J. Membrane Biol. 141, 183–192 (1994)

The Journal of
Membrane
Biology

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Amino Acid Transport System y^+L of Human Erythrocytes: Specificity and Cation Dependence of the Translocation Step

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Abstract. The transport specificity of system y^+L of human erythrocytes was investigated and the carrier was found to accept a wide range of amino acids as substrates. Relative rates of entry for various amino acids were estimated from their *trans*-effects on the unidirectional efflux of L-[^{14}C]-lysine. Some neutral amino acids, L-lysine and L-glutamic acid induced marked *trans*-acceleration of labeled lysine efflux; saturating concentrations of external L-leucine and L-lysine increased the rate by 5.3 ± 0.63 and 6.2 ± 0.54, respectively. The rate of translocation of the carrier-substrate complex is less dependent on the structure of the amino acid than binding. Translocation is slower for the bulkier analogues (L-tryptophan, L-phenylalanine); smaller amino acids, although weakly bound, are rapidly transported (L-alanine, L-serine). Half-saturation constants (±SEM) calculated from this effect (L-lysine, 10.32 ± 0.49 μM and L-leucine, 11.50 ± 0.50 μM) agreed with those previously measured in *cis*-inhibition experiments. The degree of *trans*-acceleration caused by neutral amino acids did not differ significantly in Na^+, Li^+ or K^+ medium, whereas the affinity for neutral amino acids was dramatically decreased if Na^+ or Li^+ were replaced by K^+. The observation that specificity is principally expressed in substrate binding indicates that the carrier reorientation step is largely independent of the forces of interaction between the carrier and the transport site.

Key words: Transport — Erythrocytes — Amino acids — Carrier, Kinetics — Membrane

Introduction

A new transport system that recognizes cationic and neutral (zwitterionic) amino acids was recently described in human erythrocytes and designated as system y^+L (Devés, Chávez and Boyd, 1992). The apparent affinities of system y^+L for lysine and leucine are comparably high (K_m for L-lysine, 9.5 ± 0.67 μM and K_i for L-leucine, 10.7 ± 0.72 μM); the system also interacts strongly with L-methionine, L-glutamine and with less affinity with L-phenylalanine and L-serine. It does not tolerate alpha amino-group substitution as shown by the lack of effect of *N*-methyl-L-leucine on lysine entry and does not bind compounds such as 2-amino-2-norbornane-carboxylic acid (BCH) or proline (Devés, Angelo & Chávez, 1993).

The binding specificity of system y^+L in general resembles that of system $b^{o,+}$ described in blastocysts by Van Winkle, Campione and Gorman (1988). However, an important difference exists between system $b^{o,+}$ and system y^+L regarding their interactions with monovalent inorganic cations. In the case of system $b^{o,+}$, binding of both cationic and neutral amino acids was shown to be Na^+ independent, whereas in the case of system y^+L the effect of monovalent cations differs for cationic and neutral amino acids. Lysine transport occurs at virtually the same rate in Na^+, K^+ or Li^+ medium, but the association with neutral amino acids is strictly dependent on the cation present. The inhibition constant for leucine and glutamine increases by approximately 90- and 60-fold, respectively, if Na^+ is replaced by K^+. Interestingly, Li^+ was found to be a very good substitute for Na^+ (Devés et al., 1993).

Recently, three closely related cDNA(s), isolated from rat, rabbit and human kidney, have been found to stimulate the sodium-independent transport of cationic amino acids, some neutral amino acids and cystine, after injection of the derived RNAs into *Xenopus* oocytes (Bertran et al., 1992*b;* Wells & Hediger, 1992; Bertran et al., 1993; Lee et al., 1993; Mosckovitz et al., 1993). The protein responsible for this $b^{o,+}$-like activity is a type II glycoprotein which appears to span the membrane only once; it is not yet clear, therefore, whether

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it represents a constituent element of an oligomeric transporter or an activator of the system. The protein codified by these cDNA(s) shows a significant sequence homology (≈30%) with another type II membrane glycoprotein, the heavy chain of the cell surface antigen 4F2 (4F2hc). As expected, the DNA encoding for 4F2hc also stimulates the transport of cationic and neutral amino acids (Bertran et al., 1992*a;* Wells et al., 1992).

However, the activity induced by the expression of 4F2 cDNA had a peculiar dependence on monovalent ions which distinguished it from system $b^{o,+}$ (Bertran et al., 1992*a* and Wells et al., 1992). This activity was sodium independent with respect to lysine and arginine transport, but sodium dependent with respect to neutral amino acids. This behavior matches exactly the observations made for system y$^+$L in human erythrocytes. Therefore, it is likely that a transporter with the functional characteristics of system y$^+$L may be widely distributed. This idea is supported by the recent finding of Harvey et al. (1993) showing that isolated stage VI oocytes of *Xenopus laevis,* which have been microinjected with purified poly(A)$^+$ mRNA from rat intestine, can express a lysine transport activity with similar characteristics to system y$^+$L.

Here, we have investigated the specificity of system y$^+$L with respect to the translocation step. The observations reported so far have firmly demonstrated that some neutral amino acids are able to interact strongly with the outer binding site of this transporter, but conclusive evidence regarding their ability to cross the membrane on this carrier has not yet been presented. That this may indeed be the case was suggested by the lack of inhibitory effect of external L-leucine, L-phenylalanine and L-isoleucine on the rate of exit of L-lysine from erythrocytes. In fact, these amino acids were found to *trans*-stimulate total lysine efflux by approximately 30% (Devés et al., 1992).

Our principal interest was to identify the structural features of the substrate that enable an efficient translocation of the loaded carrier and to assess the role that monovalent ions play on the transfer process itself. We have dealt, therefore, with the events that take place after the process of substrate binding.

Materials and Methods

Chemicals

Uniformly labeled L-[^{14}C]lysine was purchased from Amersham (approximately 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 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 150 mM NaCl and 4 mM KCl and incubated to reduce endogenous amino acids (2.5% hematocrit, 15 hr at 25°C). This buffer (assay buffer) was used in all experiments, unless otherwise indicated. In long incubations 0.02% chloramphenicol was included.

