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AN EVALUATION OF FACTORS LIMITING CARBON DIOXIDE EXCRETION BY TROUT RED BLOOD CELLS *IN VITRO*

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Summary

An evaluation of several potential factors limiting carbon dioxide excretion by rainbow trout (*Oncorhynchus mykiss*) red blood cells was performed *in vitro* using a recently developed radioisotopic assay. Red blood cell (RBC) CO_2 excretion was reduced by pre-treatment (30min) of blood with the carbonic anhydrase inhibitor acetazolamide (final nominal concentration 10^{-4} mol l^{-1}) or the Cl^-/HCO_3^- exchange inhibitor SITS (4-acetamido-4′-isothiocyanatostilbene-2,2′-disulphonic acid; 10^{-4} mol l^{-1}). The addition of bovine carbonic anhydrase to plasma stimulated CO_2 excretion in a dose-dependent manner, with maximal levels of CO_2 excretion achieved at a concentration of 3mg ml^{-1}. These results confirmed that carbonic anhydrase activity and/or Cl^-/HCO_3^- exchange velocity are potential limiting factors in CO_2 excretion.

Increasing the haematocrit elevated the rate of RBC CO_2 excretion, although the effect was apparent only between 0 and 15% haematocrit; the rate of CO_2 excretion was unaffected by further increases in haematocrit between 15 and 35%. Acute elevation of plasma HCO_3^- levels increased the rate of CO_2 excretion in blood but not in plasma (with or without added carbonic anhydrase). These data suggest that HCO_3^- availability may limit CO_2 excretion at higher haematocrits when the Cl^-/HCO_3^- exchange sites are most plentiful.

Lysis of RBCs and the accompanying release of intracellular carbonic anhydrase into the plasma significantly increased CO_2 excretion at all haematocrit and HCO_3^- levels, indicating that the velocity of Cl^-/HCO_3^- exchange does indeed limit trout RBC CO_2 excretion. The addition of carbonic anhydrase (3mg ml^{-1}) to lysed blood caused a further increase in the rate of CO_2 excretion but only at the low haematocrit of 5%. This result suggests that the activity of RBC carbonic anhydrase does not normally limit CO_2 excretion except at unusually low haematocrits, such as might occur during severe anaemia.

The rapid oxygenation of partially deoxygenated blood during the 3min assay caused a marked stimulation of CO_2 excretion that was concurrent with a significant decrease of RBC intracellular pH (pHi). These data indicate that the supply of Bohr protons during the oxygenation of the blood is a key factor limiting CO_2 excretion. Oxygenation of the blood prior to performing the assay also lowered RBC pHi, although CO_2 excretion was actually reduced, indicating a possible specific effect of pHi on Cl^-/HCO_3^- exchange

Key words: *Oncorhynchus mykiss*, rainbow trout, red blood cell, carbon dioxide excretion, carbonic anhydrase, hypoxia, band 3, Cl^-/HCO_3^- exchange.

activity or HCO_3^- dehydration. The results are discussed with reference to the control of carbon dioxide excretion in fish.

Introduction

In most fish, carbon dioxide is excreted across the gill into the ventilatory water owing to the rapid dehydration of plasma bicarbonate (HCO_3^-) to molecular (gaseous) CO_2 (for reviews, see Cameron and Polhemus, 1974; Randall and Daxboeck, 1984; Wood and Perry, 1985; Perry, 1986; Randall, 1990; Perry and Wood, 1989; Perry and Laurent, 1990). The dehydration reaction is catalyzed by the enzyme carbonic anhydrase contained within the red blood cells (RBCs) (Maren, 1967; Swenson and Maren, 1987; Rahim *et al.* 1988). The plasma HCO_3^- gains access to the intracellular carbonic anhydrase during the brief period when blood flows through the branchial vasculature because of the presence of a rapid anion exchanger (band 3 protein) on the RBC membrane (Romano and Passow, 1984; Hubner *et al.* 1992). During the process of CO_2 excretion, the anion exchanger extrudes Cl^- from the RBC in exchange for plasma HCO_3^-, a process termed 'the chloride shift' (Cameron, 1978; Obaid *et al.* 1979). The protons for the HCO_3^- dehydration reaction are provided largely by the dissociation of haemoglobin buffers. In theory, oxygenation of the haemoglobin should accelerate the rate of HCO_3^- dehydration, and hence overall CO_2 excretion, owing to the liberation of Bohr protons (for a review, see Jensen, 1991).

It has generally been assumed (e.g. Perry, 1986) that the process of RBC Cl^-/HCO_3^- exchange is the rate-limiting step in teleost CO_2 excretion because the velocities of the other steps (catalyzed HCO_3^- dehydration and CO_2 diffusion) are considered to be too rapid to limit overall CO_2 excretion. Surprisingly few studies, however, have evaluated experimentally the variety of potential factors limiting CO_2 excretion. This may reflect, at least in part, the inherent methodological problems associated with monitoring CO_2 excretion *in vivo* and the inappropriateness of the traditional *in vitro* techniques (e.g. Haswell and Randall, 1976) utilized to assess RBC CO_2 excretion. Recently, a radioisotopic assay has been developed (Wood and Perry, 1991; Perry *et al.* 1991) to assess RBC CO_2 excretion quantitatively *in vitro*; it utilizes physiological levels of plasma HCO_3^- and has sufficient sensitivity to detect subtle or rapidly occurring changes in the rate of HCO_3^- dehydration. In the present study, we have used the newly developed assay to evaluate several factors that could potentially limit CO_2 excretion by rainbow trout RBCs. Specifically, we have assessed the velocity of Cl^-/HCO_3^- exchange, the activity of intracellular carbonic anhydrase, the plasma HCO_3^- concentration and the availability of protons (for HCO_3^- dehydration) as variables potentially limiting carbon dioxide excretion in fish.

Materials and methods

Experimental animals

Rainbow trout [*Oncorhynchus mykiss* (Walbaum)] weighing between 175 and 250 g

(experimental *N*=251) were obtained from Linwood Acres Trout Farm (Campbellcroft, Ontario) and were transported in oxygenated water to the University of Ottawa.

