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ENERGY METABOLISM IN TROUT RED CELLS: CONSEQUENCES OF ADRENERGIC STIMULATION *IN VIVO* AND *IN VITRO*

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Summary

β-Adrenergic stimulation of salmonid red cells results in a rapid decrease (within 5 min) in the nucleotide triphosphate:haemoglobin ratio (NTP:Hb), which is thereafter maintained at a constant level, presumably through increased ATP turnover *via* matched aerobic metabolism and energy-consuming processes. Addition of the β-adrenergic agonist isoproterenol to rainbow trout red cells *in vitro* leads to a rise in intracellular pH (pHi), a corresponding decrease in extracellular pH (pHe) and an increase in red cell oxygen consumption ($\dot{M}_{O_2}$). Moreover, the extent to which red cell pHi is maintained constant in the face of an acute extracellular acidosis *in vitro* or *in vivo* is proportional to the adrenergically stimulated increase in red cell $\dot{M}_{O_2}$. In the absence of oxygen, these red cells remain capable of pH regulation, but cannot maintain NTP:Hb constant. As a result, membrane and metabolic functions become uncoupled in the stimulated deoxygenated cells.

Introduction

β-Adrenergic stimulation of the red cells of certain fish species is associated with changes in cellular energy metabolism. DeVries & Ellory (1982) observed that adrenaline stimulated a three- to fourfold increase in Na^+/K^+-ATPase activity in red cells of two antarctic fish (*Disostycus mawsoni* and *Notothenia angustata*) following capture stress. Adrenergic stimulation of the red cells of trout leads to comparable increases in both active and passive K^+ influx, indicative of an increase in Na^+/K^+-ATPase activity (Bourne & Cossins, 1982). *In vitro* preparations of *Salmo salar* whole blood have shown that adrenergic stimulation is associated with an increase in red cell aerobic metabolism, whereas anaerobic energy production is unaltered (Ferguson & Boutilier, 1988). Because the nucleotide triphosphate:haemoglobin ratio of salmonid red cells is maintained at a new steady state (following a rapid decrease), the overall conclusion is that

Key words: trout, exercise, β-adrenergic, red cell, pH, aerobic metabolism, anaerobic metabolism.

adrenaline stimulates increased NTP turnover in these cells and that aerobic metabolism predominates (Ferguson & Boutilier, 1988).

On the basis of evidence such as this, it is increasingly apparent that changes in membrane transport activity, such as those that occur in salmonid red cells following adrenergic stimulation (Bourne & Cossins, 1982; Cossins & Richardson, 1985; Nikinmaa, 1982, 1983, 1986; Baroin *et al.* 1984; Nikinmaa *et al.* 1987), are associated with changes in cellular energy metabolism. There is good evidence suggesting that this is the case in many other vertebrate cell types (Aw & Jones, 1985; Kalman, 1984; Busa & Nuccitelli, 1984; Asano *et al.* 1976). If energy balance is to be maintained, the cell's energy-producing mechanisms must be responsive to the energetic demands of membrane ion transport processes. Reciprocally, alteration of cellular energy metabolism in itself must lead to co-adaptive responses in membrane function (Hochachka, 1986*a*,*b*, 1988; Hansen, 1987) if energy balance is to be regulated.

Against this background, the present experiments were undertaken to evaluate further the coupled functions of membrane transport and energy metabolism in the red cells of the rainbow trout during adrenergic pH regulation *in vitro* and *in vivo*.

Materials and methods

Animals

Freshwater rainbow trout (0·2–0·4 kg) were obtained from a commercial supplier and maintained in the Aquatron facility at Dalhousie University. The trout were held in large (1·5 m × 1·5 m × 1 m) fibreglass tanks supplied with flowing dechlorinated tap water (9–12 °C) and fed *ad libitum* on commercially prepared pellets of trout food. The animals were maintained under laboratory conditions for at least 1 month prior to experimentation.

Surgical preparation

The trout were anaesthetized in a 3-aminobenzoic acid ethyl ester (MS-222, Sigma): fresh water mixture (1:10 000) prior to surgery. During surgery, the gills were continuously irrigated with a MS-222: fresh water mix (1:20 000; pH ≈ 7·7). The dorsal aorta was cannulated with PE 50 tubing (Clay-Adams) according to the method of Smith & Bell (1964). Immediately following the 5–10 min surgery, the fish were revived and placed in blackened Perspex boxes where they recovered for at least 24 h in flowing fresh water, temperature-controlled to 10 °C.