Treatment with NEM

Washed erythrocytes (hematocrit 2.5%) were incubated with 0.2 mM NEM at 25°C for 10 min. The reaction was terminated by addition of 2-mercaptoethanol (10 mM final concentration). The cells were then washed, packed and assayed for transport.

Measurement of Exit Rates

Cells that had been incubated overnight with assay buffer were placed in a medium containing L-[^{14}C]lysine (5.5 μM) and incubated at 50% hematocrit for 2 hr at 37°C (unless otherwise indicated). After this period, the cells were separated from the external medium by centrifugation and repeated washes with ice-cold buffer.

Aliquots of loaded cells were added at 10% hematocrit to assay buffer (37°C). Samples were withdrawn at intervals and rapidly centrifuged (45 sec, 11,000 × *g*) in tubes containing dibutylphthalate. The cells sedimented below the organic layer. The radioactivity in the supernatant was determined by scintillation counting, and the rates were calculated assuming a monoexponential decay. All determinations were performed in duplicate runs and rates were generally estimated from six time points.

Theoretical Analysis

In a cyclic carrier mechanism, unidirectional fluxes are determined not only by the rate of translocation of the carrier substrate complex, but also by the rate of reorientation of the substrate site after unloading. Thus, if substrate-loaded cells are placed in a medium that contains an unlabeled substrate analogue, which interacts with the carrier, the rate of efflux will be affected depending on the transport properties of the analogue. If the analogue carrier complex translocates inward more slowly than the free carrier, the rate of efflux will be retarded, whereas if the rate of translocation of the complex is faster than that of the free carrier, the rate will be accelerated. It follows that *trans*-effects can be used to infer the relative transport rates of unlabeled substrate analogues.

Analysis of Trans-Acceleration Experiments

On the basis of a general treatment of the carrier model (Devés & Krupka, 1979), the rate of exit of a substrate *S* into a medium that contains an unlabeled analogue *T* (v^T) can be written as:

$$v^T = \frac{v_o + v^{T_{max}}[T_o]/K_T}{1 + [T_o]/K_T} \quad (1)$$

where v_o is the rate of exit measured in the absence of analogue, v^{Tmax} the rate in the presence of a saturating concentration of exter-

nal analogue T and K_T the apparent half-saturation constant for the analogue.

This equation can be rearranged to:

$$v^T - v_o = \frac{(v^{T_{max}} - v_o)[T_o]}{K_T + [T_o]} \quad (2)$$

If v^Tmax is higher than v_o, the analogue will accelerate the rate according to a Michaelis-Menten type function. K_T reflects the interaction between the carrier and the substrate at the external site and it is theoretically equivalent to the half-saturation constant determined in entry experiments. The full expressions for the above parameters are given in the Appendix.

The ratio of the maximum acceleration caused by two analogues T and X equals the ratio of their relative maximum rates of entry (V_{max}) (*see* Appendix).

$$\frac{v^{T_{max}}/v_o}{v^{X_{max}}/v_o} = \frac{V_{maxTo}}{V_{maxXo}} \quad (3)$$

Unless otherwise indicated errors represent standard errors of the mean (SEM).

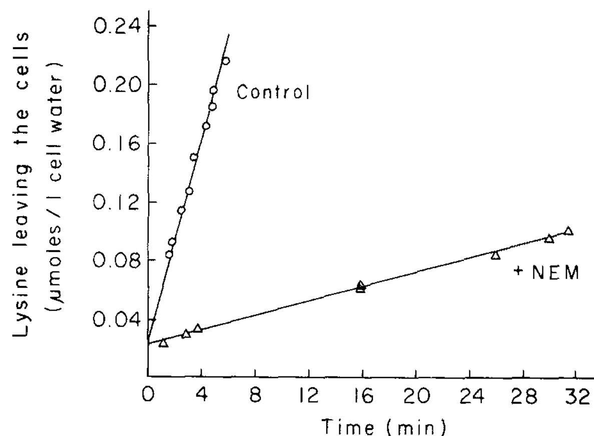

Fig. 1. L-[^{14}C]lysine exit from NEM-treated (0.2 mM, 10 min at 25°C) and untreated erythrocytes. Cells were loaded by incubation with 1.8 μM L-lysine for 40 min. The internal concentration of L-[^{14}C]lysine at the end of the incubation period was 2.1 μM (control cells) and 2.0 μM (treated cells). Exit rates (±SEM) are (μmol/liter cell water/min × 10^2): 3.5 ± 0.06 (control) and 0.25 ± 0.005 (treated cells). These results are also shown in Table 1 (Experiment 58).

Results

The rates of exit of L-[^{14}C]lysine from control and NEM-treated erythrocytes are compared in Fig. 1 and Table 1. As previously found for L-lysine entry (Devés et al., 1993), NEM was a partial inhibitor of the rate of exit of L-[^{14}C]lysine in human erythrocytes. However, whereas in entry experiments the NEM-sensitive and -insensitive components (systems y^+ and y^+L, respectively) were found to account for equivalent fractions of the total flux, the NEM-insensitive flux constitutes a relatively minor fraction of L-lysine efflux. The cause of this asymmetry is explained in the Discussion.

Subsequent experiments were carried out with the aim of investigating the substrate specificity of the translocation step in system y^+L. The experimental protocol involved measuring the effect of unlabeled substrate analogues, added to the external medium, on the rate of L-lysine exit from NEM-treated cells. The effect of the analogues on the unidirectional flux of labeled substrate provides information regarding the transport properties of the carrier-analogue complex (Devés & Krupka, 1978). *Trans*-acceleration occurs if the complex shifts to the inward-looking conformation faster than the free carrier; on the contrary, *trans*-inhibition results if the complex reorientates more slowly than the free carrier.

Addition of L-lysine to the external medium was found to induce marked acceleration of L-[^{14}C]lysine efflux, as shown in Fig. 2. Data obtained in three independent experiments, using different cell samples, follow closely the behavior predicted by Eq. (2), that is, L-lysine produces a dose-dependent acceleration that reaches saturation (Fig. 3*A*). The half-saturation constant for external L-lysine, calculated by fitting Eq. (2) to these data, was found to be 10.32 ± 0.49 μM (n = 3). On average, excess L-lysine accelerated the rate of exit by 6.2 ± 0.54 (n = 20).

