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THE EFFECT OF AZASERINE UPON THE PROLINE AND METHYL α-D-GLUCOSIDE TRANSPORT SYSTEMS OF RAT RENAL BRUSH-BORDER MEMBRANES

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An inhibitory effect of azaserine on Na^+ dependent proline and methyl α-D-glucoside transport of the rat renal brush-border membrane vesicles has been demonstrated. The inhibitory effects of azaserine were not the results of the drug disrupting the membrane vesicles as shown in osmolarity studies, nor did it affect the transport systems' affinities for Na^+. Azaserine acts as a non-competitive inhibitor for the proline transport system in renal brush-border membranes by lowering 37% and 27% in the V_{max_1} and V_{max_2}, respectively, when compared to that of control proline transport system. Azaserine had no effect upon the two K_m values for proline uptake. Azaserine inhibition of methyl α-D-glucoside transport systems was characterized by a change in both K_{m_1} and V_{max_1}. Data indicate that methyl α-D-glucoside uptake by vesicles in the presence of 7.2 mM azaserine at 22°C resulted in 66% increase in K_{m_1} value and 44% decrease in V_{max_1} as compared to that of control vesicles. There was no detectable effect upon the K_{m_2} and V_{max_2} of the methyl α-D-glucoside transport system. No effect of the drug was observed when sodium was equilibrated across the membrane, indicating that azaserine altered the driving force exerted by a sodium gradient. Azaserine only slightly affected the relative contribution of the two K_m systems to total proline uptake. Contrary to the observed effect of azaserine upon the proline transport system, azaserine exerted a distinct effect upon the relative contribution to total uptake by the two K_m systems in the low methyl α-D-glucoside concentration range. In the presence of 7.2 mM azaserine, the low-affinity, high-K_m transport system becomes the major contributor to total methyl α-D-glucoside uptake by isolated renal brush-border vesicles.

Introduction

Azaserine (*O*-diazoacetyl-L-serine) has been shown to possess marked antineoplastic and antimicrobial activity when it was first discovered in cultured filtrates of *Streptomyces fragilis* [1,2]. Therapeutic potentiation with the combination of azaserine plus β-mercaptopurine has been reported in experimental systems and in children with acute leukemia [3,4]. The first investigations on the mode of action of azaserine showed that this antibiotic affects amino acid metabolism in both mammalian and microbial systems [5–7]. Jacques et al. [8] have shown that azaserine is actively transported in tumor cells by an amino acid transport system. Recently, we have reported that azaserine inhibits glutamine transport of rat renal brush-border membrane vesicles [9]. Such transport inhibitors have been found to be useful experimental tools for investigating mechanisms of membrane transport. For example, the hallucinogen harmaline and diuretic drug amiloride were used as probes to study the transport mechanism of D-glucose, L-alanine and Na^+ in rabbit renal brush-border membrane vesicles [10,11].

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Proline and methyl α-D-glucoside, a non-metabolizable model sugar, seem to be appropriate choices of substrates for examining the in vitro effect of the transport inhibitor, azaserine, on amino acid and sugar transport. Studies in Scriver's laboratory as well as our own have delineated the properties of proline uptake by rat renal cortical slices and isolated membrane vesicles [12–17]. Methyl α-D-glucoside appears to share many of the transport characteristics, in both rats and humans, of the dietary hexoses D-galactose and D-glucose, which competitively inhibit its uptake [26–28]. In this paper, we demonstrate an inhibitory effect of azaserine on Na^+-dependent proline and methyl α-D-glucoside transport by purified rat renal brush-border membrane vesicles. Although azaserine influences the kinetic parameters of each substrate differently, its effect is related to an alteration in the electrochemical potential difference of sodium across the membrane.

Experimental procedures

Materials. L-[U-^{14}C]Proline, L-[1-^{3}H(n)]glucose and 3-*O*-[*methyl*-^{3}H]methyl-D-glucose were purchased from New England Nuclear Corp. Methyl α-D-[U-^{14}C]glucoside was obtained from Rosechem Products. L-Azaserine (*O*-diazoacetyl-L-serine) and Hepes (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid) were purchased from Calbiochem. All other chemicals were of the highest purity available.

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 saline at 4°C. Rat cortical tissue was removed using a Stadie-Riggs microtome. (Two slices of 0.5 mm cortical tissue were used from each side of kidney.) Brush-border membrane was isolated using the method of Booth and Kenny [19]. Vesicles were prepared by resuspension of the isolated brush-border membranes in hypotonic 2 mM Tris-Hepes (pH 7.4)/100 mM mannitol (buffer A).

Incubation with azaserine. Protocols described by Hsu et al. [9] were utilized to determine the effects of azaserine on transport of proline and methyl α-D-glucoside. About 3.2 mg/ml of the isolated vesicles were diluted with 6 vol. buffer and equilibrated at 22°C for 30 min. Uptake of proline and methyl α-D-glucoside by these vesicles was determined in the absence and presence of 7.2 mM azaserine. In another group of studies, the same concentration of membrane vesicles was suspended in buffer alone or in buffer containing 50 mM azaserine, immediately diluted with 6 vol. buffer prewarmed to 22°C and incubated for 30 min at 22°C before uptake of the substrates were measured. In a parallel group of studies, membrane vesicles were incubated at 37°C with buffer alone or with buffer containing 50 mM azaserine for 30 min. At the end of incubation, the vesicles were diluted with 6 vol. buffer and equilibrated to 22°C before the measurement of uptake. The final azaserine concentration in the vesicle suspension was 7.2 mM. To study the residue effect of azaserine incubation, a washing procedure was carried out as follows: Vesicles that had been preincubated with 7.2 mM azaserine at 22°C or with 50 mM azaserine for 30 min at 37°C before dilution with 6 vol. buffer were centrifuged at $35000 \times g$ for 20 min at 4°C. The pellets were washed twice with 24 vol. buffer, after which the membranes were suspended in fresh buffer and equilibrated to 22°C for measurement of substrate uptake. The final vesicle protein concentration for substrate uptake studies was 0.4–0.5 mg/ml as determined by the method of Lowry et al. [20].

