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Presence of an X_{AG}^- Carrier in Frog (*Rana esculenta*) Red Blood Cells

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Abstract. Evidence is presented that the high levels of internal L-glutamic and L-aspartic acid in frog (*Rana esculenta*) red blood cells are due to the existence of a specific carrier for acidic amino acids of high affinity (K_m = 3 μM) and low capacity (V_{max} 0.4 μmol L-Glu · Kg^{-1} dry cell mass · 10 min^{-1}). It is Na^+ dependent and the incorporation of L-glutamic acid can be inhibited by L- and D-aspartate and L-cysteic acid, while D-glutamic does not inhibit. Moreover, this glutamic uptake shows a bell-shaped dependence on the external pH. All these properties show that this carrier belongs to the system X_{AG}^- family. Besides the incorporation through this system, L-glutamic acid is also taken up through the ASC system, although, under physiological conditions, this transport is far less important, since it has relatively low affinity (K_m 39 μM) but high capacity (V_{max} 1.8 μmol L-Glu · Kg^{-1} dry cell mass · 10 min^{-1}).

Key words: L-glutamic uptake — Acidic amino acids — System X_{AG}^- — Red blood cells — Frog (*Rana esculenta*)

Introduction

The study of the uptake of dicarboxylic amino acids has deserved considerable efforts in both excitable and nonexcitable cells (for a recent review, *see* Berteloot & Maenz, 1990). Two main routes have been established for these amino acids, system X_C^-, which is sodium independent and accepts L-cystine and glutamate, but excludes aspartate (Bannai & Kitamura, 1980) and the ubiquitous system X_{AG}^-, a sodium-dependent system with high affinity for L-glutamate and L- and D-aspartate (Dall'Asta et al., 1983). In addition, minor Na^+-dependent uptake through system ASC has been recognized in different cells (Makowske & Christensen, 1982; Dall'Asta et al., 1983), which may be enhanced by low pH (Makowske & Christensen, 1982). Na^+-independent uptake through system L has also been described for L-glutamate in human fibroblasts (Dall'Asta et al., 1983).

Although red blood cells (RBC) possess high glutathione concentrations and glutamate is a component of the molecule, a specific carrier is lacking in most of these cells (Ellory et al., 1981). However, the existence of an X_{AG}^- carrier has been described in dog RBC (Ellory et al., 1981; Inaba & Maede, 1984).

In the present study, an X_{AG}^- system is reported in frog (*R. esculenta*) RBC, which correlates with the high intracellular levels of both L-aspartate and L-glutamate in these cells. Studies on amino acid carriers in RBC from nonmammalian vertebrates have been mostly limited to neutral amino acids (Ballatori & Boyer, 1988; Fincham, Wolowyk & Young, 1990; Goldstein & Brill, 1991; Gallardo, Planas & Sánchez, 1992; Gallardo & Sánchez, 1993) and there are no indications on the presence of a carrier for anionic amino acids. Moreover, we have directly tested this possibility in trout RBC and a nonmediated uptake was obtained (M.A. Gallardo and J. Sánchez, *unpublished*).

Materials and Methods

Animals were obtained from a commercial supplier (Tarragona, Spain) and maintained deprived of food at 4°C. (^{3}H)-L-glutamate was from New England Nuclear (Germany) and all other reagents were of analytical grade.

Blood was obtained from anesthetized animals (MS-222 buffered with $NaHCO_3$) by puncturing the abdominal vein with heparinized syringes. Cells were washed four times in the following buffer (in mM: NaCl 85.3, KCl 2.25, $MgSO_4$ 1, KH_2PO_4 0.44, $CaCl_2$ 2, Na_2HPO_4

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Table. Amino acid concentration in plasma and erythrocytes from frog (*R. esculenta*)

