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CHARACTERIZATION OF RED BLOOD CELL METABOLISM IN RAINBOW TROUT

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Summary

Red blood cell metabolism was studied *in vitro* using whole blood obtained by catheter from resting rainbow trout (*Oncorhynchus mykiss*). Preparations were viable as shown by stable NTP, metabolite and catecholamine levels and acid–base status, all of which remained at *in vivo* levels over the 2 h incubation period. Enzymes diagnostic of glycolysis, the tricarboxylic acid (TCA) cycle and phosphagen metabolism were all present in significant amounts in red blood cells. In direct comparisons of ^{14}C-labelled substrates at normal resting plasma concentrations, rates of CO_2 production were in the order: glucose>lactate>alanine>oleate. Total CO_2 production rates from these four oxidative substrates did not equal directly measured O_2 consumption rates, indicating that other substrates may also be important *in vivo*. Oxidative pathway K_m values for glucose (8.4 mmol l^{-1}), lactate (3.3 mmol l^{-1}) and alanine (0.8 mmol l^{-1}) were well within the normal physiological ranges of plasma concentrations. Glucose concentration did not affect lactate oxidation rates, but there was some inhibition (27%) of glucose oxidation by high lactate concentrations (20 mmol l^{-1}). The observed K_m values and competitive interactions suggest that changes in plasma concentrations associated with environmental stresses can considerably alter the relative rates of oxidation of glucose and lactate *in vivo*. Considerable pentose-phosphate shunt activity was detected in red cells, as indicated by high activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase and high CO_2 production rates from (1-^{14}C)-labelled glucose. Even in the presence of normal O_2

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levels, a significant percentage (28 %) of glucose metabolism was directed to lactate production. Taken together, these results demonstrate that rainbow trout whole blood incubated *in vitro* constitutes a dynamic and viable system for metabolic studies at the pathway level.

Introduction

In recent years, fish red blood cells (RBCs) have emerged as an exciting experimental system for studying intracellular pH (pHi) regulation and oxygen transport during physiological stress such as strenuous exercise (e.g. Nikinmaa *et al.* 1984; Wood and Perry, 1985; Primmett *et al.* 1986; Milligan and Wood, 1987), hypercapnia (e.g. Perry *et al.* 1987; Vermette and Perry, 1988; Perry *et al.* 1989) and hypoxia (e.g. Fievet *et al.* 1987; Boutilier *et al.* 1988). In particular, elevated plasma catecholamine levels have been shown to play a central role in RBC pHi homeostasis in the face of extracellular acidosis, by stimulation of proton extrusion and cell swelling *via* Na^+/H^+ exchange. This exchange does not have a direct energy requirement, but is a secondarily active mechanism dependent upon the electrochemical gradient for Na^+ entry established by Na^+/K^+-ATPase at metabolic expense (see reviews by Nikinmaa, 1986; Nikinmaa and Tufts, 1989; Motais and Garcia-Romeu, 1989). Catecholamines may thereby elevate RBC Na^+ concentrations, stimulate Na^+/K^+ transport activity and oxygen consumption (Bourne and Cossins, 1982; Ferguson and Boutilier, 1988; Ferguson *et al.* 1989) and lower intracellular nucleoside triphosphate (NTP) levels (Nikinmaa, 1983, 1986; Milligan and Wood, 1987; Ferguson and Boutilier, 1989). The reduction in fixed negative charge associated with reduced NTP levels and cell swelling contributes additionally to pHi elevation *via* the Donnan effect, and the lower concentrations of H^+ and NTP, both negative modifiers of O_2 binding to haemoglobin, favour O_2 loading in the face of extracellular acidosis.

Little is known about the metabolic substrates for these events, or even about the basic metabolic pathways of teleost RBCs. Early studies demonstrated that fish RBCs consume O_2 at a significant rate, in contrast to mammalian erythrocytes (Hunter and Hunter, 1957; Eddy, 1977), and very recent studies have concluded that aerobic metabolism accounts for more than 90 % of resting NTP production in salmonid RBCs (Ferguson and Boutilier, 1988; Ferguson *et al.* 1989). *In vitro*, RBC NTP levels are sensitive to the level of oxygenation, and RBCs incubated under anaerobic conditions produce lactate (Greaney and Powers, 1978; Tetens and Lykkeboe, 1981; Milligan and Wood, 1987; Ferguson and Boutilier, 1989; Ferguson *et al.* 1989). Measurement of RBC enzyme activities in the yellow perch (*Perca flavescens*) suggested that the tissue is largely glycolytic, but with a significant capacity for catabolism of glucose and NADPH production by the pentose-phosphate shunt (Bachand and Leray, 1975). One need for NADPH production is believed to be the continual requirement for reduced glutathione (GSH) production to protect against oxidation (e.g. Marshall *et al.* 1990).

With this background in mind, we undertook the present study utilizing rainbow

trout *Oncorhynchus mykiss* to characterize RBC metabolism in terms of: (1) ability to oxidize different substrates; (2) substrate preferences, kinetic and competitive interactions and relationship to total oxidation rates; and (3) the importance of other pathways such as the pentose-phosphate shunt. This study laid the groundwork for a companion investigation (Wood *et al.* 1990) of the effects of strenuous exercise and post-exercise recovery on RBC metabolism, and a mechanistic analysis of the relative roles of acid–base variables and catecholamine and substrate levels in the observed changes in metabolism induced by exercise.

Materials and methods

Animals and surgical preparation

Rainbow trout (*Oncorhynchus mykiss=Salmo gairdneri*; 150–400 g) were obtained from Thistle Springs Trout Farm, Ashton, Ontario, and held at The University of Ottawa in April–May or from Spring Valley Trout Farm, Petersburg, Ontario, and held at McMaster University in June–August. At both locations, the fish were housed in large fibreglass tanks at 9–12°C in running dechlorinated tapwater; no apparent effects of source or holding conditions were apparent in our measurements. Fish were fed *ad libitum* on a commercial diet, but were fasted for 24–48 h prior to use. Dorsal aortic cannulae (PE50 polyethylene tubing; Soivio *et al.* 1972) were implanted under MS-222 anaesthesia (1:10000 w/v; Sigma), and the fish allowed to recover for 24–48 h in darkened Perspex chambers served with aerated, flowing tapwater at the experimental temperature (10±1°C).