Analytical measurements

Haematocrit was determined in triplicate by spinning 50 μl samples of whole blood in microhaematocrit tubes for 3 min in an Autocrit centrifuge (Clay-Adams). Total oxygen content (C_{O_2}) of whole blood samples was determined on 25–50 μl specimens by the method of Tucker (1967), as modified by Hughes *et al.* (1982). Samples were prepared for the determination of metabolites by adding

50–100 μl of blood to twice its volume of ice-cold 8 % (w/v) perchloric acid (PCA). The PCA–blood slurries were vortexed and then kept on ice for 5 min prior to centrifugation (4 min in a Fisher microfuge). Immediately following centrifugation, the PCA supernatant was placed in liquid N_2. The haemoglobin (Hb) content of blood samples was measured in triplicate by adding 25 μl of whole blood to 2·5 ml of Drabkin's reagent (Sigma 525). Lactate, nucleotide triphosphate (NTP) and glucose concentrations were determined in duplicate using NAD/NADH-coupled enzymatic procedures (Sigma enzymes and reagents). All spectrophotometric determinations were carried out using a Gilford Response narrow bandwidth UV–VIS spectrophotometer.

Intracellular (i) and extracellular (e) pH were measured with a Radiometer G279/G2 glass capillary electrode (10 °C) coupled to a Radiometer PHM 84 pH meter. pHe was determined on 40 μl samples of whole blood whereas pHi estimates were made on haemolysates obtained from 500 μl of anaerobically centrifuged whole blood according to the freeze–thaw method of Zeidler & Kim (1977).

Experimental design

Oxygen consumption of red cells in vitro

Blood was withdrawn from resting cannulated fish and pooled in ice-chilled heparinized round-bottomed flasks. 1 ml samples were then equilibrated with a humidified 0·2 % CO_2/air mix (Wösthoff gas-mixing pumps) for 10 min in the presence of 50 μl of control solution (Cortland saline: Wolf, 1963) or isoproterenol (Sigma I 6504) in saline (final concentration of isoproterenol: 1×10^{-5} mol l^{-1}).

Following equilibration, each blood sample was drawn into a 1 ml Hamilton gas-tight syringe which was subsequently rotated along its horizontal axis (30 revs min^{-1}) at 19 ± 0·8 °C. The contents of the syringe were sampled at 0, 25 and 50 min for the determination of haematocrit, Hb and C_{O_2}. $N = 6$ for both control and isoproterenol treatments.

Metabolic responses of red cells to acid challenge and adrenergic stimulation in vitro

Blood taken from resting cannulated fish was pooled in heparinized round-bottomed flasks on ice. Samples were removed from the pool and placed in thermostatted (10 °C), intermittently rotating tonometers and equilibrated against humidified gas mixtures (Wösthoff gas-mixing pumps) of either 1·0 % CO_2/N_2 or 0·2 % CO_2/air. The composition of these gas mixtures was selected so as to resemble 'venous' and 'arterial' blood, respectively. C_{O_2} determinations showed that equilibration was completed in 5 min. After 30 min, 500 μl of blood was removed for analysis (see below) and 21 μl of either control or adrenaline solution was added to the blood remaining in the tonometer (750 μl). The control solution consisted of 0·14 mol l^{-1} HCl in Cortland saline, whereas the adrenaline solution consisted of 0·05 g of adrenaline (Sigma E 4250) dissolved in 15 ml of

control solution. The final concentration of adrenaline in the blood was 5×10^{-4} mol l^{-1}. Samples were also taken 60 min following control or adrenaline addition.

In one experimental series ($N = 16$) blood samples were used to determine pHe and pHi. In an additional series ($N = 16$) the blood samples were used to determine haematocrit, [Hb], [lactate] and [NTP].

Effects of exhaustive exercise and pharmacological agents on red cell metabolism in vivo

In vivo experiments were carried out to evaluate the functional role of adrenergically mediated red cell metabolic responses during exercise-induced stress. A gas-tight Hamilton syringe was used to remove 2 ml of blood from resting cannulated fish for the analysis of [Hb], [lactate], [NTP], C_{O_2}, pHe and pHi. Portions (approx. 100 μl) of the blood were removed and incubated for approximately 2 h at 10°C in a Hamilton syringe for the determination of oxygen content and lactate concentration changes. 30 min following the removal of blood from the resting animal (and reinfusion of an equivalent amount of Cortland saline), the fish was exercised to exhaustion by chasing it in a circular tank. Immediately following exercise-induced exhaustion, the animal was treated in one of three ways. It was injected with 250 μl kg^{-1} Cortland saline containing (1) isoproterenol ($N = 8$; 1×10^{-5} mol l^{-1} final concentration), (2) propranolol ($N = 8$; 1×10^{-5} mol l^{-1} final concentration) or (3) Cortland saline alone (i.e. sham injection, $N = 8$). 10 min after injection of the appropriate solution, a second blood sample was collected and processed as before. An additional study was carried out, the protocol of which was identical to the one just described except that no injection was made following exercise. Red cell $\dot{M}_{O_2}$, pH, pHe, haematocrit and [haemoglobin] were determined in this experiment as described above.