Amino acids	Plasma	Erythrocyte	In/Out
Taurine	21.9 ± 8.2	39.1 ± 7.2	72.5 ± 38.7
Asp	5.4 ± 0.8	1,074.2 ± 95.8	202.2 ± 32.9
Thr	8.1 ± 1.5	129.5 ± 10.8	21.6 ± 5.2
Ser	10.0 ± 0.7	16.3 ± 3.3	1.8 ± 0.4
Asn	1.9 ± 0.5	12.3 ± 2.6	7.9 ± 2.3
Glu	20.1 ± 2.7	2,211.7 ± 260.4	103.8 ± 21.1
Gln	7.3 ± 1.5	13.8 ± 2.3	1.88 ± 0.2
Gly	16.11 ± 1.4	191.8 ± 79.9	27.0 ± 4.6
Ala	15.7 ± 2.6	49.9 ± 6.6	3.5 ± 0.6
Val	19.35 ± 2.1	162.3 ± 19.7	8.1 ± 1.6
Ile	9.9 ± 2.3	12.9 ± 1.7	1.8 ± 0.3
Leu	27.1 ± 4.7	18.8 ± 2.4	1.2 ± 0.1
Tyr	5.6 ± 0.2	5.0 ± 2.4	0.9 ± 0.0
Phe	14.6 ± 1.1	29.9 ± 6.7	2.6 ± 0.6
GABA	6.9 ± 1.5	1,201.9 ± 124.1	174.2 ± 10.1
Orn	8.6 ± 1.5	11.3 ± 2.1	1.3 ± 0.1
Lys	18.7 ± 2.9	94.6 ± 4.5	6.3 ± 1.3
His	191.8 ± 6.2	9.0 ± 2.3	2.4 ± 0.7
Arg	3.8 ± 0.5	114.2 ± 10	31.7 ± 7.3

Amino acids in RBC were calculated according to Materials and Methods. Results are expressed as the mean ± SD for n = 6–8 individuals. Amino acid concentrations are in μM.

0.33, HEPES 10, $NaHCO_3$ 13, glucose 3, albumin 0.3%; pH 7.63; adjusted to 205–210 mOsm; Mommsen & Storey, 1992, slightly modified). Each time, cells were centrifuged at 340 × g, 8 min, 4°C. After each centrifugation, the upper layer containing leukocytes was discarded.

Cells were always finally resuspended at 2.5 ± 0.5% since amino acid uptake was seen to be highly dependent on the hematocrit of the suspension.

The uptake was carried out at 22.5°C in a shaking bath, in an air atmosphere.

Plasma and whole blood amino acids were analyzed by ion exchange chromatography, as described previously (Canals et al., 1992). The red blood cell amino acid concentration was calculated according to the following relationship:

$$RBC = (WB - (1 - HC) \times P)/Hc$$

where RBC is the calculated amino acid concentration, WB is the whole blood amino acid concentration, P is the plasma amino acid concentration and Hc is the whole blood hematocrit.

Results

The Table shows the free amino acid concentration in frog plasma and RBC, as well as the in/out relationship found. The most notable data were the high accumulation in cells of both aspartate and glutamate, with an in/out ratio over 100. Thus, we tested the possibility that the uptake of acidic amino acids by these cells could be carrier mediated.

Figure 1 shows the time course of the uptakes of 1 and 50 μM L-glutamic in a Na^+-containing medium. While the incorporation of 50 μM L-glutamic was nearly linear during the experimental time considered, the uptake of 1 μM L-glutamic showed a decline in the uptake rate, with a $t_{1/2}$ of about 65 min and a calculated maximal incorporation of amino acid at equilibrium of 1.2 μmol L-Glu · Kg^{-1} dry cell mass. Water content measured gravimetrically for these cells was 65% and, consequently, using 1 μM L-glutamic, the apparent internal concentration of this amino acid obtained at equilibrium was 605 nM, which is far from the physiological values measured (Table). However, the uptake obtained in a Na^+-free medium was lower than that obtained in the Na^+-containing medium, suggesting that the incorporation of L-glutamic to frog RBC is carrier mediated, at least partially.

Figure 2*A* shows the 10 min uptake of L-glutamic over a wide substrate concentration range of 1–1,000 μM in a Na^+-containing medium. Here, the uptake appears mainly as nonsaturable, indicating that at high substrate concentrations, most of the uptake is a nonmediated process. However, in a low concentration range a saturable uptake can be seen (Fig. 2*B*). The Eadie-Hofstee plot of the mediated uptake (total minus diffusion) revealed two components, indicating the possibility that more than one carrier may be involved in the incorporation of L-glutamic to these cells (Fig. 2*C*). One of them was a high affinity (K_m 3 μM), low capacity system (V_{max} 0.4 μmol L-Glu · Kg^{-1} dry cell mass · 10 min^{-1}), while the second system was of relatively low affinity (K_m 39 μM) and high capacity (V_{max} 1.8 μmol L-Glu · Kg^{-1} dry cell mass · 10 min^{-1}). Because high concentrations of different amino acids, known sub-