Uptake of substrates. The uptake of proline or methyl α-D-glucoside at 22°C was determined in the presence of a 100 mM inward sodium gradient or sodium equilibrated conditions by using filtration techniques as described by McNamara et al. [13]. Results of the substrate uptake studies are expressed as uptake in excess of 3-*O*-[*methyl*-^{3}H]methyl-D-glucose or L-[1-^{3}H(n)]glucose that are thought to enter the brush-border by diffusion [13]. In experiments to determine the effect of azaserine in the absence of a sodium gradient, vesicles were equilibrated for 30 min in 100 mM NaCl, followed by a subsequent incubation with or without 7.2 mM azaserine for an additional 30 min, after which substrate uptake was measured.

Data analysis. In studies of concentration dependence of uptake of the substrates by renal brush-border membrane vesicles, the data were plotted by the Hofstee method. Data were fitted

with the aid of a Monroe model 1775 calculator by the least-squares method. Apparent K_m and V_{max} values were determined from each experiment studied. The significance of the differences of uptake between control and azaserine-treated vesicles were analyzed by using the formula for Student's *t*-test given by Goldstein [21]. With the Hofstee parameters as initial estimates, the kinetic parameters were calculated from Eqn. 1 to give the best fit to the observed total uptake by non-linear regression analysis using a PDP-12 computer.

$$V_{total} = \frac{V_{max_1}[S]}{[S]+K_{m_1}} + \frac{V_{max_2}[S]}{[S]+K_{m_2}} \quad (1)$$

Results

Effect of osmolarity on azaserine-treated membrane vesicles.

Azaserine treatment at 37°C had no effect on the ability of brush-border membranes to form sealed vesicles as measured by the uptake of proline and 3-*O*-methyl-D-glucose. As shown in Fig. 1, uptake of 0.02 mM L-proline by control and azaserine-treated vesicles was found to be inversely proportional to medium osmolarity from 300 mosM to 548 mosM, and was thus directly related to intravesicular space. Extrapolation to infinite medium osmolarity (zero intravesicular volume) resulted in no uptake, suggesting the vesicles were osmotically active with no apparent binding component.

Effect of azaserine upon the time course of proline and methyl α-D-glucoside transport by the membrane vesicles under Na^+-gradient and Na^+-equilibrated conditions

The time course of uptake of 0.02 mM and 2.1 mM L-proline by brush-border membrane vesicles after pre-incubation with 50 mM azaserine in buffer A for 30 min at 37°C and subsequent dilution to a final concentration of 7.2 mM is shown in Fig. 2. Uptake of L-proline was measured with a 100 mM inward Na^+ gradient at 22°C after the preincubated vesicles had been diluted with buffer A. A typical 'overshoot' phenomenon of stimulated uptake was observed for both 0.02 mM and 2.1 mM L-proline. Azaserine lowered the uptake values of proline during the first 10 min of the time course studied and appeared to diminish the apparent initial rate at 3 s (Fig. 2A). For 0.02 mM proline (Fig. 2A), the maximal uptake at the end of 0.5 min incubation at 22°C by azaserine-treated vesicles was 40% of that of control membrane vesicles incubated without azaserine, whereas after 20 min of incubation, uptake was the same as that of the control ($P > 0.2$). For 2.1 mM proline (Fig. 2B), the maximal uptake at the end of 1 min incubation in the presence of azaserine was 74% of that in the absence of azaserine, with no difference in uptake between azaserine-treated vesicles and control vesicles at the end of 20 min incubation ($P > 0.1$). Also shown in Fig. 2A and B are the time curves of uptake of L-proline by brush-border vesicles equilibrated with 100 mM NaCl for 30 min prior to determination of the effect of the presence of 7.2 mM azaserine. There were no differences in the time curves of uptake of L-pro-

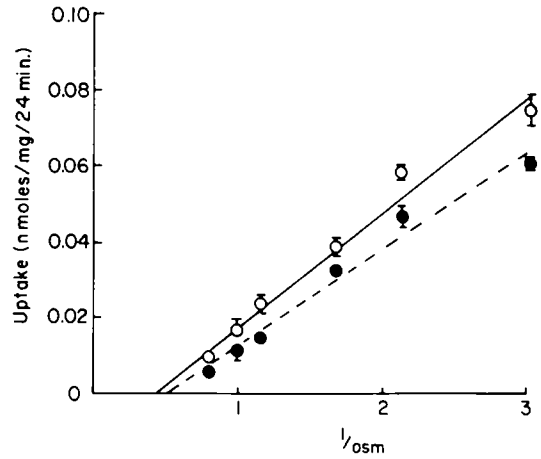

Fig. 1. Effect of osmolarity upon the uptake of 0.02 mM L-proline by brush-border membrane vesicles. Brush-border vesicles were pre-incubated at 37°C for 30 min in the absence (○) or presence (●) of 50 mM azaserine in buffer A. The pre-incubated vesicles were diluted resulting in a final azaserine concentration of 7.2 mM and equilibrated to 22°C as described in the text. The uptake was determined in the presence of 100 mM NaCl and increasing amounts of sucrose in the incubation medium. Values shown represent equilibrium values obtained after 24 min incubation at 22°C. No diffusion component was subtracted. Each data point represents the mean ± S.E. for four determinations. Data points without brackets indicate that the S.E. is included within the point. The lines were fitted to data points using linear regression analysis on a Monroe model 1775 computer.

