# 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

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$^+$]= 0.10mmol1$^{-1}$, [Cl$^-$]=0.15mmol1$^{-1}$, [Ca$^{2+}$]=0.35–0.40mmol1$^{-1}$, [K$^+$]=0.03mmol1$^{-1}$, 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 48h prior to experimentation.

### *Animal preparation*

Trout were anaesthetized in a $0.1\,gl^{-1}$ 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 48h 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 7nmol1$^{-1}$. 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 7400kBqml$^{-1}$) 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 (50μl) and total $CO_2$ ($C_{CO_2}$; 50μl) 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 0.25–6.0mgml$^{-1}$, 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. 4–6mmol 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 (7400kBqml$^{-1}$) 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 $3\,\mathrm{mg\,ml}^{-1}$ 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 $N_2$; $P_{CO_2}=0.5$kPa, $P_{O_2}=2.0$kPa) 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
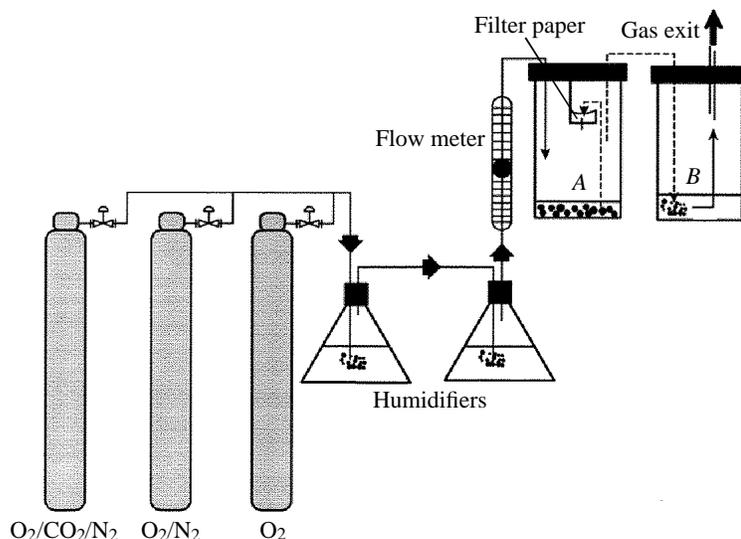
Fig. 1. A schematic representation of the arrangement used to measure whole-blood $CO_2$ excretion under open (gas flow-through) conditions (series 4). All blood was pre-incubated with a humidified gas mixture of 2% $O_2$/0.5% $CO_2$/97.5% $N_2$. At the time of assay, the blood samples were gassed with 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).

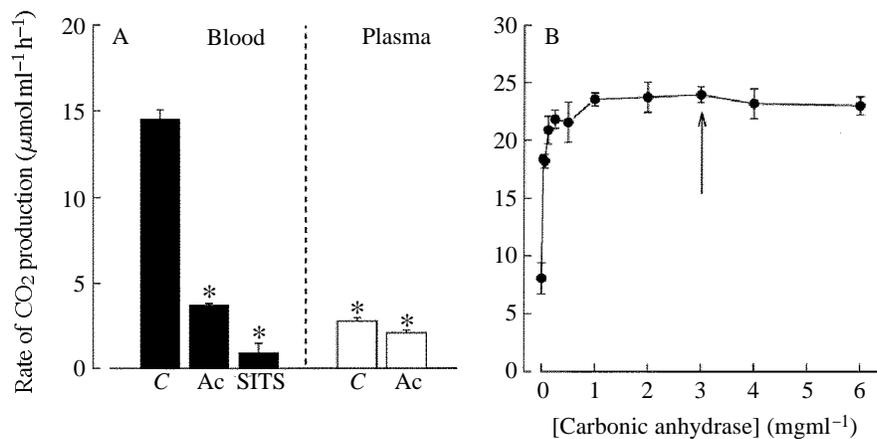The addition of bovine carbonic anhydrase to separated plasma caused a dose-