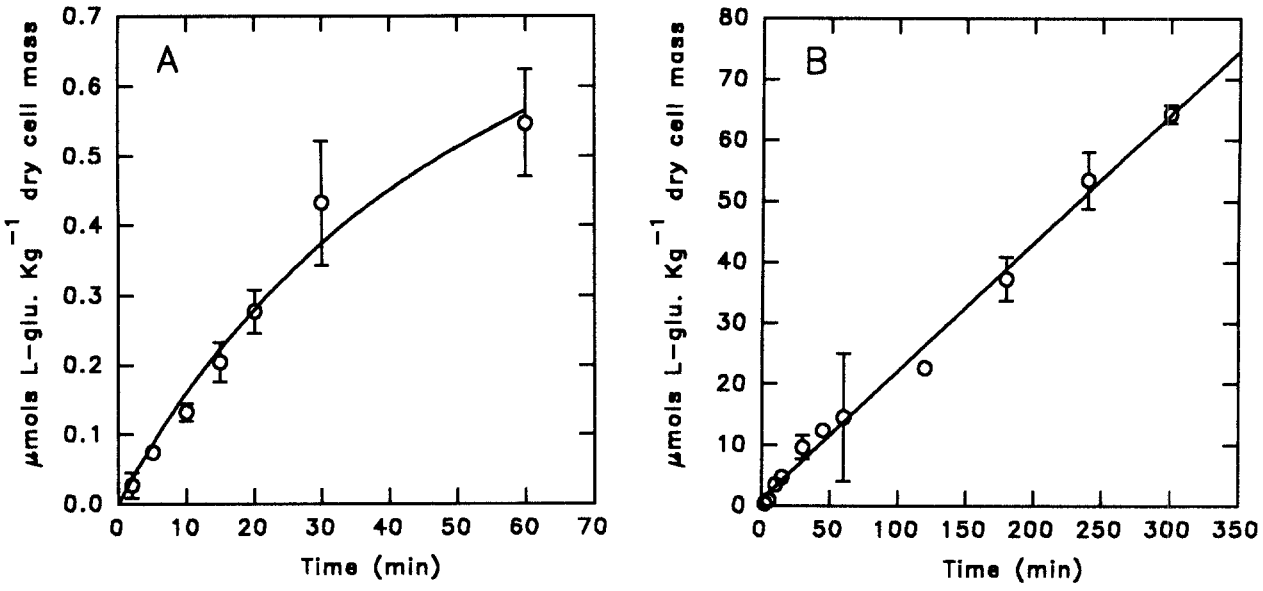

Fig. 1. Time course of L-glutamate uptake by frog RBC. Cells were suspended in the Na^+-containing medium and incubated for different times in the presence of 1 µM (*A*) or 50 µM (*B*) L-glutamate and 2 mM amino-oxyacetic acid. Each point is the mean of 3–5 individual experiments. Bars show the standard deviation.

strates of system L, system asc or system X_C^- did not inhibit the Na^+-independent uptake of 1 µM L-glutamic acid, it was concluded that there was no substantial incorporation through these systems, indicating that the two possible carriers should be Na^+ dependent.

To identify these two Na^+-dependent systems implicated in the uptake of L-glutamic acid by frog RBC, several amino acids (1 mM), were used to inhibit the incorporation of 1 µM L-glutamate. Figure 3 shows that there were two groups of amino acids able to inhibit this uptake in a Na^+-containing medium. L- and D-aspartic acid, L-cysteic acid and L-glutamic acid lowered the uptake by more than 80%, while L-alanine, L-serine and harmaline depressed it by only 20%. However, D-glutamic acid, α-amino adipic acid and N-methyl-glutamic acid had no effect on the uptake. These data suggest that L-glutamic is taken up through both the X_{AG}^- and ASC systems.

Figure 4 shows the effect of external sodium on L-glutamic acid uptake into frog RBC. Results cannot easily be fitted to either a hyperbolic or a sigmoidal plot. Hence, we have been unable to obtain these kinetic data.

Figure 5 shows the effect of varying extracellular pH on the 1 µM L-glutamic Na^+-dependent uptake, which is L-alanine resistant. Maximal incorporations were between pH 6.5–7. Lower and higher pH inhibited the uptake.

Finally, a further characterization of this carrier was attempted by consideration of *trans* effects. Results indicate that L-glutamic uptake was not *trans*-stimulable. However, this may be an artifact because we were unable to deplete internal amino acids from frog RBC, even after four hours of incubation in an amino acid-free medium (*data not shown*).