As shown in Fig. 3*B*, L-leucine was also found to *trans*-accelerate the efflux of L-lysine. The half-saturation constant for L-leucine, with Na^+ in the external medium, was 11.50 ± 0.50 μM (n = 3). It can be concluded that the rate of translocation of the lysine- and leucine-carrier complexes is faster than the rate of translocation of the free carrier.

As predicted by the kinetic analysis presented in the Appendix, the half-saturation constants measured with the present protocol were found to be equivalent to those previously estimated from the inhibitory effect of unlabeled amino acids on L-lysine entry (Table 2).

The effect of various amino acids on the rate of exit of L-[^{14}C]lysine is illustrated in Fig. 4. All these amino acids were previously shown to interact with system y^+L by their ability to inhibit L-[^{14}C]lysine entry (Devés et al., 1993 and *unpublished observations*). Their *trans*-effects on exit were assessed at two different concentrations, one of these was chosen to produce a partial effect and the other because it was saturating. Since the degree of stimulation varies with individual cell samples, the results were normalized using 1 mM lysine as internal standard. On average, the maximum acceleration ($v^{T_{max}}/v_o$) caused by L-lysine in this experiment was 6.1 ± 0.46 (n = 15). The relative effect of an analogue was found to be constant for all samples.

All the amino acids tested, except for L-tryptophan,

Table 1. Rate of L-lysine exit in NEM-treated and untreated human erythrocytes

Experiment	NEM	[L-lysine] μM	Efflux rate constant k (min^{-1}) × 10^2	Ratio k_{Cont}/k_{NEM}
58	−	2.1	1.63 ± 0.056	13.6 ± 0.58
	+	2.0	0.12 ± 0.003	
59	−	2.4	2.67 ± 0.085	16.7 ± 0.82
	+	2.4	0.16 ± 0.006	
119	−	1.3	3.16 ± 0.071	16.6 ± 0.86
	+	1.3	0.19 ± 0.009	

Cells were preincubated for 40 min with [^{14}C]lysine at 1.8 μM (Experiments 58 and 59) and 1.4 μM (Experiment 119).

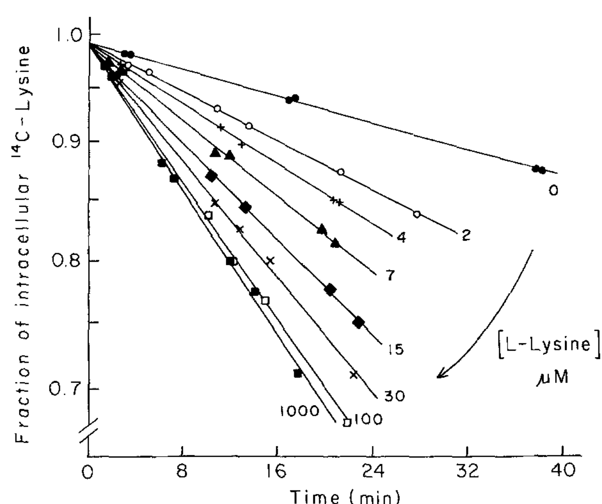

Fig. 2. Effect of unlabeled L-lysine, present in the external medium, on the rate of exit of L-[^{14}C]lysine in cells treated with NEM. The initial concentration of lysine in the intracellular medium was 4.7 μM. The ratio of the rates measured in the presence and absence of 1 mM L-lysine (±SEM) was 5.5 ± 0.18.

were able to accelerate the rate of exit of L-[^{14}C]lysine, but the relative maximum acceleration was seen to depend on substrate structure. This observation indicates that they are good substrates of system y$^+$L. Even L-tryptophan must be able to cross the membrane on this system, although at a considerably lower rate; this amino acid did not affect efflux and a nontransported ligand is expected to block the rate of exit completely (Devés & Krupka, 1978). The structure-activity relationships are examined in detail in the Discussion.

In the following experiments we investigated the role of inorganic monovalent cations (Na^+, Li^+ or K^+) on substrate translocation. As mentioned in the Introduction, neutral amino acid binding is dramatically affected by the ionic composition of the medium. It was thus important to find out whether the rate of reorientation of the loaded carrier was also dependent on the interaction with monovalent ions. Interestingly, *trans*-acceleration of lysine exit by unlabeled L-leucine was also observed in the presence of K^+ although, as expected, the concentration of leucine required to produce the effect was at least 50-fold higher than in Na^+. Progress curves for L-lysine exit in the presence of different leucine concentrations outside (50–2,500 μM) are shown in Fig. 5. The ratio of the initial rate of L-lysine exit measured in the absence or presence of 2.5 mM L-leucine was 3.5 ± 0.24.

In the same experiment L-lysine (1 mM) stimulated lysine exit by 5.9 ± 0.25. It can be appreciated by inspection of the results in Fig. 5 that at the highest L-leucine concentration, the rate of exit was nonlinear. The bending observed is likely to be reflecting increasing competition between L-leucine and the substrate at the internal site. The very high concentrations of L-leucine which are required in this experiment are sufficient to enable L-leucine uptake through system L (Rosenberg, 1981). It follows that the maximum effect of L-leucine in K^+ medium, as well as the half-saturation constant (465 ± 74 μM, n = 2) measured under these conditions, are underestimated (Table 2).

L-Leucine-dependent acceleration of L-lysine exit was also observed with Li^+ as the principal cation (Fig. 3B). The half-saturation constant for L-leucine in Li^+ medium was 4.8 ± 0.44 μM, that is, 2.4-fold lower than the inhibition constant measured in Na^+. Stronger binding of L-leucine in the presence of Li^+ was also observed in *cis*-inhibition experiments of lysine entry (Devés et al., 1993 and Table 2).

To obtain more systematic information regarding the relative carrier reorientation rates in the presence of Na^+, Li^+ or K^+, the effect of these cations was compared in the same group of cells (Fig. 6). The experiment was performed with three different amino acids in the external medium, namely, L-lysine, L-leucine and L-glutamine. As suggested by the previous experiments, the extent of the acceleration caused by neutral amino acids did not differ significantly in Na^+, Li^+ or K^+ medium, whereas the affinity for neutral amino acids was dramatically affected if Na^+ or Li^+ were replaced by K^+. The interaction of L-lysine with the carrier was unaltered by the same substitutions.

