The Journal of Experimental Biology **198**, 31–38 (1995)
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WHY IS THERE NO CARBONIC ANHYDRASE ACTIVITY AVAILABLE TO FISH PLASMA?

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Accepted 17 August 1994

Summary

Carbonic anhydrase (CA) is absent from the plasma of vertebrates. *In vitro*, CA in fish plasma will short-circuit the effect of catecholamines, which is to increase red blood cell (RBC) pH and volume, both of which enhance the affinity of hemoglobin for O_2. CA was infused into trout for a period of 6 h and injected after 48 h, during which the animal was submitted to deep hypoxia (P_{O_2}=30–35 mmHg; 4.0–4.7 kPa). O_2 content, lactate content, catecholamine levels, hematocrit, hemoglobin concentration and pHi were similar to those in the saline-infused control group. In contrast, cell volume was significantly higher and pHe, total CO_2 content and organic phosphate levels were significantly lower than in the control group. The concentration of CA was not high enough completely to short-circuit the increase in pHi and red blood cell volume caused by catecholamines. The lower pHe in the CA-infused animals could enhance the activity of the Na^+/H^+ pump, which would keep the nucleotide triphosphate levels low. pH is a balance between acid loading at the muscle and acid excretion at the gills or the kidneys; we cannot distinguish between which of these resulted in a decrease of plasma pH. In conclusion, CA in plasma did not cause the expected reduction in blood oxygen content but did have a marked effect on plasma total CO_2 content.

Key words: carbonic anhydrase, acid–base balance, rainbow trout, *Oncorhynchus mykiss*, carbon dioxide, oxygen content.

Introduction

Teleost blood plasma has no carbonic anhydrase (CA) activity and, unlike the situation in mammals, there is no CA activity on the inner surface of the respiratory epithelium (Rahim *et al.* 1988; Henry *et al.* 1988). As a result, there is no CA activity available to fish plasma at the gills and CO_2/bicarbonate hydration/dehydration reactions occur at an uncatalysed rate (Perry *et al.* 1982).

Catecholamines released into the blood, for example during extreme hypoxia (see Randall and Perry, 1992), activate a Na^+/H^+ exchanger across the red blood cell membrane, raising erythrocytic pH (pHi) (Nikinmaa, 1990; Motais *et al.* 1990). In fish erythrocytes, as in those of mammals, acid is transferred slowly from the plasma to the RBCs *via* the Jacob–Stewart cycle (Forster and Steen, 1969), that is by the cycling of CO_2 and bicarbonate (Fig. 1). Thus, Na^+/H^+ exchange removes protons from the RBCs and these will re-enter the cells *via* the Jacobs–Stewart cycle, the rate-limiting step being the uncatalysed bicarbonate dehydration reaction in the plasma. Motais *et al.* (1989*b*) and Nikinmaa *et al.* (1990) have shown that the addition of CA to the plasma *in vitro* will short-circuit the action of catecholamines on RBC pH. It has been suggested, therefore, that the absence of CA activity in plasma allows catecholamine regulation of erythrocytic pH. Fish plasma is totally devoid of any CA activity and none is available on the basal membrane of the gills. Mammalian plasma has access to CA activity at the lung endothelium, but there is no RBC pH regulation by catecholamines, as there is in fish erythrocytes.

Motais *et al.* (1989*a*), however, showed that catecholamines could still raise trout RBC pH *in vitro* even in the presence of 0.5 mg ml^{-1} CA. These CA levels are an order of magnitude higher than those normally available to mammalian plasma (Effros *et al.* 1980; Bidani *et al.* 1983). Low levels of CA activity available to plasma, therefore, may not jeopardize catecholamine-induced RBC pH regulation, and the absence of CA from fish plasma may be correlated with factors other than RBC pH regulation.

Fish bodies may consist of more than two-thirds muscle. These muscles are predominantly glycolytic and large amounts of acid are released into the blood following burst swimming. If carbonic anhydrase activity were available to the plasma, carbonic acid would be rapidly transferred into the RBCs and, in addition, the acid would titrate plasma bicarbonate and be excreted as CO_2. Both these processes would occur more slowly in the absence of carbonic anhydrase activity available to plasma.

