The conversion of plasma HCO_3^- to CO_2 by rainbow trout red blood cells *in vitro:* adrenergic inhibition and the influence of oxygenation status

Chris M. Wood and Heather Simmons[1]
Department of Biology, McMaster University, Hamilton, Ontario L8S 4K1, Canada

Abstract

This study employed a recently developed radioisotopic assay (Wood and Perry 1991) to examine the inhibition, induced by catecholamines, of the conversion of plasma HCO_3^- to CO_2 in acidotic trout blood, and the influence of oxygenation status on the response. Blood was incubated *in vitro* at P_{CO_2} = 2 torr, and 10^{-6} M noradrenaline was employed as the adrenergic stimulus. In particular we investigated whether the inhibition of plasma HCO_3^- conversion could be explained by a limited supply of H^+s for the intracellular HCO_3^- dehydration reaction because of competition by the adrenergically activated Na^+/H^+ exchanger. Hypoxia (P_{O_2} = 15 torr) was employed as a tool to intensify this competition. Hypoxia raised RBC pHi, pHe, and plasma total CO_2 concentration (C_{CO_2}) by the Haldane effect, and increased the magnitude of Na^+/H^+ activation, expressed as the change in the transmembrane pH gradient (pHe−pHi). However hypoxia did not alter the inhibition of the conversion of plasma HCO_3^- to CO_2 caused by noradrenaline. Hypoxia itself stimulated the RBC-mediated conversion of plasma HCO_3^- to CO_2 by about 20% in the presence or absence of noradrenaline. The conversion rate was strongly correlated with pHe, pHe−pHi, and plasma C_{CO_2} in these experiments, but not with pHi. We conclude that adrenergically mediated inhibition in the conversion of plasma HCO_3^- to CO_2 by trout RBCs is not due to competitive limitation on intracellular H^+s, but rather to changes in the electrochemical gradient for HCO_3^- entry and/or to CO_2 recycling from plasma to RBC. The deoxygenated condition helps to promote CO_2 excretion at the level of the RBC.

Introduction

Wood and Perry (1985) proposed that catecholamines inhibit the net rate of CO_2 excretion by salmonid red blood cells (RBCs) and presented preliminary evidence in favour of this idea (unpublished data of S.F. Perry and T.A. Heming). They argued that this phenomenon was responsible for the increase in Pa_{CO_2} commonly observed after exhaustive exercise or catecholamine infusions in salmonids ("CO_2 retention theory"), and suggested that the inhibitory action was on the entry of plasma HCO_3^- into the RBC, which is commonly believed to be the rate-limiting step in CO_2 excretion (Fig. 1; Wood and Perry 1985; Perry 1986; Perry and Wood 1989). In the ensuing years, both the original evidence and the CO_2 retention theory proved controversial (Steffensen *et al.* 1987; Tufts *et al.* 1988; Playle *et al.* 1990; Thomas and Motais 1990; Perry and Thomas 1991). However, in 1991,
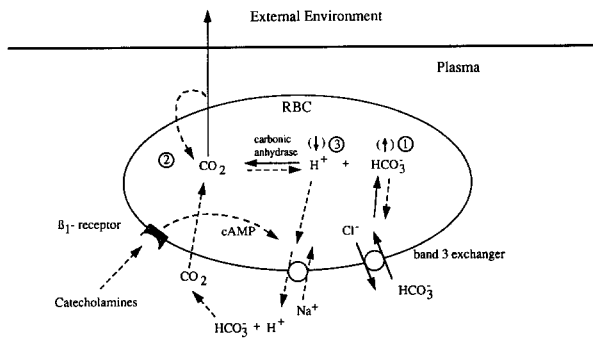
Fig. 1. A model of CO_2 excretion by trout blood and three possible mechanisms by which adrenergic activation of Na^+/H^+ exchange may cause inhibition of the *net* conversion rate of plasma HCO_3^- to excreted CO_2. Solid arrows indicate pathways which normally occur in the absence of catecholamines as blood is exposed to an external environment of lower P_{CO_2} (*i.e.* water at the gills, or the external trap in the present assay system). Dashed arrows indicate pathways which may occur upon activation of Na^+/H^+ exchange by plasma catecholamines and thereby explain reduced net conversion of plasma HCO_3^- to excreted CO_2. Mechanism (1) is a decrease or reversal of the electrochemical gradient for HCO_3^- entry; mechanism (2) is CO_2 recycling due to reduced RBC P_{CO_2}; mechanism (3) is a limitation of HCO_3^- dehydration by a shortage of intracellular H^+s. See text for further details.

Wood and Perry developed a new *in vitro* assay to directly measure the *net* rate of conversion of plasma HCO_3^- to CO_2 *via* salmonid RBCs under conditions that closely duplicated those occurring *in vivo*. The assay employs ^{14}C-labelled plasma HCO_3^- and direct collection of evolved [14]CO_2 to measure the *net* dehydration rate. Using this assay, they confirmed the original evidence, demonstrated that the inhibitory effect was β_1-adrenergic in nature, and showed that it occurred only when the blood was acidified to duplicate post-exercise conditions (Wood and Perry 1991; Perry *et al.* 1991).

At the same time, their experiments eliminated any direct inhibition of the Cl^-/HCO_3^- exchanger itself (the band 3 membrane protein; Romanow and Passow 1984, Hubner *et al.* 1992) as the cause of the phenomenon. Instead, the results indicated that the inhibition was linked to the β_1-activation of the well-known Na^+/H^+ antiporter in the red cell membrane (see Nikinmaa and Tufts 1989; Motais *et al.* 1992; Nikinmaa 1992 for reviews). For example, the extent of inhibition increased in proportion to

the change in RBC transmembrane pH gradient (pHe−pHi), and amiloride blockade of Na^+/H^+ exchange eliminated the inhibition.