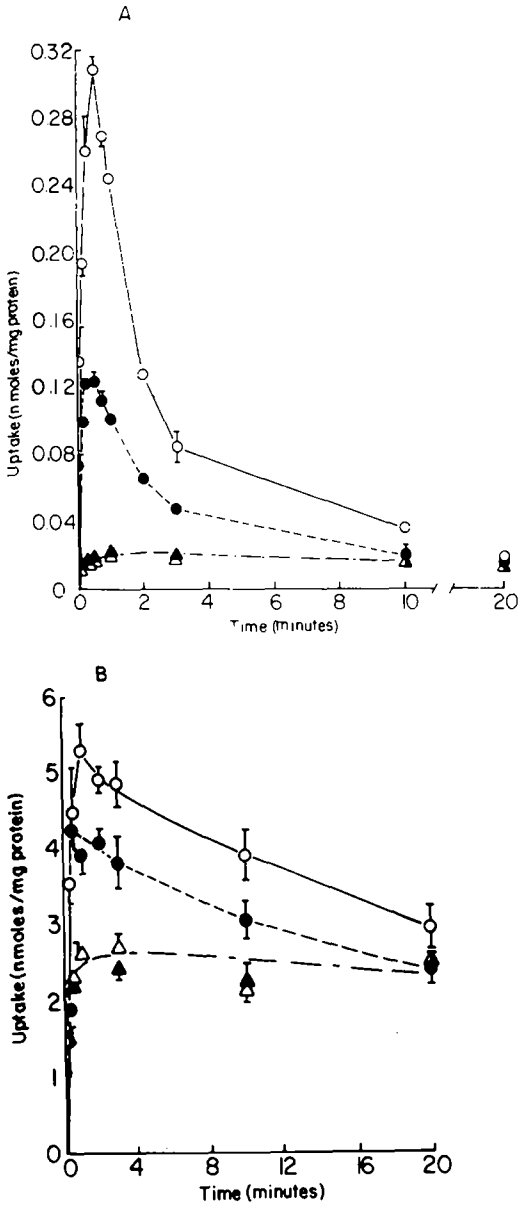

Fig. 2. Effect of azaserine upon the time course of uptake of proline under Na^+-gradient and Na^+-equilibrated conditions. Brush-border membrane vesicles were incubated at 37°C for 30 min in the absence (○) or presence (●) of 50 mM azaserine in buffer A. The vesicles were then diluted, as described in the text, resulting in a final azaserine concentration of 7.2 mM. Time course of uptake was measured under the condition of inward Na^+-gradient (100 mM) at 22°C as described in Experimental procedures. For time course of uptake in the absence of a sodium gradient, vesicles were equilibrated for 30 min in 100 mM NaCl at 22°C, followed by subsequent incubation without (△) or with (▲) 7.2 mM azaserine as described in the text before uptake of L-proline was measured. (A) shows the time course of uptake by membrane vesicles incubated with 0.02 mM L-[^{14}C]-proline. Values shown are the mean ± S.E. for six determinations under Na^+-gradient conditions, and four determinations under Na^+-equilibrated conditions. (B) illustrates the time course of uptake of 2.1 mM L-proline under conditions identical to (A). Values shown are the means ± S.E. for 14 determinations under Na^+-gradient conditions and four determinations under Na^+-equilibrated conditions. Data points without brackets indicate that the S.E. is included within the point.

line between the absence and presence of azaserine in the incubation media. Neither was the 'overshoot' phenomenon seen.

The time course of uptake of 0.02 mM and 2.1 mM methyl α-D-glucoside by brush-border vesicles after pre-incubation with 50 mM azaserine in buffer A for 30 min at 37°C is shown in Fig. 3. Similarly, under Na^+-gradient conditions, azaserine lowered the initial uptake values of methyl α-D-glucoside during the first 3 min of the time course studied. For 0.02 mM methyl α-D-glucoside (Fig. 3A), the maximal uptake at the end of 0.75 min for azaserine-treated vesicles was 49% of that of control vesicles incubated without azaserine. However, at the end of 10 min incubation, uptake of azaserine-treated vesicles was the same as that of control ($P > 0.1$). For 2.1 mM methyl α-D-glucoside (Fig. 3B), the maximal uptake at the end of 1 min incubation by azaserine-treated vesicles was 61% of that of control vesicles, whereas at the end of 10 min incubation there was no significant difference between the uptake of azaserine-treated vesicles and that of control vesicles ($P > 0.7$). Under Na^+-equilibrated conditions as shown in Fig. 3A and B, there was no difference in the time course of uptake of methyl α-D-glucoside between the absence and presence of azaserine in the incubation media.

