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L-lysine Transport in Chicken Jejunal Brush Border Membrane Vesicles

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Abstract. The properties of L-lysine transport in chicken jejunum have been studied in brush border membrane vesicles isolated from 6-wk-old birds. L-lysine uptake was found to occur within an osmotically active space with significant binding to the membrane. The vesicles can accumulate L-lysine against a concentration gradient, by a membrane potential-sensitive mechanism. The kinetics of L-lysine transport were described by two saturable processes: first, a high affinity-transport system (K_{mA} = 2.4 ± 0.7 μmol/L) which recognizes cationic and also neutral amino acids with similar affinity in the presence or absence of Na^+ (L-methionine inhibition constant K_{iA}, NaSCN = 21.0 ± 8.7 μmol/L and KSCN = 55.0 ± 8.4 μmol/L); second, a low-affinity transport mechanism (K_{mB} = 164.0 ± 13.0 μmol/L) which also recognizes neutral amino acids. This latter system shows a higher affinity in the presence of Na^+ (K_{iB} for L-methionine, NaSCN = 1.7 ± 0.3 and KSCN = 3.4 ± 0.9 mmol/L). L-lysine influx was significantly reduced with *N*-ethylmaleimide (0.5 mmol/L) treatment. Accelerative exchange of extravesicular labeled L-lysine was demonstrated in vesicles preloaded with 1 mmol/L L-lysine, L-arginine or L-methionine. Results support the view that L-lysine is transported in the chicken jejunum by two transport systems, A and B, with properties similar to those described for systems $b^{0,+}$ and y^+, respectively.

Key words: Uptake — Cationic amino acids — Intestine — Arginine — Methionine — Membrane binding

Introduction

In polarized cells, such as those present in the small intestinal epithelium, cationic amino acids are taken up by the microvillous membrane and exit into the blood across the basolateral membrane. In the intestine of vertebrates, there is evidence that L-lysine is transported in the mucosal membrane by Na^+-independent mechanisms, either exclusively (Cassano, Leszcynska & Murer, 1983; Stevens, Kaunitz & Wright, 1984; Satoh et al., 1989) or together with a Na^+-dependent component (Reiser & Christensen, 1973; Wolfram, Giering & Scharrer, 1984; Tajima et al., 1993). In the chicken rectum, L-lysine stimulates the short-circuit current (Lind, Munck & Olsen, 1980) suggesting that Na^+ has some role in the transport process.

In recent years, new transport systems for cationic amino acids have been added to the well-known y^+ system (White, 1985; MacLeod, Finley & Kakuda, 1994). These include the $B^{0,+}$ and $b^{0,+}$ systems, first described in rat blastocytes (Van Winkle, Campione & Gorman, 1988) and the y^+L, first described in human erythrocytes (Devés, Chávez & Boyd, 1992). The different systems that transport cationic amino acids can be distinguished by their kinetic properties, ion and membrane potential dependency and by their sensitivity to agents such as *N*-ethylmaleimide.

Our laboratory has been concerned with the study of the transport of D-glucose and neutral amino acids in different regions of the chicken intestine (Moretó & Planas, 1989; Moretó et al., 1991; Ferrer et al., 1994). However, little is known about the transport of cationic amino acids in the chicken, and in particular the nature of the mechanisms involved (*see* Lerner, 1984, for a review). The aim of the present work is to study the characteristics of cationic amino acid transport in the chicken jejunum, using L-lysine as substrate, due to its importance in nitrogen homeostasis, animal growth and egg production by laying hens (D'Mello, 1994). The results obtained support the view that L-lysine transport across the microvillous membrane takes place through two carrier-mediated mechanisms, with functional properties similar to those of systems $b^{0,+}$ and y^+.

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Part of this work was presented to the 13th meeting of the European Intestinal Transport Group and published in abstract form (Torras-Llort et al., 1995).

Materials and Methods

Isolation of Brush Border Membrane Vesicles (BBMV)

Brush border membrane vesicles were prepared from the jejunum of 6-wk-old male Label chickens fed a balanced diet prepared by IRTA-Mas Bové (Reus). Animals were killed in the morning by decapitation without previous starvation. A portion of the jejunum (from the end of the duodenal loop to the Meckel diverticle) was removed (about 14 g tissue), immediately flushed with ice-cold saline, opened lengthwise, frozen in liquid N_2 and then stored at −80°C.

Brush border membrane vesicles were prepared using the method originally developed by Kessler et al. (1978). All steps were carried out at 0–4°C. Briefly, the intestinal segment was thawed in 20–30 mL of a buffer containing 100 mmol/L mannitol and 2 mmol/L HEPES/TRIS (pH 7.1) and then cut into pieces of 1 cm and homogenized in a Waring blender for 5 min at low speed. The homogenate was poured through nylon stocking material and homogenized for 30 sec at high speed. $MgCl_2$ 1 mol/L was added to the homogenate to produce a final concentration of 10 mmol/L with a final volume of 150 mL. After stirring for 20 min, the suspension was centrifuged at 3,000 × *g* for 15 min, the pellet was discarded and the supernatant again centrifuged at 30,000 × *g* for 35 min. The pellet was then suspended in 100 mmol/L mannitol, 0.1 mmol/L $MgSO_4 \cdot 7H_2O$ and 2 mmol/L HEPES/TRIS (pH 7.4). The suspension was homogenized in a glass-Teflon homogenizer (40 strokes) and then centrifuged at 30,000 × *g* for 25 min. The pellet was resuspended in 300 mmol/L mannitol, 0.1 mmol/L $MgSO_4 \cdot 7H_2O$, 0.02% LiN_3 and 20 mmol/L HEPES/TRIS (pH 7.4) to a final protein concentration of 10–20 mg/mL and homogenized with a 28-gauge needle. The vesicles were frozen and stored in liquid N_2 in 150 μL aliquots, and maintained their functional properties for a period of 5 months. Each isolation batch corresponds to the jejunum of one chicken and in the Results section, ''*n*'' indicates the number of chickens or membrane preparations.

