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Na^+-independent L-Alanine Uptake by Trout Cells. Evidence for the Existence of at Least Two Functionally Different *asc* Systems

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Abstract. The Na^+-independent uptake of L-alanine has been studied in trout red blood cells, isolated hepatocytes and peripheral blood lymphocytes. The present study shows the existence of two functionally different Na^+-independent systems for short chain neutral amino acids in these cells. They are designated as *asc* systems because of their resemblance to systems described in other cell types. Besides their independence of sodium and a rough similarity in substrate preference, the most important property shared by the two carriers is a lack of *trans*-stimulation, allowing further differentiation from system L. One of them is an unusually stereospecific carrier present in red blood cells, the other is less restrictive and present in hepatocytes and peripheral blood lymphocytes. Extracellular acid pH increases the incorporation to red blood cells, while it slightly depresses the uptake in the other cells. From the data presented, it is not possible, at first, to classify these carriers as asc_1 or asc_2 systems. Moreover, the system present in red cells resembles that found in the nonerythroid cells, BSC-1, while there is no clear parallelism between the system found in hepatocytes/lymphocytes and any of those described previously.

Key words: Alanine uptake — Red blood cells — Peripheral blood lymphocytes — Isolated hepatocytes — asc System — Trout (*Salmo trutta*)

Introduction

The existence of a gradation among amino acid structures has led to a variety of membrane carriers able to move such molecules across the cell barrier. However, because there is a nearly "continuous" variation in their structures, some degree of overlap has been described in the carrying capacity of the different membrane transporters. Thus, several of these molecules are able to transport short-chain neutral amino acids. Some are Na^+ dependent, such as systems A, B^{0+}, ASC, β or Gly, while others are Na^+ independent, such as systems C or L (Christensen, 1989; Barker & Ellory, 1990). The distribution of these carriers is variable, some of them being nearly ubiquitous (ASC or L), while others show a highly restrictive presence (system C, found only in sheep red cells; Young & Ellory, 1977).

Another Na^+-independent amino acid carrier was found initially in the membranes of horse RBC (Fincham, Mason & Young, 1985). It was subsequently designated as *asc* system by agreement with Vadgama and Christensen (1985) because its preferred substrates are similar to those of system ASC, but it is active in the absence of external Na^+. Moreover, a genotypic analysis of horse erythrocytes revealed the presence of two *asc* systems, asc_1 and asc_2: system asc_1 corresponds to the original *asc* carrier, whereas the asc_2 system shows lower affinities for its substrates and is similar to the C system. Although these carriers were initially restricted to horse red cells, they are also reported in *Eptatretus stouti* (Fincham, Wolowyk & Young, 1990), pigeon (Vadgama & Christensen, 1985) and fetal rat (Vadgama, Castro & Christensen, 1987) red cells, as well as in epithelial cells of the rat pancreas (Norman & Mann, 1987) and in transformed primate kidney cells (Kuhlmann & Vadgama 1991). The presence of such a system has been indicated in trout hepatocytes (Canals et al., 1992) and erythrocytes (Gallardo & Sánchez, 1993) and its presence in skate hepatocytes may also be deduced from the work of Ballatory and Boyer (1988).

In the present study, a deeper characterization of

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this system in three different cell types from trout is presented. Moreover, we show that in this species, there are at least two functionally different *asc* carriers.

Materials and Methods

Animals and Chemicals

Brown trout (*Salmo trutta*) (weights between 250 and 400 g) were obtained from fish farms (Medi Natural, Generalitat de Catalunya) in the Pyrenees, where they were maintained in open-water circuits, directly connected to a river.

All chemicals were of analytical grade. Tritiated L-alanine (3-^{3}H) and L-leucine (4,5-^{3}H(N)) were obtained from New England Nuclear (Germany).

Isolation and Incubation of Hepatocytes

Trout were anesthetized in $NaHCO_3$-buffered MS222 and cells were obtained essentially as described by French, Mommsen and Hochachka (1981), except that hyaluronidase was omitted and 5 mM $NaHCO_3$ was used instead of 25 mM, because of the difference in the gas mixture used to equilibrate all solutions (99.5% O_2:0.5% CO_2) before use. Final pH was adjusted to 7.4. Osmolality was determined and adjusted to 305 mOsmol kg^{-1} with a micro-osmometer (mod. 3MO, Advanced Instruments). Cell viability was routinely assessed by means of the exclusion of trypan blue, and more than 95% viability was obtained. Cell integrity over time was measured by LDH release and less than 5% of initial intracellular LDH was found in the medium after 6 hr of incubation.