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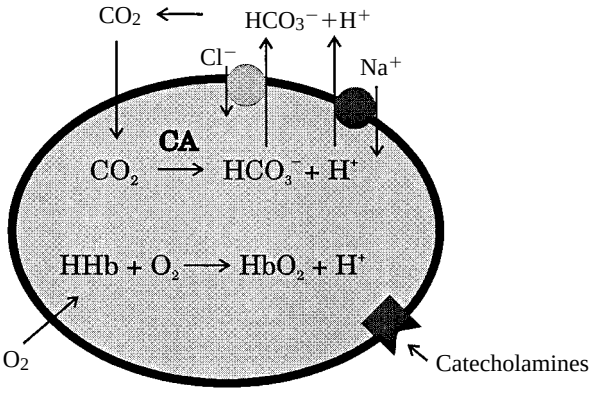

Fig. 1. The Jacob–Stewart cycle in the red blood cell. The rate-limiting step for acid cycling is the dehydration of bicarbonate in the plasma. Catecholamines, *via* an adrenergic receptor, activate the Na^+/H^+ exchanger and uncouple red cell pH from the plasma pH. CA, carbonic anhydrase; Hb, hemoglobin.

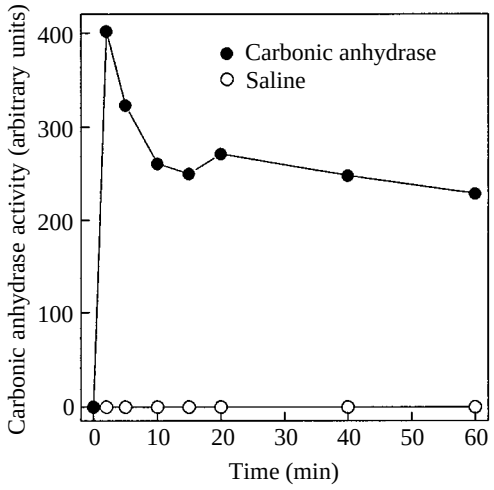

Fig. 2. Time variation of plasma carbonic anhydrase activity in trout blood after injection of bovine carbonic anhydrase (10 000 i.u. kg^{-1}), *in vivo*.

The object of the experiments reported here was to infuse carbonic anhydrase into trout to obtain activity levels similar to those available to mammalian plasma in order to determine the effect of plasma carbonic anhydrase activity on RBC pH and plasma acid–base regulation during exposure to hypoxia. Hypoxia was used as a mechanism for promoting the release of catecholamines and protons into the blood. The plasma acidosis was the result of the anaerobic production of lactic acid in the muscle and the catecholamine-induced movement of protons out of the RBCs.

Materials and methods

Freshwater rainbow trout [*Oncorhynchus mykiss* (Walbaum)], weighing 300–600 g, were obtained from a local hatchery and held outdoors at the University of British Columbia in dechlorinated Vancouver tap water (8–12 °C) for at least 2 weeks before experimentation. The animals were fed once a week with commercial trout pellets, but feeding was suspended 3 days prior to surgery. Under MS-222 anesthesia (1:10 000 in $NaHCO_3$-buffered fresh water), fish were fitted with dorsal aortic catheters according to Soivio *et al.* (1975). Following surgery, fish were allowed to recover for at least 48 h in a darkened acrylic box with recirculating water (8–10 °C).

Experimental protocol

Series 1: bolus injection (Fig. 2)

A solution containing 10 000 i.u. ml^{-1} bovine carbonic anhydrase (Sigma Chemical Co., St Louis, MO) in Cortland's saline (Wolf, 1963) was prepared for injection into each group of animals. A 500 μl blood sample was removed from the fish (time zero) and a bolus of 1 ml kg^{-1} body mass of bovine carbonic anhydrase solution was injected through the cannula followed by 500 μl of Cortland's saline. Blood samples (50 μl) were removed 1, 5, 10, 15, 20 and 60 min after CA injection, each one being replaced with the same volume of Cortland's saline. Eight fish under normoxic conditions were used in this series.

Series 2: carbonic anhydrase injection after 48 h of hypoxia or normoxia

This series was designed to check the effect of CA injection in animals under normoxic and hypoxic conditions. Eighteen animals were cannulated and allowed to recover in individual darkened Perspex boxes as described above. After the recovery period and over a period of 48 h, aerated water (P_{O_2}=155 mmHg; 20.6 kPa) for normoxic animals and deoxygenated water (P_{O_2}=35 mmHg; 4.7 kPa) for hypoxic ones flowed through the boxes. After the acclimation period, nine animals received a bolus of 1 ml kg^{-1} body mass of bovine CA and nine received a bolus of 1 ml kg^{-1} body mass of saline. Two blood samples of 2 ml each were removed from the dorsal aorta (one at 10 min and the other at 30 min after the injection). One part of each blood sample was centrifuged, and 500 μl of plasma was removed, immediately frozen in liquid nitrogen and then stored in a freezer at −80 °C for future analysis of catecholamine levels; another 500 μl of plasma was deproteinized with 70 % trichloroacetic acid and stored at −20 °C until later analysis of the amounts of lactate and electrolytes present. The remainder of the blood was used for hematocrit, hemoglobin concentration and ion determinations.