Protein and Enzyme Assays

Before uptake assays, sucrase, a brush border marker enzyme and Na^+-K^+-ATPase, a basolateral marker, were systematically assayed according to Dahlqvist (1984) and Del Castillo and Robinson (1982), respectively. Protein was determined using the BioRad protein assay, with bovine serum albumin as standard.

Uptake Assays

Rates of L-lysine uptake were assayed for time periods ranging from 1 sec to 1 hr at 37°C. Short incubation times (1–5 sec) were run taking advantage of the endogenous capacity of the experimenter to keep a constant pace. Thus, the experimenter does not await any external signal to start or stop incubation but follows his own internal rhythm which is continuously paced by an external cyclic timer (a lamp programmed to flash at 1-sec intervals). This procedure results in accurate incubation times with a very low margin of error (less than 5%, even in 1-sec incubation periods).

For each uptake 10 μL BBMV were rapidly mixed with 40 μL of incubation media containing [^{14}C]-labeled substrate and the appropriate salts and effectors. At the end of the uptake period 2 mL of ice-cold stop solution containing 150 mmol/L KSCN, 0.02% LiN_3 and 20 mmol/L HEPES/TRIS (pH 7.4) were added. The diluted samples were rapidly filtered under negative pressure through prewetted and chilled 0.22 μm cellulose acetate/nitrate filters (Millipore GSWP304F0). The filters were rinsed four times with 2 mL ice-cold stop solution, dissolved in Biogreen-6 cocktail from Sharlau (Barcelona, Spain) and radioactivity was determined by liquid scintillation counting. Nonspecific radioactivity fixation to the filters was obtained by adding ice-cold stopping solution to reaction tubes immediately before addition of the vesicles.

The data shown are the means from several experiments performed with at least three different membrane preparations, each in triplicate. The composition of the incubation medium in standard experiments was: 100 mmol/L mannitol, 0.2 mmol/L $MgSO_4 \cdot 7H_2O$, 0.02% LiN_3, 20 mmol/L HEPES/TRIS (pH 7.4) and 100 mmol/L of a salt (NaSCN, KSCN, NaCl, KCl or *N*-methyl-glucamine gluconate), the appropriate concentration of unlabeled L-lysine and of L-[^{14}C]-lysine (5–10 μmol/L) or D-[^{14}C]-glucose (100 μmol/L) as substrates. In some experiments, the salt was partially or wholly replaced by mannitol or *N*-methyl-glucamine gluconate (Glu-Glu). Intra and extravesicular media were isotonic (320 mosm/L) except for the experiments in which the effect of increasing osmolarity on substrate uptake was determined. In this case cellobiose was used to produce prescribed osmolarities in the incubation medium, which were quantified using an Advanced Instruments osmometer.

Treatment with N-ethylmaleimide (NEM)

Vesicles were preincubated with 0.2, 0.5, or 1 mmol/L NEM at 25°C for 30 min (Devés, Angelo & Chávez, 1993). After preincubation, the vesicles were diluted and centrifuged (12,000 × *g* for 25 min). The supernatant was discarded and the vesicles resuspended in incubation medium (final protein concentration 10–20 mg/ml) and then assayed for amino acid uptake. Control vesicles ran parallel to NEM samples.

Orientation of the Vesicles

Membrane orientation was studied from sucrase activity according to Del Castillo and Robinson (1982), as adapted by Jamil Dalle (1993) for the chicken intestine. Sucrase activity was determined in intact vesicles and in vesicles incubated for 30 min with a mixture of 3 μmol/L deoxycholate and 15 mmol/L EDTA.

Chemicals

All unlabeled reagents were obtained from Sigma Chemical (St. Louis, MO), except L-lysine which was from Fluka (Madrid, Spain) and the reagents used to determine enzyme activity which were from Boehringer (Mannheim, Germany). L-[U-^{14}C]-lysine and D-[U-^{14}C]-glucose were obtained from New England Nuclear Research Products (Dreieich, Germany).

Transport Equations

To estimate the kinetic parameters, relative transport rates (v/v_o, L-[^{14}C]-lysine transport with and without unlabeled inhibitor) were analyzed assuming either a one-system or a two-system model by nonlinear regression from plots generated by the Enzfitter statistical package (Biosoft, Cambridge, UK). When transport was considered to oc-

Table 1. Enzyme activities and recoveries of brush border membrane vesicles

	Recovery (%)	Specific activity	Enrichment factor
Sucrase	34.1 ± 1.8	10.45 ± 0.6	10.8 ± 2.0
(Na^+/K^+)-ATPase	0.68 ± 0.35	0.4 ± 0.2	0.20 ± 0.1

Recovery is expressed as the percentage of total activity of the original homogenate recovered in the vesicle fraction. Specific activity is defined as nmol/mg protein · sec. The enrichment factor is calculated as the ratio of specific activity of the vesicles to that of the homogenate. In this and the following tables, the values are expressed as the mean ± SE. In the present table, $n = 5$.

cur through two transport systems A and B, the equations and strategies described by Devés et al. (1992) were followed:

$$\frac{v}{v_o} = \frac{\dfrac{F}{(1 + (I/K_{iA}))} + \dfrac{1}{(1 + (I/K_{iB}))}}{F + 1} \quad (1)$$

I is the concentration of a competitive inhibitor and F the "permeability" ratio which reflects the relative contribution of each system to the total flux, when the substrate concentration is very low.

$$F = \frac{V_{maxA} \cdot K_{mB}}{V_{maxB} \cdot K_{mA}} \quad (2)$$

The kinetic analysis was performed in the presence of 10 μmol/L L-lysine concentration. In these conditions, binding accounted for only 1% of the total uptake and therefore kinetic data were not corrected.

Data Analysis

The statistical analysis of data was made using an ANOVA of the appropriate model (Guttman, 1982). On the basis of the final model, ''populational'' values and the significance of the hypotheses were rejected at an α-risk level of 0.05. Confidence intervals of estimations were fixed at 95%.

