(8)	1.08	>0.2 (NS)
L-lysine	0.02	100 ± 2.09(6)	102.05 ± 5.21(6)	0.365	>0.7 (NS)
	2.1	100 ± 4.84(5)	92.54 ± 5.04(7)	1.068	>0.3 (NS)
D-glucose	0.02	100 ± 2.45(8)	110.0 ± 4.40(7)	0.512	>0.6 (NS)
	2.1	100 ± 2.99(7)	97.35 ± 4.23(7)	0.512	>0.6 (NS)

[a] The number of determinations is indicated in the parentheses behind the mean value ± SE. *t* and *P* values indicate the difference between control and AT-125. Abbreviation: NS, not significant.

myl cycle, the uptake of cystine by tubule fragments containing intact renal cells was also assessed. Table 4 indicates that inhibition of the transpeptidase did not influence cystine uptake by the cortical cells which normally contain a gamma-glutamyl cycle.

Discussion

The renal brushborder membrane vesicle has been widely used as a model to characterize the transport systems of the proximal renal tubule for amino acids (Sacktor, 1982) as well as other substrates (Kinne, 1976; Cheng & Sacktor, 1981). From our own laboratory we have reported multiple systems for the uptake of glycine (McNamara et al., 1976), proline (McNamara et al., 1976), glutamine (Weiss et al., 1978), and cystine (Segal, McNamara & Pepe, 1977; McNamara et al., 1981). The transport properties found in membrane vesicles correspond closely to those found for isolated renal tubule fragments (Foreman et al., 1980; Hwang et al., 1983). The present studies have sought to answer the question whether uptake systems delineated in these preparations are related to the activity of gamma-glutamyl transpeptidase which is present in the brushborder membrane. Our data indicate that sodium-dependent uptake of several amino acids is unrelated to the functional capacity of this enzyme. Two substrate levels have been used in the vesicle preparations since most amino acids are transported by both high and low K_m systems (McNamara et al., 1976, 1981; Weiss et al., 1978) and uptake at both high and low concentrations was unaffected by gamma-glutamyl transpeptidase inhibition. One concentration of cystine was used but previous calculations indicate that both systems function about equally at the level employed (Foreman et al., 1980). Emphasis was placed on glutamine and cystine uptake, since both amino acids are excellent substrates for transpeptidase activity (Karkowsky & Orlowski, 1978), and Meister (1983) has postulated the possible role of the gamma-glutamyl pathway in their transport.

Though cognizant that the systems delineated for cystine uptake by rat brushborder vesicles and isolated renal tubules are similar (Foreman et al., 1980; McNamara et al., 1981), we also examined cystine uptake by the isolated tubules, a whole cell preparation where the entire pathway may be functional. Indeed, no effect of 90% transpeptidase inhibition was observed on cystine uptake by these tubules. Therefore in two model systems currently in vogue where assessment of renal amino acid transport mechanisms is directly determined, there is no evidence for gamma-glutamyl transpeptidase involvement. The lack of a role of the cycle in renal amino acid transport has also been reported during *in vivo* microperfusion experiments of rat renal tubules where glycine, glutamate and cystine absorption were uninfluenced by AT-125 treatment of the animal prior to study (Silbernagl, 1981).

McIntyre and Curthoys (1979, 1980) have summarized their data and that of others (Silbernagl & Volkl, 1978; Silbernagl et al., 1978) indicating that the role of the enzyme is the hydrolysis of glutathione in the renal tubule lumen and not transpeptidation. They point out (McIntyre & Curthoys, 1980) that transpeptidation, attachment of a gamma-glutamyl group to an amino acid, in the presence of amino acids is pH-dependent with little such activity of the enzyme at the pH of proximal tubule fluid which is 7.4 to 6.8. Hydrolysis of glutathione, how-

Table 4. Effect of AT-125 on tubular uptake of 0.025 mM L-cystine (*in vivo*, i.p. 5 mg/250 g rat)[a]

Time of incubation (min)	% of uptake mean ± SEM (no. of determinations)		*t* value	*P*
	Control	AT-125-treated		
1	100 ± 7.35(6)	92.96 ± 1.77(6)	0.934	>0.3(NS)
5	100 ± 1.92(6)	96.36 ± 2.76(6)	1.083	>0.3(NS)
15	100 ± 3.05(6)	95.24 ± 1.92(6)	1.322	>0.2(NS)

[a] *t* and *P* values indicate the difference between control and AT-125. Abbreviations: NS, not significant.

ever, is independent of pH between pH 6.0 and 8.5. It is also apparent that acceptor amino acid concentrations are low in the proximal tubule especially in the pars recta where gamma-glutamyl transpeptidase activity is maximal (Heinle, Wendel & Schmidt, 1977).

The clinical data in patients with defects in the gamma-glutamyl pathway do not indicate that aminoaciduria is a characteristic feature of these disorders (Larsson, 1981). Indeed, patients with transpeptidase deficiency have no generalized aminoaciduria and rats given AT-125 to inhibit the enzyme show only glutathionuria with increased excretion of gamma-glutamyl cystiene and cystiene-mixed disulfides (Griffith & Meister, 1979) as do affected patients. Such findings are consistent with our present observation.

The postulated involvement of gamma-glutamyl transpeptidase and the associated cycle has had an evolving formulation. As first stated (Meister, 1973), the enzyme oriented outward in the membrane performed the transfer of the gamma-glutamyl moiety of intracellular glutathione to amino acids in the tubule lumen and in the process acted as a translocater of the gamma-glutamyl amino acid into the cell where the free amino acid was subsequently liberated. The present formulation (Meister, 1983) appears to be that glutathione exits from the cell to the tubule lumen where the gamma-glutamyl portion is transferred to the receptor amino acids and the gamma-glutamyl amino acids are transported into the cell via a transport system for such compounds (Griffith, Bridges & Meister, 1979). McIntyre and Curthoys (1980) appear to negate the latter concept by citing evidence that gamma-glutamyl amino acids would be hydrolyzed in the tubule lumen since the conditions are optimal for gamma-glutamyl hydrolase activity of the transpeptidase enzyme.

The only apparent direct evidence for gamma-glutamyl transpeptidase acting as an amino acid transporter are those in which the purified hog renal enzyme was inserted into red cell membranes (Kalra et al., 1981) and artificial liposomes (Sikka & Kalra, 1980) and mediated the uptake of glutamic acid. Even here the evidence is not conclusive. In the proteoliposome, glutathione had to be present inside the vesicle to ensure accumulation of gamma-glutamyl glutamate. It may well be that the enzyme was oriented to the inside and that the amino acid diffused into the vesicle where the formation of glutamyl-glutamate occurred, trapping the labeled glutamate. This mechanism would not be considered carrier-mediated transport.

Meister (1981, 1983) has emphasized the importance of the glutamyl cycle in the renal transport of cystine and pointed out that multiple systems have evolved for amino acid transport by the kidney. Our vesicle data support the concept of at least two transport systems for cystine, one has a low affinity for cystine and another is shared with lysine, arginine and ornithine. A defect in this process would explain the amino acid excretion of these four amino acids in human cystinuria (Segal & Thier, 1983). If the gamma-glutamyl cycle and the transpeptidase-mediated formation of gamma-glutamyl cystine occurs in human cystinuric kidney, its extent is not great enough to alleviate the urinary hyperexcretion of cystine. Indeed, Griffith (1981) utilizes the function of the cycle to explain the amounts of cystine found in excess of the filtered load as being due to cystiene hydrolyzed from glutathione in the presence of a membrane-carrier defect.

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