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THE RELATIVE CONTRIBUTIONS OF RED AND WHITE BLOOD CELLS TO WHOLE-BLOOD ENERGY TURNOVER IN TROUT

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Summary

Previous studies addressing energy turnover in fish blood have ignored the possible influence of white cells. The present investigation quantified the contribution of white and red cells to whole-blood energy turnover in trout (*Oncorhynchus mykiss*) before and after adrenergic stimulation. All experiments were carried out on cells kept in their native plasma. White cells were found to have an almost twenty times higher rate of oxygen consumption than red cells. Furthermore, white cells were responsible for essentially all whole-blood lactate production. Our data therefore show that white cells account for almost half of the energy turnover in trout blood. Adrenergic stimulation elicited a significant increase in total as well as ouabain-sensitive (a Na^+/K^+-ATPase inhibitor) red cell oxygen consumption. However, the ouabain-sensitive red cell oxygen consumption amounted to approximately 23 % of the total red cell oxygen consumption, regardless of adrenergic stimulation. Therefore, energy-consuming processes other than Na^+/K^+-ATPase activity are probably involved in the increased red cell oxygen uptake after adrenergic stimulation.

Introduction

Fish red blood cell metabolism has recently been a subject of increasing interest, with research being concentrated on metabolic changes during adrenergic stimulation. Adrenaline and noradrenaline, hormones associated with exercise and stress, have been shown to increase trout blood energy turnover (Ferguson and Boutilier, 1988, 1989; Ferguson *et al.* 1989; Wood *et al.* 1990). This increase has been ascribed to an increase in Na^+/K^+-ATPase activity (Tufts and Boutilier, 1991).

Nearly all studies addressing fish red cell metabolism have been performed on whole blood. Apparently, investigators have assumed that the small proportion of white blood cells plays an insignificant part in whole-blood metabolism. Data from studies on human white cells suggest, however, that they could be of importance (Bartlett and Marlow,

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1953). With this background, we set out to quantify and describe the contribution of red and white blood cells to whole-blood energy metabolism in rainbow trout. All measurements were performed with the red and white cells together in their native plasma.

Materials and methods

Experimental animals and surgery

Rainbow trout [*Oncorhynchus mykiss* (Walbaum)] weighing 500–1000 g were obtained several months in advance of the experiments from a commercial trout farm. The fish were kept at 15 °C in recirculating tapwater, and fed *ad libitum* with fish pellets. They were exposed to a photoperiod of 12 h:12 h light:dark.

A permanent catheter was implanted in the dorsal aorta (Soivio *et al.* 1975) and led out through the upper jaw. Prior to surgery, fish were anaesthetized with benzocaine ($0.1\,g\,l^{-1}$) until ventilation ceased. During surgery, the gills were irrigated with water containing $0.02\,g\,l^{-1}$ benzocaine. After cannulation, fish were kept separate and undisturbed in perforated tubes for at least 24 h before blood sampling.

Measurements and techniques

Red cell volume varies with pH and the degree of oxygen saturation of the haemoglobin (Hb O_2-saturation). Therefore, proportions of red and white cells (v/v) were always determined on fully oxygenated blood (P_{O_2} higher than 50 kPa) at a pH of about 7.95. Under these circumstances, red cell haemoglobin concentration was $4.9\pm0.2\,mmol\,Hb\,l^{-1}$ cells (N=28). Cell volumes were measured after centrifugation of blood samples in capillary tubes for 5 min at $10\,000\,revs\,min^{-1}$. In these preparations, the number of white cells trapped among the red cells was insignificant. Column length of the white cells was measured using a microscope to ensure accurate determination. We found that the use of capillaries with a small inner diameter (0.55 mm) improved readings.