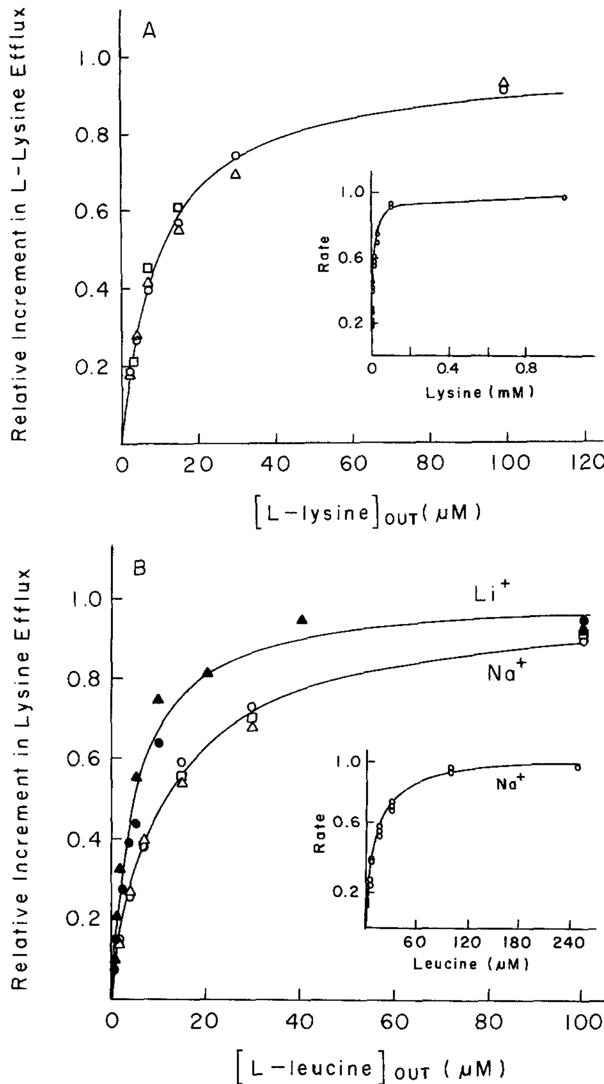

Fig. 3. The relative increment in the rate of L-[^{14}C]-lysine exit ($(v^T - v_o)/(v^{T\max} - v_o)$) induced by varying concentrations of external unlabeled amino acids is plotted against the concentration of unlabeled amino acid. Curves and transport parameters were calculated by best fit of Eq. (2). (*A*) Effect of L-lysine. The symbols denote different experiments. The maximum acceleration ($v^{T\max}/v_o$) in each cell sample (±SEM) was (○) 5.7 ± 0.10 (same data as in Fig. 2), (△) 13.1 ± 0.55 and (□) 4.0 ± 0.16. The calculated half-saturation constant is given in Table 2 ($n = 3$). The inset shows the results obtained for a wider concentration range. (*B*) Effect of L-leucine. Rates were measured with Na^+ ($n = 3$) or Li^+ ($n = 2$) in the external medium. The saline solution contained (mM) either 150 NaCl, 5 Na phosphate or 150 LiCl, 5 K phosphate. The symbols denote different experiments. The maximum acceleration ($v^{T\max}/v_o$) in each cell sample ±SEM) was (○) 9.2 ± 0.27, (△) 4.3 ± 0.16, (□) 4.6 ± 0.15, in Na^+ medium, and (▲) 4.0 ± 0.10, (●) 6.2 ± 0.19, in Li^+ medium. Calculated half-saturation constants are given in Table 2. The inset shows the results obtained for a wider concentration range (Na^+ medium).

Discussion

The evidence reported in this paper demonstrates that neutral amino acids are good substrates of system y⁺L and provides detailed information regarding the specificity of the translocation step in this carrier.

Table 2. Half-saturation constants for L-leucine and L-lysine measured in *cis*-inhibition and *trans*-acceleration experiments

Substrate	Cation	Half-saturation constant	
		Cis-inhibition[a] entry μM	*Trans*-acceleration[b] exit μM
L-lysine	Na^+	9.5 ± 0.67	10.3 ± 0.48
L-leucine	Na^+	10.7 ± 0.72	11.5 ± 0.50
L-leucine	Li^+	4.5 ± 0.26	4.8 ± 0.44
L-leucine	K^+	983 ± 80	465 ± 74[c]

[a] Taken from Devés et al., 1993.
[b] Calculated from the results in Fig. 3 by best fit of Eq. (2).
[c] Underestimated (*see* Discussion).

Relative rates of entry for a series of analogues were estimated from their *trans*-effects on the rate of exit of labeled L-lysine. The determination of transport parameters by this method is particularly useful when the solutes being investigated cross the membrane through multiple transport systems. Direct assessment of entry rates would require precise knowledge of the various routes involved in the transport of each solute and selective inhibitors to block the parallel pathways. The experimental strategy used here greatly overcomes these difficulties, since the results depend exclusively on interactions with the system under study, the only requirement being that the labeled substrate move through a single system.

In theory, the transport parameters measured from *trans*-effects on exit should coincide with the equivalent constants determined in entry experiments (Eq. A4). This prediction was experimentally confirmed. The results presented in Table 2 show that the half-saturation constants for L-lysine and L-leucine estimated from *cis*-inhibition or *trans*-acceleration experiments are in agreement.

L-Lysine, some neutral amino acids and L-glutamic acid were found to cause a marked *trans*-acceleration of L-[^{14}C]lysine exit; in the presence of saturating unlabeled L-leucine or L-lysine in the external compartment the rate increased by five- to sixfold. As mentioned in the Introduction, previous results had shown that L-leucine, L-phenylalanine and L-isoleucine were able to increase the total efflux of L-lysine in human erythrocytes by 30%. This observation is entirely consistent with the results reported here, considering that system y⁺L accounts for a small fraction ($^1/_{16}$) of total L-[^{14}C]lysine efflux (Table 1). Since system y⁺ is unaffected by neutral amino acids (Devés et al., 1992), the predicted increment of total flux in the presence of external leucine is $^1/_{16} \times 5.3$ or 0.33 as previously found.

