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UPTAKE AND METABOLISM OF GLUCOSE, ALANINE AND LACTATE BY RED BLOOD CELLS OF THE AMERICAN EEL *ANGUILLA ROSTRATA*

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Summary

The uptake and metabolism of glucose, alanine and lactate were assessed in red blood cells (RBCs) of the American eel *Anguilla rostrata*. L-Lactate was metabolized at the highest rates as assessed by O_2 consumption and CO_2 production, followed by glucose and alanine (rates were approximately half of those observed for lactate). A saturable (K_m 10.36±0.60 mmol l^{-1}, J_{max} 27.42±2.16 μmol 3-OMG l^{-1} cell water min^{-1}), sodium-independent but cytochalasin-B-sensitive carrier for D-glucose was observed, which was stereospecific and inhibited by other hexoses. These characteristics are in agreement with those reported for the GLUT-1 glucose carrier of human and Japanese eel erythrocytes. These cells also contained a saturable carrier for L-lactate in the concentration range 0–10 mmol l^{-1} (K_m 6.74±0.36 mmol l^{-1}, J_{max} 2.29±0.09 mmol lactate l^{-1} cell water min^{-1}) whereas, at higher concentrations (10–40 mmol l^{-1}), transport occurred by simple diffusion. The carrier was stereospecific, sodium-independent, fully inhibited by α-cyano-4-hydroxycinnamate, DIDS and pyruvate, but less sensitive to SITS, IBCLA and pCMBS. We suggest that this carrier is similar to the H^+/monocarboxylate carrier found in mammalian RBCs. Despite the fact that L-alanine transport did not saturate, transport was stereospecific because it was inhibited by D-alanine. These experiments do not, therefore, exclude the existence of an alanine carrier in the eel RBC. The rates of substrate uptake exceeded the ability of the RBC to metabolize the substrate (using 1 mmol l^{-1} extracellular concentration), with uptake rate/metabolic rate ratios being 2 for alanine, 5 for glucose and 151 for lactate. These experiments indicate that uptake does not limit the ability of the American eel RBC to utilize glucose, alanine or lactate, but that the mechanism(s) of substrate uptake is species-specific.

Key words: American eel, *Anguilla rostrata*, erythrocytes, transport, metabolism, glucose, lactate, alanine.

Introduction

Red blood cells (RBCs) are one of the most frequently used cell types in which to assess the uptake and subsequent metabolism of different fuels in vertebrates (Ingermann *et al.* 1985; Poole and Halestrap, 1993) because of their nature and ease of preparation. However, the studies performed in fish are scarce, with the data obtained being inconclusive in many cases (see review by Moon and Walsh, 1994). The RBCs of fish acquire about 90 % of all their energy demands by aerobic metabolism (Boutilier and Ferguson, 1989; Walsh *et al.* 1990). The substrates used for aerobic energy production have been characterized in only a few species, with the most important being glucose in rainbow trout (*Oncorhynchus mykiss*; Walsh *et al.* 1990) and brown trout (*Salmo trutta*; Pesquero *et al.* 1992) and lactate in carp (*Cyprinus carpio*; Tiihonen and Nikinmaa, 1991*a*). Despite this, few studies have examined how the transport of the main metabolites (glucose, alanine or lactate) occurs or whether transport limits metabolism.

Studies performed on metabolite carriers in fish RBCs have shown the existence of a sodium-independent, cytochalasin-B-sensitive glucose carrier in hagfish (*Eptatretus stouti*; Ingermann *et al.* 1984), river lamprey (*Lampetra fluviatilis*; Tiihonen and Nikinmaa, 1991*b*) and Japanese eel (*Anguilla japonica*; Tse and Young, 1990). In most species, however, including the brown trout (Bolis *et al.* 1971), rainbow trout (Tse and Young, 1990), carp (Tiihonen and Nikinmaa, 1991*b*) and a variety of Amazon fishes (*Arapaima gigas*, *Pterygoplichthys* sp. and *Osteoglossum bicirrhosum*; Kim and Isaaks, 1978), erythrocytes are impermeable to glucose. As for amino acids and lactate, the data available are far from conclusive, especially for lactate. Recent data from carp have

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shown the existence of a saturable, stereospecific carrier for lactate, similar to the H^+/monocarboxylate carrier of mammals (Tiihonen and Nikinmaa, 1993). In contrast, studies on skipjack tuna (*Katsuwonus pelamis*) RBCs by Moon *et al.* (1987) failed to identify a saturable lactate carrier, with the transport observed being attributable to simple diffusion. Several studies have revealed the existence of some of the known mammalian carriers for neutral amino acids, including the ASC, asc, L and Gly systems (Fincham *et al.* 1990; Gallardo *et al.* 1992; Gallardo and Sánchez, 1993) as well as the β-type carrier involved in the transport of amino acids (taurine, β-alanine) related to volume regulation (Fincham *et al.* 1987; Thoroed and Fugelli, 1993).

Despite the recent interest in these processes, there are no data on how all three metabolites are taken up and metabolized by the RBCs of a *single* fish species. Therefore, this study evaluates the uptake and metabolism of glucose, alanine and lactate in American eel RBCs and whether uptake limits the further metabolism of these compounds by the RBC. The eel was chosen because a previous study (Tse and Young, 1990) has demonstrated the presence of a specific glucose transporter in the RBCs of the Japanese eel.

Materials and methods

Fish

American eels (*Anguilla rostrata* LeSueur) were obtained from the St Lawrence River at Cornwall (Ontario) and maintained for 1 month under laboratory conditions in running dechlorinated Ottawa tap water at 10 °C. Fish were not fed during the 2 month experimental period. Experiments were undertaken during the July–August period.

Cell collection and preparation

Blood was obtained from 6–8 decapitated fish and placed into heparinized tubes. RBCs and plasma were separated by centrifugation (2 min, 1800 revs min^{-1}; Sorvall RC28S with SS-34 rotor) at 4 °C. Cells were washed twice with 10 vols of Cortland saline, followed by two additional rinses with 10 vols of modified Cortland saline (MCS), which was the normal Cortland saline plus 10 mmol l^{-1} Hepes and 0.3 % bovine serum albumin (BSA) (Albi *et al.* 1993). The buffy coat was discarded and the rinsed red cells were resuspended in MCS to achieve a final haematocrit of 20 %. The pH of all media was adjusted to 7.8, with no significant changes being observed during any experiment. The starting osmolarity of the medium was 240 mosmol l^{-1} and the maximum reached after adding the highest substrate concentration was 293 mosmol l^{-1}.

Reagents

All labelled substrates were purchased from Amersham Canada Ltd (Oakville, ON). The specific activities were: 5.66 GBq $mmol^{-1}$ L-[U-^{14}C]lactate; 5.66 GBq $mmol^{-1}$ L-[U-^{14}C] alanine; and 5.88 GBq $mmol^{-1}$ 3-*O*-methyl-D-[U-^{14}C]glucose (3-OMG). All non-radioactive substrates and inhibitors were obtained from Sigma Chemical Co. (St Louis, MO) or Boehringer Mannheim (Montréal, Québec). All other chemicals were of the highest possible purity.

