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Uptake of L-leucine by Trout Red Blood Cells and Peripheral Lymphocytes

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Abstract. The uptake of L-leucine by trout red blood cells and peripheral lymphocytes has been analyzed. The present study shows two functionally different Na^+-independent systems for apolar branched-chain amino acids. They are designated as L systems because they share some properties with the mammalian L system. The carrier present in red blood cells has low K_m values, is *trans*-stimulable and not stereospecific for leucine uptake; on the other hand, the system present in lymphocytes is stereospecific for leucine uptake and *trans*-inhibitable. Both carriers are pH sensitive in a similar fashion at low pHs, but there are important differences at higher pH values (above neutrality). These properties are compared with these of the *asc* systems previously reported in these cells.

Key words: Leucine uptake — Red blood cell (RBC) — Peripheral blood lymphocytes (PBL) — L system — Trout (*Salmo trutta*)

Introduction

The transport of neutral amino acids into eukaryotic cells is effected by several distinct systems, with overlapping substrate specificity (Christensen, 1979). Several of these carriers are Na^+ dependent, such as systems A, ASC, *Gly* and β*;* while other are Na^+ independent, such as systems L, C and *asc* (Christensen, 1989; Barker & Ellory, 1990).

Although studies on amino acid uptake have largely been limited to mammal and bird cells (Barker & Ellory, 1990), there are recent reports of carriers for neutral amino acids in several fish cells. Thus, the β, ASC, *Gly*, *asc* and L systems have been found in fish cells (hepatocytes, red blood cells or lymphocytes) (Fincham, Wolowyk & Young, 1987, 1990; Ballatori & Boyer, 1988, 1992; Canals et al., 1992; Gallardo, Planas & Sánchez, 1992; Gallardo & Sánchez, 1993; Albi et al., 1994). β, ASC, *Gly* and *asc* systems have been characterized but references to the L system are lacking.

The mammalian L system was originally described in Ehrlich ascites tumor cells as an Na^+-independent amino acid transporter (Oxender & Christensen, 1963), but it has ubiquitous distribution (Barker & Ellory, 1990), the name due to its high leucine transport rates. System L shows a broad spectrum of substrates, and is able to interact with most neutral amino acids, but it preferentially transports large apolar branched-chain and aromatic amino acids (Oxender & Christensen, 1963). The L system usually has a high transport capacity, probably due to a high level of expression, and even a poor substrate of this system may achieve a significant flux through this carrier (Ellory, 1987). It was assumed that system L is specifically inhibited by BCH 2-aminobicyclo(2,2,1)heptane-2-carboxylic acid, a nonmetabolizable amino acid analogue (Christensen et al., 1965; Christensen, 1975; Christensen, 1984), although it was later shown that the Na^+-independent T system is also inhibited by this analogue (Vadgama & Christensen, 1985). The L system is also characterized by its ability to operate in the exchange mode (Christensen, 1975); but is not purely an exchange agent, because it can transport amino acids intensely and unequivocally (Guidotti, Borghetti & Gazzola, 1978).

In the present study, system L has been characterized through the uptake of L-leucine in both red blood cells and peripheral blood lymphocytes from trout. Several differences have been found between the two subsystems.

Materials and Methods

Brown trout (*Salmo trutta*) (250–400 g) were obtained from fish farms (Medi Natural, Generalitat de Catalunya) in the Pyrenees, where they

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were maintained in open-water circuits, directly connected to a river. The experiments were carried out at different times during a whole year.

Blood was obtained by caudal puncture, diluted with heparinized RPMI 1640 (Sigma, St. Louis, MO), rinsed three times and left at 4°C to eliminate a possible catecholamine effect. Red blood cells (RBC) were separated from mononuclear white cells by centrifugation with Histopaque-1077™ (Sigma), following the procedure suggested by the supplier, slightly modified because of the high viscosity of trout blood. Adherent mononuclear cells were separated from lymphocytes (PBL) by incubation for 1 hr at 4°C. PBL and RBC were rinsed separately four times in Cortland buffer (pH 7.4) (Houston et al., 1985), slightly modified (mM: NaCl 141, KCl 3.5, $MgSO_4$ 1, NaH_2PO_4 3, $CaCl_2$ 1, pyruvic acid 2, HEPES 10, bovine serum albumin 0.3%, glucose 3). The osmolality was adjusted to 305 mOsmol.Kg^{-1}. During this process cells became depleted of amino acids (*data not shown*). When cells were used for experiments using buffers with a different ionic composition, the whole rinsing procedure was performed with the final buffer.