Sampling and handling of blood

Trout were initially infused with 0.5 ml of heparinized (50 units ml^{-1}) Cortland saline (Wolf, 1963); blood was then withdrawn slowly until the fish showed the first signs of struggling or disorientation, at which point sampling ceased. This procedure usually yielded approximately 2 ml of blood per fish, but occasionally large trout yielded up to 5 ml. In all experiments, except those for enzyme measurements, blood from several fish was pooled, heparinized and kept on ice for up to 30 min until use. This pooled blood was then placed in 20 ml glass vials in volumes of either 700 μl (for CO_2 production determinations) or 1400 μl (for O_2 consumption determinations) for gassing and incubations. The use of pooled blood allowed a paired or matrix experimental design; each pooled preparation was considered as one sample for statistical purposes.

Enzyme activity determinations

Whole blood (approximately 2 ml) was withdrawn from individual fish, centrifuged at 13000 *g* for 1 min in pre-weighed microcentrifuge tubes, plasma was decanted, and both fractions immediately frozen at −80°C for 1 month prior to assay. Thawed plasma (50 μl) was used directly in enzyme assays. Packed RBCs

were homogenized in a Brinkmann Polytron with 3 vols of ice-cold 50 mmol l^{-1} imidazole–HCl, pH 7.4 (at room temperature). This homogenate was centrifuged at 13 000 *g* for 1 min, and the supernatant (10 μl) was used directly in enzyme assays. Assays were buffered with 50 mmol l^{-1} imidazole, pH 7.4 (unless noted below). Total volume was 1 ml, and enzyme activities were monitored at 24.0±0.2°C by following the appearance/disappearance of NAD(P)H at 340 nm spectrophotometrically in an LKB 4050 Ultrospec II connected to a chart recorder (except for GNase) using procedures described by Mommsen *et al.* (1980) with slight modifications.

Malate dehydrogenase (E.C.1.1.1.37) (MDH). 0.15 mmol l^{-1} NADH, 0.5 mmol l^{-1} oxaloacetate.

Citrate synthetase (E.C.4.1.3.7) (CS). Absorbance read at 412 nm (E=13.6), buffer was 50 mmol l^{-1} sodium Hepes, pH 8.0, 0.1 mmol l^{-1} 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), 0.3 mmol l^{-1} acetylcoenzyme A, 0.5 mmol l^{-1} oxaloacetate.

Isocitrate dehydrogenase (E.C.1.1.1.42) (IDH). 0.4 mmol l^{-1} $NADP^+$, 4 mmol l^{-1} $MgCl_2$, 0.6 mmol l^{-1} threo-D(+)-isocitrate.

Glucose-6-phosphate dehydrogenase (E.C.1.1.1.49) (G6PDH). 0.4 mmol l^{-1} $NADP^+$, 7 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} glucose-6-phosphate.

6-Phosphogluconate dehydrogenase (E.C.1.1.1.44) (6PGDH). 0.8 mmol l^{-1} $NADP^+$, 20 mmol l^{-1} $MgCl_2$, 3.0 mmol l^{-1} 6-phosphogluconate.

Phosphofructokinase (E.C.2.7.1.11) (PFK). 0.12 mmol l^{-1} NADH, 2 mmol l^{-1} ATP, 50 mmol l^{-1} KCl, 10 mmol l^{-1} $MgCl_2$, 1 i.u. alpha-glycerophosphate dehydrogenase, 5 i.u. aldolase, 5 i.u. triosephosphate isomerase, 5 mmol l^{-1} fructose-6-phosphate.

Pyruvate kinase (E.C.2.7.1.40) (PK). 0.12 mmol l^{-1} NADH, 2.5 mmol l^{-1} ADP, 10 μmol l^{-1} fructose-1,6-bisphosphate, 30 mmol l^{-1} KCl, 10 mmol l^{-1} $MgCl_2$, 20 i.u. LDH, 2.5 mmol l^{-1} phosphoenolpyruvate.

Lactate dehydrogenase (E.C.1.1.1.27) (LDH). Forward direction, 0.12 mmol l^{-1} NADH, 2 mmol l^{-1} pyruvate. Reverse direction, 2.0 mmol l^{-1} NAD^+, 250 mmol l^{-1} L-lactate.

Creatine phosphokinase (E.C.2.7.3.2) (CPK). 0.2 mmol l^{-1} $NADP^+$, 1.0 mmol l^{-1} ADP, 10 mmol l^{-1} AMP, 4 mmol l^{-1} glucose, 5 mmol l^{-1} $MgCl_2$, 2 i.u. G6PDH, 5 i.u. hexokinase, 50 mmol l^{-1} creatine phosphate.

Phosphoenolpyruvate carboxykinase (E.C.4.1.1.32) (PEPCK). 0.12 mmol l^{-1} NADH, 0.5 mmol l^{-1} phosphoenolpyruvate, 20 mmol l^{-1} $NaHCO_3$, 1 mmol l^{-1} $MnCl_2$, 8 i.u. MDH, 0.2 mmol l^{-1} deoxyguanosine diphosphate.

Fructose-1,6-bisphosphatase (E.C.3.1.3.11) (FBPase). 0.2 mmol l^{-1} $NADP^+$, 15 mmol l^{-1} $MgCl_2$, 10 i.u. phosphoglucose isomerase, 2 i.u. G6PDH, 0.1 mmol l^{-1} fructose-1,6-bisphosphate.