Statistical analyses

Paired and two-sample Student's *t*-tests were used in the analyses of significance. Differences were considered significant at $P < 0{\cdot}05$. Unless specifically stated otherwise, each value is presented as the mean ± 1 S.E.M.

Results

Oxygen consumption of red cells in vitro

Isoproterenol treatment of rainbow trout red cells *in vitro* led to a marked cellular swelling (Table 1). Oxygen consumption ($\dot{M}_{O_2}$) was about 1·5 times higher in the isoproterenol-treated cells (255·2 ± 17·6 nmol O_2 g^{-1} Hb min^{-1}, $N = 6$) than in the control red cells (168·7 ± 18·0 nmol O_2 g^{-1} Hb min^{-1}, $N = 6$).

Metabolic responses of red cells to acid challenge and adrenergic stimulation in vitro

Fig. 1 shows the pHe and pHi changes of whole blood incubated in anaerobi

(1 % CO_2/N_2) and aerobic (0·2 % CO_2/air) environments. Control (i.e. acidified saline) treatment caused pHe and pHi to decrease in both the aerobic and anaerobic preparations. In the presence of adrenaline, however, pHi increased relative to the control in both cases.

Aerobic cells treated with adrenaline exhibited a decrease in [NTP] whereas

Table 1. *Mean cellular haemoglobin content (MCHC) and total oxygen content (C_{O_2}) of whole blood incubated in a closed system at 19 ± 0·8°C*

	Control		Isoproterenol	
Time† (min)	MCHC (g Hb 100 ml^{-1} RBC)	C_{O_2} (ml O_2 g^{-1} Hb)	MCHC (g Hb 100 ml^{-1} RBC)	C_{O_2} (ml O_2 g^{-1} Hb)
0	26·7 ± 0·6	1·09 ± 0·04	24·0 ± 0·6*	1·10 ± 0·03
25	26·5 ± 0·7	0·99 ± 0·04	21·2 ± 0·4*	0·98 ± 0·03
50	27·6 ± 0·8	0·90 ± 0·04	20·4 ± 0·6*	0·82 ± 0·02*

Cells were preincubated for 10 min in the presence or absence (Control) of isoproterenol. Final concentration of isoproterenol was 1×10^{-5} mol l^{-1}.

Values are presented as means ± 1 S.E.M. $N = 6$ for both groups.

* Significantly lower than control value (two-sample Student's *t*-test).

† Isoproterenol or control treatment was commenced 15 min before incubation (see Materials and methods for further details).

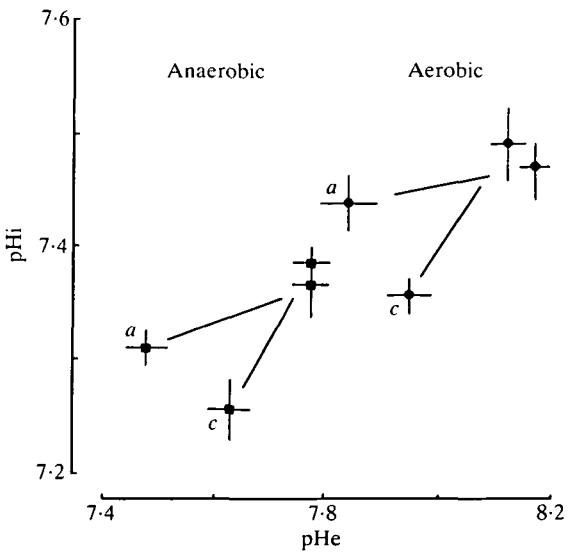

Fig. 1. pHe and pHi responses of acid-challenged cells. Whole blood was preincubated for 30 min in an anaerobic (■) (1·0 % CO_2/N_2) or an aerobic (●) (0·2 % CO_2/air) environment prior to a 60 min incubation in the presence of either a control (*c*) or adrenaline-containing (*a*) solution (final concentration of adrenaline was 5×10^{-4} mol l^{-1}). $N = 7$ for both aerobic groups and $N = 9$ for both anaerobic groups. Each point is the mean ± 1 S.E.M. (See Materials and methods and Results for further details.)