Results

Characterization of BBMV

The purity and functional integrity of the membrane preparations were assessed prior to the study of L-lysine transport. Results indicate that during the isolation procedure sucrase is enriched 11-fold while Na^+-K^+-ATPase activity is reduced 5-fold (Table 1). Studies on membrane orientation indicate that 94 ± 2% of the vesicle population is right-side oriented (out-side out oriented). The functional properties of BBMV were examined by determining D-glucose (100 μmol/L) uptake under inwardly directed NaSCN and KSCN gradients (*zero-trans:* $[salt]_o = 100$ mmol/L, $[salt]_i = 0$ mmol/L). As shown in Fig. 1, there is a transient accumulation above

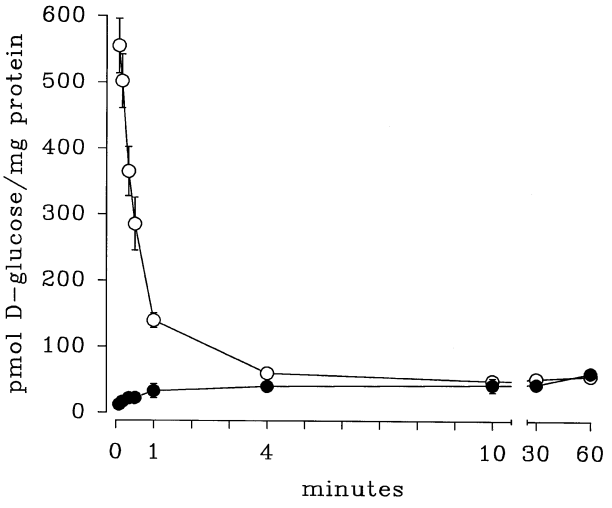

Fig. 1. Time course for uptake of 100 μmol/L D-glucose under *zero-trans* 100 mmol/L NaSCN (○) or KSCN (●) gradient. Membranes were prepared in 300 mmol/L mannitol and 20 mmol/L HEPES/TRIS, pH 7.4. Incubation media contained 100 mmol/L salt, 100 mmol/L mannitol, 20 mmol/L HEPES/TRIS, pH 7.4 and 100 μmol/L D-glucose (100 μmol/L D-[^{14}C]-glucose). Results were expressed in pmol/mg protein. Each value represents the mean ± SE of 5 membrane preparations. Only SEs that exceed size of symbol are shown.

equilibrium in the presence of Na^+ and no overshoot phenomenon in the presence of K^+, as expected.

To distinguish transport from binding, the uptake of L-lysine in equilibrium (60 min) and under standard conditions (100 mmol/L substrate) was studied as a function of the external osmolarity. Figure 2 shows that there is a linear relationship between L-lysine transport and osmolarity of the medium. Extrapolation to infinite osmolarity shows a binding of L-lysine to the membrane of 45.0 ± 3.9 pmol/mg protein (mean ± SE of three membrane preparations). These results indicate that with standard osmolarity of 320 mosm/L and after 60 min incubation, 59% of the L-lysine taken up by the BBMV is in the intravesicular space and the remaining 41% is bound to the membrane. Vesicular volume, calculated according to Berteloot (1984), was 0.63 ± 0.08 μL/mg protein. Vesicular volume calculated using D-glucose as substrate was similar (0.72 ± 0.03 μL/mg protein).

Time Course of Lysine Uptake

Figure 3 shows the uptake of 100 μmol/L-lysine in the presence of inwardly directed NaSCN, KSCN or Glu-Glu gradients (*zero-trans*). Under a Na^+ gradient, L-lysine uptake shows an overshoot with a peak accumulation at 20–30 sec equivalent to 2.8-fold the equilibrium value. Calculation of intravesicular substrate concentration after 60-min incubation and after correction of the

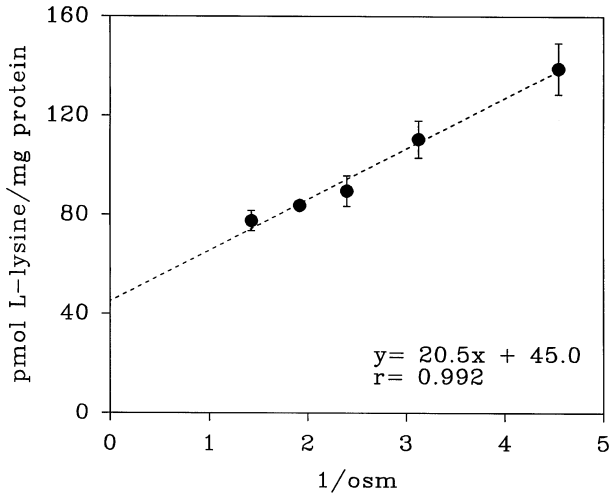

Fig. 2. Effect of increasing transmembrane osmotic gradient after 60 min equilibrium uptake of 100 μmol/L L-lysine under an inwardly directed NaSCN gradient (*zero-trans*, NaSCN 100 mmol/L). Membranes were prepared in 300 mmol/L mannitol and 20 mmol/L HEPES/TRIS, pH 7.4. Incubation media contained 100 mmol/L NaSCN, 100 mmol/L mannitol, 20 mmol/L HEPES/TRIS, pH 7.4, 100 μmol/L L-lysine (6.6 μmol/L L-[^{14}C]-lysine) and variable D-cellobiose concentrations to attain the osmolarities indicated in the figure. Results are presented as pmol/mg protein against the reciprocal of osmolarity in the incubation medium. Each value represents the mean ± SE of 3 membrane preparations. Only SEs that exceed size of symbol are shown. Regression line was calculated by the least-squares method.