All chemicals were of analytical grade. Tritiated L-leucine (4,5-^{3}H(N)) was obtained from New England Nuclear (Germany). BCH was in its commercial form (DC isomers).

For uptake experiments, both cells and solutions were preequilibrated at 15°C before experiments were started by mixing (1:1, v/v) the cell suspension with the labeled L-leucine (^{3}H, 0.3 μCi/ml cells was added) to obtain the desired concentrations and a final hematocrit of about 10%. Amino-oxyacetic acid (2 mM), a transaminase inhibitor, was used throughout. Incubations were performed in a shaking bath at 15°C, using air as the atmosphere.

Leucine uptake was stopped by diluting with Cortland buffer (1:9, v/v) containing a 10- to 50-fold excess of nonradioactive amino acids, and rinsing the cells with this solution (1:9, v/v) three times. RBC and PBL were separated each time by centrifugation (810 × *g* for 8 min at 4°C). PBL were finally lysed with 0.1% Triton X-100, while RBC were deproteinized by adding sufficient ice-cold perchloric acid to obtain a final concentration of 6%. A clear supernatant was obtained by centrifugation (3,000 × *g* for 20 min at 4°C). The radioactivity of the resulting solution in each case was counted in a well-type liquid scintillation counter (Packard, Great Britain).

Cell protein was determined by the Coomassie Blue technique (Bio-Rad). RBC hemoglobin was determined by the Drabkin reagent.

Curve-fitting of experimental data was performed by nonlinear regression analyses.

Results

Figure 1*A* shows a typical L-leucine accumulation by RBC and PBL. Data from Fig. 1*B* show that most of the uptake is Na^+ independent for both cell types. Hence, the rest of the experiments were carried out with K^+ as the main extracellular cation. The uptake of L-leucine was linear for 15 min in a concentration range between 1 and 1,000 μM (*data not shown*) and incubations of 10 min were used for subsequent experiments. For 300 μM leucine, the amount taken up at equilibrium by PBL was 15 times higher than the RBC amino acid uptake.

The concentration dependence on the rate of L-leucine uptake by these cells is shown in Fig. 2A and *B.* The uptake appears mainly nonsaturable, suggesting that at high substrate concentrations, most of the L-leucine uptake is a nonmediated process. However, a saturable uptake can be seen at low concentrations. Uptake data can be fitted to a model including a Michaelis-Menten plus a linear term by the RBC and PBL. The Eadie-Hofstee plot of the Michaelis-Menten term by both cells (*data not shown*) revealed only one component, suggesting that in each case only one carrier is involved. Although there are differences in the K_m values between RBC (84 ± 12 μM) and PBL (145 ± 62 μM), there is a more important difference between their maximal capacities: low capacity (V_{max} = 4.5 ± 0.2 pmols L-leucine. min^{-1}. mg^{-1} Hb) for RBC *vs.* high capacity (V_{max} = 47.3 ± 9.6 pmols L-leucine. min^{-1}. mg^{-1} prot) for PBL, probably related to the different needs of leucine in these cells.