Table 2. *Mean cellular haemoglobin content (MCHC), cellular NTP and lactate production rates in aerobic and anaerobic red cells from control preparations and those treated with adrenaline*

	Time (min)	Anaerobic		Aerobic	
		Control	Adrenaline	Control	Adrenaline
MCHC	30	$23{\cdot}9 \pm 0{\cdot}8^{c}$	$24{\cdot}8 \pm 0{\cdot}7^{c}$	$29{\cdot}5 \pm 0{\cdot}6$	$29{\cdot}2 \pm 0{\cdot}5$
(g Hb $100\,ml^{-1}$ RBC)	90	$22{\cdot}9 \pm 0{\cdot}3^{c}$	$18{\cdot}6 \pm 0{\cdot}7^{a,b,c}$	$28{\cdot}6 \pm 0{\cdot}3$	$27{\cdot}0 \pm 0{\cdot}4^{a,b}$
[NTP]	30	$5{\cdot}50 \pm 0{\cdot}27^{c}$	$5{\cdot}54 \pm 0{\cdot}22^{c}$	$6{\cdot}19 \pm 0{\cdot}26$	$6{\cdot}10 \pm 0{\cdot}22$
(mmol l^{-1} RBC)	90	$3{\cdot}87 \pm 0{\cdot}33^{a,c}$	$1{\cdot}94 \pm 0{\cdot}14^{a,b,c}$	$5{\cdot}95 \pm 0{\cdot}32$	$4{\cdot}96 \pm 0{\cdot}24^{a,b}$
NTP: Hb	30	$22{\cdot}96 \pm 0{\cdot}61$	$22{\cdot}32 \pm 0{\cdot}51$	$21{\cdot}01 \pm 0{\cdot}93$	$20{\cdot}98 \pm 0{\cdot}97$
(μmol g^{-1} Hb)	90	$16{\cdot}85 \pm 1{\cdot}25^{a,c}$	$10{\cdot}34 \pm 0{\cdot}47^{a,b,c}$	$20{\cdot}84 \pm 1{\cdot}22$	$18{\cdot}38 \pm 0{\cdot}96^{a,b}$
$\dot{M}_{lactate}$ (nmol g^{-1} Hb min^{-1})		$87{\cdot}8 \pm 8{\cdot}7^{c}$	$127{\cdot}9 \pm 21{\cdot}9^{c}$	$22{\cdot}0 \pm 21{\cdot}2$	$14{\cdot}0 \pm 10{\cdot}8$

A 30 min preincubation period in the respective gas environments preceded collection of the experimental sample.

Anaerobic and aerobic refer to incubation gases of 1·0 % CO_2/N_2 and 0·2 % CO_2/air, respectively.

Significant differences are designated with an a (if different from the corresponding pretreatment value), b (if different from the corresponding sham value) and c (if different from the corresponding aerobic value).

All values are expressed as the mean ± 1 S.E.M. $N = 16$. See Materials and methods section for further details.

$\dot{M}_{lactate}$, rate of lactate production; RBC, red blood cell.

control cells showed no change (Table 2). Anaerobic incubation itself led to a small, but significant, decrease in cell [NTP] with time. Adrenaline treatment of anaerobic cells led to very marked changes in [NTP], causing a 65 % decrease. Cell swelling and changes in NTP metabolism contributed to these observations as described below.

Adrenergically induced cellular swelling is evident from the decreases in MCHC shown in Table 2. Whether aerobic or anaerobic, control cells (i.e. those treated with acidified saline) displayed no significant change in MCHC over the 60 min incubation period.

A marked decrease in red cell NTP: Hb occurred during the 60 min incubation period which followed control treatment in anaerobic conditions (Table 2). This was not the case for aerobic control cells. The lowest NTP: Hb ratio was observed in anaerobic red cells treated with adrenaline. NTP: Hb of adrenaline-treated cells under aerobic conditions also fell, but to a much lesser extent. There was no significant difference between any of the pretreatment NTP: Hb values shown in Table 2.

Lactate production rates of cells incubated in anaerobic and aerobic conditions are shown in Table 2. Cells incubated in the absence of oxygen showed a significantly greater lactate production rate than their aerobic counterparts.