Uptake studies

All uptake experiments were carried out in duplicate at 10 °C. Uptake of L-[U-^{14}C]lactate, L-[U-^{14}C]alanine and 3-*O*-methyl-D-[U-^{14}C]glucose were initiated by mixing 1 vol of the cell suspension (20 % haematocrit) with 0.5 vol of incubation medium (MCS) containing both radioactive and non-radioactive substrates. For the alanine uptake studies, 1 mmol l^{-1} amino-oxyacetic acid was added 15 min prior to the experiment to inhibit the metabolism of alanine by the RBCs. The final concentrations of labelled and unlabelled substrates were 0.5 μCi ml^{-1} and 0.125–40 mmol l^{-1}, respectively. For incubations in the presence of potential inhibitors (the 3-OMG and lactate uptake experiments), cells were pre-incubated with the inhibitor for 60 min at 10 °C in the absence of substrate. Fresh stock solutions of all inhibitors were prepared daily. The vehicles used to suspend the inhibitors were MCS for D-glucose, 2-deoxyglucose, L-glucose, D-alanine, D-lactate and pyruvate, 0.5 % ethanol for α-cyano-4-hydroxycinnamate (CYN), *p*-chloromercuriphenylsulphonic acid (pCMBS) and phloretin, and 0.5 % dimethylsulphoxide (DMSO) for cytochalasin B, isobutylcarbonyl lactyl anhydride (IBCLA), 4,4′-di-isothiocyanostilbene-2,2′-disulphonate (DIDS) and disodium 4-acetamidostilbene-2,2′-disulphonate (SITS). No effects on the rate of uptake of lactate or 3-OMG were seen due to the vehicle alone (data not shown).

Incubations were stopped at pre-determined times (see below) by layering 0.15 ml of the cell suspension (13.3 % haematocrit) over 0.5 ml of dibutyl phthalate (Sigma Chemical Co.) in a 1.5 ml plastic centrifuge tube. The tube was immediately centrifuged (30 s, 10 000 revs min^{-1}; Fisher microcentrifuge 235B) and stored on ice to minimize any endogenous metabolism. The medium and oil layers were removed by suction, leaving the cell pellet. The pellet was lysed with 0.1 ml of 6 % (v/v) perchloric acid (PCA) and placed on ice for at least 15 min. The precipitates were removed by centrifugation (2 min, 10 000 revs min^{-1}) and 0.05 ml of the protein-free supernatants was measured by liquid scintillation counting (ACS II, Amersham) in an LKB Wallac 1215 Rackbeta counter, with internal standard quench correction. A correction factor for the radioactivity trapped in the extracellular space was estimated using ^{14}C-labelled polyethyleneglycol (PEG). Uptake values were obtained after correction for this extracellular trapped space (the average was 3.9 %, N=15).

To assess the possible sodium-dependence of the uptake rates, the cells were incubated using a sodium-free MCS (the osmolarity was adjusted using α-methyl-D-glucamine instead of NaCl and all other sodium salts were substituted by potassium salts). Sodium levels were assessed in sodium-free solutions, using an atomic absorption spectrophotometer (Varian Spectra AA10) with only trace concentrations (12 μmol l^{-1}) of sodium being found in solutions.

Substrate uptake is presented as μmol l^{-1} cell water, assuming that the percentage of cell water is 66 % (Tse and Young, 1990). The time course of the uptake was always determined at 10 °C using 1 mmol l^{-1} extracellular substrate concentration. Initial rates of uptake were determined using incubation periods of 30 s (lactate) and 60 min (3-OMG and alanine). Kinetic constants (the Michaelis–Menten constant, K_m, and the maximal flux, J_{max}) were determined by linear regression analysis after transforming the data using the Eadie–Hofstee method. Zero time values were substracted from all data prior to determination of kinetic constants.

CO_2 production studies

Carbon dioxide production of the cell suspension from labelled substrates was performed according to Walsh *et al.* (1990). 20 ml glass vials contained 0.6 ml of cell suspension (20 % haematocrit) prepared as described above and 0.15 ml of MCS containing the unlabelled substrate at a final concentration of 1 mmol l^{-1}. The vials were gassed with a 99.5 % O_2/0.5 % CO_2 mixture for 2 min and then sealed with a rubber septum, through which was suspended a centre well containing a glass microfibre filter (GF/A, Whatman). After a 15 min pre-incubation period, the experiment was initiated by the addition of 0.15 ml of MCS containing the labelled substrate (0.5 μCi per vial). The vials were shaken during the 2 h incubation period at 10 °C; 0.1 ml of 1 mol l^{-1} hyamine hydroxide was injected through the rubber septum, onto the filter in the centre well, and the cells were then lysed with 0.1 ml of 70 % (v/v) PCA to release the CO_2 and terminate the incubation. The sealed vials were shaken for a further 2 h at 10 °C to ensure the collection of CO_2 onto the filter.

The radioactivity trapped on the filter was determined by liquid scintillation counting (OCS, Amersham) as above. The CO_2 production rate was calculated from the specific activity of the added labelled substrate, the mass of cells used and the length of incubation, after correction for the CO_2 released from control vials (absence of cell suspension).

O_2 consumption studies

The method was performed according to Walsh *et al.* (1990). For total oxygen consumption measurements ($\dot{M}_{O_2}$), 1.2 ml samples containing 0.8 ml of cell suspension (21 % haematocrit) and 0.4 ml of substrate (1 mmol l^{-1} final concentration) or MCS (controls) were gassed with a 99.5 % O_2/0.5 % CO_2 mixture for 2 min. Samples were transferred with a syringe to an $\dot{M}_{O_2}$ measurement chamber maintained at 10 °C. The P_{O_2} was detected using a Radiometer O_2 electrode attached to a PHM blood gas analyzer and recorded using a customized data collection system consisting of a 801 AD converter and software developed by Peter Thoren, University of Gotteborg. $\dot{M}_{O_2}$ was calculated from the slope of the record over time as P_{O_2} fell from the starting value to no lower than 0.18 mmHg (24 kPa). Data from the computer package (mmHg min^{-1}) were transformed into μmol O_2 min^{-1} using the constant of oxygen solubility in plasma of 1.9861 μmol O_2 l^{-1} $mmHg^{-1}$ (Boutilier *et al.* 1984).

Analysis of plasma metabolites

Plasma was obtained after centrifugation of the blood collected into heparinized tubes as described above. After separation, the plasma was immediately deproteinized with 6 % (v/v) PCA and centrifuged (30 s, 10 000 revs min^{-1}). The deproteinized plasma was frozen at −20 °C until analyzed (within 15 days).

Plasma glucose was determined using the enzymatic colorimetric method (GOD-PAP) of Boehringer-Mannheim, using diluted plasma.

Plasma lactate was determined enzymatically in a medium containing (final concentrations): 0.5 mol l^{-1} glycine, 0.2 mol l^{-1} hydrazine sulphate, 0.5 mmol l^{-1} disodium EDTA, 3 mmol l^{-1} NAD^+ and 10 units of lactate dehydrogenase (LDH; omitted for control; 1 unit of enzyme activity is the amount of enzyme transforming 1 μmol of NADH per minute). The appearance of NADH was monitored at 340 nm (Milton Roy spectronic 1001 plus).

Plasma alanine was determined using an identical procedure with the exception that 2 units of alanine dehydrogenase (Sigma Chemical Co.; 1 unit of enzyme activity is defined as above) replaced the LDH.

Lactate dehydrogenase (E.C. 1.1.1.27) activity in red blood cells

An RBC suspension (20 % haematocrit) was obtained as described above. After centrifugation, the plasma was removed and the packed cells were homogenized in 30 vols of ice-cold 50 mmol l^{-1} imidazole–HCl, pH 7.4. The homogenate was centrifuged (30 s, 10 000 revs min^{-1}) and the supernatant (0.1 ml) was used directly in the enzyme assay. The final incubation volume (1 ml) contained 50 mmol l^{-1} imidazole–HCl (pH 7.4 or 8.4), 2 mmol l^{-1} NAD^+ and different concentrations of L-lactate (0.2–40 mmol l^{-1}), which was omitted in the controls. The LDH activity was monitored at 10 °C, following the appearance of NADH at 340 nm (Beckman DU-65 spectrophotometer).

Statistical analysis

Comparisons between kinetic variables were performed using a Student's *t*-test. The differences were considered significant at $P<0.05$.

