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GLUCOSE TRANSPORT IN CARP ERYTHROCYTES: INDIVIDUAL VARIATION AND EFFECTS OF OSMOTIC SWELLING, EXTRACELLULAR pH AND CATECHOLAMINES

KIRSTI TIIHONEN, MIKKO NIKINMAA AND JARMO LAPPIVAARA
Department of Zoology, Division of Physiology, PO Box 17, FIN-00014 University of Helsinki, Finland

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Summary

The characteristics of the uptake of 3-*O*-methyl-D-glucose (3-OMG) by carp (*Cyprinus carpio*) erythrocytes were studied *in vitro* with tracer methods. There is large individual variation in the permeability of the carp erythrocyte membrane to 3-OMG. Although transport is inhibited by cytochalasin B and phloretin, the lack of saturation kinetics for transport in a physiologically relevant concentration range suggests either that a glucose transporter does not exist or that its affinity for glucose is extremely low. The marked increase in transport after osmotic swelling and the inhibition of swelling-induced glucose transport by cytochalasin B suggest that the glucose transport pathway in carp erythrocytes (both in isotonic and hypotonic conditions) may be similar to the volume-activated channel described for flounder erythrocytes. 3-OMG transport across the carp erythrocyte membrane is increased by catecholamines by a mechanism independent of the catecholamine-induced cell swelling.

Key words: *Cyprinus carpio*, carp, erythrocyte metabolism, glucose transport, cytochalasin B, phloretin.

Introduction

Glucose transport across the erythrocyte membrane of several teleost fish is slow (Bolis *et al.* 1971; Ingermann *et al.* 1985; Tse and Young, 1990; Tiihonen and Nikinmaa, 1991*a*; Pesquero *et al.* 1992). Despite this, glucose is used as a metabolic fuel by the erythrocyte to a significant degree (Walsh *et al.* 1990; Sephton *et al.* 1991; Tiihonen and Nikinmaa, 1991*b*), because its extracellular concentration is high (several $mmol\,l^{-1}$, see Nikinmaa *et al.* 1980).

Since glucose transport into teleost erythrocytes may be rate-limiting for glucose utilization, factors that influence glucose transport may also influence metabolic rate (Booz *et al.* 1989). However, factors affecting glucose transport have been studied very little. The energy consumption of teleost erythrocytes increases markedly as a result of adrenergic stimulation (Ferguson *et al.* 1989). Catecholamines also increase the glucose permeability of teleost erythrocyte membranes (Tse and Young, 1990; Pesquero *et al.* 1992). One specific change occurring in catecholamine-treated erythrocytes is an increase in their volume (Nikinmaa, 1982). Kirk *et al.* (1992) showed that an increase in flounder (*Platichthys stellatus*) red cell volume increases the membrane permeability to glucose and to several other small organic molecules. Thus, it is possible that the catecholamine-induced increase in glucose permeability could be mediated by changes in cell volume. It is also possible that changes in extracellular pH could affect the rate of glucose transport, as in other cell types (Ismail-Beigi, 1993).

In order to clarify the factors that influence glucose permeability of teleost erythrocytes, we studied the influence of catecholamines, extracellular pH and cell swelling on the permeability to 3-*O*-methyl-D-glucose of the carp (*Cyprinus carpio*) erythrocyte membrane. Since, during the course of the experiments, it became apparent that there is marked variation in the membrane permeability to glucose between individuals, we also investigated how this individual variation is reflected in the inhibition of glucose transport by the commonly used inhibitors cytochalasin B, which binds at the inner glucose carrier site (Basketter and Widdas, 1977, 1978; Devés and Krupka, 1978), and phloretin, which binds at the outer glucose carrier site (Krupka, 1985). In many vertebrate erythrocytes, glucose uptake is inhibited by phloretin and more powerfully by cytochalasin B (Taverna and Langdon, 1973; Jung and Rampal, 1977; Goldin and Rhoden, 1978; Simons, 1983; Ingermann *et al.* 1984; Krupka, 1985; Tse and Young, 1990; Tiihonen and Nikinmaa, 1991*a*).