The degree of *trans*-acceleration was seen to vary in different cell samples; the highest acceleration produced by L-lysine was 13.1-fold and the lowest 3.7.

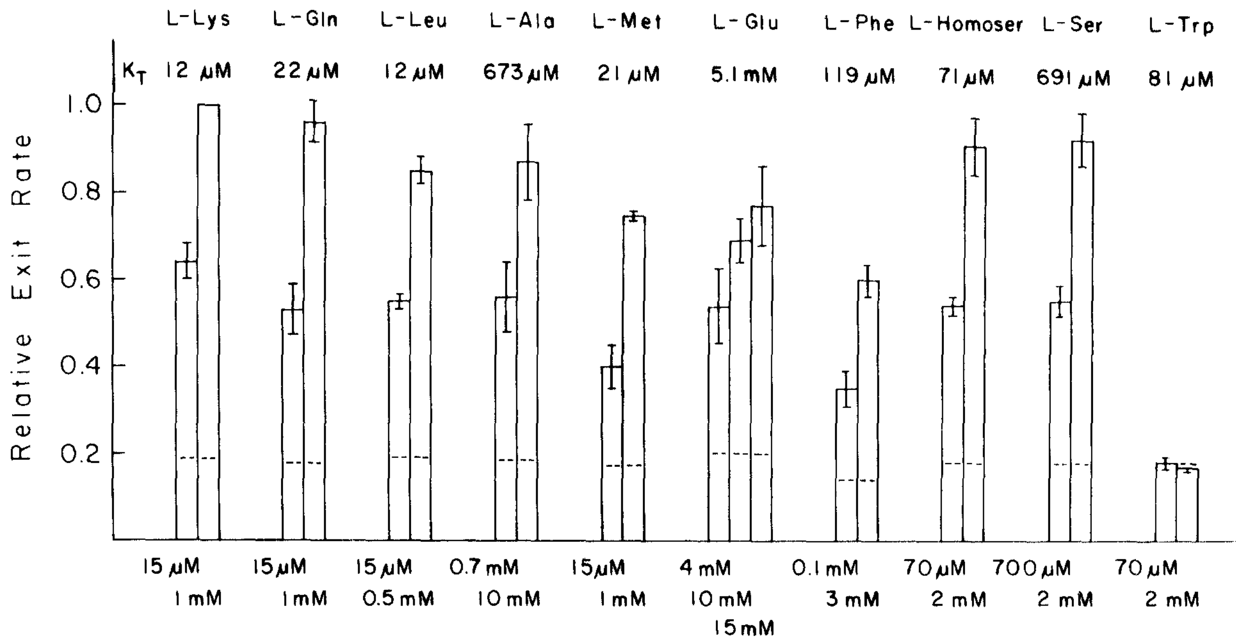

Fig. 4. Effect of L-amino acids present in the external medium, on the rate of exit of L-[^{14}C]lysine in cells treated with NEM. Rates were normalized using as reference the rate measured in the presence of 1 mM L-lysine. Error bars indicate the SDM of at least two determinations. The dotted line indicates the average value for the relative rate measured in the absence of amino acid. The ratio of the rates in the presence or absence of L-lysine 1 mM was 6.1 ± 0.46 (n = 15). The internal concentration of L-[^{14}C]lysine at the beginning of the experiment was 4–5 μM. Half-saturation constants (K_T) calculated on the basis of Eq. (2) are shown, in the case of tryptophan the constant given corresponds to the apparent inhibition constant for L-lysine entry (K_i).

This can be understood if we consider that the degree of the *trans*-acceleration depends not only on the relative rates of translocation of the free and loaded carrier (f_3/f_1, *see* Fig. 7), but also on the concentration (and transport properties) of the substrate whose exit is being followed.

From Eqs. (A2) and (A3) in the Appendix, the ratio of the exit rates in the presence or absence of analogue T is found to be:

$$\frac{v^{T_{max}}}{v_o} = \frac{\tilde{V}^T_{Si}/\tilde{K}^T_{Si}(1 + [S_i]/\bar{K}_{Si})}{\bar{V}_{Si}/\bar{K}_{Si}(1 + [S_i]/\tilde{K}^T_{Si})} \quad (4)$$

Substitution of the transport parameters (V and K) by their equivalent expression in terms of the rate constants in Fig. 7 (the definitions are given in Devés and Krupka, 1979) shows that the ratio $v^{T\max}/v_o$ can adopt the two following extreme forms:

At very low substrate concentration ($[S_i] << \bar{K}_{si}$):

$$\frac{v^{T_{max}}}{v_o} = \frac{(1 + f_{-1}/f_1)}{(1 + f_{-1}/f_3)} \quad (5)$$

Trans-acceleration will occur only if $f_3 > f_1$. In a symmetrical system, where f_1 equals f_{-1}, the ratio cannot be larger than 2, no matter how large is the f_3/f_1 ratio.

At very high substrate concentration ($[S_i] >> \bar{K}_{Si}$):

$$\frac{v^{T_{max}}}{v_o} = \frac{(1 + f_{-2}/f_1)}{(1 + f_{-2}/f_3)} \quad (6)$$

As before, *trans*-acceleration occurs only if $f_3 > f_1$, but there is no limit to the value that the flux ratio can attain, which could be very large if f_3 were much larger than f_1. In homoexchange, where $f_3 = f_{-2}$, the expression becomes:

$$\frac{v^{T_{max}}}{v_o} = 0.5\ (1 + f_{-2}/f_1) \quad (7)$$

Calculation of the $[S_i]/\bar{K}_{Si}$ ratio in our experiments is not possible because $\bar{K}_{Si}$ is unknown. $[S_i]/\bar{K}_{Si}$ cannot, however, be very low because in addition to exogenously added L-lysine (4 μM) a fraction of endogenous amino acids, which are not removed by our washing procedure, will be present. Therefore the differences which have been observed in the magnitude of the *trans*-stimulation probably result from variations of the remanent amino acid pool in individual cell samples.