Following isolation, cells were finally suspended in Hanks' solution containing 2% fatty-acid free bovine serum albumin. No loss of cell viability was observed when K^+ was used instead of Na^+ as the main extracellular cation.

Isolation and Incubation of Peripheral Lymphocytes (PBL) and Red Blood Cells (RBC)

Blood was obtained by caudal puncture and diluted with heparinized RPMI 1640 (Sigma, St Louis, MO). Red blood cells 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 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). Henceforth, this buffer will be referred to as MCB. The osmolality was adjusted to 305 mOsmol kg^{-1} as for hepatocytes.

Uptake

For uptake experiments, both cells and solutions were pre-equilibrated at 15°C before experiments were started by mixing (1:1, v/v) the cell suspension with the labeled L-alanine (^{3}H, 0.3 μCi/ml cells was added) to obtain the desired concentrations. Incubations were performed in a shaking bath at 15°C, using the indicated gas mixture as atmosphere for hepatocytes and air for both PBL and RBC. The uptake was stopped by diluting with Hanks' solution for hepatocytes or MCB for PBL and RBC. Cells were rinsed with these solutions and centrifuged (1,000 × *g*, for 8 min at 4°C) three times. Hepatocytes and 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 resulting solution in each case was counted in a well-type liquid scintillation counter (Packard, Great Britain). 1.5 mM amino-oxyacetic acid (AOA; a transaminase inhibitor) was used throughout uptake experiments.

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

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

Results

Figure 1 shows that L-alanine is incorporated to the cells studied by means of both Na^+-dependent and Na^+-independent systems. However, while for hepatocytes and RBC the main entry pathway of L-alanine was Na^+ dependent, in the case of PBL the Na^+-independent way was the principal one. The Na^+-dependent components of both hepatocytes and RBC have been identified previously as ASC systems (Canals et al., 1992; Gallardo, Planas & Sánchez, 1992; Gallardo & Sánchez, 1993) but there is no previous information about the nature of this carrier in trout PBL. Inhibition studies indicated that this Na^+-dependent system may also belong to the ASC family (there was no inhibition by α-methyl-amino-isobutyrate, but 5 mM L-cysteine depressed the Na^+-dependent uptake of 150 μM L-alanine by 76%), adding support to the hypothesis that system A is absent from cells of this species.

Figure 2 shows the concentration dependence of the Na^+-independent uptake of L-alanine by these cells and the kinetic data obtained are given in the Table. While there was no significant differences in K_m values, the maximal carrier capacities varied considerably. Hepatocytes showed the maximal capacity, while that of RBC was one order of magnitude lower.

To characterize these Na^+-independent carriers, the uptake of 150 μM L-alanine was inhibited by high concentrations (5 mM) of various amino acids (Fig. 3). The main contrast was the different ability to inhibit the uptake shown by BCH and L-leucine. In RBC these amino acids inhibit by more than 50%, while "classic" system ASC substrates show a more variable ability to depress the incorporation of L-alanine. Only L-cysteine showed an activity like those of BCH and L-leucine. The inhibition by these two amino acids was at first surprising, although a deeper analysis showed that BCH did not competitively inhibit the uptake of L-alanine by RBC (Fig. 3*D*), while L-leucine (K_i 4.4 mM) did so, with a similar strength as L-serine (K_i 2.5 mM) or L-cys-

