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GLUCOSE METABOLISM BY SEA RAVEN (*HEMITRIPTERUS AMERICANUS*) AND RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) ERYTHROCYTES

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Summary

The fate of extracellular glucose in blood isolated from sea raven (*Hemitripterus americanus*) and rainbow trout (*Onchorhynchus mykiss*) was determined. In blood from both species incubated *in vitro* at low physiological pH, the decrease in plasma glucose concentration was more than adequate to support oxygen consumption. Glucose disappearance could not be accounted for by increases in lactate, red blood cell (RBC) glucose or RBC glycogen concentrations. Rates of $^{14}CO_2$ production from [6-^{14}C]glucose over a 2 h incubation period were less than 1 % of metabolic rate. Only small amounts of label appeared in RBC protein, lipid or glycogen fractions relative to metabolic rates, but label accumulated in the intracellular acid-soluble fraction (presumably free glucose, glycolytic intermediates, amino acids, citric acid cycle intermediates, etc.) at rates consistent with oxygen consumption and glucose disappearance. The simplest explanation for the mismatch between $^{14}CO_2$ production and the other estimates of metabolic rate is that incubation times were too short for equilibration to occur. A consequence is that studies of this nature cannot use $^{14}CO_2$ production to elucidate rates of aerobic fuel utilization. By default, the data imply that glucose serves as a primary aerobic metabolic fuel for the RBCs, at least under some conditions.

Introduction

Erythrocytes of many species of fish have an aerobically based metabolism (Tipton, 1933; Hunter and Hunter, 1957; Eddy, 1977; Ferguson and Boutilier, 1988; Ferguson *et al.* 1989; Sephton *et al.* 1991). However, the fuels used by fish RBCs to support energy demand are still open to question. Sea raven (*Hemitripterus americanus*), rainbow trout (*Oncorhynchus mykiss*) and yellow perch (*Perca flavescens*) erythrocytes have glycolytic enzymes but this does not, in itself, prove that glucose is used as an aerobic fuel (Bachand and Leray, 1975; Walsh *et al.* 1990; Ferguson and Storey, 1991; Sephton *et al.* 1991). In sea raven RBCs, the rate of glucose utilization, $\dot{M}_{glucose}$, based on the disappearance of plasma glucose, was adequate to support the rate of oxygen uptake, $\dot{M}_{O_2}$, during *in vitro*

Key words: erythrocyte, red blood cell, glycolysis, sea raven, *Hemitripterus americanus*, rainbow trout, *Oncorhynchus mykiss*.

incubation, implicating glucose as a primary candidate as a carbon source. However, $^{14}CO_2$ production from [6-^{14}C]glucose, which releases $^{14}CO_2$ at the level of the citric acid cycle, accounted for only 1 % of $\dot{M}_{O_2}$ (Sephton *et al.* 1991). In blood obtained from catheterized and resting rainbow trout, plasma glucose levels did not decrease following 2 h of *in vitro* incubation, and $^{14}CO_2$ production from [U-^{14}C]glucose accounted for only 10 % of $\dot{M}_{O_2}$ (Walsh *et al.* 1990). These authors argue that fuels in addition to glucose may be utilized to support aerobic metabolism by rainbow trout RBCs.

In the present investigation, the fate of extracellular glucose in sea raven and rainbow trout RBCs is examined in further detail. Sea raven differ from active-swimming rainbow trout in their sedentary lifestyle, and their plasma glucose and lactate concentrations (Walsh *et al.* 1985; Milligan and Farrell, 1986) are much lower than those in rainbow trout blood (Wright *et al.* 1989; Walsh *et al.* 1990). As a point of departure, $\dot{M}_{O_2}$ and $\dot{M}_{glucose}$, based on plasma glucose disappearance, were measured *in vitro* in whole-blood samples. Recognizing that glucose may have fates other than oxidation, we also measured plasma lactate and RBC glycogen concentrations. Next, the discrepancy between measured rates of $^{14}CO_2$ production and $\dot{M}_{glucose}$ was addressed. Blood was incubated *in vitro* with [6-^{14}C]glucose, and $^{14}CO_2$ production, ^{14}C incorporation into RBC protein, glycogen and lipid pools and ^{14}C incorporation into the acid-soluble fraction (free glucose, glycolytic intermediates, amino acids, etc.) were determined.

The most salient findings were (a) that whole blood from both species has rates of glucose uptake quite adequate to support $\dot{M}_{O_2}$ and (b) that $^{14}CO_2$ production can account for only a small fraction of metabolic rate as most of the label remains in the intracellular acid-soluble fraction.