Materials and methods

Fish and handling of blood

The carp, *Cyprinus carpio* L., were obtained from Porla Fisheries Station and maintained under laboratory conditions (in running dechlorinated Helsinki tap water). Fish were fed a commercial diet, but were fasted for 24–96 h before use.

Animals were anaesthetized with MS-222 (0.1 g l^{-1}, 10 min) and the blood samples were taken by venipuncture.

Red blood cells (RBCs) and plasma were separated by brief centrifugation (10 000 ***g*** for 20 s). Cells were prepared for transport experiments by washing three times (for at least 2 h) with an iso-osmotic incubation medium containing (in mmol l^{-1}): 128 NaCl, 3 KCl, 1.5 $CaCl_2$, 1.5 $MgCl_2$ and 10 Hepes. The washing procedure was adequate to remove intracellular glucose. The buffy coat was also removed and the washed red cells were resuspended to a haematocrit (Hct) of 20 % in incubation medium or 10 % in medium containing inhibitor. The pH of the medium was initially adjusted to 7.5, except in the experiments in which the effects of pH on glucose transport were studied. All experiments were carried out under air at 21±1 °C.

Since anaesthesia and venipuncture may cause a release of catecholamines into the circulation, we checked that glucose transport was not affected by this in our experiments. Glucose uptake was measured after three washes (for at least 2 h) and again 24 h later. In both cases, the 3-OMG uptake rate was 0.24 mmol l^{-1} RBC h^{-1} (N=2).

In swelling experiments, cells were suspended in hypotonic (or isotonic as controls), Na^+-free (high-K^+) medium, the osmolarity of which varied down to 60 % of its normal physiological value. The isotonic medium consisted of (in mmol l^{-1}): 131 KCl, 1.5 $CaCl_2$, 1.5 $MgCl_2$ and 10 Hepes (pH 7.5). The use of K^+ as the extracellular cation minimizes the regulatory volume decrease following hypotonic swelling (Garcia-Romeu *et al.* 1991). The swelling was monitored by measuring the water content of the cells, determined as previously described without correction for extracellular trapped water (Nikinmaa and Huestis, 1984).

Transport studies

The uptake of 3-OMG was measured using the modified oil-stop procedure (Young and Ellory, 1982). The uptake of 3-*O*-[^{14}C]methyl-D-glucose was initiated by mixing the cell suspension (Hct 20 %) with an equal volume of incubation medium containing labelled substrate. In dose–response studies with inhibitors and noradrenaline, the ratio of cell suspension (Hct 10 %) to medium containing labelled substrate was 9:1 (v:v), but final incubations, in both cases, contained ^{14}C-labelled 3-OMG at an activity of approximately 10 kBq ml^{-1}. 3-*O*-[^{14}C]methyl-D-glucose (2.12 GBq $mmol^{-1}$) was obtained from Amersham International.

In the dose–response studies, a preincubation period of at least 1 h with cytochalasin B, phloretin or noradrenaline preceded the uptake experiments, which were carried out as described above. Fresh stock solutions of cytochalasin B, phloretin and noradrenaline were prepared daily. Phloretin and cytochalasin B were dissolved in dimethyl sulphoxide (DMSO) and noradrenaline was dissolved in saline. The amount of DMSO added to the incubation did not affect the rate of 3-OMG uptake.

Incubations were stopped at pre-determined times by transferring 0.2 ml of the cell suspension (Hct 10 %) to an Eppendorf tube containing 0.8 ml of ice-cold stop medium layered on top of 0.5 ml of dibutyl phthalate. Initial rates of 3-OMG uptake were determined from the first 10 min of incubation, during which the uptake was linear. The stop medium, containing 0.5 mmol l^{-1} phloretin, was prepared daily. The tube was centrifuged immediately (10 000 ***g*** for 10 s, at +4 °C). The medium and oil layers were removed by suction, leaving the cell pellet at the bottom of the tube. After the inside of the tube had been carefully wiped, the cell pellet was lysed with 0.2 ml of 0.6 mol l^{-1} perchloric acid. The radioactivity taken up by the cells was measured by liquid scintillation counting (LKB-Wallac 1211 Minibeta). A correction factor for the radioactivity trapped in the extracellular space was estimated using ice-cold cell samples, which were mixed with ice-cold substrate and centrifuged immediately. Uptake values were calculated after subtraction of these 'blank' estimates.

