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Mammalian Amino Acid Transport System y^+ Revisited: Specificity and Cation Dependence of the Interaction with Neutral Amino Acids

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Abstract. A reevaluation of the specificity of system y^+, the classical transporter for cationic amino acids is presented. System y^+ has been defined as a transporter for cationic amino acids that binds neutral amino acids with lower affinity in the presence of Na^+. The discovery of other transporters for cationic amino has suggested that some properties, originally attributed to system y^+, may relate to other transport systems. Uncertainty concerns mainly, the affinity for neutral amino acids and the cation dependence of this interaction. Neutral amino acids (13 analogues tested) were found to bind to system y^+ in human erythrocytes with very low affinity. Inhibition constants (K_{iy}, mM) ranged between 14.2 mM and >400 mM, and the strength of interaction was similar in the presence of Na^+, K^+ or Li^+ (145 mM). In choline medium, no interaction was detected up to 20 mM of the neutral amino acid. Guanidinium ion (5 mM, osmolarity maintained with choline) potentiated neutral amino acid binding; the effect was most important in the case of L-norvaline which aligned with guanidinium ion is equivalent to arginine. This suggests cooperative interaction at the substrate site. The specificity of system y^+ was shown to be clearly distinct from that of system y^+L, a cationic amino acid transporter that accepts neutral amino acids with high affinity in the presence of Na^+ and which influenced the classical definition of system y^+.

Introduction

The definition of system y^+ as a Na^+-independent transporter for cationic amino acids, which interacts less strongly with neutral amino acids (but only in the presence of Na^+), originated from the early work of Christensen and coworkers in Ehrlich cells, reticulocytes and fibroblasts (Christensen, 1964; Christensen & Antonioli, 1969; Thomas, Shao & Christensen, 1971; White, Gazzola & Christensen, 1982). Afterwards, it became the paradigm for cationic amino acid transport and was extended to nearly all other cells and tissues analyzed (reviewed in Christensen 1984, 1990; White, 1985; MacLeod & Kakuda, 1996; Closs, 1996).

In spite of this, evidence has been obtained suggesting that the classical definition of system y^+ may not reflect the properties of a single transporter. Thus, other transporters for cationic amino acids have been identified (systems $B^{0,+}$, $b^{0,+}$ and y^+L) in cells that were previously thought to posses only system y^+ activity (reviewed in Devés & Boyd, 1998). These are broad-scope transporters, which accept cationic and neutral amino acids as substrates, but differ in their cation (Na^+) dependence (Van Winkle, Christensen & Campione, 1985; Van Winkle, Campione & Gorman, 1988; Devés, Chávez & Boyd, 1992). Additionally, several members of a family of widely distributed cationic amino acid transporters (CAT) have been cloned (reviewed in Closs, 1996; MacLeod, 1996) which present some, but not all, the features ascribed to system y^+. In particular, the CAT transporters exhibit a much weaker interaction with neutral amino acids (Kim et al., 1991; Wang et al., 1991; Kakuda et al., 1993) than expected from a number of functional studies (White, 1985).

The proposal that the concept of system y^+ requires revision is further supported by a reexamination of the classical papers. We have noticed differences in the kinetic properties of the basic amino acid transporters described in Ehrlich cells and rabbit reticulocytes, (the two original experimental models for y^+ activity) which are incompatible with the idea of a single pathway. The transporter in Ehrlich cells (Christensen 1964; Christensen, Handlogten & Thomas, 1969) interacts more strongly with neutral amino acids than its counterpart in reticulocytes (Christensen & Antonioli, 1969) and the

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cation dependence of the two transporters is qualitatively different (Thomas, Shao & Christensen, 1971). In addition, whereas in Ehrlich cells the interaction is truly Na^+-dependent (that is, the affinity for neutral amino acids is reduced when Na^+ is replaced with K^+), in reticulocytes, the binding strength of the neutral analogues is similar in the presence of Na^+ or K^+. Neither transporter binds neutral amino acids effectively in the presence of choline.

The uncertainties in the properties of system y^+ reside principally in the interaction with neutral amino acids. Resolving this issue is important for two principal reasons. First, because the various transporters for cationic amino acids (systems $B^{0,+}$, $b^{0,+}$ and y^+L) differ mainly in the way they handle neutral amino acids and therefore this information is essential for the functional discrimination of these activities. Second, because the presumed interaction of a cationic amino acid transporter with neutral substrates is mechanistically interesting, considering that most amino acid transporters discriminate strongly on the basis of charge.

Here we have carried out a systematic study of the interaction of system y^+ with neutral amino acids using human erythrocytes as a model system. Our aim was to define the specificity and cation dependence of the interaction. Ad-hoc experimental strategies were used to separate the contribution of system y^+ from that of system y^+L a high affinity and low capacity system that transports cationic and neutral amino acids with equivalent affinity (Devés et al., 1992). The specificity of system y^+L has been characterized in detail (Angelo, Irarrázabal & Devés, 1996; Devés, Angelo & Rojas, 1998).

Materials and Methods

Chemicals

Uniformly labeled L[^{14}C]lysine was purchased from Amersham or Sigma (9–12 GBq $mmol^{-1}$), unlabeled amino acids and N-ethylmaleimide (NEM) from Sigma, and dibutylphthalate from Merck. All other chemicals were of commercial reagent grade.

Preparation of Cells

Human blood was obtained fresh from donors, using heparin as an anticoagulant. The cells were spun (1,400 × *g*, 5 min) and the plasma, buffy coat and upper layer of cells removed by aspiration. The red cells were washed four times with 5 mM sodium phosphate buffer (pH 6.8) containing 140 mM NaCl and 4 mM KCl and incubated to reduce endogenous amino acids (2.5% hematocrit, 0.02% chloramphenicol, 17 hr at 25°C). After this period, the cells were concentrated by centrifugation and packed (1,400 × *g*, 15 min) in the same solution.

Measurement of Entry Rates

Entry was followed as previously described (Devés et al., 1992). Packed cells were added at time zero to a solution of L-[^{14}C]lysine (10 μM) in isotonic saline (10% hematocrit, 37°C). The composition of the external medium (pH 6.8) varied depending on the experiment: "Na^+ medium", 5 mM Na^+ phosphate and 145 mM NaCl; "K^+ medium", 5 mM K^+ phosphate and 145 mM KCl; "Li^+ medium", 5 mM K^+ phosphate and 145 mM LiCl; "choline medium", 5 mM K^+ phosphate and 145 mM choline chloride; "guanidinium medium", 5 mM Na^+ phosphate, 5 mM guanidinium chloride and 140 mM choline chloride.

