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METABOLIC ENZYME ACTIVITIES, OXYGEN CONSUMPTION AND GLUCOSE UTILIZATION IN SEA RAVEN (*HEMITRIPTERUS AMERICANUS*) ERYTHROCYTES

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Summary

The organization of energy metabolism was assessed in erythrocytes of the sea raven (*Hemitripterus americanus*). Cell suspensions displayed a potential for aerobic glycolysis, shown by the presence of the glycolytic enzymes phosphofructokinase, aldolase and pyruvate kinase and the mitochondrial markers citrate synthase and malate dehydrogenase. Rates of oxygen uptake ($\dot{M}_{O_2}$) and glucose uptake $\dot{M}_{glucose}$ (as assessed by the disappearance of glucose) are closely matched in whole blood and resuspended erythrocyte preparations. Lactate does not accumulate under aerobic conditions. The enzymatic potential is well in excess of maximal rates of carbon flux in intact cells. Overall, the data reveal that sea raven erythrocytes have an aerobic metabolism that is fuelled by exogenous glucose. Calculated rates of glucose oxidation from [6-^{14}C]glucose were 1000-fold lower than rates measured directly from glucose disappearance, implying that exogenous glucose is highly diluted or mixed into the intracellular pools prior to entry into the citric acid cycle.

Introduction

Oxygen consumption by nucleated red blood cells (RBCs) of fish is a well-documented phenomenon (Tipton, 1933; Hunter and Hunter, 1957; Eddy, 1977; Ferguson and Boutilier, 1988; Ferguson *et al.* 1989). However, metabolic fuel use by fish erythrocytes is poorly understood. *In situ*, RBCs are bathed in plasma rich in glucose, lactate, fatty acids and amino acids, all potential metabolic fuels. It is probable that some fish erythrocytes are fuelled primarily by exogenous glucose. This contention is based upon the presence of a number of the prerequisite glycolytic enzymes in yellow perch *Perca flavens* (Bachand and Leray, 1975) and rainbow trout *Oncorhynchus mykiss* (Ferguson and Storey, 1991) erythrocytes, the ability of lungfish, electric eel and *Anguilla japonica* RBCs to take up glucose (Kim and Isaacks, 1978; Tse and Young, 1990) and the production of radioactive

Key words: red blood cell, erythrocyte, $\dot{M}_{O_2}$, $\dot{M}_{glucose}$, metabolic enzymes, metabolic rates, sea raven, *Hemitripterus americanus*.

CO_2 from ^{14}C-labelled glucose by lungfish, nurse shark and rainbow trout erythrocytes (Mauro and Isaacks, 1989; Walsh *et al.* 1990; Wood *et al.* 1990). Proof that glucose is a quantitatively important fuel for aerobic metabolism by erythrocytes from any fish is lacking. Indeed, Walsh *et al.* (1990) present evidence that glucose is not the primary metabolic fuel of rainbow trout RBCs. Oxygen consumption and metabolic fuel utilization were monitored in rainbow trout whole blood. The sum of the calculated oxidation rates of glucose, lactate, alanine and oleate could account for only 13 % of the oxygen consumed, with glucose being the dominant fuel. The authors conclude that other substrates may be important *in vivo*. However, interpretation of these data is limited by the unknown nature of fuels available in the extracellular space and the assumptions involved in back-calculating oxidation rates from uniformly labelled metabolites.

The purpose of the present investigation was to determine whether glucose is a dominant metabolic fuel of sea raven (*Hemitripterus americanus* Smelin) erythrocytes. The sea raven is a sedentary, marine teleost, and information on RBCs from this species could serve as a counterpoint to the much-studied salmonid RBCs. As a point of departure, the activities of key glycolytic and mitochondrial enzymes were measured to assess the potential for aerobic glycolysis. Oxygen consumption, glucose disappearance and lactate production by whole blood and resuspended cells were also assessed under a variety of conditions with glucose alone as a metabolic fuel. In addition, rates of $^{14}CO_2$ production from [6-^{14}C]glucose were measured in an attempt to monitor direct access of exogenous glucose to the citric acid cycle. A high level of isoproterenol at low extracellular pH was used to activate metabolism (Ferguson and Boutilier, 1988; Ferguson *et al.* 1989). The results show that energy metabolism in sea raven erythrocytes is fuelled to a large extent by the aerobic oxidation of glucose; however, there is probably a great deal of mixing of glucose with intracellular metabolites.