Effect of azaserine on sodium dependence of proline and methyl α-D-glucoside

Sodium dependence of proline and methyl α-D-glucoside transport were examined in vesicles incubated for 30 min at 37°C in the absence and presence of azaserine. Fig. 4 shows the effect of stepwise replacement of Na^+ by choline chloride on the initial rate of uptake of 0.02 mM L-proline in the presence of a salt gradient. From the Lineweaver-Burk plots, the apparent K_m for Na^+ for

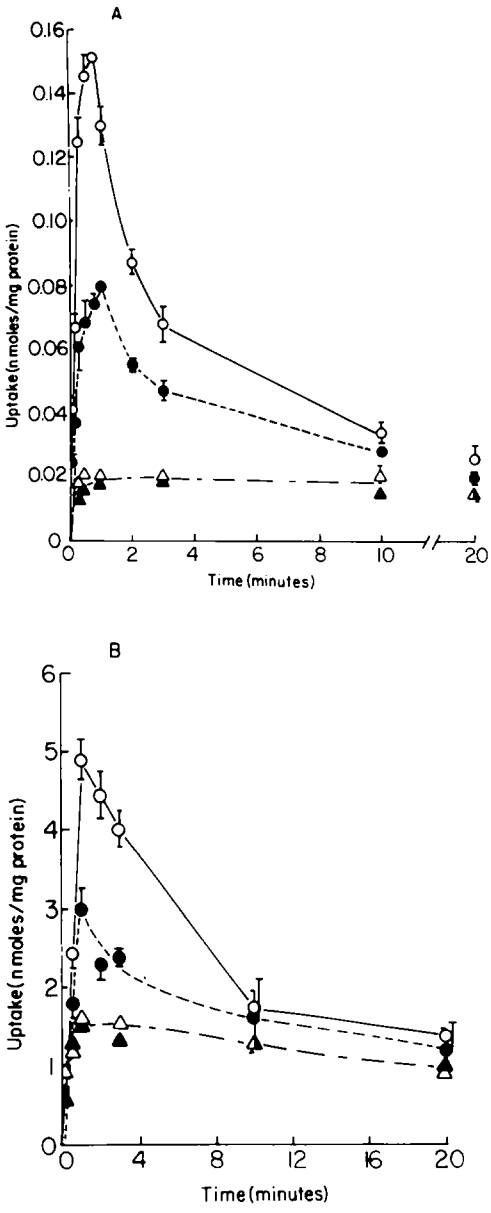

Fig. 3. Effect of azaserine upon the time course of uptake of methyl α-D-glucoside under Na^+-gradient and Na^+-equilibrated conditions. Brush-border vesicles were incubated without (○) or with (●) azaserine and time course of uptake were measured under the conditions of inward Na^+-gradient (100 mM) at 22°C as described in the legend for Fig. 2. Uptake of vesicles in the absence of a sodium gradient and in the absence (△) or presence (▲) of 7.2 mM azaserine were also measured as described in the legend for Fig. 2. (A) shows the time course of uptake of 0.02 mM ^{14}C-labeled methyl α-D-glucoside. Values shown are the means ± S.E. for six determinations under the Na^+-gradient conditions, and four determinations under Na^+-equilibrated conditions. (B) illustrates the time course of uptake of 2.1 mM ^{14}C-labeled methyl α-D-glucoside. Values shown are the means ± S.E. for eight determinations under the Na^+-gradient conditions and four determinations under Na^+-equilibrated conditions. Data points without brackets indicate that the S.E. is included within the point.

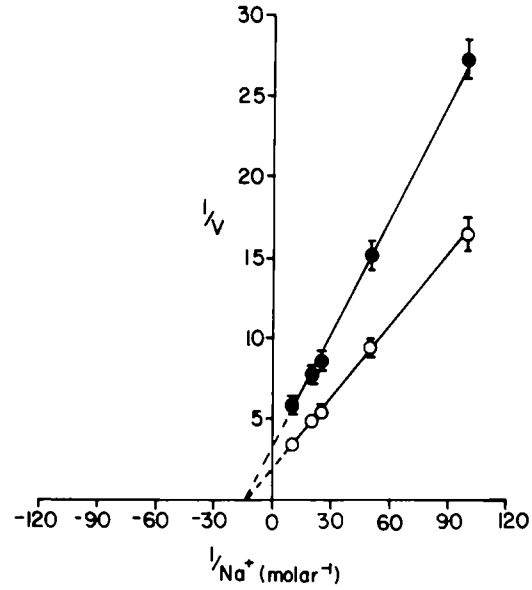

Fig. 4. Effect of Na^+ on initial rate of L-proline uptake at 22°C by brushborder membrane vesicles that had been pre-incubated at 37°C for 30 min in the absence (○) and presence (●) of 50 mM azaserine. The pre-incubated vesicles were diluted and equilibrated as described in the text. Uptake was assayed by the method described in the text under gradient conditions where 100 mM sodium was replaced stepwise by choline in the standard incubation medium. Effects of Na^+ on initial 15 s uptake of 0.02 mM L-proline were measured. Values shown are the means ± S.E. for 8–12 determinations. Data points without brackets indicate that the S.E. is included within the point.

the uptake of 0.02 mM L-proline (Fig. 4) was determined to be 71.2 mM for control vesicles. Table III shows that both low K_m and high K_m systems for proline uptake appear to have affinities for Na^+, and azaserine treatment did not alter the sodium affinity of the proline transport system in the vesicle membrane. For methyl α-D-glucoside transport by membrane vesicles, the apparent K_m for Na^+ was different for low and high concentrations of methyl α-D-glucoside uptake. Azaserine treatment also does not appear to alter the sodium affinity of the methyl α-D-glucoside transport system in the vesicle membrane.