Fish were maintained on a 12h:12h L:D photoperiod in large fibreglass aquaria supplied with flowing, aerated and dechlorinated City of Ottawa tapwater ($[Na^+]$= 0.10mmol l^{-1}, $[Cl^-]$=0.15mmol l^{-1}, $[Ca^{2+}]$=0.35–0.40mmol l^{-1}, $[K^+]$=0.03mmol l^{-1}, pH7.7–8.0). Fish were acclimated to these conditions for at least 4 weeks before experimentation. Water temperature in holding and experimental facilities varied between 10 and 12°C during the course of the experiments (May–September). Trout were fed daily to satiation using a diet of commercial trout pellets; food was withheld for 48 h prior to experimentation.

Animal preparation

Trout were anaesthetized in a 0.1 g l^{-1} solution of ethyl-*m*-aminobenzoate (MS 222; Sigma Chemical Company) adjusted to pH7.5 with $NaHCO_3$ and then placed onto an operating table to allow continuous retrograde irrigation of the gills with anaesthetic solution. To permit blood sampling, an indwelling cannula was implanted into the dorsal aorta (Soivio *et al.* 1975) using flexible polyethylene tubing (Clay-Adams PE 50; internal diameter 0.580mm, outer diameter 0.965mm). Trout were revived on the operating table by irrigation of the gills with aerated water, then transferred to individual opaque acrylic experimental chambers (volume 3l) supplied with aerated, flowing water, where they were allowed to recover from the effects of anaesthesia and surgery for at least 48 h before experimentation commenced.

Assessment of red blood cell CO_2 excretion in vitro

The technique for assessing CO_2 excretion within trout RBCs has been described in detail in a previous paper (Wood and Perry, 1991) and is therefore only briefly reiterated here. Approximately 20ml of whole blood was required for a typical single experimental run (i.e. *N*=1). Thus, it was necessary to use pooled blood obtained by slow withdrawal from the dorsal aortic cannulae of 4–5 fish (3–4ml per fish). The blood sampling was stopped if fish showed signs of agitation or struggling. Although not measured in the present study, this type of sampling protocol is known to avoid endogenously elevated catecholamine and lactate levels. Using an identical protocol, Wood and Perry (1991) reported that total plasma catecholamine (adrenaline plus noradrenaline) levels in pooled blood were always below 7nmol l^{-1}. 1ml samples of the pooled blood (stored on ice) or separated plasma were added to glass scintillation vials (20ml), which were then stoppered and gassed with a humidified gas mixture to yield a P_{CO_2} of 0.45kPa (3.38mmHg) and a P_{O_2} of 20.7kPa (155mmHg), remainder N_2 for 2h at ambient water temperature in a shaking water bath. The gas mixture was provided by a gas-mixing pump (Wösthoff model M 301a/f). In experiments where haematocrit was varied (nominal range 0–35%), the pooled blood was centrifuged (5900 ***g*** for 2min; 4°C) and appropriate volumes of homologous plasma were added or removed; separated plasma was also obtained in this manner. In all cases, the actual haematocrits were measured (see below); throughout the paper, the nominal values are reported because there was never significant deviation from the measured values.

74kBq (10 μl of 7400kBq ml^{-1}) of sodium [^{14}C]bicarbonate (in teleost Ringer; Wolf, 1963) was added to each 1ml of blood or plasma. The vial was then immediately sealed with a rubber septum, from which was suspended a plastic well containing a filter paper trap (150 μl hyamine hydroxide) for CO_2, and shaking was started. After exactly 3min of shaking, the filter was removed and assayed for ^{14}C activity. Whole-blood or plasma pH was determined and the remaining blood centrifuged (12000 ***g*** for 2min). The pellet was utilized to determine RBC pHi according to the freeze–thaw method (Zeidler and Kim, 1977). Samples of true or separated plasma were assayed for ^{14}C activity (50 μl) and total CO_2 (C_{CO_2}; 50 μl) to determine plasma HCO_3^- specific activity (disintsmin^{-1} μmol^{-1}). The CO_2 excretion rate for each assay vial was calculated by dividing filter paper ^{14}C activity by plasma specific activity and time.

Experimental protocol

Series 1

This preliminary series of experiments was designed to evaluate RBC Cl^-/HCO_3^- exchange and intracellular carbonic anhydrase levels as potential limiting factors in RBC CO_2 excretion. The experiments were performed using either separated plasma or whole blood at a constant haematocrit of 20%. The CO_2 excretion assay was performed using the naturally occurring levels of HCO_3^- in the pooled blood (4–6mmol l^{-1}). The carbonic anhydrase inhibitor acetazolamide (Sigma; final concentration 10^{-4} mol l^{-1}) dissolved in 140mmol l^{-1} NaCl was added (50 μl) to the blood/plasma 30min prior to the assay to allow it sufficient time to penetrate across the RBC membrane. The Cl^-/HCO_3^- exchange blocker SITS (4-acetamido-4′-isothiocyanatostilbene-2,2′-disulphonic acid; Sigma), dissolved in 2% dimethylsulphoxide (DMSO), or bovine carbonic anhydrase (2500Wilbur-Anderson units per mg) was added to blood/plasma immediately before the assay to yield final nominal concentrations of 10^{-4} mol l^{-1}/0.1% DMSO and 0.25–6.0mg ml^{-1}, respectively. Preliminary experiments as well as a previous study (Perry *et al.* 1991) demonstrated that addition of 50 μl of 2% DMSO was without effect on RBC CO_2 excretion; therefore, the controls in this series consisted of blood or plasma to which had been added 50 μl of 140mmol l^{-1} NaCl.