Materials and methods

Animals and blood sampling

Adult sea raven (*Hemitripterus americanus*) were collected by otter trawl off St Andrews, New Brunswick, and transported to Mount Allison University. They were held at 15°C in filtered, recirculating sea water and fed pieces of fish at regular intervals. Photoperiod was 12 h light:12 h dark.

Fish were chilled to 1°C in iced, saltwater baths for 30–45 min prior to blood sampling. This is an effective method of inducing quiescence in sea raven and avoids the complexity of drug anaesthesia. Blood was quickly drawn from the caudal artery into a chilled, heparinized syringe (100 i.u. ml^{-1} blood). After bleeding, fish were returned to a recovery tank at 15°C, where they quickly recovered swimming capability, resumed feeding and were held for at least 4 weeks before resampling.

Chemical analysis

pH was determined using a Fisher Accumet model 805MP meter. Haematocrit

was determined in duplicate using an Adams Readacrit microhaematocrit centrifuge. Whole blood samples were centrifuged at 1200*g* for 15 min at 5°C for metabolite analysis. Plasma glucose, lactate and free fatty acids were determined in duplicate using diagnostic kits from Sigma (glucose and lactate) and Boehringher-Mannheim (free fatty acids). Pyruvate was determined by the method of Czok and Lamprecht (1974). Haemoglobin was determined in duplicate on whole blood samples using a Sigma diagnostic kit.

Enzyme assays

The objective of these experiments was to assess the maximal activity of various enzymes as a qualitative index of metabolic potential. Washed erythrocytes were suspended in 9–14 vols of hypotonic extraction medium containing, in mmol l^{-1}: Tris, 50; EDTA, 1; $MgCl_2$, 2; and mercaptoethanol, 30; pH 7.4 at 5°C. Enzyme activities were assayed on very dilute crude suspensions. This was considered necessary since several glycolytic enzymes, notably phosphofructokinase and aldolase, bind to RBC membranes (Kliman and Steck, 1980; Jenkins *et al.* 1984). All enzymes were assayed spectrophotometrically by following the oxidation or reduction of pyridine nucleotides at 340 nm. An exception was citrate synthase, where the production of free coenzyme A was monitored with 5,5-dithiobis-2-nitrobenzoic acid (DTNB) at 412 nm. Assay temperature was maintained at 15±1°C with a circulating water bath.