Three samples of the suspension were withdrawn at intervals (up to approximately 4 min) and placed in tubes containing dibutylphthalate. After centrifugation (45 sec, 11,000 × *g*) the cells sedimented below the organic layer. The aqueous supernatant was taken off by aspiration and the walls of the tubes were thoroughly washed to eliminate contaminating radioactivity. Dibutylphthalate was removed and the cells were precipitated by addition of 5% trichloroacetic acid. The suspension was centrifuged, and the radioactivity in the supernatant determined by scintillation counting.

All determinations were performed in duplicate runs and the rate of transport (± SE) was estimated from linear regression analysis of six time points.

Treatment with NEM

Washed erythrocytes (hematocrit 2.5%) were incubated with NEM at 25°C for 15 min in 5 mM sodium phosphate buffer (pH 6.8) plus 145 mM NaCl and 4 mM KCl. The reaction was terminated by addition of 2-mercaptoethanol (10 mM final concentration). The cells were then washed, packed in the corresponding assay buffer as indicated for each experiment, and tested for transport (Devés, Angelo & Chávez, 1993).

Analysis

Two types of protocols were used to estimate the interaction of neutral amino acids with system y^+. In the case of analogues that bind to system y^+L with high affinity, the effect of increasing concentrations of the unlabeled neutral amino acids on the initial rate of lysine entry was studied directly. The inhibition constant was calculated from the relationship between the relative rates of L-[^{14}C] lysine entry in the presence and absence of inhibitor and the inhibitor concentration (model for two transport systems). In the case of analogues that bind to system y^+L with low affinity, the activity of system y^+L was selectively inhibited with a given concentration of unlabeled leucine (1–5 mM, depending on the conditions) and the concentration of the amino acid understudy was varied. Relative rates were calculated using as reference the rate in the presence of leucine (model for one transport system).

Model for Two Transport Systems

Assuming a Michaelis-Menten type of interaction and following the same procedure that was explained previously (Devés et al., 1992), the relative rate of transport of a substrate (S) in the presence (v) and in the absence (v_o) of an unlabeled inhibitor (I) can be written as:

$$\frac{v}{v_o} = \frac{F/(1 + [I]/K_{iyL(ap)}) + 1/(1 + [I]/K_{iy(ap)})}{F + 1} \quad (1)$$

Constant F is the ratio between the rate of transport via system y^+L and the rate of transport via system y^+, in the absence of inhibitor, and is given by the following expression:

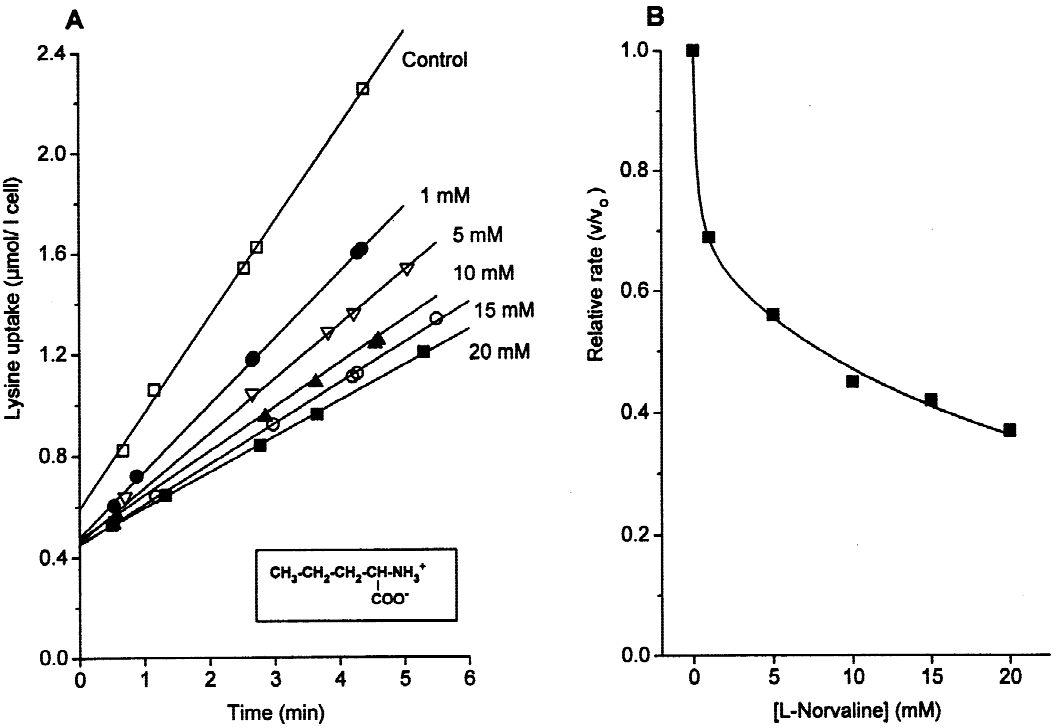

Fig. 1. Effect of L-norvaline on the rate of L-lysine entry into human erythrocytes. (*A*) Uptake of L-[^{14}C]lysine (10 μM) in the absence (□) and presence of increasing concentrations of nonradioactive L-norvaline on the *cis*-side: 1 mM (●), 5 mM (▽), 10 mM (▲), 15 mM (○), 20 mM (■) in "Na^+ medium." The rate of entry in the absence of radioactive analogue (v_o) was 0.38 ± 0.01 μmol/l cells/min. (*B*) Relative rates of entry (v/v_o) are plotted as a function of the concentration of unlabeled norvaline in the external medium. The curve was fitted to the data by nonlinear regression on the basis of Eq. 1 ($K_{iyL\text{-}NVAL(ap)}$ = 206 μM). The parameters obtained were F = 0.53 ± 0.035 and $K_{iy\text{-}NVAL}$ = 24.7 ± 2.12 mM.

$$F = \frac{V_{maxyL}\,(K_{my} + [S])}{V_{maxy}\,(K_{myL} + [S])} \tag{2}$$

where K_{my} and K_{myL} are the substrate half-saturation constants for systems y^+ and y^+L, respectively and V_{maxy} and V_{maxyL} the corresponding maximum velocities. The other two constants in equation 1, $K_{iy(ap)}$ and $K_{iyL(ap)}$, are the apparent inhibition constants for the interaction of the unlabeled analogue with system y^+ and y^+L, respectively. The full expressions for the apparent inhibition constants are as follows:

$$K_{iy(ap)} = K_{iy}\,(1 + [S]/K_{my}) \tag{3}$$

$$K_{iyL(ap)} = K_{iyL}\,(1 + [S]/K_{myL}) \tag{4}$$

K_{iy} and K_{iyL} represent the inhibition constants measured at low substrate concentration. Since the experiments were performed at a lysine concentration of 10 μM and the half-saturation constants for lysine are 112 μM (K_{my}) and 10 μM (K_{myL}) (Devés et al., 1993) we assumed that in the case of system y^+, $K_{iy(ap)} \approx K_{iy}$ (Eq. 3) and in the case of system y^+L, $K_{iyL(ap)} \approx 2\,K_{iyL}$ (Eq. 4). The apparent inhibition constants for system y^+L were calculated from previously published values of K_{iyL} (Angelo et al., 1996) and introduced in equation 1. K_{iy} and F were obtained by curve fitting.