The total contents of O_2 (C_{O_2tot}) and of CO_2 (C_{CO_2tot}) in blood samples were determined using the techniques described by Tucker (1967) and Cameron (1971) and corrected according to Bridges *et al.* (1979). We used a glass chamber mounted with both an O_2 and a CO_2 electrode, making it possible to measure C_{O_2tot} and C_{CO_2tot} in concert. A 1:1 mixture of $0.01\,mol\,l^{-1}$ HCl and Tucker's original solution was used as the reaction solution. Blood pH was measured on a BMS 2 (Radiometer) maintained at 15 °C. Samples for determination of lactate were prepared by deproteinizing blood in 12 % trichloroacetic acid (TCA) (1:1). Lactate was measured using NAD/NADH-coupled enzymatic procedures. Haemoglobin concentration was determined spectrophotometrically as cyanomethaemoglobin at 540 nm, using a millimolar extinction coefficient of 11.0 (Zilstra *et al.* 1983).

Experimental design

Approximately 6 ml of blood was drawn from a catheterized fish into a heparinized syringe. In order to measure the metabolic rates of red and white cells simultaneously, the blood sample was first divided into two samples, which were centrifuged for 5 min at $2000\,revs\,min^{-1}$. Some red and white cells were then exchanged between the two

samples. In this way, proportions of red and white cells differed between the two samples, making it possible to calculate metabolic rates of the two cell populations by use of two equations with two unknown variables:

$$M_1 = w_1 \times M_W + r_1 \times M_R ,$$

$$M_2 = w_2 \times M_W + r_2 \times M_R ,$$

where M_1 and M_2 are the total O_2 consumption, CO_2 or lactate production per litre of blood in each of the two samples, and w and r are the proportions (v/v) of white cells and red cells in the samples in question. M_R and M_W are the metabolic rates of red cells and white cells, respectively. The average cell compositions of the two samples were 13.0±0.04 % red cells with 1.4±0.5 % white cells and 19.9±5.4 % red cells with 0.5±0.1 % white cells, respectively.

Critique of methods

The use of two manipulated subsamples and two equations was the preferred experimental approach as the blood is then investigated in a composition very close to the situation *in vivo*. Unfortunately, there are disadvantages; even a small error in the determination of one of the variables involved will influence all data. Furthermore, overestimation of oxygen consumption of one cell type leads to a simultaneous underestimation of the other cell type, and *vice versa*. This problem explains the relatively large standard deviations produced in the present study, despite the fact that the techniques used to determine lactate, O_2 and CO_2 contents are very precise.

No attempt was made to distinguish between different types and ages of red or white cells. Our values, therefore, reflect the average composition of blood cells. Young red cells are smaller and lighter than mature cells (Lane, 1982; Keen *et al.* 1989; Speckner *et al.* 1989) and accordingly will be located immediately beneath white cells after centrifugation. Young red cells, furthermore, seem to have a higher metabolic rate than mature cells (Tipton, 1933). Movement of non-representative red as well as white cells during the preparation of subsamples would violate the prerequisites that validate the use of the two equations. We were aware of this problem and were very careful to avoid non-representative transfer during the preparation of subsamples.

Statistics

All data are presented as mean ±1 S.D. and two-tailed paired *t*-statistics were used to test for the significance of differences.

Experimental protocol

Metabolism of blood cells before and after adrenergic stimulation

Subsamples were prepared as described above and equilibrated in rotating tonometers for 20 min with a gas mixture of 60 % O_2, 0.3 % CO_2, balanced with N_2 (Wösthoff gas-mixing pumps). This gas mixture resulted in a blood pH close to 7.95 and virtually full Hb O_2-saturation. Subsamples were then transferred to 5 ml gas-tight Hamilton syringes, which were capped and kept rotating in a water bath at 15 °C. Thereafter, measurements

of C_{CO_2tot}, C_{O_2tot} and pH were made every 40 min for 2 h. Blood remaining in the syringes was then re-equilibrated with the same gas mixture as before. After 20 min of tonometry, isoprenaline was added to the blood (5×10^{-6} mol l^{-1} final concentration) and the blood sample was again incubated in Hamilton syringes. Owing to the increased metabolic rate, measurements of C_{O_2tot}, C_{CO_2tot} and pH were now performed every 20 min for 1 h.

Samples for the determination of blood lactate concentrations were taken three times: immediately before the first incubation in Hamilton syringes, before the addition of isoprenaline and at the end of the experiment.

This series of experiments was carried out in September and October.