The contribution of the intracellular pool to efflux is also indicated by the asymmetry observed when entry and exit rates for systems y$^+$ and y$^+$L are compared. As mentioned in the Results, in entry experiments, systems y$^+$ and y$^+$L were found to account for

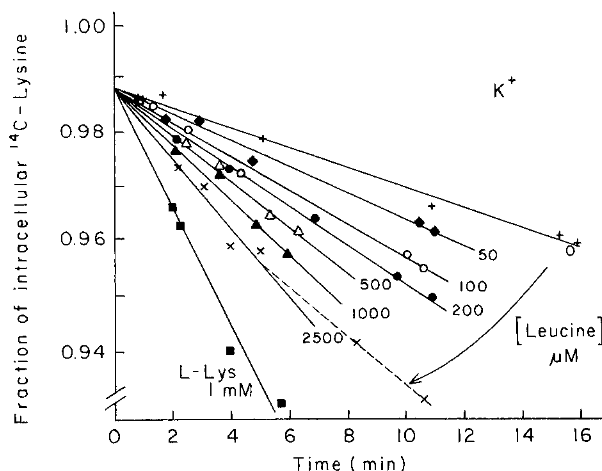

Fig. 5. Effect of L-leucine on the rate of exit of L-lysine in cells treated with NEM with K^+ in the external medium. The saline solution contained 154 mM KCl and 5 mM potassium phosphate. The initial concentration of lysine in the intracellular medium was 5.5 μM. The ratio of the rates measured in the presence and absence of 2.5 mM L-leucine was 3.5 ± 0.24.

equivalent fractions of the total flux (Devés et al., 1993), whereas in exit, system y^+L is only $^1/_{16}$ of the total flux. The apparently low activity of system y^+L in exit experiments suggests competition between L-[^{14}C]lysine and the intracellular amino acid pool; endogenous amino acids are not expected to have the same effect on system y^+ because it has a lower affinity and a more restricted substrate specificity.

If the highest value of $v^{T\max}/v_o$ which was observed (13.1) is introduced into Eq. (7), we can estimate that the f_3/f_1 ratio for L-lysine must be at least 25, that is, the lysine-loaded carrier translocates at least 25 times faster than the free carrier.

An analysis of the results reported in Fig. 4 shows that the only structural feature which appears to impair translocation is bulkiness. Most notably, L-tryptophan, the largest amino acid tested, was not able to accelerate the rate of transport at concentrations which completely inhibit entry. This implies that the tryptophan-loaded carrier and the free carrier reorient inwards at the same rate (25 times more slowly than lysine). Consistently, phenylalanine was found to be less competent in producing *trans*-acceleration (f_3/f_1 = 10.8, from Eq. 6). Small analogues, such as serine and alanine, were found to induce comparable *trans*-stimulation to the best substrates, e.g., L-lysine or L-glutamine.

In general, binding can be said to be significantly more sensitive to modifications in amino acid structure than translocation. Glutamate, for example, which is very weakly bound (approximately 500 times less strongly than lysine) is able to cross the membrane on this carrier almost as fast as lysine ($v^{\text{Glu(max)}}/v^{\text{Lys(max)}}$ = 0.9). The net negative charge, therefore, appears to interfere with substrate binding, but not with translocation. Similarly, serine and alanine, which are relatively poorly bound, possibly because of fewer interactions with the bonding points in the carrier site, moved easily with the carrier.

In conclusion, the structural requirements for binding and translocation differ. In the case of binding, there seems to be an ideal substrate dimension, reductions or increases in size result in poor binding—in the first case probably as a result of fewer interactions, in the second case because of steric hindrance. A net negative charge in the molecule also appears to be detrimental as shown by the very low affinity for glutamate. In the case of translocation, the only apparent source of perturbations appears to result from increases in size.

Similar structure-activity relationships have been observed in other facilitated diffusion systems that are able to sustain net transport (Krupka, 1990). In a study of the theoretical basis of the expression of substrate specificity in transport systems, Krupka concluded that in facilitated transport, specificity should become apparent mainly in binding. Thus, with analogues no larger than the normal substrate, the affinity, but not the maximum rate of transport, was expected to vary; whereas with larger analogues both the affinity and rate could vary. It was proposed that in these systems the transport site would fit the substrate closely and retain its shape as the carrier underwent reorientation. The theory was seen to agree with published observations on facilitated systems for choline and glucose in erythrocytes. The results presented here show that these predictions are also valid for system y^+L.

According to this theory, in systems that perform net transport, any substrate analogue capable of binding to the carrier and able to avoid repulsive interactions in the transition state should be translocated about as fast as the normal substrate. Discrimination, at least with analogues no larger than the normal substrate, should therefore be exercised in the binding step but not the translocation step, and specificity should be expressed in the affinity but not the maximum rate of transport (Krupka, 1990). The affinity of an analogue would be low if it interacted weakly with the constellation of bonding points within the carrier site as observed for alanine and serine. The system could discriminate against analogues larger than the substrate in the translocation step as well, as was observed here for tryptophan.

Another interesting observation refers to the interaction of system y^+L with monovalent cations. It was previously reported (Devés et al., 1993) that, whereas the binding of lysine was unaffected by sodium replacement, the apparent affinity of L-leucine was strongly affected by the ionic composition of the medium, being maximum in Li^+ (twofold higher than in Na^+) and minimum in K^+ (90-fold lower than in Na^+). The apparent affinity for L-glutamine also decreased approximately 60-fold when Na^+ was replaced by K^+; in this