Model for One Transport System

The activity of system y^+L was inhibited using excess leucine and the concentration of the unlabeled analogue (I) was varied. The relative rate, was calculated with reference to the rate measured in the presence of leucine (v_o). Data were analyzed according to the following equation:

$$v/v_o = 1/(1 + [I]/K_{iy}) \tag{5}$$

Results

The affinity of system y^+ for various amino acid analogues was determined by measuring the effect of external unlabeled analogues on the initial rate of entry of L-[^{14}C]lysine into human erythrocytes (*cis*-inhibition). Experimental protocols were developed to distinguish the contribution of system y^+ from that of system y^+L, a broad-scope transporter that also takes up lysine into human erythrocytes (Devés et al., 1992). An example of the type of experiment used for amino acids that are known to bind to system y^+L with high affinity is shown in Fig. 1. The uptake of L-[^{14}C]lysine (10 μM) was followed in the absence or presence of varying concentrations of unlabeled L-norvaline in the external medium (panel *A*) and the rates of entry (± SE) were calculated from the slopes of the progression curves. The relationship between the relative influx (in the presence or absence of analogue) and the concentration of L-norvaline

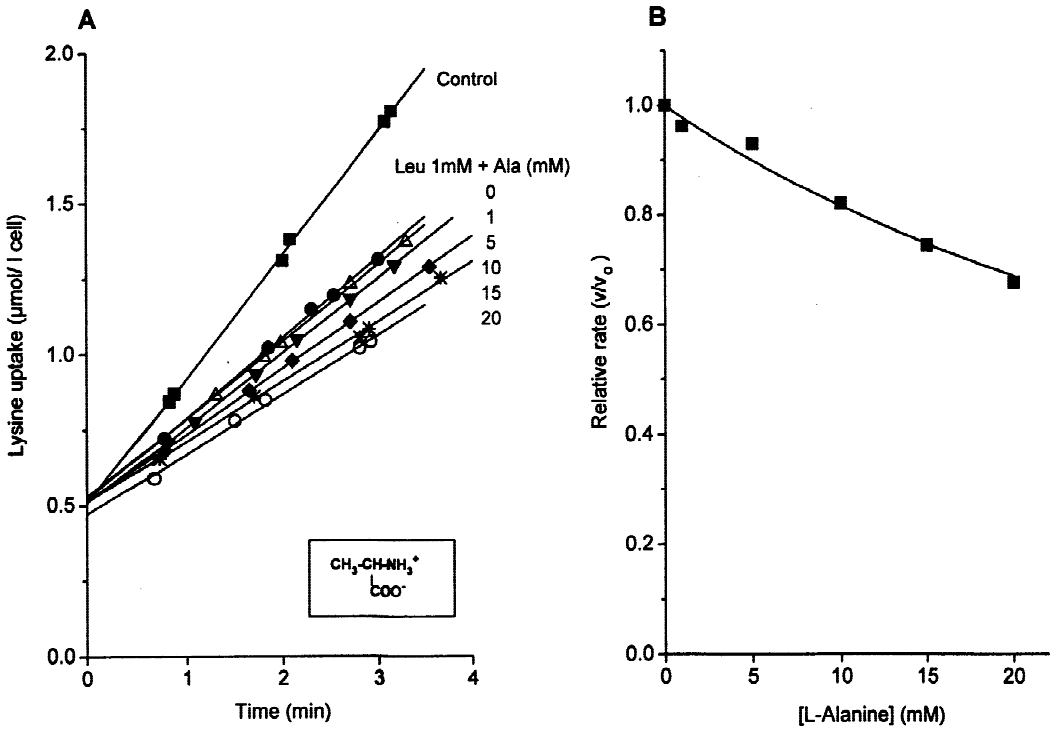

Fig. 2. Effect of L-alanine on the rate of L-lysine entry into human erythrocytes. (*A*) The activity of system y^+L was inhibited by adding 1 mM L-leucine (●) and the uptake of L-[^{14}C]lysine (10 μM) was measured in the presence of increasing concentrations of nonradioactive L-alanine on the *cis*-side (in mM): 1 (△), 5 (▼), 10 (♦), 15 (✳), 20 (○) in "Na^+ medium." The rate in the absence of neutral amino acid (■) was 0.41 ± 0.01 μmol/cell/min. (*B*) Relative rates of uptake were calculated with reference to the rate in the presence of 1 mM leucine (v_o = 0.27 ± 0.01 μmol/1 cells/min). The curve was fitted to the data by nonlinear regression using Eq. 5. The inhibition constant for L-alanine ($K_{iy\text{-}ALA}$) was 44.2 ± 2.17 mM.

(panel *B*) reflects the inhibition of the two components of the flux. The first phase of the curve represents the inhibition of system y^+L and the second phase the inhibition of system y^+. Data were analyzed on the basis of Eq. 1, which describes the inhibition of two systems operating in parallel.

The function is defined by three constants: the apparent inhibition constant for system y^+L, $K_{iyL(ap)}$ (high affinity interaction), the inhibition constant for system y^+, K_{iy} (low affinity interaction), and constant *F*, which is the ratio of the rate of lysine transport through system y^+L and system y^+, in the absence of inhibitor. Since the inhibition constants for the interaction of neutral amino acids with system y^+L (K_{iyL}) are known (Angelo et al., 1996) we have used in the analysis the reported values. It was necessary, however, to correct the constants to account for the concentration of lysine employed in this study (*see* Materials and Methods). In the case of norvaline, (K_{iyL} = 0.103 ± 0.015 mM) the corrected or apparent inhibition constant ($K_{iyL(ap)}$) was taken to be 0.206 mM (*see* Eq. 4). After introducing this constant into Eq. 1, the function was fitted to the data to calculate the other two parameters: K_{iy} = 24.7 ± 2.12 mM and F = 0.53 ± 0.035. The value of constant *F* (Eq. 2) indicates that at a concentration of 10 μM the relative contributions of systems y^+ and y^+L are 66 and 33% respectively. The average value of *F* in different experiments was 0.56 ± 0.07 (n = 38).