Optimal conditions of pH (±0.1 pH unit), substrate and cofactor concentrations were determined for each enzyme. Assay conditions, in mmol l^{-1}, were as follows.
Hexokinase (*HK*) *(EC 2.7.1.1)*: KCl, 1.5; EDTA, 0.8; $MgCl_2$, 2; KCN, 1 ATP, 1.5; creatine phosphate, 5.0; $NADP^+$, 0.2; creatine phosphokinase, 1 i.u. ml^{-1}; and glucose-6-phosphate dehydrogenase, 0.2 i.u. ml^{-1} in 50 mmol l^{-1} Tris, pH 7.6. The reaction was initiated with 10 mmol l^{-1} glucose.
Pyruvate kinase (*PK*) *(EC 2.7.1.40)*: KCl, 150; $MgSO_4$, 10; KCN, 1; NADH, 0.2; ADP, 0.32; and lactate dehydrogenase, 10 i.u. ml^{-1} in 50 mmol l^{-1} imidazole, pH 7.4. The reaction was initiated with 2 mmol l^{-1} phosphoenolpyruvate.
Phosphofructokinase (*PFK*) *(EC 2.7.1.11)*: KCl, 50; $MgCl_2$, 5; KCN, 1; AMP, 3.0; ATP, 0.5; NADH, 0.15; glycerol-3-phosphate dehydrogenase, 1 i.u. ml^{-1}; triose phosphate isomerase, 10 i.u. ml^{-1}; and aldolase, 1 i.u. ml^{-1} in 50 mmol l^{-1} Tris, pH 8.2. The reaction was initiated with 6 mmol l^{-1} fructose 6-phosphate.
Aldolase (*ALD*) *(EC 4.1.2.13)*: KCN, 1; $MgCl_2$, 1; glycerol-3-phosphate dehydrogenase, 0.5 i.u. ml^{-1}; triose phosphate isomerase, 15 i.u. ml^{-1}; and NADH, 0.2 in 50 mmol l^{-1} Tris, pH 8.0. The reaction was initiated with 2 mmol l^{-1} fructose 1,6-diphosphate.
Lactate dehydrogenase (*LDH*) *(EC1.1.1.27)*: KCN, 1; and NADH, 0.15 in 50 mmol l^{-1} Tris, pH 7.6. The reaction was initiated with 2 mmol l^{-1} pyruvate.
Citrate synthase (*CS*) *(EC 4.1.3.7)*: KCN,1; DTNB, 0.25; and acetyl coenzyme A, 0.3 in 50 mmol l^{-1} Tris, pH 8.0. The reaction was initiated with 0.2 mmol l^{-1} oxaloacetate.
Malate dehydrogenase (*MDH*) *(EC 1.1.1.3.7)*: KCN, 1; KCl, 80; and NADH, 0.2

in 50 mmol l^{-1} Tris, pH 8.0. The reaction was initiated with 1 mmol l^{-1} oxaloacetate.

All biochemicals were obtained from Boehringer-Mannheim or Sigma Chemical Company.

Resuspension of red blood cells

RBCs were isolated by centrifugation at 1200 *g* for 15 min at 5°C. Plasma was decanted and the erythrocytes were washed twice with medium containing, in mmol l^{-1}: NaCl, 150; KCl, 5; $CaCl_2$, 3; and $MgSO_4$, 2, pH 7.8 at 5°C. Metabolic rates were assessed in resuspended cells so that glucose concentration, haematocrit and pH could be controlled and the influence of alternative exogenous metabolic fuels avoided. Elapsed time between blood removal and time zero of metabolic studies was about 2 h.

In most experiments RBCs were suspended in medium containing, in mmol l^{-1}: Tris, 50; NaCl, 150; KCl, 5; $CaCl_2$, 3; $MgSO_4$, 2; glucose, 1; and 3 % (w/v) bovine serum albumin (BSA). The medium was gassed with 0.5 % CO_2:99.5 % air for 30 min at 15°C, and pH was adjusted with HCl or NaOH to 7.1, 7.4 or 7.8±0.1 at 15°C. Bicarbonate levels in Tris Ringer were not determined, but probably approached concentrations predicted by the Henderson–Hasselbalch equation, that is 2.2, 4.5 and 11 mmol l^{-1} at pH values of 7.1, 7.4 and 7.8, respectively. When present, isoproterenol (Sigma) was added to a final concentration of 10^{-5} mol l^{-1} to activate metabolism fully. Final resuspension was at a haematocrit of 20±1 %.

In some experiments RBCs were resuspended in a medium containing, in mmol l^{-1}: NaCl, 150; KCl, 5; $CaCl_2$, 3; $MgSO_4$, 2; Na_2HPO_4, 2.3; NaH_2PO_4, 0.2; $NaHCO_3$, 11; glucose, 1; and 3 % (w/v) BSA. The medium was gassed with 0.5 % CO_2:99.5 % air for 30 min and pH was adjusted 7.8±0.1 at 15°C. Final resuspension was at a haematocrit of 20±1 %.

Oxygen and glucose consumption experiments were conducted in sealed systems. Under these conditions the sum of CO_2 and bicarbonate necessarily increases. Preliminary experiments showed that pH did not decrease by more than 0.1 unit during the course of the incubations in sealed systems. It was considered important to regulate pH since it is a prime determinant of glycolysis, especially at the level of phosphofructokinase.