Effect of Hb O_2-saturation on whole-blood O_2 consumption after adrenergic stimulation

Three separate whole-blood samples were divided into two subsamples (with identical cell composition) and kept in rotating tonometers at either 60 or 6 kPa O_2 (0.4 % CO_2, balance N_2), resulting in virtually complete and approximately 65 % Hb O_2-saturation, respectively. Isoprenaline was added to the tonometers (5×10^{-6} mol l^{-1} final concentration) and blood was then immediately incubated in Hamilton syringes. After incubation, C_{O_2tot} was measured a minimum of eight times during the following hour. This series of experiments was carried out in October.

Energy requirement of the Na^+/K^+-ATPase before and after adrenergic stimulation

Only O_2 consumption was measured in this series of experiments. Oxygen consumption was calculated from the decrease in P_{O_2} of anaerobically incubated blood using an O_2 solubility coefficient of 0.250 μmol l^{-1} blood kPa^{-1} (Christoforides and Hedley-Whyte, 1969). A glass Tucker chamber was mounted with an oxygen electrode (Radiometer, E 5046-0) and thermostatted at 15 °C. The output of the oxygen electrode was recorded on a Radiometer servograph for later analysis. Application of this technique requires that Hb O_2-saturation does not change. Blood samples (prepared as above) were accordingly equilibrated with 0.2 % CO_2 and 70 % O_2, balanced with N_2, in rotating tonometers before incubation in the Tucker chamber, resulting in a blood pH above 8.0 and oxygen tensions around 60 kPa. Experiments were therefore performed at blood P_{O_2} values well above 30 kPa and at high oxygen affinities (to take into account the Bohr effect). Oxygen consumption was first determined on aliquots of both subsamples before and after adrenergic stimulation. Adrenergic stimulation took place in the Tucker chamber by injection of 2 μl of noradrenaline (final concentration of 5×10^{-6} mol l^{-1}). Ouabain was then added to the remaining blood in the tonometers (10^{-3} mol l^{-1}, final concentration). After another 10 min of tonometry, oxygen consumption was determined on both ouabain-treated subsamples before and after adrenergic stimulation. These experiments were carried out in December.

Results

Metabolism of blood cells before and after stimulation

Fig. 1 shows an example of the progression in blood C_{O_2tot}, C_{CO_2tot} and pH before and after adrenergic stimulation in a blood sample enclosed in a gas-tight syringe. All three

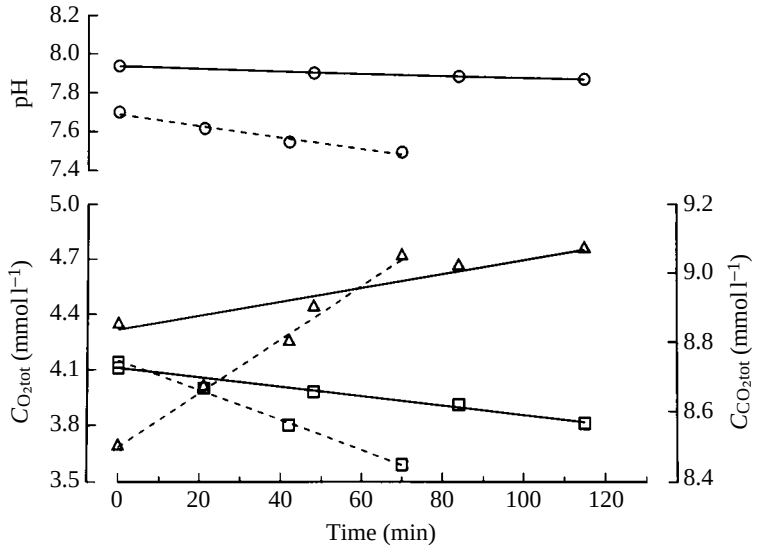

Fig. 1. Development of extracellular pH (○), total O_2 content (C_{O_2tot}) (□) and total CO_2 content (C_{CO_2tot}) (△) of blood incubated in a gas-tight syringe at 15 °C. The continuous line is before, and the broken line is after, stimulation with 5×10^{-6} mol l^{-1} isoprenaline.

variables changed linearly over time, reflecting constant metabolism. The reduced levels of C_{CO_2tot} and pH after re-equilibration of blood were probably due to lactate production during the preceding incubation and adrenergic stimulation.