An example of the type of experiment used for analogues that interact with low affinity with system y^+L is shown in Fig. 2. In this case, system y^+L was inhibited with 1 mM leucine and, keeping this condition constant, the concentration of the amino acid under study was varied. L-leucine is the neutral amino acid that interacts more strongly with system y^+L ($K_{iyL\text{-}LEU(ap)} \approx 20$ μM) and at a concentration of 1 mM it inhibits this activity completely in the presence of Na^+. The effect of L-alanine on the initial rate of lysine entry (in the presence of 1 mM leucine) is shown in Fig. 2*A*. Relative rates of entry (calculated with reference to the flux in the presence of 1 mM leucine) were plotted as a function of the alanine concentration in panel *B*. Results were analyzed on the basis of Eq. 5. The calculated inhibition constant ($K_{iy\text{-}ALA}$) was 44.2 ± 2.1 mM.

Using these protocols, the interaction of system y^+ with a number of L-amino acids was investigated. Figure 3 compares the effect of glutamine, tryptophan, leucine, serine and homoserine on the relative rates of lysine entry (10 mM) through system y^+. Although all these experiments were performed using the protocol illus-

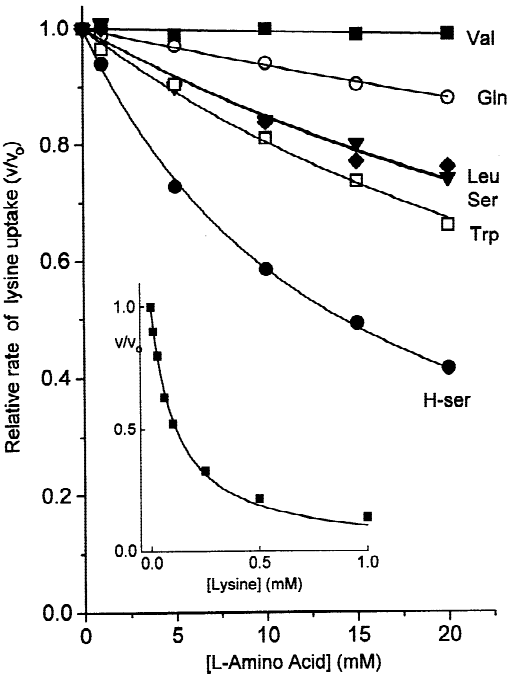

Fig. 3. Relative influx of lysine through system y^+ in human erythrocytes in the presence of increasing concentrations of unlabeled L-amino acids in the external compartment ("Na^+ medium"): glutamine (○), serine (▼), leucine (♦), tryptophan (□), homoserine (●), and lysine (■) (insert). The effect of neutral amino acids was tested using the protocol illustrated in Fig. 1, and the relative rates of system y^+ shown were calculated subtracting the contribution of system y^+L. The K_{iy} values calculated on the basis of Eq. 1 are listed in Table 1. Unlabeled lysine was added in the presence of 1 mM leucine to inhibit system y^+L; $K_{iy\text{-}LYS}$, calculated on the basis of Eq. 5, was 0.116 ± 0.005 mM.

trated in Fig. 1, for the purpose of presentation, we calculated the rate of entry through system y^+ by subtracting the contribution of system y^+L from the total flux at each analogue concentration. The inhibition constants obtained for these and other amino acids in the presence of Na^+ are given in Table 1. The effect of unlabeled lysine on system y^+ is shown, as reference, in the insert of Fig. 3.

As mentioned in the Introduction, it is generally believed that the interaction of system y^+ with neutral amino acids is Na^+-dependent. However, a reexamination of the original reports has shown that the cation dependence of this interaction differs in Ehrlich cells and reticulocytes, the two model systems for y^+ activity. To clarify this problem we carried out a detailed study of the cation dependence of neutral amino acid binding.

The effect of L-homoserine on the rates of entry of lysine in the presence of Na^+, K^+, Li^+ or choline is compared in Fig. 4. The results show that the interaction of homoserine with system y^+ is very similar in the presence of Na^+, K^+ or Li^+ and undetectable in the presence of choline. Table 2 lists the inhibition constants measured for homoserine and two other amino acids in media

Table 1. Inhibition constants (K_{iy},mM) for the interaction of neutral amino acids and lysine with system y^+ in the presence of Na^+ in the external medium

L-Amino Acid	K_{iy} (mM)	L-amino Acid	K_{iy} (mM)
Homoserine	14.2 ± 0.4	Tryptophan	41.0 ± 2.3
Alanine	44.2 ± 2.1	Methionine	48.4 ± 3.9
Leucine	54.6 ± 5.9	Serine	56.6 ± 8.2
Isoleucine	103 ± 2.5	Glutamine	146 ± 3.5
Glycine	332 ± 41	Valine*	>400

The K_{iy} values were determined using the protocols described in Fig. 1 (homoserine, glutamine, serine, leucine, tryptophan, methionine) or in Fig. 2 (valine, isoleucine, alanine, glycine).
* Value was estimated assuming that 5% inhibition would have been detected.

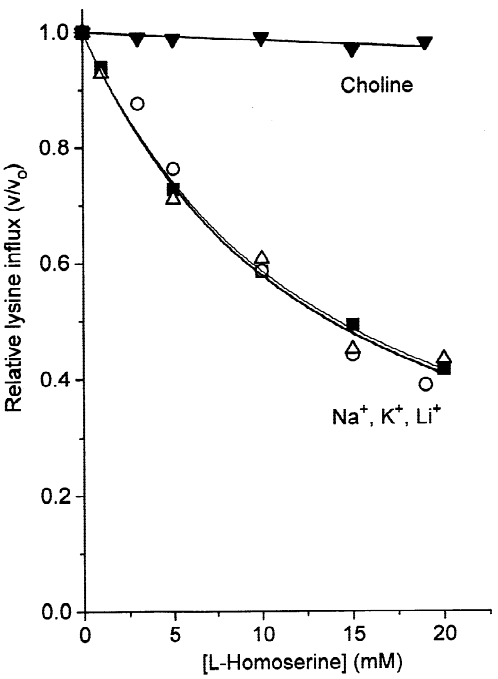

Fig. 4. Comparison of the effect of increasing concentrations of unlabeled L-homoserine, added to the *cis*-side, on the relative rate of L-[^{14}C]lysine entry through system y^+ in different ionic media, K^+ (○), Li^+ (△) or choline (▼). The results in the presence of Na^+ (■) (from Fig. 3) are also included as reference. In K^+ or choline medium, system y^+L was inhibited with a fixed concentration of 5 mM unlabeled L-leucine and the relative rates were calculated from the slopes of the uptake curves in the presence of varying concentrations of homoserine. The effect of homoserine in the presence of Li^+ was studied using the protocol described in Fig. 1, and the relative rates shown were calculated by subtracting the contribution of system y^+L. The calculated inhibition constants (K_{iy}) are listed in Table 2.

of different ionic composition. The same pattern was observed in all three cases. As has been reported by others (White, 1985), the lysine flux measured in the absence of neutral amino acids was similar in the presence of alkali metal ions or choline (*data not shown*).