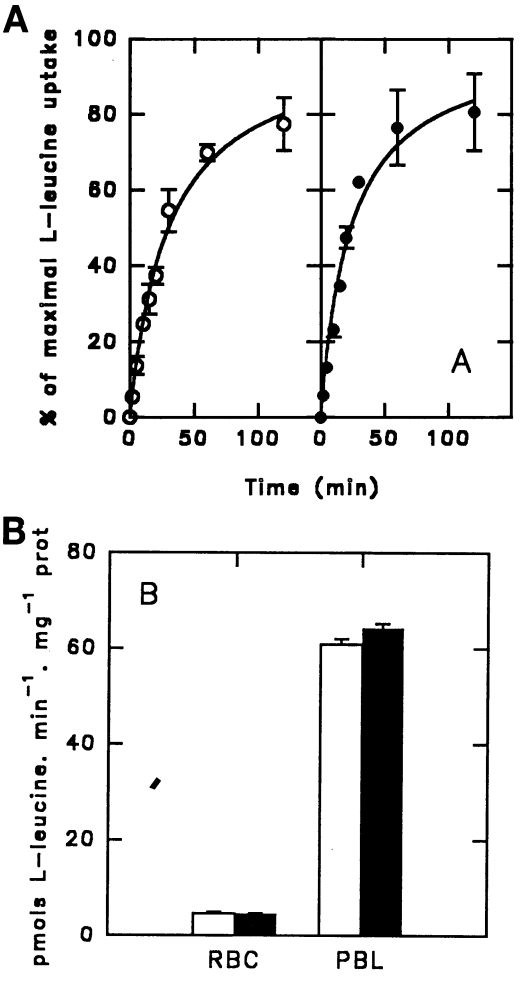

Fig. 1. (*A*) Time courses of L-leucine by trout red blood cells (RBC) (○) and peripheral blood lymphocytes (PBL) (●). Cells were suspended in buffers containing potassium as the main cation and were incubated for different times in the presence of 10 μM L-leucine. Results are expressed as the percentage of the maximal L-leucine uptake. (*B*) 300 μM L-leucine initial rates by RBC and PBL in the presence of sodium (□) or potassium (■) as main cation. Cells were incubated for 10 min before the uptake was stopped as described in Material and Methods. Each point is the mean of three individual experiments. Bars are standard deviation of the mean.

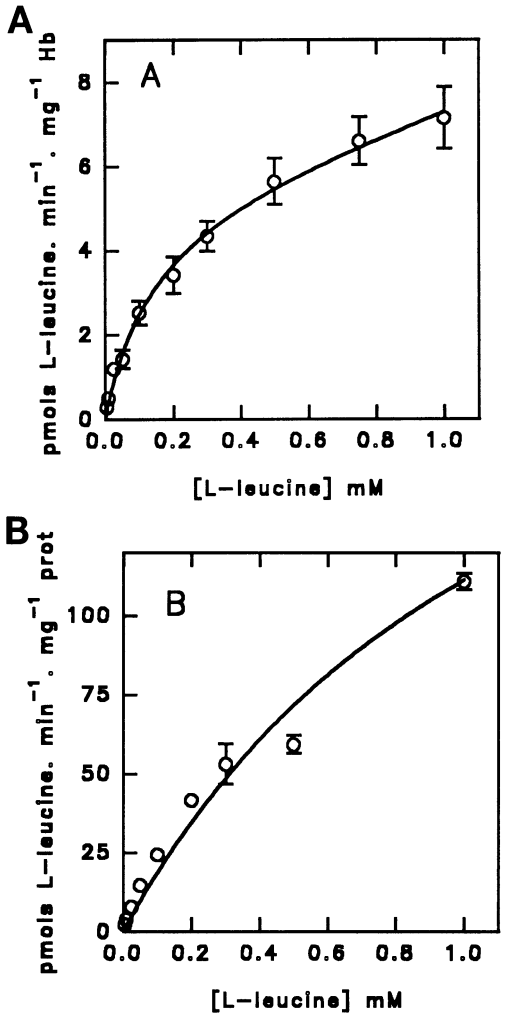

Fig. 2. (*A* and *B*) Concentration dependence of the rate of L-leucine uptake by trout RBC (*A*) and PBL (*B*). Cells were incubated for 10 min before the uptake was stopped. Each point is the mean of 3–5 individual experiments. Bars show the standard deviation.

