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L-SERINE UPTAKE BY TROUT (*SALMO TRUTTA*) RED BLOOD CELLS: THE EFFECT OF ISOPROTERENOL

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Summary

We studied the uptake of L-serine by trout red blood cells and the effect of a β-adrenergic agonist (isoproterenol) on this process. The results obtained indicate that L-serine is taken up by these cells by means of a concentrative process. The uptake seems to be mediated both by a sodium-dependent process and by a sodium-independent process. The sodium-dependent uptake is mediated by a transport system that probably belongs to the ASC system family. Isoproterenol exerts an inhibitory effect on L-serine uptake. This effect is dose-dependent. It is proposed that the inhibitory effect of isoproterenol is mediated by a rise in the intracellular sodium concentration and/or changes in cell volume.

Introduction

It has been shown that red blood cells (RBCs) may play an important role in amino acid metabolism, being involved in the biosynthesis of glutathione (Ellory *et al.* 1983), interorgan transport (Christensen, 1982) and peptide degradation following amino acid efflux (Tucker and Young, 1981). Some amino acids have been implicated in the regulation of cell volume, particularly in fish red blood cells (Fincham *et al.* 1987; Goldstein and Boyd, 1978).

In all these processes, membrane transport steps appear to be central. On the basis of their Na^+ dependence and substrate specificity, several amino acid transport systems have been described in RBCs (Eavenson and Christensen, 1967), as well as in other cell systems (Le Cam and Freychet, 1977; Shotwell *et al.* 1983). One of these transport systems uses L-alanine, L-serine and L-cysteine as preferred substrates and requires Na^+ (Christensen *et al.* 1967; Kilberg *et al.* 1981). This system, called the ASC system, was first described in Erlich cells, but it has been found in other cells (Christensen *et al.* 1967). Interestingly, a similar system has been found in human RBCs (Young *et al.* 1983, 1988) and in rabbit reticulocytes (Thomas and Christensen, 1971), as well as in pigeon erythrocytes

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(the ASCP system; Eavenson and Christensen, 1967). However, no references have been found to the existence of a similar system in fish RBCs.

Fish RBCs are particularly sensitive to high levels of circulating catecholamines, which occur under hypoxic conditions (Tetens and Christensen, 1987) and after exhaustive exercise (Ristori and Laurent, 1985), inducing a rise in the sodium concentration inside the cells (Baroin *et al.* 1984). The cell swelling was shown to be coupled to Na^+/H^+ and Cl^-/HCO_3^- exchanges under physiological conditions (Nikinmaa and Huestis, 1984). The effect also seems to be mediated by a rise in the cyclic AMP level, through the interaction of the hormone with membrane receptors of the β type.

In the present study, evidence is presented that an amino acid transport system similar to the ASC(P) system exists in trout RBCs and that catecholamines may alter its carrier ability by changing the internal sodium concentration.

Materials and methods

Brown trout (*Salmo trutta*) (body mass between 250 and 400 g) were obtained from a fish farm (Departament Medi Ambient, Generalitat de Catalunya) in the Pyrenees (Bagà, Spain) and were acclimatized to the laboratory conditions (a closed water circuit, filled with deionized water and with controlled $[NH_4^+]$ and $[O_2]$, maintained at 15°C) for at least 1 week before the experiments were performed.

Blood was obtained by caudal puncture and diluted with heparinized RPMI 1640 (Sigma Co., USA). Owing to the high metabolic activity of white blood cells, these cells, as well as thrombocytes, were removed by centrifugation with Histopaque-1077 (Sigma Co., USA), following the procedure suggested by the supplier, slightly modified to obviate the high viscosity of trout blood. Once separated, the RBCs were rinsed four times with a slightly modified Cortland buffer (pH 7.4) (Houston *et al.* 1985) (NaCl 141 mmol l^{-1}, KCl 3.5 mmol l^{-1}, $MgSO_4$ 1 mmol l^{-1}, NaH_2PO_4 3 mmol l^{-1}, $CaCl_2$ 1 mmol l^{-1}, pyruvic acid 2 mmol l^{-1}, Hepes 10 mmol l^{-1}, bovine serum albumin 0.3 %, glucose 3 mmol l^{-1}). This buffer will be referred to as MCB. The osmolality was adjusted to 305 mosmol kg^{-1}. During this process the RBCs became depleted of L-serine (data not shown). When cells were used for experiments using buffers with a different ionic composition, the whole rinsing procedure was performed using the final buffer.