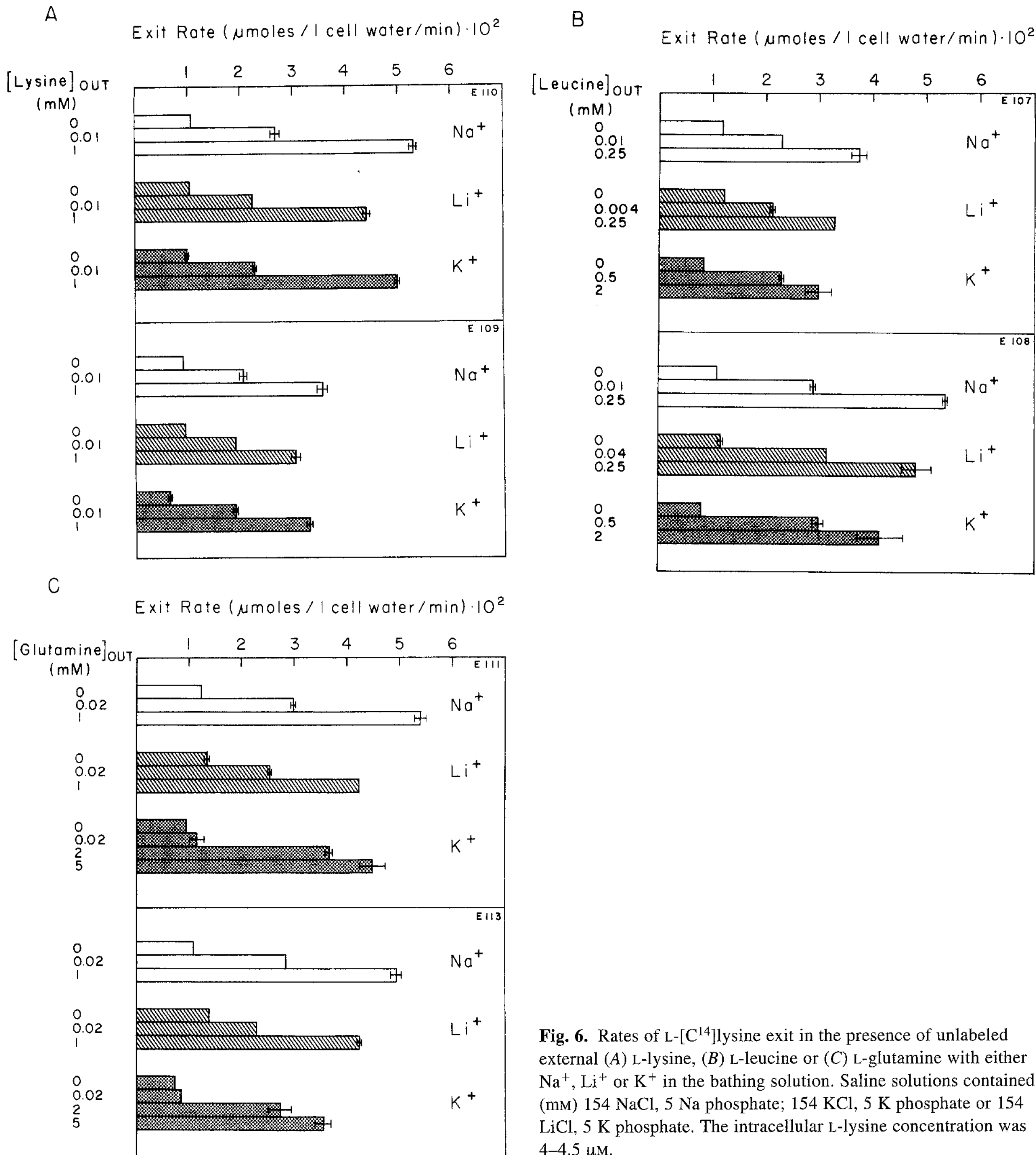

Fig. 6. Rates of L-[C[14]]lysine exit in the presence of unlabeled external (*A*) L-lysine, (*B*) L-leucine or (*C*) L-glutamine with either Na^+, Li^+ or K^+ in the bathing solution. Saline solutions contained (mM) 154 NaCl, 5 Na phosphate; 154 KCl, 5 K phosphate or 154 LiCl, 5 K phosphate. The intracellular L-lysine concentration was 4–4.5 μM.

case, Li^+ was equivalent to Na^+. Therefore, replacement of external Na^+ by K^+ converts the broad spectrum transporter into a cationic amino acid transporter. The requirement of a co-ion for the efficient recognition of neutral amino acids suggests that this ion may be a functional substitute of the ε amino group of lysine, as previously proposed by Christensen and Antonioli (1969) for the transport of cationic amino acids in Ehrlich cells. The experiments reported here confirm our earlier findings (using a different experimental strategy) and show, in addition, that the rate of translocation is not significantly affected by sodium replacement. L-leucine and L-glutamine were able to induce marked *trans*-acceleration in K^+ medium, but as expected from previous results a substantially higher concentration of neutral amino acids was required to produce the effect.

It can be concluded that inorganic monovalent cations are affecting the binding step, but not the

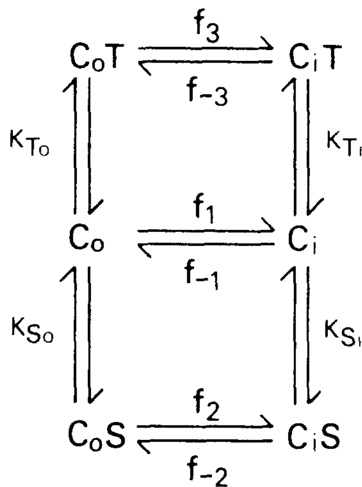

Fig. 7. Kinetic scheme for the simple carrier model, involving two substrates *S* and *T*. The free carrier exists in two forms, outward-facing (C_o) and inward-facing (C_i). Substrates in the external solution form a complex with C_o (C_oS and C_oT), substrates in the internal solution with C_i. The substrate dissociation constants for *S* and *T* are K_{So} and K_{To} on the outer face of the membrane and K_{Si} and K_{Ti} on the inner face, respectively. The rate constants for carrier reorientation are $f_1, f_{-1}, f_2, f_{-2}, f_3$ and f_{-3}.

translocation step. This finding is in agreement with the conclusion reached from the structure-activity relationships for different analogues, and indicates that the carrier reorientation step is largely independent of the forces of interaction between the carrier and the transport site. It also suggests that the ion must be affecting a region in the carrier that determines substrate binding without actively participating in the carrier site reorientation. The induction of distinct conformational changes in an enzyme, dialkylglycine decarboxylase, by K^+ or Na^+ has recently been reported (Toney et al., 1993).

