J. exp. Biol. ***194****, 225–253 (1994)*
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CARBONIC ANHYDRASE INJECTION PROVIDES EVIDENCE FOR THE ROLE OF BLOOD ACID–BASE STATUS IN STIMULATING VENTILATION AFTER EXHAUSTIVE EXERCISE IN RAINBOW TROUT

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Accepted 23 May 1994

Summary

This study tests the hypothesis that the increase in blood P_{CO_2} and associated respiratory acidosis after exhaustive exercise play an important role in stimulating ventilation during post-exercise recovery in fish. Injection of bovine carbonic anhydrase ($10\,mg\,kg^{-1}$) into the bloodstream of rainbow trout caused a persistent 40 % increase in the HCO_3^- dehydration capacity of the blood. The treatment was designed to increase CO_2 excretion and therefore to reduce P_{CO_2} build-up and acidosis after exercise. Aerobic and anaerobic swimming performance were not affected by carbonic anhydrase, and there were only very minor effects on arterial blood acid–base status in resting fish. However, carbonic anhydrase attenuated post-exercise increases in P_{aCO_2} and decreases in pHa by about 50 % without altering arterial O_2 variables, red cell swelling or the intracellular pH of the brain or muscle tissues. The effects on arterial pH (pHa) resulted largely from alleviation of the increase in P_{aCO_2}. In accordance with the original hypothesis, normal post-exercise hyperventilation was greatly attenuated, through reductions in both ventilatory stroke volume and frequency, and excess post-exercise O_2 consumption was reduced. Post-exercise increases in plasma levels of adrenaline and noradrenaline were also reduced by the carbonic anhydrase treatment. Overall, there was a strong correlation between increases in relative ventilation and decreases in pHa after exhaustive exercise. The results provide functional significance for the phenomenon of P_{aCO_2} elevation and associated respiratory acidosis after exercise and are consistent with other recent studies indicating an important secondary drive to ventilation in fish based on arterial acid–base status, in addition to the primary drive based on arterial O_2 levels.

Introduction

It is now well established that severe, exhaustive exercise causes an increase in arterial P_{CO_2} in fish and that this is a major contributor to blood acidosis early in the recovery period. The cause remains unclear and controversial, with various workers advocating

Key words: *Oncorhynchus mykiss*, trout, carbonic anhydrase, ventilation, respiratory acidosis, catecholamines, exercise, red blood cell, CO_2 excretion.

adrenergic inhibition of HCO_3^- flux through the red blood cells (RBCs), the titration of plasma HCO_3^- to CO_2 by protons extruded from RBCs and/or muscle tissue, or disequilibria associated with gill transit time limitations as major causes (see Wood and Perry, 1985; Perry, 1986; Wood, 1991; Randall and Perry, 1992; Thomas and Perry, 1992; Nikinmaa and Vihersaari, 1993). The present paper addresses the possible functional significance of this response, rather than its origin, because, at first glance, a phenomenon that exacerbates extracellular acidosis at a time of intense excess post-exercise O_2 consumption (EPOC, classically termed 'O_2 debt'; Scarabello *et al.* 1992) appears to be maladaptive.

In particular, we tested the hypothesis first advanced by Wood and Perry (1985) that an important purpose of this post-exercise respiratory acidosis is to help to maintain hyperventilation during recovery from exhaustive exercise, thereby ensuring correction of the O_2 debt. The classical view has been that ventilation in fish is linked solely to an O_2 drive (Randall, 1982; Shelton *et al.* 1986). However, in the last few years there has been increasing evidence for an important secondary drive linked to pH and/or P_{CO_2} in both elasmobranchs (Heisler *et al.* 1988; Heisler, 1989; Graham *et al.* 1990; Wood *et al.* 1990) and teleosts (Perry and Wood, 1989; Kinkead and Perry, 1991). Such a pH/P_{CO_2}-based drive could play an important role in stimulating ventilation during the post-exercise period, when arterial O_2 levels are relatively normal and input from proprioceptors sensing muscle activity is presumably minimal.

In order to test this hypothesis, our goal was to alleviate the post-exercise respiratory acidosis by injection of bovine carbonic anhydrase into the circulation prior to exercise. Blood plasma normally lacks this enzyme; added carbonic anhydrase should increase the HCO_3^- dehydration capacity of the blood, thereby increasing CO_2 excretion and reducing P_{CO_2} build-up and acidosis after exercise. Our original rationale was that addition of exogenous carbonic anhydrase to the extracellular compartment should compensate for inhibited access of plasma HCO_3^- to RBC carbonic anhydrase caused by exercise-induced catecholamine mobilization (Wood and Perry, 1991; Perry *et al.* 1991). Shortly after the initiation of this project, two reports appeared showing that exogenous carbonic anhydrase added to trout blood *in vitro* would also attenuate the decrease in extracellular pH caused by adrenergic activation of RBC Na^+/H^+ exchange (Motais *et al.* 1989; Nikinmaa *et al.* 1990). The mechanism appears to be that carbonic anhydrase catalysis of HCO_3^- dehydration relieves a strong acid disequilibrium in the plasma caused by the protons extruded *via* Na^+/H^+ exchange. Thus, added carbonic anhydrase might alleviate both classical respiratory acidosis (due to $P_{a}{}_{CO_2}$ increase) and 'disequilibrium acidosis'.

In order to validate the experimental approach, it was first necessary to determine the extent to which exogenous carbonic anhydrase persisted in trout blood *in vivo*, its effects on resting blood acid–base and O_2 status, and its influence on both aerobic and anaerobic exercise performance. The next step was to evaluate its effectiveness in alleviating $P_{a}{}_{CO_2}$ elevation and the associated acidosis, and to determine whether arterial blood O_2 variables were altered. The final step was to determine the effects of carbonic anhydrase on post-exercise ventilation and O_2 consumption and to investigate possible causative mechanisms, such as alterations in blood acid–base status itself, plasma catecholamine

mobilization (Randall and Taylor, 1991) and brain acid–base status (Wood *et al.* 1990). The results provide functional significance in terms of ventilatory control for the phenomenon of $P_{a\mathrm{CO_2}}$ elevation and associated respiratory acidosis after exercise.

Materials and methods

Experimental animals and standard treatments

Experiments were performed on 102 adult rainbow trout [*Oncorhynchus mykiss* (Walbaum); 150–450 g] and 30 juvenile rainbow trout (1.5–4.5 g), obtained from Spring Valley Trout Farm, Petersburg, Ontario, and held at seasonal temperatures in flowing dechlorinated Hamilton tapwater (moderately hard water; composition as in Scarabello *et al.* 1992). The fish were acclimated to the experimental temperature (15±1 °C) for at least 7 days prior to experiments. All of the adult fish (series 1, 2, 4, 5, 6 and 7) were fitted with an indwelling dorsal aortic catheter for blood sampling and treatment injection (Soivio *et al.* 1972) and allowed to recover for 24–48 h in darkened individual fish boxes prior to testing. A standard protocol to induce exhaustion was used in all exercise experiments with adult fish. Each trout was transferred, without air exposure, to a 500 l tank and then vigorously chased for 6 min, following which the fish was returned to its chamber. Juvenile fish (series 3) were exercised aerobically as described below using a 150 l acrylic swimming respirometer (Graham and Wood, 1981).

In those experiments involving carbonic anhydrase injection, a standard dose of 10 mg kg^{-1} of bovine erythrocytic carbonic anhydrase (Sigma) dissolved in 1 ml kg^{-1} of Cortland saline was selected, on the basis of preliminary experiments with a range of doses. This represents approximately 25000 Wilbur-Anderson units kg^{-1} (1 Wilbur-Anderson unit causes the pH of 0.012 mol l^{-1} veronal to drop from 8.3 to 6.3 in 1 min at 0 °C). The carbonic anhydrase was washed in with a further 1 ml kg^{-1} of Cortland saline. Saline-injected control fish received 2 ml kg^{-1} of Cortland saline only.

Series 1

This series was designed to assess the stability of the chosen dose of carbonic anhydrase in the blood *in vivo* over the intended time course of subsequent experiments. Carbonic anhydrase activity was quantified by measurement of the HCO_3^- dehydration rate of the blood using the [^{14}C]bicarbonate technique of Wood and Perry (1991). This *in vitro* assay measures the rate at which plasma HCO_3^- is converted to evolved CO_2 when the blood is exposed to an external gas phase where the P_{CO_2} is essentially zero.

Fish (N=6) were injected with 10 mg kg^{-1} carbonic anhydrase *via* the dorsal aortic catheter at a time corresponding to just *after* control 1 (C1) in series 2, 4, 5 and 6. One hour later, a time corresponding to control 2 (C2), a 1.0 ml blood sample was withdrawn for measurement of HCO_3^- dehydration rate. A second 1.0 ml sample was withdrawn at 3.5 h, a time corresponding to 2 h post-exercise in these series. A pre-injection reference sample (1.0 ml) corresponding to C1 was taken from a separate group of trout (N=8). This approach was used so as to avoid depleting the haematocrit of the injected fish to the point (approximately 15 %) where the endogenous HCO_3^- dehydration rate due to the presence of RBCs becomes dependent upon haematocrit (Wood and Perry, 1991; Perry and

Gilmour, 1993). Blood samples were heparinized (175 i.u. ml^{-1}), tonometered for 15 min with a humidified precision gas mixture (P_{CO_2}=2 mmHg, P_{O_2}=155 mmHg, balance N_2) at 15±1 °C, and then processed through the [^{14}C]bicarbonate assay. The assay was performed exactly as described by Wood and Perry (1991), except that the radioisotope (2 μCi=74 kBq) was added in a different injection volume (50 μl, rather than 20 μl) of 5 mmol l^{-1} HCO_3^- Cortland saline. 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 of Amersham ACS II) and filter papers (in 10 ml of customized cocktail containing 2.0 g of PPO + 0.1 g of POPOP in 0.8 l of toluene + 0.2 l of 95 % ethanol).

Series 2

This series was designed to assess the influence of carbonic anhydrase injection on arterial blood acid–base, CO_2 and O_2 status in *resting* fish (N=8) over the intended time course for sampling in the exercise experiments. Fish were not exercised in this experiment, and carbonic anhydrase (10 mg kg^{-1}) was injected immediately after the C1 sample. Blood samples (600 μl) were withdrawn at C1 (1.5 h prior to intended exercise), at C2 (0.5 h prior to intended exercise) and 0, 0.25, 0.5, 1 and 2 h after the period of intended exercise. Samples were analysed for pHa, plasma Ca_{CO_2}, Pa_{O_2}, whole-blood Ca_{O_2}, haemoglobin, haematocrit and plasma lactate. Approximately 300 μl of blood from each sample was returned to the fish, together with sufficient non-heparinized Cortland saline to compensate for the volume deficit.