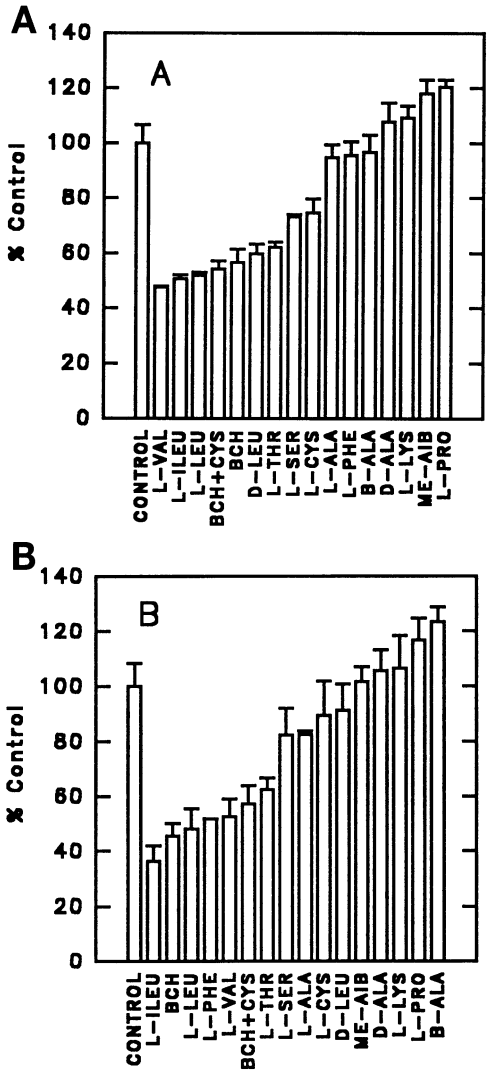

Fig. 3. Inhibition of different amino acids (5 mM) of the 300 μM L-leucine by trout RBC (*A*) and PBL (*B*). Cells were incubated for 10 min in Na^+-free media. Values are expressed as a percentage of control uptake. Each point is the mean of three individual experiments. Bars are standard deviation of the mean.

To characterize these Na^+-independent carriers, the uptake of 300 μM L-leucine was *cis*-inhibited by high concentrations (5 mM) of several amino acids (Fig. 3). The main difference between the cells was the inhibition caused by D-leucine and L-phenylalanine. The RBC carrier (Fig. 3*A*) did not show stereospecificity and the L-phenylalanine caused a poor inhibition. In contrast, the PBL carrier (Fig. 3*B*) showed stereospecificity and it was inhibited by L-phenylalanine. Both carriers were inhibited by most of the neutral amino acids but large apolar branched-chain amino acids were the strongest inhibitors, suggesting that L-leucine was taken up through L system by trout RBC and PBL.

A further analysis of inhibition kinetics was carried out for BCH (inhibitor of the L system, Christensen et al., 1965) (Fig. 4), L-cysteine [a good substrate of Na^+-independent systems *asc* and L (Oxender & Christensen, 1963; Fincham, Mason & Young, 1985) and used as a nearly specific inhibitor for the *asc* system] (Fig. 5) and L-alanine [a good substrate of trout Na^+-independent *asc* systems (Albi et al., 1994)] (Fig. 6). Results show that both BCH and L-cysteine were competitive inhibitors of the L-leucine uptake by trout RBC (*A*) and PBL (*B*). However, L-alanine was not. RBC K_is (mM) for BCH, L-cysteine and L-alanine were respectively 0.52 ± 0.01, 2.66 ± 0.1 and higher than 50. PBL K_is (mM) were 0.98 ± 0.17, 2.42 ± 0.45 and 22 ± 3. Absolute uptake rates in Figs. 4*A*, 5*A* and 6*A* for RBC and 4*B*, 5*B* and 6*B* for PBL varied somewhat, probably owing to the different season of the year when these experiments were carried out.

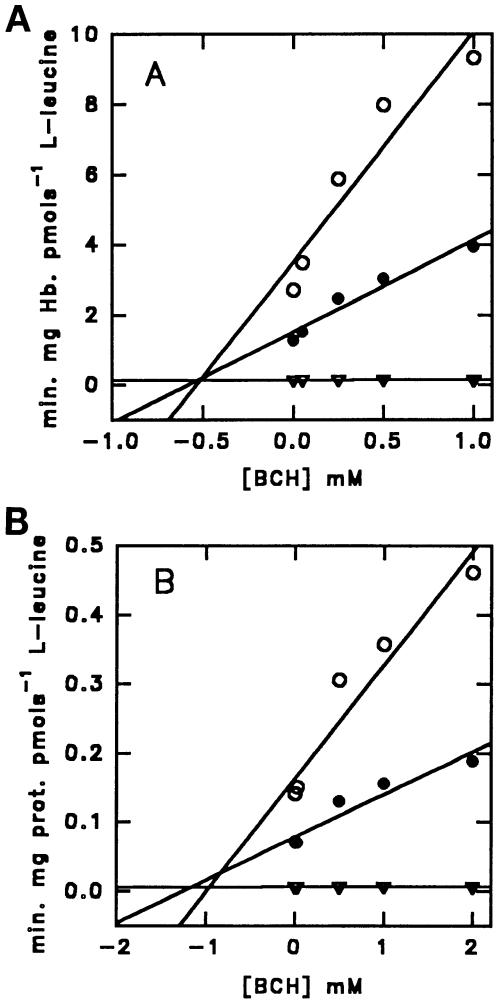

Fig. 4. Kinetics of BCH on the uptake of L-leucine [10 (○), 25 (●) and 1,000 (▽) μM by trout RBC (*A*) and PBL (*B*). The K_is values are given in the text.