Series 2

In this series of experiments, the effects of haematocrit and acutely altered plasma HCO_3^- concentration were evaluated. Four final concentrations of HCO_3^- were utilized; the naturally occurring concentration (i.e. 4–6 mmol l^{-1}, hereafter referred to as 5 mmol l^{-1} HCO_3^- blood/plasma), 10mmol l^{-1} HCO_3^-, 15mmol l^{-1} HCO_3^- or 30mmol l^{-1} HCO_3^-. Plasma HCO_3^- levels were elevated abruptly at the start of the assay by 'spiking' the blood/plasma with 10–30 μl of sodium [^{14}C]bicarbonate (7400kBq ml^{-1}) prepared in teleost Ringer containing 500mmol l^{-1} $NaHCO_3$ instead of the usual teleost Ringer. Each HCO_3^- concentration (N=6 for each concentration) was tested at eight different haematocrits ranging from zero (plasma) to 35% (0, 2.5%, 5%, 10%, 15%, 20%, 25%, 35%). The nominal HCO_3^- levels were verified by analyzing the true plasma C_{CO_2}.

Series 3

In this series, the activity of the RBC membrane Cl^-/HCO_3^- exchanger and the levels of intracellular carbonic anhydrase were evaluated as limiting factors in RBC CO_2 excretion. This was accomplished by comparing the rates of CO_2 production in (i) control blood (N=6), (ii) lysed blood (N=6) and (iii) lysed blood containing an additional $3\,mg\,ml^{-1}$ bovine carbonic anhydrase (N=6). The blood cells were haemolyzed by subjecting the blood to high-frequency sonication for 10s using a micro ultrasonic cell disrupter (Kontes). Preliminary experiments demonstrated that this protocol was effective at causing total cellular lysis (i.e. no intact RBCs remained). The cell lysis or the addition of carbonic anhydrase to the lysed blood was performed immediately before the CO_2 excretion assay commenced. This series of experiments was performed using four haematocrits (0, 5%, 20%, 35%) and four levels of plasma HCO_3^- (5, 10, 15, $30\,mmol\,l^{-1}$).

Series 4

In this series, the effects of rapid oxygenation of the blood on RBC CO_2 excretion were assessed. The aim of these experiments was to simulate the situation *in vivo* whereby prebranchial deoxygenated blood is rapidly oxygenated as the blood flows through the gill vasculature. In theory, the oxygenation process should facilitate CO_2 excretion owing to the associated liberation of Bohr protons from haemoglobin (the Haldane effect) and the resultant acceleration of RBC HCO_3^- dehydration. In order to test this theory experimentally, it was necessary to modify the RBC CO_2 excretion assay so as to operate under open conditions in which the appropriate gases were continually flowing in and out of the assay vessel. This was achieved by connecting a second CO_2 trap [a 20ml glass scintillation vial containing 1.5ml of a CO_2-absorbing solution (Carbo-Trap 2; Baker)] in series with the primary reaction vessel (see Fig. 1). Thus, in these experiments RBC CO_2 excretion was calculated using the summed ^{14}C activities from the filter paper and the Carbo-Trap solution. Pilot experiments demonstrated that a single additional CO_2 trap was sufficient to remove all of the $^{14}CO_2$ from the flowing gas provided that the flow of gas was kept at $120\,ml\,min^{-1}$. At higher gas flow rates, the single additional trap was not capable of removing all of the $^{14}CO_2$. Thus, it was critical in these experiments to monitor the gas flow rate continually using flow meters (see Fig. 1).

The experimental design involved pre-incubating 1ml samples of pooled blood (as above) with a gas mixture (0.5% CO_2, 2.0% O_2, remainder N_2; P_{CO_2}=0.5kPa, P_{O_2}=2.0kPa) that was intended to simulate *in vivo* venous blood gas tensions in rainbow trout. One group was subjected to rapid oxygenation at the commencement of the 3min CO_2 excretion assay by switching the inflowing gas to pure O_2, while the other group was kept deoxygenated by switching the inflowing gas to 2% O_2 in N_2 at the beginning of the assay. CO_2 was purposely omitted from these gases to simulate the washout of CO_2 occurring at the gill.

To try to distinguish between the rapid effects of oxygenation in supplying H^+ for intracellular HCO_3^- dehydration and the possible effects of the RBC pHi change *per se* (or other long-term effects associated with oxygenation), an additional set of experiments

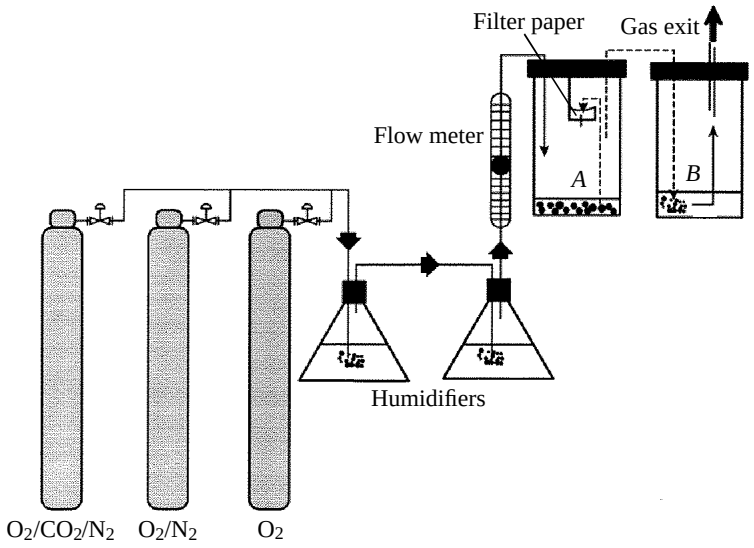

Fig. 1. A schematic representation of the arrangement used to measure whole-blood CO_2 excretion under open (gas flow-through) conditions (series 4). All blood was pre-incubated with a humidified gas mixture of 2% O_2/0.5% CO_2/97.5% N_2. At the time of assay, the blood samples were gassed with either 2% O_2/98% N_2 (deoxygenated blood) or pure O_2 (oxygenated blood). The flow of humidified gas was monitored and kept constant using flow meters. The gas was supplied to two 20ml vials connected in series. The first vial (*A*) contained the blood sample (1.0ml) and a filter paper (soaked in hyamine hydroxide) CO_2 'trap' while a second vial (*B*) containing 1.5ml of Carbo-Trap served as an additional CO_2 'trap'. The dashed lines represent the movement of gaseous $^{14}CO_2$. CO_2 excretion was determined by summing the $^{14}CO_2$ activities of the filter paper and the Carbo-Trap solution. See text for further details.

was performed in which RBC CO_2 excretion was assessed in blood pre-equilibrated with hypoxic (P_{O_2}=2.0kPa, P_{CO_2}=0.5kPa) or normoxic (P_{O_2}=20.8kPa, P_{CO_2}=0.5kPa) gases. In these experiments, the unmodified RBC CO_2 excretion assay was used.