U-^{14}C-labelled L-serine and other chemicals were obtained from Sigma Co. (USA). 8-Bromoadenosine-3′,5′-cyclic monophosphate (Br-cAMP) was from Boehringer Mannheim (Germany).

Both cells and solutions were pre-equilibrated at 15°C before the experiments were started by mixing the RBC suspension with the L-serine (1:1, v/v) to obtain the desired concentrations and a final haematocrit of about 10 %. 0.04 μCi of U-^{14}C-labelled L-serine for each millilitre of cell suspension was added to the cold L-serine solution before mixing with cells. Incubations were performed in a

shaking bath at 15 °C, using air as the gas phase. The experiments were performed in the presence of 2 mmol l^{-1} amino-oxyacetic acid to avoid L-serine metabolization.

Radioactive L-serine uptake was stopped by diluting with MCB (1:9, v/v) containing a 10- to 50-fold excess of cold amino acid and rinsing the cells with this solution (1:9, v/v) three times; the RBCs were separated each time by centrifugation (810 *g* for 8 min at 4 °C). Cells suspensions were deproteinized by adding sufficient ice-cold perchloric acid to obtain a final concentration of 6 %. Deproteinization was developed in ice and a clear supernatant was obtained by centrifugation (1825 *g* for 20 min at 4 °C). The radioactivity in this supernatant was measured by using a liquid scintillation counter.

Cell water content was determined gravimetrically and RBC sodium measurements were performed by flame photometry following Mahé *et al.* (1985).

Curve fitting of experimental data was performed by means of non-linear regression systems (Graph-Pad 2.0 and Sigma Plot 4.0).

Results and discussion

Serine uptake by trout red blood cells

Eukaryotic cells take up neutral amino acids by three main transport systems, A, ASC and L, as well as by other minor transport systems, Gly, β and N (Shotwell *et al.* 1983). These transport systems have been found in a variety of cells and tissues, including Erlich ascites tumour cells (Christensen *et al.* 1967), fish (Ballatori and Boyer, 1988) and mammalian hepatocytes (Kilberg *et al.* 1981; Le Cam and Freychet, 1977) and other tissues, although they do not seem to be present simultaneously in all cells types. Some variations of these main transport systems have been found, such as the Na^+-independent asc system present in RBCs from different species (Fincham *et al.* 1985, 1990; Vadgama and Christensen, 1985).

The time course of 150 μmol l^{-1} L-serine uptake by RBCs in a Na^+-containing medium is shown in Fig. 1. The concentration of amino acid in the intracellular water was 2.8 times higher than that outside the cell after 3 h of incubation, i.e. this amino acid is taken up in a concentrative way. Physiological values for the RBC/plasma L-serine concentration ratio range between 1.0 and 5.0, as a function of seasonal and physiological changes (M. A. Gallardo, J. Planas and J. Sánchez, unpublished data). The uptake was almost linear for 15 min and subsequent experiments used incubation times of 10 min.

The effect on the amino acid uptake of different L-serine concentrations in the incubation medium was measured in media containing either Na^+ or K^+ [potassium was substituted for sodium as the main cation in the sodium-independent experiments (Fincham *et al.* 1987)]. Fig. 2 shows that at low L-serine concentrations [below 50 μmol l^{-1}, i.e. approximately physiological levels, which ranged between 50 and 100 μmol l^{-1} (M. A. Gallardo, J. Planas and J. Sánchez, unpublished data)] the transport is mainly Na^+ dependent, while at higher

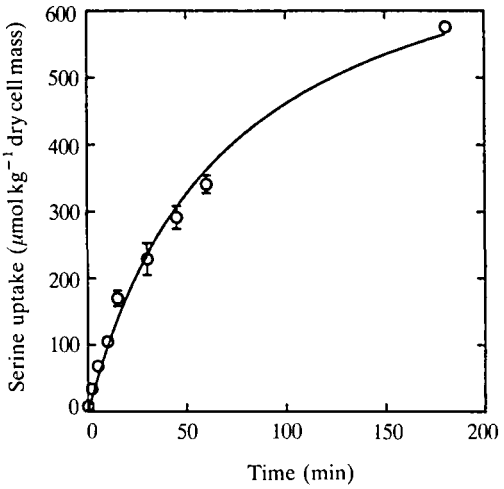

Fig. 1. Time course of L-serine uptake by trout red blood cells (RBCs). Cells were suspended in MCB and incubated for different times in the presence of 150 μmol l^{-1} L-serine and 2 mmol l^{-1} amino-oxyacetic acid. Each point is the mean of 3–5 individual experiments. Bars are standard deviation of the mean.