Series 3: continuous carbonic anhydrase infusion at the onset of hypoxia

For measurements at rest, one blood sample (1.2 ml) was taken from the dorsal aorta and immediately analyzed for plasma pH (pHe), blood and plasma total CO_2, hematocrit, hemoglobin and oxygen content. The remainder of the blood was centrifuged, plasma was separated from RBCs and both were frozen in liquid nitrogen for future analysis to estimate levels of lactate, catecholamines, adenylates and guanylates and red blood cell pH (pHi). These fish were not used for the

hypoxia experiments because we did not want any stress prior to the infusion and the hypoxia exposure. At the same time that hypoxia was induced (P_{O_2}=30–35 mmHg; 4.0–4.7 kPa), one group of fish was infused with saline and another with approximately 3 ml of carbonic anhydrase solution of 10 000 i.u. (to achieve 0.3 g l^{-1} in the animal) for the duration of the hypoxic exposure (6 h). Blood samples were taken as described above at 10, 30, 120 and 360 min.

Analytical procedures

Plasma CA activity was determined using the boat technique as described by Haswell and Randall (1976); this consists of adding 2 ml of bicarbonate solution (0.2 mol l^{-1} $NaHCO_3$ in aqueous solution with 0.02 mol l^{-1} NaOH) to one side of an Erlenmeyer flask with a partial septum on the bottom ('boat'), and 2 ml of 0.2 mol l^{-1} phosphate buffer solution, pH 6.8, to the other side. Samples (100 μl of plasma) were introduced into the buffer and the Erlenmeyer flask was connected to a pressure transducer (Statham P23BB, AST/Servo System Inc., Newark, NJ) connected to a chart recorder. After a 3 min period of temperature equilibration, the Erlenmeyer flask was shaken to mix the solutions and the increase in gas pressure was recorded. Enzyme activity in arbitrary units was then calculated according to Haswell and Randall (1976) considering the slopes for the catalyzed and uncatalysed reactions.

Plasma pH was determined with a microcapillary electrode (Radiometer G279/G2) coupled to a PHM84 pH meter. Total CO_2 measurements were carried out using a gas chromatography method (Boutilier *et al.* 1985) on samples obtained anaerobically. Total CO_2 inside the erythrocytes was obtained by subtracting total plasma CO_2 from total blood CO_2, taking into account the hematocrit. P_{CO_2} was calculated from the Henderson–Hasselbach equation using the solubility coefficient and pK calculated according to Boutilier *et al.* (1984). Hematocrit was measured using microhematocrit tubes centrifuged at 12 000 revs min^{-1} for 5 min. Hemoglobin concentration (Hb) was measured using Drabkin's reagent from a Sigma kit (no. 525-A). Mean cellular hemoglobin concentration (MCHC) was calculated as [Hb]/Hct. Blood and plasma levels of Na^+, K^+ and Ca^{2+} were determined by flame photometry (Perkin-Elmer, model 2380). Blood levels of all cations were corrected according to the hematocrit values in order to estimate the levels inside the red blood cell. The oxygen content of the blood was determined using the method of Tucker (1967). Whole-blood lactate levels were analyzed using the L-lactate dehydrogenase/NAD method (Sigma kit no. 826-B). Analyses of plasma catecholamine levels were performed by HPLC with electrochemical detection, using a Brownlee Spheri-5 reverse-phase column (Technical Marketing, Richmond, British Columbia), a Bioanalytical Systems LC-4A amperometric detector (Mandel Scientific, Rockwood, Ontario) and a Spectra-Physics SP8700 solvent delivery system (Terochem Laboratories Ltd, Edmonton, Alberta), as described by Primmett *et al.* (1986). The freeze–thaw method of Zeidler and Kim (1977) was used to measure pHi.

Statistical methods

Statistical significance of data for the 48 h experimental series was determined using Kruskall–Wallis one-way analysis of variance (ANOVA) or Student's *t*-test, as appropriate, with a fiducial limit of significance of 5 %. When no significant difference was detected between 10 and 30 min samples after the drug administration, the data were pooled and a mean and respective standard error were calculated and submitted to further statistical analysis.

