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SHORT COMMUNICATION

SUBSTRATE UTILIZATION BY CARP (*CYPRINUS CARPIO*) ERYTHROCYTES

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Compared with the anucleated mammalian erythrocytes, which rely on glycolysis for most of their energy production, the nucleated erythrocytes of teleost fish are highly aerobic (Ferguson and Boutilier, 1988; Ferguson *et al.* 1989; Eddy, 1977).

Rainbow trout (*Oncorhynchus mykiss*) erythrocytes possess significant amounts of enzymes associated with glycolysis and the tricarboxylic acid (TCA) cycle (Bachand and Leray, 1975; Walsh *et al.* 1990; Ferguson and Storey, 1991) and are able to oxidize glucose. However, the erythrocytes of several fish, including rainbow trout (*Salmo gairdneri* Richardson; Tse and Young, 1990), brown trout (*Salmo trutta*; Bolis *et al.* 1971) and carp (*Cyprinus carpio*; Tiihonen and Nikinmaa, 1991) are essentially impermeable to glucose. Thus, other substrates utilized *via* the TCA cycle may be important in energy metabolism. The TCA cycle can, in addition to glucose, utilize glycolytic intermediates, monocarboxylic acids, amino acids and substrates of the pentose phosphate pathway.

Tuna (*Katsuwonus pelamis*) erythrocytes are permeable to lactate (Moon *et al.* 1987). Lactate may enter the TCA cycle after conversion to pyruvate in a reaction catalyzed by lactate dehydrogenase, which is present at high activity in trout erythrocytes (Ferguson and Storey, 1991). Pyruvate itself may be an important energy source in fish erythrocytes since extracellular pyruvate is able to maintain nucleoside triphosphate (NTP) levels of rainbow trout erythrocytes (Houston *et al.* 1985).

In addition, amino acids may be used, as high levels of free amino acids are present in fish plasma (carp about 6–9 $mmol\,l^{-1}$; Ogata and Arai, 1985). Glutamine is used as a substrate in rabbit reticulocytes (Rapoport *et al.* 1971). The high erythrocyte to plasma concentration ratios of glutamic acid, aspartate and isoleucine in carp (Dabrowski, 1982; Ogata and Arai, 1985) may indicate effective transport of these amino acids into the cells.

Nucleosides, metabolized *via* the pentose phosphate pathway, are evidently the

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major energy source for glucose-impermeable mammalian erythrocytes (Kim *et al.* 1980; Jarvis *et al.* 1980). The enzymes of the pentose phosphate pathway have been found in erythrocytes of *Perca flavescens* (Bachand and Leray, 1975) and *Oncorhynchus mykiss* (Walsh *et al.* 1990), suggesting the possibility that nucleosides and 5-carbon sugars may be used in the energy metabolism.

We have examined the substrate utilization of carp erythrocytes using different substrates. Glucose and pyruvate concentrations were measured and represent experiments in resting animals; amino acid concentrations were taken from Dabrowski (1982) and Ogata and Arai (1985). Lactate concentrations were taken from Jensen *et al.* (1987). Data for nucleoside concentrations were not available for carp, so it was assumed that they would be similar to inosine concentration in pig plasma (Jong and Goldstein, 1974; Jarvis *et al.* 1980). The dihydroxyacetone phosphate (DHAP; 0.1 mmol l^{-1}) and ribose (1 mmol l^{-1}) concentrations used were probably higher than those present in carp, since the measured value for DHAP in perch erythrocytes was 15.5 μmol l^{-1} (Leray and Bachand, 1975) and that for ribose in rat plasma was 0.36 mmol l^{-1} (Green *et al.* 1949).

The carp, *Cyprinus carpio* L. (0.8–1.2 kg, N=23), were obtained from Porla Fisheries Research Station and maintained under laboratory conditions (air-saturated, dechlorinated Helsinki tap water at pH 7.0–7.4, at 12–14 °C) for a minimum of 1 month before experimentation. Animals were anaesthetized with MS-222 (0.1 g l^{-1}, 5 min) and the blood samples were taken by venipuncture. Erythrocytes and plasma were separated by centrifugation (1 min, 10 000 ***g***), and pooled erythrocytes from 2–3 fish were washed twice with saline. The composition (in mmol l^{-1}) of the physiological saline was: 128 NaCl, 3 KCl, 1.5 $CaCl_2$, 1.5 $MgCl_2$ and 15 Tris.