Discussion

Frog RBC contain an unexpectedly high amount of dicarboxylic acids, L-aspartate and L-glutamate. Only GABA concentration is of the same order of magnitude and these three amino acids account for 80% of total amino acid content in these cells. Although different fish species show an RBC/plasma ratio between 10 and 30 for L-glutamic or L-aspartic (Ogata & Arai, 1985; Fincham et al., 1990) and in rats this ratio is 4–10 (Viñas, 1986), there is no indication that these gradients may be achieved through the existence of a specific carrier for acidic amino acids. However, in the present study this ratio is at least 100, thus the existence of a membrane transporter for these amino acids in frog RBC is not surprising even though no concentrated uptake of L-glutamate was observed in this study. However, this anomalous situation has been reported for other amino acids, such as glycine, in different cell systems (Fincham et al., 1990; Gallardo & Sánchez, 1993). Moreover, under the same experimental conditions, L-alanine became three times concentrated in these cells (M.A. Gallardo, M.I. Ferrer, J.L. Albi, J. Sánchez, *unpublished data*), thus ruling out experimental artifacts, which might have impaired the uptake.

The results obtained in the present study indicate the existence of a membrane carrier in frog RBC that is

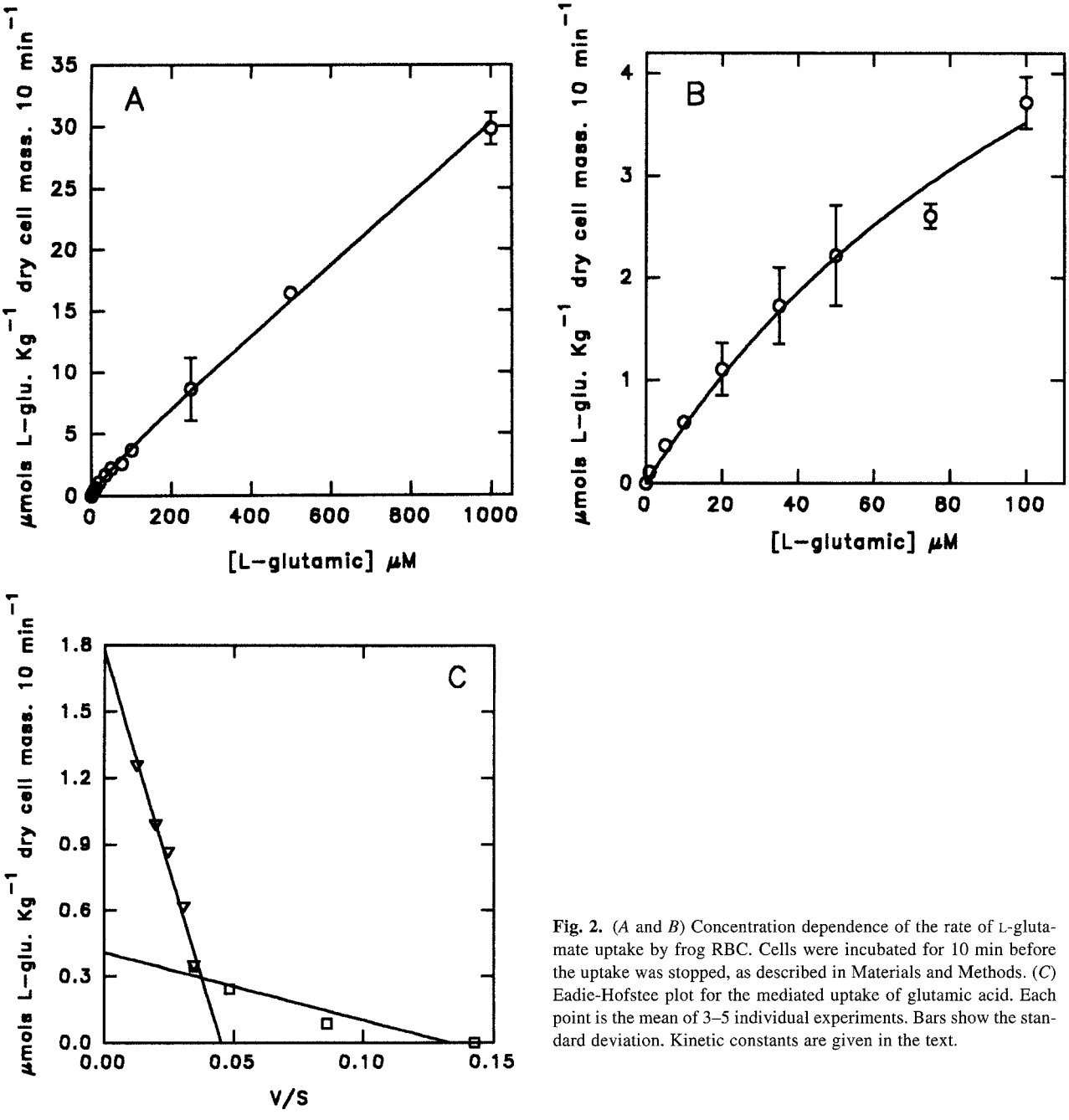

Fig. 2. (*A* and *B*) Concentration dependence of the rate of L-glutamate uptake by frog RBC. Cells were incubated for 10 min before the uptake was stopped, as described in Materials and Methods. (*C*) Eadie-Hofstee plot for the mediated uptake of glutamic acid. Each point is the mean of 3–5 individual experiments. Bars show the standard deviation. Kinetic constants are given in the text.

