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A study of the substrate specificity of Na^+-dependent and Na^+-independent neutral amino acid transport systems in dog intestinal brush-border membrane vesicles using L-alanine analogues

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Abstract

Neutral amino acids are mainly transported across the intestinal brush-border membrane by two Na^+-dependent systems (system B^0 and system $B^{0,+}$) and one Na^+-independent system (system $b^{0,+}$). To investigate potential differences in substrate specificity between these systems, we screened ten different alanine analogues for their ability to inhibit the transport of L-alanine in dog intestinal brush-border membrane vesicles. The results suggested that a phenyl group directly attached to the α-carbon has different effects on the Na^+-dependent and Na^+-independent transport systems, with an increased affinity for the former and a decreased affinity for the latter. Based on these inhibition studies, we investigated [^{14}C]L-phenylglycine transport kinetics in comparison with L-alanine. Similar to L-alanine, L-phenylglycine transport followed at least three routes, however, the K_m of the Na^+-dependent transport system was lower and the K'_m of the Na^+-independent system was higher than the corresponding values for L-alanine. These results corroborated the conclusions drawn from the inhibition studies. Based on these data, we conclude that different sizes of immediate parts to the α-carbon in functional groups of amino acid analogues have differential effects on the interaction of these amino acid analogues with the Na^+-dependent and Na^+-independent transport systems for neutral amino acids.

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Introduction

Some amino acid analogues that have pharmacological effects, such as L-α-methyldopa and N^G-nitro-L-arginine, are known to be transported across intestinal epithelial cells by neutral amino acid transport systems (Osiecka et al 1987; Hatanaka et al 1999a). Neutral amino acids are mainly transported across the intestinal brush-border membrane by three systems: system B^0 (Na^+-dependent system for neutral amino acids), system $B^{0,+}$ (Na^+-dependent system for neutral and basic amino acids) and system $b^{0,+}$ (Na^+-independent system for neutral and basic amino acids) (Ganapathy et al 1996). In a previous study, we found that N^G-nitro-L-arginine uptake into intestinal brush-border membrane vesicles (BBMVs) was inhibited not only by neutral amino acids, but also basic amino acids, in the presence and absence of Na^+(Hatanaka et al 1999a). The results suggested that N^G-nitro-L-arginine is also transported by these three amino acid transport systems across the intestinal brush-border membrane similar to neutral amino acids, although it is not an essential amino acid. In that study, we also reported that even

N^G-nitro-L-arginine methyl ester, the carboxyl methyl ester of N^G-nitro-L-arginine, is transported both by the Na^+-dependent and Na^+-independent amino acid transporters. Thus, it is not clear whether these transport systems can be differentiated in terms of their interaction with neutral amino acids. Therefore, as a first step to investigate this issue, we carried out inhibition screening, using dog intestinal BBMVs, for L-alanine transport with various L-alanine analogues, because L-alanine has a simple chemical structure and is the most-studied neutral amino acid involved in membrane transport.

Materials and Methods

Chemicals

L-[^{3}H]-Alanine (sp. act. 1.92 TBq mmol^{-1}) and L-[^{14}C]-phenylglycine (2.04 GBq mmol^{-1}) were purchased from Amersham (Little Chalfont, Bucks, UK) and Moravek Biochemicals (Brea, CA, USA), respectively. All other chemicals were at least of analytical grade and were obtained from Aldrich (Milwaukee, WI, USA) and Tokyo Kasei Kogyo Co., Ltd (Tokyo, Japan). 4-(1-Methyl-1-aminoethyl)benzoate was synthesized from methyl 4-(1-methyl-1-nitroethyl)benzoate by reduction of the nitro group and hydrolysis of the carboxymethyl group.

Preparation of BBMVs

The studies were approved by the Animal Welfare Ethics Committee of Chugai Pharmaceutical Co., Ltd. BBMVs were isolated from the small intestine of dogs by the calcium precipitation method as described previously (Hatanaka et al 1999a). BBMVs were suspended in suspension buffer (100 mM mannitol and 10 mM Hepes, pH 7.5 adjusted with KOH), to a concentration of approximately 15 (mg protein) mL^{-1}. Samples of the final suspension were stored at −80°C until use.