Effect of azaserine on the kinetic parameters of proline and methyl α-D-glucoside uptake studies

McNamara et al. [13] have previously reported that the kinetics of proline transport by brush-border membrane vesicles indicate the presence of two saturable systems for uptake. As shown in Fig. 5A, the uptake of proline by brush-border membrane vesicles at 22°C in the absence and presence of 7.2 mM azaserine was concentration-dependent and saturable. The Hofstee plot for the concentration dependence of 15 s proline uptake shows a two-limbed curve that indicates the presence of multiple transport systems [22] for the vesicles in the absence of azaserine. The Hofstee plot for proline uptake by vesicles in the presence of 7.2 mM azaserine at 22°C (Fig 5A) indicated a decrease of 37% in V_{max_1} ($P < 0.01$) and a 27% decrease in V_{max_2} ($P < 0.05$) but no effect upon the two K_m values ($P > 0.1$ and $P > 0.5$ for K_{m_1} and K_{M_2}, respectively). The transport parameters determined from the Hofstee plots are presented in Table I. It should be emphasized that the difference in V_{total} for proline by control vesicles and vesicles in the presence of 7.2 mM azaserine at 22°C are statistically significant at all concentrations studied ($P < 0.001$ to $P < 0.01$ except for 1.77 mM proline where $P < 0.05$). Included in Table I 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 the final concentration of 7.2 mM azaserine. Pre-incubation of membrane vesicles at 37°C for 30 min in buffer alone slightly increased the V_{max_1} by only 21% ($P < 0.02$) as compared to the V_{max_1} of vesicles preincubated at 22°C. The values of V_{max_2} and the two K_m values were not affected. The Hofstee plot for proline uptake by vesicles pre-incubated with 50 mM azaserine at 37°C retained a two-limbed curve with lower intercept values at the ordinate than the control suggesting the inhibitory effect of azaserine upon the V_{max} of both proline transport systems. The same degree of inhibition of the proline transport systems was observed whether 7.2 mM azaserine was added directly, or vesicles were preincubated with 7.2 mM azaserine at 22°C for 30 min, or vesicles pre-incubated with 50 mM azaserine at 37°C and subsequently diluted to the final concentration of 7.2 mM azaserine. There were no statistical differences in the kinetic parameters due to the different treatments with azaserine ($P > 0.1$, > 0.6, > 0.05, and > 0.5 for K_{m_1}, V_{max_1}, K_{m_2} and V_{max_2}, respectively). Thus, temperature did not seem to affect the mode of action of azaserine upon the proline transport system in vesicles, while the prior incubation with azaserine at 37°C is a prerequisite to obtain any perceivable effect upon the activity of the membrane marker

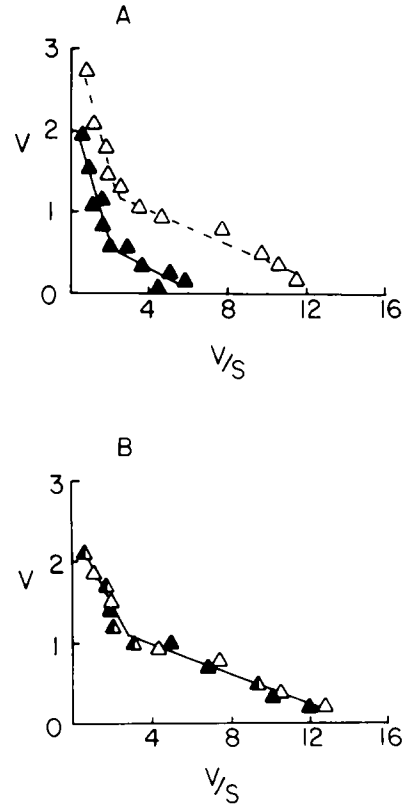

Fig. 5. Hofstee plots for the concentration-dependence of L-proline uptake by brush-border vesicles. Uptake after 15 s of incubation at 22°C with an 100 mM Na^+ inward gradient was measured over a range of 0.0184–3.913 mM L-proline. The velocity of uptake, V, was measured as nmol/mg protein per 15 s and S is the proline concentration in the incubation medium. The lines represent the best fit for the data by using linear regression analysis. (A) shows the uptake of L-proline in the absence (△) and presence (▲) of 7.2 mM azaserine. Values given are the means ± S.E. for four determinations. (B) illustrates the effect of azaserine removal upon the concentration-dependence of proline uptake by brushborder vesicles that were pre-incubated at 22°C in the absence (△) and presence (▲) of 7.2 mM azaserine before removal of the azaserine by repeated resuspension in buffer. Values given are the means ± S.E. for four determinations.

TABLE I

KINETIC PARAMETERS OF PROLINE UPTAKE

The number of determinations are indicated in the parentheses after the mean value ± S.E.