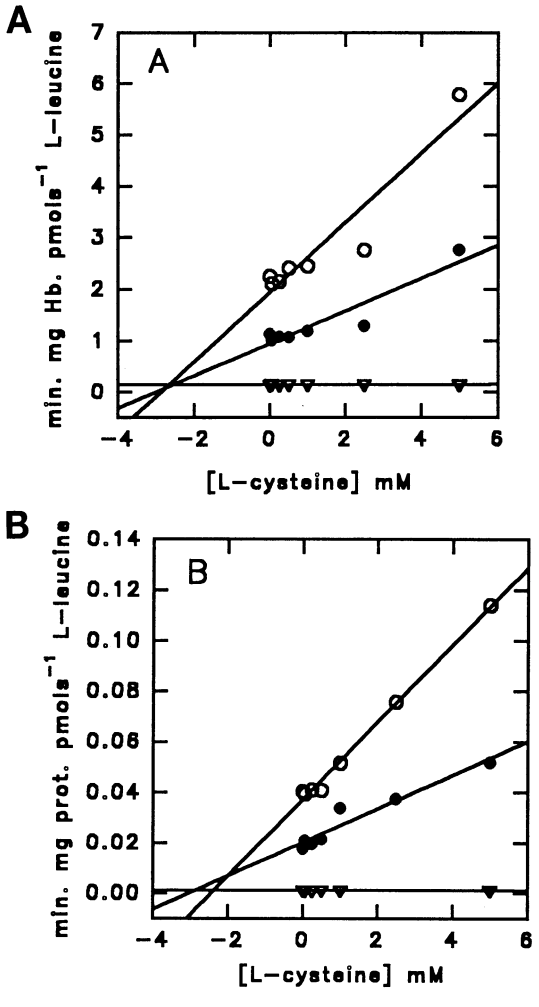

Fig. 5. Kinetics of L-cysteine on the uptake of L-leucine [10 (○), 25 (●) and 1000 (▽) μM] by trout RBC (*A*) and PBL (*B*). The K_is values are given in the text.

The inhibitions obtained above pose some questions on the specificities of the carriers studied. The concentration dependence of the rate of total Na^+-independent L-leucine uptake, BCH-inhibitable L-leucine uptake and L-cysteine-inhibitable L-leucine uptake by trout RBC is shown in Fig. 7. The incorporation of BCH-resistant L-leucine was linear, suggesting that BCH completely inhibits the carrier-mediated uptake of L-leu and although L-Cys did not fully inhibit the carrier-mediated uptake of L-leu, there were no significant differences between the K_ms for the BCH- or L-Cys-sensitive L-leucine uptake (*data not shown*) and the K_m described for the RBC L system.

In addition to the experiments described above, the activity of L systems was characterized by means of *trans* and pH effects. *Trans* effects were measured by preloading cells with nonradioactive 300 μM L-leucine for different times to achieve different intracellular L-leucine concentrations; tracer L-leucine was then added externally and the amino acid uptake was allowed for 10 min. Figure 8 shows the effect of these different L-leu concentrations on the rate of BCH-sensitive tracer uptake. The RBC L system became *trans*-stimulable, but the PBL L system was *trans*-inhibitable.

Finally, Fig. 9 shows the effect of varying extracellular pH on the BCH-sensitive 300 μM L-leucine uptake. The RBC L system presents minimal uptakes at nearly neutral pHs, while both lower and higher pHs activated the uptake. On the other hand, the L system from PBL was less sensitive to external pH than the RBC L system, but it was activated when the pH was below 6.5.