Metabolic rate experiments

Oxygen consumption

Whole blood was gently swirled in a flask for 10 min at 15°C. Whole blood or resuspended erythrocytes were transferred to a glass syringe, sealed to exclude any gas bubbles and held at 15°C in a shaking water bath. 50 μl of sample was withdrawn anaerobically with a Hamilton syringe at 0, 30 and 45–60 min for resuspended erythrocytes and at 0, 60, 90 and 120 min for whole blood. The oxygen content of each sample was determined in a water-jacketed chamber maintained at 15°C following the method of Tucker (1967) with a YSI model 16582

oxygen meter equipped with a YSI Clarke-type electrode. Linearity between oxygen depletion and time was taken as evidence that cell suspensions were viable.

Glucose disappearance

Blood was collected from individual animals and divided into four parts. Whole blood or resuspended cells were incubated in capped micro-centrifuge tubes at 15 °C and glucose concentrations were determined on individual tubes at times up to 180 min.

$^{14}CO_2$ production from [6-^{14}C]glucose

Samples of whole blood or resuspended erythrocytes (2 ml) were placed in each of several 25 ml incubation flasks and swirled in a shaking water bath at 15 °C for 10 min. 1.25 μCi of D-[6-^{14}C]glucose (Dupont Canada) was added to each flask and flasks were closed with a rubber stopper which held a centre well containing 0.25 ml of methoxyethanol:ethanolamine (7:1, Eastman Kodak) to trap evolved $^{14}CO_2$. 1 ml of 5 mol l^{-1} H_2SO_4 was injected into individual flasks at 0, 60, 90 or 120 min to terminate the incubation. Each flask was swirled for a further 30 min to allow complete recovery of $^{14}CO_2$. The centre wells and their contents were counted in Aquasol II (Dupont Canada) in an LKB liquid scintillation counter. Although some CO_2 may be trapped during the incubation period, the linearity of $^{14}CO_2$ production with respect to time implies that any alterations in the CO_2/bicarbonate balance did not impact upon glucose metabolism.

Pyruvate and lactate production rate

Washed erythrocytes were resuspended in Tris Ringer at pH 7.8. Medium was equilibrated with either 0.5 % CO_2:99.5 % air or 0.5 % CO_2:99.5 % N_2; gassing of the head space above the suspension was continued throughout the experiment. This protocol was selected to ensure adequate oxygen delivery to one group of cells during the course of the incubation. Samples (1 ml) were withdrawn at intervals up to 150 min and lactate and pyruvate concentrations determined. pH remained constant within 0.05 unit during the course of the experiment.

Data analysis

The slopes of the oxygen content, metabolite content and disints min^{-1} recovered *versus* time plots were determined by linear regression analysis for each experiment and used to calculate $\dot{M}_{O_2}$, (nmol O_2 consumed h^{-1}), $\dot{M}_{glucose}$ (μmol consumed h^{-1}), $\dot{M}_{lactate}$ (μmol produced h^{-1}) and $^{14}CO_2$ (disints min^{-1} collected h^{-1}). In the radioisotope studies initial glucose concentration was determined for each experiment and used to calculate the specific substrate activity. Data are expressed as $\dot{M}_{[^{14}C]glucose}$ (nmol oxidized h^{-1}). Individual haematocrits were used to normalize to 1 ml of RBC for each experiment.

All data are expressed as mean±S.E.M. Statistical significance was assessed with either a one-way analysis of variance (ANOVA) or a two-sample Student's *t*-test. A P value of less than 0.05 was considered significant.

Results

Blood chemistry

Whole-blood pH at 15°C was 7.67±0.04 (N=36). Haematocrit ranged from 10.5 to 26.0%, with an average value of 16.9±0.5% (N=36). Plasma levels of glucose, lactate and free fatty acids, in mmol l^{-1}, were 1.58±0.18 (N=18), 0.11±0.04 (N=11) and 0.19±0.06 (N=8), respectively. Mean haemoglobin content was 74.5±5.7 g l^{-1} (N=6): 1 g of fresh RBC was equivalent to 0.26±0.016 g dry mass (N=4).