Glutamate dehydrogenase (E.C.1.4.1.3) (GDH). 0.12 mmol l^{-1} NADH, 1 mmol l^{-1} ADP, 250 mmol l^{-1} ammonium chloride, 0.1 mmol l^{-1} EDTA 14 mmol l^{-1} alpha-ketoglutarate.

Glutaminase (GNase). 150 mmol l^{-1} potassium phosphate, 50 mmol l^{-1} Tris

(pH 8.6), 0.2 mmol l^{-1} EDTA, 20 mmol l^{-1} glutamine, incubated for 30 min, terminated with perchloric acid. Glutamate was subsequently determined in neutralized extracts by the glutamate dehydrogenase assay.

After addition of extract and monitoring of control absorbance changes, reactions were initiated by adding a small volume (25–100 μl) of the last item listed (usually one of the substrates). In all cases control activity was less than 5 % and was subtracted from the activity with substrate.

CO_2 production determinations

Carbon dioxide production of pooled whole blood was measured from [U-^{14}C]L-lactate (90–105 mCi mmol^{-1}; ICN), [U-^{14}C]D-glucose (310 mCi mmol^{-1}; ICN), [U-^{14}C]L-alanine (135 mCi mmol^{-1}; ICN), [1-^{14}C]D-glucose (60 mCi mmol^{-1}; Amersham) and [1-^{14}C]oleate (57 mCi mmol^{-1}; NEN) according to the methods of French *et al.* (1981) as adapted by Walsh *et al.* (1988). The protocol required a modified CO_2 trapping system. Briefly, 20 ml glass vials were set up in a shaking water bath; each vial contained 700 μl of blood plus 150 μl of saline with 125 i.u. of heparin, unlabelled substrate, and any other effectors. The vials were sealed with a rubber septum, fitted with inflow and outflow ports for gassing, and a suspended well containing a fluted glass filter paper (the primary trap). The outflow port was connected *via* a short length of PE50 tubing to the bottom of a similar vial filled with a mixture of 1 ml of Carbotrap II (Baker) and 1 ml of ethanol. This secondary trap served to collect any $^{14}CO_2$ liberated by gassing during the incubation.

To control acid–base status, the vials were continually gassed as open systems with a humidified 99.75 % air/0.25 % CO_2 mixture (representative of typical *in vivo* resting Pa_{CO_2}=0.28 kPa) *via* a Wösthoff 301a-F gas-mixing pump. After an initial 15 min equilibration period, the run (usually 2 h) was started by the addition of labelled substrate (approximately 0.5 μCi per vial) to the blood by injection through the septum. At the end of the run, gassing was stopped, 100 μl of 1 mol l^{-1} hyamine hydroxide was injected onto the filter in the centre well, and the blood was acidified with 0.1 ml of 70 % perchloric acid to stop metabolism and liberate $^{14}CO_2$. The sealed vials were then shaken vigorously at room temperature for a further 1.5 h to ensure complete collection of $^{14}CO_2$ in the primary trap. Combined radioactive $^{14}CO_2$ from both traps was used to calculate total CO_2 production rate from the single labelled substrate, based on the specific activity, length of incubation, wet mass of RBCs and a correction for $^{14}CO_2$ release from simultaneously run chemical control vials. Specific activity was measured by counting the stock label for ^{14}C radioactivity and measuring the total substrate concentration in dummy vials. Chemical control vials contained heparin, labelled and unlabelled substrates and saline or plasma; no difference was observed between these two treatments. Dummy vials (no radioactivity) were set up in parallel for measurement of metabolites, catecholamines, haemoglobin and acid–base parameters at the beginning and end of the 2 h run. All vials were shaken gently during the incubation and temperature was maintained at 10±1°C. In one experiment, specific activity of [^{14}C]lactate, produced by RBCs *in vitro* from [U-^{14}C]glucose,

was measured in the residual perchloric acid extract following CO_2 collection by the methods of Roca *et al.* (1985) with no further modification.

O_2 consumption determinations

For total oxygen consumption measurements (M_{O_2}), pooled whole blood samples (1400 μl) were additionally heparinized (125 i.u. in 50 μl of saline) and then gassed with 99.75 % air/0.25 % CO_2 for 15 min as outlined above. Samples were then transferred by gas-tight Hamilton syringe to an M_{O_2} measurement chamber thermostatted to 10±1 °C. This consisted of a 1.35 ml glass chamber, similar to that described by Tucker (1967), which was fitted with a Radiometer E5046 P_{O_2} electrode and stirring flea. The P_{O_2} output was displayed on a Brown Boveri flatbed chart recorder. M_{O_2} was calculated from the slope of the record over time as P_{O_2} fell from the starting value (approximately 20.7 kPa) to no lower than 18.0 kPa, a region over which trout haemoglobin is fully saturated (Eddy, 1971). The system was calibrated empirically by measuring M_{O_2} simultaneously in 16 pairs of identical samples (selected from a wide range of experimental treatments) by the present method, and by the method described by Ferguson *et al.* (1989). In the latter, the decline in total O_2 content over a 2 h period in a sealed syringe was measured by the method of Tucker (1967). There was a linear proportional relationship between the two methods (r=0.78, N=16, P<0.001) with the P_{CO_2} method yielding values that were on average 34.5 % of the total O_2 method. The advantage of the present technique is that determinations are complete in about 15 min, whereas the O_2 content method takes 2 h at 10 °C to detect reliable changes, or necessitates a rise in temperature to speed up the determination (see Ferguson *et al.* 1989). A minor disadvantage is that the system becomes less reliable at lower haematocrits; samples with haematocrits less than 14 % were not used.