Kinetic analyses

To estimate the apparent inhibition constant, K_i, the results were fitted by least-squares analysis to the equation:

$$V = [V_o K_i/(K_i + I)] + V_r, \quad (1)$$

where V_o is the inhibitor-sensitive rate, I is the inhibitor concentration, V_r is the residual inhibitor-insensitive rate, K_i is the inhibition constant (Simons, 1983) and V is the measured rate. The inhibitor-insensitive rate was determined in the presence of 10 μmol l^{-1} cytochalasin B and 50 μmol l^{-1} phloretin.

In concentration-dependence experiments, the linear relationship between the extracellular 3-OMG concentration and the initial uptake rate suggested simple diffusion. This was confirmed with a Lineweaver–Burk plot (1/V *versus* 1/[S], where S is the substrate), which showed that the line passes through the origin.

Results

The rate of 3-OMG uptake into carp erythrocytes is slow: the half-time for 3-OMG equilibration varied between 30 min and 3 h in most carp erythrocytes. A representative time course of 3-OMG equilibration (3 mmol l^{-1}) across carp erythrocyte membrane is shown in Fig. 1. The uptake remained linear for at least 10 min (Fig. 1, inset). The marked variation in the 3-OMG uptake amongst individuals is shown in Table 1. In one of the eight fish tested, the rate of 3-OMG uptake was one-tenth of that in most of the other fish.

As for the rate of 3-OMG transport, the inhibition of transport by cytochalasin B also varied markedly amongst individuals. Among the eight carp tested in this experiment, one fish showed neither cytochalasin-B-sensitive nor phloretin-sensitive 3-OMG uptake, whereas in others the cytochalasin inhibition varied between 30 and 85 % (Table 1). Maximal inhibition caused by phloretin was about 60 %. In the fish that showed inhibition by cytochalasin B and phloretin, transport was 50 % inhibited by approximately 0.5 μmol l^{-1} cytochalasin B and by approximately 50 μmol l^{-1} phloretin

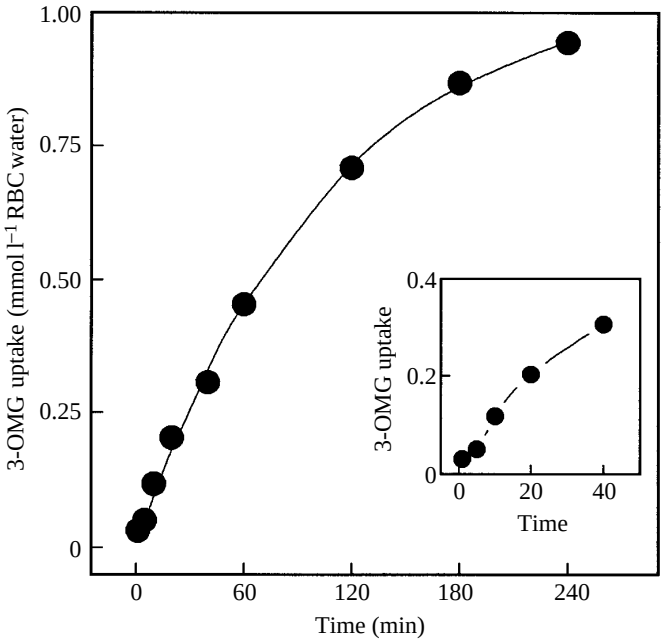

Fig. 1. Representative time course of 3-OMG uptake by carp erythrocytes (extracellular concentration 3 mmol l^{-1}, 20 °C). Each point represents the mean of duplicate samples. Inset: 3-OMG uptake at the earlier time intervals.