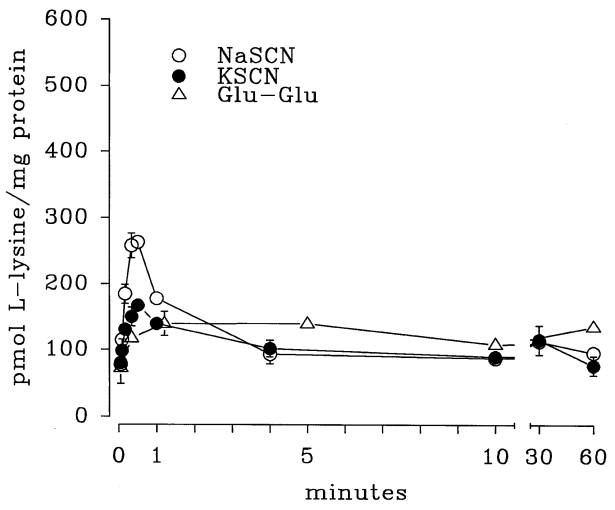

Fig. 3. Time course for uptake of 100 μmol/L L-lysine under *zero-trans* 100 mmol/L NaSCN (○), KSCN (●) or *N*-methyl-glucamine gluconate (△) gradient. Intravesicular and incubation media conditions were the same as described in legend of Fig. 1 except that incubation media contained 100 μmol/L L-lysine (6.6 μmol/L L-[^{14}C]-lysine). Results are expressed in pmol/mg protein. Each value represents the mean ± SE of 3–5 membrane preparations. Only SEs that exceed size of symbol are shown.

amount bound, yields a value of 101 μmol/L, which does not differ from the substrate concentration in the medium. The transient concentration of L-lysine at the peak of the overshoot is about 450 μmol/L (4.5-fold accumulation).

In the presence of a KSCN gradient, there was also an overshoot after 20–30 sec incubation, although somewhat smaller than that obtained with the NaSCN gradient. L-lysine concentration at min 60, was the same as in the NaSCN condition. However, in the presence of a poorly permeant salt, such as Glu-Glu, no overshoot phenomenon was observed.

Initial Rates of L-lysine Transport

L-lysine transport was determined under short-time incubation (1–5 sec) and the results showed that initial rates of uptake were linear in the first 3 sec at least (*see* Fig. 4). This indicates that substrate influx is not yet masked by efflux or by changes in vesicular volume, and ion gradients are not dissipated. The slope of the regression line corresponds to the initial rate of transport, while the intercept of this line with the ordinate represents substrate binding to the external surface of the vesicles (Murer et al., 1984). Figure 4 shows that there are no

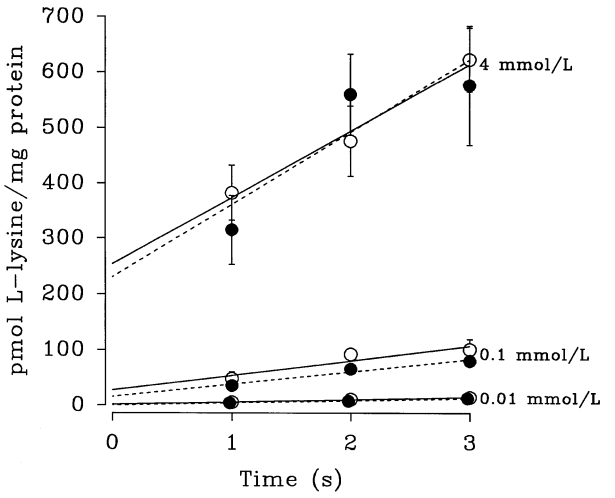

Fig. 4. L-lysine influx (1–3 sec) in the presence of three substrate concentrations, under *zero-trans* 100 mmol/L NaSCN (○, solid line) or KSCN (●, broken line) gradient. Vesicles were prepared as described in legend of Fig. 1 and incubated in the presence of increasing unlabeled L-lysine concentrations (6.6 μmol/L L-[^{14}C]-lysine). Each value represents the mean ± SE of 3–5 membrane preparations. Only SEs that exceed size of symbol are shown. Lines were drawn to linear regression fit, and coefficients of determination were between 0.87 and 0.99.

differences between L-lysine influx in the presence of either Na^+ or K^+ and that the degree of binding of L-lysine to the membrane correlates well with the external concentration of the amino acid.

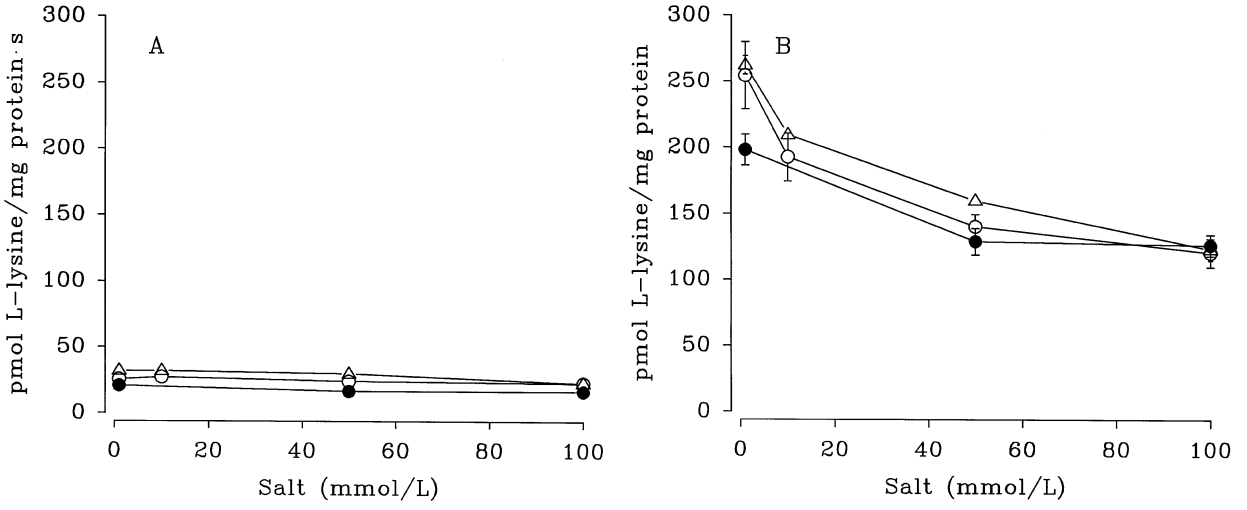

Fig. 5. Effect of Na^+ and K^+ gradient on 100 μmol/L L-lysine uptake (6.6 μmol/L L-[^{14}C]-lysine): (*A*) influx of L-lysine after 3- or 5-sec incubation normalized per sec. (*B*) 60 min incubation. Vesicles were prepared as described in legend of Fig. 1 and incubated in the presence of increasing *zero-trans* NaSCN (○) or KSCN (●) concentrations (1–100 mmol/L). Osmolarity was maintained with: mannitol (○, ●) or *N*-methyl-glucamine gluconate (△). Each value represents the mean ± SE of 2–4 membrane preparations. Only SEs that exceed size of symbol are shown.