Transport studies

Transport studies were performed using a rapid filtration technique, as described previously (Hatanaka et al 1999a). Transport was initiated by adding 100 μL of the incubation buffer (100 mM mannitol, 10 mM Hepes and 100 mM NaCl or KCl, pH 7.5 adjusted with KOH) containing labelled (0.2 μCi/100 μL for L-phenylglycine and 1 μCi/100 μL for L-alanine) and unlabelled substrates at various concentrations to 10 μL of the BBMV suspension after pre-incubation for 2 min at 37°C for the L-phenylglycine kinetic study and 25°C for the L-alanine inhibition study. At the stated times, the transport was stopped by adding 1 mL of the ice-cold stop solution (150 mM NaCl and 20 mM Hepes/Tris pH 7.5). The BBMVs were collected on a Millipore HAWP membrane filter and kept under suction while being washed with 5 mL of the ice-cold stop solution. The *cis*-inhibitory effects on L-alanine uptake (0.5 mM, 15 s, 25°C) were examined by the addition of various compounds in the incubation buffer at concentrations of 5, 10 or 20 mM, in accordance with our previous study (Hatanaka et al 1999a).

Calculation of kinetic parameters of L-phenylglycine uptake

The kinetic parameters of L-phenylglycine initial uptake were calculated using the following equations, as described previously (Hatanaka et al 1999a):

$$V_1 = (V_{max}[S]/(K_m+[S]))+(V'_{max}[S]/(K'_m+[S]))+k_d[S]$$
$$V_2 = (V'_{max}[S]/(K'_m+[S]))+k_d[S]$$

where V_1 is the total velocity with the Na^+ gradient, V_2 is the total velocity with the K^+ gradient, [S] is the concentration of L-phenylglycine, V_{max} and K_m are the maximal velocity and Michaelis constant of the Na^+-dependent carrier-mediated transport, respectively, V'_{max} and K'_m are the maximal velocity and Michaelis constant of Na^+-independent carrier-mediated transport, respectively, and k_d is the coefficient of non-saturable transport.

Statistical analysis

The significance of differences was determined using Student's *t*-test. A value of $P < 0.05$ was considered statistically significant.

Results

Screening for substrate specificity of Na^+-dependent and Na^+-independent neutral amino acid transport systems in dog intestinal BBMVs

We screened ten different L-alanine analogues (Figure 1) for inhibition of L-alanine (0.5 mM) transport across dog intestinal BBMVs to determine whether Na^+-dependent and Na^+-independent neutral amino acid transport systems have different substrate specificity