At present, the explanation of the phenomenon remains elusive. Perry *et al.* (1991) proposed two possibilities which are outlined in Fig. 1. The first (1) was that activation of Na^+/H^+ exchange would cause an immediate decrease or reversal in the electrochemical gradient for the inward movement of HCO_3^- from plasma to RBC. This would result from a rise in intracellular $[HCO_3^-]$ which would accompany the increase in RBC pHi owing to physicochemical buffering and the presence of intra-erythrocytic carbonic anhydrase. The second (2), somewhat related explanation, was based on the observation of Motais *et al.* (1989) that significant "CO_2 recycling" could occur from plasma to RBC after activation of Na^+/H^+ exchange. By this explanation (see Thomas and Perry 1992), intracellular P_{CO_2} would fall due to carbonic anhydrase-mediated formation of intracellular HCO_3^-, some of which would leave by Cl^-/HCO_3^- exchange. The normal P_{CO_2} gradient from RBC to plasma would be reversed for some period, during which CO_2 would tend to enter rather than leave the RBC. A portion of the CO_2 formed at the uncatalysed rate in the plasma by the reaction of HCO_3^- with extruded H^+ would also diffuse back into the RBC along the local P_{CO_2} gradient. In both scenarios, a reduced net dehydration rate of plasma HCO_3^- would occur.

However, an entirely separate third possibility (3) is raised by the proposal of Randall and Brauner (1991) that Na^+/H^+ exchange and the intracellular HCO_3^- dehydration reaction may compete for the same pool of H^+s. In this case, activation of Na^+/H^+ exchange would inhibit the HCO_3^- dehydration reaction itself by "starving" it of H^+s. Based on work with mammalian RBCs, the explanation would appear unlikely because the rate of HCO_3^- dehydration catalyzed by carbonic anhydrase appears to be limited by the availability of buffers that can donate H^+ ions to the active site of the enzyme, rather than by the availability of free H^+ ions (Jonsson *et al.* 1976; Silverman and Vincent 1983). However, it is possible that the situation in teleost RBCs may be very different; teleost

hemoglobins have much lower buffer capacities than mammalian hemoglobins (Jensen 1989), and of course mammalian red cells lack the Na^+/H^+ antiporter. Therefore, the primary goal of the present study was to directly evaluate this explanation (3) by changing the oxygenation state of the hemoglobin. Hypoxia is well known to increase the intensity of the RBC Na^+/H^+ activation for any given plasma catecholamine level (Motais *et al.* 1987; Salama and Nikinmaa 1988; Nikinmaa and Jensen 1992; Reid and Perry 1991; Reid *et al.* 1993). At the same time, hypoxia raises RBC pHi by the Haldane effect because deoxygenated hemoglobin is a more effective binder of H^+s than oxygenated hemoglobin (Jensen 1989). We reasoned that if competition for intracellular H^+s were the dominant factor, then this competition would be more severe and catecholamine-induced inhibition of net HCO_3^- dehydration would be much greater in hypoxic blood as measured by the assay of Wood and Perry (1991).

Materials and methods

Experimental animals

Adult rainbow trout (*Oncorhynchus mykiss*; 150–400 g) of both sexes were obtained from Spring Valley Trout Farm, Petersburg, Ontario, acclimated to the experimental temperature of $15 \pm 1°C$ in dechlorinated Hamilton tapwater (moderately hard water; composition as in Milligan and Wood 1986), and starved for 7 days prior to experiments. The fish were fitted with indwelling dorsal aortic catheters (Soivio *et al.* 1972) and allowed to recover for 24–48h in darkened individual fish boxes.

Sampling and handling of blood

In order to avoid endogenously elevated catecholamine and lactate levels, blood was obtained from the arterial catheters without disturbance to the fish, exactly as described by Wood and Perry (1991). For each test, blood was pooled from 5–8

fish, heparinized at 150 i.u.ml^{-1} (ammonium heparin; Sigma), and stored on ice for 2h prior to use. At this point, the pooled blood was routinely assayed for whole blood pH (pHe = 7.9–8.1) true plasma total CO_2 content (C_{CO_2} = 6.6–7.4 mmol.l^{-1}), and hematocrit. The latter was adjusted to 18–20% by gentle centrifugation (500 × g for 1 min), addition or removal of homologous plasma as necessary, and resuspension.

Based on the measured pHe, C_{CO_2}, and the known relationship between true plasma non-bicarbonate buffer capacity (β) and hematocrit (Wood *et al.* 1982), sufficient 140 mmol.l^{-1} HCl was added very gradually to lower pHe to approximately 7.3. This value was chosen because it is typical of post-exercise acidosis (Milligan and Wood 1986) and because it yields maximum activation of the Na^+/H^+ antiporter under adrenergic stimulation (Borgese *et al.* 1987; Salama and Nikinmaa 1989). Plasma for "plasma only" assays was obtained by centrifugation (500 × g for 5 min) at this point. The blood was then divided into 1 ml aliquots in 20 ml glass scintillation vials which were placed in a shaking water bath at $15 \pm 1°C$. The scintillation vials were sealed with rubber stoppers fitted with entry and exit ports, the former served with a continual flow of a humidified gas mixture from either a normoxic or hypoxic gas source. Samples were gassed and shaken for at least 1h prior to experiment to ensure complete equilibration.