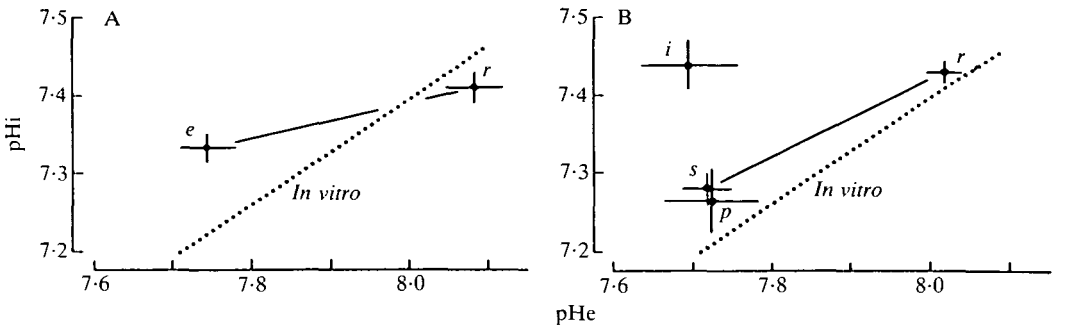

Fig. 2. (A) Data from an *in vivo* experiment which was undertaken to determine the response of red cell pHi and $\dot{M}_{O_2}$ to exercise-induced stress. The relationship between pHe and pHi previously determined *in vitro* for aerobic control-treated whole blood (Fig. 1) is shown by a dotted line constructed from the means. Unlike the protocol for B, these fish were exercised to exhaustion (*e*) without any sham or pharmacological treatment (see text for details). Linear regression analysis established that there was a significant difference between the slopes of the two lines. (B) pHi and pHe responses following exhaustive exercise for isoproterenol-, sham- and propranolol-treated fish (designated *i*, *s* and *p*, respectively). Consolidated rest values are designated *r*. Linear regression analysis established that there was no significant difference between slopes of the *in vitro* and the 'sham' or the *in vitro* and the 'propranolol' pH relationships. (See Materials and methods and Results for details.)

Neither preparation showed any significant change in lactate production as a result of incubation with adrenaline.

In the light of the different equilibration gases used in this study, the question arises as to whether the observed differences in the aerobic and anaerobic responses are a function of C_{O_2} or C_{CO_2} influences (Nikinmaa *et al.* 1987; Borgese *et al.* 1987). In a companion study (Ferguson & Boutilier, 1989) we have evaluated the metabolic and ionic responses of trout red cells in oxygenated and deoxygenated environments at the same CO_2 tensions and conclude that the differences in the response of aerobic and anaerobic cells presented here are mostly attributable to differences in C_{O_2}.

Effects of exhaustive exercise and pharmacological agents on red cell metabolism in vivo

The changes in pHe and pHi following sham, isoproterenol or propranolol injection into exercised fish are shown in Fig. 2B. An additional experiment (same protocol but no injection) was conducted to determine the whole-blood pH response of the trout to the stress of exhaustive exercise alone (Fig. 2A). It is clear that these fish respond to exercise-induced stress with regulation of red cell pH. MCHC fell and red cell $\dot{M}_{O_2}$ increased by about 50% during red cell pH regulation. The plasma and red cell pH of both sham- and propranolol-treated fish decreased following exercise (Fig. 2B). Unlike the fish which received no injection following exercise (Fig. 2A), the sham- and propranolol-treated fish displayed a

Table 3. *Properties of blood removed before (Rest) and after exhaustive exercise with drug treatment (see Materials and methods for details)*

		Exercise		
	Rest	Sham	Isoproterenol	Propranolol
Whole-blood lactate ($mmol\,l^{-1}$)	0·79 ± 0·10 (24)	4·85 ± 0·62 (8)*	5·16 ± 0·73 (8)*	5·32 ± 0·35 (8)*
C_{O_2} ($ml\,O_2\,g^{-1}\,Hb$)	1·32 ± 0·04 (23)	1·26 ± 0·06 (8)	1·37 ± 0·08 (7)	1·16 ± 0·09 (8)*
MCHC ($g\,Hb\,100\,ml^{-1}\,RBC$)	28·3 ± 0·5 (24)	27·0 ± 0·9 (8)*	24·4 ± 0·7 (8)*	26·8 ± 1·3 (8)
RBC NTP ($mmol\,l^{-1}$)	7·03 ± 0·28 (24)	6·23 ± 0·21 (8)*	4·72 ± 0·39 (8)*	6·46 ± 0·56 (8)
RBC NTP ($\mu mol\,g^{-1}\,Hb$)	24·8 ± 1·1 (24)	23·3 ± 1·2 (8)	19·5 ± 1·8 (8)*	24·2 ± 1·9 (8)
RBC $\dot{M}_{lactate}$ ($nmol\,g^{-1}\,Hb\,min^{-1}$)	2·11 ± 2·35 (18)	1·90 ± 0·46 (6)	−1·0 ± 0·7 (7)	−0·2 ± 0·6 (5)
RBC $\dot{M}_{O_2}$ ($nmol\,O_2\,g^{-1}\,Hb\,min^{-1}$)	102·5 ± 7·8 (20)	84·7 ± 12·8 (8)	131·2 ± 10·7 (6)*	91·6 ± 12·0 (6)

Rest values were pooled as there was no significant difference between the means of the rest values of the three groups of fish used in this study.

* Significantly different from the rest value (paired *t*-test, $P < 0·05$).

Each value is presented as the mean ± 1 S.E.M.

See Materials and methods section for further details.

N values are given in parentheses.

C_{O_2}, total O_2 concentration; MCHC, mean cellular haemoglobin content; RBC, red blood cell; $\dot{M}_{lactate}$, rate of lactate production; $\dot{M}_{O_2}$, rate of oxygen utilization.

pHe/pHi relationship which was not significantly different from the one obtained from unstimulated aerobic cells *in vitro*. In fish injected with isoproterenol, however, pHi remained constant, despite a considerable extracellular acidosis (Fig. 2B).