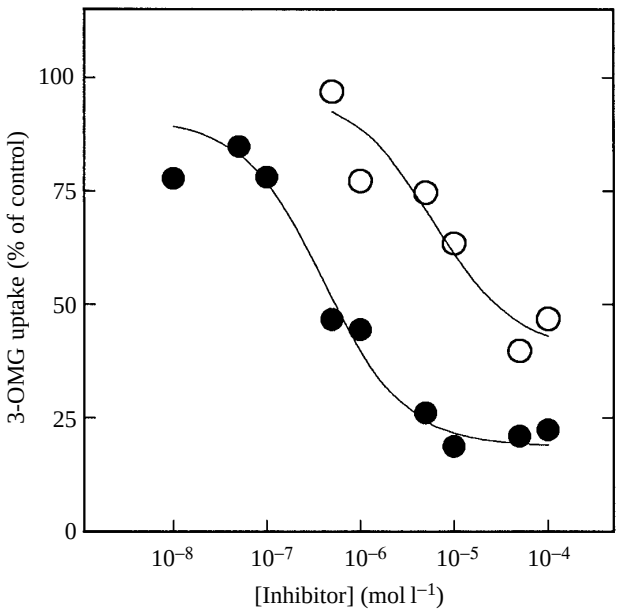

Fig. 2. Inhibition of 3-OMG uptake (extracellular concentration 3 mmol l^{-1}) by carp erythrocytes by cytochalasin B (●) and phloretin (○). Values are means of 3–5 determinations from different fish. Erythrocytes from one carp (not shown here) were totally devoid of cytochalasin-B-sensitive and phloretin-sensitive 3-OMG transport. Lines are fitted by using equation 1.

(Fig. 2). The apparent inhibition constant, K_i, for cytochalasin B was 0.38 μmol l^{-1} and that for phloretin was 6.0 μmol l^{-1}.

Fig. 3 shows the concentration-dependence of the initial rate

Table 1. *3-OMG uptake by erythrocytes from eight individual carp*

Fish mass (kg)	3-OMG uptake (mmol l^{-1} RBC h^{-1})		Difference
	− cytochalasin B	+ cytochalasin B	
0.93	0.083	0.094	0
0.82	1.007	0.208	0.799
0.66	0.682	0.425	0.257
0.60	0.433	0.091	0.342
0.35	0.557	0.083	0.474
0.29	0.771	0.439	0.332
0.19	0.853	0.262	0.591
0.13	0.764	0.196	0.568

Initial uptake rates of 3-OMG were measured at an extracellular concentration of 3 mmol l^{-1} in the presence and in the absence of 10 μmol l^{-1} cytochalasin B.

Each value is a single measurement.

'Difference' represents the cytochalasin-B-sensitive uptake rate.

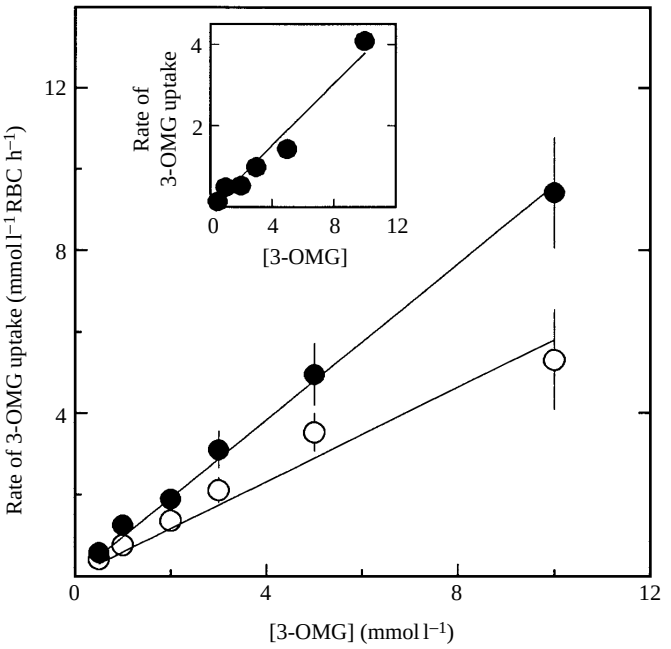

Fig. 3. Concentration-dependence of 3-OMG uptake by carp erythrocytes. Initial rates of 3-OMG uptake were measured in the absence (●) and in the presence (○) of 10 μmol l^{-1} cytochalasin B. Each point is the mean of 7–8 individual experiments. Error bars denote ± S.E.M. The inset shows the cytochalasin-B-sensitive 3-OMG uptake based on the same experiments.