Twelve complete runs were performed in total. Each run consisted of 4 treatments on the same blood pool: normoxic + saline; hypoxic + saline; normoxic + noradrenaline ($10^{-6}M$); hypoxic + noradrenaline ($10^{-6}M$), facilitating the use of paired statistical tests. A few additional data points from incomplete runs were included in the correlation analyses. Normoxic (P_{O_2} = 155 torr, P_{CO_2} = 2 torr, balance N_2) and hypoxic gases (P_{O_2} = 15 torr, P_{CO_2} = 2 torr, balance N_2) were provided by gas mixing pumps (Wosthoff 301/a–f) and analyzed precision gas mixtures. Hypoxaemia (typical of venous blood or arterial blood during environmental hypoxia) rather than total anoxia was employed as we wished to avoid the possibility of an additional H^+ source from RBC glycolysis. Blood lactate measured in two runs was stable at less than

1 mmol.l^{-1}. Noradrenaline rather than adrenaline was used because it is the more potent natural agonist on this β_1-adrenergic response. The assay was started 5 min after the addition of noradrenaline (or saline vehicle) because the greatest inhibition of RBC CO_2 excretion rate was seen at this time (Wood and Perry 1991).

The assay

The assay measuring the conversion of plasma HCO_3^- to CO_2 *via* RBCs was performed as described by Wood and Perry (1991; see their Fig. 1) with minor modification. In brief, 50 μl of either 140 mmol.l^{-1} NaCl or 2×10^{-5} M L-noradrenaline bitartrate (Sigma) in 140 mmol.l^{-1} NaCl was added to an individual blood sample exactly 5 min prior to the start of the assay. Shaking and appropriate normoxic or hypoxic gassing was maintained until the start. At this point, 2 μCi (50 μl of 40 μCi.ml^{-1}) sodium [^{14}C]bicarbonate (ICN Biomedicals Inc.) in 5 mmol.l^{-1} HCO_3^- Cortland saline (Wolf 1963) was added to the blood sample. The vial was immediately resealed with a new rubber stopper containing a $^{14}CO_2$ trap so as to form a closed system (no gassing), and shaking was continued for exactly 3 min. The CO_2 trap was a fluted filter paper (Whatman GF/A 2.4 cm glass microfibre filter) impregnated with 150 μl of 1 mol.l^{-1} hyamine hydroxide in methanol.

At the termination of the assay, the filter was immediately removed and assayed for ^{14}C radioactivity, and the blood was drawn into a gas-tight Hamilton syringe for immediate measurement of pHe. The remaining blood was then centrifuged (12,000 × g for 2 min). The red cell pellet was frozen in liquid N_2 for later determination of RBC pHi by the freeze-thaw lysate method (Zeidler and Kim 1977). The plasma was decanted anaerobically for measurements of C_{CO_2} (2 × 50 μl) and ^{14}C radioactivity (2 × 50 μl), and thereby plasma specific activity (dpm.umol^{-1}). The net rate of conversion of plasma HCO_3^- to CO_2 (*i.e.* dehydration rate in umol.ml^{-1}.h^{-1}) for each 1 ml sample assayed was calculated by dividing filter paper ^{14}C radioactivity (dpm, corrected for $^{14}CO_2$ trap-

ping efficiency) by plasma specific activity (dpm.umol^{-1}) and time (0.05h).

Analytical techniques

Whole blood (extracellular) pHe and RBC pHi were determined with a micro-capillary electrode (G297/G2) thermostatted to the experimental temperature and connected to a PHM-71 Mk2 acid-base analyzer (Radiometer-Copenhagen). Plasma C_{CO_2} was measured with a Capni-con Total CO_2 Analyzer, Model II (Cameron Instruments) calibrated with NaHCO$_3$ standards in the range of interest (0–4 mmol.l^{-1}). Scintillation counting with quench correction on an LKB Rackbeta 1217 Counter was employed to measure ^{14}C radioactivity in plasma (50 μl in 10 ml Amersham ACS II) and filter papers (in 10 ml of customized cocktail containing 2.0 g PPO + 0.1 g POPOP in 0.8 l of toluene + 0.21 of 95% ethanol).

Data are expressed as means ± 1 SEM (N = 12). In view of the paired experimental design, statistical significance (p < 0.05) was assessed using Student's Paired t-test (two-tailed). In correlation analyses, regression lines were fitted by the method of least squares, and the significance of the simple correlation coefficient determined.

Results

There was no effect of any of the experimental treatments on the rate of conversion of HCO_3^- to CO_2 in separated plasma, which was 3.16 ± 0.08 umol.ml^{-1}.h^{-1}. Therefore, for each run, the respective plasma rates were subtracted from the whole blood rates to yield RBC conversion rates, which are the values reported.