Analytical procedures

Blood samples for acid–base and metabolite measurements were drawn from the incubation vials through the septum into Hamilton gas-tight syringes. Whole-blood pH (pHe) was measured using a Radiometer PHM-71 meter and G97-G2 micro-capillary electrode thermostatted to the experimental temperature. Samples were removed for NTP and haemoglobin (Hb) analysis; the remainder of the sample was then transferred to a pre-weighed micro-centrifuge tube. Plasma was separated and an RBC pellet obtained by centrifugation at 13 000 *g* for 2 min. Plasma was decanted for determination of catecholamine, glucose, lactate and alanine levels; packed cell mass and haematocrit were determined gravimetrically. RBC pHi was measured by the freeze–thaw method of Zeidler and Kim (1977), using the same electrode. Total carbon dioxide content (C_{CO_2}) was measured on 100 μl of true plasma using a Corning 965 CO_2 analyzer. Plasma P_{CO_2} and [HCO_3^-] were calculated using the Henderson–Hasselbalch equation and appropriate dissociation and solubility constants listed in Boutilier *et al.* (1984). Whole-blood NTP, haemoglobin (Hb) and plasma lactate concentrations were measured

spectrophotometrically using commercial kits (Sigma), and standard enzymatic methods were used to measure plasma alanine (Graßl and Supp, 1983) and plasma glucose and RBC glycogen concentrations (Keppler and Decker, 1974). A separate plasma sample was mixed with 25 mmol l^{-1} each of reduced glutathione and EGTA, stored at −80°C for no more than 3 days, and analyzed for catecholamines (epinephrine and norepinephrine) by HPLC (Woodward, 1982, as applied by Vermette and Perry, 1988). Biochemicals were purchased from Sigma or Boehringer–Mannheim. All other chemicals were reagent grade.

Statistics

All values are presented as means±1 S.E.M. (N). Statistically significant differences ($P<0.05$) were assessed using Student's two-tailed t-test, paired or unpaired format as appropriate to the design of the experiments.

Results and Discussion

Significant activities of enzymes of glycolysis (pyruvate kinase, lactate dehydrogenase), the tricarboxylic acid cycle (malate dehydrogenase), the pentose-phosphate shunt and NADPH metabolism (glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, isocitrate dehydrogenase) and phosphagen metabolism (creatine phosphokinase) were detected in rainbow trout RBCs (Table 1), indicating a potential for activity of all these pathways. These values are substantially lower than those observed in salmonid liver (Mommsen, 1986), consistent with the lower metabolic rates (see below). We attempted to measure six other enzymes (glutamate dehydrogenase, citrate synthase, phosphoenolpyruvate carboxykinase, fructose 1,6-bisphosphatase, phosphofructokinase and glutaminase), but could detect no activity. However, with the exception of glutaminase (which is assayed non-spectrophotometrically), this lack of detection could be due to the relatively low homogenate volumes we were forced to use to avoid spectral interference by Hb; alternatively, some enzymes may be freeze-labile. Significant activities of malate dehydrogenase, lactate dehydrogenase, creatine phosphokinase and pyruvate kinase were also detected in blood plasma (Table 1). Since the Hb concentration ratio between plasma and cell homogenates was considerably lower than the comparable ratio of enzyme activities, it is unlikely that these plasma enzymes were derived from post-sampling lysis of RBCs. Enzyme activities have been reported previously in fish plasma, and they appear to increase with stress (Wells *et al.* 1986). It would be interesting to determine the isozyme types and tissue origins of the enzymes found in rainbow trout plasma before and after stress.

Our treatments of rainbow trout blood resulted in preparations with high viability, shown by stable NTP values and NTP/Hb ratios (Table 2) comparable to values from *in vivo* studies (e.g. Milligan and Wood, 1987). The flow-through gassing system maintained blood P_{O_2} and acid–base parameters (Table 2) very close to measured resting *in vivo* values in this same batch of fish (Wood *et al.*

Table 1. *Activities of several enzymes in rainbow trout blood cells and plasma*

Pathway Enzyme	Activity (μmol min^{-1} g^{-1} cell wet mass)	Activity (μmol min^{-1} ml^{-1} plasma)
Glycolysis		
Pyruvate kinase (PK) (E.C.2.7.1.40)	7.40±0.67	0.27±0.11
Lactate dehydrogenase (LDH) (E.C.1.1.1.27)		
Pyruvate→lactate	6.84±0.78	0.32±0.15
Lactate→pyruvate	1.98±0.08	---
Tricarboxylic acid cycle		
Malate dehydrogenase (MDH) (E.C.1.1.1.37)	7.95±0.60	0.76±0.09
Pentose-phosphate shunt and NADPH metabolism		
Glucose-6-phosphate dehydrogenase (G6PDH) (E.C.1.1.1.49)	6.09±0.36	ND
6-Phosphogluconate dehydrogenase (6PGDH) (E.C.1.1.1.44)	2.58±0.21	ND
Isocitrate dehydrogenase (IDH) (E.C.1.1.1.42)	1.10±0.13	ND
Phosphagen kinase		
Creatine phosphokinase (CPK) (E.C.2.7.3.2)	1.25±0.10	0.22±0.17

Values are means±1 S.E.M., N=6 fish for cells, N=3 fish for plasma. ND, not detectable. ---, not determined.

Hb content (g dl^{-1}) in cell homogenate supernatant=6.585±0.179; in plasma=0.045±0.024.