Enzyme activities

The maximal *in vitro* activities of key metabolic enzymes are given in Table 1. Background rates of oxidation of pyridine nucleotides were high, even in the presence of cyanide, in all homogenates and for all enzymes assayed. We were unable to obtain an accurate estimate for HK activity, as background rates of NADPH oxidation were extremely variable and often in excess of the rate of NADPH generated in the assay medium. An HK activity of 0.25–0.30 μmol ml^{-1} RBC min^{-1} is our best estimate in one animal only. The enzymes associated with glycolysis (phosphofructokinase, aldolase, pyruvate kinase and lactate dehydrogenase) ranged in activity from 0.15 to 1.03 μmol ml^{-1} RBC min^{-1}. The mitochondrial enzyme citrate synthase was always detectable at low activities. Malate dehydrogenase, which has a cytosolic and mitochondrial distribution, was the most active of the enzymes measured.

Oxygen consumption

$\dot{M}_{O_2}$ of whole blood was twofold (P<0.05) greater than $\dot{M}_{O_2}$ of erythrocytes suspended in medium having the same pH as whole blood (i.e. pH 7.8) (Table 2). There was no difference in $\dot{M}_{O_2}$ between RBCs resuspended in Tris Ringer or phosphate–bicarbonate Ringer at pH 7.8. Altered pH alone did not have a statistically significant effect on oxygen consumption of RBCs resuspended in Tris Ringer over the range 7.1–7.8. At pH 7.4 the inclusion of 10^{-5} mol l^{-1} isoproterenol resulted in a significant increase in oxygen consumption by RBCs resuspended in Tris Ringer.

Table 1. *Activity of metabolic enzymes from sea raven erythrocytes at 15°C*

Enzyme	Activity (μmol ml^{-1} RBC min^{-1})
Phosphofructokinase	0.69±0.16
Aldolase	0.15±0.03
Pyruvate kinase	1.00±0.09
Lactate dehydrogenase	1.03±0.14
Malate dehydrogenase	14.92±0.98
Citrate synthase	0.38±0.14

Data represent the mean±S.E.M., N=6 for each enzyme.

Table 2. *Rates of oxygen consumption ($\dot{M}_{O_2}$) of sea raven whole blood or erythrocytes resuspended in Ringer's solution*

Medium	pH	$\dot{M}_{O_2}$ (μmol ml^{-1} RBC h^{-1})
Whole blood (N=6)	7.7±0.17	4.09±0.81
Tris Ringer (N=8)	7.8	1.80±0.43
Phosphate–bicarbonate Ringer (N=5)	7.8	2.09±0.52
Tris Ringer (N=8)	7.4	2.05±0.38
Tris Ringer plus 10^{-5} mol l^{-1} isoproterenol (N=11)	7.4	3.88±0.34*
Tris Ringer (N=3)	7.1	2.30±0.56

* Statistically different from the value for RBCs resuspended at pH 7.4 without isoproterenol, $P<0.05$.

Haematocrit was 15.8±1.0 % in whole blood and 20 % in resuspended preparations.

Glucose concentration was 1.78±0.33 mmol l^{-1} in whole blood and 1 mmol l^{-1} in resuspended preparations.

Assay temperature was 15°C.

All data are mean±S.E.M.

Numbers in parentheses indicate the number of experiments each with blood from a different specimen.

In some cases blood from the same animal was utilized under a number of conditions.

Table 3. *Rates of glucose consumption for sea raven whole blood and erythrocytes resuspended in Ringer's solution*

Medium	pH	Rate of glucose consumption (μmol ml^{-1} RBC h^{-1})
Whole blood	7.58±0.09	0.71±0.13
Tris Ringer	7.8	0.66±0.18
Tris Ringer	7.4	0.65±0.19
Tris Ringer plus 10^{-5} mol l^{-1} isoproterenol	7.4	0.49±0.09

Haematocrit was 24.5±1.1 % in whole blood and 20 % in resuspended preparations.