Materials and methods

Animals and blood sampling

Adult sea raven (*Hemitripterus americanus* (Gmelin)) (700–1500 g) were collected by otter trawl off St Andrews, New Brunswick, Canada, and transported to Mount Allison University. Fish were held at 12–15 °C in filtered, recirculating sea water and fed pieces of frozen fish or live mummichugs (*Fundulus heteroclitus*) twice a week.

Rainbow trout [*Oncorhynchus mykiss* (Walbaum)] (500–1200 g) were obtained from Leger Fish Farm, Cormier Village, New Brunswick (experiment 1) and Victoria-Carleton Fish Hatchery, Centreville, New Brunswick (experiments 2–4). Fish were held at 12–15 °C in running well water and fed commercial trout pellets daily. The photoperiod for all fish was 12 h:12 h light:dark.

Blood was collected from sea raven following chilling in ice-cold sea water (Sephton *et al.* 1991). Blood was obtained from rainbow trout by chilling fish for 10–15 min in ice-cold water containing a very low level of MS222 (1:100 000 w/v, Sigma). Blood was withdrawn quickly from caudal vessels into chilled, heparinized (100 i.u. ml^{-1}) syringes. This method of blood removal and animal treatment yields RBCs which are responsive to catecholamine stimulation of glucose utilization (Sephton and Driedzic, 1994).

Experimental protocols

Experiment 1: parallel determination of $\dot{M}_{O_2}$ *and* $\dot{M}_{glucose}$

Whole blood was placed in chilled 25 ml flasks and swirled in a 15 °C water bath for 15 min. $\dot{M}_{O_2}$ and $\dot{M}_{glucose}$ were determined on separate samples of the same blood sample. A 3–5 ml sample designated for $\dot{M}_{O_2}$ determination was withdrawn into a chilled, sealed, gas-tight glass syringe. The remaining blood was incubated in an open flask to ensure that RBCs were not oxygen-limited over the longer time course required for measurement of glucose consumption. Samples were typically taken at 0, 30, 60, 90 and 120 min.

Experiment 2: carbohydrate budget

Whole blood was placed in a chilled flask in a shaking water bath at 15 °C and gassed with 0.25 % CO_2:balance air for 10–15 min prior to determination of initial values. Blood was incubated with gassing for 2 h and plasma glucose, plasma lactate and glycogen and glucose concentrations in the RBC pellet were measured at 30 min intervals in each sample. Glucose and glycogen concentrations were measured in RBCs isolated by centrifugation at 1200 ***g*** for 15 min at 5 °C. Sea raven erythrocytes were washed twice with a medium containing (in mmol l^{-1}): NaCl, 150; KCl, 5; $CaCl_2$, 3; $MgSO_4$, 2; pH 7.8 at 5 °C. Trout erythrocytes were washed twice in a medium containing (in mmol l^{-1}): NaCl, 125; KCl, 5; $CaCl_2$, 1.5; $MgSO_4$, 1; imidazole, 20, pH 7.2 at 5 °C. Glucose concentration in the RBC pellet was considered to approximate intracellular RBC glucose concentration.

Experiment 3: [6-^{14}C]glucose oxidation and ^{14}C incorporation into protein, glycogen and lipid pools

$^{14}CO_2$ is produced when [6-^{14}C]glucose is oxidized through the citric acid cycle. Glucose labelled at C-6, however, may be cycled into glycogen, lipid or amino acid pools prior to the liberation of $^{14}CO_2$.

Whole blood was collected and gassed as described for experiment 2. Thereafter, 1.85×10^5 Bq of D-[6-^{14}C]glucose (Dupont Canada) and 9.5 ml of whole blood were mixed, transferred to a gas-tight glass syringe and incubated at 15 °C for 2 h. Initial specific activities were $1.73\times10^6\pm0.23\times10^6$ disints min^{-1} μmol $^{-1}$ glucose for sea raven and $0.52\times10^6\pm0.07\times10^6$ disints min^{-1} μmol $^{-1}$ glucose for rainbow trout. At the end of the incubation period, 2 ml of blood was removed and placed in a chilled 25 ml flask. The flask was closed with a rubber stopper, which held a centre well containing 0.25 ml of methoxyethanol:ethanolamine (7:1, Eastman:Kodak). 1 ml of 10 mol l^{-1} H_2SO_4 was added by injection through the stopper and the flask was swirled for 1 h to collect evolved $^{14}CO_2$. The well and its contents were counted in 10 ml of CytoScint (ICN Biomedicals, Inc.) in a liquid scintillation counter. RBCs were isolated from the blood remaining in the syringe as described for experiment 2. Separate samples of washed cells were placed in preweighed centrifuge tubes and frozen in liquid nitrogen for protein, lipid and glycogen extraction.