Analytical procedures

Haematocrit was determined by centrifuging approximately 80 μl of blood in a heparinized capillary tube for 10min at 5000 ***g***. Whole-blood or plasma pH (pHe) and RBC intracellular pH (RBC pHi) were determined with a Radiometer micro-capillary pH electrode (G299A) maintained at the experimental temperature (10–12°C) in a Radiometer BMS3 Mk2 blood micro-system. Plasma C_{CO_2} was determined on 50 μl samples using a Corning model 965 CO_2 analyzer. Plasma HCO_3^- levels were calculated using the Henderson–Hasselbalch equation and the appropriate constants listed in Boutilier *et al.* (1984).

Plasma and filter paper ^{14}C activities were determined by liquid scintillation counting (Packard TR 2500) and automatically corrected for quenching. Plasma (50 μl), Carbo-Trap (1.5ml) and filter papers were counted using a commercial scintillation cocktail. The

plasma and filter papers were counted in 10ml of ACS II (Amersham), whereas the Carbo-Trap was counted in 18ml of OCS II so as to reduce colour quenching.

Statistical analysis

All values shown are means ± 1 standard error of the mean (S.E.M.). For multiple comparisons, the results have been statistically analyzed using factorial analysis of variance followed by Fisher's LSD multiple-comparison test. For two-sample comparisons, the unpaired two-tailed Student's *t*-test was used. In both cases, 5% was taken as the fiducial limit of significance.

Results

Series 1

Incubation of blood with the carbonic anhydrase inhibitor acetazolamide caused a 72 % reduction in the rate of CO_2 production (Fig. 2A) to yield a rate that was equivalent to that of separated plasma; acetazolamide did not influence the rate of CO_2 production in the absence of RBCs. Treatment of blood with the Cl^-/HCO_3^- exchange blocker SITS caused a 91% reduction in overall CO_2 production (Fig. 2A). Surprisingly, the rate of whole-blood CO_2 production after SITS treatment was lower than the rate observed using separated plasma. SITS did not affect the rate of CO_2 production in separated plasma (data not shown).

The addition of bovine carbonic anhydrase to separated plasma caused a dose-

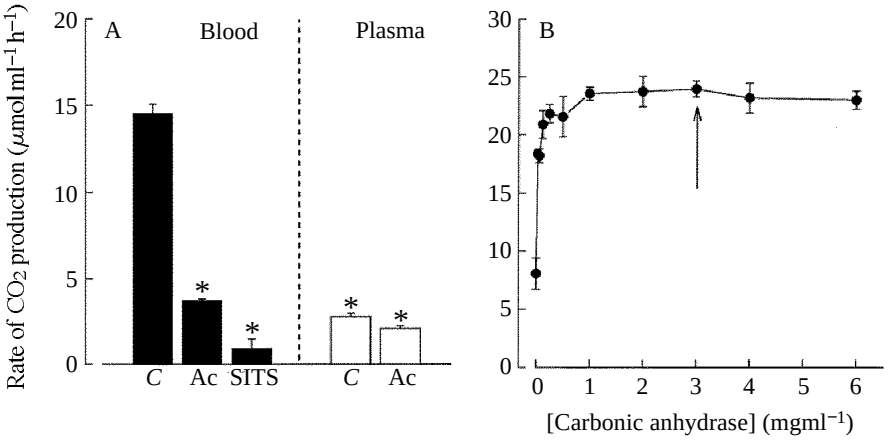

Fig. 2. (A) The effects of the carbonic anhydrase inhibitor acetazolamide (Ac) or the Cl^-/HCO_3^- exchange blocker SITS on the rate of CO_2 production in whole blood (haematocrit 20%; filled bars) or separated plasma (open bars; acetazolamide only); *C* refers to control (untreated) blood or plasma; * indicates a significant difference ($P<0.05$) from the rate of CO_2 production in control whole blood. (B) The effects of bovine carbonic anhydrase on the rate of CO_2 production in separated plasma; the arrow denotes the dose of bovine carbonic anhydrase (3mgml^{-1}) utilized in subsequent experiments. Values are mean ± S.E.M. (N=6).

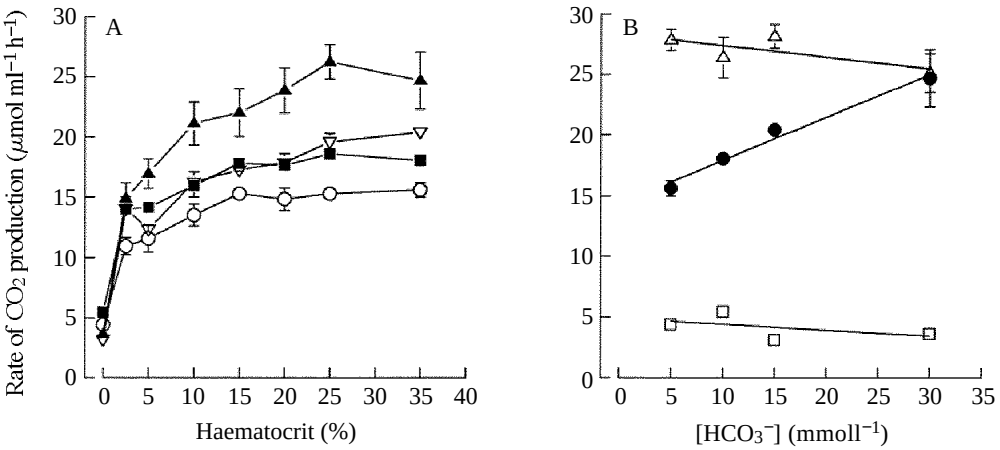

Fig. 3. (A) The interactive effects of whole-blood haematocrit (0–35%) and plasma HCO_3^- levels (5–30mmol l^{-1}) on the rate of CO_2 production (○, 5mmol l^{-1} HCO_3^-; ■, 10mmol l^{-1} HCO_3^-; ▽, 15mmol l^{-1} HCO_3^-; ▲, 30mmol l^{-1} HCO_3^-. (B) The relationship between plasma [HCO_3^-] and the rate of CO_2 production in whole blood (●), separated plasma (□) or separated plasma containing 3mgml^{-1} bovine carbonic anhydrase (△). There was a significant (P<0.05) correlation between plasma [HCO_3^-] and the rate of CO_2 production in whole blood (y=0.35x+14.4; r=0.99). Values are mean ± S.E.M. (N=6).