Results

The cells were not left overnight in order to eliminate the possibility of catecholamines affecting the variables studied, since eel red cells are known to be relatively unresponsive to catecholamines (Perry and Reid, 1992). The low uptake rates observed for 3-OMG relative to those in the literature also support this contention (see below).

3-OMG uptake

The time course of the uptake of the non-metabolizable analogue of D-glucose, 3-*O*-methyl-D-glucose (3-OMG), was carried out using a concentration of 1 mmol l^{-1} and an

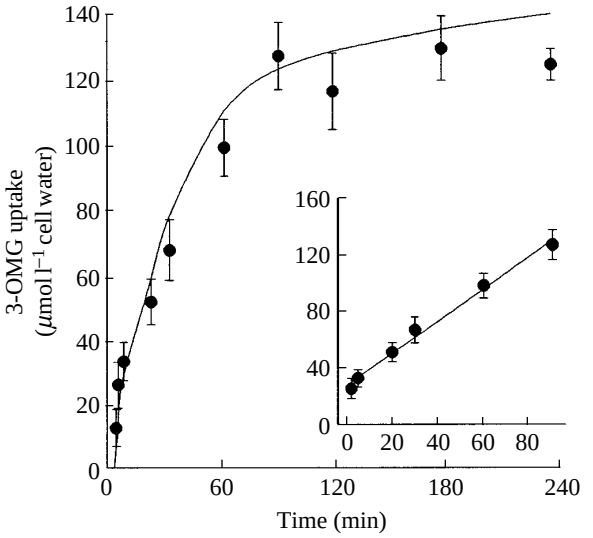

Fig. 1. Time course of 3-OMG uptake by American eel red blood cells. Uptake of 3-OMG (1 mmol l^{-1} extracellular concentration; 10 ˚C) was measured in the presence of 0.5 μCi of 3-*O*-methyl-D-[U-^{14}C]glucose. Incubations were terminated by centrifuging 0.15 ml of the mixture through 0.5 ml of dibutyl phthalate in a 1.5 ml plastic tube. Deproteinized pellet supernatant radioactivity was counted by scintillation counting and uptake was corrected for trapped extracellular space. Each value is the mean ± S.E.M. of four experiments. The line was drawn by eye as a best fit approximation. Inset: 3-OMG uptake between 2 and 90 min.

incubation period of 0–240 min. A steady state was reached after 90 min of incubation (Fig. 1), with the rate of uptake being linear from 2 to 90 min (Fig. 1, inset). The curve does not pass through zero time (Fig. 1, inset), so we cannot exclude a faster (<2 min) uptake component of 3-OMG transport in eel RBCs. A 60 min sampling period was, however, selected to coincide with the linear component of the time curve.

The concentration-dependence of the 3-OMG uptake was measured between 0.125 and 40 mmol l^{-1} and with an incubation time of 60 min. The uptake saturated, with the maximum being achieved at 20 mmol l^{-1} 3-OMG (Fig. 2). Using the Eadie–Hofstee regression analysis, K_m and J_{max} were estimated to be 10.36±0.60 mmol l^{-1} and 27.42±2.16 μmol 3-OMG l^{-1} cell water min^{-1}, respectively.

The effects of two well-known inhibitors of glucose uptake were tested in these RBCs (Fig. 2A). Cytochalasin B (10 μmol l^{-1}) significantly decreased both the K_m (2.70±0.79 mmol l^{-1}) and the J_{max} (2.23±0.27 μmol 3-OMG l^{-1} cell water min^{-1}) of 3-OMG uptake. Phloretin (1 mmol l^{-1}) significantly increased the K_m to 20.5±1.2 mmol l^{-1}, but decreased the J_{max} to 12.97±2.37 μmol 3-OMG l^{-1} cell water min^{-1}. There was no sodium-dependency of the carrier as assessed in uptake experiments in sodium-free medium; no differences were observed when compared with the normal sodium-containing medium (Fig. 2A).

Finally, when the effects of two other hexoses, D-glucose and 2-deoxyglucose, as competitors of 3-OMG uptake were

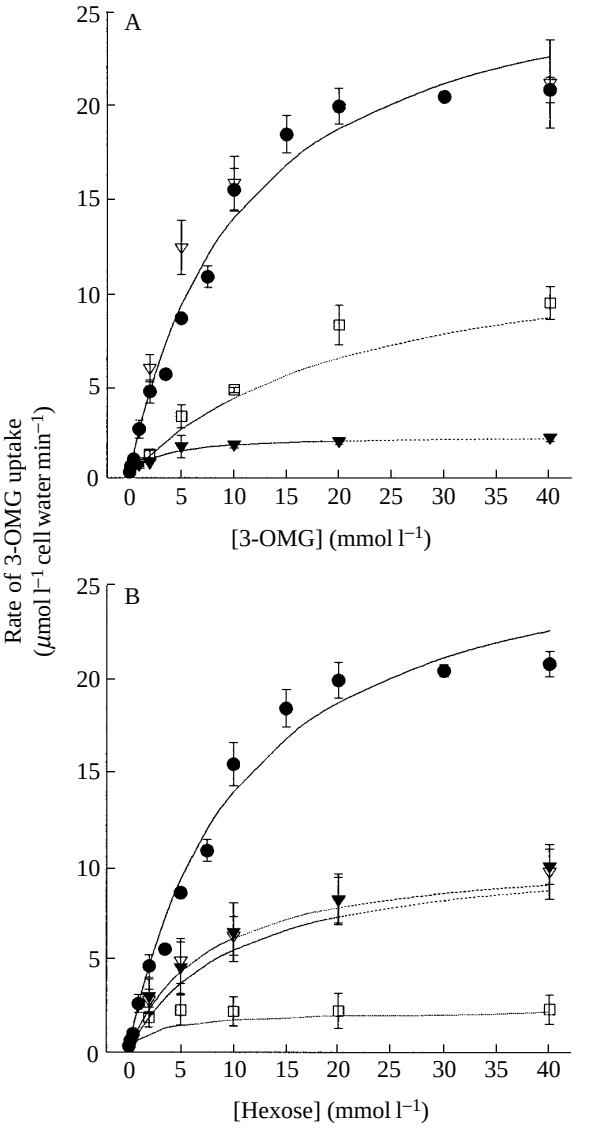

Fig. 2. Concentration-dependence of 3-OMG uptake by American eel red blood cells. Uptake was measured as described in Fig. 1 at 60 min and at different 3-OMG concentrations. Each value is the mean ± S.E.M. of eight experiments for 3-OMG uptake and three for all other substrates. (A) Initial uptake rate of 3-OMG in the presence (filled circles) or in the absence (open triangles) of added sodium, and 3-OMG in the presence of 1 mmol l^{-1} phloretin (open squares) and 10 μmol l^{-1} cytochalasin B (filled triangles). (B) Initial uptake rate of 3-OMG (filled circles) and 3-OMG in the presence of different concentrations of D-glucose (filled triangles), 2-deoxyglucose (open triangles) and L-glucose (open squares). The lines were drawn by eye as a best fit approximation.

tested (Fig. 2B), the results failed to indicate a change in K_m (9.6±2.1 mmol l^{-1} and 7.3±1.8 mmol l^{-1}, for D-glucose and 2-deoxyglucose, respectively), but J_{max} significantly decreased by approximately 60 % (10.75±1.66 and 10.59±2.63 μmol 3-OMG l^{-1} cell water min^{-1} for D-glucose and 2-deoxyglucose, respectively). The stereospecificity of the carrier was also assessed, with the rate of transport of L-glucose