Statistical significance of data for 6 h of hypoxia was determined using a two-way ANOVA followed by a Dunnett test when comparing the hypoxia values with the resting values and an unpaired *t*-test when comparing CA-infused values with control values, both with a statistical significance level of 5 %. Data are presented as mean ± S.E.M.

Results

In resting animals, catecholamine and lactate levels were similar to resting levels measured by others (Nilsson, 1983; Tetens *et al.* 1988; Hart *et al.* 1989; Perry *et al.* 1989; Thomas and Perry, 1991; McDonald and Milligan, 1992). In both saline- and CA-infused fish, catecholamine levels increased significantly after 10 min of deep hypoxia and remained elevated even after 48 h of exposure to hypoxia (Table 1). In both groups, lactate concentration was significantly elevated relative to that in the resting fish at 10 min and kept increasing for the next 6 h (Fig. 3). After 48 h of exposure to hypoxia, the lactate level had dropped but was still above resting values. Exposure to hypoxia for 48 h had no significant effect on plasma and RBC Na^+, K^+ and Ca^{2+} levels, except that there was a significant difference in RBC Ca^{2+} concentration between normoxic and hypoxic CA-injected animals (data not shown). This is consistent with the proposition that Ca^{2+} is extruded from RBCs during hypoxia in trout (Smallwood *et al.* 1983).

Infusion of CA caused an increase in pHe but had no effect on pHi in normoxic fish (Table 2). Saline infusion was without effect on pH in normoxic fish (Table 2). Hypoxia had little effect on plasma pH in saline-infused fish (Fig. 4). In the group of fish infused with CA, however, plasma pH initially increased and then decreased for the next 6 h of hypoxic exposure. Injection of CA after 48 h of exposure to hypoxia caused a similar increase in plasma pH to that seen if carbonic anhydrase was infused at the beginning of the exposure to hypoxia (Table 2). Erythrocytic pHi did not vary significantly during hypoxia in either treatment (Fig. 4; Table 2).

In normoxia, CA and saline infusion had no effect on the total CO_2 content in the plasma or in the red blood cells (data not shown). In hypoxia, total CO_2 content of the plasma decreased significantly more in the CA-treated animals than in the saline-treated ones (Fig. 5A). After 48 h of exposure, however, CA injection had much less of an effect on plasma total CO_2 (Table 3). No significant changes were observed in RBC total CO_2 during exposure to hypoxia with or without the

Table 1. *Plasma catecholamine concentrations for fish infused with saline or carbonic anhydrase during normoxia and at specific time after exposure to hypoxia*

		Hypoxia				
	Normoxia	10 min	30 min	120 min	360 min	48 h
Adrenaline (ng ml^{-1})	4.1±1.4					
Saline		16.2±5.3*	12.2±2.7*	21.6±6.6*	22.4±9.3*	22.3±15.6*
CA		10.2±2.2*	14.1±4.3*	23.1±9.5*	19.3±6.9*	3.3±0.6†
Noradrenaline (ng ml^{-1})	3.99±1.40					
Saline		22.7±7.8*	17.2±3.8*	25.2±6.2*	17.6±7.9*	14.5±10.0*
CA		11.1±2.1*	18.1±6.9	31.1±15.9*	23.7±10.7	6.6±2.5*

*Significantly different from normoxic control; †significant difference between saline- and carbonic-anhydrase-infused hypoxic animals.
Values are mean ± S.E.M., *N*=7.
CA, carbonic anhydrase.

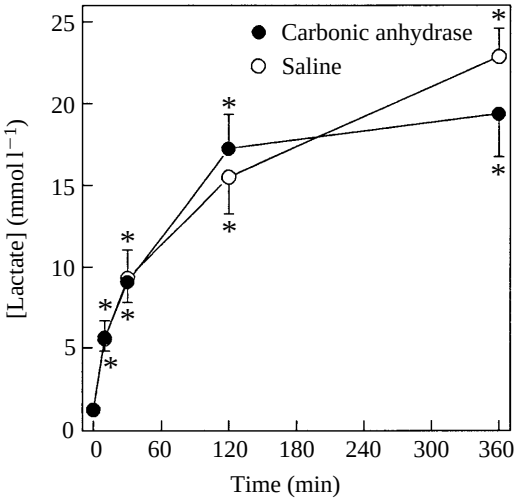

Fig. 3. Levels of lactate in rainbow trout infused with saline or carbonic anhydrase in hypoxic conditions. *Significant difference between normoxic and hypoxic animals. Values are mean ± S.E.M., *N*=7.