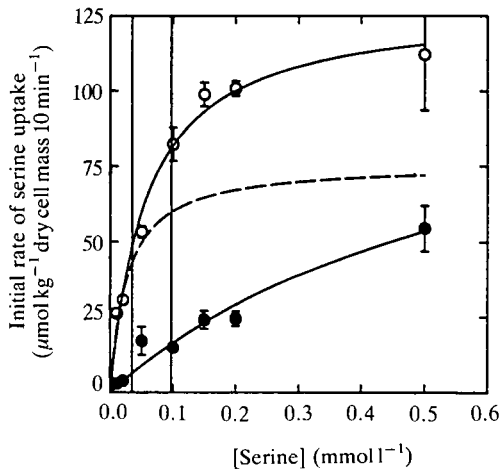

Fig. 2. Concentration dependence of L-serine uptake by trout RBCs. Initial rates were measured in the presence of buffers containing sodium (160 mmol l^{-1}) or potassium (160 mmol l^{-1}). Cells were incubated for 10 min before the uptake was stopped, as described in Materials and methods. (○) Total uptake. (●) Sodium-independent uptake. (---) Sodium-dependent uptake. The box indicates the physiological range of plasma L-serine concentrations. Each point is the mean of 3–5 individual experiments. Bars are standard deviation of the mean. Kinetic constants for Na^+-dependent L-serine uptake are given in the text.

concentrations, the Na^+-independent component becomes important, accounting for between one-quarter and one-third of the total uptake. Although it has not been tested in the present study, this sodium-independent component may be related to the presence of an asc system or an L system. The asc system has been described in hagfish (*Eptatretus stouti*) (Fincham *et al.* 1990), pigeon (Vadgama and Christensen, 1985) and horse (Fincham *et al.* 1985) RBCs, as well as in rabbit reticulocytes (Wheeler and Christensen, 1967). The L system is known to transport small amounts of L-serine in Chinese hamster ovary cells (Shotwell *et al.* 1981). When the rates of uptake in the K^+-containing medium were subtracted from those in Na^+-containing medium, essentially all of the sodium-dependent uptake was saturable. The apparent K_m of this saturable component was 26.06 μmol l^{-1} and the V_{max} was 75.88 μmol kg^{-1} dry mass 10 min^{-1}.

These results indicate that some kind of specific transport system for L-serine exists in trout RBCs and further experiments were performed to obtain information about some characteristics of this transport system. The sodium dependence of the L-serine transport was explored by modifying the external sodium concentration. This was varied between 5 and 180 mmol l^{-1}, and 10 μmol l^{-1} L-serine was used as the initial concentration to exclude most of the sodium-independent component of the transport. The amount of L-serine taken up by the cells rose continuously as a hyperbolic function. Kinetic variables were as follows: apparent K_m=122 mmol l^{-1} and V_{max}=23.03 μmol kg^{-1} dry mass 10 min^{-1} (Fig. 3).

It has been postulated that harmaline, an inhibitor of sodium cotransport systems (Sepulveda and Robinson, 1974), inhibits the human and horse erythro-

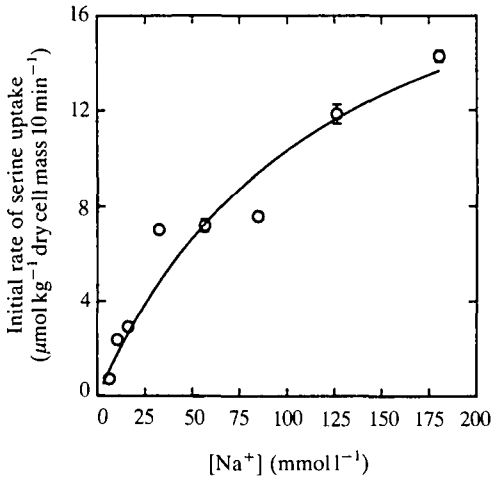

Fig. 3. Na^+ dependence of L-serine uptake by trout RBCs. The amino acid concentration used was 10 μmol l^{-1}. Potassium was used throughout to replace sodium and osmolality was maintained constant (305 mosmol kg^{-1}). The initial rates were measured after 10 min incubations as a function of external sodium concentration. Each point is the mean of 3–5 individual experiments. Bars are standard deviation of the mean. Kinetic constants for the uptake are given in the text.