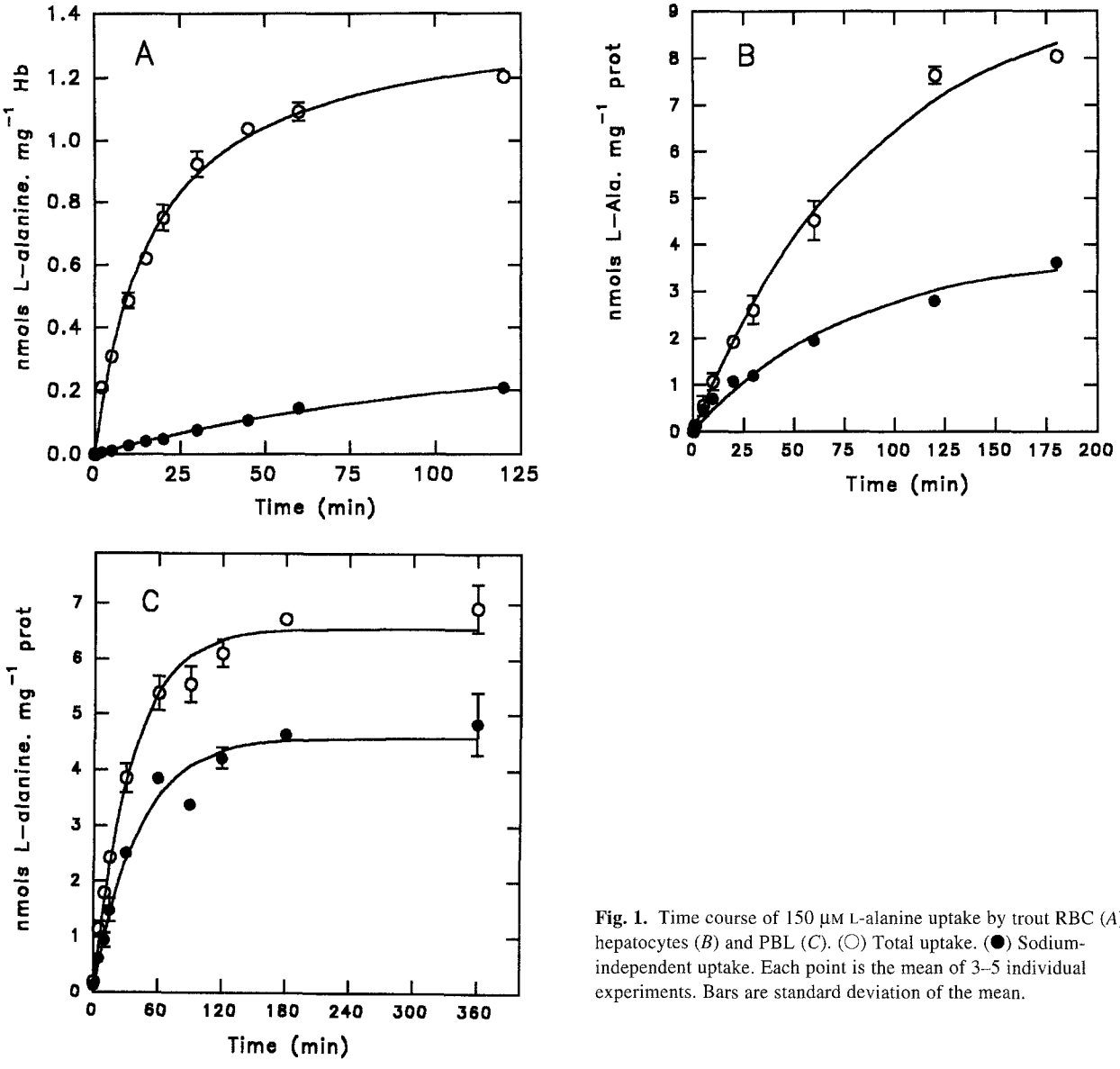

Fig. 1. Time course of 150 μM L-alanine uptake by trout RBC (*A*), hepatocytes (*B*) and PBL (*C*). (○) Total uptake. (●) Sodium-independent uptake. Each point is the mean of 3–5 individual experiments. Bars are standard deviation of the mean.

teine (K_i 2.1 mM). The opposite was found in the other two cells, where both BCH and L-leucine have a lower inhibitory action, while L-serine, L-cysteine, L-alanine and glycine were able to inhibit the uptake of 150 μM L-alanine by more than 70%. Moreover, the distinct behavior of the RBC carrier with respect to the hepatocyte/PBL carrier was clearer when the stereospecific requirements were considered. Thus, K_i values for D-alanine were 29 mM in RBC and 3 mM in hepatocytes. Furthermore, in RBC K_i for AIB was 45 mM, while in lymphocytes this value was 2.7 mM (Fig. 4). Absolute uptake rates in Fig. 4*A* and *B* varied somewhat, probably owing to the different season of the year when these experiments were carried out.

Although the inhibition obtained with BCH in RBC proved to be noncompetitive, that of L-leucine was competitive, which could indicate an uptake of L-alanine through system L. To examine this, *trans*-effects were tested, since in these cells, the uptake of L-leucine through this system is subjected to *trans*-stimulation (Fig. 5*A*). Results for L-alanine indicated that the uptake was never stimulated; in contrast, some degree of *trans*-inhibition can be observed, thus ruling out the possibility that the main way for the Na^+-independent uptake of L-alanine is through system L (Fig. 5*B*). No substantial variations in the uptake were obtained in both hepatocytes and PBL, under the same experimental conditions.