Discussion

As indicated in the introduction, system L preferentially transports large apolar branched-chain and aromatic

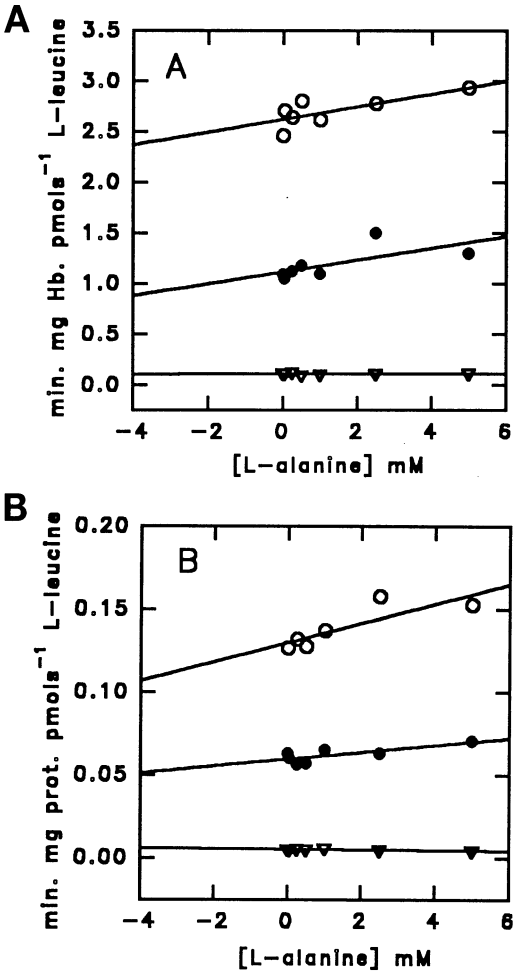

Fig. 6. Kinetics of L-alanine on the uptake of L-leucine [10 (○), 25 (●) and 1000 (▽) μM] by trout RBC (*A*) and PBL (*B*). The K_is values are given in the text.

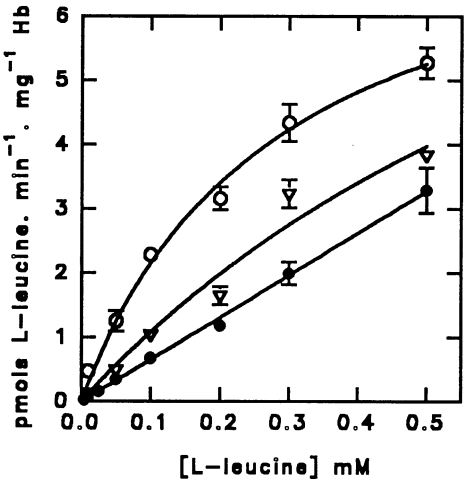

Fig. 7. Concentration dependence of the rate of L-leucine uptake by trout RBC. Cells were incubated for 10 min before the uptake was stopped. (○) Total uptake. (●) Uptake in the presence of 5 mM BCH. (▽) Uptake in the presence of 5 mM L-cysteine. Each point is the mean of three individual experiments. Bars show the standard deviation.

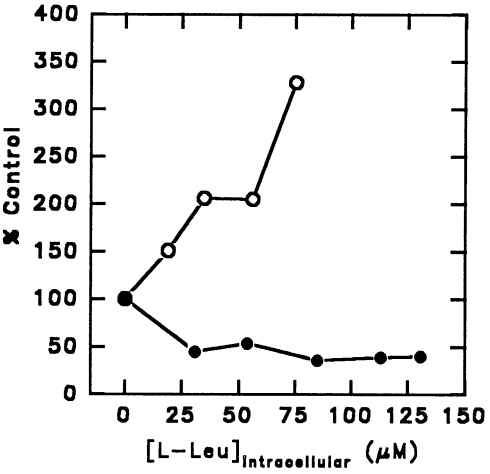

Fig. 8. *Trans* effects on the rate of L system uptake by trout RBC (○) and PBL (●). They were preloaded with ''cold'' L-leucine for different times to achieve different intracellular concentrations, washed twice and incubated for 10 min with 300 μM labeled L-leucine. The rate of L system uptake was measured as L-leucine total uptake minus L-leucine uptake in the presence of 5 mM BCH. Results are expressed as a percentage of the control uptake (without preloading). Each point is the mean of three individual experiments.

amino acids (Oxender & Christensen, 1990). Its presence in trout cells was shown previously (Gallardo & Sánchez, 1993) and here it has been characterized through the uptake of L-leucine.