Under normoxia, noradrenaline (10^{-6}M) significantly inhibited the rate of conversion of plasma HCO_3^- to CO_2 *via* trout RBCs (10.21 ± 0.98 umol.ml^{-1}.h^{-1}) by 36.1 ± 5.7% (Fig. 2A). Under hypoxia, noradrenaline significantly reduced the rate of conversion by 30.0 ± 3.7%. Neither these relative reductions nor the absolute reductions (−4.03 ± 0.82 umol.ml^{-1}.h^{-1} in normoxia *vs.* −3.78 ± 0.63 umol.ml^{-1}.h^{-1} in hypoxia) were
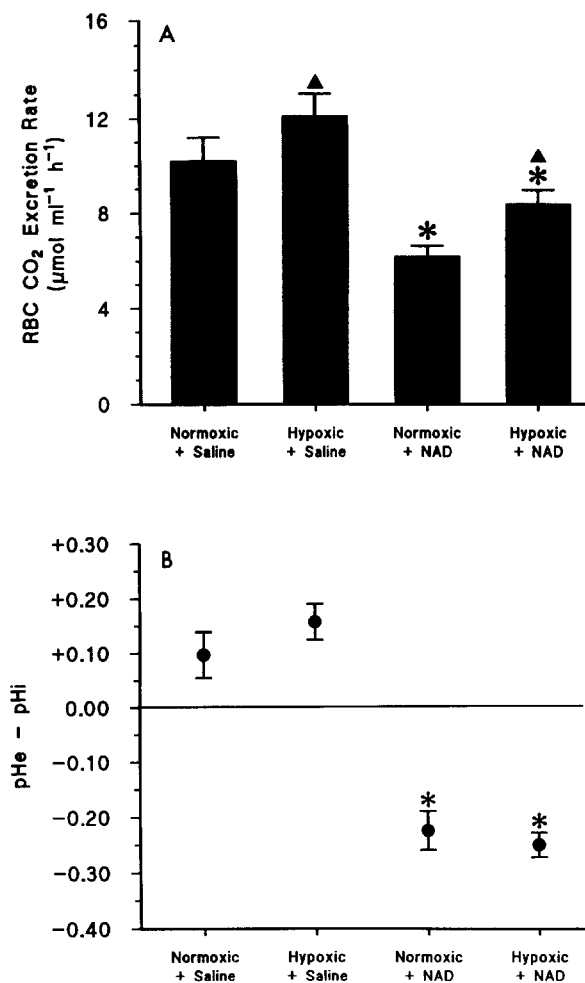
Fig. 2. The effects of normoxia + saline, hypoxia + saline, normoxia + 10^{-6} M noradrenaline (NAD), and hypoxia + 10^{-6} M NAD on (A) the rate of conversion of plasma HCO_3^- to CO_2 via trout RBCs ("RBC CO_2 excretion rate"), and (B) the pH gradient (pHe−pHi) across the RBC membrane. Means ± 1 SEM (N = 12). * indicates a significant difference (p<0.05) due to the presence of NAD relative to the appropriate saline treatment. ▲ indicates a significant difference due to the presence of hypoxia relative to the appropriate normoxic treatment.

significantly different between the normoxic and hypoxic regimes. We therefore conclude that hypoxia does not alter the magnitude of adrenergic inhibition of plasma HCO_3^- conversion to CO_2 via trout RBCs. However, to our surprise, hypoxia itself significantly elevated the conversion rate by 19.0% to 12.13 ± 0.92 umol.ml^{-1}.h^{-1}. This stimulatory effect of hypoxia persisted in the presence of noradrenaline, relative to normoxic

samples similarly treated with noradrenaline.

The reduction in HCO_3^- dehydration caused by noradrenaline was associated with a reversal of the pHe−pHi gradient across the RBC membrane from the normal value of about +0.100 units to about −0.225 units (Fig. 2B). This reflects activation of Na$^+$/H$^+$ exchange in the RBC membrane. The response was similar under normoxic and hypoxic conditions but the actual *change* in pHe−pHi gradient in response to noradrenaline was significantly greater under hypoxia (−0.408 ± 0.022 units) than under normoxia (−0.320 ± 0.024 units). This reflects the greater intensity of Na$^+$/H$^+$ activation in hypoxaemic blood.

The noradrenaline-induced reversal of the pHe−pHi gradient was due to a large decrease in pHe (about 0.275 units; Fig. 3A) and a small increase in pHi (about 0.050 units; Fig. 3B). Both extracellular pHe (7.270±0.022 vs. 7.151±0.026) and RBC pHi (7.113±0.014 vs. 7.055±0.019) were significantly elevated by hypoxia relative to normoxia in the absence of noradrenaline. In the presence of noradrenaline, pHe fell and pHi increased as in the saline treated samples, but the relative differences from the latter were maintained.

Plasma C_{CO_2} was not altered by noradrenaline during either normoxia or hypoxia. However, hypoxia itself caused a significant increase in mean plasma C_{CO_2} from about 2.0 to about 2.4 mmol.l^{-1} (Fig. 3C) in both saline and noradrenaline treatments.