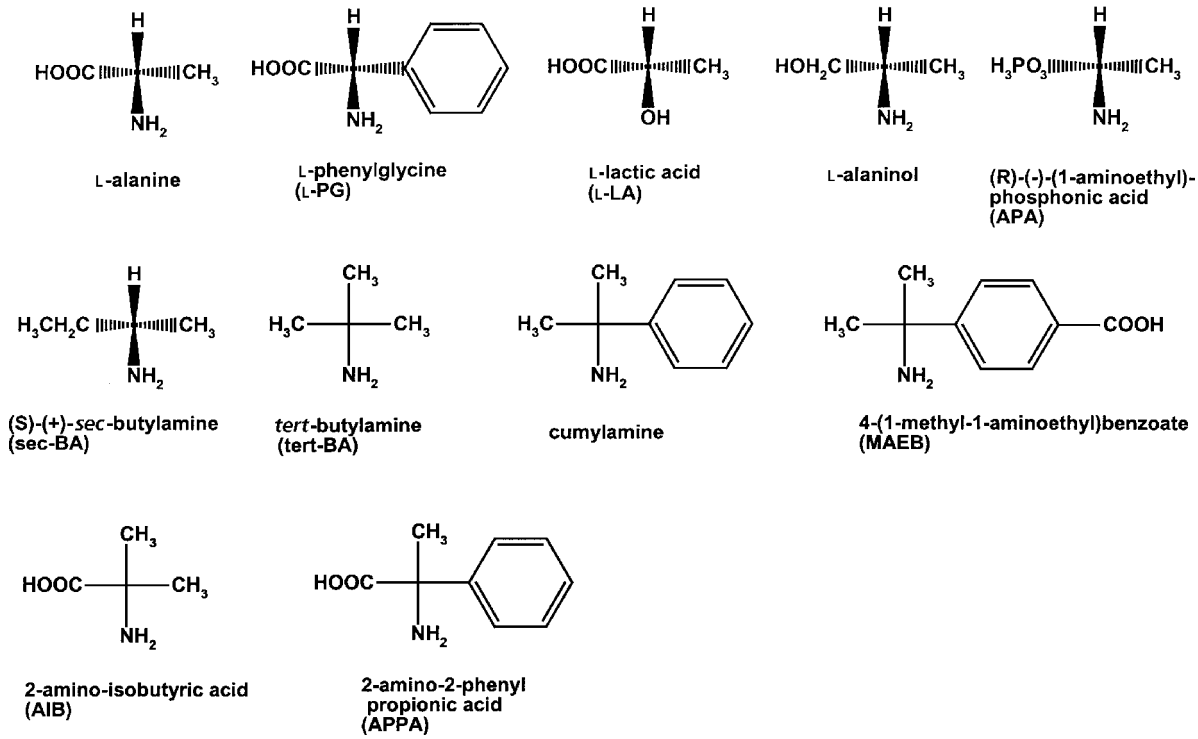

Figure 1 Chemical structures of L-alanine analogues.

(Figure 2).

L-Phenylglycine, cumylamine and 2-amino-isobutyric acid inhibited only Na^+-dependent L-alanine uptake, whereas *tert*-butylamine inhibited both Na^+-dependent and Na^+-independent uptake in a dose-dependent manner, with complete inhibition at 20 mM. *S*-(+)-*sec*-Butylamine and L-alaninol had no effect. *R*-(-)-(1-Aminoethyl)-phosphonic acid, 4-(1-methyl-1-aminoethyl)benzoate and 2-amino-2-phenyl propionic acid stimulated the uptake of L-alanine. L-Lactic acid also stimulated the uptake, but only at low concentrations. At higher concentrations, L-lactic acid inhibited the uptake. The reasons for the stimulatory effect of these compounds are not clear.

L-Phenylglycine uptake across dog intestinal BBMVs

The uptake values for L-phenylglycine (0.5 mM) at 15 s and 5 min incubation with Na^+ gradient were 3.49 and 1.05 nmol (mg protein)$^{-1}$, respectively; the corresponding values with K^+ gradient were 1.15 and 0.81 nmol (mg protein)$^{-1}$, respectively. These results indicate an overshoot phenomenon only with Na^+ gradient, which we also observed for L-alanine (Hatanaka et al 1999b) and N^G-nitro-L-arginine (Hatanaka et al 1999a) in previous studies under similar conditions.

The initial uptake (15 s) of L-phenylglycine was measured at various concentrations, ranging from 0.333 to 10 mM, with inwardly directed Na^+ or K^+ gradients (Figure 3). The uptake was saturable under both gradients, indicating that L-phenylglycine is transported across the intestinal brush-border membrane by both Na^+-dependent and Na^+-independent carrier-mediated pathways, similar to L-alanine transport.

We determined the kinetic parameters for L-phenylglycine uptake, resolving the total uptake into three components similar to our previous studies (Hatanaka et al 1999a, b). The V_{max}, K_m, V'_{max}, K'_m and k_d for the L-phenylglycine initial uptake were 9.66 ± 1.24 nmol (mg protein)$^{-1}$/15 s, 2.63 ± 0.86 mM, 5.17 ± 1.93 nmol (mg protein)$^{-1}$/15 s, 1.66 ± 1.05 mM and 0.452 ± 0.145 μL (mg protein)$^{-1}$/15 s, respectively.

Discussion

We investigated the substrate specificity of neutral amino acid transport systems in the intestinal brush-border membrane by screening ten different L-alanine analogues for their ability to inhibit Na^+-dependent or