Series 3

The exhaustive exercise protocol used in series 4, 5, 6 and 7 induces both aerobic and anaerobic exercise. To assess whether the aerobic exercise performance was affected by carbonic anhydrase injection, critical swimming speeds (U_{crit}) were determined in saline and carbonic-anhydrase-injected trout using the methods of Brett (1964). Juvenile, rather than adult, trout were used for this experiment as the maximum speed of the swimming respirometer (Graham and Wood, 1981) was not sufficient to exhaust the adult fish. Fish were freeze-branded (Mighell, 1969) approximately 2 weeks prior to testing so that each fish could be identified visually when tested as part of a group in the respirometer. Approximately 1 h prior to testing, the fish were weighed and injected *via* the caudal vein with carbonic anhydrase (10 mg kg^{-1}) or saline alone, using the same total volumes of saline per unit mass as for adult fish. A modified 50 μl Hamilton syringe with a shortened needle was employed. Data were taken only from fish in which the injection was made cleanly into the vessel with a minimum of struggling and which exhibited no post-injection blood loss or necrosis.

After approximately 1 h in the respirometer, the flow was started at 6 cm s^{-1} (approximately 1 body length s^{-1}; BL s^{-1}) and increased in steps of 6 cm s^{-1} at 1 h intervals thereafter. Respiratory frequency (f_R) was recorded visually during each period. The exact time of exhaustion was recorded; fish were considered to be exhausted once they became impinged on the rear screen and were unresponsive to gentle prodding with a blunt rod. Exhausted fish were removed through a siphon tube (30 mm i.d.) while the test with the other fish remaining in the group continued until the last fish had stopped

swimming. Fork length was measured (typically 6–7 cm) and critical swimming speed (U_{crit}, in $BL\,s^{-1}$) for each fish was calculated according to Brett (1964). Three runs involving five fish from each treatment (10 fish per run) were performed and the results were combined (N=15 per treatment).

Series 4

This series with adult fish was designed to assess whether anaerobic exercise performance was affected by injection of carbonic anhydrase, by measuring the accumulation of lactate in white muscle. A second goal was to provide a preliminary indication whether carbonic anhydrase injection was successful in alleviating the normal post-exercise increase in P_{aCO_2}. Blood samples (600 μl, with replacements of 300 μl blood plus 300 μl saline) were drawn at C1, followed immediately by injection of carbonic anhydrase (N=6) or saline (N=6). A second pre-exercise sample was taken at C2. Exhaustive exercise was commenced 24 min later. At the end of 6 min of exercise (0 h post-exercise), a third blood sample was taken. The fish was then immediately killed by a blow on the head; a sample of epaxial white muscle was immediately excised from below the dorsal fin and freeze-clamped in liquid N_2. Blood samples were analysed for pHa, plasma C_{aCO_2}, P_{aO_2}, whole-blood C_{aO_2}, haemoglobin, haematocrit and plasma lactate. Muscle samples were analyzed for lactate and water content.

Series 5

This series concentrated on the blood acid–base, CO_2 and O_2 changes in the arterial blood of trout during the first 2 h of post-exercise recovery in the presence or absence of carbonic anhydrase. The blood sampling (600 μl, with replacements of 300 μl of blood plus 300 μl of saline) and measurement protocols (pHa, plasma C_{aCO_2}, P_{aO_2}, whole-blood C_{aO_2}, haemoglobin, haematocrit, plasma lactate) were identical to those of series 2. Fish were injected with either carbonic anhydrase (10 mg kg^{-1}; N=11) or saline (N=8) immmediately after the C1 sample. Exhaustive exercise (6 min) was commenced 24 min after the C2 sample. During recovery, blood samples were drawn at 0, 0.25, 0.5, 1 and 2 h post-exercise.

Series 6

The primary objective of this series was to determine whether carbonic anhydrase injection altered the magnitude or pattern of post-exercise hyperventilation and O_2 consumption. A secondary goal was to determine whether plasma catecholamine mobilization was altered. For this experiment, it was not possible to use trout fitted with ventilation masks for direct measurement of ventilatory water flow rate ($\dot{V}_W$) in a Van Dam chamber (e.g. Playle *et al.* 1990) because the mask interfered with normal swimming. We therefore we adopted an indirect approach based on the Fick principle (Dejours, 1973).

Trout of a uniform mass (about 350 g) were used for these experiments. In addition to the dorsal aortic catheter (PE 50, with a PE 160 grommet), each fish was fitted with a buccal catheter (PE 160, with a PE 240 grommet) for measurement of inspired O_2 tension (P_{IO_2}) and one opercular catheter on each side (PE 60, with a PE 200 grommet) for measurement of expired O_2 tension (P_{EO_2}) (Fig. 1). The catheters were positioned as

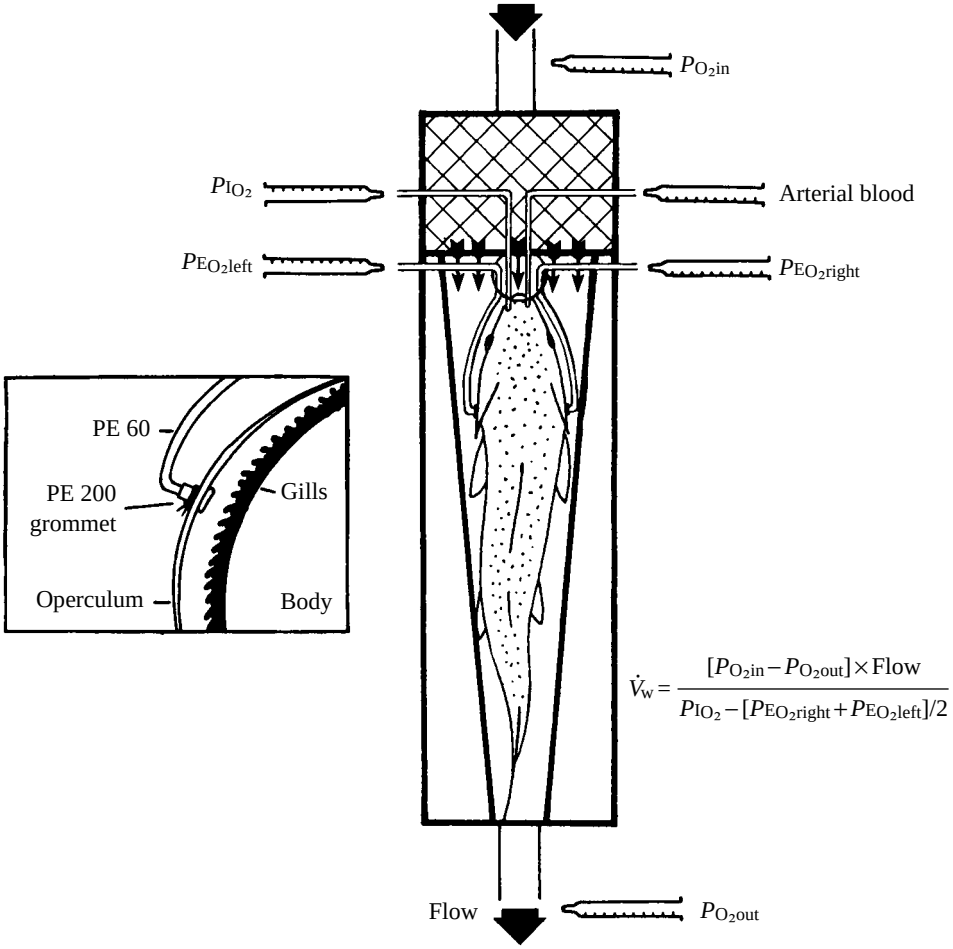

Fig. 1. Dorsal view of a rainbow trout, with water- and blood-sampling catheters attached, in the respirometry chamber used for determination of ventilatory water flow rate ($\dot{V}w$) by the Fick principle. The equation used is given. The inset illustrates the placement of an opercular catheter.

described by Holeton and Randall (1967; see their Fig. 1), but a 90° bend was heat-moulded into the PE60 immediately outside the grommet to allow the opercular catheters to trail backwards during exercise or forward during respirometry (Fig. 1). After surgery, the fish was allowed to recover for 48 h in a custom-built respirometry chamber fitted with sampling ports for measurement of O_2 tension in inflowing (P_{O_2in}) and outflowing water (P_{O_2out}). The respirometer was designed for minimum deadspace by tapering to fit the fish's body; total volume was 870 ml (approximately 520 ml with the fish in place). Water flow rate was $1\,ml\,g^{-1}\,min^{-1}$ for resting measurements and $2\,ml\,g^{-1}\,min^{-1}$ for post-exercise measurements. Water samples for O_2 measurements from the five sampling sites (P_{O_2in}, P_{O_2out}, P_{IO_2}, left P_{EO_2}, right P_{EO_2}) were siphoned continually (at a head of −1 cm) into glass sampling syringes.

Ventilatory water flow ($\dot{V}_W$) was calculated by the Fick principle, as illustrated in Fig. 1. Respiratory frequency (f_R) was counted visually, and respiratory stroke volume $V_{S,R}$ was calculated:

$$V_{S,R} = \dot{V}_W / f_R . \quad (1)$$

O_2 uptake ($\dot{M}_{O_2}$) was calculated as:

$$\dot{M}_{O_2} = \text{Flow} \times (P_{O_2 in} - P_{O_2 out}) \times \alpha O_2 , \quad (2)$$

where αO_2 was the O_2 solubility in fresh water at 15 °C (Boutilier *et al.* 1984). The percentage utilization of O_2 from water flowing over the gills (U_{WO_2}) was estimated as:

$$U_{WO_2} = \left[\frac{P_{IO_2} - [\text{right } P_{EO_2} + \text{left } P_{EO_2}]/2}{P_{IO_2}} \right] \times 100\,\% . \quad (3)$$

The experimental protocol was similar to that of series 2 and 5 with injections of 10 mg kg^{-1} carbonic anhydrase (N=12) or saline (N=15) immediately after C1, the C2 samples taken 1 h later, and 6 min of exhaustive exercise started 24 min after C2. Complete sets of water samples for respirometry, plus blood samples (700 μl with replacements of 300 μl of blood plus 400 μl of saline) were taken at C1 and C2 and at 0.25, 0.5, 1 and 2 h post-exercise. In this series, it was not feasible to take 0 h post-exercise samples because of the time needed to re-establish measurement conditions in the respirometry appararatus. Blood variables measured were pHa, plasma C_{aCO_2}, P_{aO_2}, haematocrit and plasma catecholamine concentrations, the latter in eight carbonic-anhydrase-injected and seven saline-injected fish only. Plasma for catecholamine analysis was immediately stored at −80 °C in the presence of 25 mmol l^{-1} reduced glutathione and 25 mmol l^{-1} EGTA.