1990). Equilibration was complete within 15 min; an important precaution was to ensure that the inflowing gas was fully humidified at the experimental temperature to prevent changes in the hydration state of the blood. Plasma catecholamine, glucose and lactate levels (Table 2) were slightly higher than prior studies from the same laboratory (Perry *et al.* 1989; Wright *et al.* 1989) but comparable to *in vivo* values measured on this same batch of trout (Wood *et al.* 1990). Plasma alanine levels were in the range of salmonid values (e.g. Mommsen *et al.* 1980). RBC intracellular glycogen levels were very low in comparison to values for rainbow trout liver (Perry *et al.* 1988; Wright *et al.* 1989), but detectable, whereas intracellular alanine, lactate and glucose were not detectable (Table 2). All measured variables appeared to be stable throughout the 120 min incubation period, except plasma lactate which increased significantly. Note, in particular, the stability of epinephrine and norepinephrine. In confirmation, we found that when much higher levels of epinephrine (350 nmol l^{-1}) were added to blood *in vitro*, the

Table 2. *Profile of oxidative metabolism and metabolites in blood obtained from resting catheterized rainbow trout*

Total oxygen consumption rate

$1.43\pm0.07\ \mu mol\ g^{-1}$ cell wet mass h^{-1} (20)*

CO_2 production ($\mu mol\ g^{-1}$ cell wet mass h^{-1}) from:

Lactate	Glucose	Alanine	Oleate
0.040±0.005	0.135±0.057	0.006±0.001	0.002±0.001 (6)

[Substrate] (mmol l^{-1} plasma)

	Lactate	Glucose	Alanine	Oleate
0 min	0.764±0.057†	8.228±0.495	0.474±0.030	0.25‡
120 min	0.890±0.076†	8.221±0.509	0.480±0.026	

Acid–base status (120 min)

pHe	pHi	pHe−pHi	C_{CO_2} (mmol l^{-1})	P_{CO_2} (kPa)	P_{O_2} (kPa)
7.855±0.024	7.336±0.021	0.519	6.93±0.45	0.3±0.02	20.1±0.27

Blood metabolites§

	NTP ($\mu mol\ g^{-1}$ cell wet mass)	NTP/Hb ($\mu mol\ g^{-1}$)	Epinephrine (nmol l^{-1} plasma)	Norepinephrine (nmol l^{-1} plasma)	Glycogen ($\mu mol\ g^{-1}$ cell wet mass)
0 min	4.24±0.26	21.2±1.2	16.79±5.82	3.13±0.53	0.244±0.034
120 min	4.36±0.23	21.8±1.2	13.44±6.17	2.24±0.62	0.265±0.023

Values are means±1 S.E.M., $N=8$ preparations (unless noted in parentheses), where each preparation is blood pooled from 6–8 fish.

* This is equivalent to 95.81 nmol g^{-1} Hb min^{-1} (Wood *et al.* 1990).

† Significantly different (two-tailed, paired Student's *t*-test, $P<0.02$).

‡ Value from Bilinski and Gardner (1968) used for specific activity calculations.

§ Intracellular lactate, alanine and glucose were not detectable ($N=8$).

loss rate was only about 10 % h^{-1}. This conflicts with the recent report of Tetens *et al.* (1988) that catecholamines are extremely labile in trout blood incubated *in vitro*; the reason for this difference is unknown.

Overall metabolic rates of RBCs (Table 2) were low in comparison to other highly active preparations such as trout hepatocytes (Mommsen *et al.* 1988) and fish gill cells (Perry and Walsh, 1989). O_2 consumption rates (M_{O_2}) were comparable to previous measurements on salmonid erythrocytes (Eddy, 1977; Ferguson and Boutilier, 1988; Ferguson *et al.* 1989). Assuming typical values for whole-trout metabolic rate, haematocrit and blood volume (e.g. Milligan and Wood, 1982), the RBCs would account for 2–3 % of resting whole-animal M_{O_2}.

Rainbow trout RBCs were presented (separately) with several different labelled substrates, at typical resting plasma levels, to determine their ability to oxidize

these fuels to CO_2. Under resting conditions, glucose was clearly the dominant of the four substrates, followed by lactate; both were oxidized at substantially higher rates than alanine and oleate (Table 2). Interestingly, the combined resting CO_2 production rates from glucose, lactate, alanine and oleate were substantially lower than measured O_2 consumption rates (Table 2). These data suggest that other substrates are also important in supporting RBC metabolism *in vivo*. The extremely low level of intracellular glycogen would appear to exclude it as a significant contributor. However, since there are many amino acids in fish plasma, and several types of fatty acids, the total quantitative contribution of these substrate types may be important to total metabolic rate. In mammalian red blood cells, adenosine may be metabolized to several purine catabolites and pentose sugars; the pentoses can then enter glycolysis (and ultimately the citric acid cycle) *via* the pentose-phosphate shunt (for a review, see Joshi and Palsson, 1989). The presence or absence of this pathway should be examined in fish red blood cells. It is likely that other metabolic substrates may also be important in fish red blood cells. However, one methodological caution is appropriate: measurement of $^{14}CO_2$ production has the *potential* to underestimate metabolic rates artificially, compared to direct measurement of oxygen consumption, if recycling of metabolically generated CO_2 into synthetic pathways (*via* HCO_3^-) occurs. Carbon skeleton synthesis has not been evaluated in fish red blood cells, so we cannot assess the extent of recycling. One appropriate additional experiment to evaluate adenosine metabolism would be to test if added adenosine stimulates oxygen consumption rate.

This first metabolic screening of RBCs was performed on blood from resting fish, with relatively low substrate levels in the plasma (Table 2). These concentrations can change markedly *in vivo* during stresses such as hypoxia (Dunn and Hochachka, 1987; Wright *et al.* 1989; Boutilier *et al.* 1988), hypercapnia (Perry *et al.* 1988) and strenuous exercise (Wood and Perry, 1985; Milligan and Wood, 1987; Wood *et al.* 1990). Therefore, we wished to determine the kinetic and competitive relationships for glucose, lactate and alanine in order to clarify possible substrate preferences *in vivo*. As oxidation rates for oleate were so low, this substrate was not examined further.