dependent elevation of CO_2 production between 0 and 1.0mgml^{-1}; at levels greater than 1.0mgml^{-1} there were no further increases in the rate of CO_2 production (Fig. 2B). In all subsequent experiments employing carbonic anhydrase, a dose of 3mgml^{-1} was utilized.

Series 2

Increasing the haematocrit caused an elevation of the rate of CO_2 production, although this effect was apparent only between 0 and 15% haematocrit; the rate of CO_2 production was unaffected by further increases in haematocrit between 15 and 35% (Fig. 3A).

Although there was considerable overlap of data (compare 10 and 15mmol l^{-1} HCO_3^-), it is nevertheless clear that acutely elevating plasma HCO_3^- levels increased the rate of CO_2 production in whole blood (Fig. 3A). This stimulatory effect of elevated [HCO_3^-] was absent in separated plasma while being most pronounced at the higher haematocrits (Fig. 3A). Fig. 3B illustrates the result of a separate group of experiments in which HCO_3^- levels were varied between 5 and 30mmol l^{-1} in (i) high-haematocrit blood (35%), (ii) separated plasma, and (iii) separated plasma containing 3mgml^{-1} carbonic anhydrase. In the whole blood, the rate of CO_2 production was a positive linear function of the plasma HCO_3^- concentration. However, CO_2 production was not correlated with HCO_3^- levels in separated plasma either with or without exogenous carbonic anhydrase.

Series 3

Haemolysis of the blood caused significant increases in the rate of CO_2 production at

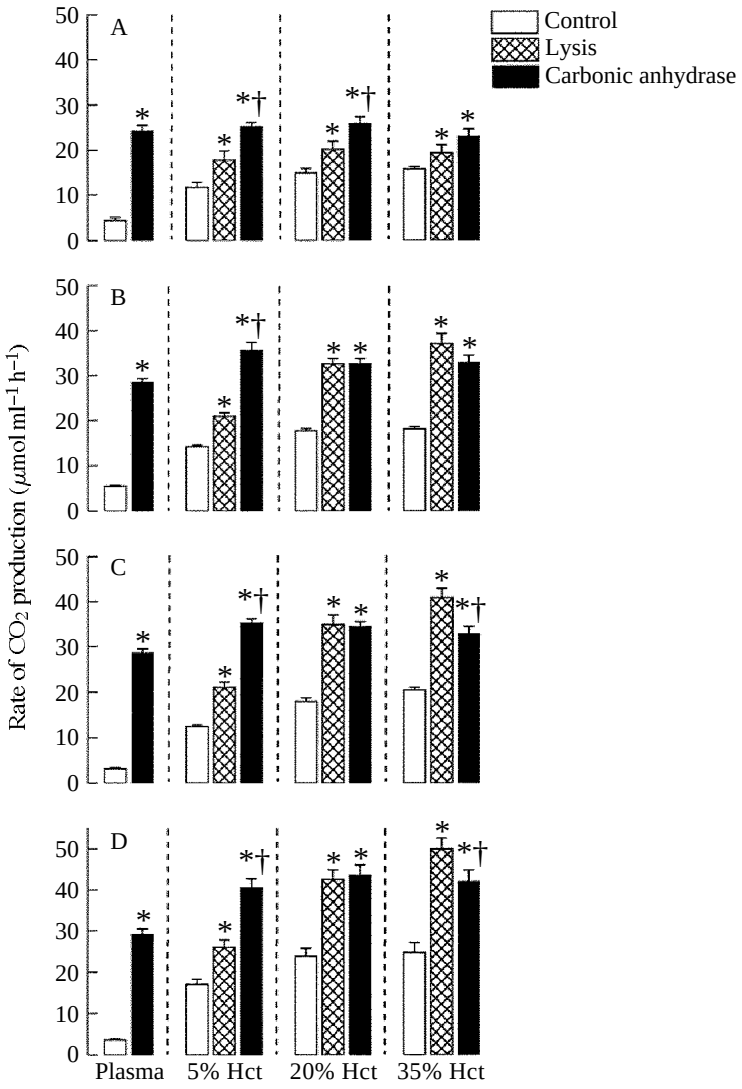

Fig. 4. CO_2 production in plasma or whole blood [5%, 20%, 35% haematocrit (Hct)] under control (untreated) conditions (open bars; *N*=6), after total RBC haemolysis (cross-hatched bars; *N*=6) or after the addition of 3mgml^{-1} bovine carbonic anhydrase to haemolyzed blood (filled bars; *N*=6). The experiments were performed using (A) 5mmol l^{-1} HCO_3^-, (B) 10mmol l^{-1} HCO_3^-, (C) 15mmol l^{-1} HCO_3^- and (D) 30mmol l^{-1} HCO_3^-. * indicates a significant difference (P<0.05) from the corresponding control value; † indicates a significant difference from the corresponding value in the haemolyzed blood (P<0.05). Values are mean ± S.E.M.

all haematocrits (5%, 20%, 35%) and extracellular HCO_3^- levels (5, 10, 15 and 30mmol l^{-1}; Fig. 4). The stimulatory effect of haemolysis on CO_2 production was greatest at the highest haematocrits and HCO_3^- levels (compare panels A and D in