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).
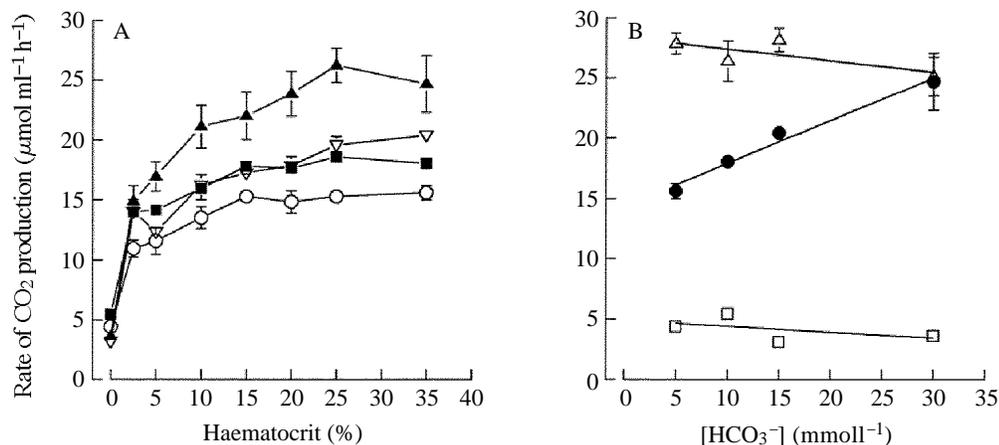
Fig. 3. (A) The interactive effects of whole-blood haematocrit (0–35%) and plasma $HCO_3^-$ levels (5–30mmol$l^{-1}$) on the rate of $CO_2$ production ($\bigcirc$, 5mmol$l^{-1}$ $HCO_3^-$; $\blacksquare$, 10mmol$l^{-1}$ $HCO_3^-$; $\triangledown$, 15mmol$l^{-1}$ $HCO_3^-$; $\blacktriangle$, 30mmol$l^{-1}$ $HCO_3^-$. (B) The relationship between plasma [$HCO_3^-$] and the rate of $CO_2$ production in whole blood ($\bullet$), separated plasma ($\square$) or separated plasma containing 3mgml$^{-1}$ bovine carbonic anhydrase ($\triangle$). 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
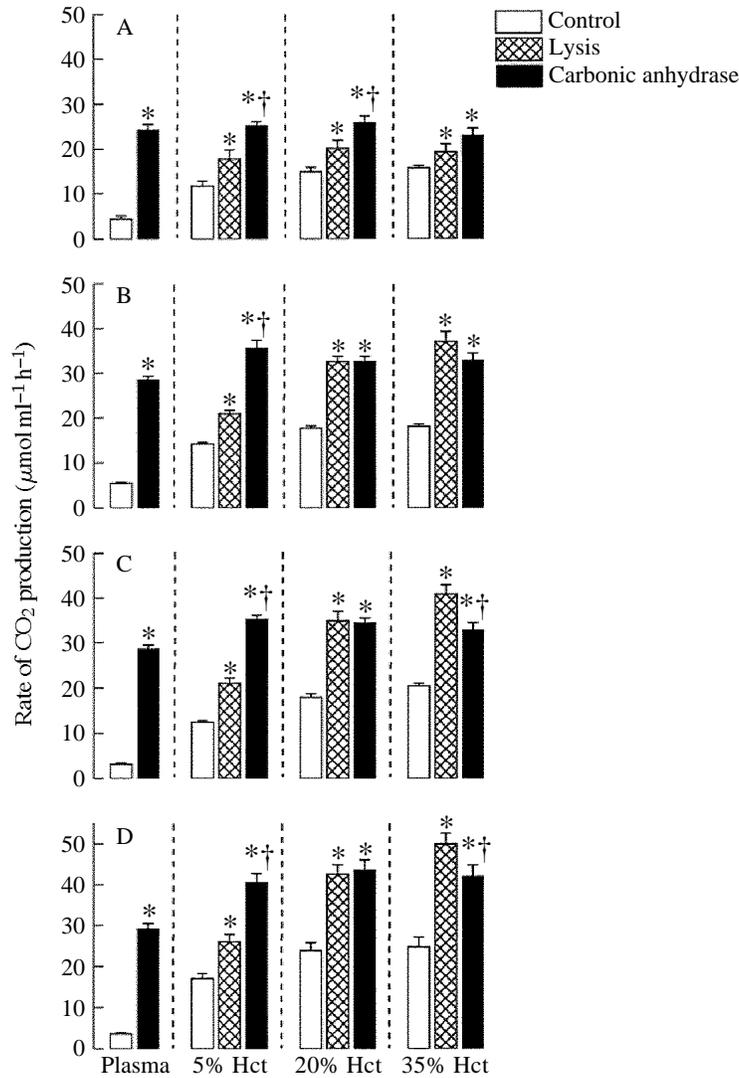
Fig. 4. $CO_2$ production in plasma or whole blood [5%, 20%, 35% haematocrit (Hct)] under control (untreated) conditions (open bars; $N$=6), after total RBC haemolysis (cross-hatched bars; $N$=6) or after the addition of $3\,\mathrm{mg\,ml^{-1}}$ bovine carbonic anhydrase to haemolyzed blood (filled bars; $N$=6). The experiments were performed using (A) $5\,\mathrm{mmol\,l^{-1}}$ $HCO_3^-$, (B) $10\,\mathrm{mmol\,l^{-1}}$ $HCO_3^-$, (C) $15\,\mathrm{mmol\,l^{-1}}$ $HCO_3^-$ and (D) $30\,\mathrm{mmol\,l^{-1}}$ $HCO_3^-$. * 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 $30\,\mathrm{mmol\,l^{-1}}$; 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
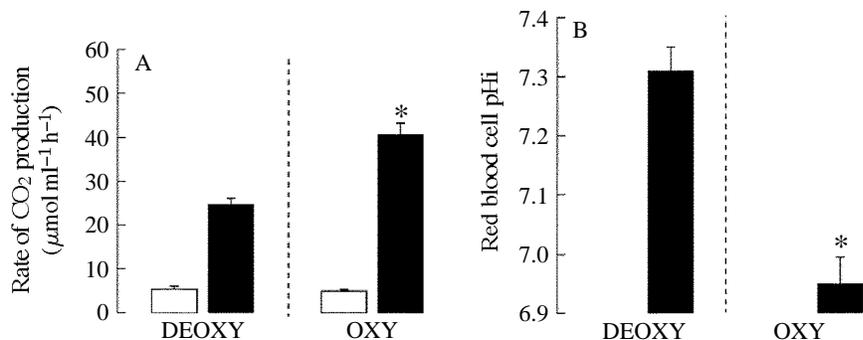
Fig. 5. The effects of rapid oxygenation of separated plasma (open bars) or whole blood (Hct 20%) on (A) the rate of $CO_2$ production and (B) red blood cell intracellular pH (pHi). * indicates a significant difference ($P<0.05$; $N=6$) from the corresponding value in the deoxygenated blood/plasma. Values are mean ± S.E.M.