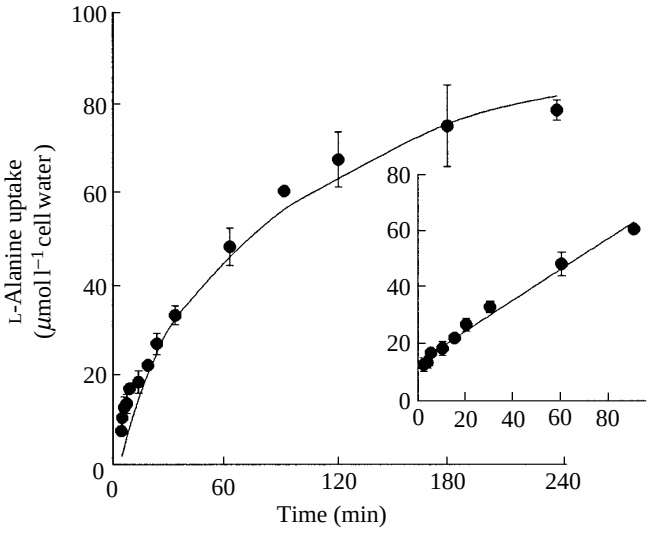

Fig. 3. Time course of alanine uptake by American eel red blood cells. Uptake of L-alanine (1 mmol l^{-1} extracellular concentration; 10 °C) was measured as described in Fig. 1, except that 0.5 μCi of L-[U-^{14}C]alanine was added. Each value is the mean ± S.E.M. of four experiments. The line was drawn by eye as a best fit approximation. Inset: alanine uptake between 0.5 and 90 min.

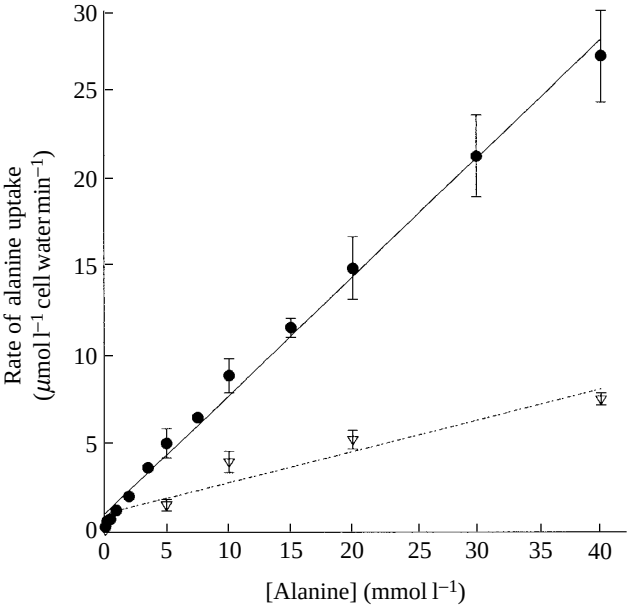

Fig. 4. Concentration-dependence of alanine uptake by American eel red blood cells. Initial uptake rate of L-alanine was measured as described in Fig. 3 at 60 min in the presence of different concentrations of L-alanine (filled circles) and D-alanine (open triangles). Each value is the mean ± S.E.M. of eight experiments for L-alanine uptake and four experiments for D-alanine uptake. Lines were drawn by computer using linear regression analysis.

being lower than that of any of the sugars tested and similar to values produced by the action of cytochalasin B (Fig. 2B).

Alanine uptake

Using 1 mmol l^{-1} L-alanine as substrate, the time course of the uptake, from 0 to 240 min, reached a plateau after 90 min of incubation, with the uptake rate being linear from 0.5 to 90 min (Fig. 3). As with 3-OMG, 60 min was chosen as the measurement period for all subsequent uptake experiments.

No saturation was observed when L-alanine uptake was evaluated between 0.125 and 40 mmol l^{-1} (Fig. 4). Even though the line appears to deviate from linearity at low concentrations, Eadie–Hofstee regression analysis was inconclusive as to whether saturation occurred at alanine concentrations below 2 mmol l^{-1}. When the uptake of D-alanine was assessed, a linear rate was also observed, with the 40 mmol l^{-1} value being approximately one-quarter of that observed for L-alanine uptake (Fig. 4). This linear uptake rate for L-alanine was the same whether sodium-containing or sodium-free incubation medium was used (data not shown).

Lactate uptake

The time course of L-lactate uptake was evaluated between 0 and 120 min. A steady state was observed after 5 min, with a linear response over the first 1.5 min (Fig. 5). Thus, a time of 30 s was chosen for all subsequent uptake experiments. The line does not go through time zero in Fig. 5 (inset), possibly because of a technical problem. Lactate uptake was very rapid, so a 1 or 2 s delay in stopping the incubation could be enough

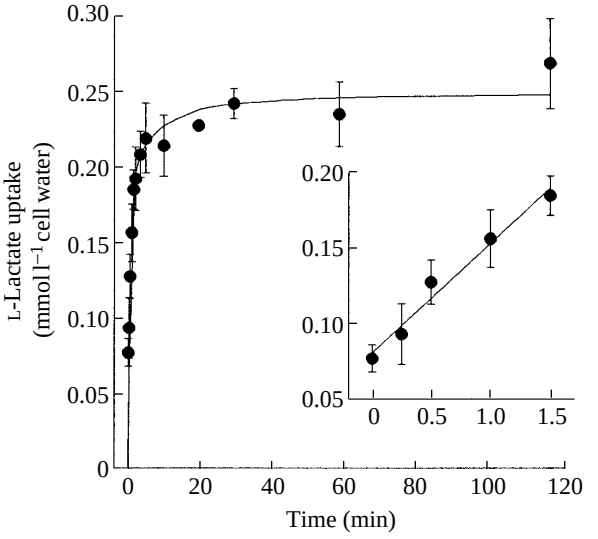

Fig. 5. Time course of L-lactate uptake by American eel red blood cells. Uptake of L-lactate (1 mmol l^{-1} extracellular concentration; 10 °C) was measured as described in Fig. 1, except that 0.5 μCi of L-[U-^{14}C]lactate was used. Each value is the mean ± S.E.M. of four experiments. The line was drawn by eye as a best fit approximation. Inset: L-lactate uptake between 0 and 1.5 min.

to account for the presence of some radioactivity inside the cells, thus producing a zero time value higher than zero.

The kinetic curve with L-lactate as substrate can be divided into two distinct phases (Fig. 6), the first from 0.125 to

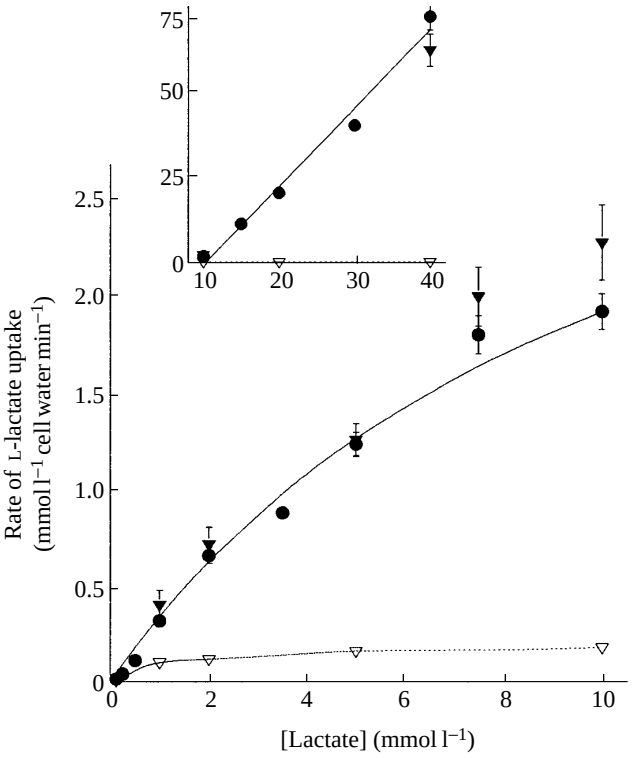

Fig. 6. Concentration-dependence of L-lactate uptake by American eel red blood cells. Uptake was measured as described in Fig. 5 at 30 s. Each value is the mean ± S.E.M. of twelve experiments for L-lactate uptake, four experiments for D-lactate uptake and three experiments for the uptake in the absence of extracellular sodium. Initial uptake rate of L-lactate in the presence (filled circles) or in the absence (filled triangles) of added sodium, and L-lactate uptake in the presence of different concentrations of D-lactate (open triangle). The lines were drawn by eye as a best fit approximation. Inset: lactate uptake between 10 and 40 mmol l^{-1}.