Under all substrate conditions tested, the slowest oxidation rates were always obtained for alanine (Fig. 1). Over most of the range of comparable substrate levels (except extremely high values), lactate oxidation rates exceeded glucose oxidation rates (Fig. 1). However, it must be remembered that resting levels of lactate in plasma were much lower than resting levels of glucose, which explains why glucose oxidation was greater than lactate oxidation under resting conditions. There are stress conditions (e.g. exercise, hypoxia) where plasma lactate may equal or exceed plasma glucose concentration. From the kinetic relationships in Fig. 1, lactate oxidation would clearly exceed glucose oxidation under such circumstances. For this reason, we decided to compare lactate and glucose oxidation rates in direct competition experiments using the ranges seen after exhaustive exercise (Wood *et al.* 1990). Variation in glucose concentration from 0

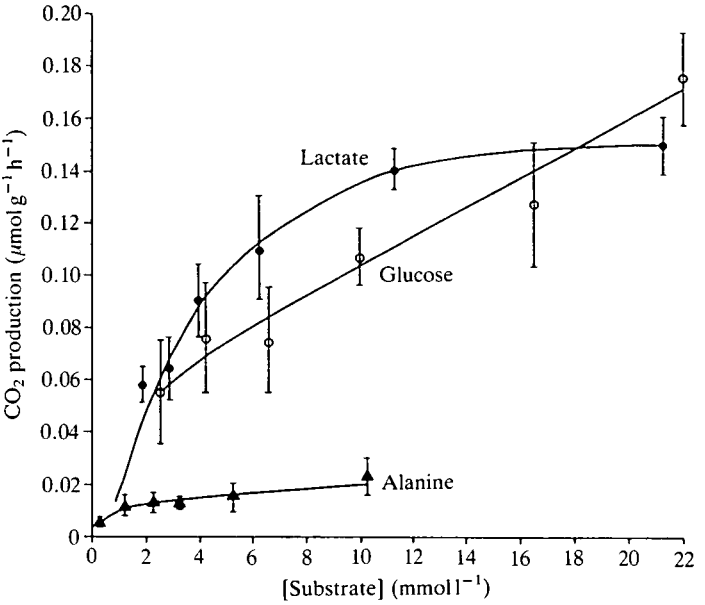

Fig. 1. Effect of substrate concentration on CO_2 production rates from alanine, glucose and lactate by rainbow trout blood. Values are means±1 S.E.M. (N=3). For alanine and lactate, variations in substrate concentration were accomplished by adding known amounts of concentrated stock solutions to whole blood from resting fish (i.e. with low initial concentrations). For glucose, cells were first centrifuged and resuspended in saline to reduce high levels of native glucose before addition of stock. K_m and V_{max} values (determined from Lineweaver–Burke plots for each substrate and preparation) are, respectively: lactate, 3.33±0.62 mmol l^{-1} and 0.148±0.019 μmol g^{-1} h^{-1}; glucose 8.41±1.25 mmol l^{-1} and 0.202±0.077 μmol g^{-1} h^{-1}; alanine, 0.76± 0.09 mmol l^{-1} and 0.018±0.005 μmol g^{-1} h^{-1}.

to 10 mmol l^{-1} did not affect the rate of lactate oxidation at either high or low lactate concentration. However, lactate had a small inhibitory effect on glucose oxidation at high concentrations of lactate and glucose (Table 3). Thus, there are circumstances when oxidative substrate preference, at least between glucose and lactate, may change.

To check whether alterations in lactate concentration affected total RBC M_{O_2}, plasma lactate was varied from control levels to 20 mmol l^{-1} in one experiment on a single pool of blood (not shown). There was no change in M_{O_2}, reflecting the fact that lactate oxidation accounted for only a small fraction (<10 %) of M_{O_2} over this entire range.

The dependence of lactate and alanine oxidation rates on substrate concentrations yielded unequivocal hyperbolic relationships, which were well described by standard Michaelis–Menten analysis (Fig. 1). In both cases, K_m values were well within the physiological range for the respective substrate concentrations in the blood, and the upper end of the physiological range produced near V_{max} velocities *in vitro*. However, glucose oxidation did not follow such a clear

Table 3. *Competitive effects of glucose and lactate on oxidation rates in blood from rainbow trout*

[Lactate] ($mmol\,l^{-1}$)	[Glucose] ($mmol\,l^{-1}$)	CO_2 production ($\mu mol\,g^{-1}$ cell wet mass h^{-1})
From lactate		
20	10	0.127±0.040
20	0	0.121±0.021
1	10	0.028±0.006
1	0	0.030±0.004
From glucose		
20	10	0.122±0.053*
0	10	0.166±0.066*
20	6	0.103±0.036
0	6	0.105±0.031

Values are means±1 S.E.M., N=3 preparations, where each preparation is blood pooled from 5–7 fish. Cells were centrifuged and resuspended in saline three times to remove native glucose and lactate.

*Significantly different (two-tailed, paired Student's t-test, $P<0.05$).

hyperbolic curve, but continued to increase even at supraphysiological levels (Fig. 1). The K_m (again in the physiological range) and V_{max} estimates are, therefore, at best approximate. This lack of saturation, plus our demonstration of the presence of significant activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in RBCs (Table 1), led us to suspect that CO_2 was being produced from glucose *via* the pentose-phosphate shunt as well as by the tricarboxylic acid cycle.

To examine this possibility, we measured glucose oxidation with both uniformly labelled and (1-C)-labelled glucose. The ratio of CO_2 production from (1-C)-labelled glucose ($0.128\pm0.047\,\mu mol\,g^{-1}\,h^{-1}$) to CO_2 production from uniformly labelled glucose ($0.390\pm0.159\,\mu mol\,g^{-1}\,h^{-1}$) was 0.403±0.011 ($N$=6), indicating an appreciable pentose-phosphate shunt activity (Wood and Katz, 1958). Prior studies (Ferguson *et al.* 1989) and our initial data (Table 2) also indicated that a measurable quantity of lactate was produced in rainbow trout blood during *in vitro* incubation, despite highly oxygenated conditions. Therefore, in the same experiment, we also measured the production of total lactate and [^{14}C]lactate from uniformly labelled glucose. Lactate production was $0.051\pm0.031\,\mu mol$ lactate $g^{-1}\,h^{-1}$ (N=6) or approximately 28% of total glucose metabolism to CO_2 and lactate. This result directly confirms initial observations that significant lactate production from glucose can occur in rainbow trout RBCs despite the presence of high O_2 concentrations and partial pressures. Cancerous cells also exhibit this 'excess aerobic glycolysis' (Lehninger, 1982), and interesting parallels may become evident upon further study of the regulatory aspects of glycolysis in fish red blood cells.