Series 7

The primary goal of this final series was to test whether the ventilatory differences attributable to carbonic anhydrase injection in series 6 could be correlated with differences in the intracellular pH (pHi) of the brain. An additional objective was to determine whether post-exercise intracellular acidoses in red and white muscle were affected by carbonic anhydrase. Single, terminal blood and tissue samples for pHi measurements were taken from three treatment groups: saline-injected at rest (N=8); saline-injected, sampled 0.25 h after exhaustive exercise (N=7); and carbonic-anhydrase-injected (10 mg kg^{-1}), sampled 0.25 h after exhaustive exercise (N=7). In each case, the injection was administered at a time corresponding to C1.

Intracellular pHi was measured by the DMO (5,5-dimethyl-2,4-oxazolidinedione) technique; the method has been described in detail and critically evaluated previously (Milligan and Wood, 1985, 1986*a*,*b*; Wright *et al.* 1988). In brief, 12–13 h prior to the planned time of killing, trout were injected, *via* the dorsal aortic catheter, with a 1 ml kg^{-1} dose of 7 μCi ml^{-1} (=259 kBq ml^{-1}) [^{14}C]DMO (New England Nuclear; specific activity 1850 mBq mmol^{-1}) plus 28 μCi ml^{-1} (=1036 kBq ml^{-1}) [^{3}H]mannitol (New England Nuclear; specific activity 101.4 GBq mmol^{-1}) in 140 mmol l^{-1} NaCl, flushed in with an equal volume of Cortland saline. Mannitol was used as the extracellular space marker as it

penetrates the blood–brain barrier better than do other labels (Munger *et al.* 1991). At sampling, a 2 ml blood sample was withdrawn from the dorsal aortic catheter, and then the fish was immediately killed by a blow on the head. The whole brain, a sample of epaxial white muscle just below the dorsal fin and a sample of adductor mandibularis red muscle from the opercular region were excised and processed for pHi measurement. Arterial pH, plasma water content and plasma [^{3}H] and [^{14}C] radioactivities were measured on the blood samples. Water content and [^{3}H] and [^{14}C] radioactivities were measured on digested tissue samples. Digestion, scintillation counting and water content measurements on plasma and tissue samples for pHi determination were carried out exactly as described by Milligan and Wood (1986*b*) and Wright *et al.* (1988). Intracellular pH in each tissue sample was calculated using the relevant equations from Wright *et al.* (1988), employing pHa as representative of extracellular pH (pHe).

Analytical techniques

Haematocrit was determined by centrifuging 60 μl of blood in a heparinized capillary tube for 5 min at 5000 ***g***. Haemoglobin was measured by the cyanmethaemoglobin procedure (Blaxhall and Daisley, 1973), using Sigma reagents. Mean cell haemoglobin concentration (MCHC), an index of RBC swelling, was calculated as [haemoglobin] (g ml^{-1}) divided by haematocrit (ml ml^{-1}). Blood pHa, $P_{a_{O_2}}$ and $P_{a_{CO_2}}$ were measured directly using Radiometer electrodes thermostatted to the experimental temperature and displayed on PHM71 Mk2 acid–base analyzers. Whole-blood total oxygen concentration ($C_{a_{O_2}}$) and true plasma total carbon dioxide concentration ($C_{a_{CO_2}}$) were determined by the methods of Tucker (1967) and Cameron (1971), respectively, again using Radiometer electrodes. True plasma was obtained by breaking the sealed tubes used for haematocrit determination. To adjust for differences in haemoglobin concentration ([Hb]) and physically dissolved O_2 concentrations between fish, haemoglobin-bound O_2 per unit haemoglobin was calculated as:

$$[O_2]/[Hb] = (C_{a_{O_2}} - P_{a_{O_2}} \times \alpha O_2)/[Hb], \quad (4)$$

where $[O_2]/[Hb]$ is in μmol g^{-1}, $C_{a_{O_2}}$ in mmol l^{-1}, $P_{a_{O_2}}$ in mmHg, [Hb] in g l^{-1}, and αO_2 is the appropriate solubility coefficient for O_2 in trout plasma from Boutilier *et al.* (1984). An *approximate* conversion is 58 μmol O_2 g^{-1} Hb represents 4 mol O_2 mol^{-1} Hb (assuming 100 % saturation under ideal conditions).

Accurate P_{CO_2} measurements are difficult at the low tensions and temperatures present in fish blood. To obtain maximum accuracy, the recommendations of Boutilier *et al.* (1978) were followed to avoid 'memory' effects in the electrode. The electrode was pre-equilibrated to a P_{CO_2} close to the expected value and the sample was replaced at 2 and 4 min, with a final equilibration time of 6 min. For comparative purposes, $P_{a_{CO_2}}$ was also calculated from the Henderson–Hasselbalch equation using appropriate values for αCO_2 and pK′ for rainbow trout plasma from Boutilier *et al.* (1984) and for mammalian plasma extrapolated to fish temperatures from Severinghaus *et al.* (1956).

The 'metabolic acid load' ($[\Delta H^+_m]$) to the blood plasma after exercise was calculated in the cumulative fashion outlined by Milligan and Wood (1986*a*), using the following equation for each interval:

$$[\Delta H^{+}_{m}] = [HCO_3^{-}]_1 - [HCO_3^{-}]_2 - \beta(pHa_1 - pHa_2) \quad (5)$$

and summing (taking account of the sign) for each period from C1 onwards. Plasma $[HCO_3^{-}]$ was calculated from the direct measurements of Pa_{CO_2} and true plasma Ca_{CO_2}, and β, the non-bicarbonate buffer value for true plasma (in slykes, mmol HCO_3^{-} l^{-1} pH $unit^{-1}$) was estimated from the blood haemoglobin concentration ([Hb], g 100 ml^{-1}) at each time using the regression equation derived by Wood *et al.* (1982) for rainbow trout blood at 15 °C:

$$\beta = -1.271[Hb] - 2.31 . \quad (6)$$

Lactate in blood plasma and muscle tissue was measured enzymatically (L-lactate dehydrogenase/NADH method) on neutralized 6 % perchloric acid extracts using Sigma reagents, as described by Milligan and Wood (1986*b*). Muscle lactate concentrations were corrected for trapped extracellular lactate and expressed per litre of intracellular fluid using the extracellular fluid volume space estimates of Milligan and Wood (1986*b*), which were confirmed by the direct measurements of series 7 in the present study. Catecholamines (adrenaline and noradrenaline) were determined on alumina-extracted plasma samples by HPLC (Waters 510 pump, reverse phase C-18 column) with electrochemical detection (Waters M460).

All data are expressed as means ±1 S.E.M. (*N*). Statistically significant differences ($P \leqslant 0.05$) were assessed using paired or unpaired Student's two-tailed *t*-tests, as appropriate to the design of the experiments. Whenever multiple comparisons were involved, the *t*-value was adjusted by the Bonferroni procedure.

Results

Series 1

Injection of 10 mg kg^{-1} of bovine carbonic anhydrase into resting trout elevated the HCO_3^{-} dehydration rate of the blood by about 40 % (Table 1). There was essentially no attenuation of this increase over the 3.5 h time course of the intended experiments, indicating that carbonic anhydrase is quite stable when infused into the circulatory system.

Series 2

The effects of carbonic anhydrase injection on arterial acid–base and O_2 status were evaluated in resting fish over the time course of the intended experiments. Some small but significant changes were seen. These included an increase in pHa, a decrease in plasma $[HCO_3^{-}]$ and a decrease in Pa_{CO_2}, effects that persisted more or less throughout the whole experimental period (Table 2). Ca_{O_2} increased at the first sample after injection (C2), but decreased gradually thereafter. These effects reflected initial increases in haematocrit and [haemoglobin] and subsequent declines due to blood sampling (not shown); Pa_{O_2}, $[O_2]$/[Hb], MCHC and plasma [lactate] did not change (Table 2).

Saline injection controls were not performed in this experiment; however, it was evident that the changes associated with carbonic anhydrase injection were all exhibited

Table 1. *The influence of carbonic anhydrase injection (10 mg kg^{-1})* in vivo *on the HCO_3^- dehydration rate of trout blood*

Sample designation	Time	HCO_3^- dehydration rate (μmol ml^{-1} blood h^{-1})	Haematocrit (%)
C1	Pre-injection (N=8)	24.11±0.92	19.6±0.9
C2	1 h post-injection (N=6)	34.50±1.57*	20.8±1.9
2 h	3.5 h post-injection (N=6)	33.58±1.11*	14.7±1.9*

The fish were not exercised in this experiment but the sample designations C1, C2 and 2 h correspond to the sample times used in other experimental series.

Values are means ± 1 S.E.M.

Significantly different (P<0.05) from C1 value.

by the time of the C2 sample (Table 2). Therefore, the resting data of series 4, 5 and 6 (i.e. the C1 and C2 samples), where saline controls were performed, were employed to evaluate whether the observed changes in resting fish were specific to carbonic anhydrase. The results (Table 3) indicated that the effects could only be partially attributed to the actions of carbonic anhydrase. Saline injection alone caused significant decreases in $P_{a_{CO_2}}$ and [HCO_3^-], though these effects were only half the size of the changes induced by carbonic anhydrase. These small changes might have resulted from dilution or a small increase in ventilation induced by disturbance. $C_{a_{O_2}}$ increased slightly but not significantly with both treatments, probably reflecting the same phenomenon as the significant increase in $C_{a_{O_2}}$ in series 2 (Table 2), and indicating that the effect was not specific to carbonic anhydrase. However, a small increase in pHa was seen only with carbonic anhydrase. We conclude that, in resting fish, carbonic anhydrase injection induces only very minor decreases in $P_{a_{CO_2}}$ and [HCO_3^-] and increases in pHa, changes that are close to the limit of reliable detection.

Series 3

Carbonic anhydrase injection had no effect on aerobic swimming performance, as assessed by the U_{crit} of juvenile trout (Table 4). There were also no significant effects on respiratory frequency (f_R) at any stage in the aerobic swim test; the maximum values of f_R, and the frequencies in the period during which exhaustion finally occurred, are illustrated as representative values in Table 4.