10 mmol l^{-1} and the second from 15 to 40 mmol l^{-1} (higher concentrations were not investigated). This second phase clearly showed a linear rate of uptake, in contrast to the first phase, during which L-lactate uptake was saturable, with an estimated K_m of 6.74±0.36 mmol l^{-1} and a J_{max} of 2.29±0.09 mmol lactate l^{-1} cell water min^{-1}. The carrier for L-lactate is stereospecific, as judged by the negligible rate of D-lactate uptake (Fig. 6). No changes were observed in the rate of uptake when sodium was omitted from the incubation medium (Fig. 6).

The effects of several compounds on the uptake rate were assessed using four different concentrations of L-lactate (2, 5, 10 and 40 mmol l^{-1}) and 6–10 different concentrations of each inhibitor. The concentrations of substances that produced a 50 % inhibition (IC_{50}) of L-lactate uptake rate are shown in Table 1. The IC_{50} was not reached by any of the inhibitors tested when the L-lactate concentration used was 40 mmol l^{-1}.

The effect of α-cyano-4-hydroxycinnamate (CYN) on L-lactate uptake was a dose-dependent inhibition at 2 and 5 mmol l^{-1} lactate (Fig. 7A). The IC_{50} value increased more than 16 times between these two L-lactate concentrations

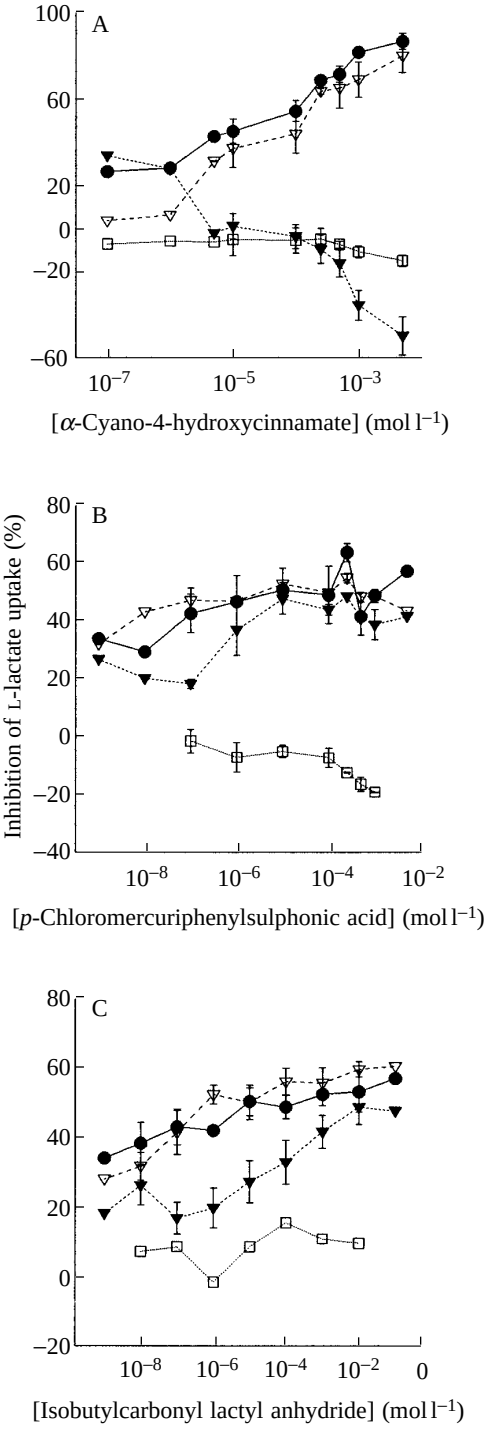

Fig. 7. Effect of inhibitor concentrations on the uptake of L-lactate at 2 mmol l^{-1} (filled circles), 5 mmol l^{-1} (open triangles), 10 mmol l^{-1} (filled triangles) and 40 mmol l^{-1} (open squares) in American eel red blood cells. Uptake was measured as described in Fig. 6. Each value is the mean ± S.E.M. of three experiments. Results are presented as a percentage of the control uptake rate in the absence of inhibitor. (A) Effects of α-cyano-4-hydroxycinnamate; (B) effects of *p*-chloromercuriphenylsulphonic acid; (C) effects of isobutylcarbonyl lactyl anhydride.

Table 1. *Concentrations of different compounds (μmol l^{-1}) causing a 50 % inhibition (IC_{50}) of the initial uptake rate of different concentrations of lactate into red blood cells of American eel*

	[Lactate] (mmol l^{-1})		
Inhibitor	2	5	10
α-Cyano-4-hydroxycinnamate	18.7	314	NR
DIDS	110	140	1590
SITS	100	51	NR
Isobutylcarbonyl lactyl anhydride	200	200	NR
p-Chloromercuriphenylsulphonic acid	10	100	NR
Pyruvate	21740	45200	67600

NR, the substance inhibited lactate uptake by less than 50 %. At 40 mmol l^{-1} lactate none of the inhibitors achieved 50 % inhibition of lactate uptake.

IC_{50} values were determined by linear regression analysis of the curves in Figs 7 and 8.

(Table 1). The inhibition of L-lactate uptake ranged from essentially zero at 0.1 μmol l^{-1} CYN to 90 % at 5 mmol l^{-1} CYN. More striking, however, was that at 10 mmol l^{-1} L-lactate, the effect of CYN was as an activator of L-lactate uptake (nearly 60 % at 5 mmol l^{-1} CYN) rather than an inhibitor. There were no effects at any CYN concentrations using 40 mmol l^{-1} L-lactate.

The effect of *p*-chloromercuriphenylsulphonic acid (pCMBS) on L-lactate uptake was assessed at concentrations from 1 nmol l^{-1} to 5 mmol l^{-1} (Fig. 7B). No clear dose-dependent pattern emerged at any of the concentrations of L-lactate evaluated, although the IC_{50} value did increase 10-fold between 2 and 5 mmol l^{-1} L-lactate (Table 1).

A dose–response curve was plotted for isobutylcarbonyl lactyl anhydride (IBCLA) on the uptake of 2, 5 and 10 mmol l^{-1} L-lactate, though the magnitude of the changes was negligible (from 30 % inhibition at 1 nmol l^{-1} to 50 % inhibition at 200 mmol l^{-1} IBCLA, Fig. 7C) and the IC_{50} value did not change (Table 1). No changes were observed in the uptake of 40 mmol l^{-1} L-lactate.

A dose-dependent inhibition was seen using SITS (Fig. 8A) and the IC_{50} value actually decreased at higher L-lactate concentrations (Table 1). Inhibition of L-lactate uptake ranged from approximately 20 % at 1 nmol l^{-1} to approximately 60 % at 2 mmol l^{-1} SITS. Again, no inhibition was observed, at any of the different concentrations of SITS used, when the uptake of 40 mmol l^{-1} L-lactate was studied.

The effect of DIDS on lactate uptake was a clear dose-dependent inhibition of uptake at 2, 5 and 10 mmol l^{-1} lactate (Fig. 8B); IC_{50} values increased at these higher L-lactate concentrations (Table 1). The inhibition ranged from approximately 10 % at 0.1 μmol l^{-1} to approximately 90 % at 5 mmol l^{-1} DIDS. Again, the uptake rate of 40 mmol l^{-1} L-lactate remained unchanged with all the concentrations of inhibitor assessed.