A final characterization of these Na^+-independent uptakes was obtained by analyzing the effect of extracellular pH on the uptake of 150 μM L-alanine. Figure 6 shows a strong sensitivity of alanine uptake by red blood cells to acidic pHs, compared to the uptake by hepatocytes or PBL, where it had a small inhibitory effect.

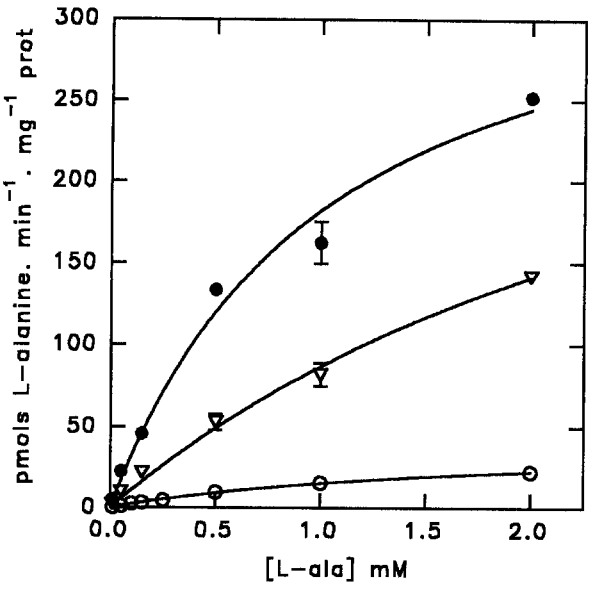

Fig. 2. Concentration dependence of the Na^+-independent uptake of L-alanine by trout RBC (○), hepatocytes (●) and PBL (▽). Cells were incubated for 10 min before the uptake was stopped as described in Materials and Methods. Each point is the mean of 3–5 individual experiments. Bars are standard deviation of the mean.

Discussion

In previous studies, we have suggested the existence of a membrane carrier for short-chain neutral amino acids in trout hepatocytes (Canals et al., 1992) and RBC (Gallardo & Sánchez, 1993) with some properties of an *asc* system. In the present study, its presence has been fully demonstrated in these cells, as well as in peripheral blood lymphocytes from this species. Moreover, in these leukocytes this system appears as the main uptake pathway for L-alanine, in contrast with the other two cell types, in which the main entrance is Na^+ dependent.

In trout, the *asc* carrier does not appear to be a homogeneous structure and some of its properties varied between the different cells, suggesting that functionally different carriers may be involved. Correspondence between present data and those published on the properties of *asc* systems in different cells is not easy to establish. For example, there are strong variations in their pH dependence, ability to be *trans*-stimulated, affinity for substrates, etc. The main reason for these variations is that the full range of the *asc* activities cannot be accounted for by two isoforms.

The *asc* system found in trout RBC shares most of its properties with those of the *asc* systems found in other erythroid cells (Fincham et al., 1985; Vadgama & Christensen, 1985; Vadgama et al., 1987; Fincham et al., 1990; Fincham, Ellory & Young, 1992): it is stereoselective, excluding D-isomers, as well as the α-methylated analogue of alanine, α-amino-isobutyrate. However, longer chains such as L-threonine and L-valine are good inhibitors, as is L-leucine. At first glance, the inhibitory action of L-leucine in addition to that of BCH, may appear to indicate that L-alanine is actually taken up via system L. However, although the inhibition of L-leucine proved to be competitive, that of BCH was noncompetitive. Moreover, system L is *trans*-stimulable, while the Na^+-independent uptake of L-alanine was *trans*-inhibited, thus ruling out the possibility that L-alanine may be incorporated through system L, at least as the main uptake pathway. This high degree of inhibition by L-leucine on the uptake of substrates of *asc* systems was also found in other erythroid (Vadgama & Christensen, 1985; Vadgama et al. 1987) and nonerythroid (Kuhlmann & Vadgama, 1991) cells. However, the C system from sheep (Young & Ellory, 1977) and *asc* systems from thoroughbred horse (Fincham et al., 1985) and Przewalski's horse (Fincham et al., 1992) RBC do not interact with L-leucine, and the L-alanine efflux through the *asc* system from *Eptatretus* RBC is only weakly stimulated by this amino acid (Fincham et al., 1990). In any case, despite the competitive interaction of L-leucine with the *asc* system, this amino acid is normally taken up through system L in trout RBC (J.L. Albi, M.A. Gallardo, and J. Sánchez, *unpublished data*).