Exercise-stimulated increases in whole-blood [lactate] were the same regardless of treatment (Table 3). In fish treated with isoproterenol, blood O_2 content was unchanged by exercise. However, isoproterenol treatment did lead to red cell swelling and a metabolic decline in [NTP] (i.e. NTP:Hb declined). Red cells of sham-treated fish exhibited a reduction of NTP concentration but this was due solely to dilution caused by cellular swelling (i.e. MCHC decreased) and not due to reduction in NTP:Hb. Of the three treatments given to exercised fish, only the injection of isoproterenol induced a significant change in red cell aerobic energy production. Net red cell lactate production was not significantly changed by any of the treatments.

Discussion

Aerobic and anaerobic metabolism of nucleated erythrocytes

The mature nucleated red blood cells of salmonids and other lower vertebrates

possess the metabolic capabilities for both anaerobic and aerobic NTP production (Eddy, 1977; Greaney & Powers, 1978; Scheid & Kawashiro, 1975; Ferguson & Boutilier, 1988). Under aerobic conditions, NTP equivalents are produced almost exclusively *via* aerobic respiration (Tables 2 and 3; Ferguson & Boutilier, 1988). Using data from Table 3, for example, the relative contributions of aerobic and anaerobic energy production can be estimated at 430 and 2 nmol NTP g^{-1} Hb min^{-1}, respectively, in red cells collected from resting animals (a P/O ratio of 2 for NADH-linked oxidation reactions was assumed: see Hochachka & Somero, 1984). In other words, energetic equivalents derived *via* aerobic pathways account for over 99 % of the total energy production of the red cell. This underlines the importance of oxygenation and oxidative metabolism to red cell function both *in vivo* and *in vitro*.

Red cells incubated under anaerobic conditions (Table 2) are obviously limited to anaerobic production of NTP *via* glycolytic lactate formation. Yet we do not observe anything close to the anticipated increase in lactate production which would be necessary to meet the NTP production deficit created by the absence of aerobic energy production. To do this $\dot{M}_{lactate}$ would have to increase to well over 400 nmol g^{-1} Hb min^{-1}. Whole-blood glucose values remained high throughout the incubation period (approx. 6 mmol l^{-1}, data not presented).

Furthermore, NTP depletion in anaerobic red cells does not proceed at the rate which would be expected if cellular energy demands remained at the aerobic levels. From $\dot{M}_{lactate}$ and $\dot{M}_{O_2}$ data collected from resting cannulated fish (Table 3) we estimated that the steady-state NTP demand in aerobic cells is of the order of 430 nmol g^{-1} Hb min^{-1}. If energy demand were to remain at this level in anaerobic cells, cellular [NTP] would have fallen by essentially 50 % by the end of the 30 min incubation period that preceded data collection in experiment 2 (see Materials and methods). Yet at the end of the 90 min incubation period the cells maintained under anaerobic conditions had an NTP:Hb value which was 80 % of that observed for their aerobic counterparts.

These data strongly suggest that salmonid red cells may be well adapted for hypoxia and/or anoxia tolerance. In general, hypoxia-tolerant cells are characterized by an ability to employ biochemical and physiological strategies which permit enhanced survival during oxygen limitation. The most successful of these strategies are (i) metabolic arrest and (ii) the stabilization of membrane function (Hochachka, 1986*a*,*b*, 1988), both of which serve to minimize imbalances between NTP synthesis rates and NTP consumption rates of membrane-bound NTPases. When these two processes are matched (i.e. coupled) in the face of oxygen limitation, hypoxia tolerance is achievable. The relatively weak Pasteur effect and low NTP consumption rates noted in sham-treated anaerobic red cells (Table 2) are indicative of some degree of hypoxia tolerance. Indirect evidence for this tolerance is available from the work of Tetens & Lykkeboe (1981), who found that rainbow trout red cell ATP levels were maintained equally well in 7 % and 2 % O_2 environments. In addition, the lack of any accelerated lactate production rate upon adrenergic stimulation (in comparison to the increase in $\dot{M}_{O_2}$ typically

observed in aerobic cells, e.g. Table 1) suggests that metabolic arrest strategies may be functioning.

Adrenergic stimulation of red cells in aerobic and anaerobic conditions

As observed for the red cells of the Atlantic salmon (Ferguson & Boutilier, 1988), adrenergic stimulation of aerobically incubated red cells of rainbow trout results in an increased $\dot{M}_{O_2}$ (Table 1). We have observed that adrenaline stimulates a relatively rapid (within 5 min) decline in NTP: Hb of Atlantic salmon red cells, after which increased $\dot{M}_{O_2}$ is accompanied by the maintenance of NTP: Hb at a new, but slightly lower, steady-state level for as long as 4 h (Ferguson & Boutilier, 1988). These data indicate that adrenergic stimulation is associated with increased metabolic turnover of NTP in aerobic red cells.