specific for acidic amino acids. In general terms, competition analysis allows us to identify this carrier as an X^-_{AG} system. The extra negative charge of acidic amino acids is a strong requirement for this carrier, and L-glutamate, L-cysteate, L-aspartate react with it. Interestingly, this system shows an anomalous stereoselectivity, accepting D-aspartate but not D-glutamate (Gazzola et al., 1981; Berteloot & Maenz, 1990).

The low K_m values found, one order of magnitude below plasma L-glutamate concentration, reinforce the identification of this carrier as an X^-_{AG} system (*see ref.* Berteloot & Maenz, 1990, for K_m values in different systems) and indicate that "in vivo," this system always operates under saturating conditions. The characteristic "bell-shaped" effect of extracellular pH on the L-glutamate uptake, which has been found in several cell systems for the pH dependence of the X^-_{AG} system, was also obtained in the present study.

A more confusing situation appeared when the Na^+ dependence of L-glutamate uptake was considered. Activation of the uptake by external sodium showed a sigmoidal shape for cation concentrations above 50 mM,

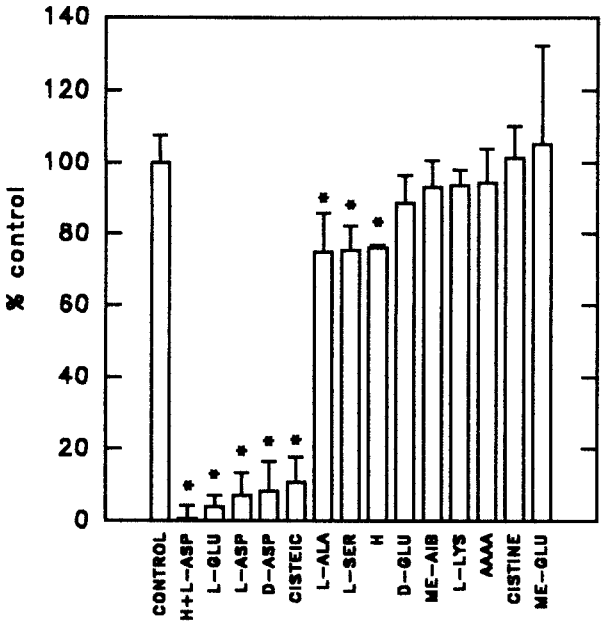

Fig. 3. Effect of different amino acids (1 mM) on the Na^+-dependent uptake of 1 μM L-glutamate by frog RBC. Cells were incubated for 10 min in either Na^+-containing or K^+-containing medium. The difference between both uptakes is shown. Values are the mean of 3–6 individual experiments and bars show the standard deviation. (*) Values significantly different from the control value in the absence of added amino acids ($P < 0.001$). *H:* harmaline, *AAAA:* α-aminoadipic acid, *ME-GLU:* N-methyl-glutamic acid.