Series 4

Carbonic anhydrase injection also had no effect on anaerobic swimming performance, as assessed by the intracellular concentrations of lactate in white muscle (Table 5) immediately after exhaustive exercise (time 0 h). This experiment also provided a preliminary indication that carbonic anhydrase injection reduced the extent of post-exercise $P_{a_{CO_2}}$ build-up and pHa depression, both of which were significantly attenuated

Table 2. *The influence of carbonic anhydrase injection (10 mg kg^{-1})* in vivo *on arterial blood variables in resting rainbow trout*

	Sample designation						
	C1	C2	0 h	0.25 h	0.5 h	1.0 h	2.0 h
Time post-injection (h)	Pre	1.0	1.5	1.75	2.0	2.5	3.5
pHa	7.708±0.025	7.817±0.019*	7.832±0.010*	7.853±0.010*	7.870±0.021*	7.838±0.020*	7.793±0.026
[HCO_3^-] (mmol l^{-1})	6.71±0.17	5.72±0.23*	5.72±0.23*	5.51±0.25*	5.68±0.21*	5.99±0.14*	4.99±0.16*
Pa_{CO_2} (mmHg)	3.68±0.27	2.98±0.16*	3.14±0.32	2.80±0.15*	2.75±0.16*	2.88±0.22*	3.27±0.29
Pa_{O_2} (mmHg)	87.2±5.0	92.1±4.1	92.2±7.1	104.4±4.2	102.9±4.0	100.2± 4.1	99.1±9.4
Ca_{O_2} (mmol l^{-1})	2.48±0.15	2.99±0.10*	2.67±0.13	2.45±0.12	2.24±0.13	2.13±0.18	1.98±0.16*
[O_2]/[Hb] (μmol g^{-1})	41.67±1.28	43.96±1.03	45.59±1.60	45.40±1.60	43.73±2.17	42.60±1.61	44.97±2.70
MCHC (g ml^{-1})	0.2780±0.0071	0.2803±0.0060	0.2781±0.0033	0.2863±0.0064	0.3023±0.0111	0.2979±0.0083	0.2815±0.0082
[lactate] (mmol l^{-1})	0.19±0.06	0.36±0.11	0.43±0.14	0.46±0.17	0.49±0.16	0.56±0.17	0.79±0.22

Means ± 1 S.E.M. (N=8).

*Significantly different (P<0.05) from C1 value.

The fish were not exercised in this experiment, but the sample designations C1, C2, 0, 0.25, 0.5, 1.0 and 2 h correspond to the sample times used in other experimental series.

Table 3. *A comparison of the effects of saline and carbonic anhydrase injections (10 mg kg^{-1})* in vivo *on arterial blood variables in resting rainbow trout*

	Saline		Carbonic anhydrase	
	C1	C2	C1	C2
pHa (N=29)	7.760±0.011	7.762±0.012	7.774±0.013	7.820±0.010*
$[HCO_3^-]$ (mmol l^{-1}) (N=29)	6.37±0.17	6.01±0.16*	6.49±0.24	5.51±0.22*
Pa_{CO_2} (mmHg) (N=29)	3.26±0.14	3.13±0.13*	3.36±0.13	3.08±0.12*
Ca_{O_2} (mmol l^{-1}) (N=15)	2.93±0.19	3.06±0.24	2.88±0.21	3.04±0.18

Means ± 1 S.E.M.
*Significantly different ($P \leqslant 0.05$) from C1 value.
Saline or carbonic anhydrase injections were administered immediately after the C1 sample, and the C2 sample was taken 1 h later.

relative to those of saline-injected controls (Table 5). Plasma lactate and ΔH_m^+ concentrations were not affected.

Series 5

This series extended the results of series 4, demonstrating that carbonic anhydrase injection reduced the post-exercise increase in Pa_{CO_2} by about 50 % relative to saline-injected controls. This difference persisted through 1 h of recovery (Fig. 2A). In concert, the post-exercise fall in pHa was also attenuated over the same time course (Fig. 2B). Decreases in plasma Ca_{CO_2} (not shown) and $[HCO_3^-]$ (Fig. 2C) were slightly greater in the carbonic anhydrase treament, but the differences were significant only at one time point (0.25 h; Fig. 2C). These data suggest that carbonic anhydrase attenuated mainly the respiratory component (i.e. elevated Pa_{CO_2}) of post-exercise acidosis, with minimal effect on the metabolic component (i.e. from fixed acidic equivalents). This was confirmed by calculation

Table 4. *The influence of carbonic anhydrase injection (10 mg kg^{-1})* in vivo *on aerobic exercise performance as assessed by critical swimming speed (U_{crit}), maximum respiratory frequency during swimming and respiratory frequency during the period in which exhaustion occurred*

	Saline (N=15)	Carbonic anhydrase (N=15)	P
U_{crit} (BL s^{-1})	6.42±0.26	6.08±0.26	NS
f_{Rmax} (min^{-1})	164.8±7.6	156.9±6.8	NS
f_{Rex} (min^{-1})	147.8±6.2	140.9±5.6	NS

Means ± 1 S.E.M.
NS, not significant; BL, body length.

Table 5. *The influence of carbonic anhydrase injection (10 mg kg^{-1})* in vivo *on anaerobic exercise performance as assessed by intracellular concentrations of lactate in white muscle and assorted plasma variables immediately after exhaustive exercise (time 0 h)*

	Saline (N=7)	Carbonic anhydrase (N=6)
Muscle [lactate] (mmol l^{-1})	55.61±3.83	53.52±6.44
Plasma [lactate] (mmol l^{-1})	4.12±0.55	2.89±0.35
pHa	7.377±0.049	7.517±0.016*
Pa_{CO_2} (mmHg)	6.36±0.20	5.39±0.25*
[ΔH^+_m] (mmol l^{-1})	4.75±0.85	4.46±0.95

Means ± 1 S.E.M.
*Significantly different ($P \leqslant 0.05$) from saline value.

of the plasma [ΔH^+_m], which exhibited no significant differences between the two treatments at any time after exercise (Table 6). Plasma [lactate] changes were also similar in the two groups early in recovery. Curiously, plasma [lactate] elevations were lower in carbonic-anhydrase-treated fish from 0.5 h onwards, a difference which reached 40 % by 2 h.

Despite these marked effects on blood-acid base variables, carbonic anhydrase treatment had virtually no effect on blood O_2 variables. Post-exercise changes in Pa_{O_2} (Fig. 3A), Ca_{O_2} (not shown) and [O_2]/[Hb] (Fig. 3B) were very similar, apart from a significantly lower Pa_{O_2} in the carbonic anhydrase group at 2 h (Fig. 3A). Haematocrit (not shown), [haemoglobin] (not shown) and MCHC (Fig. 3C) also underwent identical fluctuations in the two treatments, with a marked haemoconcentration (elevated haematocrit and [haemoglobin]) and red cell swelling (lowered MCHC) over the first 0.5 h after exercise. Indeed, in none of the experimental series was there any significant difference in [haemoglobin], haematocrit or MCHC between carbonic-anhydrase-injected fish and saline-injected controls.

Series 6

The primary focus of this series was on ventilatory effects (Fig. 4). However, blood acid–base responses were recorded for reference, and responses to carbonic anhydrase were very similar to those of series 5. These included attenuation of both the post-exercise acidosis (Fig. 5A) and the post-exercise elevation in Pa_{CO_2} (Fig. 6A), effects that were actually slightly greater and longer-lasting than in series 5 (Fig. 2). The carbonic-anhydrase-injected fish were significantly different from the saline-injected controls through 2 h post-exercise, at which time blood acid–base status had been restored close to resting levels.

Carbonic anhydrase injection had no effect on ventilatory variables at rest, but greatly attenuated the normal post-exercise hyperventilation (Fig. 4). In saline-injected control fish, $\dot{V}_W$ increased to about six times resting levels at 0.25 h post-exercise and remained elevated at 4.75 times resting levels through 2 h post-exercise (Fig. 4A). In contrast, $\dot{V}_W$ only increased about threefold at 0.25 h post-exercise and had returned to resting levels by 2 h in trout treated with carbonic anhydrase. This difference was due to a large

attenuation of the post-exercise increase in $\dot{V}_{S,R}$ (Fig. 4B) and a smaller reduction in the post-exercise increase in f_R (Fig. 4C). Differences between the two groups were significant throughout the post-exercise period.

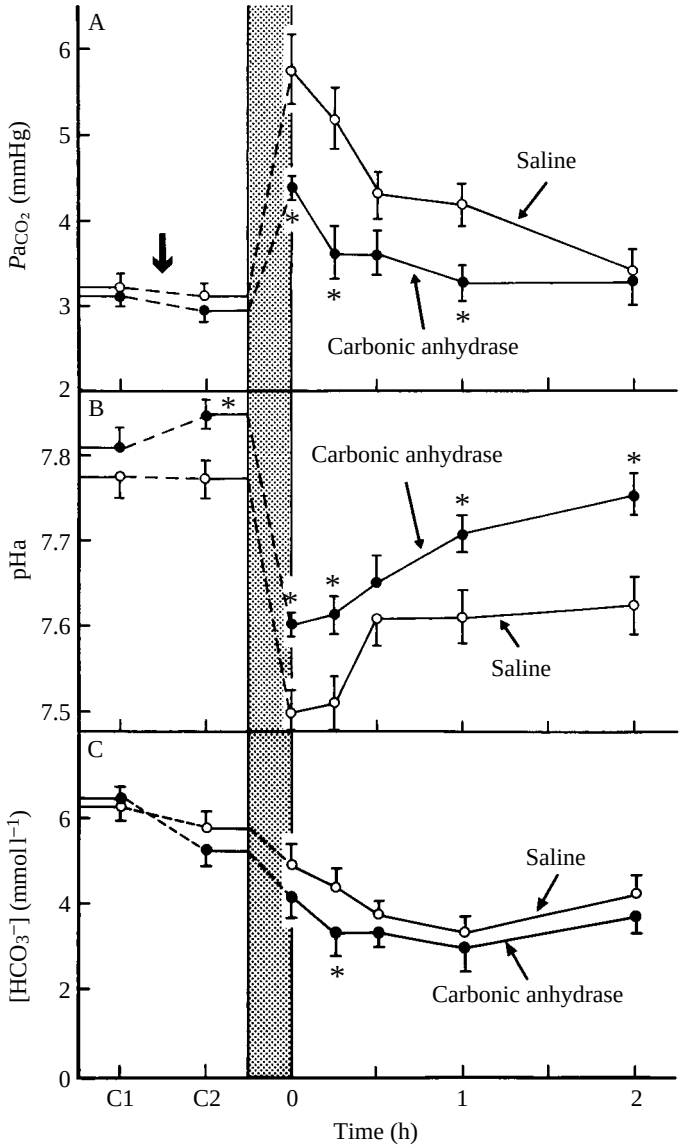

Fig. 2. The effects of 6 min of exhaustive exercise (vertical bar) on (A) the arterial carbon dioxide tension (P_{aCO_2}), (B) the arterial pH (pHa) and (C) the true plasma bicarbonate concentration ($[HCO_3^-]$) in the blood of rainbow trout injected intravascularly with either saline (open circles, N=8) or carbonic anhydrase (10 mg kg^{-1}, filled circles, N=11). Injections (arrow) were given immediately after the first control sample (C1) and the second control sample (C2) was taken 1 h later. Values are means ±1 S.E.M. Asterisks indicate significant differences ($P \leqslant 0.05$) between the saline and carbonic anhydrase treatments at the same sample time.