The existence of a clear-cut distinction between the *asc* and L systems has been accepted for cells from higher vertebrates, but this may not be the case for fish cells. In the two types tested, PBL and RBC, leucine is taken up at low concentrations mainly through carrier-mediated systems operating without the help of the transmembrane Na^+ gradient. As for the *asc* systems found in trout cells (Albi et al., 1994), the properties of leucine uptake differ somewhat between the two cell types considered, suggesting functionally different L systems, the activity found in RBC being more similar to the system L from higher vertebrates (high affinities for the substrate, *trans*-stimulation and broad substrate specificity; Oxender & Christensen, 1963; Christensen, 1975; Barker & Ellory, 1990), while the carrier present in PBL was not *trans*-stimulable and showed stereospecificity. Instead, the *asc* system present in trout PBL does not show stereospecificity (Albi et al., 1994). The activities of the *asc* systems found in erythroid and some nonerythroid cells (Kuhlmann & Vagdama, 1991; Albi et al., 1994), but not in trout PBL (Albi et al., 1994) show a characteristic dependence on the external pH, being highly activated at low pHs. In the present study, a similar situ-

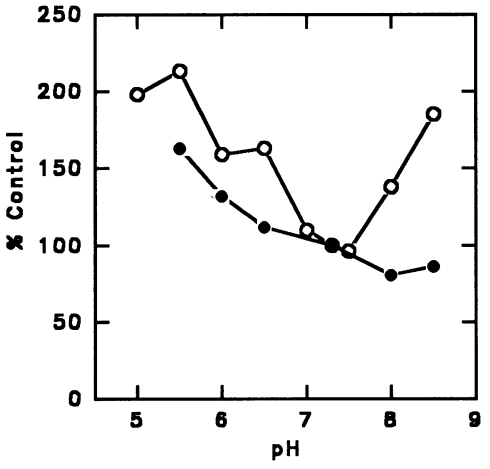

Fig. 9. Effect of extracellular pH on the rate of L system uptake of 300 μM L-leucine by trout RBC (○) and PBL (●). Cells were incubated for 10 min. Results are expressed as a percentage of the uptake measured at pH 7.4. The rate of L system uptake was measured as described the Fig. 8. Each point is the mean of three individual experiments.

ation was found, although with lower slopes, in spite of the fact that the L system from high vertebrate cells is only slightly dependent on external pH (Barker & Ellory, 1990). Nevertheless, the results were slightly more complex in RBC, where an activation at pHs above neutrality was found also, obtaining an inverted bell-shape for the plot, similar to that found for some other amino acid carriers (e.g., the X_{AG} system; Berteloot & Maenz, 1990; Gallardo, Ferrer & Sánchez, 1994). The different behavior of this carrier in the two cell types may be explained by differences in the carrier and/or in the membrane environment near to the carrier. However, none of these possibilities have been tested in the present study.

All these data raised the possibility that L-leu was taken up through an *asc* system in PBL. However, the lack of competitive inhibition by L-ala, a good substrate for this system (Albi et al., 1994) together with the competitive inhibition found for BCH does not support this, confirming a separate entity for the uptake of large apolar branched-chain amino acids.

As can be deduced from the data presented here and in a previous study (Albi et al., 1994), in lower vertebrates, Na^+-independent amino acid carriers exhibit less specificity in their characteristics than their homologs in higher vertebrates. Moreover, there are important differences in the properties of Na^+-dependent amino acid carriers of fish cells (Canals et al., 1992; Gallardo et al., 1992; Canals, Gallardo & Sánchez, 1993; Gallardo & Sánchez, 1993) with respect to those found in mammals (Barker & Ellory, 1990). These systems will need further attention, to ascertain the physiological significance of their presence and structural studies will be needed to establish whether they are different entities.