Experiment 4: incorporation of [6-^{14}C]glucose into acid-soluble products

Whole blood was collected and gassed as described for experiment 2. Thereafter, 6.012×10^4 Bq of D-[6-^{14}C]glucose and 3.0 ml of whole blood were mixed, transferred to a

gas-tight glass syringe and incubated in a shaking water bath at 15 °C for 2 h. Initial specific activities were $1.46\times10^6\pm0.28\times10^6$ disints min^{-1} μmol^{-1} glucose for sea raven and $0.41\times10^6\pm0.08\times10^6$ disints min^{-1} μmol^{-1} glucose for rainbow trout. At the end of the incubation period, 2 ml of blood was removed and RBCs were isolated and washed as described in experiment 3. 1 ml of 10 mol l^{-1} H_2SO_4 was added to the washed RBCs, vortex-mixed and held on ice for 5 min. The acid supernatant was obtained by centrifugation at 2300 ***g*** for 15 min at 5 °C. The remaining pellet was washed with 5 ml of distilled water, centrifuged at 2300 ***g*** for 15 min at 5 °C, and resuspended in 5 ml of Protosol (Dupont Canada). Samples of acid supernatant, pellet wash and solubilized pellet were counted separately in 10 ml of CytoScint.

Analytical procedures

Oxygen content, metabolite levels, pH and haematocrit

Whole blood was incubated in a gas-tight glass syringe in a shaking water bath at 15 °C. 50 μl of blood was withdrawn anaerobically at set intervals with a Hamilton syringe and oxygen content was determined in a water-jacketed chamber maintained at 15 °C according to the method of Tucker (1967).

Whole-blood samples were centrifuged at 1200 ***g*** for 15 min at 5 °C to separate plasma from packed erythrocytes. Plasma [glucose] was determined in duplicate either using a Sigma diagnostic kit (Glucose HK, no.16-UV) or in chilled $HClO_4$ extracts adjusted to neutral pH with 1 mol l^{-1} Mops and 2 mol l^{-1} KOH, according to the method of Bergmeyer *et al.* (1983). Plasma [lactate] was determined in duplicate using a Sigma diagnostic kit (no. 726-UV/826-UV).

RBC glucose and glycogen levels were determined in duplicate in neutralized $HClO_4$ extracts. Packed erythrocytes were diluted 1:4 (w/v) with chilled 6 % (w/v) $HClO_4$, centrifuged at 10 000 ***g*** at 5 °C for 10 min, and the pH of the supernatant was neutralized as described for plasma extracts. A sample was taken for the determination of RBC [glucose] using the method of Bergmeyer *et al.* (1983). A second sample was taken to determine RBC glycogen content by a modification of the method of Keppler and Decker (1983). One volume of extract was hydrolyzed in 4.2 volumes of a solution containing 9.5 mg ml^{-1} amyloglucosidase and 0.1 mol l^{-1} potassium bicarbonate at 40 °C for 2 h. Hydrolysis was terminated by the addition of 2 volumes of 0.6 mol l^{-1} $HClO_4$, the pH was neutralized with solid potassium bicarbonate, and glucose content was determined as described above. Glycogen content was calculated by subtracting the glucose content of the unhydrolyzed extract from the glucose content of the hydrolyzed extract. Because of the low glycogen concentrations in RBCs, a reagent blank containing 1 volume of neutralized $HClO_4$ was run in parallel with the RBC samples. Neutralized $HClO_4$ served as an assay blank in the glucose assays.

pH was determined using a Fisher Accumet model 805MP meter. Haematocrit was determined in duplicate using an Adams Readacrit microhaematocrit centrifuge.

Incorporation of ^{14}C

^{14}C-labelled protein, glycogen and lipid were extracted from separate 0.1–0.5 ml samples of packed RBCs.

Protein was isolated by vortexing erythrocytes with 1 ml of 10 % (w/v) ice-cold trichloroacetic acid (TCA), followed by centrifugation at 2300 ***g*** for 10–15 min at 5 ˚C. The pellet was washed twice with 10 % TCA and once with 5 ml of diethyl ether. The washed pellet was dried at 35 ˚C under a stream of air and then digested at 100 ˚C for 10–15 min in 1 ml of 1 mol l^{-1} NaOH. The digested protein was taken up in 4–9 ml of distilled water and protein concentration was determined using a Biorad DC protein assay kit. Beef haemoglobin (MCB Manufacturing Chemicals, Inc.), treated in the same fashion, was used as a standard.

Glycogen was extracted as described previously by Sephton *et al.* (1990), based on the method of Walaas and Walass (1950). Briefly, RBCs were digested in 0.3 ml of boiling 30 % (w/v) KOH for 10 min, cooled and 0.2 ml of 2 % aqueous Na_2SO_4 and 2 ml of absolute ethanol were added. Glycogen was precipitated by centrifugation at 2300 ***g*** for 15 min. The pellet was resuspended in 66 % ethanol, centrifuged again, and finally resuspended in 1–2 ml of distilled water. pH was neutralized and glycogen was assayed following the method of Bergmeyer *et al.* (1983).