Carbon dioxide production was measured from ^{14}C-labelled substrates both when the labelled substrate was the only one present in labelled and unlabelled forms and when other potential substrates were present in unlabelled form. Radiochemicals, which were obtained from Amersham, were L-[U-^{14}C]lactic acid sodium salt (154 mCi mmol $^{-1}$), [1-^{14}C]pyruvic acid (32 mCi mmol $^{-1}$), [U-^{14}C]adenosine (519 mCi mmol $^{-1}$), L-[U-^{14}C]glutamic acid (270 mCi mmol $^{-1}$), L-[U-^{14}C]glutamine (270 mCi mmol $^{-1}$), L-[U-^{14}C]aspartic acid (220 mCi mmol $^{-1}$), L-[U-^{14}C]isoleucine (324 mCi mmol $^{-1}$) and D-[U-^{14}C]glucose (304 mCi mmol $^{-1}$). The incubations were started by the addition of a tracer concentration of labelled (0.5 μCi per vial) and physiological concentration of unlabelled substrate to the cell suspension (1 ml, haematocrit, Hct, about 20 %). Medium concentrations of the unlabelled substrates are given in Table 1. For the measurements of carbon dioxide production from the single labelled substrate in the presence of other substrates, the cells were incubated in saline containing (in mmol l^{-1}): 128 NaCl, 3 KCl, 1.5 $CaCl_2$, 1.5 $MgCl_2$, 15 Tris, 2.2 D-glucose, 0.6 L-lactic acid, 1 D-ribose, 0.05 pyruvic acid, 0.1 L-aspartic acid, 0.05 L-glutamic acid, 0.1 L-glutamine, 0.1 L-isoleucine, 0.01 guanosine, 0.01 adenosine, 0.01 inosine and 0.1 dihydroxyacetone. The pH of the salines was initially adjusted to 7.8. During the incubation the pH of the medium decreased to 7.65±0.05 (S.E.M.). The Warburg flask was

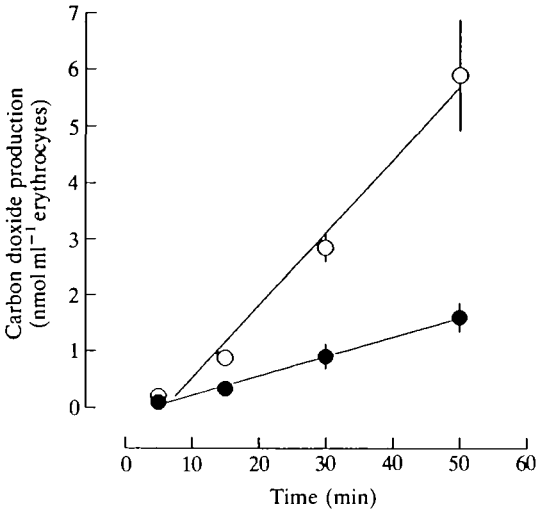

Fig. 1. Time course of carbon dioxide production from 0.05 mmol l^{-1} glutamate in carp erythrocyte, when glutamate was the only substrate present (○) and when other potential substrates were also present (●). Values are reported as mean±S.E., N=5

immediately sealed with a rubber plug and shaken gently during the incubation at 20±1 °C.

The cells were incubated for 50 min, during which period $^{14}CO_2$ release from the substrate was linear, as shown in Fig. 1 for glutamate. Samples were taken at 5, 15, 30 and 50 min. The incubations were terminated by acidification of the cell suspension with 0.2 ml of 1 mol l^{-1} H_2SO_4. The $^{14}CO_2$ formed was collected in the centre well containing a filter paper (Whatman no. 1), soaked with 10 % KOH, for an hour to allow complete recovery of $^{14}CO_2$. The radioactivity was then determined using a liquid scintillation counter (LKB-Wallac 1211 MiniBeta).

The CO_2 production rate was calculated from the disints min^{-1} values obtained for each substrate. These were corrected for the different proportions of erythrocytes in each measurement using the Hct value, and transformed to moles of substrate by using the activity of the substrate in the incubation. This value was then transformed to moles of CO_2 by multiplying it by the number of carbons in the studied substrate. The CO_2 production rate of the studied substrate was then estimated from the time course of evolved carbon dioxide. All value are presented as means±S.E.M. (N). The statistical significance was calculated using paired t-test.

As shown in Table 1, lactate appears to be the predominant energy source of carp erythrocytes. Its utilization clearly exceeded that of the other potential substrates, especially when other substrates were present. In contrast to trout erythrocytes (Walsh *et al.* 1990), lactate oxidation rates exceeded glucose oxidation rates in carp erythrocytes.