Finally, the effect of pyruvate on the L-lactate uptake rate also showed a dose-dependent inhibition pattern at all concentrations studied, even at 40 mmol l^{-1} L-lactate

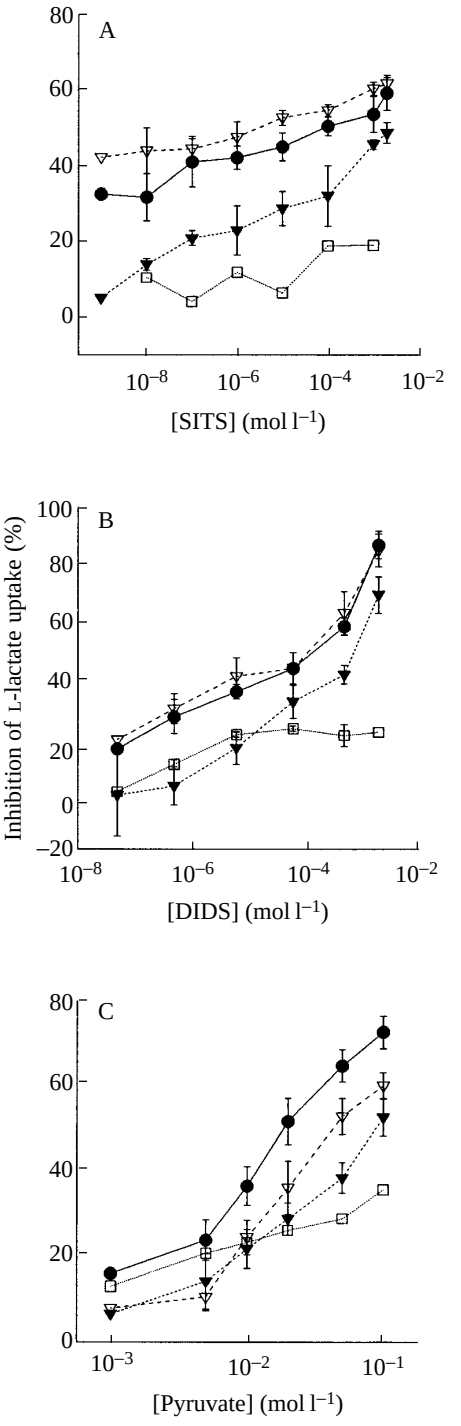

Fig. 8. Effect of inhibitor concentrations on the uptake of L-lactate at 2 mmol l^{-1} (filled circles), 5 mmol l^{-1} (open triangles), 10 mmol l^{-1} (filled triangles) and 40 mmol l^{-1} (open squares) in American eel red blood cells. Uptake was measured as described in Fig. 6. Each value is the mean ± S.E.M. of three experiments. Results are presented as a percentage of the control uptake rate in the absence of inhibitor. (A) Effects of disodium 4-acetamidostilbene-2,2′-disulphonate (SITS); (B) effects of 4,4′-di-isothiocyanostilbene-2,2′-disulphonate (DIDS); (C) effects of pyruvate.

Table 2. *Oxidative metabolism by red blood cells and plasma metabolite levels of American eels*

Variable	Substrate			
	Glucose	Alanine	Lactate	Control
CO_2 production	182.1±44.2	113.6±20.4	377.8±38.8	
(μmol CO_2 l^{-1} cell water h^{-1})	(8)	(8)	(8)	
O_2 consumption	332.7±20.2	306.5±43.8	615.1±41.1	87.6±12.5
(μmol O_2 l^{-1} cell water h^{-1})	(2)	(2)	(2)	(2)
Plasma concentration	9.40±0.44	0.46±0.07	1.17±0.06	
(mmol l^{-1})	(7)	(7)	(7)	

All values are means ± S.E.M. of (*N*) preparations; see Materials and methods for procedures.
All substrates were 1 mmol l^{-1} final concentration.

Table 3. *Comparison between the uptake and oxidation rates of different substrates at 1 mmol l^{-1} extracellular concentration by American eel red blood cells*

Substrate	Uptake rate (μmol l^{-1} cell water h^{-1})	Oxidation rate (μmol l^{-1} cell water h^{-1})	Uptake rate/oxidation rate
Glucose	155.9±28.3 (8)	30.36±7.36 (8)	5.1
Alanine	74.12±6.84 (8)	37.86±6.80 (8)	2.0
Lactate	19013±1188 (12)	125.9±12.9 (8)	151

Data were obtained from Table 2 (oxidation) and Figs 2, 4 and 6 (uptake).
Oxidation rates were calculated from CO_2 production, assuming that 1 mol of lactate or alanine produces 3 mol of CO_2 and that 1 mol of glucose produces 6 mol of CO_2.
Values are the means ± S.E.M. of (*N*) preparations.

(Fig. 8C). The IC_{50} value paralleled these changes (Table 1). The inhibition by pyruvate ranged from less than 10 % at 1 mmol l^{-1} to 40–90 % (depending on the L-lactate concentration) at 100 mmol l^{-1} pyruvate.

CO_2 production studies

American eel RBCs oxidized L-lactate at higher rates than either glucose or alanine, as judged by CO_2 production from 1 mmol l^{-1} substrate (Table 2).

In order to determine whether substrate oxidation was limited by uptake rates, the data were transformed into μmol substrate l^{-1} cell water h^{-1} to compare them with the uptake rate of 1 mmol l^{-1} substrate (taken from Figs 2, 4 and 6). The comparison (Table 3) clearly showed that the rate of metabolism was not limited by the rate of uptake, with ratios ranging from 2 (alanine) to 151 (lactate).

O_2 consumption studies

The same substrate order that was noted for CO_2 production was observed for the O_2 consumption rates (Table 2). The rate of O_2 consumption in the presence of 1 mmol l^{-1} L-lactate was double that in the presence of either glucose or L-alanine. It is interesting to note that the control cells (no added substrate) also consumed O_2, reflecting endogenous metabolism by the RBCs.

Plasma metabolites and lactate dehydrogenase activity in red blood cells

The levels of glucose, alanine and lactate in plasma (Table 2) were evaluated to contrast them with the kinetic constants of the respective carriers. The glucose levels (9.40±0.44 mmol l^{-1}) very closely approximated the estimated K_m for 3-OMG uptake (10.36±0.60 mmol l^{-1}). For L-lactate, plasma levels were four times lower (1.17±0.06 mmol l^{-1}) than the estimated K_m of the carrier (6.74±0.36 mmol l^{-1}). L-Alanine content was considerably lower than that of either glucose or L-lactate.

The activity of LDH in eel RBCs was measured in the lactate oxidase direction to evaluate the ability of these cells to convert lactate to pyruvate. The results (Fig. 9) showed that the optimal enzyme activity (V_{opt}), 0.96±0.03 mmol L-lactate l^{-1} cell water min^{-1} at pH 7.4 or 1.14±0.05 mmol L-lactate l^{-1} cell water min^{-1} at pH 8.4, correlated with the J_{max} of L-lactate uptake (2.29±0.09 mmol L-lactate l^{-1} cell water min^{-1}). It was also interesting to note that enzyme activity saturated at lactate concentrations above 10 mmol l^{-1}, the concentration at which the uptake of L-lactate changed from carrier transport to simple diffusion (Fig. 6).