Table. Kinetic constants for the Na^+-independent uptake of L-alanine by trout red blood cells, hepatocytes and lymphocytes

Experimental condition	K_m (mM)	V_{max} (pmol mg^{-1} prot. min^{-1})
Red blood cells	0.9 ± 0.1	15.2 ± 1.1
Hepatocytes	0.4 ± 0.1	157.1 ± 20.1
Lymphocytes	0.5 ± 0.1	73.2 ± 5.3

Values are expressed as the mean ± SD (n = 3).

Although the dependence of the *asc* system activity on the extracellular pH has been shown to be variable among the several cells studied, the carrier from trout RBC shows the same pattern as fetal rat erythroid cells (Vadgama et al., 1987) or BSC-1 kidney-derived cells (Kuhlmann & Vadgama, 1991), but opposite to the *asc* system from pigeon RBC (Vadgama & Christensen, 1985). Unfortunately, comparison of the present study with those by Young and co-workers was not easy since the latter did not systematically study the effect of pH on the activity of horse *asc* systems and sheep system C.

When the *asc* system was first described in horse RBC (Fincham et al., 1985), an approximate association between this system and the Na^+-dependent ASC system was established. Most of this similarity was based on a common preference for neutral amino acids of intermediate size and by their interaction with dibasic

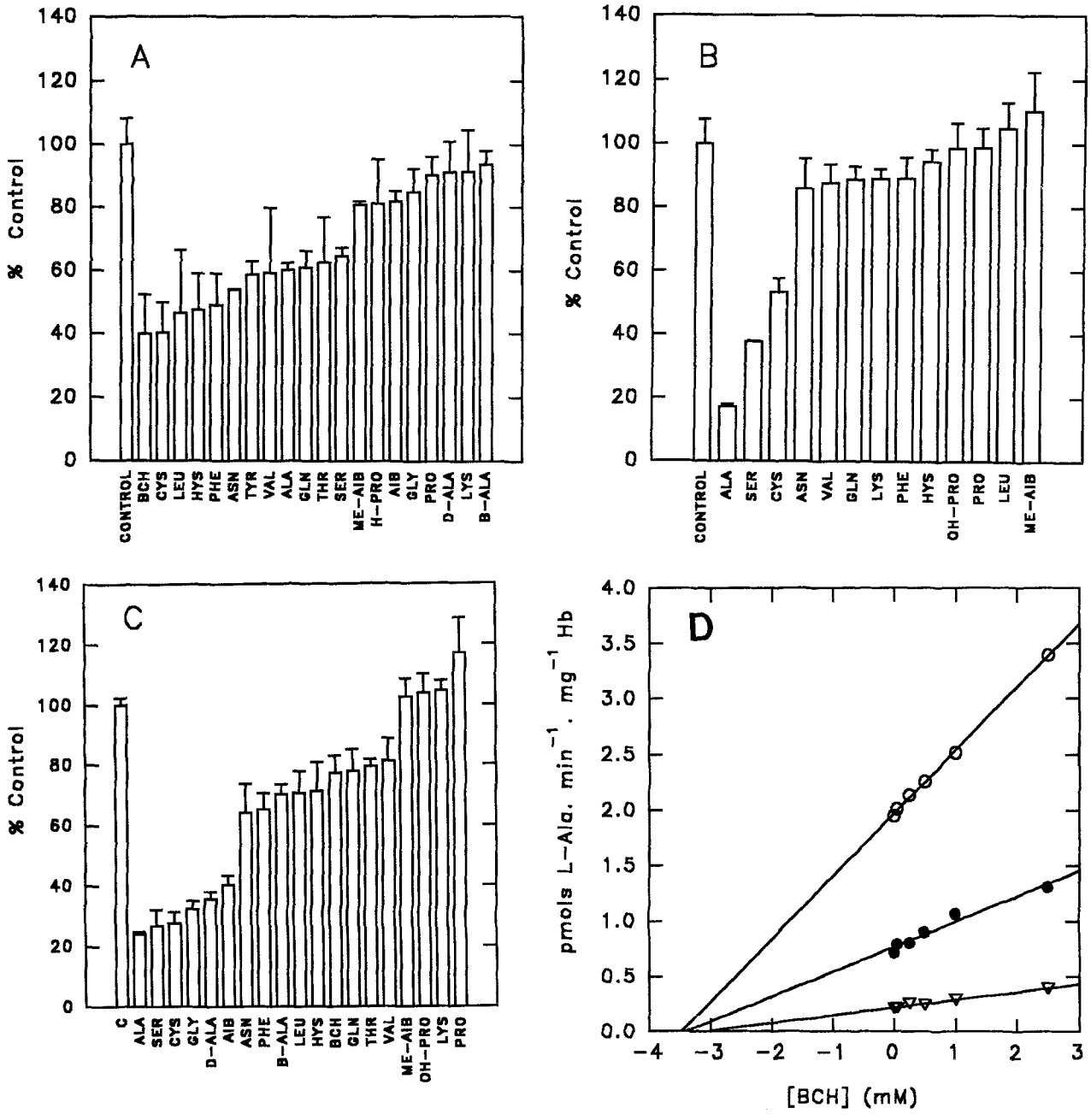

Fig. 3. Inhibition by different amino acids (5 mM) of the Na^+-independent uptake of 150 μM L-alanine by trout RBC (*A*), hepatocytes (*B*) and PBL (*C*). Cells were incubated for 10 min in Na^+-free media. Values are expressed as a percentage of control uptake. Each point is the mean of three individual experiments. Bars are standard deviation of the mean. (*D*) Kinetics of the effect of BCH on the Na^+-independent uptake of L-alanine [10 (○), 25 (●), 100 (▽) μM] by RBC.

amino acids. Moreover, there is a similar interaction of both systems with harmaline (Young, Mason & Fincham, 1988). However, the growing knowledge on the widespread presence of system *asc* in different cell types has restricted these initial considerations to some of the *asc* systems, namely those present in horse and the corresponding system C from sheep RBC. The carrier present in trout RBC does not share this substrate preference, as seen from the inhibition by different amino acids. Moreover, this system is not *trans*-stimulable, while those present in horse and sheep RBC are subjected to *trans*-acceleration.

The L-alanine uptake through the Na^+-independent system by hepatocytes and PBL shows an inhibitory pattern that is very similar to that of the ASC system in trout hepatocytes (Canals et al., 1992). From this point