Table 2. Inhibition constants (K_{iy}, mM) for the interaction of system y^+ with L-amino acids in different ionic media

Cation	Inhibition constant K_{iy} (mM)		
	L-Leucine	L-Norvaline	L-Homoserine
Na^+	54.6 ± 5.9*	24.7 ± 2.1	14.2 ± 0.4*
K^+	53.1 ± 12.9	17.7 ± 0.3	13.6 ± 1.0
Li^+	50.6 ± 1.0	21.8 ± 0.6	12.8 ± 0.2
Choline	>400	>400	>400

The K_{iy} values were calculated from six independent determinations by nonlinear regression analysis on the basis of either equation 1 (Na^+ or Li^+) or Eq. 5 (K^+ or choline).
* Also shown in Table 1.

It has been proposed that Na^+ could facilitate the interaction of neutral amino acids with system y^+ by binding to the substrate site at the locus that normally accepts the positively charged terminal group of the cationic amino acid (Christensen & Handlogten, 1969; Christensen, 1984), but the hypothesis has not been experimentally demonstrated. With this question in mind we studied the effect of guanidinium ion, the terminal group of arginine, on the interaction of system y^+ with neutral amino acids.

The effect of guanidinium ion (5 mM) on the binding of L-homoserine and L-norvaline to system y^+ is shown in Fig. 5. The addition of 5 mM guanidinium to a choline medium, stimulated homoserine binding to an extent comparable to that observed in Na^+, K^+ or Li^+ media, but more striking was the effect of guanidinium on norvaline interaction. The inhibition constant for norvaline, which in the presence of 145 mM alkali metal ions was approximately 20 mM, was reduced to 1.55 ± 0.02 mM in the presence of guanidinium, i.e., ≈ 15-fold. This is specially interesting because norvaline and guanidinium aligned generate a structure that is equivalent to arginine. The cooperative effect between guanidinium and norvaline binding can also be appreciated in the complementary experiment (Fig. 6), which shows that guanidinium ion is inhibitory only in the presence of norvaline.

The apparent affinities (log $1/K_{iy}$) of system y^+ for the *L*-amino acids α-aminobutyric acid, norvaline and norleucine, measured in Na^+ and guanidinium media, are plotted against the number of carbon atoms in Fig. 7. Interestingly, the effect of guanidinium was dramatic only in the case of L-norvaline.

Figure 8 compares the apparent affinities of system y^+ and system y^+L (Angelo et al., 1996) for leucine, norvaline and homoserine in the presence of Na^+ and K^+ and Fig. 9 compares the interaction of system y^+ and

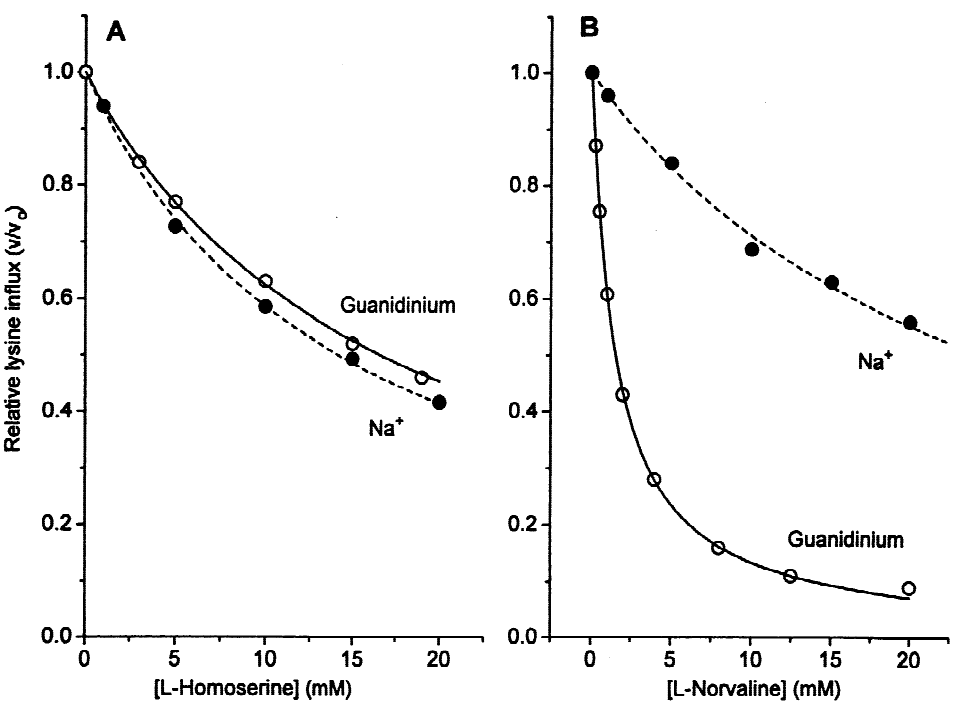

Fig. 5. Effect of increasing concentrations of unlabeled L-homoserine (*A*) and L-norvaline (*B*) on the rate of entry of lysine through system y^+, in the presence of guanidinium ion. System y^+L was inhibited with 2 mM leucine. The concentration of guanidinium ion was 5 mM and the isotonicity of the external medium was kept constant with choline chloride. The curves were fitted using Eq. 5 and the inhibition constants calculated were $K_{iy\text{-}HSER}$ = 16.6 ± 0.19 mM and $K_{iy\text{-}NVAL}$ = 1.55 ± 0.02 mM. The effect of L-homoserine (from Fig. 3) and L-norvaline (from Fig. 1) in the presence of Na^+ are indicated by the dotted line and are included as reference.