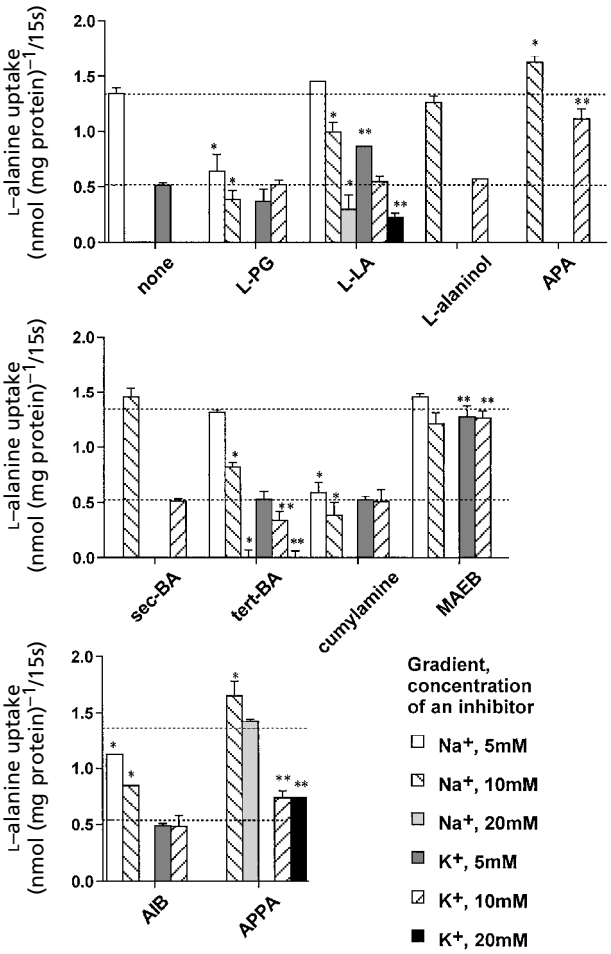

Figure 2 *cis*-Inhibitory effects of various L-alanine analogues on L-alanine uptake by dog intestinal BBMVs. Each value represents the mean±s.e. of three to four measurements (12 measurements for the control group). The column without a bar and an error bar shows "not tested" at the concentration of the inhibitor. The two horizontal dotted lines indicate the control values (no inhibitor) with Na^+ gradient (upper) and K^+ gradient (lower). Values with $*P < 0.05$ and $**P < 0.05$ indicate significant differences compared with the control values in the presence and the absence of the Na^+ gradient, respectively. The abbreviations for the inhibitors are shown in Figure 1.

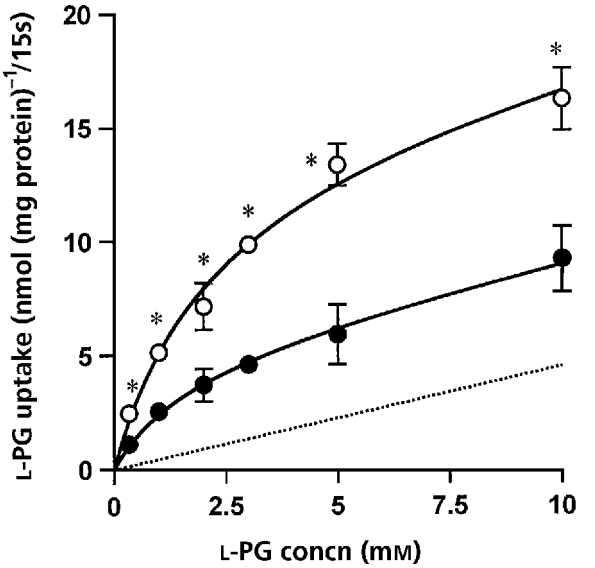

Figure 3 Concentration dependence of the initial uptake rate (15 s) of L-phenylglycine (L-PG) by dog intestinal BBMVs in the presence (○) or absence (●) of the Na^+ gradient, (……) diffusion. The concentration–initial uptake rate curves and the lines expressing simple diffusion were obtained by non-linear regression. Each value in the presence of the Na^+ gradient with $*P < 0.05$ is significantly different compared with the corresponding value in the absence of the Na^+ gradient. Each point represents the mean±s.e. of three to six measurements.

Na^+-independent L-alanine uptake. In this study, we used dog intestinal BBMVs because they have more distinct system $B^{0,+}$ activity than those of other species (Hatanaka et al 1999a, b).