Table 6. *The influence of carbonic anhydrase injection (10 mg kg^{-1})* in vivo *on plasma metabolic acid load ([ΔH_m^+]) and plasma lactate concentrations in exhaustively exercised rainbow trout*

	Sample designation						
	C1	C2	0 h	0.25 h	0.5 h	1.0 h	2.0 h
[ΔH_m^+] (mmol l^{-1})							
Saline (*N*=8)	0	0.55±0.20	3.67±0.48	4.04±0.31	3.72±0.50	4.32±0.59	3.00±0.63
Carbonic anhydrase (*N*=11)	0	0.68±0.28	3.88±0.32	4.53±0.50	4.17±0.40	3.53±0.34	2.18±0.59
[Lactate] (mmol l^{-1})							
Saline (*N*=8)	0.53±0.09	0.48±0.05	3.23±0.43	5.47±0.75	8.30±1.33	10.67±2.15	11.17±2.49
Carbonic anhydrase (*N*=11)	0.37±0.06	0.62±0.11	2.76±0.24	4.45±0.52	5.21±0.76*	6.73±0.91*	6.66±1.22*

Means ± 1 S.E.M.
*Significantly different ($P \leqslant 0.05$) from comparable value in the saline treatment.
Carbonic anhydrase or saline injections were administered immediately after the C1 sample.

Carbonic anhydrase injection had no effect on resting $\dot{M}_{O_2}$, but altered the post-exercise increase in $\dot{M}_{O_2}$ (Fig. 5B). Despite the large attenuation of the hyperventilatory response, $\dot{M}_{O_2}$ was only slightly (and not significantly) lower in the carbonic anhydrase treatment at

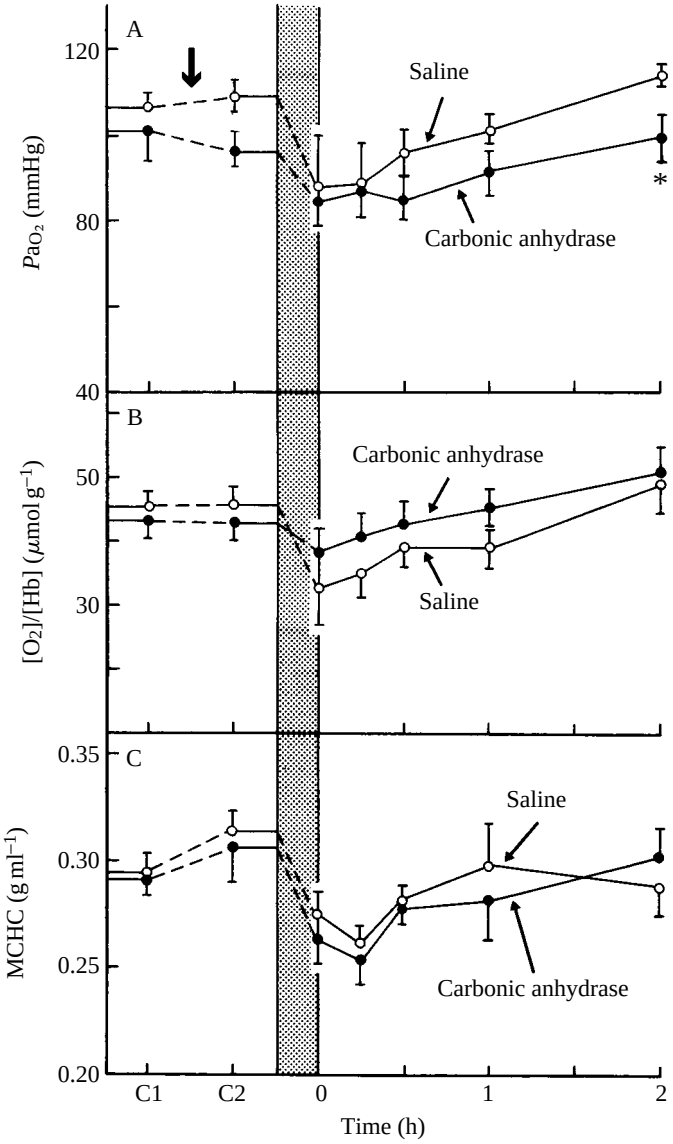

Fig. 3. The effects of 6 min of exhaustive exercise (vertical bar) on (A) the arterial oxygen tension (Pa_{O_2}), (B) the haemoglobin-bound oxygen per unit haemoglobin ($[O_2]/[Hb]$) and (C) the mean cell haemoglobin concentration (MCHC) in the blood of rainbow trout injected intravascularly with either saline (open circles, N=8) or carbonic anhydrase (10 mg kg^{-1}, filled circles, N=11). Injections (arrow) were given immediately after the first control sample (C1) and the second control sample (C2) was taken 1 h later. Values are means ±1 S.E.M. Asterisks indicate significant differences ($P \leqslant 0.05$) between the saline and carbonic anhydrase treatments at the same sample time.

0.25 h and 0.5 h post-exercise than in saline-injected control fish (Fig. 5B). However, at 1 h and 2 h, the difference became significant. Indeed, by 2h, $\dot{M}_{O_2}$ was no longer elevated above resting levels, in marked contrast to the controls. In accordance with these differences in $\dot{V}_W$ and $\dot{M}_{O_2}$, carbonic anhydrase greatly attenuated the post-exercise

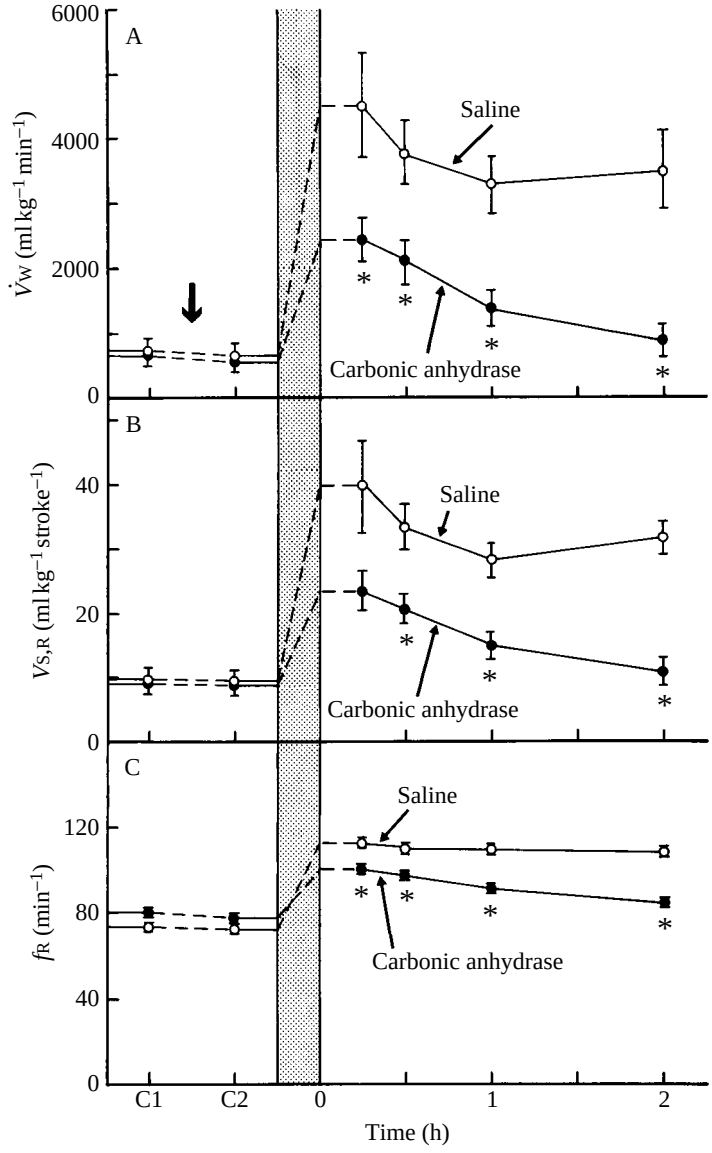

Fig. 4. The effects of 6 min of exhaustive exercise (vertical bar) on (A) the ventilatory water flow ($\dot{V}_W$), (B) the respiratory stroke volume ($V_{S,R}$) and (C) the respiratory frequency (f_R) of rainbow trout injected intravascularly with either saline (open circles, N=12) or carbonic anhydrase (10 mg kg^{-1}, filled circles, N=15). Injections (arrow) were given immediately after the first control sample (C1) and the second control sample (C2) was taken 1 h later. Values are means ±1 S.E.M. Asterisks indicate significant differences ($P \leqslant 0.05$) between the saline and carbonic anhydrase treatments at the same sample time.

decrease in U_{WO_2}, an effect that was significant throughout the recovery period (Fig. 5C). P_{aO_2} (not shown) followed a pattern similar to that seen in series 5 (Fig. 3A), with virtually identical values in the two groups until 0.25 h post-exercise, followed by a small