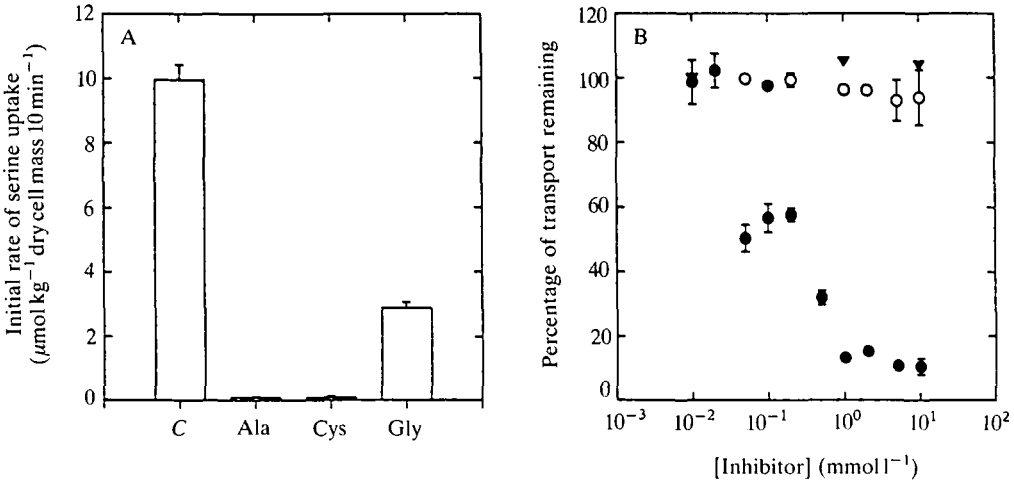

Fig. 4. Inhibition by different amino acids of the uptake of L- serine by trout RBCs. Cells were incubated for 10 min in sodium-containing medium as for the experiment shown in Fig. 1. (A) L-Serine uptake, at 10 μmol l^{-1}, was measured in the presence of 10 mmol l^{-1} L-alanine (Ala) or L-cysteine (Cys) which, in addition to L-serine, are considered to be preferred substrates for the ASC system. The effect of 10 mmol l^{-1} glycine (Gly), which has been considered to be a substrate for this system, is also presented. *C* is the control. (B) Effect of variable external concentrations of L- alanine (●), α-(methyl)aminoisobutyric acid (▼) and L-lysine (○) on the uptake of 10 μmol l^{-1} L-serine. Each point is the mean of three individual experiments. Bars are standard deviation of the mean.

cyte ASC system in a noncompetitive way with respect to amino acid concentration (Young *et al.* 1988). Using 10 μmol l^{-1} L-serine, the presence of 5 mmol l^{-1} harmaline reduced L-serine uptake by 45±0.4 % (S.D., N=5) with respect to controls, while 10 μmol l^{-1} harmaline had no effect on the uptake.

To investigate the substrate specificity of the L-serine transport system in trout RBCs, various amino acids were tested as inhibitors of the transport. The presence of a 1000-fold excess of either L-alanine or L-cysteine fully inhibited the uptake of L-serine (10 μmol l^{-1} initial concentration), while 10 mmol l^{-1} L-glycine caused a 70 % inhibition (Fig. 4A). A more detailed study of the inhibiting effect of amino acids on L-serine transport was undertaken by using increasing concentrations (0–10 mmol l^{-1}) of L-alanine (related to transport systems A and ASC), α-(methyl)aminoisobutyric acid (MeAIB) (related to system A) or L-lysine [it has been reported that dibasic amino acids can act as inhibitors of the ASC system in pigeon RBCs (Eavenson and Christensen, 1967)]. According to the data shown in Fig. 4B, L-alanine showed a marked inhibitory effect, half-maximal inhibition occurring at 160 μmol l^{-1} [as judged from curve-fitting (curve not shown)], while MeAIB had no effect and L-lysine had only a small inhibitory effect at high concentrations.