Fig. 4). The addition of $3 \, \text{mg ml}^{-1}$ 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
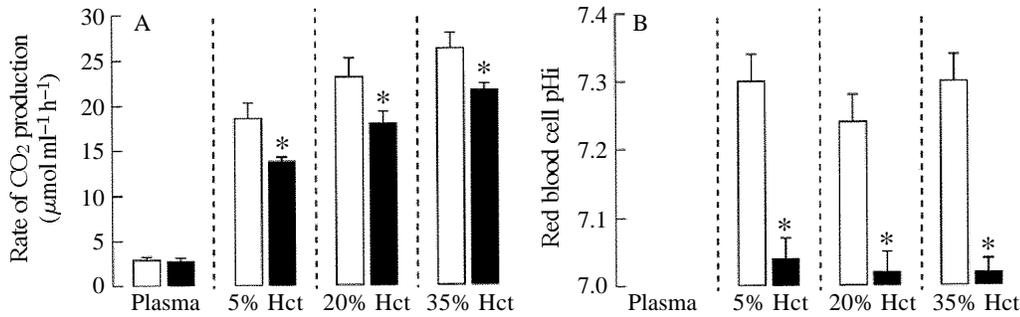
Fig. 6. (A) The rate of $CO_2$ production and (B) red blood cell intracellular pH (pHi) in separated plasma or whole blood [5%, 20%, 35% haematocrit (Hct)] pre-equilibrated and assayed under deoxygenated (open bars; $N=6$) or oxygenated (filled bars; $N=6$) conditions using a constant $[HCO_3^-]$ of $5 \text{mmol l}^{-1}$. * 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 $1 \text{mmol l}^{-1}$ (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, $120 \, \text{ml} \, \text{min}^{-1}$. 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 ($3 \, \text{mg} \, \text{ml}^{-1}$) 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 CO2 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 CO2 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 ($200\,mmol\,l^{-1}$). 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 CO₂ 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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