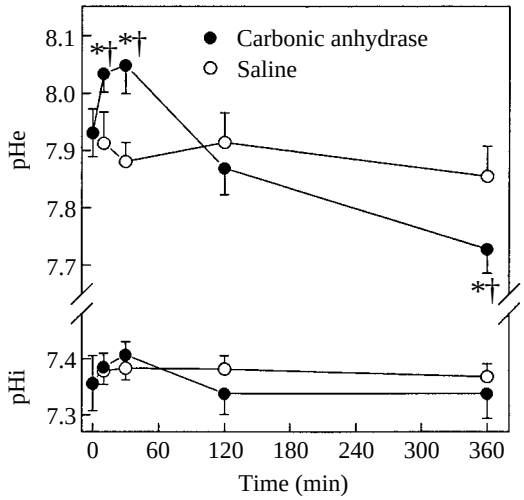

Fig. 4. Effects of carbonic anhydrase infusion in hypoxic rainbow trout on plasma pH (pHe) and intracellular pH (pHi). *Significant difference between normoxic and hypoxic animals. †Significant difference between saline- and carbonic-anhydrase-infused hypoxic animals. Values are mean ± S.E.M., *N*=7.

Table 2. *Plasma pH and red blood cell pH of fish injected with saline or carbonic anhydrase during normoxia*

	Time after injection	
	10 min	30 min
pHe		
Saline	7.98±0.06	8.02±0.05
Carbonic anhydrase	7.99±0.03	8.12±0.06*
pHi		
Saline	7.27±0.05	7.28±0.05
Carbonic anhydrase	7.28±0.03	7.30±0.04

*Significantly different from saline control.
Values are mean ± S.E.M., *N*=8.

infusion of CA (data not shown). The calculated equilibrium P_{CO_2} decreased significantly in the CA-infused group compared with both normoxic and hypoxic values (Fig. 5B). P_{CO_2} showed a small decrease in the saline-infused group during hypoxia compared with normoxic values, but this was not significant.

Initially, the hematocrit increased to a peak at 30 min (Fig. 6A) and was still elevated after 48 h of exposure to hypoxia (Table 3). Hemoglobin concentration was constant during the first 6 h of exposure to hypoxia (Fig. 6B), but was elevated after 48 h of exposure in the saline-injected, but not the CA-injected, group of fish (Table 3). The ratio of hemoglobin and hematocrit (MCHC), which is an indication of RBC volume, differed between the two groups (Fig. 6C). Initially, hypoxia produced an increase in RBC volume (as indicated by the decrease in MCHC), which was significantly greater than the normoxic values in both treatments. In the saline treatment, RBC volume remained constant for the next 6 h. In the CA-infused group, RBC volume continued to increase and became significantly different from that of the saline group at 6 h. After 48 h of exposure to hypoxia, RBC

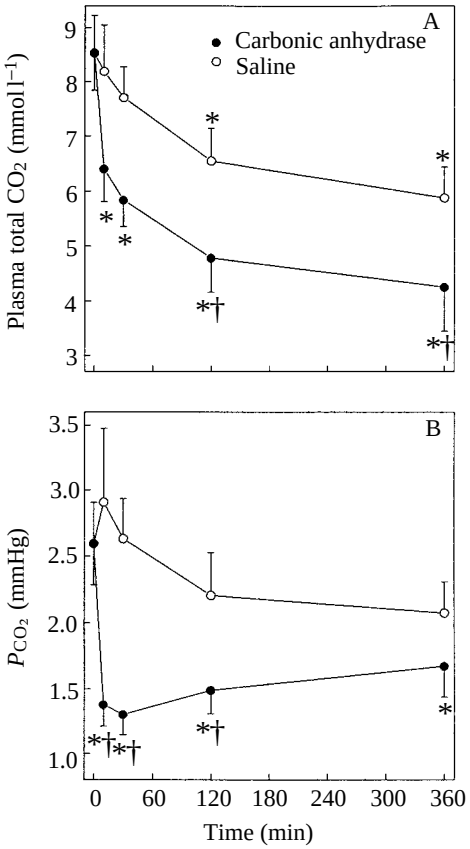

Fig. 5. Effects of carbonic anhydrase infusion in hypoxic rainbow trout on (A) plasma total CO_2 and (B) CO_2 partial pressure (P_{CO_2}). *Significant difference between normoxic and hypoxic animals. †Significant difference between saline- and carbonic-anhydrase-infused hypoxic animals. Values are mean ± S.E.M., *N*=7.