In the present study we have demonstrated that L-serine uptake by trout RBCs is mediated by a saturable high-affinity sodium-dependent transport system, as

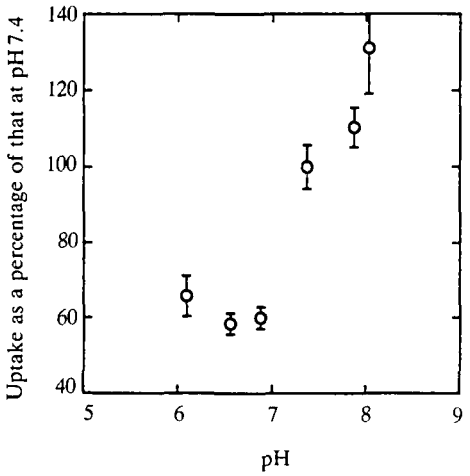

Fig. 5. Effect of extracellular pH on the rate of uptake of L- serine by trout RBCs. Cells were incubated for 10 min. Results are expressed as a percentage of the uptake measured at pH 7.4. Each point is the mean of three individual experiments. Bars are standard deviation of the mean.

well as by a sodium-independent system. The properties of the sodium-dependent transport system are similar to those of the ASC system; for example, inhibition by other neutral short-chain amino acids (alanine and cysteine); partial inhibition by glycine; only a small inhibitory effect of dibasic amino acids; and MeAIB had no effect (Shotwell *et al.* 1983). K_m values for both sodium and L-serine are near their physiological values (M. A. Gallardo, J. Planas and J. Sánchez, unpublished data). Discrimination between the A and ASC systems is difficult from a kinetic point of view, because the properties of the ASC system are very similar to those of the A system; however, this system seems to be almost inactive or nonexistent in mature RBCs (Eavenson and Christensen, 1967).

The pH dependence of the ASC system is highly variable in different cell types. In rabbit reticulocytes and erythrocytes (Winter and Christensen, 1965), as well as Ehrlich cells (Christensen *et al.* 1967), it is barely sensitive to external pH. However, the activity of this system from pigeon erythrocytes shows a marked dependence on external pH (Eavenson and Christensen, 1967). The pH dependence of L-serine (10 μmol l^{-1}) uptake by trout RBCs was studied in the pH range 6–8, in a Na^+-containing medium (Fig. 5). The results showed a clear dependence of the rate of uptake on the external pH. It would be of interest to know the pH dependence of system A, if present in other fish tissues, but at present there is a lack of information on this.

Effect of isoproterenol on the L-serine uptake by trout red blood cells

Addition of catecholamines to an isotonic suspension of fish red blood cells causes the cell volume to increase, owing to a net uptake of sodium and chloride and osmotically obligated water (Baroin *et al.* 1984). It has been proposed that

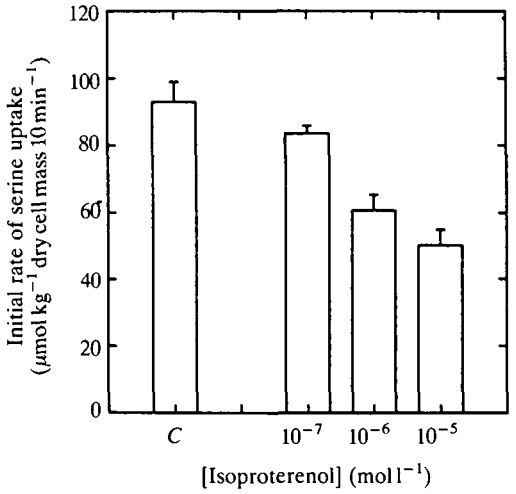

Fig. 6. Effect of isoproterenol at three different concentrations on 150 μmol l^{-1} L-serine uptake by trout RBCs. *C* is the control. Cells were incubated for 10 min. Each bar is the mean of three individual experiments. Bars are standard deviation of the mean.

these cells take up sodium one-for-one with Cl^-, as well as by Na^+/H^+ and Cl^-/HCO_3^- exchange (Nikinmaa and Huestis, 1984; Cossins and Richardson, 1985; Nikinmaa, 1990). This sodium-increasing effect at physiological concentrations of adrenergic agonists seems to be exclusive to fish RBCs (Nikinmaa and Huestis, 1984).

The presence of different concentrations of isoproterenol ($10^{-7}-10^{-5}$ mol^{-1}) in the suspension medium had an inhibitory effect on the L-serine uptake; the effect was dose-dependent (Fig. 6).