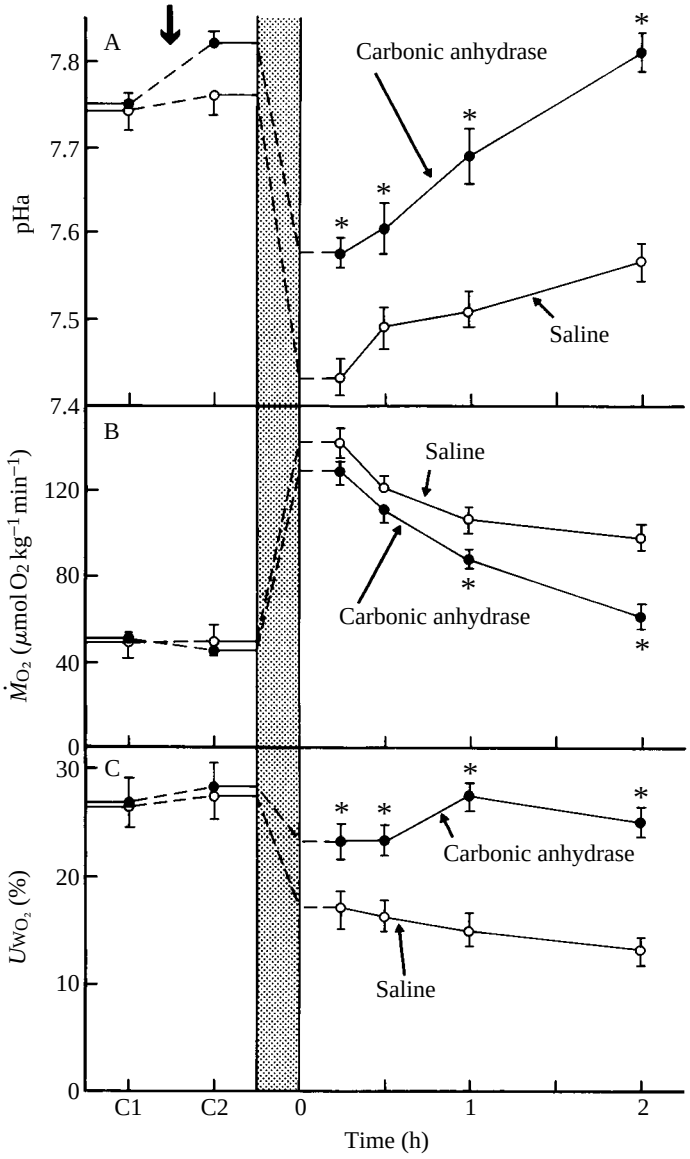

Fig. 5. The effects of 6 min of exhaustive exercise (vertical bar) on (A) the arterial pH (pHa), (B) the oxygen uptake ($\dot{M}_{O_2}$) and (C) the percentage utilization of oxygen from water flowing over the gills (U_{WO_2}) of rainbow trout injected intravascularly with either saline (open circles, N=12) or carbonic anhydrase (10 mg kg^{-1}, filled circles, N=15). Injections (arrow) were given immediately after the first control sample (C1) and the second control sample (C2) was taken 1 h later. Values are means ±1 S.E.M. Asterisks indicate significant differences ($P \leqslant 0.05$) between the saline and carbonic anhydrase treatments at the same sample time.

difference thereafter. $P_{a_{O_2}}$ was significantly lower in the carbonic anhydrase group than in the saline group at 0.5 h (75.6±3.6 *versus* 95.7±6.4 mmHg) and 1 h (86.3±3.0 *versus* 104.4±5.4 mmHg), but not at 2 h.

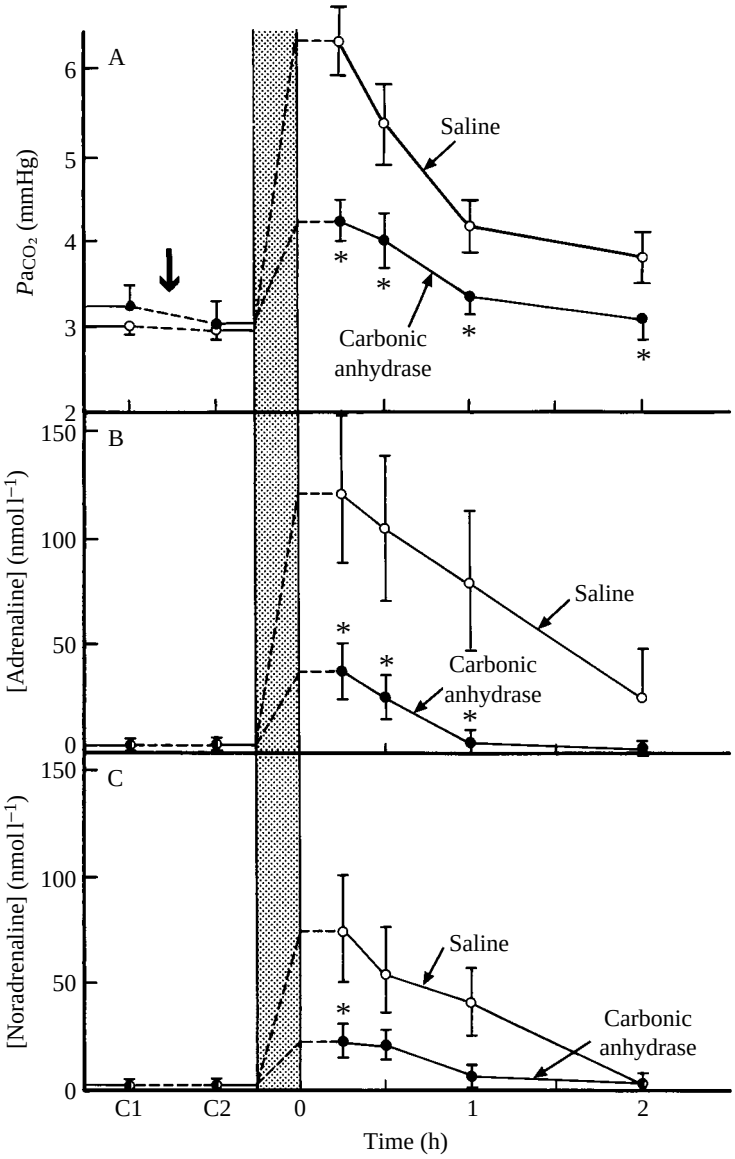

Fig. 6. The effects of 6 min of exhaustive exercise (vertical bar) on (A) the arterial carbon dioxide tension ($P_{a_{CO_2}}$) (B) the plasma adrenaline concentration and (C) the plasma noradrenaline concentration of rainbow trout injected intravascularly with either saline (open circles, N=12 for $P_{a_{CO_2}}$, N=7 for catecholamines) or carbonic anhydrase (10 mg kg^{-1}, filled circles, N=15 for $P_{a_{CO_2}}$, N=8 for catecholamines). Injections (arrow) were given immediately after the first control sample (C1) and the second control sample (C2) taken 1 h later. Values are means ±1 S.E.M. Asterisks indicate significant differences ($P \leqslant 0.05$) between the saline and carbonic anhydrase treatments at the same sample time.

The mobilization of plasma catecholamines was markedly affected by carbonic anhydrase injection (Fig. 6B,C). Peak levels of adrenaline (approximately 120 nmol l^{-1}) and noradrenaline (approximately 80 nmol l^{-1}) at 0.25 h post-exercise in control fish were reduced to approximately one-third of these levels by the carbonic anhydrase treatment. Although catecholamine levels declined thereafter in both groups, a significant difference for adrenaline persisted through 1 h of recovery.

Series 7

Intracellular pH (pHi) was measured in saline-injected fish at rest, and in both saline-injected and carbonic-anhydrase-injected animals at 0.25 h after exercise (Fig. 7). At rest, extracellular pH (i.e. pHa) was about 7.8, and intracellular pH was higher in brain (approximately 7.6) than in red or white muscle (both approximately 7.25). After exercise, the pH values of all compartments fell significantly. As before, the extracellular acidosis (i.e. fall in pHa) after exercise was reduced by carbonic anhydrase. Brain pHi fell by about 0.2 units after exercise, but this response was unaffected by carbonic anhydrase. In red and white muscle, pHi fell by about 0.25 and 0.45 units, respectively, but again this response was not significantly altered by the carbonic anhydrase treatment.

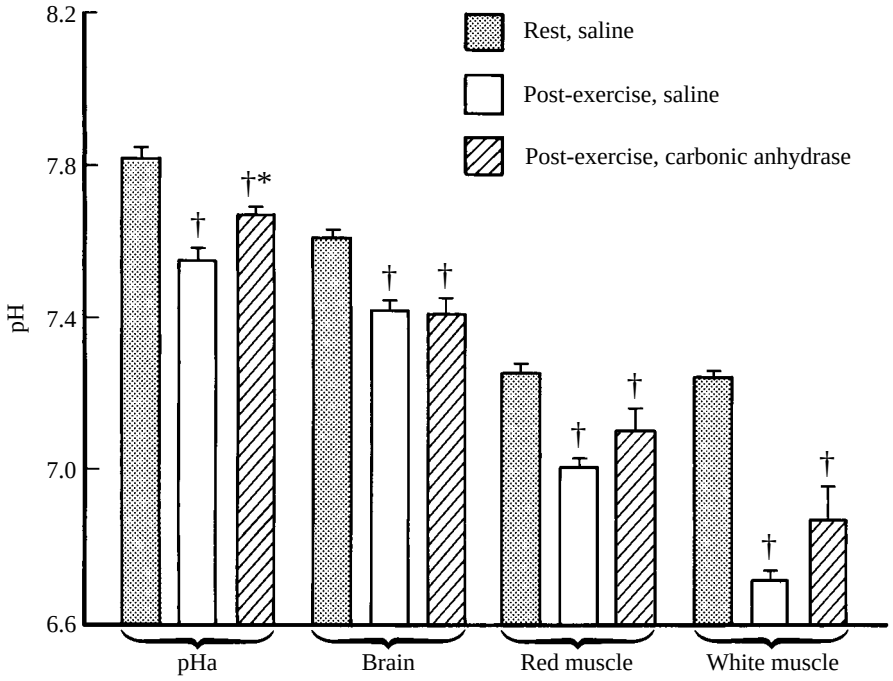

Fig. 7. Simultaneous measurements of arterial pH (pHa) and intracellular pH (pHi) in brain, red muscle and white muscle in rainbow trout at rest (saline injected, *N*=8) or at 0.25 h after exhaustive exercise (saline-injected, *N*=7, or carbonic anhydrase injected, *N*=7). In each case, injections were given at a time corresponding to C1 in other experiments. Values are means ±1 S.E.M. Daggers indicate significant differences ($P \leqslant 0.05$) with respect to the rest, saline-injected group. Asterisks indicate a significant difference ($P \leqslant 0.05$) between the two exercise groups.