Table 3. *Blood variables for fish injected with a single dose of carbonic anhydrase or saline after 48 h of exposure to hypoxia*

	Saline	Carbonic anhydrase
Lactate (mmol l^{-1})	94.4±23.0*	80.5±17.0*
pHe	7.83±0.03	8.04±0.05†
pHi	7.35±0.04	7.43±0.03
Plasma total CO_2 (mmol l^{-1})	6.28±0.17*	5.695±0.24*†
P_{CO_2} (mmHg)	2.20±1.22	1.40±0.14*†
Hematocrit (%)	34.3±1.7*	32.1±1.7*
Hemoglobin (g dl^{-1})	9.79±0.66*	7.92±0.28†
MCHC (g dl^{-1})	26.3±1.5	25.1±1.0

*Significantly different from normoxia control; †significant difference between saline- and carbonic-anhydrase-infused hypoxic animals.

Values are mean ± S.E.M., *N*=9.

volume was not significantly different from the initial normoxic value in both the saline- and CA-infused groups (Table 3).

In both treatments, O_2 content dropped dramatically within the first 10 min. It then remained constant with no significant

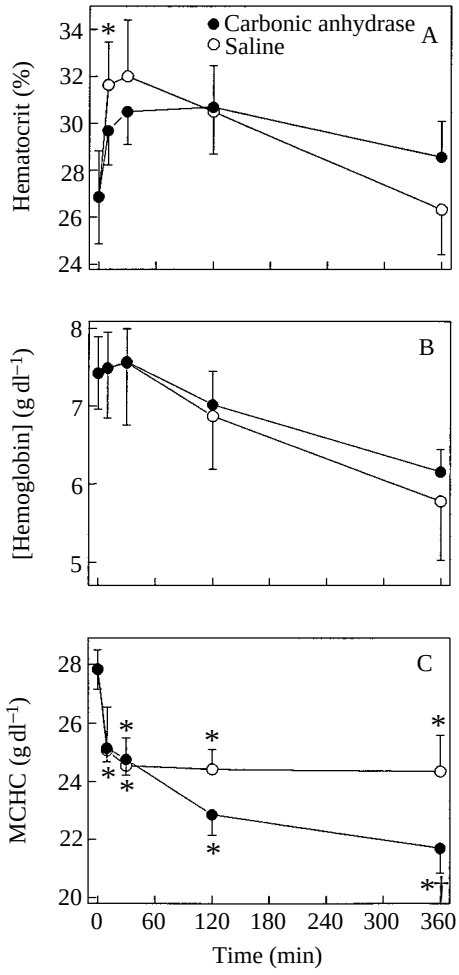

Fig. 6. Effects of carbonic anhydrase infusion in hypoxic rainbow trout on (A) hematocrit, (B) hemoglobin concentration and (C) mean cell hemoglobin concentration (MCHC). *Significant difference between normoxic and hypoxic animals. †Significant difference between saline- and carbonic-anhydrase-infused hypoxic animals. Values are mean ± S.E.M., *N*=7.

difference between the two groups for the next 6 h of hypoxia (Fig. 7A). After 48 h of exposure to hypoxia, blood oxygen content was still reduced, but there was a small increase in blood oxygen content 30 min after the injection of CA (Fig. 7B). A bolus injection of CA in normoxic fish resulted in a small reduction in oxygen content of arterial blood 30 min after the injection (Fig. 7B).

Discussion

The concentration of CA was still 60 % of the initial value 60 min after a bolus injection, indicating a slow rate of removal of CA activity from the plasma (Fig. 2). CA was infused continuously during the 6 h of hypoxia and the calculated concentration of CA in the plasma, assuming no loss, was nearly an order of magnitude higher than that available to plasma in the mammalian lung (Effros *et al.* 1980). Thus, the plasma CA activity, assuming some loss, was probably similar to that available to plasma flowing through the mammalian