Separate samples of dissolved protein and glycogen were counted in 10 ml of CytoScint.

Lipid was extracted following the method of Johnston (1971). Briefly, the erythrocytes were extracted three times in 2:1 (v/v) chloroform:methanol in a ground-glass homogenizer. The first extraction used 20 ml g^{-1}; subsequent extractions used 10 ml g^{-1}. The fourth extraction was with 10 ml g^{-1} of 1:2 (v/v) chloroform:methanol. The filtered extracts were combined, washed with 0.2 volumes of distilled water and the aqueous layer was decanted. The washed extract was transferred to a preweighed scintillation vial and dried at 35 ˚C under a stream of N_2. The vial was reweighed and the dried lipid was then taken up in 10 ml of Betamax (ICN Biomedicals, Inc) for scintillation counting.

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

Data analysis

The slopes of the oxygen and glucose content *versus* time plots were determined by linear regression analysis for each experiment and used to calculate $\dot{M}_{O_2}$ (μmol O_2 consumed h^{-1}) and $\dot{M}_{glucose}$ (μmol glucose consumed h^{-1}). Initial plasma glucose concentration was determined for each radioisotope experiment and used to calculate specific activity. $^{14}CO_2$ collected was used to express $\dot{M}_{[^{14}C]glucose}$ (nmol oxidized h^{-1}) based on the initial extracellular glucose specific activity. [^{14}C]glucose incorporated into protein, glycogen or lipid was expressed as nmol incorporated h^{-1}. [^{14}C]glucose incorporated into acid-soluble products (acid supernatant + pellet wash) and insoluble fractions was expressed as μmol incorporated h^{-1}. Average haematocrit values over the course of each incubation were used to normalize to millilitres of RBC for each experiment.

All data are expressed as means ± 1 S.E.M. A paired-sample *t*-test was used to assess statistically significant changes (P<0.05) of variables within blood samples over time.

Results

Experiment 1: parallel determination of $\dot{M}_{O_2}$ *and* $\dot{M}_{glucose}$

Whole-blood oxygen content and plasma glucose concentrations during *in vitro* incubation in rainbow trout and sea raven blood are presented in Fig. 1. For rainbow trout blood, over the 90 min incubation period, the change in O_2 content was approximately 4 ml O_2 100 ml^{-1} blood or 180 μmol O_2 100 ml^{-1} blood; the change in glucose content was approximately 1.6 μmol glucose ml^{-1} plasma or 104 μmol glucose 100 ml^{-1} blood once haematocrit is factored into the calculation. For sea raven blood, the change in O_2 content was approximately 1 ml O_2 100 ml^{-1} blood or 45 μmol O_2 100 ml^{-1} blood. Changes in glucose concentration are less apparent from the cumulative data, but in every

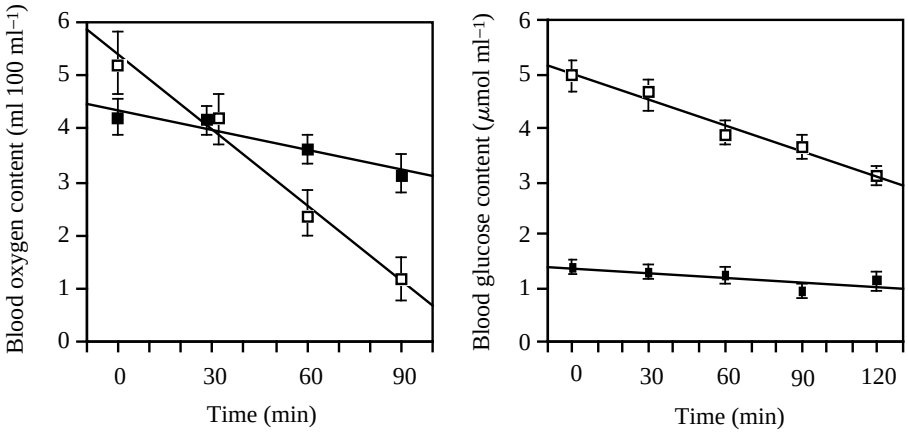

Fig. 1. Blood oxygen content (ml O_2 100 ml^{-1} blood) *versus* time and plasma glucose concentration (μmol ml^{-1} plasma) *versus* time for rainbow trout (*N*=5; open symbols) and sea raven (*N*=6; filled symbols) during *in vitro* incubation.