Before stimulation with isoprenaline, white cell oxygen consumption was seventeen times higher than that of red cells (Table 1). Red cell oxygen consumption doubled after adrenergic stimulation, whereas white cell oxygen consumption showed no significant change. Only white cells showed a net production of lactate, and the rate was not affected by adrenergic stimulation.

Table 1. *Oxygen consumption and lactate production (μmol l^{-1} cells min^{-1}) of red and white cells from trout at 15 °C before and after stimulation with isoprenaline (5×10^{-6} mol l^{-1})*

	Unstimulated	Stimulated
Red blood cells		
Oxygen consumption	23±10	49±11*
Lactate production	–5±15**	7±20**
White blood cells		
Oxygen consumption	390±130	520±240
Lactate production	170±380	190±240

*Significantly higher than unstimulated cells ($P\leqslant0.001$).
**Not significantly different from zero.
Values are mean ± 1 S.D. (N=10).

Table 2. *Respiratory quotient (RQ) of rainbow trout blood cells at 15 °C before and after stimulation with isoprenaline (5×10^{-6} mol l^{-1})*

	Unstimulated	Stimulated
Red blood cells	0.83±0.35	1.08±0.62
White blood cells	0.65±0.52	1.17±1.44
Subsamples	0.86±0.19	1.02±0.29*

*Significantly higher than unstimulated control subsample ($P\leqslant0.01$).
Values are mean ± 1 S.D. (N=10).

Red cell Hb O_2-saturation did not affect whole-blood O_2 consumption after β-adrenergic stimulation. The mean difference in O_2 consumption between blood with low and high Hb O_2-saturation was only 0.3±0.3 μmol l^{-1} blood min^{-1} (N=3, range 0.1–0.6 μmol l^{-1} blood min^{-1}), representing only 2 % of total O_2 consumption.

The respiratory quotient (RQ) was similar for red and white cells (Table 2). These averages are encumbered with large standard deviations and we have therefore also presented RQ values for manipulated subsamples. Adrenergic stimulation increased RQ, although only significantly so for the subsamples.

Whole-blood energy turnover

Fig. 2 shows an estimation of whole-blood ATP turnover before and after adrenergic stimulation. The estimate is based on results from this study (Table 1) and red and white cell proportions of 20 % and 1 % (v/v), respectively. These proportions are representative of the average *in vivo* blood composition. Oxygen consumption and net lactate production were converted to ATP turnover by conversion factors of 3 and 1 respectively (P/O ratio

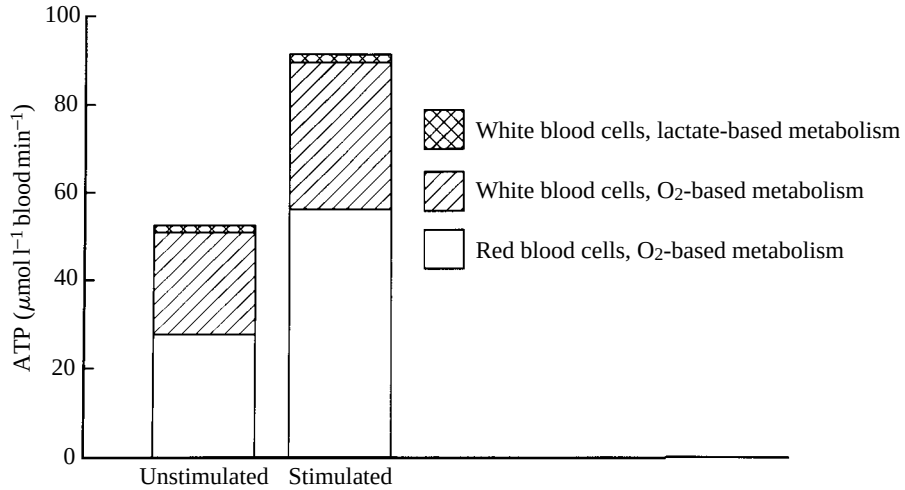

Fig. 2. Estimated total ATP turnover in trout whole blood at 15 °C. The estimate is based on a P/O ratio of 3, glucose as substrate for lactate production, and white and red cell proportions of 1 % and 20 %, respectively, in the blood (v/v).