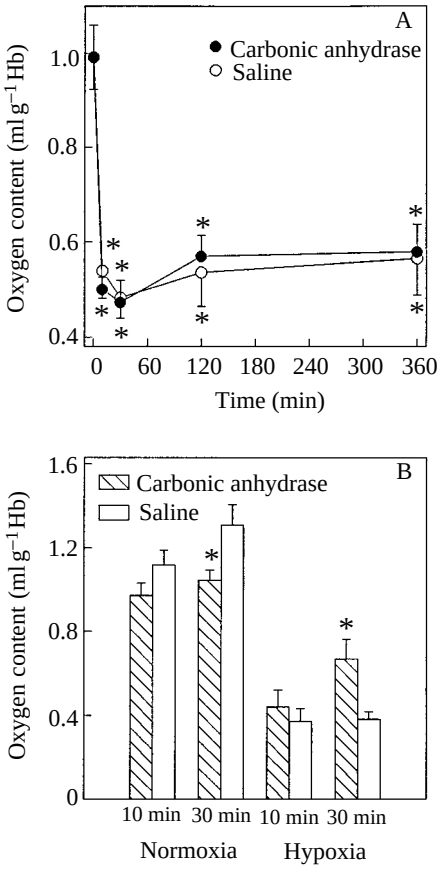

Fig. 7. Effects of carbonic anhydrase infusion on oxygen content during the first 6 h of hypoxia (A) and in normoxic and hypoxic rainbow trout after 48 h of acclimation (B). *Significant difference between normoxic and hypoxic animals. Values are mean ± S.E.M., N=7.

lung. This activity of CA was insufficient to alter the regulatory effects of catecholamines on RBC pH because there was no difference in either RBC pH or blood oxygen content between the saline- and CA-infused groups during the first 6 h of hypoxic exposure. The fact that there was a catecholamine effect is indicated by the marked increase in RBC volume in both groups. These results are in contrast to the *in vitro* experiments of Nikinmaa *et al.* (1990), who added $3\,g\,l^{-1}$ (rather than $0.3\,g\,l^{-1}$) CA to the blood and short-circuited the effects of catecholamines on RBC pH. Motais *et al.* (1989*b*), also *in vitro*, used a lower concentration ($0.5\,g\,l^{-1}$) than Nikinmaa, but similar to ours, and observed an increase of pHi in response to the addition of catecholamines. Based on the data of Motais *et al.* (1989*b*), it seems that to short-circuit the rise in pHi the concentration of CA needed is higher than that used in this study and, therefore, than that available to mammalian plasma in the lungs. The enzyme carbonic anhydrase has one of the highest known turnover numbers and for that reason the concentration of CA might not be the limiting factor. The difference in the results between the study of Nikinmaa *et al.* (1990) and the present study and that of Motais *et al.* (1989*b*) and the present study could be due the to difference in experimental conditions, especially plasma buffering, which would affect catalysed bicarbonate dehydration rates. In our experiments, infusion of CA caused a rise in pHe during normoxia (Table 2). This rise in pHe was enhanced during hypoxia (Fig. 4). Thus, the changes in pHe were different between saline- and CA-infused fish. In all cases, pHi was unaffected. Interpretation of the differential effects of catecholamines on pHi during hypoxia in CA- and saline-infused fish is confounded by these differences in pHe. Nevertheless, oxygen content was similar in both groups, indicating a comparable hemoglobin oxygen-affinity.

The increase in CA activity in fish plasma in these experiments had a marked effect on plasma total CO_2 content. $P_{a}CO_2$ in the saline-infused fish is determined by the rate of chloride/bicarbonate exchange across the RBC membrane and by ventilation (Perry, 1986). In the CA-infused group, $P_{a}CO_2$ will be determined largely by ventilation because plasma bicarbonate dehydration is catalyzed and CO_2 does not have to pass through the anion exchanger to be excreted. The end result is that the equilibrium $P_{a}CO_2$ level is higher in the saline-infused fish than in the CA-infused fish. Hypoxic exposure results in an increase in ventilation and therefore a small reduction in $P_{a}CO_2$ (Thomas *et al.* 1988), which contributes to the reduction in total CO_2 observed in fish exposed to hypoxia (Fig. 5A). The subsequent decrease in total CO_2 in CA-infused fish is probably related to acid titration of plasma bicarbonate. The increase in plasma lactate concentration during hypoxia was the same in both saline- and CA-infused groups (Fig. 3), indicating that approximately the same amount of metabolic acid was produced by both groups. The total CO_2 in the blood decreased more rapidly in the CA- than in the saline-infused group (Fig. 5A), presumably because plasma bicarbonate is titrated to a greater extent by the acid released by anaerobic metabolism in the CA-infused animals. This rapid removal of protons *via* bicarbonate dehydration resulting from CA infusion, either during the initial phase of hypoxia or after 48 h of hypoxic exposure, resulted in a marked alkalosis not seen in the saline-infused group. The bicarbonate concentration in the CA-infused group was much lower than that of the saline-infused group after 30 min of hypoxic exposure, but the subsequent rate of change of bicarbonate concentration was much the same in both groups for the next 6 h. During this time, blood pH in saline-infused fish remained constant; that is, the rate of proton entry into the blood was the same as the rate of proton excretion. In the CA-infused animals, blood pH fell after the initial alkalosis, so that the rate of proton entry exceeded the rate of proton excretion from the blood. It is possible, therefore, that CA infusion may have resulted in increased acid removal from muscle.