Discussion

The use of carbonic anhydrase in vivo

Carbonic anhydrase injection was employed to test whether $P_{a_{CO_2}}$ elevation and the associated respiratory acidosis were involved in driving post-exercise hyperventilation. Our goal was to reduce the post-exercise increase in $P_{a_{CO_2}}$ and the resulting fall in pHa without directly interfering with either exercise performance or other responses to exercise. Overall, the approach was highly successful. The chosen dose ($10\,mg\,kg^{-1}$) was selected to compensate for an observed 35 % relative inhibition of the HCO_3^- dehydration rate (i.e. the rate of conversion of plasma HCO_3^- to CO_2) observed in blood samples taken from trout after exhaustive exercise (Wood, 1994). The exogenous carbonic anhydrase actually raised the blood HCO_3^- dehydration rate by about 40 % and persisted without reduction in the bloodstream *in vivo* throughout the 3.5 h experimental period (Table 1). The effects on resting blood acid–base and O_2 status were very small (Tables 2, 3). Exercise performance, assessed by both aerobic (U_{crit}; Table 4) and anaerobic (white muscle [lactate]; Table 5) criteria, was not impaired. Post-exercise changes in arterial O_2 variables (Fig. 3A,B), MCHC (Fig. 3C; indicative of RBC swelling), haematocrit and [haemoglobin] were largely unaffected, indicating that alterations in the primary O_2-based drive to ventilation did not occur to complicate the results. Most importantly, the treatment achieved about a 50 % reduction in the extent of post-exercise $P_{a_{CO_2}}$ elevation (Figs 2A, 6A; Table 5) and pHa depression (Figs 2B, 5A, 7; Table 5). However, there was one significant, and unexpected, complicating feature associated with the carbonic anhydrase treatment: the mobilization of plasma catecholamines associated with exhaustive exercise was reduced by about 65 % (Fig. 6B,C). The potential implications of this effect are discussed below.

Measurement versus *calculation of* $P_{a_{CO_2}}$

Throughout this study, $P_{a_{CO_2}}$ was measured, rather than calculated, to counter the argument that the elevation in post-exercise $P_{a_{CO_2}}$ is an artefact of using the Henderson–Hasselbalch equation at a time of 'disequilibrium acidosis'. In every series, $P_{a_{CO_2}}$ increased significantly after exercise, whether the values were measured directly or calculated indirectly using the Henderson–Hasselbalch equation from measurements of plasma $C_{a_{CO_2}}$ and pHa. Furthermore, this was true whether or not the fish were treated with carbonic anhydrase (which should relieve 'disequilibrium acidosis'; Motais *et al.* 1989; Nikinmaa *et al.* 1990), and irrespective of whether the pK′ values of Severinghaus *et al.* (1956) or Boutilier *et al.* (1984) were used in the calculations. Fig. 8 shows a representative data set, from series 5, and illustrates several interesting features. First, the post-exercise elevation of $P_{a_{CO_2}}$ was in fact larger and longer-lasting when measured directly than when calculated. Second, this was true whether or not the fish had been treated with carbonic anhydrase. Third, even when calculated in the presence of exogenous carbonic anhydrase, the post-exercise increase in $P_{a_{CO_2}}$ was still significant. Finally, under all conditions, $P_{a_{CO_2}}$ estimates calculated using the pK′ values of Severinghaus *et al.* (1956) were closer to measured values than were estimates calculated using the pK′ values of Boutilier *et al.* (1984). The latter was surprising, as the

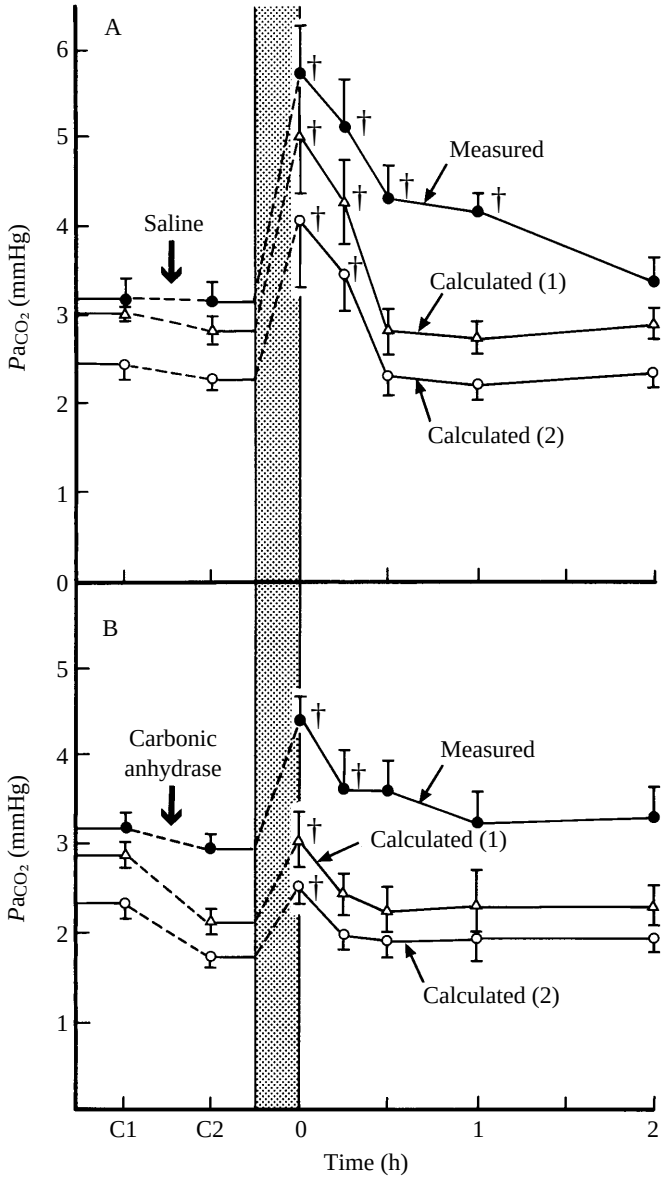

Fig. 8. A comparison amongst the directly measured values of arterial carbon dioxide tension (P_{aCO_2}; filled circles), values calculated from measurements of pHa and true plasma C_{aCO_2} by means of the Henderson–Hasselbalch equation using pK′ values from Severinghaus (1956; open triangles), and values calculated from the same measurements of pHa and C_{aCO_2} by means of the Henderson–Hasselbalch equation using pK′ values from Boutilier *et al.* (1984; open circles). In A, the trout were infused with saline (N=8), and in B with carbonic anhydrase (10 mg kg^{-1}; N=11) immediately after the first control sample (C1). In both cases, the second control sample (C2) was taken 1 h later. Values are means ±1 S.E.M. Daggers indicate significant differences ($P \leqslant 0.05$) after exercise from the corresponding C2 values. The vertical bar shows the period of exercise.

Severinghaus pK′ values are for mammalian plasma extrapolated to fish temperatures, while the Boutilier *et al.* (1984) pK′ values are based on rainbow trout plasma assayed at the correct temperature. Nevertheless, we conclude that the post-exercise increase in $P_{a_{CO_2}}$ is a real phenomenon and that the use of calculated $P_{a_{CO_2}}$ values will give a conservative estimate of the response.

The influence of carbonic anhydrase on $P_{a_{CO_2}}$ *and pHa*

The entry of plasma HCO_3^- into the RBCs through the band 3 HCO_3^-/Cl^- exchange protein for subsequent dehydration by erythrocytic carbonic anhydrase is generally considered to be the rate-limiting step in CO_2 excretion (Perry, 1986; Perry and Laurent, 1990). Recently, Perry and Gilmour (1993) have confirmed this *in vitro* using the radioisotopic assay of Wood and Perry (1991). In particular, they showed that lysis of trout RBCs, which makes erythrocytic carbonic anhydrase directly available to plasma HCO_3^-, will increase the rate of CO_2 excretion over a wide range of blood haematocrits and plasma HCO_3^- levels. However, in the present study, the addition of exogenous carbonic anhydrase to the blood plasma of resting trout *in vivo* had only very minor effects on $P_{a_{CO_2}}$, pHa and plasma HCO_3^- (Tables 2, 3). This result suggests that access of plasma HCO_3^- to erythrocytic carbonic anhydrase *via* Cl^-/HCO_3^- exchange is *not* an important limiting factor at rest *in vivo*. If there is an 'apparent diffusive limitation' on branchial CO_2 transfer as suggested by some models (e.g. Malte and Weber, 1985; Piiper, 1989), it most probably occurs at some other point in the system.

In contrast, after exhaustive exercise, the present results indicate that access of plasma HCO_3^- to erythrocytic carbonic anhydrase becomes an important factor in the apparent diffusive limitation on CO_2 excretion. The attenuation of $P_{a_{CO_2}}$ elevation by approximately 50 % by exogenous carbonic anhydrase occurs at a time of elevated CO_2 production by the tissues (Steffensen *et al.* 1987; Milligan and McDonald, 1988; Scarabello *et al.* 1992), elevated cardiac output (Neumann *et al.* 1983) and catecholamine mobilization (Fig. 6). *In vitro*, catecholamines cause a β_1-adrenergic inhibition of the HCO_3^- dehydration rate of trout RBCs (i.e. the rate of conversion of plasma HCO_3^- to CO_2) as measured by the [^{14}C]bicarbonate assay (Wood and Perry, 1991; Perry *et al.* 1991; Wood and Simmons, 1994). Blood sampled from exhaustively exercised trout also exhibits a reduction in HCO_3^- dehydration rate, compared with that of resting fish, when assayed *in vitro* under the same conditions (Wood, 1994). This contrasts with the observations of Tufts *et al.* (1988), who used a very different assay procedure; possible reasons for this difference have been discussed by Wood and Perry (1991) and Perry *et al.* (1991). Therefore, we speculate that, *in vivo*, catecholamine mobilization during and after exhaustive exercise similarly inhibits the rate of HCO_3^- dehydration through the RBCs and that this limitation, at a time of increased CO_2 loading to the system from metabolism and reduced gill transit time, results in an increase in $P_{a_{CO_2}}$.

It might be questioned why carbonic anhydrase injection only attenuated and did not abolish the increase in $P_{a_{CO_2}}$. The probable explanation is that insufficient carbonic anhydrase was used to block the phenomenon completely. Assuming a distribution volume about equal to the blood volume (approximately 50 ml kg^{-1}; Milligan and Wood,

1982), the concentration of carbonic anhydrase achieved *in vivo* was about 0.2 mg ml^{-1}, and the resulting activity measured (Table 1) agreed closely with an *in vitro* dose–response curve presented by Perry and Gilmour (1993). According to these authors, the level used here represents only about 30 % of a saturating concentration, or about a 40 % increase above the resting *in vivo* level for whole blood, in agreement with the present data (Table 1). At this time of increased CO_2 load to the system, it may be necessary to add more carbonic anhydrase than is needed to compensate for the 35 % inhibition relative to resting levels (Wood, 1994).