Effect of Monovalent Cations

The dependence of initial rates of L-lysine transport on Na^+ and K^+ was studied by incubating the vesicles with extravesicular NaSCN or KSCN, respectively, at external concentrations ranging from 1 to 100 mmol/L and a 0 mmol/L intravesicular salt concentration. In one group of experiments, the external osmolarity was maintained with mannitol whereas in another, NaSCN and KSCN were substituted by Glu-Glu in order to keep the ionic strength constant. In both experimental groups there is no evidence of Na^+ dependency nor K^+ dependency (Fig. 5*A*). L-lysine binding was not significantly affected by salt substitution (*see below*).

However, values obtained at equilibrium (60-min incubation) show that the amount of L-lysine in the vesicles decreases as Na^+ or K^+ concentration increases. Figure 5*B* shows that when mannitol completely substitutes Na^+, the amount of L-lysine at equilibrium is about 260 pmol/mg, a value similar to that obtained with Glu-Glu (263 pmol/mg), supporting the view that the increase in the concentration of diffusible ions in the incubation medium decreases vesicular L-lysine binding.

Effect of Membrane Potential

Since L-lysine transport is not affected by the Na^+ gradient, the overshoot obtained with KSCN and its different magnitude when compared to the NaSCN condition (Fig. 3) may be attributed to an effect of the membrane potential. Initial transport (1–3-sec slopes) were quantified in the presence of 100 mmol/L Cl^-, SCN^- or gluconate salts or in the presence of the equivalent osmolar concentration of mannitol, to generate different membrane diffusion potentials through the membrane (Kimmich et al., 1985).

The higher permeability of SCN^- when compared to Cl^- was verified in experiments in which D-glucose (100 μmol/L) transport was studied (Table 2). The results indicate an increase on D-glucose influx in the presence of NaSCN when compared to NaCl. Transport was significantly reduced in the presence of K^+ salts as predicted for a Na^+-dependent mechanism and no differences were observed between KSCN, KCl or mannitol. No effect of SCN^- is observed on L-lysine influx with either Na^+ or K^+ present and values do not differ from those obtained in the presence of the nondiffusible salt Glu-Glu or mannitol. The *y* intercepts of the slopes were not affected by the different salts tested, thus indicating no differences on substrate binding.

To further investigate the electrogenic nature of L-lysine transport, initial uptake was studied using a valinomycin-induced K^+ diffusion potential. In chicken enterocytes, valinomycin increases 40-fold the K^+ permeability (Kimmich et al., 1985). BBMV were preloaded (1 hr, 21°C) with 100 mmol/L KCl and incubated in the presence of 10 mmol/L KCl, 90 mmol/L *N*-methyl-glucamine chloride and 100 μmol/L L-lysine, either in the presence of 15 μmol/L valinomycin or the corresponding volume of ethanol ($[KCl]_i$ = 100 mmol/L, $[KCl]_o$ = 10 mmol/l ± valinomycin). The induction of a K^+ diffusion potential through the membrane of the vesicles was validated by studying D-glucose transport in the same vesicle preparation but with NaCl instead of

Table 2. Effects of different salts on 100 μmol/L L-lysine and D-glucose influx

Salt (100 mmol/L)	D-glucose (pmol/mg protein · sec)	L-lysine (pmol/mg protein · sec)
NaSCN	111 ± 8.2	25.6 ± 2.4
NaCl	54.5 ± 4.5	26.6 ± 2.9
KSCN	2.4 ± 0.5	21.8 ± 1.4
KCl	3.0 ± 0.5	19.0 ± 1.6
Mannitol	2.7 ± 0.9	24.7 ± 2.2
Glu-Glu		27.8 ± 4.6

Statistical analysis for L-lysine:
NaSCN = KSCN = NaCl = KCl = Mannitol = Glu-Glu ($P > 0.05$)

Vesicles were incubated in the presence of 100 mmol/L zero-*trans* of the corresponding salt ($n = 3$). L-lysine fluxes were calculated from the slopes of 1–3 sec initial uptakes and D-glucose fluxes were measured after 5-sec incubation. Boths were normalized per sec to enable comparison.

N-methyl-glucamine chloride. After 3-sec incubation, valinomycin caused a 2-fold increase on D-glucose uptake, an effect which is maintained after 20-sec incubation (Table 3). L-lysine influx in the presence of the potassium ionophore was not yet affected after 3-sec incubation but significantly enhanced (1.5-fold) after 20-sec incubation. Equilibrium substrate accumulation was not different for either substrate when compared to standard conditions.

Indirect evidence of the effect of the membrane potential was also obtained when L-lysine influx (10 μmol/L) in the presence of D-glucose (10 mmol/L) under *zero-trans* NaSCN conditions was determined (Fig. 6). The results show a strong inhibition (60%, 3-sec incubation) of amino acid influx which may be attributed to the dissipation of the membrane potential caused by Na^+-glucose cotransport.

Substrate Specificity of L-lysine Transport

The interaction between L-lysine and other amino acids was studied in *cis*-inhibition experiments (KSCN gradient), using a low substrate concentration (10 μmol/L) and a 1,000-fold excess (10 mmol/L) of unlabeled L-neutral and L-dibasic amino acids. As shown in Fig. 6, L-lysine and L-arginine are the most powerful inhibitors of L-lysine influx (99 and 96% inhibition, respectively). Neutral amino acids also exerted a significant inhibition of L-lysine influx. Inhibitory effects ranged from 78% (L-alanine) to 90% (L-phenylalanine).