It has been proposed that β effects of catecholamines on fish RBCs operate by raising the intracellular levels of cyclic AMP. When 0.5 mmol l^{-1} 8-bromocyclic AMP (a phosphodiesterase-resistant cyclic AMP analogue) was added to the cells, before the addition of amino acid to obviate its slow diffusion rate, a fall in the rate of uptake was observed (data not shown). No additive effects between cyclic AMP and isoproterenol were observed, suggesting that the effect of the β-analogue on L-serine transport is mediated by a rise in the cyclic AMP level.

Two possible mechanisms may be responsible for the effect of isoproterenol on the L-serine uptake. Catecholamines change the intracellular sodium concentration (Nikinmaa and Tufts, 1989), leading to a change in the sodium electrochemical gradient, which acts as the driving force for the amino acid internalization. A similar situation has been observed in the transport of L-alanine by isolated rat hepatocytes when the transmembrane Na^+ electrochemical gradient is eliminated by gramicidin D (Kristensen and Folke, 1986). An alternative (but not exclusive) explanation is the well-known effect of volume-induced changes in amino acid efflux, described for skate (*Raja erinacea*) (Goldstein, 1989; Goldstein

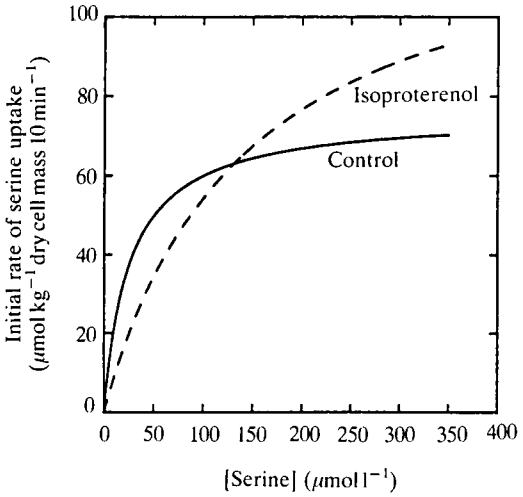

Fig. 7. Effect of 10^{-6} mol l^{-1} isoproterenol on the concentration dependence of sodium-dependent L-serine uptake by trout RBCs. Values were obtained as in Fig. 2. Cells were incubated for 10 min with MCB or with MCB plus isoproterenol. Kinetic constants are given in the text. These curves are equivalent to those shown in Fig. 2 and show the difference between total and sodium-independent serine uptake.

and Brill, 1990), eel (*Anguilla japonica*) and starry flounder (*Platichthys stellatus*) (Fincham *et al.* 1987) erythrocytes. Hypo-osmotic or iso-osmotic cell swelling increased the rate of release of taurine from these cells.

Adrenergic stimulation of salmonid RBCs can acidify the extracellular medium, although only by 0.1 pH unit, when 10 mmol l^{-1} Hepes is used as the buffer (Motais *et al.* 1989). Such a change may contribute to a drop in the rate of uptake of the amino acid such as that shown in Fig. 6.

The effect of 10^{-6} mol l^{-1} isoproterenol on the concentration dependence of the L-serine uptake was studied. Results shown in Fig. 7 indicated that the β-agonist induced a rise in the apparent K_m of the sodium-dependent uptake (from 26.06 μmol l^{-1} for control cells to 139.43 μmol l^{-1} for isoproterenol-treated cells). V_{max} was also affected by isoproterenol, rising from 75.88 to 127.87 μmol kg^{-1} dry mass 10 min^{-1}.

The influence of isoproterenol on the sodium-dependent L-serine uptake should be measured for the incorporation of other amino acids into fish RBCs, although this has not been investigated here. It is known that catecholamines exert a gluconeogenic effect in the trout liver (Wright *et al.* 1989). Moreover, some amino acids (e.g. L-serine and L-alanine) may act as gluconeogenic substrates (Walton and Cowey, 1982) or as oxidative substrates in fish (French *et al.* 1981). Thus, it is suggested that, under stressful conditions, whatever the mechanism may be, the concentration of amino acids in the RBCs may fall, facilitating their uptake by the liver or other organs to obtain glucose and/or energy. One question, at present being investigated, is whether there is a change, induced by isoproterenol, in the equilibrium concentrations of amino acids within the erythrocytes.

It is interesting that both CO_2 and glucose production by trout hepatocytes are higher when L-serine instead of L-alanine is used as substrate (French *et al.* 1981). However, to our knowledge, an increase in the amino acid uptake by fish liver or other cells following adrenergic stimulation has not been demonstrated.

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