Anaerobic red cells also exhibit adrenergic pH regulation (Fig. 1); however, in this case it is not accompanied by enhanced production of NTP, at least of the magnitude shown by aerobic cells (Tables 1, 2 and 3; Ferguson & Boutilier, 1989). Indeed, no apparent increase in NTP production occurs, since $\dot{M}_{lactate}$ does not increase in anaerobically incubated cells treated with adrenaline (Table 2). Nevertheless, we can presume that some NTP-consuming processes become accelerated. For example, there is evidence that the adrenergically stimulated acceleration of Na^+/H^+ exchange and concomitant Na^+ accumulation (Nikinmaa, 1986) evokes enhanced Na^+/K^+-ATPase activity (Bourne & Cossins, 1982; Ferguson & Boutilier, 1989). This leads us to believe that, in deoxygenated cells, membrane function and cellular metabolism become uncoupled following adrenergic stimulation. That is to say, metabolic energy production is not matched by metabolic demand on a moment-to-moment basis.

Under anaerobic conditions, suppression of an increased $\dot{M}_{lactate}$ in response to adrenergic stimulation (Table 2) would appear to be an appropriate means of limiting intracellular H^+ production at a time when the cell must defend itself against extracellular protons. The consequence of enhanced cell swelling and NTP: Hb decline on cellular NTP concentration is shown in Table 2. Adrenaline-treated anaerobic cells exhibit a marked fall in [NTP] which may, in the case of energy metabolism, seem counter-productive to normal cellular function. Nevertheless, this is not a totally inappropriate environment for the Hb molecule when, upon transit through the gill, there is a need for maintaining Hb O_2-carrying capacity so as to repay organismic oxygen debt. Thus, a depleted NTP pool (Table 2) and elevated pHi (Fig. 1) should only serve to enhance O_2 loading at the gill, thereby preserving the red cell's role in O_2 uptake.

Red cell metabolism and pH regulation in vivo

Extracellular acidification is positively correlated with adrenaline release in *Salmo gairdneri* (Boutilier *et al.* 1986). In the present study, exhaustive exercise leads to an acute extracellular acidosis (Fig. 2A,B) which, on the basis of the relationship given in Boutilier *et al.* (1986), can be calculated to promote a 10-fold increase in plasma adrenaline levels. Indeed, in a parallel study conducted by Tang

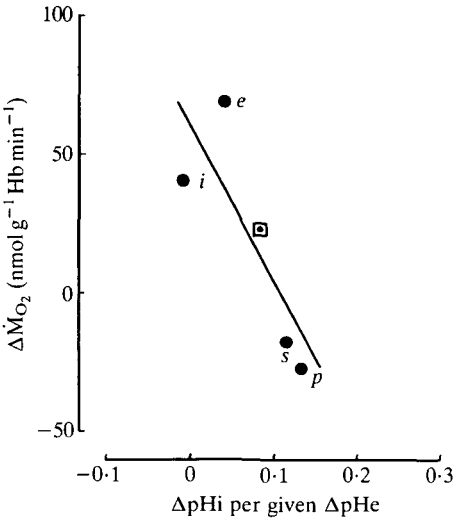

Fig. 3. $\dot{M}_{O_2}$ *vs* pHi relationship for data obtained in Fig. 2. Means were taken for *in vivo* samples for which both $\Delta\dot{M}_{O_2}$ and ΔpHi were available. pHi values per given pHe values were determined in the following manner. Regression lines of pHe/pHi relationships (Fig. 2) were used to determine, by interpolation, the change in pHi which occurs as pHe decreases from 8·00 to 7·75. Points were calculated from data collected from isoproterenol (*i*)-, sham (*s*)- and propranolol (*p*)-treated fish as well as exercised (*e*) fish receiving no treatment. The point designated ⊡ was calculated from adrenaline-treated anaerobically incubated cells (i.e. the NTP consumption above that which occurred in sham-treated anaerobic cells was converted to O_2 equivalents; see text for details of calculation).

& Boutilier (1988), with fish from the same stocks as those used in this study, exhaustive exercise always led to catecholamine release in the pH-dependent fashion noted above. For this reason, we think it unlikely that the 'lack' of the adrenergic pH response in the pharmacological experiment (Fig. 2B) was due to non-release of endogenous β-adrenergic agonists.

Our study (Fig. 2A) on the effects of burst-exercise-induced stress and the concomitant extracellular acidosis on the pH response of red cells *in vivo* confirms the findings of many other studies (Boutilier *et al.* 1986; Primmett *et al.* 1986; Milligan & Wood, 1987). It is obvious from Fig. 2A and the other published data that salmonids are capable of mitigating the effects of plasma acidosis on red cell function *via* adrenergic regulation of red cell pH. Given this, it is noteworthy that the red cells of the sham-treated fish (Fig. 2B) displayed neither pHi compensation nor the elevated $\dot{M}_{O_2}$ observed in the earlier experiment (see below and Fig. 3). The reason(s) for this is unclear. The earlier experiment shown in Fig. 2A was carried out 5 months before the pharmacological study (Fig. 2B, Table 3) and was conducted with animals obtained from a different commercial source from that used in the other experiments presented in this paper.