Overall, there was a highly significant positive correlation (r = 0.71) between pHe and the conversion rate of plasma HCO_3^- to CO_2 via RBCs in individual blood samples (Fig. 4A). In contrast, there was no significant correlation between RBC pHi (r = −0.18) and the conversion rate (Fig. 4B). These data indicate that pHe rather than pHi was by far the more important influence contributing to the strong positive correlation (r = 0.65) between the transmembrane pH gradient (pHe−pHi) and the conversion rate (Fig. 5A). However, there was an equally strong positive correlation (r = 0.64) between plasma C_{CO_2} and the observed rate (Fig. 5B) even though noradrenaline had no effect on C_{CO_2} (Fig. 3C). Clearly, more than one factor was involved in determining the rates of conversion of
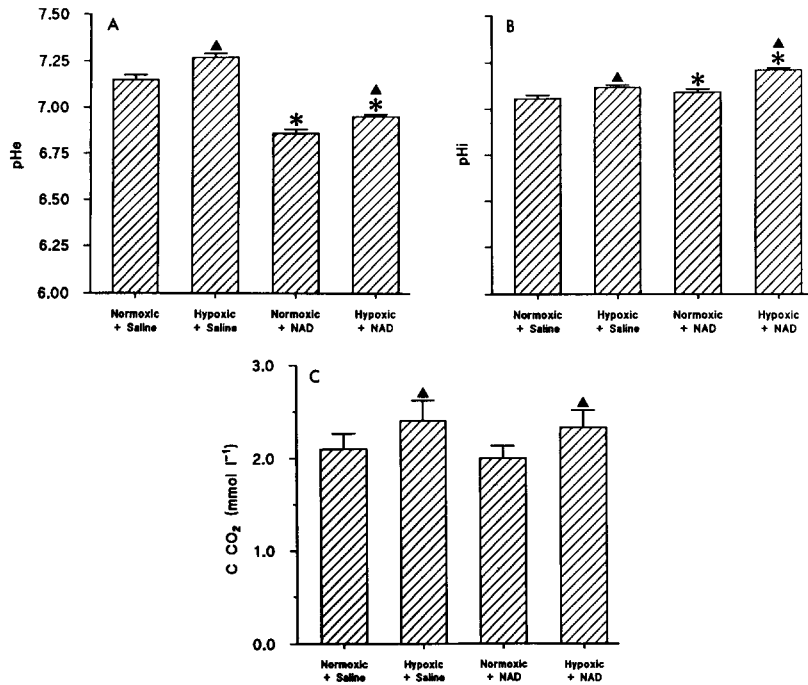
Fig. 3. The effects of normoxia + saline, hypoxia + saline, normoxia + 10^{-6} M noradrenaline (NAD), and hypoxia + 10^{-6} M NAD on (A) extracellular pHe, (B) RBC intracellular pHi, and (C) plasma total CO_2 concentration (C_{CO_2}) of trout blood. Means $\pm$ 1 SEM (N = 12). * indicates a significant difference (p < 0.05) due to the presence of NAD relative to the appropriate saline treatment. ▲ indicates a significant difference due to the presence of hypoxia relative to the appropriate normoxic treatment.

plasma HCO_3^- to CO_2 by trout RBCs in these experiments.

Discussion

Comparison with previous studies

Measured rates of conversion of plasma HCO_3^- to CO_2 in separated plasma and via RBCs in trout blood in the present study were similar in magnitude to those determined earlier by Wood and Perry (1991) and Perry *et al.* (1991) under similar conditions with the same assay system. As discussed by these authors, the observed rates lie in the general range of estimated values for rainbow trout *in vivo*, and are far below the greatly elevated values yielded by the unphysiological conditions present in the "boat" assay used by earlier workers (Heming and Randall 1982; Tufts *et al.* 1988). The 30–36% inhibition of HCO_3^- dehydration induced by 10^{-6} M

noradrenaline (Fig. 2A) was lower than the 54% inhibition reported by Perry *et al.* (1991), but the *change* in the pHe–pHi gradient (Fig. 2B) was also lower by about the same proportion in the present study. Intra-specific differences in the extent of adrenergic activation of Na^+/H^+ exchange are well documented and may result from differences in season, previous oxygen regime, and hormonal status (Nikinmaa and Tufts 1989; Reid and Perry 1991; Thomas and Perry 1992).

The more intense activation of Na^+/H^+ exchange in hypoxaemic blood (Fig. 2B) was in accord with previous observations and can be attributed to an increase in the number of surface-associated β_1-adrenergic receptors, resulting increased c-AMP production, and a greater responsiveness of Na^+/H^+ exchange to this c-AMP (Motais *et al.* 1987; Salama and Nikinmaa 1988; Reid and Perry 1991; Reid *et al.* 1993). It has been suggested that the oxygenation status of haemoglobin itself can serve as a signal transducer by
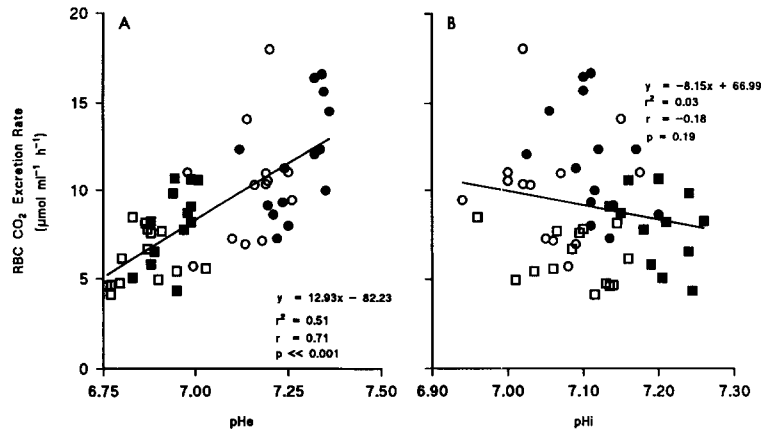
Fig. 4. (A) The relationship between extracellular pHe and "RBC CO_2 excretion rates" in trout blood. (B) The relationship between RBC intracellular pHi and the rate of conversion of plasma HCO_3^- to CO_2 *via* trout RBCs ("RBC CO_2 excretion rate"). o = normoxia + saline, ● = hypoxia + saline, □ = normoxia + 10^{-6} M noradrenaline (NAD), and ■ = hypoxia + 10^{-6} M NAD. The equations of the regression lines and the significance of the correlation coefficients are given.
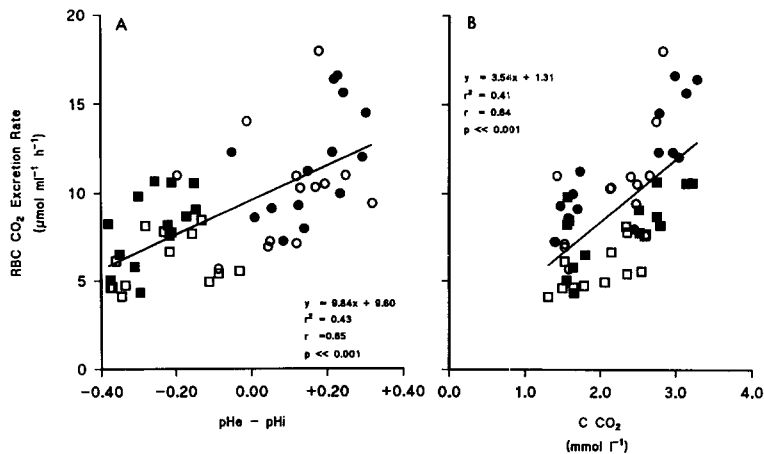
Fig. 5. (A) The relationship between the pH gradient (pHe–pHi) across the RBC membrane and "RBC CO_2 excretion rates" in trout blood. (B) The relationship between plasma total CO_2 concentration (C_{CO_2}) and the rate of conversion of plasma HCO_3^- to CO_2 via trout RBCs ("RBC CO_2 excretion rate"). o = normoxia + saline, ● = hypoxia + saline, □ = normoxia + 10^{-6} M noradrenaline (NAD), and ■ = hypoxia + 10^{-6} M NAD. The equations of the regression lines and the significance of the correlation coefficients are given.