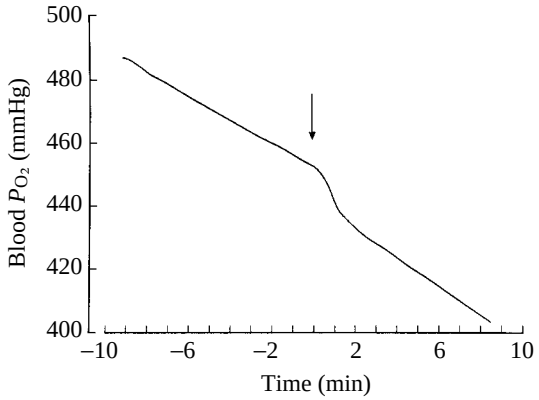

Fig. 3. Recording of the output from the O_2 electrode during incubation of blood in a Tucker cell at 15 °C. The arrow indicates injection of 2 μl of noradrenaline into the chamber (5×10^{-6} mol l^{-1}, final concentration). 1 mmHg=0.133 kPa.

of 3 and glucose as substrate). White cells accounted for almost half of the whole-blood ATP turnover, despite their sparse occurrence. After adrenergic stimulation, the share of the ATP turnover of the white cells decreased to approximately one-third. Lactate production never accounted for more than 3 % of total ATP turnover of blood or 6 % of white blood cell ATP turnover.

Energy requirement of the Na^+/K^+-ATPase before and after adrenergic stimulation

In this series of experiments, blood oxygen consumption was calculated from the decrease in P_{O_2} of anaerobic incubated blood samples. Prior to incubation, blood samples were equilibrated with a very high P_{O_2} (above 30 kPa) and low P_{CO_2} (plasma pH above 8.0) in order to minimize unloading of oxygen from haemoglobin. A typical recording of the decreasing P_{O_2} in anaerobically incubated blood is presented in Fig. 3. Adrenergic stimulation caused an immediate drop in P_{O_2}. Thereafter, P_{O_2} decreased at a faster, albeit steady, rate, indicating increased metabolism. The sudden drop in P_{O_2} corresponds to a less than 1 % change in Hb O_2-saturation and may be explained by the binding of oxygen to haemoglobin. Increased intracellular pH after stimulation will cause an increase in haemoglobin oxygen-carrying capacity due to the Root effect and thereby promote binding of oxygen. This interpretation is supported by the observation that the size of the P_{O_2} drop increased with decreasing pH.

Ouabain caused decreases in oxygen consumption of 21 % in red cells and 6 % in white cells (Table 3). Adrenergic stimulation increased oxygen consumption in both control and ouabain-treated red cells by approximately 50 % and ouabain reduced oxygen consumption of adrenergically stimulated red cells by 24 %.

Discussion

The present study shows that white and red blood cells from rainbow trout are extremely different in terms of energy turnover. In fact, comparing our data with other

Table 3. *Effect of ouabain (10^{-3} mol l^{-1}) on trout blood cell oxygen consumption (μmol O_2 l^{-1} cells min^{-1}) before and after adrenergic stimulation (5×10^{-6} mol l^{-1} noradrenaline) at 15 °C*

	Unstimulated	Stimulated
Red blood cells		
Control	19±5	29±7*
Ouabain	15±4	22±5**,***
White blood cells		
Control	350±100	400±110
Ouabain	330±90	380±100**

*Significantly higher than unstimulated control cells ($P\leqslant0.02$).
**Significantly higher than unstimulated ouabain-treated cells ($P\leqslant0.02$).
***Significantly lower than stimulated control cells ($P\leqslant0.02$).
Values are mean ± 1 S.D. (N=10).