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References

BACHAND, L. AND LERAY, C. (1975). Erythrocyte metabolism in the yellow perch (*Perca flavescens* Mitchill). I. glycolytic enzymes. *Comp. Biochem. Physiol.* B **50**, 567–570.

BILINSKI, E. AND GARDNER, L. J. (1968). Effects of starvation on free fatty acid level in blood plasma and muscular tissues of rainbow trout (*Salmo gairdneri*). *J. Fish. Res. Bd Can.* **25**, 1555–1560.

BOURNE, A. R. AND COSSINS, A. R. (1982). On the instability of K^+ influx in erythrocytes of the rainbow trout, *Salmo gairdneri*, and the role of catecholamine hormones in maintaining *in vivo* influx activity. *J. exp. Biol.* **101**, 93–104.

BOUTILIER, R. G., DOBSON, G., HOEGER, U. AND RANDALL, D. J. (1988). Acute exposure to graded levels of hypoxia in rainbow trout (*Salmo gairdneri*): metabolic and respiratory adaptations. *Respir. Physiol.* **71**, 69–82.

BOUTILIER, R. G., HEMING, T. A. AND IWAMA, G. K. (1984). Physicochemical parameters for use in fish respiratory physiology. In *Fish Physiology*, vol. XA (ed. W. S. Hoar and D. J. Randall), pp. 401–429. New York: Academic Press.

DUNN, J. F. AND HOCHACHKA, P. W. (1987). Turnover rates of glucose and lactate in rainbow trout during acute hypoxia. *Can. J. Zool.* **65**, 1144–1148.

EDDY, F. B. (1971). Blood gas relationships in the rainbow trout, *Salmo gairdneri*. *J. exp. Biol.* **55**, 695–711.

EDDY, F. B. (1977). Oxygen uptake by rainbow trout blood, *Salmo gairdneri*. *J. Fish Biol.* **10**, 87–90.

FERGUSON, R. A. AND BOUTILIER, R. G. (1988). Metabolic energy production during adrenergic pH regulation in red cells of the Atlantic salmon, *Salmo salar*. *Respir. Physiol.* **74**, 65–76.

FERGUSON, R. A. AND BOUTILIER, R. G. (1989). Metabolic–membrane coupling in red blood cells of trout: the effects of anoxia and adrenergic stimulation. *J. exp. Biol.* **143**, 149–164.

FERGUSON, R. A., TUFTS, B. L. AND BOUTILIER, R. G. (1989). Energy metabolism in trout red cells: consequences of adrenergic stimulation *in vivo* and *in vitro*. *J. exp. Biol.* **143**, 133–147.

FIEVET, B., MOTAIS, R. AND THOMAS, S. (1987). Role of adrenergic-dependent H^+ release from red cells in acidosis induced by hypoxia in trout. *Am. J. Physiol.* **252**, R269–R275.

FRENCH, C. J., MOMMSEN, T. P. AND HOCHACHKA, P. W. (1981). Amino acid utilization in isolated hepatocytes from rainbow trout. *Eur. J. Biochem.* **113**, 311–317.

GRAßL, M. AND SUPP, M. (1983). L-Alanine. Determination with alanine amino transferase and lactate dehydrogenase. In *Methods of Enzymatic Analysis*, 3rd edition, vol. 8 (ed. H. U. Bergmeyer), pp. 345–349. Deerfield Beach: VCH Publishers.

GREANEY, G. S. AND POWERS, D. A. (1978). Allosteric modifiers of fish hemoglobins: *in vitro* and *in vivo* studies of the effect of ambient oxygen and pH on erythrocyte ATP concentrations. *J. exp. Zool.* **203**, 339–350.

HUNTER, A. S. AND HUNTER, F. R. (1957). A comparative study of erythrocyte metabolism. *J. cell. comp. Physiol.* **49**, 479–502.

JOSHI, A. AND PALSSON, B. O. (1989). Metabolic dynamics in the human red cell. I. A comprehensive kinetic model. *J. theor. Biol.* **141**, 515–528.

KEPPLER, D. AND DECKER, K. (1974). Glycogen determination with amyloglucosidase. In *Methods of Enzymatic Analysis*, 2nd edition, vol. 3 (ed. H. U. Bergmeyer), pp. 1127–1131. New York: Academic Press.

LEHNINGER, A. L. (1982). *Principles of Biochemistry*. New York: Worth Publishing.

MARSHALL, W. S., BRYSON, S. E. AND SAPP, M. M. (1990). Volume regulation in glutathione-treated brook trout (*Salvelinus fontinalis*) erythrocytes. *Fish Physiol. Biochem.* (in press).

MILLIGAN, C. L. AND WOOD, C. M. (1982). Disturbances in hematology, fluid volume

distribution and cardiovascular function associated with low environmental pH in the rainbow trout, *Salmo gairdneri*. *J. exp. Biol.* **99**, 397–415.

MILLIGAN, C. L. AND WOOD, C. M. (1987). Regulation of blood oxygen transport and red cell pHi after exhaustive activity in rainbow trout (*Salmo gairdneri*) and starry flounder (*Platichthys stellatus*). *J. exp. Biol.* **133**, 263–282.

MOMMSEN, T. P. (1986). Comparative gluconeogenesis in hepatocytes from salmonid fishes. *Can. J. Zool.* **64**, 1110–1115.

MOMMSEN, T. P., FRENCH, C. J. AND HOCHACHKA, P. W. (1980). Sites and patterns of protein and amino acid utilization during spawning migration of salmon. *Can. J. Zool.* **58**, 1785–1799.