Incubation conditions	Azaserine (mM)	Hofstee		Calculated	
		K_m (mM)	V_{max} (nmol/mg per 15 s)	K_m	V_{max}
22°C	0	0.09 ± 0.01 (4)	1.43 ± 0.06 (4)	0.05	0.72
		0.64 ± 0.12 (4)	3.06 ± 0.26 (4)	1.80	3.0
37°C, 30 min	0	0.09 ± 0.01 (8)	1.73 ± 0.04 (8)	0.04	0.95
		0.60 ± 0.04 (8)	3.81 ± 0.13 (8)	1.70	3.80
22°C	0	0.08 ± 0.01 (4)	1.30 ± 0.01 (4)	0.05	0.85
	(washed)	0.44 ± 0.04 (4)	2.42 ± 0.14 (4)	1.60	1.90
37°C, 30 min	0	0.12 ± 0.02 (8)	2.06 ± 0.87 (8)	0.04	0.95
	(washed)	0.57 ± 0.24 (8)	4.20 ± 0.63 (8)	2.50	5.50
22°C	7.2	0.14 ± 0.03 (4)	0.90 ± 0.11 (4)	0.05	0.35
		0.78 ± 0.15 (4)	2.22 ± 0.22 (4)	1.70	2.35
37°C, 30 min	50 → 7.2	0.09 ± 0.01 (8)	0.98 ± 0.11 (8)	0.05	0.52
		0.49 ± 0.02 (8)	2.42 ± 0.22 (8)	1.70	2.95
22°C	7.2	0.10 ± 0.01 (4)	1.39 ± 0.10 (4)	0.05	0.85
	(washed)	0.32 ± 0.01 (4)	2.35 ± 0.13 (4)	1.60	1.90
37°C, 30 min	50 → 7.2	0.13 ± 0.02 (8)	1.78 ± 0.77 (8)	0.06	0.78
	(washed)	0.76 ± 0.23 (8)	4.14 ± 0.70 (8)	2.30	5.30

enzyme activity, γ-glutamyltranspeptidase [20,23], to which azaserine is bound.

To determine any residual transport inhibition by azaserine, experiments were performed in which membranes were washed to remove unbound azaserine. The Hofstee plot for concentration dependent proline uptake by vesicles, that had been pre-incubated with 7.2 mM azaserine at 22°C and then washed, was a two-limbed curve (Fig. 5B) with kinetic parameters similar to those of the control preparation (Table I). The washing procedure had no apparent effect for the two proline transport systems of the control vesicles without azaserine ($P > 0.2$, > 0.1, > 0.1 and > 0.05 for K_{m_1}, V_{max_1}, K_{m_2} and V_{max_2}, respectively) as compared to the vesicles without washings and in the absence of azaserine. After removal of the 7.2 mM azaserine by repeated resuspension of the vesicles, it is important to note that the V_{max} values for proline uptake were completely recovered. When vesicles that had been pre-incubated at 37°C for 30 min with 50 mM azaserine were washed to remove unbound azaserine, again there was the total recovery of two transport systems of proline uptake. This is evidenced by the superimposable, two-limbed Hofstee plots for initial uptake of proline by the vesicles (data not shown here).

To examine the effect of azaserine upon the substrate specificity of the membrane transport system, methyl α-D-glucoside was selected to represent the sugar family to compare with proline uptake which represents the amino acid transport. Our laboratory [18,23] has previously indicated the existence of sodium-dependent transport systems of methyl α-D-glucose in rat kidney cortex slices. As shown in Fig. 6A, the uptake of methyl α-D-glucoside by brush-border membrane vesicles at 22°C in the absence and presence of 7.2 mM azaserine was concentration-dependent and saturable. The Hofstee plot for the concentration dependence of 15 s methyl α-D-glucoside uptake shows a two-limbed curve that suggests the presence of multiple transport systems for the vesicles in the absence of 7.2 mM azaserine. The Hofstee plot for methyl α-D-glucoside uptake by vesicles in the presence of 7.2 mM azaserine at 22°C, as also shown in Fig. 6A, resulted in 66% increase K_{m_1} value ($P < 0.01$) and 44% decrease in V_{max_1} ($P <$