Many factors are known to influence the adrenergically mediated responses of

vertebrate cells. For example, the adrenergic responses of trout gill are sensitive to neurohypophyseal hormones such as arginine vasotocin, which suppresses basal and agonist-stimulated adenylate cyclase activity by as much as 40 and 100%, respectively (Guibbolini & Lahlou, 1987). In mammals, hormones such as these, and others including insulin, are also known to antagonize cyclic AMP formation in stimulated cells (Houslay *et al.* 1984). Of course, the hormonal milieu of the extracellular space is itself influenced by many factors such as the time of day, diet, activity, age, reproductive cycle (Chester-Jones *et al.* 1987; Kuhn *et al.* 1986) – any or all of which may express themselves differently in fish not sharing the same genetic or environmental history. Seasonal differences in adrenergic responses are documented for the ventilatory systems of both eel (Peyraud-Waitzenegger, 1979; Peyraud-Waitzenegger *et al.* 1980) and carp (Waitzenegger & Serfaty, 1967) as well as for the erythrocyte pH regulatory system of trout (Nikinmaa & Jensen, 1986). The latter finding has been questioned by Milligan *et al.* (1988), who could find no influence of season and temperature on the pH response of adrenergically stimulated trout red cells. By far the most intense investigation of the factors which influence receptor–agonist sensitivity have used mammalian cell models: even so, the mechanisms in these cells and those of the lower vertebrates are far from being understood (reviewed by Lefkowitz & Caron, 1986). Nevertheless, it is apparent that (de)sensitization is not an 'on–off' modulation of the cell's response to agonist stimulation. The observation of an adrenergic response in red cells of fish treated with heightened levels of agonist (i.e. isoproterenol injection) suggests to us that the desensitization observed in this study involves a rightward shift in the red cell dose–response curve.

In Atlantic salmon, a positive correlation is found *in vitro* between the adrenergically mediated changes in $\dot{M}_{O_2}$ and the extent to which red cell pH is regulated. Regulation of red cell pH following exhaustive exercise (Fig. 2A) and exhaustive exercise complemented with isoproterenol injection (Fig. 2B) is accompanied by an increase in red cell $\dot{M}_{O_2}$ (Fig. 3). Also, red cell oxyhaemoglobin content is maintained constant, albeit at a lower NTP:Hb ratio (Table 3). The red cells of sham- and propranolol-treated fish exhibited no increase in $\dot{M}_{O_2}$, nor did they exhibit the pHi regulation observed in those cells in which an increased $\dot{M}_{O_2}$ was stimulated. Adjustments in red cell energy metabolism are, apparently, an important complement to pHi regulation.

The apparent metabolic cost of adrenergic pHi regulation is presented in Fig. 3. Clearly, a positive correlation exists between the change in red cell oxygen consumption and the extent to which pHi is regulated. Estimates of the actual amount of oxygen consumed when pHi is regulated (e.g. Fig. 3) have shown that it is only a small fraction (<4%) of that gained by the stabilization of pHi and, therefore, of haemoglobin oxygenation (Ferguson & Boutilier, 1988). In other words, the so-called 'adrenergic pH response' of salmonid erythrocytes is a highly efficient process in terms of oxygen gained for delivery to the tissues.

Theoretical calculations can be made for the anaerobic adrenaline-treated cells to see if the relationship shown in Fig. 3 also holds true for these cells. Upon

adrenergic stimulation, the NTP consumption rate for these cells was 98·3 nmol g^{-1} Hb min^{-1} higher than that observed for sham-treated anaerobic cells ($\dot{M}_{lactate}$ was not significantly different between the two; Table 2). Assuming a carbohydrate such as glucose as the preferred substrate, a P/O ratio of 2 for NADH-linked oxidations, and therefore 4·2 mol of ATP equivalents yielded per mol of oxygen consumed (Hochachka & Somero, 1984), we can determine the change in oxygen consumption that would have been required to maintain NTP levels in adrenaline-treated cells at the level observed for sham-treated anaerobic cells. This value turns out to be 23 nmol O_2 g^{-1} Hb min^{-1}, which fits well with the *in vivo* aerobic data in Fig. 3.

Whether the changes in $\dot{M}_{O_2}$ represent exclusively the cost of pHi regulation must await further experimentation. Nevertheless, it appears that energy-consuming (membrane) functions are indeed coupled with cellular energy-yielding processes, such that red cell function is conserved under conditions which threaten organismic oxygen transfer.

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