The other potentially important monocarboxylic acid, pyruvate, also plays an important role in carp erythrocyte metabolism, although its concentration in this study was only about one-tenth of the lactate concentration. In the presence of

Table 1. *CO_2 production rates from different substrates by carp erythrocytes*

Substrate	CO_2 production rates (nmol ml^{-1} erythrocytes h^{-1})		External substrate concentration (mmol l^{-1})
	−other substrates	+other substrates	
L-Lactate	86.3±10.0	98.1±4.1	0.6
Pyruvate	58.2±17.8	33.7±6.3	0.05
Glutamine	26.1±7.9	19.2±5.1	0.1
D-Glucose	17.0±4.7	9.5±2.4	2.2
Glutamate	7.8±1.3**	2.0±0.4	0.05
Aspartate	2.5±0.6	2.4±0.5	0.1
Adenosine	1.8±0.7*	0.5±0.1	0.01
Isoleucine	0.8±0.5 (N=4)	0.11±0.09 (N=4)	0.1

* Significantly different from rate in the presence of other substrates (paired Student's t-test, $P<0.05$).

** Significantly different from rate in the presence of other substrates (paired Student's t-test, $P<0.01$).

Values are means±S.E., N=5 (unless noted in parentheses).

other substrates its utilization decreased almost to half (Table 1.). Because the oxidation rates of these monocarboxylic acids in carp erythrocytes are high, it is evident that their transport into carp erythrocytes is quite rapid. The presence of a specific monocarboxylate carrier in erythrocyte membranes has been demonstrated in many mammals (e.g. Deuticke *et al.* 1978). In human erythrocytes, pyruvate uptake inhibits lactate uptake competitively (Halestrap, 1976). Thus, the inhibition of pyruvate utilization in the presence of other substrates is probably caused by the inhibition of pyruvate transport by the high external lactate concentration.

Under the experimental conditions used, the most important external amino acid of carp erythrocyte energy metabolism appears to be glutamine. In the presence of other substrates, about 10 times as much CO_2 is formed from glutamine as from glutamate and aspartate. The oxidation rate for isoleucine was even slower and barely detectable (Table 1). Carp erythrocytes clearly prefer extracellular glutamine to glutamate as an energy source. The difference was especially marked in the presence of other substrates. The external glutamine concentration was twice as great as the glutamate concentration, but CO_2 production from glutamine was 10 times greater than that from glutamate in the presence of other substrates. Ferguson and Storey (1991) have found moderate glutamate dehydrogenase activity in trout erythrocytes. Thus, glutamate can be fed into the TCA cycle *via* alpha-ketoglutarate. Since glutamine must be converted into glutamate before it is oxidatively deaminated to yield alpha-ketoglutarate, the higher production of CO_2 from glutamine than glutamate in this study, as in rabbit reticulocytes (Rapoport *et al.* 1971), indicates that glutamine permeability is greater than that of glutamate.

Despite the low glucose permeability of carp red cells, they are able to oxidize glucose. It is apparent that the high plasma concentrations are adequate to cause a large enough glucose flux into the cells to fuel measurable glucose oxidation. Thus, as shown for trout, carp erythrocytes must contain a full set of glycolytic enzymes. However, in the presence of other substrates, glucose utilization was only one-tenth of lactate utilization, indicating that glucose is probably a minor subtrate *in vivo*. This situation contrasts with observations on rainbow trout, the red cells of which also have a low glucose permeability: Walsh *et al.* (1990) observed that glucose oxidation rate was greater than that of lactate.

In contrast to the glucose-impermeable mammalian red cells in which nucleosides appear to be the primary fuel (Kim *et al.* 1980, Jarvis *et al.* 1980), adenosine utilization by the nearly glucose impermeable carp red cells was very small, and the utilization was further inhibited by the presence of other substrates (Table 1). This suggests that the pentose phosphate pathway is not utilized for energy production to any great extent in resting situations. Similarly, the lack of inhibition of lactate utilization by the presence of other substrates, including the glycolytic intermediate dihydroxyacetone at a relatively high ($0.1\,mmol\,l^{-1}$) concentration, suggests that the role of glycolytic intermediates in energy production is small.

We did not examine the CO_2 production from alanine or other short-chain amino acids or from short-chain fatty acids. However, Walsh *et al.* (1990) have found that oxidation rates for alanine and especially oleate in trout erythrocytes are quite low (6 and 2 $nmol\,g^{-1}$ cell wet mass h^{-1}, respectively, at medium concentrations of $0.48\,mmol\,l^{-1}$ for alanine and $0.25\,mmol\,l^{-1}$ for oleate).

In conclusion, carp erythrocytes preferentially use substrates other than glucose as an energy source. They prefer lactate and pyruvate and have also a capacity for amino acid, especially glutamine, oxidation.

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