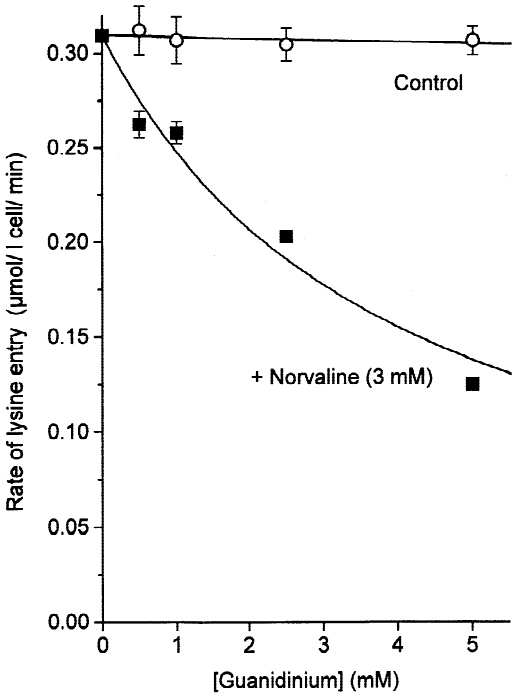

Fig. 6. Effect of increasing concentrations of guanidinium ion on L-lysine (10 µM) influx in the absence or presence of 3 mM unlabeled L-norvaline in the external compartment. The isotonicity of the medium was kept constants with choline chloride and the solution was buffered with 5 mM Na^+ phosphate (pH 6.8). System y^+L was inhibited with 2 mM L-leucine, when the concentrations of guanidinium was between 2.5 and 5 mM, and with 5 mM L-leucine, when the concentration was between 0.5 and 1 mM.

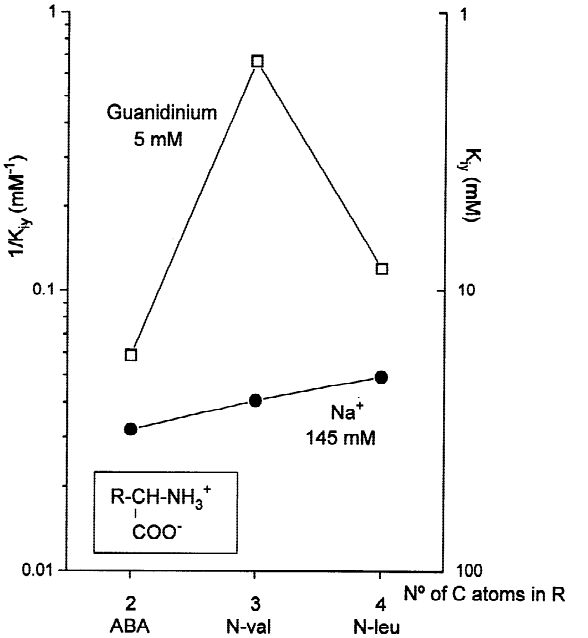

Fig. 7. Structure-activity relation of the interaction of system y^+ with three L-amino acids with linear apolar lateral chain (α-aminobutyric acid (ABA), L-norvaline and L-norleucine) in the presence of 5 mM guanidinium ion (□) or Na^+ (●) in the external medium. The apparent affinity ($1/\log K_{iy}$) is plotted against the number of carbon atoms of the side chain (R). The K_{iy} in the presence of guanidinium were measured using the protocol from Fig. 2 (with 2 mM L-leucine) and the K_{iy} in the presence of Na^+ were measured using the protocol from Fig. 1. The K_{iy} (mM) measured in the presence of guanidinium ion (G) or sodium (Na^+) were: ABA, (G) 17.0 ± 1.1 (Na^+) 31.2 ± 1.1; norvaline, (G) 1.5 ± 0.02, (Na^+), 24.7 ± 2.1 (value from Fig. 1); norleucine, (G) 8.2 ± 0.4, (Na^+) 20.2 ± 0.4.

system y^+L with norvaline in the presence of Na^+, K^+, choline and guanidinium ion.

Discussion

It has been suggested that the classical cationic amino acid transporter, system y^+, and the broad-scope and cation modulated transporter, system y^+L, have mechanistic similarities, and some authors have referred to system y^+L as a "y^+-like" system (Bertran et al., 1992; Palacín, 1994) or as a "variant of system y^+" (Van Winkle, 1993). The two transporters were assumed to exhibit similarities in three properties: (i) the ability to bind neutral amino acids, in addition to cationic amino acids, (ii) the Na^+-independent interaction with cationic amino acids and (iii) the Na^+ requirement for neutral amino acid binding. However, the results reported here show that, even though the interaction of the two systems with cationic amino acids is qualitatively similar, their specificities for neutral amino acids differ markedly.

The affinity of system y^+ for neutral amino acids was found to be extremely low. L-homoserine showed the highest potency with an inhibition constant for lysine influx (K_{iy}) equal to 14.2 ± 0.39 mM. Since the affinity for cationic amino acids (lysine, arginine and ornithine) is considerably higher (K_m = 50–120 µM) (Christensen & Antonioli, 1969; White et al., 1982; Devés et al., 1993; Forray et al., 1995), system y^+ can be considered selective for cationic amino acids. This is in contrast with the observations with system y^+L which binds both neutral and cationic amino acids with high affinity (L-leucine and L-lysine exhibit half-saturation constants of approximately 10 µM).

It should be noted that two activities (b^+_1 and b^+_2) that resist inhibition by 30 mM homoserine, and thus appear to be even more selective towards cationic amino acids than system y^+ have been detected in preimplantation mouse blastocytes and 1-cell conceptuses. These activities constitute a small fraction (≈ 2–3%) of the lysine flux at low substrate concentrations (Van Winkle & Campione, 1990).