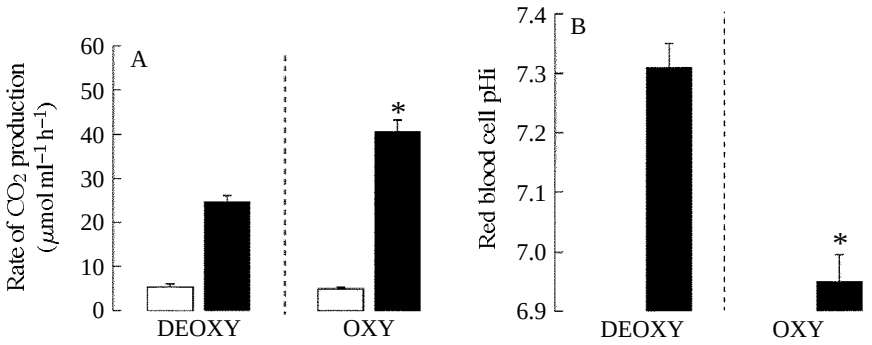

Fig. 5. The effects of rapid oxygenation of separated plasma (open bars) or whole blood (Hct 20%) on (A) the rate of CO_2 production and (B) red blood cell intracellular pH (pHi). * indicates a significant difference (P<0.05; N=6) from the corresponding value in the deoxygenated blood/plasma. Values are mean ± S.E.M.

Fig. 4). The addition of 3mgml^{-1} bovine carbonic anhydrase to previously lysed blood caused a pronounced stimulation of CO_2 production at all HCO_3^- levels in the low-haematocrit (5%) blood (Fig. 4). At higher haematocrits, there was a slight stimulation (Fig. 4A), no effect or a slight inhibition (Fig. 4C,D).

Series 4

The rapid oxygenation of whole blood (20% haematocrit) caused a pronounced increase in the rate of CO_2 production in comparison to the rate of CO_2 production from partially deoxygenated blood (approximately 50% haemoglobin O_2-saturation; Fig. 5A). Rapid oxygenation of deoxygenated blood elicited a decrease in RBC pHi from 7.30±0.05 to 6.95±0.05 (Fig. 5B). The rate of CO_2 production from separated plasma was unaffected by oxygen status (Fig. 5A).

In contrast, the rate of CO_2 production from blood that had been oxygenated prior to the assay was consistently lower than that from deoxygenated blood at the various haematocrits tested (Fig. 6A). In all cases, the RBC pHi was markedly lower in the oxygenated blood (Fig. 6B).

Discussion

Critique of the methods

The goal of this study was to evaluate as potentially limiting factors several of the multiple components of the CO_2 excretion pathway in fish. Included within the analysis were (i) the rate of RBC Cl^-/HCO_3^- exchange, (ii) the rate of the catalyzed dehydration of HCO_3^- to CO_2 by carbonic anhydrase and (iii) the availability of the substrates HCO_3^- and H^+ for the dehydration reaction. This was accomplished *in vitro* by using a sensitive radioisotopic assay (Wood and Perry, 1991; Perry *et al.* 1991) that monitors the evolution of $[^{14}C]CO_2$ from $[^{14}C]HCO_3^-$. As discussed in detail by Wood and Perry (1991), this technique offers several significant advantages over other commonly used

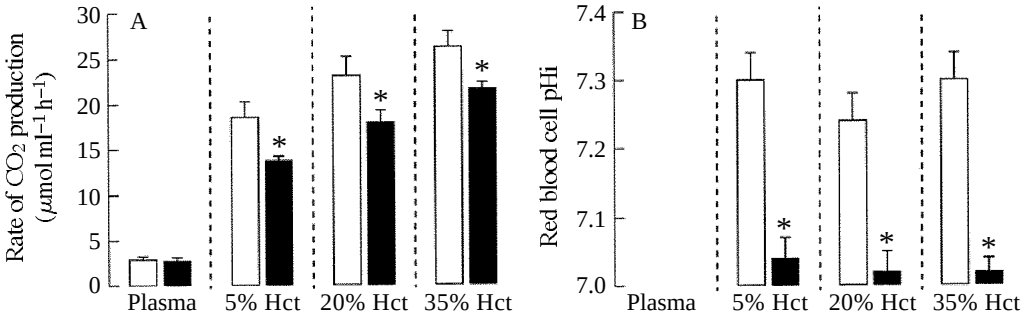

Fig. 6. (A) The rate of CO_2 production and (B) red blood cell intracellular pH (pHi) in separated plasma or whole blood [5%, 20%, 35% haematocrit (Hct)] pre-equilibrated and assayed under deoxygenated (open bars; *N*=6) or oxygenated (filled bars; *N*=6) conditions using a constant [HCO_3^-] of 5mmol l^{-1}. * indicates a significant difference (P<0.05) from the corresponding value in deoxygenated blood. Values are mean ± S.E.M.

methods (Heming and Randall, 1982; Tufts *et al.* 1988) because it avoids non-physiological buffer solutions, inappropriate acid–base values and abnormally high HCO_3^- gradients between the plasma and the RBC interior. The use of a high-capacitance CO_2 trap within the assay vessel (see Fig. 1 in Perry and Wood, 1991) maintains the P_{CO_2} gradient between the blood compartment and the gas phase and simulates the situation of blood flowing through the branchial vasculature *in vivo* where the ventilatory water P_{CO_2} is nearly zero. However, this assay, like all *in vitro* methods for determining the rates of CO_2 transfer between tissue, fluid and gas compartments, probably does not exactly simulate the kinetics of CO_2 exchange *in vivo* (see review by Klocke, 1988). This is probably a consequence of inadequate convection of the blood, leading to formation of unstirred layers which, in turn, may restrict the diffusion of CO_2. In addition, the 3min assay time is considerably longer than the residence time of blood within the gill (0.5–2.5s). Despite the relatively long duration of the assay, plasma [HCO_3^-] is reduced only by approximately 1mmoll^{-1} (Wood and Perry, 1991), which is similar to the reduction in plasma [HCO_3^-] during CO_2 excretion *in vivo* (Perry, 1986). Thus, it is unlikely that extracellular [HCO_3^-] or intracellular [Cl^-] (based on a 1:1 stoichiometric exchange of Cl^- for HCO_3^-) is a limiting factor for HCO_3^- dehydration during the *in vitro* assay.