values for fish tissue, trout white cells are the most metabolically active cells described in fish, whereas red cells are among the least active (Itazawa and Oikawa, 1983). The overwhelming difference in oxygen consumption between the two cell types implies that white cells are of great importance in whole-blood energy turnover, in spite of their sparse occurrence (Fig. 3). All previous studies on whole-blood metabolism in rainbow trout have disregarded white cells, and whole-blood oxygen consumption has therefore been attributed to red cells alone. Consequently, these studies have overestimated red cell metabolism. Only Tufts and Boutilier (1991) have reported a trout red cell oxygen consumption as low as in the present study (Table 4). Data from the sea raven (*Hemitripterus americanus*) suggest that white cells play an equally important role in whole-blood oxygen consumption in this species (Sephton *et al.* 1991). These authors found that washed red cell oxygen consumption only accounted for 45 % of the consumption of whole blood.

For red cells, the relative cost of the Na^+/K^+-ATPase was 23 % of the total oxygen consumption (Table 3). Similar values have been determined for hepatocytes and gill tissue of teleost fish (Shwarzbaum *et al.* 1992; Stagg and Shuttleworth, 1982), and these are within the range reported by Clausen *et al.* (1991) on various mammalian tissues. The relative cost of the Na^+/K^+-ATPase was considerably lower for white cells (Table 3).

All blood lactate production could be ascribed to white cells (Table 1). The lack of red cell lactate production is challenged by experiments on blood where the amount of white cells was reduced to less than 10 % of the original level. In this preparation, HCO_3^- disappeared at a rate of 4±2 μmol l^{-1} cells min^{-1} at constant P_{CO_2} (N=30, T. Wang, unpublished results). This rate is 2–3 times higher than predicted from the remaining white cells. Proton-liberating processes other than white cell lactate production must therefore take place. A red cell net lactate production of 2–3 μmol l^{-1} cells min^{-1} could make up for the difference, but degradation of sulphur-containing amino acids would also titrate HCO_3^-. Sephton *et al.* (1991) found that washed red cells from sea raven showed

Table 4. *Fish blood cell oxygen consumption (μmol O_2 l^{-1} cells min^{-1}) at 15 °C*

Species	Unstimulated	Stimulated	Reference
Oncorhynchus mykiss			
Whole blood	80.4		Eddy (1977)
	30.9	46.8	Ferguson *et al.* (1989)
	38.6		Wood *et al.* (1990)
	43.9		Walsh *et al.* (1990)
	23.7	33.2	Tufts and Boutilier (1991)
	30.2	42.8	Tufts and Boutilier (1991)
Red cells	23.0	49.0	Present study
Salmo salar			
Whole blood	40.9	81.4	Ferguson and Boutilier (1988)
Hemitripterus americanus			
Whole blood	68.2		Sephton *et al.* (1991)
Washed red cells	30.0	64.6	Sephton *et al.* (1991)

Values have been corrected to 15 °C with a Q_{10} of 2.77 [Eddy, 1977 (Fig. 1); Ferguson and Boutilier, 1988 (Table 1)].

Conversion from nmol O_2 g^{-1} Hb min^{-1} to μmol O_2 l^{-1} cells min^{-1} is based on red cell Hb content of 276 g l^{-1} (Ferguson and Boutilier, 1989; Wood *et al.* 1990).

either minimal or no lactate production. In hen blood, whole-blood lactate production can also be ascribed to white cells (Bell and Culbert, 1968).

Energy turnover after stimulation

The estimated increase in whole-blood oxygen consumption of 90 % after adrenergic stimulation (Fig. 2) is comparable to earlier studies on rainbow trout (Ferguson and Boutilier, 1989; Ferguson *et al.* 1989; Tufts and Boutilier, 1991; Table 4). The increase in oxygen consumption upon stimulation was very rapid, taking only 1–2 min for full expression (Fig. 3). After this initial increase, the oxygen consumption remained constant for at least 1 h (Figs 1, 3). However, during the first hour after stimulation, a net degradation of red cell ATP takes place (Nikinmaa, 1983; Tetens, 1987), which reduces cellular ATP content to approximately 75 % of the original value (Tetens, 1987). The rate of ATP degradation declines exponentially (Tetens, 1987) and total blood energy turnover may thus peak a few minutes after adrenergic stimulation.