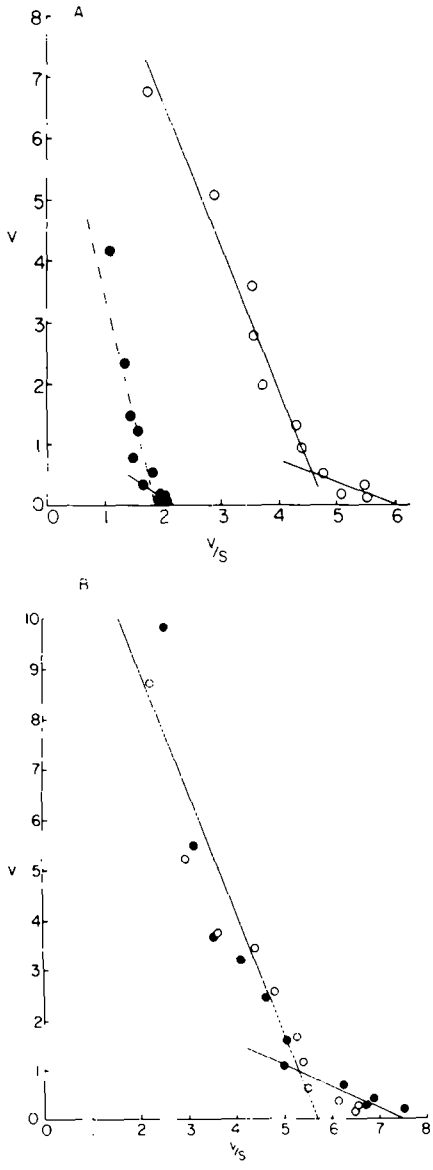

Fig. 6. Hofstee plots for the concentration dependence of methyl α-D-glucoside uptake by brush-border vesicles. Uptake after 15 s incubation at 22°C with an 100 mM Na^+ inward gradient was measured over a range of 0.0215–3.917 mM methyl α-D-glucoside. The lines represent the best fit for the data by using linear regression analysis. (A) shows the uptake of methyl α-D-glucoside in the absence (○) and presence (●) of 7.2 mM azaserine. Values given are the means ± S.E. for 4–7 determinations. (B) illustrates the effect of azaserine removal upon the concentration-dependence of methyl α-D-glucoside uptake by brush-border vesicles that were pre-incubated at 22°C in the absence (○) and presence (●) of 7.2 mM azaserine before removal of the azaserine by repeated resuspension in buffer. Values given are the means ± S.E. for 8–12 determinations.

0.05) as compared to that of control vesicles. However, azaserine had no significant effect upon the K_{m_2} ($P > 0.1$) and V_{max_2} ($P > 0.2$) of the methyl α-D-glucoside transport system. Though data points seem to fit a single line, it was not a best fit. This could not be done either by the least-squares analysis or by the non-linear regression analysis. The transport parameters determined from the Hofstee plots are presented in Table II. The calculated and best fit kinetic parameters are also included in the table as described in detail under the proline transport studies. To determine any residual effect of azaserine, membranes were washed to remove unbound azaserine and those results are shown in Fig. 6B. The washing procedure had no apparent effect upon the two methyl α-D-glucoside transport systems in the control vesicles without azaserine ($P > 0.9$, > 0.8, > 0.2 and > 0.5 for K_{m_1}, V_{max_1}, K_{M_2} and V_{max_2}, respectively) as compared to vesicles without washings and in the absence of azaserine. After removal of the 7.2 mM azaserine by repeated resuspension of the vesicles, there was recovery of the two transport systems of methyl α-D-glucoside uptake. This is evidenced by kinetic parameters presented in Table II and the superimposed, two-limbed Hofstee plots for the initial uptake of methyl α-D-glucoside by the vesicles.

Effect of azaserine upon the relative contribution to total uptake by the low K_m and high K_m systems of proline and methyl α-D-glucoside

By using observed kinetic parameters determined from the Hofstee plots as an initial estimate, the kinetic parameters for the two component systems for either proline or methyl α-D-glucoside were calculated by non-linear regression analysis as previously described [9]. The relative contribution of each system was measured as nmol substrate per mg vesicle protein per 15 s, then calculated and expressed as percentage of the total uptakes at the designated substrate concentrations for proline (Fig. 7) and methyl α-D-glucoside (Fig. 8). Results indicate that pre-incubation with 50 mM azaserine only slightly affected the contribution of each system to proline uptake. Contrary to the observed effect of azaserine upon the proline transport systems, azaserine exerted a distinct effect upon the relative contribution to total uptake by the two K_m systems in the low methyl

TABLE II

KINETIC PARAMETERS OF METHYL α-D-GLUCOSIDE UPTAKE

The number of determinations are indicated in parentheses after the mean value ± S.E. In each case, incubation temperature was 22°C.

Azaserine (mM)	Hofstee		Calculated	
	K_m (mM)	V_{max} (nmol/mg per 15 s)	K_m	V_{max}
0	0.38 ± 0.05 (7)	2.42 ± 0.46 (7)	0.35	1.28
	2.30 ± 0.20 (7)	11.06 ± 1.89 (7)	5.19	13.15
0 (washed)	0.37 ± 0.15 (8)	2.70 ± 1.17 (8)	0.39	1.55
	2.04 ± 0.10 (12)	12.20 ± 0.3 (12)	5.20	16.70
7.2	0.62 ± 0.06 (4)	1.36 ± 0.05 (4)	0.65	0.15
	4.17 ± 1.10 (4)	7.69 ± 1.65 (4)	5.85	9.75
7.2 (washed)	0.38 ± 0.02 (12)	2.98 ± 0.52 (12)	0.39	1.25
	2.45 ± 0.31 (12)	13.44 ± 1.80 (12)	5.50	19.80

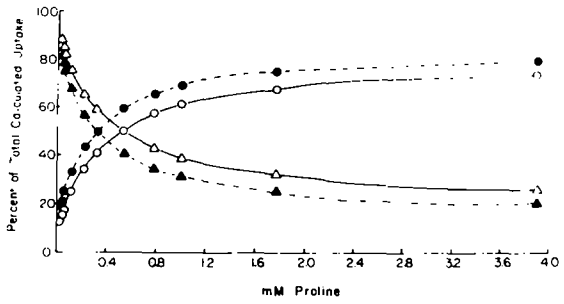

Fig. 7. The relative contribution to total uptake by the proline transport system of brush-border membrane vesicles. Membrane vesicles were preincubated at 37°C for 30 min in the presence (▲,●) and absence (△,○) of 50 mM azaserine, then diluted and equilibrated as described in the text. By using observed kinetic parameters determined from the Hofstee plots as the initial estimates, the kinetic parameters for the low-K_m (△,▲) and high-K_m (○,●) systems for proline uptake were calculated by PDP-12 computer. The relative contribution of each system was determined from Eqn. 1 in the text. Values shown are means ± S.E. for eight determinations.