Among the L-alanine analogues used in this study, L-lactic acid and *tert*-butylamine showed dose-dependent inhibition of both Na^+-dependent and Na^+-independent L-alanine uptake. L-Lactic acid is known to be transported by the monocarboxylate transport system (Takanaga et al 1995). However, it also affected the Na^+-dependent and Na^+-independent amino acid transport systems. This interaction was unexpected because L-lactic acid is an anion and does not have an amino group. The reasons for the interaction of this anion with the transport systems for neutral amino acids are not known. *tert*-Butylamine at a concentration of 20 mM completely inhibited L-alanine uptake via Na^+-dependent and Na^+-independent systems, whereas *S*-(+)-*sec*-butylamine had no effect at 10 mM, despite being an isomer of *tert*-butylamine.

Cumylamine and L-phenylglycine showed specific inhibition only on Na^+-dependent uptake. The structural difference between *tert*-butylamine and cumylamine, and between L-alanine and L-phenylglycine, is that the side chain is either a methyl or a phenyl group. From these results, we conclude that a phenyl group directly attached to α-carbon causes differential effects on the interactions with Na^+-dependent and Na^+-independent neutral amino acid transport systems.

L-phenylglycine was expected to be transported by amino acid transporters, because it is an α-amino acid, whereas cumylamine was expected only to inhibit amino acid transporters. We investigated L-phenylglycine transport directly to confirm whether the results of the inhibition studies reflected differences in transport kin-

Table 1 Comparison of kinetic parameters of neutral amino acid transport in intestinal brush-border membrane vesicles.

	L-Phenylglycine	L-Alanine[a]	L-Alanine[b]	L-Phenylalanine[b]
K_m (mM)	2.63 (0.86)	10.3 (4.0)	9	8.8
K'_m (mM)	1.66 (1.05)	0.14 (0.4)	0.32	0.23
V_{max} (nmol (mg protein)$^{-1}$/15 s)	9.66 (1.24)	16.7 (4.1)	1.50	9.33
V'_{max} (nmol (mg protein)$^{-1}$/15 s)	5.17 (1.93)	0.76 (0.45)	0.44	1.02
k_d (μL (mg protein)$^{-1}$/15 s)	0.45 (0.15)	0.63 (0.07)	0.29	0.36
K_m/K'_m	1.6	72.4	28.1	38.3

Values in the parentheses are standard deviations in non-linear least square regression analysis. [a]Data from Hatanaka et al 1999b. [b]Data from Stevens et al 1982 using rabbit jejunal BBMVs.

etic parameters between L-alanine and L-phenylglycine for transport via Na^+-dependent and Na^+-independent systems (Figure 3). The kinetic parameters showed that L-phenylglycine transport follows at least three routes, similar to L-alanine (Hatanaka et al 1999b) and L-phenylalanine (Stevens et al 1982). However, the K_m of the Na^+-dependent transport system was lower, and the K'_m of the Na^+-independent system was higher than the corresponding results for L-alanine in dog intestinal BBMVs (Hatanaka et al 1999b) (Table 1). These differences in the kinetic parameters of L-alanine and L-phenylglycine are supported by the results of the inhibition study of L-alanine uptake by L-phenylglycine, indicating that the inhibition study with BBMVs is useful as the rapid and simple screening for selectivity of substrates transported by several transporters.

To compare the selectivity of neutral amino acids for interaction with Na^+-dependent and Na^+-independent transport systems, we calculated K_m/K'_m ratios (Table 1). Generally, Na^+-dependent transport is of low affinity, whereas Na^+-independent transport is of high affinity. However, both transport systems for L-phenylglycine have the same order of K_m and K'_m values, because the substitution of the methyl group in L-alanine with a phenyl group increased the affinity for the Na^+-dependent system and at the same time decreased the affinity for the Na^+-independent system. As a result, the K_m/K'_m ratio of L-phenylglycine was much lower than the corresponding values for L-alanine and L-phenylalanine in rabbit jejunal BBMVs (Stevens et al 1982), and dog intestinal BBMVs (Hatanaka et al 1999b). These results indicate that the direct attachment of bulky groups, such as the phenyl group, to α-carbon causes drastic differences in selectivity between the Na^+-dependent and Na^+-independent transport systems.