Discussion

3-OMG uptake

A wide range of permeabilities to glucose have been reported in fish RBCs, from almost impermeable (brown trout, Bolis *et al.* 1971; rainbow trout, Tse and Young, 1990) to significant glucose permeabilities (see Introduction; Moon and Walsh, 1994). However, not all species that demonstrate rapid

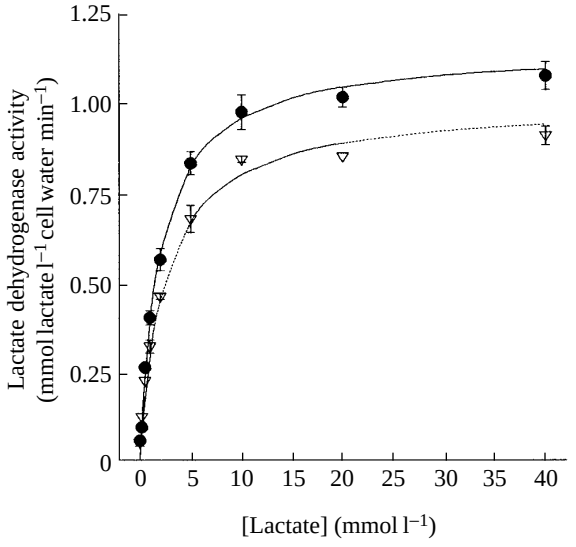

Fig. 9. Lactate dehydrogenase activity (lactate oxidase direction) in American eel red blood cells. Enzyme activity was measured at pH 8.4 (filled circles) and pH 7.4 (open triangles) in a medium containing 50 mmol l^{-1} imidazole–HCl, 2 mmol l^{-1} NAD^+ and the given L-lactate concentrations at 10 °C. Each value is the mean ± S.E.M. of four experiments. The lines were drawn by eye as a best fit approximation.

glucose transport possess a specific glucose transporter such as those in electric eel and lungfish (Kim and Isaaks, 1978) and carp (Tiihonen and Nikinmaa, 1991*b*). Carrier-saturated transport has been described only for river lamprey (Tiihonen and Nikinmaa, 1991*b*), Pacific hagfish (Ingermann *et al.* 1984; Young *et al.* 1994) and Japanese eel (Tse and Young, 1990) RBCs. Other vertebrates, such as mammals, possess permeable (human, Kim *et al.* 1983) or impermeable (pig, Young *et al.* 1985) RBCs. Certainly 3-OMG does not equilibrate across the eel RBC membrane (Fig. 1; 1 mmol l^{-1} and 0.14 mmol l^{-1}, extracellular and intracellular, respectively) in a manner similar to that previously reported by Pesquero *et al.* (1992), although the presentation of other data does not permit comparisons (Tiihonen and Nikinmaa, 1991*b*; Canals *et al.* 1992; Albi *et al.* 1993). Tse and Young (1990) found that, in some individual eels, 3-OMG did equilibrate across the red blood cell membrane, at least to 89 % of the level in the extracellular water. The lack of equilibration may relate to specific membrane features (e.g. transporters, diffusion, protein and phospholipid composition) rather than to the experimental transport technique.

The American eel RBC clearly demonstrated saturable 3-OMG transport over the concentration range 0–40 mmol l^{-1} (Fig. 2), in contrast to the data obtained in river lamprey, where two different transport components have been described (Tiihonen and Nikinmaa, 1991*b*). A clear stereospecificity was observed because L-glucose transport was negligible (10-fold lower than the rate of 3-OMG transport) (Fig. 2B). D-Glucose and 2-deoxyglucose would be expected to function as competitive inhibitors of 3-OMG uptake. However, in our study, both D-glucose and 2-deoxyglucose changed the J_{max} of 3-OMG transport (Fig. 2), which is in agreement with other studies carried out in fish RBCs (Ingermann *et al.* 1984; Tse and Young, 1990; Young *et al.* 1994). The lack of a K_m effect may relate to the extended preincubation of cells with D-glucose and 2-deoxyglucose. As expected from similar studies in mammals (Ingermann *et al.* 1985), 3-OMG uptake rates were independent of extracellular sodium.

In the present study, J_{max} was markedly decreased in the presence of 1 mmol l^{-1} phloretin (twofold) and 10 μmol l^{-1} cytochalasin B (10-fold) (Fig. 2A), which lends further support to our hypothesis that a GLUT-1 carrier exists on the American eel RBC, in agreement with similar effects of both inhibitors previously observed in Japanese eel (Tse and Young, 1990) and Pacific hagfish (Young *et al.* 1994) RBCs. The effectiveness of cytochalasin B strengthens the hypothesis for a sodium-independent glucose transport process, since this substance does not inhibit the sodium-dependent carrier (Ingermann *et al.* 1984). The different effects of phloretin and cytochalasin B on the kinetic characteristics of 3-OMG transport may arise from their different sites of action on the glucose carrier, i.e. on the inward and outward conformation of the hexose permeation site for cytochalasin B and phloretin, respectively (Ingermann *et al.* 1985).

The relatively slow uptake of 3-OMG (Fig. 1) was not surprising and is similar to that observed in other species (Ingermann *et al.* 1985), including the Japanese eel (half-time of 15 min at 20 °C; Tse and Young, 1990). The uptake rate of 5 mmol l^{-1} 3-OMG in American eel RBCs is six times slower and the apparent K_m is 8.5 times higher than values reported for the Japanese eel (Tse and Young, 1990). It is unlikely that these differences are due to assay differences since the two studies used similar conditions, except for the assay temperature (10 °C *versus* 20 °C). However, the J_{max} of the American eel RBC 3-OMG carrier is four times higher than values reported in other species in which no carrier has been described, such as *Monopterus albus* and rainbow trout (Tse and Young, 1990) and brown trout (Pesquero *et al.* 1992). Such species differences can only relate to genetic differences and possibly to the control of transport, which was not the subject of this study.

These studies support the sodium-independent GLUT-1 transport of glucose in the American eel RBC, as reported in mammalian RBCs and more recently in Pacific hagfish (Young *et al.* 1994).

Alanine uptake

The uptake of L-alanine by American eel RBCs displayed neither saturation (Fig. 4) nor sodium-dependency (data not shown). These results are compatible with a simple diffusion model for L-alanine uptake by the American eel RBC. However, the lack of an L-alanine carrier is inconsistent with the result obtained using D-alanine (Fig. 4). This isomer reduced L-alanine uptake fourfold. Thus, these data are consistent with one characteristic of a carrier, i.e. stereospecificity, but this uptake is not saturable. No experiment undertaken during this study provided evidence for

a saturable transporter, even at L-alanine concentrations of 2 mmol l^{-1} and below, where the curve appears to deviate from linearity (Fig. 4). It was noted (Fig. 3, inset) that the curves do not pass through zero time; this may suggest a faster L-alanine uptake component that could have different kinetic properties from the one illustrated in Fig. 4 and described in studies using hepatocytes by Canals *et al.* (1992) showing two different L-alanine uptake processes. More work is needed to investigate these contradictory results.

Lactate uptake

L-Lactate is transported into adult mammalian and avian RBCs by simple diffusion, by the anion exchanger known as the band 3 protein or by a monocarboxylate carrier (using H^+ or Na^+ as cotransported ions) (Poole and Halestrap, 1993). Previous studies using fish have reported lactate uptake to be by simple diffusion in tuna RBCs (Moon *et al.* 1987) or by a combination of all possible transporters in carp RBCs, depending upon L-lactate concentrations (Tiihonen and Nikinmaa, 1993).