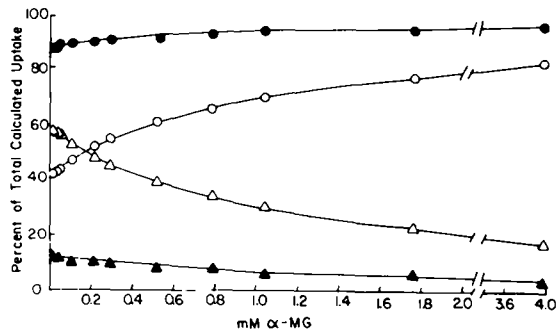

Fig. 8. The relative contribution to total uptake by the methyl α-D-glucoside transport system of brush-border membrane vesicles. The low-K_m (△,▲) and high-K_m (○,●) systems were calculated for methyl α-D-glucoside uptake by brush-border membrane vesicles in the presence (▲,●) and absence (△,○) of 7.2 mM azaserine without preincubation. Data points were obtained as described in the legend of Figure 7. Values shown are means ± S.E. for 4–7 determinations.

α-D-glucoside concentration range. Data show the contribution of low-K_m system to total uptake of 0.02 mM methyl α-D-glucoside by vesicles in the presence of 7.2 mM azaserine was decreased from 58% to 12%, while the contribution of high-K_m system to total methyl α-D-glucoside uptake at the same media concentration was doubled from 42% to 88%. The contribution of the low K_m system to total uptake of 3.9 mM methyl α-D-glucoside in the presence of azaserine was only 3% as compared to 17% in the absence of azaserine. It seems that, in the presence of 7.2 mM azaserine, the low-affinity, high-K_m transport system becomes the major contributor to total methyl α-D-glucoside uptake by isolated renal brush-border vesicles.

Discussion

In this paper, we demonstrate an effect of azaserine on brush-border membrane transport processes in the absence of any disruptive effect on membrane vesicles. Specifically, azaserine in-

hibited the Na^+-dependent transport for L-proline and methyl α-D-glucoside. Azaserine inhibition of Na^+-dependent transport of both proline and methyl α-D-glucoside was reversible and not enhanced by pre-incubation of the membrane with azaserine. Azaserine clearly acts as a non-competitive inhibitor for the proline transport system in renal brushborder membranes by lowering the V_{max_1} and V_{max_2}. However, azaserine inhibition of methyl α-D-glucoside transport systems was characterized by a change in both K_{m_1} and V_{max_1}. In the presence of azaserine, the calculated value of K_{m_1} for methyl α-D-glucoside was increased by 86% while the calculated V_{max_1} was decreased by 88%. The K_{m_2} and V_{max_2} of the methyl α-D-glucoside transport system were not affected in the presence of the azaserine. Our earlier findings indicated that azaserine is a competitive inhibitor of a high-affinity glutamine uptake system without affecting the low-affinity component [9]. From the diversified kinetic effects of azaserine on sugar and amino acid uptake, it appears that there is a substrate specificity in the response of transport systems to the chemical and indicates the independence of these transport mechanisms.

Despite the varied kinetic effects under sodium gradient conditions there is no influence of azaserine on proline and methyl α-D-glucoside uptake under sodium equilibration. This implies that the transport systems are not affected directly and that the effect observed is due to an alteration of the driving force of the electrochemical difference of sodium across the membrane. The underlying mechanism of the effect is unknown. It is not due to a change in affinity of the transport systems for sodium (Table III). The striking thing is that, as a result of the alteration of the driving force of the electrochemical difference of Na^+ across the membrane created by the presence of azaserine, the Na^+ related transport systems of several substrates appear to have rather specific changes in their kinetic parameters. It may be that the azaserine interferes with sodium gradient formation or membrane potential which affects the configuration of various membrane proteins involved in transport in different ways.

Since azaserine has unique properties as a reversible transport inhibitor as well as inhibitor of membrane peptidase [3,24] it is a useful chemical

TABLE III

EFFECT OF AZASERINE ON THE APPARENT K_m FOR Na^+ OF L-PROLINE AND METHYL α-D-GLUCOSIDE UPTAKE IN THE PRESENCE OF Na^+ GRADIENT

Membrane substrate concentration	Absence (−) or presence (+) of azaserine	K_m for Na^+ (mM)
0.02 mM proline	−	71.2
	+	74.2
2.1 mM proline	−	8.4
	+	8.7
0.02 mM methyl α-D-glucoside	−	28.2
	+	30.5
2.1 mM methyl α-D-glucoside	−	15.5
	+	14.7

to probe and elucidate the mechanisms of underlying Na^+-dependent transport in renal brush border membrane vesicles and its relationship with the membrane peptidase activity. Kenny et al. [25] have found that the dipeptidyl peptidase IV released dipeptides of the form X-Pro-Y or X-Ala-Y from its substrate and postulated the enzyme could be connected with the transport of these amino acids. γ-Glutamyltranspeptidase has also been postulated to be involved in amino acid transport [26]. However, glutamine uptake was independent of the inhibition of γ-glutamyltranspeptidase activity by azaserine [9]. Hydrolysis of glutamine to glutamate was completely inhibited by preincubation with and subsequent removal of azaserine as determined by paper chromatography, yet glutamine uptake by these vesicles was unaffected. Only in the presence of azaserine is Na^+-stimulated transport of amino acids as well as sugar inhibited.

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