RBC volume increased upon catecholamine stimulation in both groups. This can be accounted for by the entry of ions, mainly Na^+ and Cl^-, with water following osmotically (Fievet *et al.* 1988; Baroin *et al.* 1984). CA infusion, however, caused a further increase in RBC volume after 30 min of exposure to hypoxia. This could be due to enhanced activity of the Na^+/H^+ exchanger in CA- compared with saline-infused fish. Such an enhancement has been shown to occur following a fall in blood

pH (Nikinmaa, 1990) such as that seen following CA infusion in hypoxic fish (Fig. 4). The levels of organic phosphates remained depressed in the CA-infused group (Val *et al.* 1995), probably because of increased ATP utilization by the Na^+/H^+ exchanger. Red blood cell volume approached that seen in normoxic animals in both groups after 48 h of exposure to hypoxia (Table 3). Catecholamine levels were still elevated, but the absence of a volume increase is to be expected as Thomas *et al.* (1991) observed a desensitization of the RBC response to catecholamines following chronic exposure to hypoxia.

RBC swelling resulted in the initial increase in hematocrit observed when fish were exposed to hypoxia; any release of red blood cells from the spleen was offset by RBC removal during sampling. Fewer samples were taken from the fish exposed to hypoxia for 48 h and, in these animals, there was an increase in both hematocrit and hemoglobin concentration, indicating catecholamine-induced release of RBCs from the spleen.

Hypoxia, as expected, resulted in a marked drop in oxygen content of the blood. Infusion of CA had no effect except in the 30 min sample after CA infusion following 48 h of exposure to hypoxia. Although not significantly different, ATP and GTP levels tended to be lower (Val *et al.* 1995) and RBC pH higher in CA- compared with saline-infused animals, so it is possible that hemoglobin oxygen-affinity was higher in CA-infused fish. Thus, at a given oxygen level, arterial blood oxygen content would be higher in the CA-infused fish exposed to hypoxia.

Why then is there no carbonic anhydrase activity available to blood plasma in fish? It is not simply to permit catecholamine-induced pH regulation of the red blood cell, because this could occur in the presence of low levels of CA. In general, the absence of CA activity reduces the titration of bicarbonate when protons are liberated from muscle into the blood, and the red blood cell is protected from this acidosis because the acid is transferred to the RBC more slowly than it would be if CA were present. In addition, the transfer of acid across the gill epithelium is not short-circuited by a rapid bicarbonate hydration/dehydration reaction in the plasma causing a rapid cycling of CO_2 between water and plasma. The uncatalysed CO_2/bicarbonate reaction in the plasma therefore facilitates blood pH regulation due to acid transfer across the gill epithelium.

In the presence of plasma CA activity, there is probably a reduced bicarbonate flux through the red blood cell during the oxygenation and deoxygenation process. Bicarbonate dehydration consumes the protons produced by hemoglobin oxygenation. A reduced bicarbonate flux through the red blood cell will result in larger oscillations in erythrocytic pH during oxygenation of the hemoglobin and may impair blood oxygen saturation in fish with a marked Root shift. A reduction in oxygen content was observed in fish infused with CA during normoxia (Fig. 7B). This was not seen in hypoxic fish because of the reduced hemoglobin oxygen-saturation imposed by the hypoxic conditions. Thus, the absence of CA activity in the plasma is coupled to oxygen transfer in fish, ensuring a large bicarbonate flux through the RBCs to remove protons produced upon oxygenation and preventing these protons from generating a Root shift and interfering with further binding of oxygen to hemoglobin.

Fish tolerate a plasma acidosis but protect the RBCs from pH changes and maintain oxygen transfer by having no CA activity available to the plasma. This also prevents large changes in plasma bicarbonate levels and allows the animal to adjust these levels in order to regulate pH. This is important because fish do not regulate pH by changes in $P_{a_{CO_2}}$ *via* ventilation. Mammals do have the capacity to adjust $P_{a_{CO_2}}$ *via* ventilation and have carbonic anhydrase activity available to the plasma as it flows through the lungs.

This work was supported by NSERC research grants to D.J.R.

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