of uptake of 3-OMG in the presence and in the absence of 10 μmol l^{-1} cytochalasin B. In both cases, the rate of uptake rises linearly and saturation kinetics are not apparent at the concentrations used (0.5–10 mmol l^{-1}). The cytochalasin-B-sensitive part of the 3-OMG uptake (Fig. 3, inset) is also a linear function of the extracellular concentration of substrate.

The rate of 3-OMG uptake by carp erythrocytes was stimulated by 10^{-8} mol l^{-1} noradrenaline, and at 10^{-6} mol l^{-1}

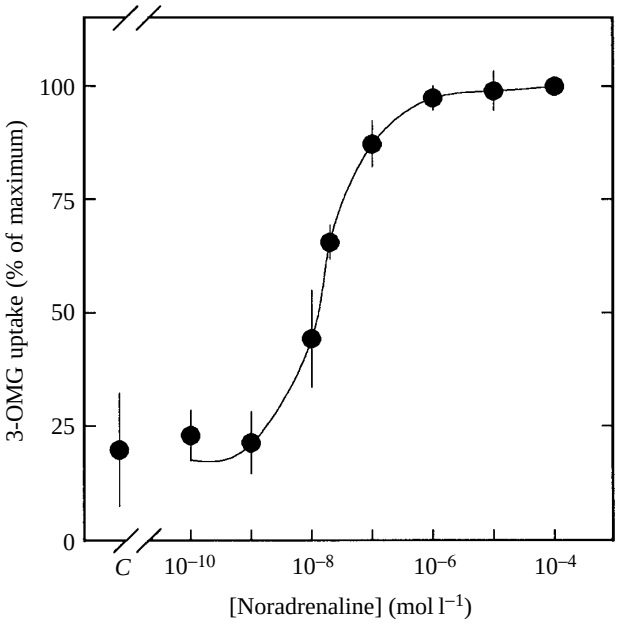

Fig. 4. Concentration-dependence of catecholamine stimulation of 3-OMG uptake by carp erythrocytes. Cells were preincubated with varying concentrations of noradrenaline for at least 1 h. Uptake is expressed as a percentage of the maximum rate of uptake (at 10^{-4} mol l^{-1} noradrenaline). The data are averaged from five experiments on cells from different fish. Error bars denote ± S.E.M. and *C* denotes control experiments. The line is fitted by eye.