Finally, the results reported in this paper may have an important physiological bearing. Compartmental analysis of lysine transport across the vascularly perfused small intestine and sheets of ileum has shown that the rate-limiting step in lysine *trans*-epithelial movement is exit across the basolateral membrane (Munck & Schultz, 1969; Cheeseman, 1983). Furthermore, it has been found that leucine increases the *trans*-epithelial flux of lysine by six- to tenfold (Cheeseman, 1983). It was proposed that countertransport between lysine and leucine at the basolateral side may be responsible for this effect. However, the validity of this explanation was questioned arguing that the effective leucine concentrations (10–100 μM) were too low (Cheeseman, 1992). The finding that system y^+L is able to sustain lysine–leucine exchange at low concentrations supports the countertransport hypothesis.

We wish to thank Dr C.A.R. Boyd for helpful discussions and Prof. H.N. Christensen for sharing with us very relevant bibliographic material. We are grateful to FONDECYT (1282/91) and DTI (B 2674) (Chile) for financial assistance.

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APPENDIX

The expression for the rate of exit of substrate S in the presence of a substrate analogue (T), present in the external medium, derived on the basis of the carrier model in Fig. 7, is given by:

$$v^T = \frac{(\bar{V}_{Si}/\bar{K}_{Si})[S_i] + (\tilde{V}^T_{Si}/\tilde{K}^T_{Si})[S_i]([T_o]/\bar{K}_{To})}{(1 + [S_i]/\bar{K}_{Si}) + ([T_o]/\bar{K}_{To})(1 + [S_i]/\tilde{K}^T_{Si})} \tag{A1}$$

This equation was obtained by simplification of a general transport equation published elsewhere (Devés & Krupka, 1979) assuming that labeled substrate is present only inside of the cell ($[S_o] = 0$) and unlabeled analogue T only outside ($[T_i] = 0$). The constants in this equation are experimental parameters whose equivalent expressions, in terms of the individual rate constants in Fig. 7, can be found in earlier publications (Devés & Krupka, 1979).

The convention followed in naming the transport parameters is intended to suggest their significance directly, for in each case the nature of the experiment is denoted by subscripts and superscripts attached to V and K. Subscripts designate the substrate or analogue involved (S or T) and also its location, where i and o represent the inner and outer compartments, respectively. Symbols over the constants specify the conditions of the experiments, either zero-*trans* (e.g., $\bar{K}$) where no substrate analogue is present in the opposite compartment; or infinite-*trans* (e.g., $\tilde{K}$) where a saturating concentration of substrate or analogue is present in the *trans* compartment. $\bar{V}_{Si}$ is the maximum rate of exit of substrate S in the absence of analogue in the external medium (zero-*trans* flux) and $\bar{K}_{Si}$ is the corresponding half-saturation constant. $\tilde{V}^T_{Si}$ is the maximum rate of exit of substrate S into a medium containing a saturating concentration of analogue T and $\tilde{K}^T_{Si}$ the corresponding half-saturation constant. $\bar{K}_{To}$ is the half-saturation constant for the analogue in zero-*trans* influx experiment.

If the following definitions are considered, Eq. (A1) yields Eq. (1) (Materials and Methods):

(a) The rate of exit of substrate S in the absence of analogue T in the external medium:

$$v_o = \frac{(\bar{V}_{Si}/\bar{K}_{Si})[S_i]}{1 + [S_i]/\bar{K}_{Si}} \tag{A2}$$

where $\bar{V}_{Si}$ and $\bar{K}_{Si}$ represent the maximum rate and half-saturation constants for exit measured under zero-*trans* conditions, respectively.

(b) The rate of exit of substrate S in the presence of a saturating concentration of analogue T in the external medium:

$$v^{T_{max}} = \frac{(\tilde{V}^T_{Si}/\tilde{K}^T_{Si})[S_i]}{1 + [S_i]/\tilde{K}^T_{Si}} \tag{A3}$$

Where $\tilde{V}^T_{Si}$ and $\tilde{K}^T_{Si}$ represent the maximum rate and half-saturation constants measured under infinite-*trans* conditions, respectively.

(c) The half-saturation constant for analogue T:

$$K_T = \bar{K}_{To}\frac{(1 + [S_i]/\bar{K}_{Si})}{(1 + [S_i]/\tilde{K}^T_{Si})} \tag{A4}$$

K_T is identical to the half-saturation constant (or inhibition constant) measured in entry experiments (Devés & Krupka, 1979). If the internal substrate concentration is low ($S_i << \bar{K}_{Si}$), it becomes the half-saturation constant for zero-*trans* entry ($\bar{K}_{To}$).

From Eq. (A3) the ratio of $v^{T_{max}}$ for two different analogues T and X can be written as:

$$\frac{v^{T_{max}}}{v^{X_{max}}} = \frac{\tilde{V}^T_{Si}/\tilde{K}^T_{Si}(1 + [S_i]/\tilde{K}^X_{Si})}{\tilde{V}^X_{Si}/\tilde{K}^X_{Si}(1 + [S_i]/\tilde{K}^T_{Si})} \tag{A5}$$

Substitution of the ratio $(\tilde{V}^T_{Si}/\tilde{K}^T_{Si})/(\tilde{V}^X_{Si}/\tilde{K}^X_{Si})$ by the corresponding microscopic contants (Devés & Krupka, 1979) shows that this ratio is equivalent to the relative rates of zero-*trans* entry ($\bar{V}_{To}/\bar{V}_{Xo}$) for analogues T and X, and therefore Eq. (A5) can also be expressed as:

$$\frac{v^{T_{max}}}{v^{X_{max}}} = \frac{\bar{V}_{To}(1 + [S_i]/\tilde{K}^X_{Si})}{\bar{V}_{Xo}(1 + [S_i]/\tilde{K}^T_{Si})} = \frac{V_{maxTo}}{V_{maxXo}} \tag{A6}$$

Interestingly, the ratio $v^{T_{max}}/v^{X_{max}}$ is found to be equivalent to the ratio of the maximum rates of entry for the two analogues (V_{maxTo}/V_{maxXo}), measured under the same experimental conditions. If the substrate concentration is very low ($S_i << \bar{K}_{Si}$), the ratio of $v^{T_{max}}$ for two analogues equals the ratio of their maximum transport rates of zero *trans* entry. If, on the contrary, the substrate concentration is very high ($S_i >> \bar{K}_{Si}$), it equals the ratio of their maximum rates of entry in an infinite *trans* experiment. This can be verified on the basis of the analysis given in Devés and Krupka (1979).