Kinetics of L-lysine Transport

The effect of increasing concentrations of L-lysine and L-methionine in the incubation medium, on 10 μmol/L L-[C^{14}]-lysine entry is shown in Fig. 7. L-lysine and L-methionine cause a significant reduction on L-lysine influx, but with different inhibitory profiles. In the presence of Na^+, L-lysine influx values are better adjusted to a model consisting of two transport systems than to a model based on a single transport mechanism. When the results were analyzed for two transport systems (named A and B, in accordance with Devés et al., 1992), the estimated inhibition constants for L-methionine were 21.0 μmol/L (K_{iA}) and 1.7 mmol/L (K_{iB}) (Table 4). Since *F* represents the ''permeability'' ratio, calculated according to Eq. (2) of Materials and Methods, the value of 0.62 obtained indicates that at low substrate concentrations, system B contributes more than system A to L-lysine entry. The kinetic analysis of the data ob-

Table 3. Effect of a valinomycin-induced K^+ diffusion potential on 100 μmol/L L-lysine and D-glucose uptake

	D-glucose uptake (pmol/mg protein)		L-lysine uptake (pmol/mg protein)	
	3 sec	20 sec	3 sec	20 sec
Control	245 ± 21	298 ± 9	62 ± 14	80 ± 20
+ Valinomycin	502 ± 25	649 ± 26	49 ± 12	138 ± 18

Vesicles were preloaded (1 hr, 21°C) with 100 mmol/L KCl and incubated in the presence of 10 mmol/L KCl, 90 mmol/L NaCl and 15 μmol/L valinomycin or the corresponding volume of ethanol. In L-lysine experiments, Glu-Cl substituted NaCl (90 mmol/L). D-glucose uptake was enhanced 2-fold by valinomycin both after 3- and 20-sec incubation. The effect of valinomycin on L-lysine uptake was only observed after 20-sec incubation (1.7-fold, $P < 0.05$, $n = 3$).

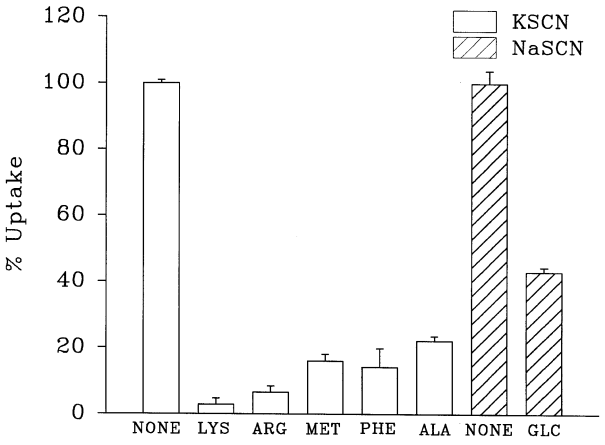

Fig. 6. *Cis*-inhibition of L-lysine influx by other amino acids and by D-glucose. Membrane vesicles were prepared in standard conditions (*see* legend of Fig. 1) and incubated for 3 sec in the presence of 100 mmol/L KSCN, 10 μmol/L L-[^{14}C]-lysine and 10 mmol/L of the unlabeled amino acid or 100 mmol/L NaSCN and 10 mmol/L unlabeled D-glucose (Glc). Each value represents the mean ± SE of three membrane preparations. Only SEs that exceed size of symbol are shown.

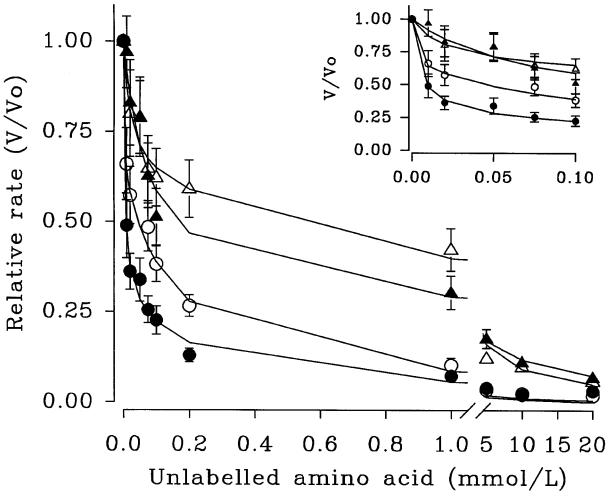

Fig. 7. Relative rates of L-[^{14}C]-lysine (10 μmol/L) influx in the presence of varying concentrations of unlabeled L-lysine (○, ●) and L-methionine (△, ▲), under 100 mmol/L *zero-trans* NaSCN (empty symbols) or KSCN (filled symbols) gradient. Vesicles were prepared as described in legend of Fig. 1. The results were fitted according to Eq. (1) and the lines represent the best fit to two transport systems. Each value represents the mean ± SE of 3–5 membrane preparations. Only SEs that exceed size of symbols are shown.

tained in the presence of K^+ (Table 4), shows that the inhibition constants of L-methionine are K_{iA} = 55 μmol/L and K_{iB} = 3.4 mmol/L, with an *F* value of 1.63. However, it should be considered that K_i obtained with L-methionine in the presence of Na^+ are probably overestimated since inhibition may be due to both competitive and charge movement effects.

The estimated half-saturation constants for L-lysine in the presence of Na^+ were K_{mA} = 2.4 μmol/L and K_{mB} = 164.0 μmol/L and the ratio of maximal influxes (V_{maxA}/V_{maxB}) was found to be 0.009. Thus, at saturating substrate concentrations, the activity of system B would exceed that of system A by more than 100 times. Substitution of Na^+ by K^+ had no effect on affinity but modified the maximum transport capacity ratio, which was increased 3.7-fold. The results of K_i calculation using L-arginine as inhibitor, indicate that this amino acid interacts with two transport mechanisms like L-lysine, but with different affinity (Table 4).

NEM Treatment

The sulfhydryl-binding reagent NEM has been shown to selectively inactivate L-lysine transport through system y^+, both in epithelial (Furesz, Moe & Smith, 1995) and in nonepithelial cells (Devés et al., 1993). Preliminary experiments showed that the maximal inhibition of L-lysine influx under *zero-trans* 100 mmol/L KSCN gradient was obtained with 0.5 mmol/L NEM and this was the concentration used in further experiments. Self-inhibition kinetics of L-lysine transport (5 μmol/L L-[^{14}C]-lysine) in control vesicles indicated the presence of two transport systems (K_{mA} 8.5 μmol/L and K_{mB} 156.0 μmol/L); however, in vesicles pretreated with NEM only one system could be identified (K_m 7.7 μmol/L). These results strongly suggest the existence of a y^+-like carrier system in the chicken intestine.