MOMMSEN, T. P., WALSH, P. J., PERRY, S. F. AND MOON, T. W. (1988). Interactive effects of catecholamines and hypercapnia on glucose production in isolated trout hepatocytes. *Gen. comp. Endocr.* **70**, 63–73.

MOTAIS, R. AND GARCIA-ROMEU, F. (1989). Effects of catecholamines and cyclic nucleotides on Na^+/H^+ exchange. In *Na^+/H^+ Exchange* (ed. S. Grinstein), pp. 255–270. Boca Raton: CRC Press.

NIKINMAA, M. (1983). Adrenergic regulation of hemoglobin oxygen affinity in rainbow trout red cells. *J. comp. Physiol.* **152**, 67–72.

NIKINMAA, M. (1986). Control of red cell pH in teleost fishes. *Ann. Zool. Fennici* **23**, 223–235.

NIKINMAA, M., CECH, J. J. AND MCENROE, M. A. (1984). Blood oxygen transport in stressed striped bass (*Morone saxatilis*): role of beta-adrenergic responses. *J. comp. Physiol.* **154**, 365–369.

NIKINMAA, M. AND TUFTS, B. (1989). Regulation of acid and ion transfer across the membrane of nucleated erythrocytes. *Can. J. Zool.* **67** (in press).

PERRY, S. F., KINKEAD, R., GALLAUGHER, P. AND RANDALL, D. J. (1989). Evidence that hypoxemia promotes catecholamine release during hypercapnic acidosis in rainbow trout (*Salmo gairdneri*). *Respir. Physiol.* **77**, 351–364.

PERRY, S. F., MALONE, S. AND EWING, D. (1987). Hypercapnic acidosis in the rainbow trout (*Salmo gairdneri*). I. Branchial ionic fluxes and blood acid–base status. *Can. J. Zool.* **65**, 888–895.

PERRY, S. F. AND WALSH, P. J. (1989). Metabolism of isolated fish gill cells. *J. exp. Biol.* **144**, 507–520.

PERRY, S. F., WALSH, P. J., MOMMSEN, T. P. AND MOON, T. W. (1988). Metabolic consequences of hypercapnia in the rainbow trout, *Salmo gairdneri*: β-adrenergic effects. *Gen. comp. Endocr.* **69**, 439–447.

PRIMMETT, D. R. N., RANDALL, D. J., MAZEAUD, M. M. AND BOUTILIER, R. G. (1986). The role of catecholamines in erythrocyte pH regulation and oxygen transport in rainbow trout during exercise. *J. exp. Biol.* **122**, 139–148.

ROCA, P., GIANOTTI, M. AND PALOU, A. (1985). Enzymatic lactate-specific radioactivity determination in biological samples. *Analyt. Biochem.* **148**, 190–193.

SOIVIO, A., WESTMAN, K. AND NYHOLM, K. (1972). Improved method of dorsal aorta catheterization: haematological effects followed for three weeks in rainbow trout. *Finnish Fish. Res.* **1**, 11–21.

TETENS, V. AND LYKKEBOE, G. (1981). Blood respiratory properties of rainbow trout, *Salmo gairdneri*: responses to hypoxia acclimation and anoxic incubation of blood *in vitro*. *J. comp. Physiol.* **145**, 117–125.

TETENS, V., LYKKEBOE, G. AND CHRISTENSEN, N. J. (1988). Potency of adrenaline and noradrenaline for beta-adrenergic proton extrusion from red cells of rainbow trout, *Salmo gairdneri*. *J. exp. Biol.* **134**, 267–280.

TUCKER, V. A. (1967). Method for oxygen content and dissociation curves on microliter blood samples. *J. appl. Physiol.* **23**, 410–414.

VERMETTE, M. G. AND PERRY, S. F. (1988). Adrenergic involvement in blood oxygen transport and acid–base balance during hypercapnic acidosis in the rainbow trout, *Salmo gairdneri*. *J. comp. Physiol.* **158**, 107–115.

WALSH, P. J., MOMMSEN, T. P., MOON, T. W. AND PERRY, S. F. (1988). Effects of acid–base variables on *in vitro* hepatic metabolism in rainbow trout. *J. exp. Biol.* **135**, 231–241.

WELLS, R. M. G., MCINTYRE, R. H., MORGAN, A. K. AND DAVIE, P. S. (1986). Physiological

stress responses in big gamefish after capture: observations on plasma chemistry and blood factor. *Comp. Biochem. Physiol.* A **84**, 565–571.

WOLF, K. (1963). Physiological salines for freshwater teleosts. *Progve Fish Cult.* **25**, 135–140.

WOOD, C. M. AND PERRY, S. F. (1985). Respiratory, circulatory, and metabolic adjustments to exercise in fish. In *Circulation, Respiration and Metabolism* (ed. R. Gilles), pp. 1–22. Berlin: Springer-Verlag.

WOOD, C. M., WALSH, P. J., THOMAS, S. AND PERRY, S. F. (1990). Control of red blood cell metabolism in rainbow trout after exhaustive exercise. *J exp. Biol.* **154**, 491–507.

WOOD, H. G. AND KATZ, J. (1958). The distribution of C14 in the hexose phosphates and the effect of recycling in the pentose cycle. *J. biol. Chem.* **233**, 1279–1282.

WOODWARD, J. J. (1982). Plasma catecholamines in resting rainbow trout, *Salmo gairdneri* Richardson, by high pressure liquid chromatography. *J. Fish Biol.* **21**, 429–432.

WRIGHT, P. A., PERRY, S. F. AND MOON, T. W. (1989). Regulation of hepatic gluconeogenesis and glycogenolysis by catecholamines in rainbow trout during environmental hypoxia. *J. exp. Biol.* **147**, 169–188.

ZEIDLER, R. AND KIM, D. H. (1977). Preferential hemolysis of postnatal calf red cells induced by internal alkalinization. *J. gen. Physiol.* **70**, 385–401.