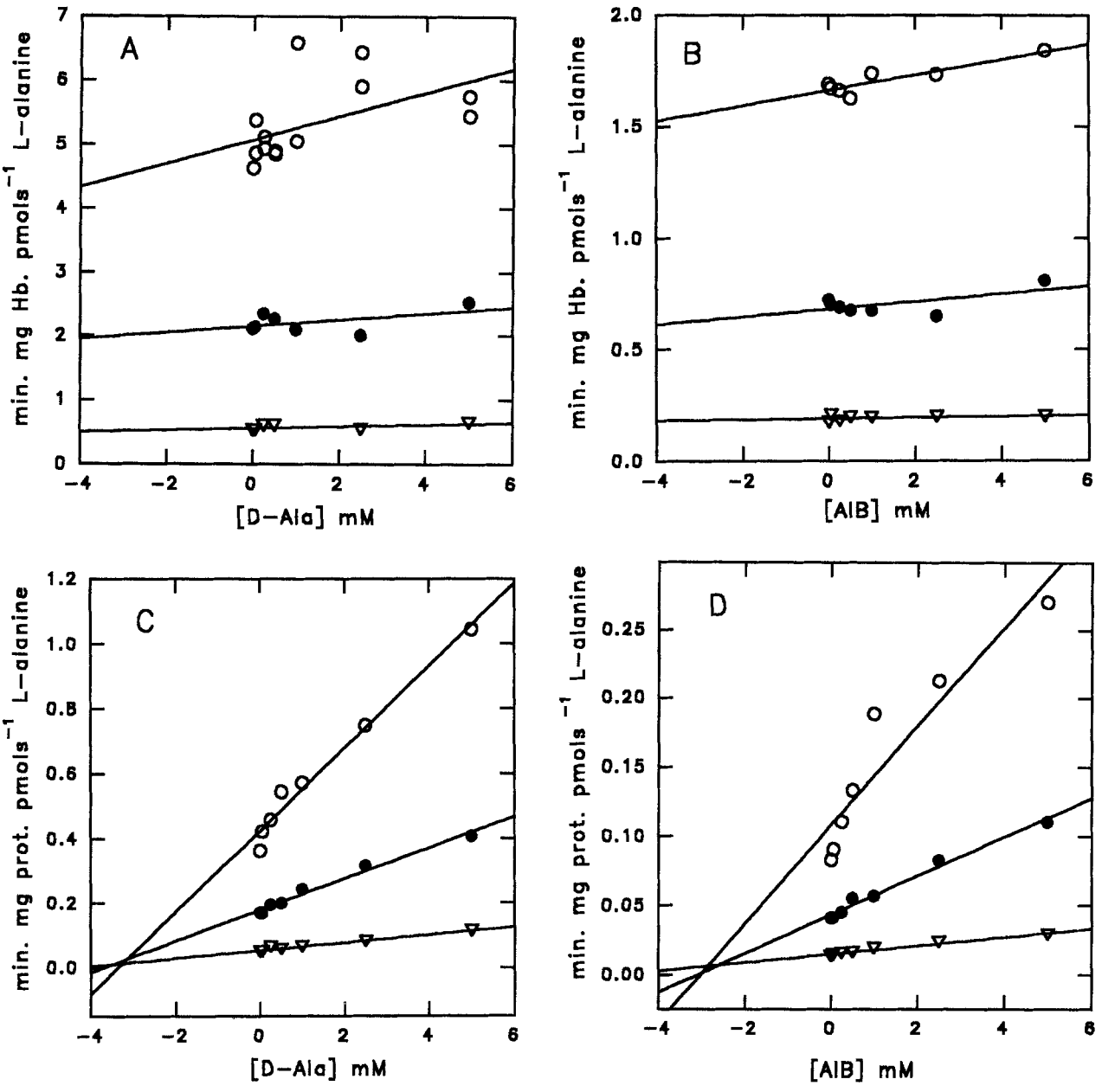

Fig. 4. Kinetics of the effect of D-alanine (*A*) and AIB (*B*) on the Na^+-independent uptake of L-alanine (10, 25, 100 μM) by RBC. Kinetics of the inhibition of D-alanine on the Na^+-independent uptake of L-alanine (10, 25, 100 μM) by hepatocytes (*C*) and of AIB on the Na^+-independent uptake of L-alanine (10, 25, 100 μM) by PBL (D). The K_i values are given in the text.

of view, the *asc* system in hepatocytes and PBL resembles the *asc* systems initially described in horse RBC. However, this similarity is only apparent and D-alanine and α-amino-isobutyrate had no effect on the influx of L-alanine in horse or sheep RBC, while in both hepatocytes and PBL they showed K_i values of about 3 mM, one order of magnitude below those of trout RBC, indicating important differences in their stereoselectivity. A different behavior of the carrier in nonerythroid cells can be also observed in its dependence on extracellular pH. Acidification of the incubation medium decreased the uptake in both hepatocytes and PBL, the effect being more marked in the former cell type. Once again, the *asc* activity in hepatocytes/PBL was not *trans*-stimulable as in trout RBC.

Although a Na^+-independent carrier, with different properties from the L system has been fully demonstrated in several cell systems for amino acids of intermediate size, there is also growing evidence that it is not a homogeneous entity. There are important differences