conformational interactions with membrane proteins (Motais *et al.* 1987; Nikinmaa and Jensen 1992). While RBC O_2 content was not measured, the hypoxia ($P_{O_2} = 15$ torr) applied under the acidic conditions of the present study likely lowered the O_2 saturation of the hemoglobin from about 85% to 30% based on the O_2 dissociation curves of Eddy (1971) for rainbow trout blood.

The small increase in pHi (Fig. 3B) associated with this partial deoxygenation was due to the Hal-

dane effect as the deoxygenated hemoglobin bound more H^+ ions (Salama and Nikinmaa 1988; Jensen 1989). It is interesting that in an open system where P_{CO_2} but not pHe was directly controlled, the rise in pHe (Fig. 3A) associated with deoxygenation was considerably greater than that in pHi (Fig. 3B). This reflects a Donnan equilibration of H^+s (in the absence of noradrenaline) across the RBC membrane in combination with the different buffer capacities of the extracellular and intracellular com-

partments (Wood *et al.* 1982). The increased pHe at constant P_{CO_2} is associated with an elevation of plasma HCO_3^-, thereby explaining the increased plasma C_{CO_2} (Fig. 3C).

The effects of hypoxia on the conversion of plasma HCO_3^- to CO_2 via RBCs

Despite the reduced availability of free intracellular H^+s associated with the Haldane effect and a more intense activation of Na^+/H^+ exchange, the noradrenaline-induced inhibition of HCO_3^- dehydration was not intensified by hypoxia (Fig. 2A). This result, in combination with the general lack of correlation between pHi and the observed rate (Fig. 4B), would appear to eliminate explanation (3) of Fig. 1 – that adrenergic inhibition of the conversion of plasma HCO_3^- to CO_2 is due to a shortage of intracellular H^+s for the HCO_3^- dehydration reaction (Randall and Brauner 1991). Likely, as in mammals (Jonsson *et al.* 1976; Silverman and Vincent 1983), the ability of intracellular buffers (*i.e.* hemoglobin) to transfer H^+ ions to the active site on carbonic anhydrase is more than adequate to prevent H^+ availability from becoming a limiting factor.

This therefore leaves the two original explanations offered by Perry *et al.* (1991) – (1) an alteration in the electrochemical gradient for HCO_3^- entry and/or (2) recycling of CO_2 from plasma to RBC. Neither of these possibilities can be eliminated by the present results, and indeed there is no reason why they should be mutually exclusive. The strong positive relationship between pHe–pHi and the conversion rate (Fig. 5B) is exactly as expected from both explanations (1) and (2) and is in good agreement with the data of Wood and Perry (1991). Similarly, the strong positive relationship between plasma C_{CO_2} and the conversion rate (Fig. 5B) suggests that external substrate (HCO_3^-) concentration is also important, but in the absence of intracellular C_{CO_2} measurements and the likely presence of non-equilibrium P_{CO_2} in both the RBC and plasma compartments after adrenergic stimulation, the HCO_3^- gradients (1) cannot be calculated. From experimental manipulations of plasma HCO_3^- concentrations, Wood and Perry (1991) concluded that the HCO_3^- gradient had a small influence under resting (Donnan equilibrium) conditions, but potentially a very large influence under non-equilibrium conditions.

An interesting variant on this idea is that the adrenergic inhibition of plasma HCO_3^- conversion is in some way a direct consequence of the non-equilibrium acid load acutely added to the plasma by Na^+/H^+ exchange. Unfortunately, the idea is difficult to test experimentally. When the RBC's add acid to the plasma, then their intracellular pH and HCO_3^- rise instantaneously (explanation 1) and intracellular P_{CO_2} falls instantaneously (explanation 2). However when small amounts of exogenous acid are added to the plasma by the investigator, then both pHe, and to a lesser extent, pHi fall, and P_{CO_2} increases throughout the system. We have found that the conversion of plasma HCO_3^- to CO_2 measured by the assay actually *increases* rather than decreases in the first few minutes because the whole process is driven by the P_{CO_2} gradient from RBC to atmosphere. Over the longer term, the measured rate decreases slightly, because of the change in the HCO_3^- gradient.