Accelerative Exchange

Finally, L-lysine influx was studied in vesicles preloaded with 1 mmol/L test amino acid and incubated with tracer amounts (10 μmol/L) of radioactive L-lysine in the presence of valinomycin. The results indicate that L-lysine present at the *trans* side significantly increases its own transport by more than 80% after only 3-sec incubation (Table 6); L-arginine and L-methionine also stimulate L-lysine uptake (37% and 26% respectively), after 1 min incubation. This indicates that L-lysine transport shows accelerative exchange by both cationic and neutral amino acids.

Discussion

One of the problems of studying L-lysine uptake is its capacity to bind to the membrane, as this may represent a significant component of the total uptake. This phenomenon has already been noted by Cassano et al. (1983), Wolfram et al. (1984) and McNamara, Rea and Segal (1986) in brush border membrane vesicles and by Reiser and Christensen (1973) in isolated epithelial cells. After equilibration, there is significant binding resulting from the sum of external plus internal L-lysine fixation. The value obtained by extrapolation to infinite osmolarity is 45 pmol/mg protein (concentration of L-lysine 100 μmol/L), lower than that obtained by Stieger et al. (1983) (125 pmol/mg; 100 μmol/L L-lysine). Moreover, the amount of L-lysine at equilibrium increases as NaSCN and KSCN are progressively substituted by either mannitol or Glu-Glu, as illustrated in Fig. 5*B*. Such increase in accumulation may be a consequence of the reduction of diffusible salts inside the vesicle which may compete with L-lysine for binding sites (low penetrating osmolytes such as mannitol or Glu-Glu do not induce L-lysine efflux from the vesicles) or be the result of a standing Donnan potential, as suggested by McNamara et al. (1986).

Uptake of L-lysine was studied with inwardly directed gradients of Na^+ and K^+ thiocyanate salts and in the presence of the slow diffusible Glu-Glu, and the results show that there is an overshoot only in vesicles incubated with Na^+ and K^+ ions. The overshoot in the presence of Na^+, though greater than that obtained with K^+, is small compared to that obtained with D-glucose (258 and 555 pmol/mg protein, respectively) and takes

Table 4. Inhibition of L-lysine transport by L-methionine and L-arginine

		K_{iA} (μmol/L)	K_{iB} (mmol/L)	*F*
+L-methionine	NaSCN	21.0 ± 8.7	1.7 ± 0.3	0.62 ± 0.1
	KSCN	55.0 ± 8.4*	3.4 ± 0.9*	1.63 ± 0.1*
+L-arginine	KSCN	3.1 ± 0.6	1.3 ± 0.2	†

Inhibition constants were determined under NaSCN or KSCN *zero-trans* gradient ($n = 4$). K_i (inhibition constant) and the ''permeability'' ratio *F* (*see* Eq. 2 of Materials and Methods) was calculated by nonlinear regression analysis from the values of relative rates (v/v_o). (†) Estimation of K_i in the presence of L-arginine was done with an *F* value fixed to 1.63. (in L-methionine data * $P < 0.05$ in each column).

Table 5. Kinetic constants of L-lysine transport

	Salt	K_{mA} (μmol/L)	K_{mB} (mmol/L)	V_{maxA}/V_{maxB}
L-lysine	NaSCN	2.4 ± 0.7	164.0 ± 13.0	0.009
	KSCN	2.7 ± 0.6	131.0 ± 20.4	0.033

Kinetic constants were determined under NaSCN or KSCN *zero-trans* gradient. K_m (half-saturation constant) was calculated by nonlinear regression analysis from the values of relative rates (v/v_o) and with the ''permeability'' ratio calculated in Table 4 taken as fixed value. The ratio of maximum rates (V_{maxA}/V_{maxB}) was calculated from the ''permeability'' ratio (*F*). The statistical analysis showed no significant differences between the kinetic constants in the presence or in the absence of Na^+ ($P \geqslant 0.05$, $n = 4$–5).

Table 6. Effect of 1 mmol/L amino acid efflux on influx of 10 μmol/L L-[^{14}C]-lysine when KCl was equilibrated across the membrane with valinomycin

	Control	+Lysine	+Arginine	+Methionine
3 sec	7.4 ± 0.7	13.7 ± 1.6*	7.8 ± 0.7	5.8 ± 0.8
20 sec	14.0 ± 0.8	22.4 ± 2.3*	17.0 ± 1.1*	13.7 ± 1.5
1 min	16.8 ± 1.0	30.5 ± 2.6*	23.0 ± 2.3*	20.4 ± 3.2*

Vesicles were preloaded (1 hr, 21°C) with 100 mmol/L KCl and 1 mmol/L of unlabeled amino acid. After 50 min of preloading, 15 μmol/L valinomycin was added to the preincubation medium to ensure equilibrated conditions. The incubation medium contains 100 mmol/L KCl, 15 μmol/L valinomycin and 10 μmol/L L-[^{14}C]-lysine. Results were expressed in pmol/mg protein ($n = 3$–4, *$P < 0.05$).

place after 20–30 sec incubation while with D-glucose it takes place at 5-sec incubation or before. This indicates that L-lysine influx in BBMV of the chicken jejunum is much slower than that of D-glucose, in agreement with observations of Cassano et al. (1983) in rat intestinal BBMV.

The existence of an overshoot may indicate an ion dependence or a membrane potential effect. The possibility of ion-dependent L-lysine transport was studied in two ways: on the one hand, by determination of initial rates in the presence of Na^+, K^+, mannitol or Glu-Glu and, on the other, by measuring initial uptakes at NaSCN and KSCN concentrations comprised between 1 and 100 mmol/L. The results of both experiments clearly show that L-lysine influx by jejunal BBMV is ion independent. This agrees with results obtained in the intestine of other animal species (Reiser & Christensen, 1973; Cassano et al., 1983; Stevens et al., 1984; Satoh et al., 1989) but is in conflict with other studies in which an effect of Na^+-gradient was found (Wolfram et al., 1984; Tajima et al., 1993).