The original assay of Perry and Wood (1991) was designed to operate in a sealed reaction vessel so as to prevent loss of evolved [^{14}C]CO_2 into the atmosphere. Consequently, the assay (as designed) could not be used to evaluate the effects of rapid oxygenation of the blood (and the associated liberation of Bohr protons) on RBC CO_2 excretion owing to the requirement for a fully open system. Thus, for this component of the study, the assay was modified to operate under open conditions. This was achieved by adding a second CO_2 trap in series with the original reaction vessel (see Fig. 1). Gas was allowed to flow in the system without the loss of [^{14}C]CO_2 to the atmosphere because the capacitance of the second trap (1.5ml Carbo-Trap solution) was sufficient to remove all of the [^{14}C]CO_2 from the inflowing gas, provided that the flow rate of the gas was kept at,

or below, 120mlmin^{-1}. Interestingly, the rate of whole-blood CO_2 excretion was approximately doubled under open conditions compared with closed conditions (compare Figs 5 and 6). This probably reflects an increase in the delivery (owing to convection) of the evolved CO_2 to the two traps rather than the addition of the second trap *per se*, because Wood and Perry (1991) demonstrated that the capacity of the single trap for CO_2 was well in excess of the rate of RBC CO_2 production. It is likely, therefore, that the open system provides a better estimate of the true rate of RBC CO_2 excretion. However, in the present study, no comparisons of data generated by the two different techniques were required and, indeed, it would be incorrect to compare the absolute levels of CO_2 excretion in the two methods.

Red blood cell Cl^-/HCO_3^- exchange as a limiting factor in CO_2 excretion

Numerous previous studies have provided indirect (Wood *et al.* 1982; Heming and Randall, 1982) or direct (Cameron, 1978; Obaid *et al.* 1979; Perr *et al.* 1982, 1991; Tufts *et al.* 1988) evidence for the involvement of RBC Cl^-/HCO_3^- exchange in CO_2 excretion in fishes. Owing to the slow velocity of Cl^-/HCO_3^- exchange in relation to other steps in overall CO_2 excretion (CO_2 diffusion, HCO_3^- dehydration), it has largely been assumed that RBC Cl^-/HCO_3^- exchange is a rate-limiting process in piscine CO_2 excretion (Perry, 1986; Piiper, 1989) as it is in mammals (see review by Bidani and Crandall, 1988).

The present study provides the first experimental data to support the theory of RBC Cl^-/HCO_3^- exchange as a rate-limiting factor in fish CO_2 excretion. The near total inhibition of RBC CO_2 production with the Cl^-/HCO_3^- exchange blocker SITS (Fig. 2) confirmed the requirement of this pathway to provide substrate (HCO_3^-) for intracellular H_2CO_3 dehydration. Although this experiment, in itself, did not demonstrate that Cl^-/HCO_3^- exchange is rate-limiting, it was nevertheless crucial to show that the production of CO_2 in this particular radioisotopic assay was indeed derived from the dehydration of plasma HCO_3^- *via* Cl^-/HCO_3^- exchange. When the need for this step was eliminated, by ultrasonic disruption of the RBC membrane, there was a marked stimulation of CO_2 excretion (Fig. 4). With a few exceptions (see below), the levels of CO_2 excretion obtained after membrane disruption were identical to those obtained after the addition of saturating levels (3mgml^{-1}) of bovine carbonic anhydrase, indicating that maximal rates of CO_2 production had been achieved. These data clearly indicate that the velocity of the Cl^-/HCO_3^- exchange pathway was not sufficient to allow maximal rates of CO_2 production and that this pathway must be a rate-limiting factor under the conditions of the *in vitro* assay. It is also important to note that non-HCO_3^- buffers, normally present in high concentrations in the RBC, would be released into the plasma during lysis and would still supply H^+ for HCO_3^- dehydration (see Bidani and Heming, 1991). Haemolysis of the blood also served to eliminate the RBC membrane as a barrier to CO_2 diffusion. It is extremely unlikely, however, that the increased rate of CO_2 excretion in haemolyzed blood was a result of the elimination of this diffusion barrier, because it is generally accepted that the diffusion of CO_2 across tissue barriers is extremely rapid and not rate-limiting (Piiper, 1989; Klocke, 1988).

It seems likely that the results of this *in vitro* study can be extrapolated to the *in vivo*

situation. First, *in vivo* the 'effective' diffusing capacity (overall diffusive conductance) of the gill for CO_2 is less than that predicted from the CO_2 diffusing capacity (Piiper, 1989). Second, the catalytic capacity of the dogfish (*Squalus acanthias*) RBC carbonic anhydrase is about 100 times greater than the measured rate of CO_2 excretion (Swenson and Maren, 1987). These apparent diffusion limitations for branchial CO_2 transfer (Malte and Weber, 1985) are most readily explained by the slow velocity of Cl^-/HCO_3^- exchange. Cameron (1978) determined the time constant of Cl^-/HCO_3^- exchange to be 400ms in rainbow trout blood. The transit time of blood in the gill is probably 0.5–2.5s, so it is conceivable that RBC Cl^-/HCO_3^- exchange continues in the post-branchial arterial blood.

Red blood cell carbonic anhydrase activity as a limiting factor in CO_2 excretion

The addition of acetazolamide to blood lowered the rate of CO_2 excretion to values observed using separated plasma (Fig. 2), confirming the essential role of intracellular carbonic anhydrase in these *in vitro* experiments. More importantly, the results of this study suggest that under certain conditions the activity of RBC carbonic anhydrase may be rate-limiting for overall CO_2 excretion. First, the rate of CO_2 excretion in haemolyzed blood was significantly greater (with the exception of the $5 mmol\,l^{-1}$ HCO_3^- blood) at the higher haematocrit values of 20 and 35% than at 5% (Fig. 4). Second, the addition of $3 mg\,ml^{-1}$ bovine carbonic anhydrase to previously haemolyzed blood significantly increased the rate of CO_2 excretion consistently at the low haematocrit value of 5% (Fig. 4). These results suggest that the activity of carbonic anhydrase may be rate-limiting in CO_2 excretion, but only at the unusually low haematocrits that might occur during severe anaemia (e.g. Wood *et al*. 1982). This potential limitation by intracellular carbonic anhydrase activity of CO_2 excretion at low haematocrits is consistent with the notion that fish RBCs contain significantly less carbonic anhydrase than do mammalian RBCs (Maren, 1967). It is unlikely, however, that CO_2 excretion is limited by RBC carbonic anhydrase activity at normal haematocrits (20–30% in rainbow trout).