Table 1. *Rates of oxygen consumption and glucose utilization at 15 °C by sea raven and rainbow trout blood*

	Sea raven	Rainbow trout
$\dot{M}_{O_2}$ (μmol O_2 ml^{-1} RBC h^{-1})	1.33±0.20	3.63±0.63
$\dot{M}_{glucose}$ (μmol glucose ml^{-1} RBC h^{-1})	0.45±0.14	1.77±0.14
pH		
t=0 min	7.46±0.08	7.31±0.05
t=120 min	–	7.61±0.06*

All values are means ± S.E.M., *N*=6 for sea raven and *N*=5 for rainbow trout.

*Value for *t*=0 is significantly different from value for *t*=120 min (two-tailed, paired Student's *t*-test, $P<0.01$).

individual experiment there was a decline in glucose content. On average, the change in glucose level was approximately 0.2 μmol glucose ml^{-1} plasma or 16 μmol glucose 100 ml^{-1} blood.

Mean metabolic rates, normalized per millilitre of RBCs, on the assumption that these cells are the major contributors to the metabolic events, are presented in Table 1. Haematocrit was typically 19 % for sea raven and 34 % for rainbow trout. Given that 6 μmol of O_2 is required to oxidize 1 μmol of glucose, the [glucose] decrease was in excess of that required to support aerobic metabolism by RBCs from either species. There was a significant rise in rainbow trout whole-blood pH during the course of the experiment in which glucose levels were monitored.

Experiment 2: simultaneous measurements of glucose, glycogen and lactate levels

The most likely fate of extracellular glucose, other than oxidation, is incorporation into lactate or glycogen. Therefore, carbohydrate budgets were assessed over the course of a 2 h *in vitro* incubation. Initial and final values only are presented in Table 2, as the pattern for the change in [glucose] was similar to data presented in Fig. 1.

Plasma [glucose] declined during *in vitro* incubation of blood from both species. $\dot{M}_{glucose}$ for sea raven RBCs calculated from regression equations was 0.46±0.10 μmol ml^{-1} RBC h^{-1}, which was the same as the rate observed in experiment 1. For rainbow trout, however, $\dot{M}_{glucose}$ was 0.45±0.10 μmol ml^{-1} RBC h^{-1} in experiment 2, which was lower than the value obtained in experiment 1. This is possibly the result of variation between the different populations of fish used in the two studies.

There was no significant change in lactate concentration in blood from either species between 0 and 120 min of incubation, nor was there a consistent direction in either lactate production or accumulation. Lactate was produced in two of five sea raven experiments

Table 2. *Carbohydrate budget in sea raven and rainbow trout blood following 2 h of* in vitro *incubation at 15 °C*

	Sea raven		Rainbow trout	
	t=0 min	*t*=120 min	*t*=0 min	*t*=120 min
Plasma [glucose] (μmol ml^{-1})	1.94±0.30	1.71±0.28*	6.26±0.97	5.70±1.00*
Plasma [lactate] (μmol ml^{-1})	0.83±0.27	0.32±0.20	2.74±0.62	2.66±0.62
RBC [glucose] (μmol ml^{-1} RBC)	0.84±0.40	0.49±0.17	0.91±0.60	0.76±0.24
RBC [glycogen] (μmol ml^{-1} RBC)	3.92±1.15	1.00±0.48	0.43±0.13	0.31±0.16
pH	7.52±0.06	7.69±0.02*	7.39±0.01	7.62±0.04*

All values are means ± S.E.M., *N*=5.
*Significantly different from value at *t*=0 (two-tailed, paired Student's *t*-test, $P<0.05$).

and in two of five rainbow trout experiments. In blood samples where lactate was produced, the net accumulation was small (typically 0.2 μmol ml^{-1}), relative to glucose disappearance (typically 0.5–1.0 μmol ml^{-1}).

Initial RBC glucose values were not significantly different from final values, although there was a tendency for levels to decline. The initial glycogen level was nine times higher in sea raven than in trout RBCs. In sea raven, [glycogen] declined with time in all experiments, but mean final [glycogen] values were not significantly different from mean initial [glycogen] values. Glycogen concentrations did not change with time in rainbow trout erythrocytes and oscillated between 0.2 and 0.8 μmol ml^{-1} RBC during the incubations.

The decline in plasma [glucose] that occurred during *in vitro* incubation of sea raven and rainbow trout blood was not associated with increases in intracellular glucose, glycogen or plasma lactate concentrations.

Initial and final blood pH values indicate that blood was acidotic in both species at the onset of the experiment.