Following adrenergic stimulation, red cell oxygen consumption determined from decreases in P_{O_2} in anaerobically incubated blood (Table 3) was lower than when calculated from the fall in C_{O_2tot} in blood kept in gas-tight syringes (Table 1). Part of this difference may be explained by the unloading of oxygen from haemoglobin as P_{O_2} declined during those experiments where oxygen consumption was calculated from the decrease in P_{O_2}. In order to minimize this oxygen unloading, all measurements were performed at high P_{O_2} and high pH to ensure complete Hb O_2-saturation before incubation. However, the immediate drop in P_{O_2} following adrenergic stimulation indicates that the stimulation-induced increase in intracellular pH resulted in additional

binding of oxygen to haemoglobin. Nevertheless, the two methods resulted in similar values for white cell oxygen consumption. Explanations other than differences in experimental design should accordingly be considered. Both the capacity of the red cell Na^+/H^+ exchanger and the activity of the Na^+/K^+-ATPase are low during winter (Cossins and Kilbey, 1989; Raynard and Cossins, 1991). The experiments where oxygen consumption was determined by measurements of total blood oxygen content were performed in early autumn, while experiments using P_{O_2} changes were carried out during winter when the potential increase in Na^+/K^+-ATPase activity should be at a minimum. Thus, the different estimates of red cell oxygen consumption obtained in the two sets of experiments may be explained by seasonal variations in the Na^+/K^+-ATPase activity. Additionally, high oxygen tension (as used in the Tucker-chamber-based experiments) attenuates the response of the Na^+/H^+ exchanger (Motais *et al.* 1987). The predictably smaller increase in red cell sodium concentration upon stimulation may represent a smaller stimulus to the Na^+/K^+-ATPase than was elicited at the much lower P_{O_2} present in the syringe-based experiments. This possibility is, however, not supported in the experiments that show no effects of Hb O_2-saturation on whole-blood O_2 consumption after adrenergic stimulation.

Tufts and Boutilier (1991) found that ouabain totally abolished the catecholamine-mediated increase in oxygen consumption in whole blood from trout. Increased Na^+/K^+-ATPase activity therefore seemed to account for the increased blood oxygen consumption. Our results are in contrast to this conclusion, as noradrenaline caused a significant increase in oxygen consumption of ouabain-treated cells (Table 3). Our data therefore suggest that other energy-demanding processes are stimulated by adrenergic stimulation. Unfortunately, our experimental protocol does not allow identification of these processes. Unlike the experiments of Tufts and Boutilier (1991), our experiments were carried out at high Hb O_2-saturation. This distinction is, however, unlikely to explain the contradictory results since Hb O_2-saturation did not influence O_2 consumption after adrenergic stimulation.

Respiratory quotient

The present results on RQ both before and after adrenergic stimulation (Table 2) are in agreement with data on whole blood from *Salmo salar* (Tufts *et al.* 1991) and indicate mixed substrate oxidation. Similar values of 0.69 and 0.9 have been reported for blood from penguins and ducks (Nicol *et al.* 1988; Scheid and Kawashiro, 1975). Most studies conclude that nucleated blood cells, although capable of oxidizing amino acids (Mauro and Isaacks, 1989; Tiihonen and Nikinmaa, 1991), use glucose or monocarboxylic acids as the main substrate (Walsh *et al.* 1990; Wood *et al.* 1990; Sephton *et al.* 1991; Tiihonen and Nikinmaa, 1991). These studies, therefore, suggest a RQ of approximately 1.0, which is not supported by direct measurements. Our measurements, in conjunction with previous reports, strongly suggest that substrates other than glucose and lactate are oxidized in trout red and white blood cells. The tendency towards increased RQ after stimulation, however, suggests that glucose is the major substrate during periods of increased energy demand.

In conclusion, the present study shows that the contribution of white cells to trout

whole-blood metabolism is substantial. They account for at least 80 % of net lactate production and approximately half of the energy turnover in trout whole blood. White blood cells play, however, only a minor (if any) role in the increased blood energy turnover upon adrenergic stimulation.

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