Attenuation of the post-exercise depression of pHa by carbonic anhydrase could result not only from the reduced $P_{a_{CO_2}}$ build-up (i.e. reduction of classical respiratory acidosis), but also from alleviation of 'disequilibrium acidosis' (see Introduction; Motais *et al.* 1989; Nikinmaa *et al.* 1990). For example, Motais *et al.* (1989) found that injection of a saturating dose of carbonic anhydrase (50-fold higher than that used here) into either a tonometer of trout blood or the extracorporeal blood loop of an intact rainbow trout greatly attenuated the decrease in blood pH caused by adrenergic activation of RBC Na^+/H^+ exchange. The $[\Delta H^+_m]$ calculation (equation 5) is a useful diagnostic tool because it uses the assumption of equilibrium conditions to subtract the respiratory component from the total acidosis. The remaining $[\Delta H^+_m]$ was the same in the saline and carbonic anhydrase treatments (Tables 5, 6). This suggests that, in the present experiments, the influence of carbonic anhydrase was explained mainly by its action in reducing respiratory acidosis.

Measurement of ventilation

It was necessary to use an indirect method based on the Fick principle (Dejours, 1973) in order to measure changes in $\dot{V}_W$ in trout which would freely exercise to exhaustion. This approach has been used in numerous studies and has been critically evaluated in a few. The principal limitation is the requirement for six different measurements (Fig. 1), the most problematic of which are the $P_{E_{O_2}}$ values. The approach works best in those species where the anatomy ensures that a well-mixed expired sample can be collected (e.g. skate; Graham *et al.* 1990). In trout, which have large, non-discrete opercular openings, the method has been criticized because of variability in $P_{E_{O_2}}$ over time and with respect to exact opercular catheter location (Davis and Watters, 1970). In our experience, the former is not a serious problem and can be minimized by continually siphoning through the catheters at a low rate, rather than aspirating samples from time to time. We sought to minimize the latter source of variability by standardizing the catheter location and by measuring $P_{E_{O_2}}$ separately for each opercular chamber. The resulting $\dot{V}_W$ determinations for trout at rest (approximately 700 ml kg^{-1} min^{-1}; Fig. 4A) were about twice those routinely measured in our laboratory at this temperature by use of ventilation masks on fish in Van Dam chambers (e.g. Playle *et al.* 1990). While it is possible that *absolute* values of $\dot{V}_W$ and $V_{S,R}$ were somewhat overestimated by the Fick approach used there, there is no reason to believe that the *relative* changes seen after exercise were in error.

The influence of carbonic anhydrase on post-exercise ventilation and $\dot{M}_{O_2}$

The most important finding of the present study was the marked attenuation of post-

exercise hyperventilation and EPOC caused by the administration of carbonic anhydrase prior to exercise (Figs 4, 5). This finding provides adaptive significance for the phenomenon of 'CO_2 retention' and strong support for the original hypothesis of Wood and Perry (1985) that 'the rise in $P_{a_{CO_2}}$ and/or the associated fall in plasma pH may help maintain hyperventilation during the post-exercise period, thereby ensuring correction of the O_2 debt'. The present experiments were not designed to separate the relative importance of elevations in $P_{a_{CO_2}}$ and decreases in pHa in driving hyperventilation and, in any event, the two are not independent variables.

Nevertheless, it was of interest to examine the relationships between ventilation and changes in $P_{a_{CO_2}}$, and between ventilation and changes in pHa. For this analysis, the $\dot{V}_W$

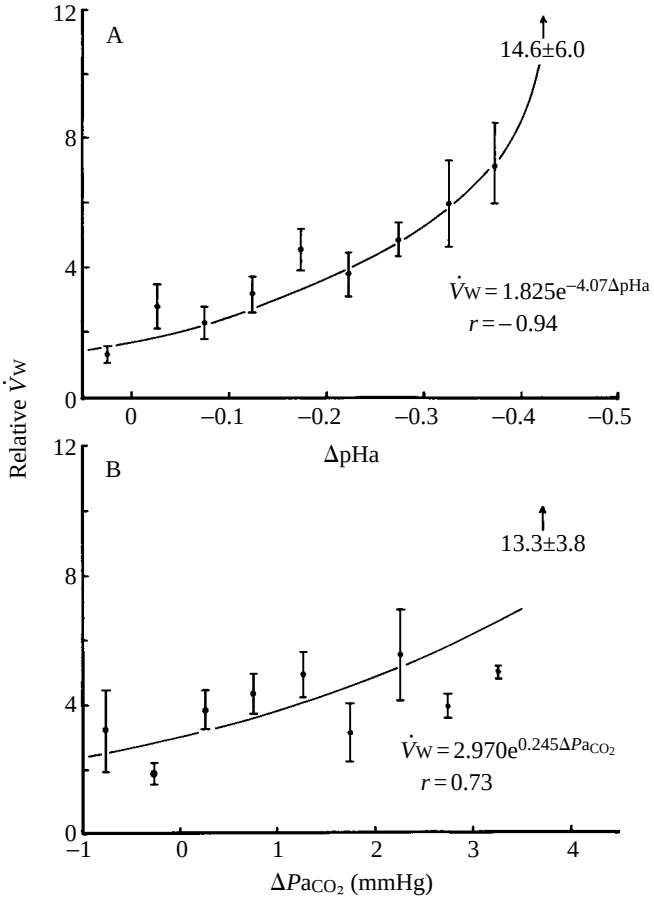

Fig. 9. Relationships between relative ventilatory water flow rate after exercise (actual value of $\dot{V}_W$ divided by value measured in the same fish at C2) and (A) the change in arterial pH (ΔpHa, with respect to the value measured in the same fish at C2); and (B) the change in arterial carbon dioxide tension ($\Delta P_{a_{CO_2}}$, with respect to the value measured in the same fish at C2). The analyses are each based on 91–98 data points from 12 carbonic-anhydrase-injected fish and 15 saline-injected fish. Means ±1 S.E.M. (N=3–29 at each interval).

for each fish of series 6 at each post-exercise time was expressed as a simple ratio to its $\dot{V}_W$ at C2, and absolute changes in $P_{a_{CO_2}}$ and pHa were calculated relative to their values at C2. Data were grouped over intervals of 0.5 mmHg $P_{a_{CO_2}}$ and 0.05 pH units respectively. In each case, the relationship between relative $\dot{V}_W$ and $\Delta P_{a_{CO_2}}$ or ΔpHa could be expressed as a simple exponential and was highly significant (Fig. 9). However, the correlation with ΔpHa ($r=-0.94$; Fig. 9A) was stronger than that with $\Delta P_{a_{CO_2}}$ ($r=0.73$; Fig. 9B). In this regard, it is interesting that two separate studies on elasmobranchs (dogfish, Heisler *et al.* 1988; Heisler, 1989; skate, Graham *et al.* 1990; Wood *et al.* 1990) found correlations between changes in arterial acid–base status and relative $\dot{V}_W$ under conditions where the primary O_2 drive to ventilation was more or less constant. In both, the correlation with ΔpHa was stronger than that with $\Delta P_{a_{CO_2}}$, as in the present investigation. Indeed, in the study of Heisler (1989), the slope of the exponential relating ΔpHa and relative $\dot{V}_W$ (−3.35) was very similar to that of the present study (−4.07; Fig. 9A).

Heisler (1989) suggested that a secondary respiratory drive might be provided by 'pH sensitive structures in, or closely related to, the arterial bloodstream'. The acid–base status of the brain is important in this regard in higher vertebrates (Shelton *et al.* 1986), and Wood *et al.* (1990) found that increases in ventilatory flow rates in the skate correlated with depressions in the pHi of the brain, as well as with depressions in pHa. However, in the present study, there was no difference in the mean pHi of the brain between carbonic-anhydrase- and saline-infused fish (Fig. 7) at a time when pHa was much lower and $\dot{V}_W$ much higher (Fig. 4A) in the former. Therefore, it appears more likely that the detector system in the trout responds directly to the pHa of the arterial blood.

The role of catecholamines

Our original idea was that carbonic anhydrase acted to alleviate the consequences of catecholamine mobilization – i.e. the elevation in $P_{a_{CO_2}}$ and plasma acidosis associated with β_1-adrenergic activation of RBC Na^+/H^+ exchange (Perry *et al.* 1991). Therefore, the reduction in $\dot{V}_W$ would be related directly to the alleviation of these effects. However, this interpretation is complicated by the unexpected finding of much lower plasma levels of catecholamines after exercise in carbonic-anhydrase-treated fish (Fig. 6B,C). Regressions between relative $\dot{V}_W$ and plasma adrenaline concentration or noradrenaline concentration or the sum of these two catecholamines (similar to those of Fig. 9) yielded much weaker but significant correlations ($r=0.27$–0.41, $N=52$–57). This raises several alternative scenarios. (i) Because these fish mobilized lower levels of catecholamines, there was less adrenergic stimulation of the RBCs and therefore less $P_{a_{CO_2}}$ build-up and acidosis, and therefore less stimulation of $\dot{V}_W$. (ii) Because these fish became less acidotic, they mobilized lower levels of catecholamines, and there was less effect on the RBCs, etc. (iii) Because these fish mobilized lower levels of catecholamines, there was less direct stimulation of $\dot{V}_W$ by catecholamines themselves. From the present results, we cannot eliminate any of these possibilities.

The role of *circulating* catecholamines in direct ventilatory stimulation remains highly controversial, at least in trout (Aota *et al.* 1990; Playle *et al.* 1990; Kinkead and Perry, 1990, 1991; Kinkead *et al.* 1991, 1993; Randall and Taylor, 1991; Perry *et al.* 1992;

Randall and Perry, 1992). The nature of the stimuli for catecholamine release is another area of controversy (see Randall and Perry, 1992; Thomas and Perry, 1992, for surveys of current ideas). Nevertheless, there certainly exists evidence that the extent of acidosis and the extent of catecholamine mobilization are related (Boutilier *et al.* 1986; Tang and Boutilier, 1988). We therefore favour some combination of the above scenarios such that the lower degree of acidosis and the reduced catecholamine mobilization were closely matched to one another by feedback loops in the carbonic-anhydrase-injected fish, resulting in a lower post-exercise ventilation. Whatever the exact details here, our overall conclusion is that $P_{a}CO_2$ elevation and the associated respiratory acidosis play an important role in driving hyperventilation after exhaustive exercise.

We thank Rod Rhem and Marnie Goldstein for excellent technical assistance. Supported by NSERC research grants to C.M.W.

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