Experiment 3: [6-^{14}C]glucose oxidation to $^{14}CO_2$ and ^{14}C incorporation into intracellular pools

To define better the fate of glucose, RBCs were offered [^{14}C]glucose as a metabolic fuel. The rates of oxidation of [6-^{14}C]glucose based on $^{14}CO_2$ production and incorporation of ^{14}C into protein, glycogen and lipid for sea raven and rainbow trout RBCs are given in Table 3. In both species, label appeared in all four fractions assayed. In sea raven, the rates of glucose incorporation into protein, lipid and glycogen were similar, and were approximately three times higher than rates of oxidation to CO_2. In rainbow trout, the rates of incorporation into protein and glycogen were approximately six times higher than the rate of oxidation, and the rate of incorporation into lipid was approximately three times higher than the rate of oxidation. Total $\dot{M}_{[^{14}C]glucose}$, calculated from the sum of incorporation of ^{14}C into the various intracellular pools, represented only about 5 % of $\dot{M}_{glucose}$ measured by direct analysis of glucose disappearance from the plasma (compare experiments 2 and 3).

Protein and lipid contents were similar in sea raven and rainbow trout erythrocytes. Glycogen levels in trout RBCs were too low to be measured accurately by the assay employed in this experiment.

Experiment 4: incorporation of [6-^{14}C]glucose into acid-soluble products

In an effort to account for the mismatch between $\dot{M}_{glucose}$ assessed by direct chemical analysis and the combined rates of $^{14}CO_2$ production and ^{14}C incorporation from [6-^{14}C]glucose, accumulation of ^{14}C into RBC acid-soluble and acid-insoluble fractions was measured (Table 4). The rate of ^{14}C incorporation into the acid-soluble fraction (free glucose, glycolytic intermediates, citric acid cycle intermediates, free amino acids, etc.) was eight times higher than the rate of incorporation into the acid-insoluble fraction in both species.

The rates of [^{14}C] incorporation into the acid-soluble fraction accounted for 33 % and 74 % of $\dot{M}_{glucose}$, respectively, assessed by direct chemical analysis (compare experiments

Table 3. *[6-^{14}C]glucose oxidation to $^{14}CO_2$ and incorporation of ^{14}C into protein, glycogen and lipid pools of sea raven and rainbow trout erythrocytes following 2 h of* in vitro *incubation of whole blood at 15 °C*

	Sea raven	Rainbow trout
$\dot{M}_{[^{14}C]glucose}$ (nmol oxidized ml^{-1} RBC h^{-1})	1.82±0.52	1.76±0.51
[^{14}C] incorporation into protein (nmol ml^{-1} RBC h^{-1})	6.47±1.48	11.53±2.60
(nmol mg^{-1} protein h^{-1})	0.07±0.04	0.06±0.01
[^{14}C] incorporation into glycogen (nmol ml^{-1} RBC h^{-1})	4.52±1.81	13.21±1.88
(nmol mg^{-1} glycogen h^{-1})	19.95±5.59	–
[^{14}C] incorporation into lipid (nmol ml^{-1} RBC h^{-1})	5.33±1.93	6.33±1.18
(nmol mg^{-1} lipid h^{-1})	0.72±0.28	0.72±0.21
Total $\dot{M}_{[^{14}C]glucose}$ (nmol ml^{-1} RBC h^{-1})	18.14±4.77	32.83±4.76
Protein content (mg ml^{-1} RBC)	138.6±29.8	89.4±2.3
Glycogen content (mg ml^{-1} RBC)	0.31±0.14	–
Lipid content (mg ml^{-1} RBC)	7.95±1.39	9.92±1.20

All values are means ± S.E.M., *N*=5 in all cases.

Table 4. *^{14}C incorporation from [6-^{14}C]glucose into acid-soluble and acid-insoluble fractions of sea raven and rainbow trout erythrocytes following 2 h of* in vitro *incubation of whole blood at 15 °C*

	Sea raven	Rainbow trout
[^{14}C] acid-soluble fraction (μmol incorporated ml^{-1} RBC h^{-1})	0.153±0.017	0.334±0.099
[^{14}C] acid-insoluble fraction (μmol incorporated ml^{-1} RBC h^{-1})	0.018±0.002	0.043±0.015

All values are means ± S.E.M., *N*=5.

2 and 4) for sea raven and rainbow trout RBCs. Rates of incorporation of [^{14}C] into the acid-insoluble fraction were consistent with rates of incorporation into the protein plus lipid pools (compare experiments 3 and 4).