The supply of HCO_3^- as a limiting factor in CO_2 excretion

In a preliminary series of experiments, Wood and Perry (1991) showed, using an identical assay, that the relationship between RBC CO_2 excretion and haematocrit was curvilinear but that acute elevation of plasma $[HCO_3^-]$ could further increase CO_2 excretion rates. In the present study, we have elaborated on those initial observations by performing a detailed analysis of the interactive effects of haematocrit and plasma $[HCO_3^-]$ on CO_2 excretion. The results confirm the curvilinear relationship between haematocrit and CO_2 excretion and the stimulatory effects of acute elevation of plasma $[HCO_3^-]$ (Fig. 3). The stimulatory effect of increasing haematocrit between 0 and 15% can be explained by the combined effects of the increasing levels of carbonic anhydrase in the blood and the greater numbers of Cl^-/HCO_3^- exchangers. Although carbonic anhydrase levels continue to increase at haematocrits above 15%, there was no further stimulatory effect on CO_2 excretion because the levels were presumably already in excess. Despite excessive carbonic anhydrase activity and increasing numbers of Cl^-/HCO_3^- exchange sites, the rate of CO_2 excretion remained constant as haematocrit

was raised above 15%. The absence of any further stimulation of CO_2 excretion at haematocrits above 15% may be related to insufficient quantities of HCO_3^-. Interestingly, the addition of HCO_3^- to plasma (with or without bovine carbonic anhydrase) did not affect CO_2 excretion, which suggests that the single mechanism underlying the stimulatory effect of increased [HCO_3^-] was an increase in the velocity of Cl^-/HCO_3^- exchange and that mass action effects were insignificant. These results demonstrate that rapid elevations of plasma HCO_3^- levels *in vivo* may transiently affect CO_2 excretion. The effect would be expected to be of short duration because the normal HCO_3^- gradient across the RBC membrane would be quickly re-established.

In contrast to the present study, Tufts *et al.* (1988) demonstrated a linear relationship between haematocrit and CO_2 excretion *in vitro* with no attenuation at the higher haematocrits. This may reflect the unusually high levels of HCO_3^- added to the blood at the onset of the assay (200mmol l^{-1}). It is likely, therefore, that the velocity of Cl^-/HCO_3^- exchange was not limited at the higher haematocrits by insufficient substrate, unlike in the present study. Similarly, Tufts *et al.* (1988) reported that the addition of SITS caused CO_2 excretion to be inhibited by only 41% (compared to 91% in the present study); this difference may also reflect the high levels of HCO_3^- used by Tufts *et al.* (1988).

The supply of H^+ as a limiting factor in CO_2 excretion

The conversion of HCO_3^- to CO_2 *via* the dehydration of H_2CO_3 requires a supply of protons. The protons are derived from the dissociation of non-HCO_3^- buffers (see Bidani and Heming, 1991, for a discussion of the importance of non-HCO_3^- buffers in CO_2 excretion). In addition to the buffering process, which is independent of oxygen, there is also a supplementary source of H^+ (Bohr protons) derived during oxygenation of the haemoglobin molecule (the Haldane effect). In mammals, this oxylabile component may constitute as much as 50% of overall CO_2 excretion (Klocke, 1988). Teleost fish are known to possess large Haldane effects (see review by Jensen, 1991); thus, the oxylabile component of CO_2 excretion is expected to be significant.

The results of this study permit for the first time a partitioning of O_2-independent and O_2-dependent CO_2 excretion in trout. The rapid oxygenation of partially deoxygenated blood (to simulate blood arriving at the gills) caused a 37% increase in the rate of CO_2 excretion *in vitro*. Thus, it would appear that the oxylabile component is approximately 40% of overall CO_2 excretion in this *in vitro* assay and that, by extrapolation, the O_2-dependent linked supply of Bohr protons is an important factor limiting CO_2 transfer *in vivo.*

Unlike the situation in mammals (see Klocke, 1988), the O_2-dependent component of CO_2 excretion in fish probably does not include a significant contribution from oxylabile carbonate because carbamino CO_2 in fish is considered to be less important than in mammals (Jensen, 1991). However, the radioisotopic assay used in these experiments only measures the excretion of CO_2 derived from plasma HCO_3^- and not from other potential sources such as oxylabile carbamate bound to haemoglobin.

In order to differentiate between the O_2-dependent release of Bohr protons and other effects of oxygenation, such as changes in RBC pHi, a series of experiments was

performed in which CO_2 excretion was determined in blood that had previously been deoxygenated or oxygenated. These results showed that prior oxygenation of the blood caused a pronounced reduction in the rate of CO_2 excretion in association with large decreases in RBC pHi (Fig. 6). These results suggest that one or more steps in the net conversion of plasma HCO_3^- to CO_2 (Cl^-/HCO_3^- exchange or H_2CO_3 dehydration) is influenced by intracellular pH.

This study has identified several variables that can affect the rate of CO_2 excretion, including haematocrit, plasma HCO_3^- concentration, velocity of Cl^-/HCO_3^- exchange and the extent of haemoglobin oxygenation. Under normal physiological conditions, *in vivo*, it is unlikely that haematocrit or plasma HCO_3^- concentration is a limiting factor for CO_2 excretion. During severe anaemia (Hct<5%), however, the low activity of carbonic anhydrase in the blood may limit CO_2 excretion. On the basis of extrapolation of the present results, it would seem that the two factors limiting CO_2 excretion *in vivo* are the velocity of RBC Cl^-/HCO_3^- exchange and the supply of Bohr protons during the oxygenation of haemoglobin.

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