Therefore, if the overshoot is not to be attributed to an ion effect it has to be the result of a membrane potential effect. The effects of the membrane potential on transport systems are well documented in the chicken (Kimmich et al., 1995) as well as in other animal species (Acevedo & Armstrong, 1987). To check this hypothesis, initial rates of L-lysine uptake in vesicles incubated with NaCl and NaSCN were compared. In these conditions an inside negative transient potential is expected because SCN^- is 9-fold more permeable than Cl^- in chicken enterocytes (Kimmich et al., 1985). The results using D-glucose as substrate, showing a 2-fold increase in uptake, are consistent with a transient inside negative diffusion potential. However, no increase in the influx of L-lysine with SCN^- gradients was observed.

A second approach was to induce a diffusion potential, preloading the vesicles with KCl in the presence of valinomycin, which is considered the most effective strategy for the demonstration of the electrogenic properties of a transport system (Murer et al., 1984). In these conditions, initial (3 sec) uptakes were again not affected but there was a significant 70% increase in uptake after

20-sec incubation. McNamara, Rea & Segal (1992), in rat renal brush border membrane vesicles, obtained a 77% increase in L-lysine uptake in similar conditions, and also reported that such effects take some time (about 15 sec in their study) to develop. Finally, *zero-trans* gradient experiments comparing NaSCN and KSCN (Fig. 3) confirm that the initial L-lysine uptake is unchanged while a significant effect is observed after 20-sec incubation. The delay in the effects observed would suggest that the potential is not modifying the influx of L-lysine, but instead is slowing one of the efflux routes. Since efflux can take place across the same or different pathways as those used for influx, it is difficult to assign the effect of potential to the transport system(s) mediating L-lysine influx. The experiments with D-glucose added some complication to the interpretation of the data. The simultaneous incubation of L-lysine with D-glucose in the presence of Na^+ induces a Na^+ influx (with the consequent depolarization of the membrane) resulting in a pronounced decrease in L-lysine influx (Fig. 6). This demonstrates that the initial rate of L-lysine uptake is indeed responsive to membrane potential.

It therefore has to be concluded that L-lysine transport is sensitive to membrane potential albeit the uptake rate requires significant time before the internal concentration of L-lysine rises above the control conditions.

The results of this group of experiments support the view that L-lysine uptake is Na^+ and K^+ independent and potential sensitive. Consequently, the possible presence in the chicken jejunum of one (or more) Na^+-dependent transport system(s) such as $B^{0,+}$ (Ganapathy, Brandsch & Leibach, 1994) was not further considered.

Na^+-independent transport of L-dibasic amino acids across the plasma membrane of mammalian cells has been shown to be mediated by at least three different transport mechanisms: systems y^+, y^+L and $b^{0,+}$. Systems y^+ and y^+L also accept neutral amino acids but only in the presence of Na^+. It has been shown that Na^+ increases severalfold the affinity of neutral amino acids for system y^+ (White, 1985) and 60–90 times for system y^+L (Devés et al., 1992). System $b^{0,+}$ is able to transport dibasic amino acids as well as neutral amino acids with similar affinity in the presence and in the absence of Na^+ (Van Winkle, 1993; Palacín, 1994).

The kinetic analysis of L-lysine transport in the chicken jejunum, indicates the presence of a high-affinity carrier, here called system A, and a low-affinity transport system, called system B. The low half-saturation and inhibition constants obtained for system A and the way it interacts with neutral amino acids, approaches this transport mechanism to system $b^{0,+}$. The presence in the brush border of a transport system with similar characteristics to system $b^{0,+}$ has already been demonstrated (Herzberg, Sheerin & Lerner, 1971; Paterson, Sepúlveda & Smith, 1981; Munck, 1985).

In a recent review, Munck and Munck (1994) conclude that $b^{0,+}$ is the only transport system for cationic amino acids, ruling out the presence of alternative Na^+-independent pathways such as y^+. This view is in conflict with the belief that y^+ is an ubiquitous cationic transporter, present at both the apical and basolateral membranes of the enterocyte (Hopfer, 1987; Ganapathy et al., 1994; Mailliard, Stevens & Mann, 1995). The properties of our system B, described in the present study, are: an affinity for L-lysine which is lower than that of $b^{0,+}$; its capacity to recognize neutral amino acids with a slightly higher affinity in the presence than in the absence of sodium and, finally, its sensitivity to NEM, which all point to the identification of system B with system y^+.

The presence of a Na^+-gradient does not affect the K_m for L-lysine transport through either system. However, Na^+ reduces the F constant and the V_{max} ratio. Such changes may be explained either by a lower participation of system A ($b^{0,+}$) or by an increase of the contribution of system B (y^+). Since the y^+ system is a high-capacity transport mechanism, strongly affected by membrane potential (White, 1985), the increase in V_{maxB} could be explained as an effect of an inside negative potential due to the different permeability of Na^+ and K^+. Similar effects were described by Eleno, Devés and Boyd (1994) in the human placenta and by Kavanaugh (1993) in *Xenopus* oocytes. It is worth noting that the influence of a Na^+-gradient on L-lysine uptake at high substrate concentrations is very small (*see* Fig. 4).

In the search for further evidence supporting the existence of a transport system shared by cationic and neutral amino acids, accelerative exchange experiments were carried out. L-lysine influx was measured in short-circuited vesicles preloaded with 1 mmol/L unlabeled amino acids. Accelerative exchange is rapidly manifested for unlabeled L-lysine (already significant after only 3 sec incubation) and for L-arginine (after 20 sec), and with some delay in the case of L-methionine (60-sec incubation). The time needed for the effects of L-arginine and L-methionine to be significant, correlates well with the differences observed on carrier affinity for L-dibasic and L-neutral amino acids. Preloaded L-lysine may exit the vesicle through two transport systems; L-arginine will interact with the same mechanisms but with a lower affinity for system B (y^+), and L-methionine will interact with a much lower affinity for both transport systems. These results contrast with those of Cassano et al. (1983) who were not able to demonstrate heteroexchange of L-lysine influx by neutral amino acids in the rat intestine.

In conclusion, the results shown in the present study support the view that the small intestine of the chicken can absorb L-lysine by two different systems: one, apparently specific for cationic amino acids identified as

system y^+, and a second one, shared with neutral amino acids, identified as system $b^{0,+}$.

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