Discussion

Initial blood variables

Plasma glucose and lactate levels in sea raven recorded here were similar to those

reported previously (Walsh *et al.* 1985; Milligan and Farrell, 1986; Sephton *et al.* 1991). Plasma glucose levels in rainbow trout were within the range of values reported for this species, although plasma lactate values were higher than those obtained from resting fish *via* indwelling catheters (Ferguson *et al.* 1989; Wright *et al.* 1989; Walsh *et al.* 1990; Wood *et al.* 1990; Pagnotta and Milligan, 1991). Plasma glucose and lactate levels are about three times higher in rainbow trout blood than in sea raven blood. Total oxygen content for rainbow trout blood was lower than reported by others (Eddy, 1971; Ferguson *et al.* 1989; Wright *et al.* 1989). This difference was probably the result of low pH. Differences in blood variables between the two species in this study may relate to higher activity patterns and subsequent higher levels of metabolism at the whole-animal level in rainbow trout than in sea raven.

Initial whole-blood pH for both species was lower than arterial pH values (Milligan and Farrell, 1986; Ferguson *et al.* 1989; Wright *et al.* 1989; Walsh *et al.* 1990) and increased during the course of *in vitro* incubation. Low initial pH levels were possibly due to a combination of factors, including temperature, arterial *versus* mixed blood obtained by caudal puncture and the method of blood sampling, which probably stressed the animals. Blood pH for the sea raven experiments was always within the range experienced post-exercise (Milligan and Farrell, 1986), while for rainbow trout, blood pH reached post-exercise values during the course of *in vitro* incubation. The low pH values should not alter the general conclusions of this paper which, as discussed below, contend that glucose is a fuel for energy metabolism in fish RBCs. If it has any effect, low extracellular pH should impair glucose utilization at the level of phosphofructokinase, a rate-controlling step in glycolysis; thus, the experimental protocol could have led to an underestimate of glucose use. The lack of a substantial impact of low pH on glucose utilization by trout RBCs is apparent from earlier work in which $^{14}CO_2$ production from glucose was not altered in blood obtained immediately following exercise compared with blood from resting animals, despite a drop in pH and an increase in plasma [lactate] (Wood *et al.* 1990). Although, as argued below, $^{14}CO_2$ production is not a measure of absolute glucose utilization in studies of this nature, it is probably a mirror of relative rates of glucose metabolism.

Oxygen consumption and [glucose] decrease

Oxygen content declined significantly during *in vitro* incubation for both species. $\dot{M}_{O_2}$ by sea raven blood was three times lower than reported previously (Sephton *et al.* 1991). Literature values for $\dot{M}_{O_2}$ of trout RBCs extend over a three- to fourfold range once assay temperature is taken into consideration (Eddy, 1977; Ferguson *et al.* 1989; Tufts and Boutilier, 1991; Walsh *et al.* 1990). The current value is well within the established range. $\dot{M}_{O_2}$ of fish RBCs may be influenced by a variety of factors such as age, sampling techniques, season, etc.

Glucose concentration fell significantly in both sea raven and rainbow trout plasma during *in vitro* incubation. A similar finding has been noted with eel RBCs (B. Pelster, J. M. T. Hicks and W. R. Driedzic, unpublished results). In sea raven plasma, the mean [glucose] decline was comparable with values cited in previous work (Sephton *et al.* 1991). Walsh *et al.* (1990) reported no change in rainbow trout plasma glucose

concentration between time zero and a sample taken 2 h later. A number of possibilities exist to explain the difference between the two studies. One explanation is the 5 °C higher assay temperature utilized here. A second possibility is that the cells used in the current study were metabolically hyperactive as a result of the sampling technique. A further consideration is that decreases in glucose content are small relative to assay variability, so sampling at a single time point runs the risk of missing any changes. The present analysis, which considered multiple samples over time, reveals a decrease in glucose concentration.

In experiment 1, $\dot{M}_{O_2}$ and $\dot{M}_{glucose}$ were assessed on blood from the same animals. The experimental conditions were not exactly the same since a sealed system is required for the measurement of oxygen utilization and an open system is required to allow enough time for any [glucose] changes to be measurable without creating a hypoxic environment. In a glucose-supported aerobic metabolism, 6 mol of oxygen are consumed per mol of glucose oxidized. The decrease of [glucose] in plasma of both sea raven and rainbow trout was 2–3 times greater than that required to support aerobic metabolism. Although it is possible that differences in incubation conditions contributed to the mismatch, it is overwhelmingly clear that enough glucose disappeared potentially to support aerobic metabolism. Comparisons of $\dot{M}_{O_2}$ with radioisotope studies, which were conducted in sealed systems but with blood from different animals, confirm that glucose is an important metabolic fuel.