The relative affinity of system y^+ and system y^+L for various neutral analogues is also unrelated. For example, the inhibition constants of system y^+ for L-leucine and L-serine are comparable (Table 1), but differ more

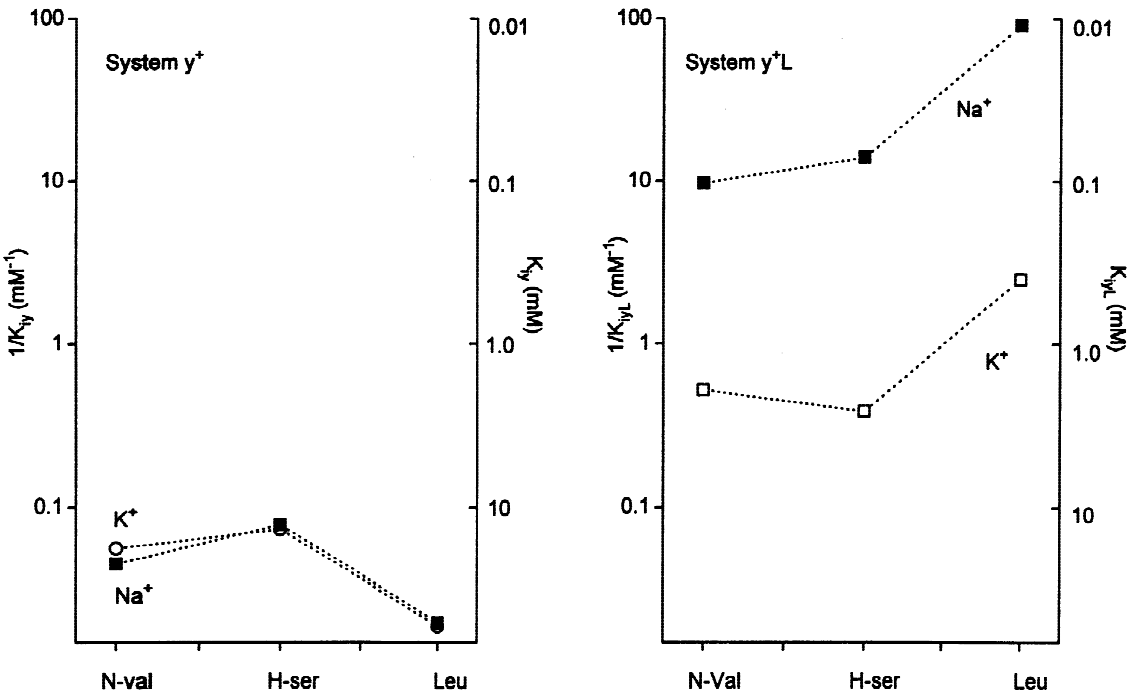

Fig. 8. Comparison of the apparent affinity (log $1/K_i$) of systems y^+ and y^+L for the amino acids L-norvaline, L-homoserine and L-leucine in the presence of Na^+ or K^+ in the external medium. The values of the inhibition constants for system y^+ (K_{iy}) are the same shown in Table 2 and the inhibition constants for system y^+L (K_{iyL}) are from Angelo et al., 1996.

than 44-fold in the case of system y^+L ($K_{iyL\text{-}LEU}$, 0.011 ± 0.0007 mM; $K_{iyL\text{-}SER}$, 0.49 ± 0.08 mM). Differences are also seen in the binding of L-glutamine which is a good substrate of system y^+L ($K_{iyL\text{-}GLN}$ = 0.027 ± 0.003 mM) and a very poor ligand of system y^+. Although the elongation of the lateral chain makes a positive contribution to the binding of neutral amino acids to both carriers, the effect is more important in the case of system y^+L. Thus, the inhibition constants for L-alanine and L-norleucine differ by ≈ 2-fold in the case of system y^+ (Table 1 and Fig. 7) and by ≈ 9-fold in the case of system y^+L (Angelo et al., 1996). It should be noticed that it is often stated that system y^+ exhibits a preference for small neutral amino acids over larger analogues (Closs, 1996; White, 1985). There are no systematic studies in the literature justifying this conclusion and we have not found this to be the case.

An important finding of this investigation is that the binding of neutral amino acids to system y^+ is not strictly Na^+-dependent as generally stated (Christensen, 1984; White, 1985; Palacín, 1994; Closs, 1996; MacLeod & Kakuda, 1996). In fact, as shown in Figs. 4, 8, 9 and Table 2, the interaction of neutral amino acids with system y^+, is very similar in the presence of Na^+, K^+ or Li^+. The affinity of system y^+ for neutral amino acids was reduced when any of these alkali metal ions was replaced by choline. This is in contrast with the observations with system y^+L, for in this case Na^+ replacement with K^+ markedly decreases the affinity of the transporter for neutral amino acids. From a physiological point of view, a Na^+-dependent system is one that exhibits a different behavior in the presence of Na^+ as compared to K^+, since these are the cations that are relevant in the generation of substrate gradients. The inability of system y^+ to discriminate between Na^+ and K^+ has been overlooked in previous investigations, because Na^+ has been usually replaced with choline and not K^+ (in order to prevent possible membrane potential effects).

The cation dependence observed in this paper is consistent with the activity described by Christensen & Antonioli (1969) in rabbit reticulocytes, but differs from that reported in Ehrlich cells (Thomas et al., 1971). In Ehrlich cells, the interaction of the transporter with a series of neutral amino acids was strongest in Li^+, important in Na^+ and undetectable in K^+, a behavior which is totally consistent with the ionic dependence later described for system y^+L (Devés et al., 1993; Angelo et al., 1996).

As found for erythrocytes, cationic amino acids appear to cross the cell membrane of Ehrlich cells using two different transport systems (Christensen & Liang, 1966). We conclude that the activity that has been better characterized, has been incorrectly attributed to system y^+ and corresponds to a broad-scope transporter similar or identical with system y^+L. The other fraction, is more selective for cationic amino acids and could be analo-