This structure–transport relationship, however, does not seem to be applicable to α-methyl amino acids. 2-Amino-isobutyric acid also inhibited only the Na^+-dependent L-alanine uptake, but not completely even at 10 mM, whereas 2-amino-2-phenyl propionic acid showed comparatively less affinity for both Na^+-dependent and Na^+-independent transport systems. Attachment of two methyl groups to α-carbon (2-amino-isobutyric acid) also causes changes in the selectivity for Na^+-dependent and Na^+-independent transport systems to some extent. However, the methyl and phenyl groups directly attached to α-carbon (2-amino-2-phenyl propionic acid) may be too large to allow the molecule to fit into the binding pockets of neutral amino acid transport systems. There seems to be a threshold for L-phenylglycine and 2-amino-2-phenyl propionic acid in their affinity for the Na^+-dependent transport system.

Thus, the bulkiness and lipophilicity of immediate parts to α-carbon in the functional groups is likely to be one of the important factors that define the affinity of an amino acid to the binding pocket of the amino acid transport systems. The order of bulkiness and lipophilicity among the amino acids used in this study is as follows: L-alanine $<$ 2-amino-isobutyric acid $<$ L-phenylglycine $<$ 2-amino-2-phenyl propionic acid. Among them, 2-amino-isobutyric acid and L-phenylglycine showed selectivity for Na^+-dependent amino acid transport, but the latter had a much stronger effect. This is in accord with the order of bulkiness and lipophilicity of immediate parts to α-carbon in the functional groups. In contrast, the selectivity for Na^+-independent transport was less in those amino acids than in L-alanine. These results suggest that different sizes of immediate parts to the α-carbon in functional groups of amino acid analogues have differential effects on the interaction of these amino acid analogues with the Na^+-dependent and Na^+-independent transport systems for neutral amino acids. However, the effect of 2-amino-2-phenyl propionic acid on the Na^+-dependent

system does not follow this conclusion. The reason for this may be that 2-amino-2-phenyl propionic acid is too large to allow the molecule to fit into the binding pockets of neutral amino acid transport systems. That is, the optimal size of immediate parts to α-carbon in the functional groups of neutral amino acids to have an increased affinity to the Na^+-dependent transport system is that of the phenyl group as in L-phenylglycine. On the other, that optimal size for the Na^+-independent transport system is that of the methyl group as in L-alanine.

Conclusions

We found that direct attachment of a phenyl group to α-carbon causes different effects on the Na^+-dependent and Na^+-independent neutral amino acid transport systems in terms of affinity. The phenyl group increases substrate affinity for the Na^+-dependent system and decreases substrate affinity for the Na^+-independent system. It is suggested that different sizes of immediate parts to the α-carbon in functional groups of amino acid analogues have differential effects on the interaction of these amino acid analogues with the Na^+-dependent and Na^+-independent transport systems for neutral amino acids. We also showed that the inhibition study with BBMVs is useful as a rapid and simple screening for the selectivity of substrates transported by transporters with different ion dependencies.

References

Ganapathy, V., Brandsch, M., Leibach F. H. (1996) Intestinal transport of amino acids and peptides. In: Johnson, L. R. (ed.) *Physiology of the gastrointestinal tract*, 4th edn. Raven Press, New York, pp. 1773–1794

Hatanaka, T., Nabuchi, Y., Ushio, H. (1999a) Transport of N^G-nitro-L-arginine across intestinal brush border membranes by Na^+-dependent and Na^+-independent amino acid transporters. *Pharm. Res.* **16**: 1770–1774

Hatanaka, T., Nabuchi, Y., Ushio, H. (1999b) Na^+-Dependent and Na^+-independent transport of L-arginine and L-alanine across dog intestinal brush border membrane vesicles. *Comp. Biochem. Physiol. B* **123**: 105–113

Osiecka, L., Cortese, M., Porter, P. A., Borchardt, R. T., Fix, J. A., Gardner, C. R. (1987) Intestinal absorption of α-methyldopa: in vitro mechanistic studies in rat small intestinal segments. *J. Pharmacol. Exp. Ther.* **242**: 443–449

Stevens, B. R., Ross, H. J., Wright, E. M. (1982) Multiple transport pathways for neutral amino acids in rabbit jejunal brush border vesicles. *J. Membr. Biol.* **66**: 213–225

Takanaga, H., Tamai, I., Inaba, S., Sai, Y., Higashida, H., Yamamoto, H., Tsuji. A. (1995) cDNA cloning and functional characterization of rat intestinal monocarboxylate transporter. *Biochem. Biophys. Res. Commun.* **217**: 370–377