The time course of L-lactate uptake by American eel RBCs showed a rapid attainment of a steady state (Fig. 5), comparable to the 15 s needed in most mammalian cells (Poole and Halestrap, 1991), but much more rapid than the time needed in carp RBCs (Tiihonen and Nikinmaa, 1993). The L-lactate concentration differed across the membrane at equilibrium (Fig. 5; 1 mmol l^{-1} extracellular, 0.25 mmol l^{-1} intracellular). Although equilibrium was achieved for lactate in carp RBCs (Tiihonen and Nikinmaa, 1993), the lack of an equilibrium for eel lactate transport again may reflect the characteristics of this membrane, as suggested for 3-OMG transport. A saturable uptake in eel RBCs was demonstrated up to a concentration of 10 mmol l^{-1} lactate (Fig. 6), whereas at concentrations above 15 mmol l^{-1} lactate, the linear relationship between uptake and concentration supports a simple diffusion model. The transport of L-lactate was approximately 10 times faster than that of D-lactate at concentrations below 15 mmol l^{-1} L-lactate (Fig. 6), as has been reported in human erythrocytes (Poole and Halestrap, 1993), suggesting that transport is stereospecific. Stereospecificity is strictly associated with the monocarboxylate carrier, not with the band 3 exchanger (Poole and Halestrap, 1993), strengthening the hypothesis that the L-lactate carrier in the American eel RBC is of the monocarboxylate type. Most strikingly, the rate of D-lactate transport did not increase at a rate identical to that of L-lactate at concentrations of lactate above 15 mmol l^{-1} (Fig. 6). A possible explanation for this discrepancy may be that since D-lactate, unlike L-lactate, is not metabolized by the cells, an equilibrium may exist across the RBC membrane, blocking uptake. Tiihonen and Nikinmaa (1993) reported two components of L-lactate transport in carp RBCs. Despite not evaluating carrier stereospecificity, Tiihonen and Nikinmaa (1993) observed a saturable component up to 5 mmol l^{-1} lactate and a diffusional component from 10 to 40 mmol l^{-1} lactate. Comparing the eel and carp data, it is interesting to note that whereas the estimate of K_m is higher in carp than in eel (6.74 mmol l^{-1} for eel, 2.76 mmol l^{-1} for carp), the estimated J_{max} is higher in eel than in carp (2.29 and 0.317 mmol lactate l^{-1} cell water min^{-1}, respectively). These values of K_m differ from plasma lactate levels in these two species (1.2 mmol l^{-1} in eel, 4.2 mmol l^{-1} in carp).

Inhibitors of L-lactate transport are also important in characterizing the specific transporters used, although the typical inhibitors of the monocarboxylate carrier in human RBCs, α-cyano-4-hydroxycinnamate and stilbene-disulphonates, also inhibit the band 3 exchanger (Poole and Halestrap, 1991, 1993), making it difficult to ascertain which one is working. In the eel RBC, the transport of 2 and 5 mmol l^{-1} L-lactate was inhibited by CYN (Fig. 7A), SITS (Fig. 8A) and DIDS (Fig. 8B). The observation that at 40 mmol l^{-1} L-lactate the effect of these inhibitors and others used was negligible (nearly 0 % for all the inhibitors, except pyruvate) supports the increased importance of the passive diffusion of the undissociated acid at these higher concentrations, in a way similar to that suggested in mammalian β-cells (Best *et al.* 1992). The IC_{50} values of the inhibitors (Table 1) were in the range described for the monocarboxylate carrier of mammal RBCs by Poole and Halestrap (1991). The reduced effect and lower dose–response inhibition by SITS compared with DIDS are typical of mammalian erythrocytes (Poole and Halestrap, 1991). The effects of pCMBS are not clear, as no dose–response relationship was observed, even though an IC_{50} value of 10 μmol l^{-1} at 2 mmol l^{-1} L-lactate was obtained (Table 1), a value very similar to that described for human erythrocytes (Poole and Halestrap, 1991). The high IC_{50} value (200 μmol l^{-1}, using 2 mmol l^{-1} lactate; Table 1) of IBCLA makes this inhibitor useless for characterizing the transport mechanism of L-lactate in this system. The monocarboxylate carrier of carp RBCs was inhibited with both CYN and pCMBS (Tiihonen and Nikinmaa, 1993). As the actions of pCMBS and IBCLA are through covalent modification (Poole and Halestrap, 1993), and thus involve specific amino acids of the carrier molecule, these species differences may be great enough to explain the different responses of the carrier. This would not be the first case in which a family of monocarboxylate carriers, with different sensitivities for the classical inhibitors, has been suggested (Poole and Halestrap, 1993).

Other evidence concerning the nature of the carrier comes from the effect of pyruvate, which can be transported through the same carrier (Poole and Halestrap, 1993). Pyruvate decreased L-lactate uptake, even at L-lactate concentrations of 40 mmol l^{-1} (Fig. 8C), in a manner similar to the competitive inhibition described for the monocarboxylate carrier of human erythrocytes (Poole and Halestrap, 1991) and other cells (McDermott and Bonen, 1993). Although the transport of L-lactate in American eel RBCs was not sodium-dependent (Fig. 6), we cannot assume proton-dependency because the effects of a pH gradient on the uptake rate were not tested. Thus, further experiments are required to ascertain the effect

of H^+ movement in the function of this carrier. However, most of the characteristics reported support the hypothesis that the carrier for L-lactate in American eel RBCs is similar to the monocarboxylate carrier found in most vertebrate red blood cells, at least at physiological lactate concentrations.

Metabolism

When we compared the values of O_2 consumption and CO_2 production (Table 2), a 1.6- to 2.6-fold difference (depending on the substrate assessed) between these values was observed; i.e. the cells appear to use more oxygen than carbon dioxide produced. Similar data (showing even higher differences) have been previously reported for brown (Pesquero *et al.* 1992) and rainbow (Walsh *et al.* 1990) trout. An explanation for this observation may include (i) different measurement methods for oxygen (electrochemical) and CO_2 (radioactive), and (ii) significant dilution and/or mixing of exogenously added substrates prior to their entry into the Krebs cycle, as previously reported in sea raven (*Hemitripterus americanus*) RBCs (Sephton *et al.* 1991).

Pesquero *et al.* (1992) reported that oxygen consumption was substrate-independent in brown trout RBCs. This is not the case for American eel RBCs, since each substrate was metabolized at a different rate, in agreement with results reported by Walsh *et al.* (1990) for rainbow trout RBCs. The RBCs of American eel oxidized lactate in preference to glucose (Table 3), which is the predominant fuel in rainbow (Walsh *et al.* 1990) and brown (Pesquero *et al.* 1992) trout RBCs. The rate of metabolism of each substrate assessed (using $1\,mmol\,l^{-1}$ substrate) was not limited by transport, since in all cases the transport rates were higher than the maximum rate at which substrate could be metabolized (Table 3). However, the ratio between the uptake rate and the oxidation rate was dependent on the substrate assessed, exceeding 150 for L-lactate but being only 2 for alanine. In the other study in which these rates were compared, Tiihonen and Nikinmaa (1993) also observed that the use of the main metabolite of carp RBC, L-lactate, was not limited by its transport rate, with the ratio of uptake rate to metabolic rate being 200.

It is also apparent that lactate oxidase (LDH activities in the lactate to pyruvate direction) is sufficiently active not to limit lactate oxidation (Fig. 9). However, eel RBCs also oxidized glucose, which is not surprising considering the high levels of glucose in plasma (almost 10 times those of lactate; Table 2), but at 25 % of the rate of lactate oxidation (Table 3). As the rate of transport of glucose is considerably lower than that observed for lactate (125 times), the metabolism of glucose is more likely to be limited by uptake rate than is that of lactate. The substrate oxidised slowest was alanine, in agreement with results obtained in rainbow trout (Walsh *et al.* 1990). These results support the contention that glucose and lactate (depending on the species studied), but not alanine, are the principal substrates oxidized by fish RBCs, although this conclusion cannot be generalized to unlabelled substrates.

These experiments demonstrate that substrate oxidation by American eel RBCs is generally not limited by the rate of substrate transport, and that substrate transport mechanisms are species-dependent. The American eel RBC transports glucose using a cytochalasin-B-sensitive transporter with low maximal rates and L-lactate using a monocarboxylate carrier, at least over physiological substrate concentrations. Alanine is apparently transported by simple diffusion in the absence of a neutral amino acid carrier. These major differences between the American eel and other species may be related to many factors, but attempting to determine these factors may be extremely difficult.

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