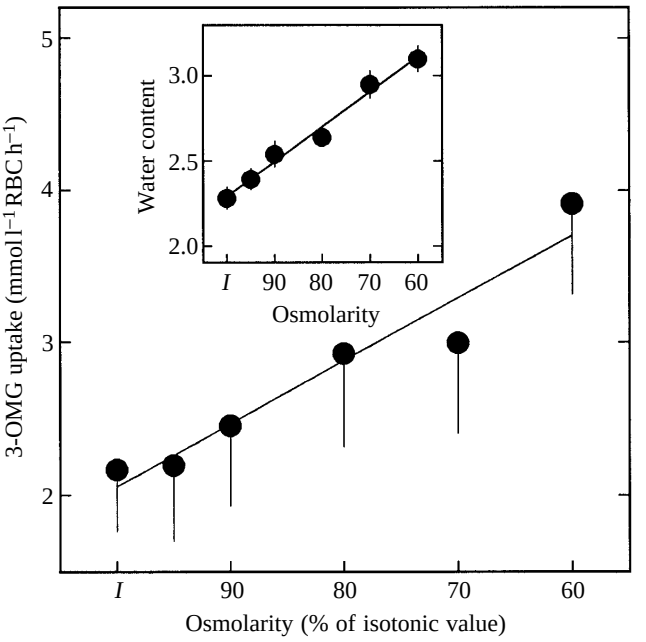

Fig. 5. Effect of osmolarity on the initial rate of 3-OMG (extracellular concentration 3 mmol l^{-1}) uptake by carp erythrocytes. The osmolarity of the media varied down to 60 % of its isotonic value (*I*). The inset shows the effect of osmolarity on the erythrocyte water content (measured as g RBC water g^{-1} RBC dry mass). Values are means from 6–7 experiments on cells from different carp. Error bars denote ± S.E.M.

noradrenaline the stimulation was almost maximal (Fig. 4). Since the dose–response curves for catecholamine stimulation of glucose transport were similar in different individuals, we conclude (1) that the washing procedure had effectively removed any influence of endogenous catecholamines on glucose transport and (2) that the individual variation was not caused by different catecholamine levels in different fish after sampling.

Osmotic swelling also caused a clear increase in the total 3-OMG uptake in carp erythrocytes (Fig. 5). Both the cytochalasin-sensitive and the cytochalasin-insensitive fluxes were increased (Table 2). In fact, the percentage inhibition of transport by cytochalasin was greater after osmotic swelling (60 %) than in isotonic medium (50 %).

Although there was a clear increase in the 3-OMG fluxes as a function of osmotic swelling, the catecholamine-induced increase in 3-OMG uptake cannot be explained by the volume changes. In the presence of 10 μmol l^{-1} noradrenaline and at low extracellular pH (6.7–7.2), carp erythrocyte water content increased from 2.58±0.10 to 2.78±0.06 g RBC water g^{-1} RBC dry mass (mean ± S.E.M., *N*=8) compared with control cells at the same pH. At the same time, the rate of 3-OMG uptake increased from 0.96±0.29 to 2.61±0.44 mmol l^{-1} RBC h^{-1} (mean ± S.E.M., *N*=8), i.e. much more than would be expected on the basis of the volume increase (see also Fig. 5). The mean rates of uptake are different at $P<0.05$.

The effect of extracellular pH on 3-OMG transport showed large individual variation (Fig. 6): in some animals, the influx clearly increased with decreasing pH; in others, there was either no change or a decrease. It is thus clear that the acidification-induced increase in cellular water content (Fig. 6, inset) was not adequate to cause a general increase in the rate of 3-OMG uptake as extracellular pH decreased. Furthermore, the increase in 3-OMG influx in catecholamine-treated cells cannot be caused by the decrease in extracellular pH that is commonly observed after catecholamine stimulation (e.g. Nikinmaa, 1982).

Table 2. *Effect of cytochalasin B on 3-OMG uptake by carp erythrocytes in the isotonic and hypotonic media*

Conditions	3-OMG uptake (mmol l^{-1} RBC h^{-1})	Difference
Isotonic	0.685±0.091	0.337
Isotonic + cytochalasin B	0.349±0.112	
Hypotonic	1.514±0.092	0.916
Hypotonic + cytochalasin B	0.598±0.144	

Initial rates of 3-OMG uptake were measured at an extracellular concentration of 3 mmol l^{-1} in the presence and in the absence of 10 μmol l^{-1} cytochalasin B as described in Materials and methods.
The hypotonic medium was 60 % of that at isotonicity.
Values (±S.E.M.) are means of four experiments from four carps.
'Difference' represents the cytochalasin-B-sensitive uptake rate.

