J. exp. Biol. 154, 475–489 (1990)
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CHARACTERIZATION OF RED BLOOD CELL METABOLISM IN RAINBOW TROUT

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Accepted 20 June 1990

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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Key words: rainbow trout, *Oncorhynchus mykiss*, red blood cell, catecholamines, metabolism, lactate, glucose, alanine, pentose phosphate shunt, enzymes.

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