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References

Albi, J.L., Canals, P., Gallardo, M.A., Sánchez, J. 1994. Na^+-independent L-alanine uptake by trout cells. Evidence for the existence of at least two functionally different *asc* systems. *J. Membrane Biol.* **140:**189–196

Ballatori, N., Boyer, J.L. 1988. Characteristics of L-alanine uptake in freshly isolated hepatocytes of elasmobrach *Raja erinacea. Am. J. Physiol.* **254:**R801–R808

Ballatori, N., Boyer, J.L. 1992. Taurine transport in skate hepatocytes. I. Uptake and efflux. *Am. J. Physiol.* **262:**G451–G460

Barker, G.A., Ellory, J.C. 1990. Review article: The identification of neutral amino acid transport systems. *Experimental Physiology* **75:**3–26

Berteloot, A., Maenz, D.D. 1990. Acidic amino acid transport in mammalian cells and tissues. *Comp. Physiol.* **7:**130–185

Canals, P., Gallardo, M.A., Blasco, J., Sánchez, J. 1992. Uptake and metabolism of L-alanine by freshly isolated trout (*Salmo trutta*) hepatocytes: the effect of fasting. *J. Exp. Biol.* **169:**37–52

Canals, P., Gallardo, M.A., Sánchez, J. 1993. Effect of amino acid deprivation of L-alanine uptake through the ASC system in freshly isolated trout hepatocytes. *J. Exp. Biol.* **178:**269–274

Christensen, H.N. 1975. Recognition sites for mateial transport and information transfer. *Current Topics In Membranes and Transport* **6:**227–258

Christensen, H.N. 1979. Membrane transport. *Advances in Enzymology* **49:**41–101

Christensen, H.N. 1984. Organic ion transport during seven decades: the amino acids. *Biochim. Biophys. Acta* **779:**255–269

Christensen, H.N. 1989. Distinguishing amino acid transport systems of a given cell or tissue. *Methods in Enzymology* **173:**576–616

Christensen, H.N., Oxender, D.L., Liang, M., Vatz, K.A. 1965. The use of N-methylation to direct the route of mediated transport of amino acids. *J. Biol. Chem.* **240:**3609–3616

Ellory, J.C. 1987. Amino acid transport systems in mammalian red cells. *In:* Amino acid transport in animal cells. D.L. Yudilevich and C.A.R. Boyd, editors. pp. 47–86. Manchester University Press, Manchester

Fincham, D.A., Mason, D.K., Young, J.D. 1985. Characterization of a novel Na^+-independent amino acid transporter in horse erythrocytes. *Biochem. J.* **227:**13–20

Fincham, D.A., Wolowyk, M.W., Young, J.D. 1987. Volume-sensitive taurine transport in fish erythrocytes. *J. Membrane Biol.* **96:**45–56

Fincham, D.A., Wolowyk, M.W., Young, J.D. 1990. Characterization of amino acid transport in red blood cells of a primitive vertebrate, the pacific hagfish (*Eptatretus stouti*). *J. Exp. Biol.* **154:**355–370

Gallardo, M.A., Ferrer, M.I., Sánchez, J. 1994. Presence of an X_{AG} carrier in frog (*Rana esculenta*) red blood cells. *J. Membrane Biol.* **139:**97–102

Gallardo, M.A., Planas, J., Sánchez, J. 1992. L-serine uptake by trout (*Salmo trutta*) red blood cells. The effect of isoproterenol. *J. Exp. Biol.* **163:**85–95

Gallardo, M.A., Sánchez, J. 1993. Glycine uptake by trout (*Salmo trutta*) red blood cells. *J. Membrane Biol.* **134:**251–259

Guidotti, G.G., Borghetti, A.F., Gazzola, G.C. 1978. The regulation of amino acid transport in animal cells. *Biochim. Biophys. Acta* **515:**329–366

Houston, A.H.m McCullough, C.A.M., Keen, J., Maddalena, C., Edward, J. 1985. Rainbow trout red cell in vitro. *Comp. Biochem. Physiol.* **81A:**555–565

Kuhlmann, M.K., Vadgama, J.V. 1991. Characterization of threonine transport into a kidney epithelial cell line (BSC-1). Evidence for the presence of Na^+-independent system ASC. *J. Biol. Chem.* **266:**15042–15047

Oxender, D.L., Christensen, H.N. 1963. Evidence for two types of mediation of neutral amino acid transport in Ehrlich cells. *Nature* **197:**765–767

Vadgama, J.V., Christensen, H.N. 1985. Characterization of amino acid transport during erythroid cell differentiation. *J. Biol. Chem.* **262:**13273–13284