Glucose concentration was 1.75±0.29 mmol l^{-1} in whole blood and 1 mmol l^{-1} in resuspended preparations.

Assay temperature was 15°C.

All data are mean±S.E.M. Blood was sampled from five individuals and tested under all conditions.

Glucose consumption

$\dot{M}_{glucose}$ (calculated from the direct disappearance of glucose) by erythrocytes incubated in whole blood and resuspended in Tris Ringer is given in Table 3. It should be noted that the units for oxygen consumption and $\dot{M}_{glucose}$ are both expressed as μmol ml^{-1} RBC h^{-1}. There was no significant difference in $\dot{M}_{glucose}$

amongst the four experimental conditions (one-way ANOVA). That is, $\dot{M}_{glucose}$ was comparable in whole blood and resuspended cells. Lowered pH alone or the presence of isoproterenol at pH 7.4 did not significantly enhance $\dot{M}_{glucose}$ by resuspended cells.

$^{14}CO_2$ production from labelled glucose

The rate of oxidation of exogenous glucose calculated from $^{14}CO_2$ production was 0.79 ± 0.18 nmol ml^{-1} RBC h^{-1} ($N=6$) for whole blood and 0.52 ± 0.13 nmol ml^{-1} RBC h^{-1} ($N=5$) for RBCs resuspended in phosphate–bicarbonate Ringer. These experiments were conducted on the same blood samples and run simultaneously with the data presented in Table 2. Glucose oxidation rates determined in this fashion are about 1000-fold lower than rates calculated from $\dot{M}_{O_2}$, assuming glucose is the only metabolic fuel, or from $\dot{M}_{glucose}$ measured directly from decreases in glucose concentration.

Lactate production

Erythrocytes incubated under aerobic conditions did not produce lactate in two of four preparations and showed only marginally detectable levels of lactate production in the remaining two preparations. Under anaerobic conditions, four of five preparations had a mean lactate production of 1.02 μmol ml^{-1} RBC h^{-1}. Pyruvate concentrations were low and variable in all preparations. RBCs did not produce significant quantities of pyruvate as an end product of glucose metabolism under either aerobic or anaerobic conditions.

Discussion

Glucose, lactate and free fatty acid levels in plasma of resting sea raven reported here are similar to those reported previously (Milligan and Farrell, 1986; Walsh *et al.* 1985). The extremely low level of lactate in resting sea raven blood implies that this metabolite is not a dominant fuel of RBCs under these conditions. Moreover, peak levels of lactate following strenuous activity reach only about 0.6 mmol l^{-1} (Milligan and Farrell, 1986), suggesting that even under these conditions lactate is not a quantitatively important metabolic fuel for sea raven erythrocytes. This contention must be viewed with caution as low extracellular levels of a potential substrate do not necessarily mean low catabolic rates. The potential for fatty acid oxidation by sea raven RBCs is yet to be assessed. In rainbow trout RBCs the rate of CO_2 production from oleate is very low relative to the rate from glucose (Walsh *et al.* 1990).

HK is considered to be the rate-limiting enzyme in mammalian RBCs, where its activity is much lower than those of other glycolytic enzymes (Kaneko, 1974). Similarly, in yellow perch erythrocytes HK activity is 10–100 times lower than the activities of PFK, ALD or PK (Bachand and Leray, 1975). Our inability to assess HK activity in sea raven erythrocytes probably reflects very low levels of this enzyme and implies that this is a potential rate-limiting locus. Activities of the