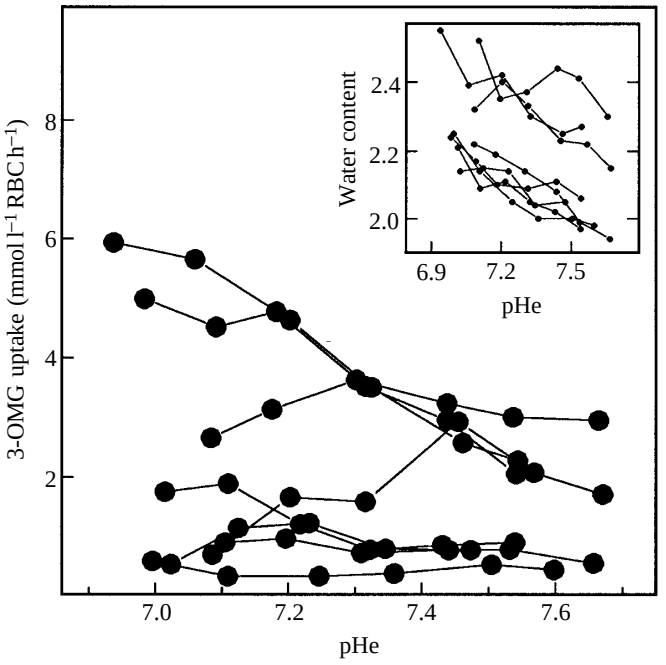

Fig. 6. Effect of extracellular pH (pHe) on the initial rate of 3-OMG (extracellular concentration 3 mmol l^{-1}) uptake by carp erythrocytes. The inset shows the effect of extracellular pH on carp erythrocyte water content (measured as g RBC water g^{-1} RBC dry mass). Each point represents a single sample. Points connected by lines represent measurements with one carp.

Discussion

The uptake of 3-OMG by carp erythrocytes is slow, as in many other teleost fish studied. Transport was much faster than in our earlier experiments with carp erythrocytes in which the half-time for 3-OMG equilibration was about 10 h (Tiihonen and Nikinmaa, 1991*a*). However, as is obvious from Table 1, there is marked variation in the rate of 3-OMG uptake amongst individuals. In one of the eight fish tested, the rate of 3-OMG uptake was one-tenth of that in most of the other fish, a value comparable to those of our earlier study. Considerable differences in the rate of 3-OMG uptake in the erythrocytes of individual eels (*Anguilla japonica*) have also been found (Tse and Young, 1990).

In accordance with our earlier study, phloretin inhibited 3-OMG influx into carp erythrocytes. Furthermore, 3-OMG transport into carp erythrocytes was even more powerfully inhibited by cytochalasin B. However, as for the rate of 3-OMG transport, the inhibition of transport by cytochalasin B also varied markedly amongst individuals. Transport-deficient eels have also been found (Tse and Young, 1990).

In the specimens that showed inhibition by cytochalasin B and phloretin, transport was 50 % inhibited by approximately 0.5 μmol l^{-1} cytochalasin B and by approximately 50 μmol l^{-1} phloretin. These values are near the concentrations required for 50 % inhibition of transport in eel erythrocytes (cytochalasin B, 0.125 μmol l^{-1}; phloretin, 80 μmol l^{-1}; Tse and Young, 1990). The apparent inhibition constant, K_i, measured for cytochalasin B was 0.38 μmol l^{-1} and that for phloretin was 6.0 μmol l^{-1}. Similar K_i values have been reported for cytochalasin B inhibition of glucose transport in other vertebrate erythrocytes (hagfish, 0.7 μmol l^{-1}, Ingermann *et al.* 1984; pigeon, 0.23 μmol l^{-1}, Simons, 1983; human, 0.3–0.7 μmol l^{-1}, Taverna and Langdon, 1973; Jung and Rampal, 1977; Goldin and Rhoden, 1978; common eel, 0.1 μmol l^{-1}, Tse and Young, 1990). In contrast, the phloretin inhibition constant in carp erythrocytes is lower than that for inhibition of glucose transport in common eel (48 μmol l^{-1}; Tse and Young, 1990) or pigeon (28 μmol l^{-1}; Simons, 1983) erythrocytes, but not as low as that in human erythrocytes (0.24 μmol l^{-1}; Krupka, 1985).