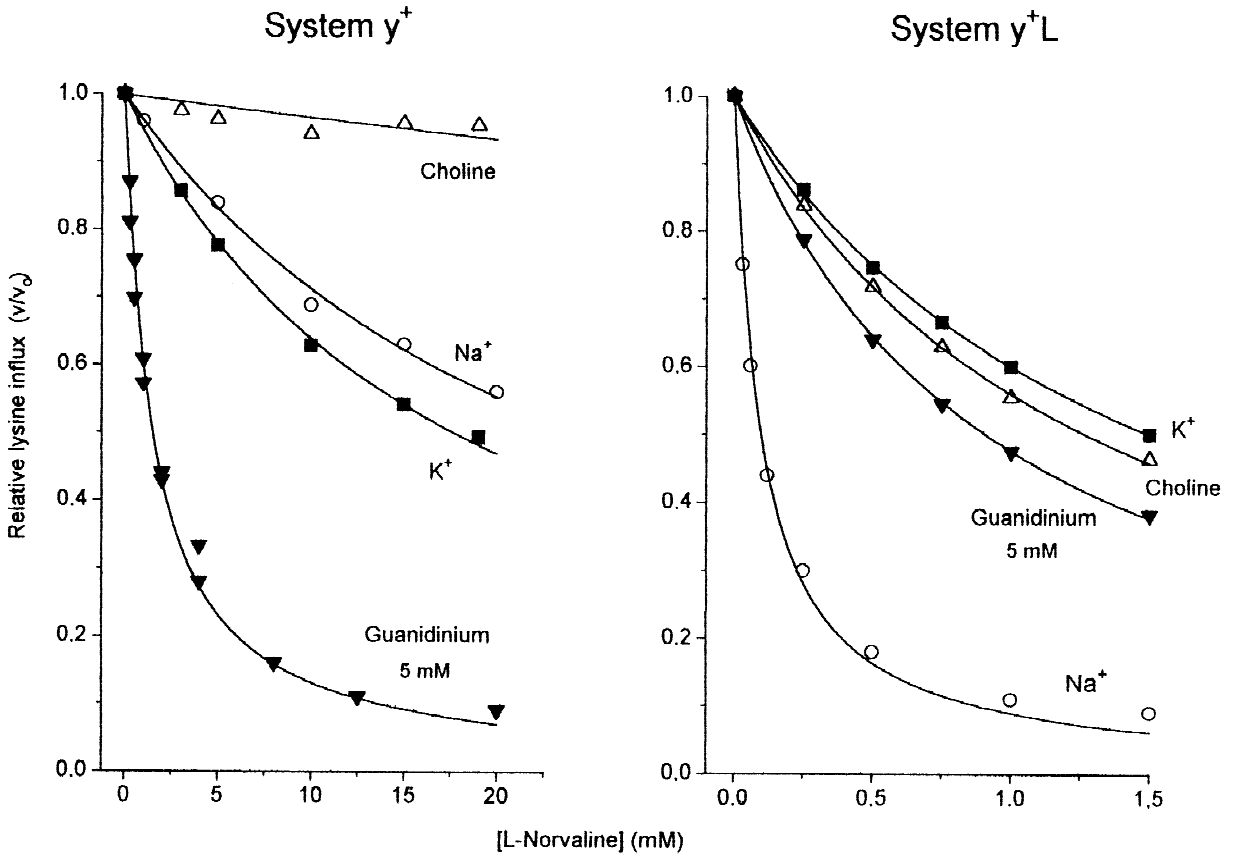

Fig. 9. Comparative effect of L-norvaline added to the *cis*-side on L-[^{14}C]lysine influx through systems y^+ and y^+L in human erythrocytes in media with Na^+ (○), K^+ (■), guanidinium (▼) or choline (△). (*A*) Effect of L-norvaline on system y^+ (L-[^{14}C]lysine, 10 μM) was tested after inhibiting system y^+L with 2 mM leucine (in guanidinium) or with 5 mM leucine (in K^+ or choline). The data in Na^+ medium were recalculated from Fig. 1. (*B*) The effects of L-norvaline on system y^+L (L-[^{14}C]lysine, 1 μM) were measured in cells pretreated with 0.2 mM NEM during 15 min. The curves were adjusted to the data by nonlinear regression analysis according to Eq. 5. The inhibition constants for system y^+L ($K_{iyL\text{-}NVAL}$) were: 0.10 ± 0.004 mM (Na^+); 1.49 ± 0.01 mM (K^+); 1.27 ± 0.01 mM (choline) and 0.91 ± 0.01 mM (guanidinium). The inhibition constants for system y^+ are given in Table 2 and Fig. 6.

gous to the system described here. It must be remembered that the existence of two transporters for cationic amino acids in Ehrlich cells was proposed by Christensen 30 years ago (Christensen & Liang, 1966), but was later dismissed and replaced by the unifying concept of system y^+ (Thomas et al., 1971, reviewed in Devés & Boyd, 1998).

The observations reported here are also consistent with observations made with the mCAT-1 and mCAT-2 cationic amino acid transporters following expression of the corresponding cRNA in *Xenopus* oocytes. In electrophysiological studies with mCAT-1, it has been observed that high concentrations of L-homoserine are required to induce a small entry current in the presence of Na^+, the current was not observed in choline medium (Wang et al., 1991). With mCAT-2 it has been shown that the currents are more significant in the presence of L-homoserine or L-methionine and undetectable in the presence of L-glutamine and L-valine (Kakuda et al., 1993). The ionic dependence of the CAT transporters has not been characterized in detail.

The presumed requirement of external Na^+ for the interaction of system y^+ with neutral analogues led to the proposal that Na^+ would exert this effect by binding to the position that is normally occupied by the terminal cationic group of the basic amino acid. According to this hypothesis, system y^+ would be able to recognize the quasi-substrate formed by Na^+ and the neutral amino acid. It was also proposed that the hydroxyl group or another polar group in the lateral chain would determine the degree of interaction between the amino acid and the Na^+ ion (reviewed in Christensen, 1984).

With the objective of testing the proposal that cations can bind to the substrate site in the locus that normally recognizes the terminal group of the basic amino acid, and as a result stimulate neutral amino acid binding, we investigated the effect of guanidinium ion on the binding of neutral amino acids. Guanidinium ion (5 mM)

was found to stimulate the binding of all neutral amino acids assayed (relative to choline), but the stimulation was found to be specially important for norvaline (Figs. 5, 6 and 7). Considering that the pair L-norvaline-guanidinium form a molecular structure equivalent to arginine and that this dramatic effect is not observed with the preceding and following homologues (α-aminobutyric acid (ABA) and L-norleucine respectively), these results suggest that a conformational change occurs during substrate binding. It is proposed that guanidinium exerts its effect by binding directly to the substrate and that the neutralization of the charge produces a conformational change that manifests itself as a cooperative effect.

The results in Fig. 9 show that in the case of system y^+L the effect of guanidinium ion is not as important as in the case of system y^+. In addition, there is no specific preference for norvaline since the potency of the analogues, in the presence of 5 mM guanidinium, increases with chain length: $K_{iyL\text{-}ABA}$, 6.3 mM; $K_{iyL\text{-}VAL}$, 0.91 mM and $K_{iyL\text{-}LEU}$, 0.19 mM.

In conclusion, we propose that the name "system y^+" be maintained for the Na^+-independent transporter that shows a marked preference for basic amino acids and whose structural selectivity and ion dependence are described in this paper. System y^+ would be responsible for a great proportion of the basic amino acid flux across the reticulocyte (Christensen & Antonioli, 1969) and the fibroblast membrane (White et al., 1982) and its molecular equivalent would be the CAT family (Closs, 1996). This activity is clearly different from that of system y^+L (Devés et al., 1992), which does account for the activity characterized in Ehrlich cells (Christensen, 1964).

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