glycolytic enzymes PFK, ALD, PK and LDH in sea raven erythrocytes are similar (i.e. within the same order of magnitude) to those in yellow perch (Bachand and Leray, 1975) and rainbow trout (Walsh *et al.* 1990; Ferguson and Storey, 1991). The activities of PFK, ALD, PK and LDH are 100- to 500-fold lower in these fish than in frog (*Rana ridibunda*) erythrocytes (Kaloyianni-Dimitriades and Beis, 1984). Frog erythrocytes have only one or two mitochondria per cell, and CS activity is below detectable levels, in contrast with sea raven and rainbow trout erythrocytes, in which CS activity is comparable to the activities of the glycolytic enzymes. In addition, both sea raven and trout exhibit vigorous MDH activity, which may be associated either directly with the citric acid cycle or with the transport of reducing equivalents across the mitochondrial membrane *via* the malate–aspartate shuttle (Safer, 1975). The difference in enzyme profiles between fish and frog erythrocytes is consistent with frogs having an anaerobic metabolism similar to that found in mammalian red blood cells (Kaloyianni-Dimitriades and Beis, 1984), while fish erythrocytes have an aerobic metabolism.

Energy metabolism by sea raven RBCs is supported fully by aerobic metabolism, as shown by the lack of lactate production. A typical $\dot{M}_{O_2}$ value for sea raven erythrocytes is $3\,\mu mol\,ml^{-1}\,RBC\,h^{-1}$. This converts to about $260\,\mu l\,O_2\,g^{-1}$ dry mass h^{-1}, a value in the same range as those reported for other teleosts and the elasmobranch *Dasyatis* (Boutilier and Ferguson, 1989). *In vitro* oxygen consumption by sea raven erythroctyes is not stimulated by pH change alone, over the range 7.1–7.8. With the addition of pharmalogical levels of isoproterenol at pH 7.4, however, $\dot{M}_{O_2}$ doubled. Ferguson and Boutilier (1988) noted a twofold increase in $\dot{M}_{O_2}$ when *Salmo salar* blood was incubated *in vitro* at lowered pH and in the presence of adrenaline. Ferguson *et al.* (1989) observed a 1.5-fold increase in $\dot{M}_{O_2}$ in rainbow trout red blood cells in the presence of isoproterenol. Our results support the contention that erythrocyte $\dot{M}_{O_2}$ can increase by twofold under conditions of adrenergic stimulation at low pH. The important point for the present investigation is that the activation of oxygen consumption shows that the metabolism of the resuspended cells is not limited by fuel availability in the absence of isoproterenol. $\dot{M}_{O_2}$ of erythrocytes in whole blood is twofold higher than $\dot{M}_{O_2}$ of erythrocytes resuspended in medium approximating the ionic composition of sea raven plasma at pH 7.8. Enhanced $\dot{M}_{O_2}$ in whole blood may reflect the effect of blood-borne catecholamines or other unknown stimulatory agents.

The amounts of oxygen necessary to oxidize exogenous glucose totally to CO_2 may be calculated by multiplying the values in Table 3 by 6. In whole blood there is a close match between measured oxygen consumption ($4.09\,\mu mol\,ml^{-1}\,RBC\,h^{-1}$) and the calculated amount of oxygen needed to oxidize glucose ($4.26\,\mu mol\,ml\,RBC\,h^{-1}$). This is especially remarkable since these experiments were conducted on different samples of blood. At pH values of 7.8 and 7.4, in the absence of isoproterenol, glucose disappearance exceeds that which could be fully oxidized, suggesting that glucose carbon is being sequestered into the RBC carbon pool. In resuspended cells at pH 7.4 and in the presence of isoproterenol measured

$\dot{M}_{O_2}$ (3.88 μmol ml^{-1} RBC h^{-1}) exceeds calculated oxygen consumption required to oxidize exogenous glucose fully (2.94 μmol ml^{-1} RBC h^{-1}). The resuspended cells must be mobilizing endogenous fuel under these conditions. In any case, exogenous glucose is the dominant energy source.

Sea raven erythrocytes have limited anaerobic potential even under oxygen-limiting conditions. $\dot{M}_{lactate}$ for resuspended cells at pH 7.8 was 1.02 μmol ml^{-1} RBC h^{-1} when incubated under anoxia. This is similar to the glycolytic flux for sea raven RBCs under aerobic conditions and to rates of lactate production by rainbow trout RBCs under anaerobic conditions (Ferguson *et al.* 1989).