Although the general pattern of 3-OMG transport inhibition is similar to that of erythrocytes from other vertebrates, suggesting the presence of a specific glucose transport pathway in the membrane of carp erythrocytes, two other findings indicate that the inhibition of glucose transport by cytochalasin B and phloretin may be due to their non-specific interactions with the cell membrane. Fig. 3 shows the concentration-dependence of the initial rate of uptake of 3-OMG in the presence and in the absence of 10 μmol l^{-1} cytochalasin B. In both cases, the rate of uptake rises linearly and saturation kinetics are not apparent over the concentration range used (0.5–10 mmol l^{-1}). This finding is similar to our earlier observations (Tiihonen and Nikinmaa, 1991*a*). Also, the cytochalasin-B-sensitive part of 3-OMG uptake (Fig. 3, inset) is a linear function of the extracellular concentration. These results suggest either that most of the 3-OMG transport across the carp erythrocyte membrane occurs by simple diffusion (utilizing no specific carrier) or that the 3-OMG affinity of the putative glucose carrier is low and far above the physiological glucose concentration. If no specific carrier exists, the inhibition of glucose transport by cytochalasin B may be the result of its interactions with the membrane skeleton. Cytochalasin B binds to actin, an important component of the membrane skeleton, causing structural changes in this protein (Lin and Spudich, 1974; Spudich and Lin, 1972).

The reasons for the marked individual variation in both the total and the cytochalasin-sensitive 3-OMG transport across the carp erythrocyte membrane are not clear. The variability of cytochalasin-B-sensitive uptake in common eel erythrocytes correlated neither with season nor with intracellular ATP levels (Tse and Young, 1990). Tse and Young (1990) suggested that the transport variation could be under genetic control. In the present study, there was no clear correlation between the size of the fish and the 3-OMG transport activity. Thus, the variation in the uptake rates may be genetic or they may reflect changes during erythrocyte maturation: there is a loss of glucose transporters in, for example, avian erythroblasts during maturation (Mathew *et al.* 1994).

Noradrenaline stimulated 3-OMG uptake by carp erythrocytes at a concentration of 10^{-8} mol l^{-1}. Thus, the plasma noradrenaline concentrations of anoxic carp, at least, are sufficient to stimulate 3-OMG uptake (Mazeaud and

Mazeaud, 1981). 3-OMG uptake was stimulated by noradrenaline concentrations similar to those for other adrenergic responses, for example cyclic AMP accumulation and cell swelling (Salama and Nikinmaa, 1990). However, the relationships between the other adrenergic responses and 3-OMG uptake is unknown.

Kirk *et al.* (1992) observed that in flounder erythrocytes the rate of cytochalasin-B-insensitive transport of glucose is markedly increased by osmotic swelling and that the transport probably occurs *via* a volume-activated channel that also transports various other small organic molecules, such as taurine and nucleosides. In the present study, we observed that osmotic swelling caused a clear increase in the total 3-OMG uptake. Both the cytochalasin-sensitive and cytochalasin-insensitive fluxes were increased. In fact, the percentage inhibition of transport by cytochalasin was greater after osmotic swelling (60 %) than in isotonic medium (50 %). One possible explanation of these findings is that transport in both isotonic and hypotonic media occurs *via* the same pathway, which is volume-activated and inhibited by cytochalasin B (and may be similar to the volume-activated channel studied by Kirk *et al.* 1992). Transport *via* such a relatively non-specific channel would explain the lack of saturation kinetics.

In conclusion, the present results show large individual variation in the permeability of the carp erythrocyte membrane to 3-OMG. Although transport is inhibited by cytochalasin B and phloretin, the lack of saturation kinetics for transport over a physiologically relevant concentration range suggests either that a glucose transporter does not exist or that the affinity of the transporter for glucose is extremely low. The marked increase in transport after osmotic swelling and the inhibition of swelling-induced glucose transport by cytochalasin B suggest that the glucose transport pathway in carp erythrocyte (in both isotonic and hypotonic conditions) may be similar to the volume-activated channel described for flounder erythrocytes by Kirk *et al.* (1992). 3-OMG transport across the carp erythrocyte membrane is also increased by catecholamines by a mechanism independent of the catecholamine-induced cell swelling.

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