Alternative fates of glucose

A decrease in plasma glucose concentration does not in itself prove its use as an aerobic fuel, as fates other than oxidation are possible. During aerobic *in vitro* incubation, neither sea raven nor rainbow trout blood produced lactate in consistent and substantial quantities. This was shown previously for sea raven (Sephton *et al.* 1991), although low levels of lactate production have been noted for rainbow trout blood (Ferguson *et al.* 1989; Walsh *et al.* 1990). Initial glycogen levels were about nine times higher in sea raven RBCs than in rainbow trout RBCs, the content of which was comparable to that reported by Walsh *et al.* (1990). While RBC glycogen levels declined markedly during *in vitro* incubation in sea raven cells, mean initial glycogen levels were not significantly different from mean final levels. Our data rule out the possibility that glucose disappearance from plasma may be accounted for by lactate production, glycogen storage or accumulation of intracellular glucose in RBCs from either species. *In vitro* incubation of RBCs with [6-^{14}C]glucose resulted in the appearance of ^{14}C in protein and lipid pools. However, the rate of incorporation was extremely low compared with the rate of [glucose] decrease. By default, the logical conclusion to derive from the data is that glucose is degraded to support aerobic metabolism of both sea raven and rainbow trout RBCs under the incubation conditions of these studies.

Mismatch between $^{14}CO_2$ production and metabolic rates

[6-^{14}C]glucose releases $^{14}CO_2$ during passage of intermediates through the citric acid cycle and, hence, is a measure of aerobic oxidation of this metabolic fuel. Glucose oxidation over the 2 h incubations for RBCs from both species was approximately

2 nmol ml^{-1} RBC h^{-1} when measured in this fashion. This level is only a small fraction (less than 1 %) of the metabolic rate assessed by either direct measurement of glucose decrease or oxygen consumption. This general finding of low rates of $^{14}CO_2$ production relative to metabolic rates is consistent with previous reports not only for sea raven (Sephton *et al.* 1991) and rainbow trout (Walsh *et al.* 1990) but also for RBCs from brown trout (Pesquero *et al.* 1992). In the present study, the labelled carbon does not accumulate in intracellular protein, glycogen or lipid at high levels. Instead, the carbon remains in the acid-soluble fraction, which would include free intracellular glucose, glycolytic intermediates, citric acid cycle intermediates, amino acids, etc. The rate of incorporation of glucose into the acid-soluble fraction is similar to the rate of uptake of the non-metabolizable sugar 3-*O*-methyl-glucose by rainbow trout RBCs (Tse and Young, 1990). The simplest explanation to account for the mismatch between $^{14}CO_2$ production and metabolic rate is that equilibration times are far in excess of the 2 h incubation period. Regardless of the reason for the mismatch, a consequence is that $^{14}CO_2$ production cannot be utilized as a measure of the oxidative rate of glucose. This limitation would probably also apply to measurement of metabolic rates for amino acids and other metabolites (Tiihonen and Nikinmaa, 1991).

The rate at which labelled glucose is incorporated into the acid-soluble pool provides a reasonable estimate of the metabolic rate. For instance, if for every labelled glucose molecule remaining in the acid-soluble fraction an unlabelled glucose molecule was oxidized to CO_2, this could account for 70 % and 55 % of the $\dot{M}_{O_2}$ of sea raven and rainbow trout RBCs, respectively, and 33 % and 74 %, respectively, of the decline in glucose levels measured by direct chemical analysis for sea raven and rainbow trout RBCs. Given the variability in metabolic rate amongst different populations of fish, the variability in incubation conditions (open *versus* closed systems) and the difference in animal treatment, this is a close match between $^{14}CO_2$ incorporation and metabolic rate and suggests that glucose is a prime metabolic fuel of aerobic metabolism. This situation is similar to that described for rates of exogenous fatty acid oxidation in various preparations of rat tissues, where $^{14}CO_2$ production accounted for only 1–28 % of the total oxidation rate of [^{14}C]palmitate. The sum of $^{14}CO_2$ and ^{14}C-labelled acid-soluble products, such as acetylCoA, acetylcarnitine and citric acid cycle intermediates, was a more accurate estimate of fatty acid oxidation (Veerkamp *et al.* 1986).

General conclusions

Indirect evidence shows that at low pH glucose is an important aerobic fuel for sea raven and rainbow trout RBCs. This contention is based upon the decrease in plasma glucose concentration during *in vitro* incubation and an inability to account for this glucose in lactate or intracellular fractions. The uptake of labelled glucose into the acid-soluble pool approximates the metabolic rate. The use of glucose as a metabolic fuel is consistent with the presence of glycolytic and citric acid cycle enzymes in sea raven (Sephton *et al.* 1991) and rainbow trout (Walsh *et al.* 1990; Ferguson and Storey, 1991) RBCs. The present study investigated metabolism under pH conditions similar to those that may occur following challenges of exercise or during hypoxia. The metabolic fuel preferences of RBCs under normoxic and resting conditions may, however, differ.

Moreover, the present experimental design does not rule out the possibility that other metabolites at very low concentrations, such as adenosine or amino acids, could serve as fuels if blood levels could be maintained. These questions are currently being addressed.

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