The clear stimulation by hypoxia of the net conversion rate of plasma HCO_3^- to CO_2 via RBCs in both the presence and absence of noradrenaline (Fig. 2A) was an unexpected finding, but one which has been observed independently by Perry and Gilmour (1993). There is obvious functional importance. *In vivo*, this phenomenon will help enhance CO_2 excretion at the gills during environmental hypoxia, thereby contributing to lowered Pa_{CO_2}, raised RBC pHi, and improved conditions for O_2 loading (Tetens and Christensen 1987; Thomas and Motais 1990; Perry and Thomas 1991). We suggest two possible explanations for the mechanism involved. The first is that the higher plasma HCO_3^- associated with the Haldane effect in hypoxaemic blood (Figs. 3C) increases the supply of external substrate and therefore possibly the electrochemical gradient for HCO_3^- entry. The strong positive correlation between the HCO_3^- conversion rate and both pHe (but not pHi) and extracellular C_{CO_2} (Figs. 4,5) supports this idea. The second is the interesting possibility of a direct effect of hemo-

globin oxygenation status on the activity of the band 3 Cl^-/HCO_3^- exchanger. In mammalian RBCs it is well established that the cytoplasmic domain of band 3 preferentially binds to deoxygenated hemoglobin (Salhany *et al.* 1980; Walder *et al.* 1984; Chetrite and Cassoly 1985, but it remains unclear whether this interaction has any influence on the rate of anion exchange (Jennings 1989). The trout RBC may be a useful system for future studies on this question.

In the present study, we have addressed the cause behind the adrenergic inhibition of plasma HCO_3^- conversion seen in trout blood *in vitro*. *In vivo*, the situation is undoubtedly more complex, and two features deserve special attention in future studies. The first is the extent to which a redistribution of HCO_3^- transport occurs as a result of elevated pHi and RBC swelling associated with hypoxia and adrenergic stimulation. Both factors will ensure that a greater proportion of total blood HCO_3^- is inside the RBCs right from the point of loading at the tissues. Therefore at the gills (and in the assay), a greater proportion of total amount of HCO_3^- dehydrated to excreted CO_2 could originate from intracellular HCO_3^- stores. This would not be detected by the present assay system where the label is added to the plasma compartment. The second is the fact that the release of Bohr protons by hemoglobin oxygenation should promote the overall HCO_3^- dehydration rate as blood passes through the gills (Jensen 1989). Support for this idea has recently been provided by Perry and Gilmour (1993) using a modified version of the present assay system, but it is unclear how adrenergic stimulation would interact with this process.

Acknowledgements

References cited

Borgese, F., Garcia-Romeu, F. and Motais, R. 1987. Ion movements and volume changes induced by catecholamines in erythrocytes of rainbow trout. J. Physiol. 382: 145–157.

Chetrite, G. and Cassoly, R. 1985. Affinity of hemoglobin for the cytoplasmic fragment of human erythrocyte membrane band 3. J. Mol. Biol. 185: 639–644.

Eddy, F.B. 1971. Blood gas relationships in the rainbow trout, *Salmo gairdneri*. J. Exp. Biol. 55: 695–711.

Heming, T.A. and Randall, D.J. 1982. Fish erythrocytes are bicarbonate permeable: problems with determining carbonic anhydrase activity using the modified boat technique. J. Exp. Zool. 219: 125–128.

Hubner, S., Michel, F., Rudloff, V. and Applehans, H. 1992. Amino acid sequence of band-3 protein from rainbow trout erythrocytes derived from c-DNA. Biochem. J. 285: 17–23.

Jennings, M.L. 1989. Structure and function of the red blood cell anion transport protein. Annu. Rev. Biophys. Chem. 18: 397–430.

Jensen, F.B. 1989. Hydrogen ion equilibria in fish haemoglobins. J. Exp Biol. 143: 225–234.

Jonsson, B.H., Steiner, H., and Lindskog, S. 1976. Participation of buffer in the catalytic mechanism of carbonic anhydrase. FEBS Letters 64: 310–314.

Milligan, C.L. and Wood, C.M. 1986. Intracellular and extracellular acid-base status and H^+ exchange with the environment after exhaustive exercise in the rainbow trout. J. Exp. Biol. 123: 93–121.

Motais, R., Borgese, F., Fievet, B. and Garcia-Romeu, F. 1992. Regulation of Na^+/H^+ exchange and pH in erythrocytes of fish. Comp. Biochem. Physiol. 102A: 597–602.

Motais, R., Fievet, B., Garcia-Romeu, F. and Thomas, S. 1989. Na^+/H^+ exchange and pH regulation in red blood cells: role of uncatalyzed H_2CO_3 dehydration. Am. J. Physiol. 256: C728–C735.

Motais, R., Garcia-Romeu, F. and Borgese, F. 1987. The control of Na^+/H^+ exchange by molecular oxygen in trout erythrocytes. J. Gen. Physiol. 90: 197–207.

Nikinmaa, M. 1992. Membrane transport and control of hemoglobin-oxygen affinity in nucleated erythrocytes. Physiol. Rev. 72: 301–321.

Nikinmaa, M. and Jensen, F.B. 1992. Inhibition of adrenergic proton extrusion in rainbow trout red cell by nitrite-induced methaemoglobinaemia. J. Comp. Physiol. B. 162: 424–429.

Nikinmaa, M. and Tufts, B.L. 1989. Regulation of acid and ion transfer across the membrane of nucleated erythrocytes. Can. J. Zool. 67: 3039–3045.