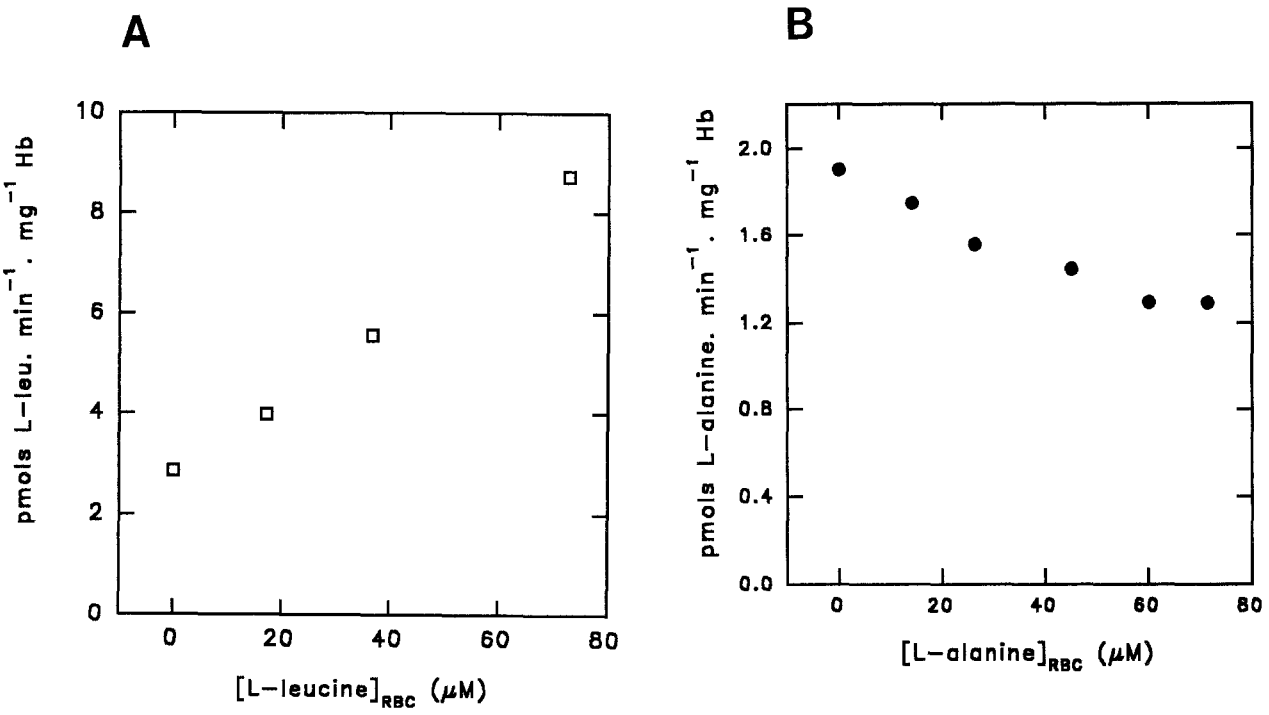

Fig. 5. *Trans*-effects on the Na^+-independent uptake of L-leucine and L-alanine by trout red blood cells. They were preloaded with either 10 mM "cold" L-leucine or L-alanine for different times until different intracellular concentrations were achieved and were washed twice. The uptake of 150 μM labeled L-alanine or 300 μM L-leucine was measured for 10 min.

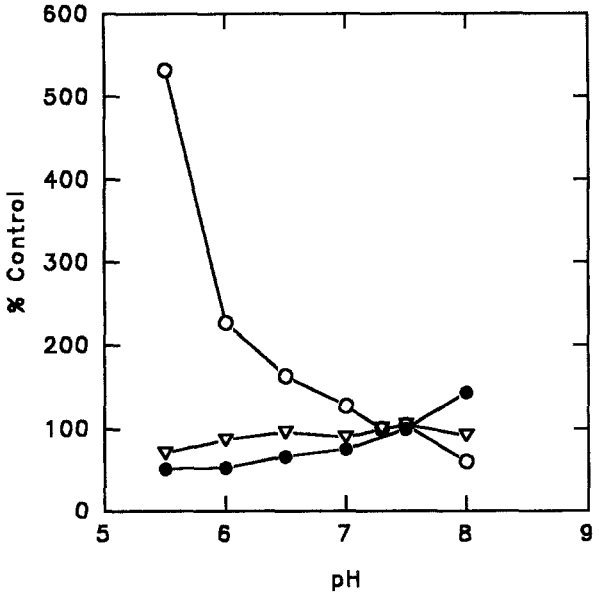

Fig. 6. Effect of extracellular pH on the rate of Na^+-independent uptake of 150 μM L-alanine by trout cells: RBC (○), hepatocytes (●) and PBL (V). Cells were incubated for 10 min. Results are expressed as a percentage of the uptake measured at pH 7.4. Each point is the mean of three individual experiments. Bars are standard deviation of the mean.

between the activities studied, not only among species, but as in the present case, between different cells from the same species. Further, there is no clear indication of their physiological role; Na^+-dependent systems with higher capacities and affinities for the substrate are present together where tested. Trout PBL are an exception, because system *asc* appears as the main route of L-alanine movements, and it will be of interest to determine whether the activity of this system may be modulated by changes in cell function, as occurs with system A and, to a lesser extent, system L in activated mammalian lymphocytes (Borghetti, Kay & Wheeler, 1979).

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J.L. Albi, P. Canals and M.A. Gallardo contributed equally to this study.

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