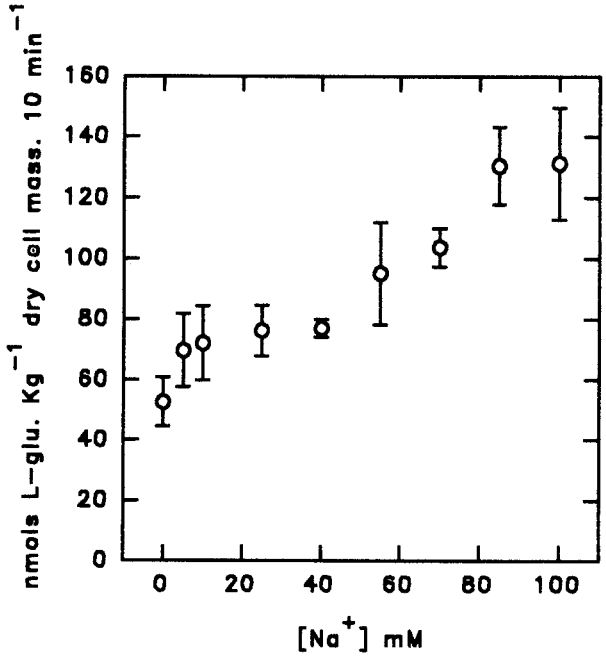

Fig. 4. Na^+ concentration dependence of the uptake of 1 μM L-glutamate by frog RBC. Potassium was used throughout to replace sodium, and osmolality was maintained constant (205 mOsm). The rates were measured after 10 min of incubation. Each point is the mean of six individual experiments. Bars are standard deviation.

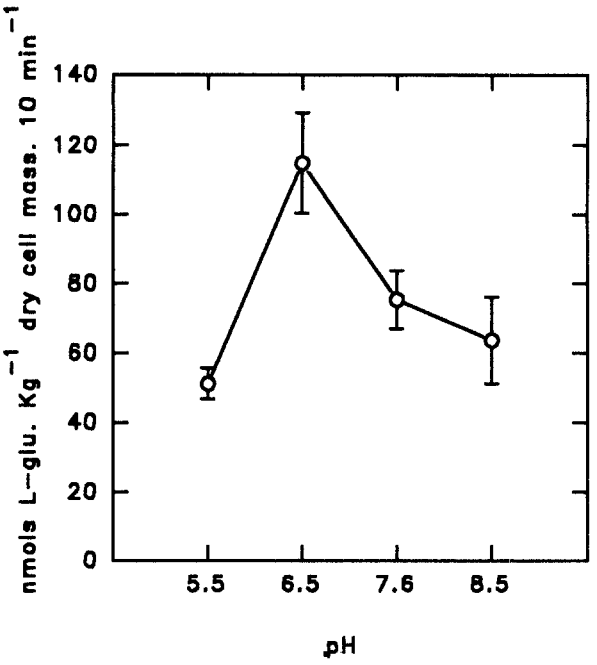

Fig. 5. Effect of extracellular pH on the rate of uptake of L-glutamate through the X^-_{AG} system by frog RBC. Cells were incubated for 10 min. Each point is the mean of three individual experiments. Bars are standard deviation.

while there were no changes at lower values, except for an increase in incorporation in the presence of an extremely low Na^+ concentration with respect to its absence, the significance of which remains obscure. In any case the sigmoidal part of the curve indicates a stoichiometry above 1:1, which has been observed by some other authors (Harvey & Ellory, 1989), although it has been indicated that this kind of relationship is not proof of higher-order sodium dependences (Berteloot & Maenz, 1990).

As seen in Fig 3, besides the inhibition of acidic amino acids, L-glutamate uptake is also inhibited by short-chain neutral amino acids such as L-Ala, L-Ser and L-Cys, which are recognized substrates of the ASC system. Uptake of L-glutamate through system ASC was recognized several years ago (Makowske & Christensen, 1982) and at low pH values, most L-glutamate may be incorporated through this system. Interestingly, in the present study, the uptake through this system was not constant, and some kind of environmental dependence of the activity of system ASC seems to be present in frog RBC, as has been observed for the uptake of glycine in trout RBC (Gallardo & Sánchez, 1993). Thus, from the beginning of this study in February to its completion at the end of April, activity of the ASC system dropped continuously.

Harmaline has been used as a competitive inhibitor of Na^+ in the activity of the ASC system (Young, Mason & Fincham, 1988) and in the present study it was

able to inhibit the uptake of L-glutamate to the same extent as L-alanine. The lack of inhibition of harmaline on the uptake of L-glutamate through the X^-_{AG} system suggests that the actual role of Na^+ is not the same in the different Na^+-dependent carriers.

The physiological significance of such a specific carrier for L-glutamate in frog RBC is not clear. Dietetic behavior does not seem the main reason for the presence of the X^-_{AG} system in carnivore RBC, because this system is lacking in RBC from trout, which have the same kind of diet as frogs. In any case, most of the properties showed by the carrier in frog RBC coincide with those described for mammalian carrier, indicating a remarkably high degree of maintenance of its structure.

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