Perry, S.F. 1986. Carbon dioxide excretion in fishes. Can. J. Zool. 64: 565–572.

Perry, S.F. and Wood, C.M. 1989. Control and coordination of gas transfer in fishes. Can. J. Zool. 67: 2961–2970.

Perry, S.F., Wood, C.M., Thomas, S. and Walsh, P.J. 1991. Adrenergic inhibition of carbon dioxide excretion by trout red blood cells *in vitro* is mediated by activation of Na^+/H^+ exchange. J. Exp Biol. 157: 367–380.

Perry, S.F. and Thomas, S. 1991. The effects of endogenous or exogenous catecholamines on blood respiratory status during acute hypoxia in rainbow trout (*Oncorhynchus mykiss*). J. Comp. Physiol. B. 161: 489–497.

Playle, R.C., Munger, R.S. and Wood, C.M. 1990. Effects of catecholamines on gas exchange and ventilation in rainbow

trout (*Salmo gairdneri*). J. Exp. Biol. 152: 353–367.

Randall, D.J. and Brauner, C. 1991. Effects of environmental factors on exercise in fish. J. Exp. Biol. 160: 113–126.

Reid, S.D., LeBras, Y. and Perry, S.F. 1993. The *in vitro* effect of hypoxia on the trout erythrocyte β-adrenergic signal transduction system. J. Exp. Biol. 176: 103–116.

Reid, S.D. and Perry, S.F. 1991. The effects and physiological consequences of raised levels of cortisol on rainbow trout (*Oncorhynchus mykiss*) erythrocyte β-adrenoreceptors. J. Exp. Biol. 158: 217–240.

Romano, L. and Passow, H. 1984. Characterization of anion transport system in trout red blood cell. Am. J. Physiol. 246: C330–C338.

Salhany, J.M., Cordes, K.A. and Gaines, E.D. 1980. Light-scattering measurements of hemoglobin binding to the erythrocyte membrane. Evidence for transmembrane effects related to a disulfonic stilbene binding to band 3. Biochemistry 19: 1447–1454.

Salama, A. and Nikinmaa, M. 1988. The adrenergic responses of carp (*Cyprinus carpio*) red cells: effects of P_{O_2} and pH. J. Exp. Biol. 136: 405–416.

Salama, A. and Nikinmaa, M. 1989. Species differences in the adrenergic responses of fish red cells: studies on whitefish, pike perch, trout and carp. Fish. Physiol. Biochem. 6: 167–173.

Silverman, D.N. and Vincent, S.H. 1983. Proton transfer in the catalytic mechanism of carbonic anydrase. CRC Critical Rev. Biochem. 14: 207–255.

Soivio, A., Westman, K. and Nyholm, K. 1972. Improved method of dorsal aorta catheterization: haematological effects followed for three weeks in rainbow trout (*Salmo gairdneri*). Finnish Fish. Res. 1: 11–21.

Steffensen, J.F., Tufts, B.L. and Randall, D.J. 1987. Effect of burst swimming and adrenaline infusion on O_2 consumption and CO_2 excretion in rainbow trout *Salmo gairdneri*. J. Exp. Biol. 131: 427–434.

Tetens, V. and Christensen, N.J. 1987. Beta-adrenergic control of blood oxygen affinity in acutely hypoxia exposed rainbow trout. J. Comp. Physiol. B. 156: 668–675.

Thomas, S. and Motais, R. 1990. Acid-base balance and oxygen transport during acute hypoxia in fish. *In* Animal Nutrition and Transport Processes 2. Transport, Respiration and Excretion: Comparative and Environmental Aspects. Comp. Physiol. Volume 6, pp. 76–91. Edited by J.P. Truchot and B. Lahlou. Karger, Basel.

Thomas, S. and Perry, S.F. 1992. Control and consequences of adrenergic activation of red blood cell Na^+/H^+ exchange on blood oxygen and carbon dioxide transport. J. Exp. Zool. 263: 160–175.

Tufts, B.L., Ferguson, R.A. and Boutilier, R.G. 1988. *In vivo* and *in vitro* effects of adrenergic stimulation on chloride/bicarbonate exchange in rainbow trout erythrocytes. J. Exp. Biol. 140: 301–312.

Walder, J.A., Chatterjee, R., Steck, T.L., Low, P.J., Musso, G.F., Kaiser, E.T., Rogers, P.H. and Arnone, A. 1984. The interaction of hemoglobin with the cytoplasmic domain of band 3 of the human erythrocyte membrane. J. Biol. Chem. 259: 10238–10246.

Wolf, K. 1963. Physiological salines for freshwater teleosts. Progr. Fish Cult. 25: 135–140.

Wood, C.M. and Perry, S.F. 1991. A new *in vitro* assay for carbon dioxide excretion by trout red blood cells: effects of catecholamines. J. Exp Biol. 157: 349–366.

Wood, C.M. and Perry, S.F. 1985. Respiratory, circulatory, and metabolic adjustments to exercise in fish. *In* Circulation, Respiration, Metabolism. pp. 2–22. Edited by R. Gilles. Springer-Verlag, Berlin.

Wood, C.M., McDonald, D.G. and McMahon, B.R. 1982. The influence of experimental anaemia on blood acid-base regulation *in vivo* and *in vitro* in the starry flounder (*Platichthys stellatus*) and the rainbow trout (*Salmo gairdneri*). J. Exp. Biol. 96: 221–237.

Zeidler, R. and Kim, D.H. 1977. Preferential hemolysis of post-natal calf red cells induced by internal alkalinization. J. Gen. Physiol. 70: 385–401.