$\dot{M}_{O_2}$ and $\dot{M}_{glucose}$ assessed by direct measurements of glucose disappearance exceed the predicted rates of glucose consumption, calculated from $^{14}CO_2$ production from labelled glucose, by a factor of 1000. The simplest explanation for the shortfall is that labelled glucose is being diluted prior to its entry into the citric acid cycle. Glucose labelled at C-6 may be cycled into glycogen, lipid or amino acid pools (at pyruvate or acetyl CoA) before the liberation of $^{14}CO_2$. In sea raven heart, the apparent oxidation of exogenous glucose accounts for only 2 % of $\dot{M}_{O_2}$, as [^{14}C]glucose is incorporated into lipid and glycogen (Sephton *et al.* 1990). In rainbow trout RBCs, CO_2 production calculated from $^{14}CO_2$ liberated by the oxidation of uniformly labelled glucose could account for only one-tenth of the oxygen consumed (Walsh *et al.* 1990). Again, this shortfall was possibly due to mixing of partially oxidized glucose into intracellular pools. The calculated rate of glucose oxidation is about 25 times higher in rainbow trout than in sea raven RBCs. In the rainbow trout studies, uniformly labelled glucose would liberate $^{14}CO_2$ from the C-1 position if glucose entered the hexose monophosphate shunt or from the C-3 and C-4 positions in the decarboxylation of pyruvate to acetyl CoA. In the present experiments with sea raven, $^{14}CO_2$ would be produced only upon entry of acetyl CoA into the citric acid cycle. The difference in labelling pattern, coupled with mixing into endogenous substrate pools, probably accounts for the large difference in apparent rates of glucose oxidation, despite similar rates of oxygen consumption by sea raven and rainbow trout RBCs.

Maximal $\dot{M}_{glucose}$ expressed by sea raven RBCs is 0.71 μmol ml^{-1} RBC h^{-1}. The catalytic potential of the glycolytic enzymes is well in excess of the maximal glycolytic flux rates. For instance, PFK activity for sea raven erythrocytes was 0.69 μmol ml^{-1} RBC min^{-1}, which converts to 41 μmol ml^{-1} RBC h^{-1}. The excess in catalytic potential in sea raven RBCs is similar to that noted for mammalian erythrocytes (Kaneko, 1974). Glycolytic enzymes bind to many subcellular structures, including erythrocyte membranes (Kliman and Steck, 1980; Jenkins *et al.* 1984), and alterations in the proportion of membrane-bound and cytosolic enzyme components have been implicated in metabolic regulation in other tissues. For example, the proportion of bound PFK and ALD increase in fish skeletal muscle following exercise with increasing anaerobic glycolytic flux (Brooks and Storey, 1988). Enzyme assays were made on crude erythrocyte suspensions in this study and the partitioning of PFK, ALD, PK and LDH between cytosolic and

membrane-bound fractions was not determined. If glycolytic enzymes are partitioned between cytosol and cell membrane fractions in sea raven RBCs, this could contribute to the low rates of glycolytic flux relative to maximal enzyme activities. In rainbow trout, binding of glycolytic enzymes to particulate matter is very low (<5 %) and does not change between rest and immediately post-exercise conditions (Ferguson and Storey, 1991). But the rate of glucose oxidation by rainbow trout RBCs is the same in these two states (Wood *et al.* 1990); hence, alterations in binding would not be anticipated.

In conclusion, sea raven erythrocytes have the enzymatic potential for aerobic glycolysis. In resuspended cells $\dot{M}_{O_2}$ is not stimulated by pH alone over the range 7.1–7.8; however, at pH 7.4, $\dot{M}_{O_2}$ doubles with β-adrenergic stimulation. $\dot{M}_{O_2}$ and $\dot{M}_{glucose}$ are closely matched in whole blood and resuspended erythrocyte preparations, and provide the first evidence for the use of glucose as the primary metabolic fuel for fish erythrocytes. Exogenous glucose, however, does not have direct access to the citric acid cycle and is probably extensively recycled into intracellular pools.

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