The conversion of plasma HCO$_3^-$ to CO$_2$ by rainbow trout red blood cells in vitro: adrenergic inhibition and the influence of oxygenation status

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Abstract

This study employed a recently developed radioisotopic assay (Wood and Perry 1991) to examine the inhibition, induced by catecholamines, of the conversion of plasma HCO$_3^-$ to CO$_2$ in acidotic trout blood, and the influence of oxygenation status on the response. Blood was incubated in vitro at P$_{CO_2}$ = 2 torr, and 10$^{-6}$ M noradrenaline was employed as the adrenergic stimulus. In particular we investigated whether the inhibition of plasma HCO$_3^-$ conversion could be explained by a limited supply of H$^+$ for the intracellular HCO$_3^-$ dehydration reaction because of competition by the adrenergically activated Na$^+/H^+$ exchanger. Hypoxia (P$_{O_2}$ = 15 torr) was employed as a tool to intensify this competition. Hypoxia raised RBC pHi, pHe, and plasma total CO$_2$ concentration (C$_{CO_2}$) by the Haldane effect, and increased the magnitude of Na$^+/H^+$ activation, expressed as the change in the transmembrane pH gradient (pHe−pHi). However hypoxia did not alter the inhibition of the conversion of plasma HCO$_3^-$ to CO$_2$ caused by noradrenaline. Hypoxia itself stimulated the RBC-mediated conversion of plasma HCO$_3^-$ to CO$_2$ by about 20% in the presence or absence of noradrenaline. The conversion rate was strongly correlated with pHe, pHe−pHi, and plasma C$_{CO_2}$ in these experiments, but not with pHi. We conclude that adrenergically mediated inhibition in the conversion of plasma HCO$_3^-$ to CO$_2$ by trout RBCs is not due to competitive limitation on intracellular H$^+$, but rather to changes in the electrochemical gradient for HCO$_3^-$ entry and/or to CO$_2$ recycling from plasma to RBC. The deoxygenated condition helps to promote CO$_2$ excretion at the level of the RBC.

Introduction

Wood and Perry (1985) proposed that catecholamines inhibit the net rate of CO$_2$ excretion by salmonid red blood cells (RBCs) and presented preliminary evidence in favour of this idea (unpublished data of S.F. Perry and T.A. Heming). They argued that this phenomenon was responsible for the increase in Pa$_{CO_2}$ commonly observed after exhaustive exercise or catecholamine infusions in salmonids ("CO$_2$ retention theory"), and suggested that the inhibitory action was on the entry of plasma HCO$_3^-$ into the RBC, which is commonly believed to be the rate-limiting step in CO$_2$ excretion (Fig. 1; Wood and Perry 1985; Perry 1986; Perry and Wood 1989). In the ensuing years, both the original evidence and the CO$_2$ retention theory proved controversial (Steffensen et al. 1987; Tufts et al. 1988; Playle et al. 1990; Thomas and Motais 1990; Perry and Thomas 1991). However, in 1991,
the extent of inhibition increased in proportion to
membrane (see Nikinmaa and Tufts 1989; Motais
well-known inhibition was linked to the ,Sl-activation of the
Phenomenon. Instead, the results indicated that the
Passow 1984, Hubner
itself (the band 3 membrane protein; Romanow and
any direct inhibition of the CI-/HCO3 exchanger
conditions (Wood and Perry 1991; Perry
blood was acidified to duplicate post-exercise
ature, and showed that it occurred only when the
that the inhibitory effect was 3,-adrenergic in na-
they confirmed the original evidence, demonstrated
CO2 recycling due to reduced RBC PCO2; mechanism (3) is a limitation
of HCO3 dehydration by a shortage of intracellular H+s.
See text for further details.

Wood and Perry developed a new in vitro assay to
directly measure the net rate of conversion of plasma HCO3- to excreted CO2. Solid arrows indicate pathways
which normally occur in the absence of catecholamines as blood is exposed to an external environment of lower P CO2 (i.e. water at the gills, or the external trap in the present assay system). Dashed arrows indicate pathways which may occur upon activation of Na+/H+ exchange by plasma catecholamines and thereby explain reduced net conversion of plasma HCO3- to excreted CO2. Mechanism (1) is a decrease or reversal of the electrochemical gradient for HCO3- entry; mechanism (2) is CO2 recycling due to reduced RBC P CO2; mechanism (3) is a limitation of HCO3- dehydration by a shortage of intracellular H+s. See text for further details.

At the same time, their experiments eliminated
any direct inhibition of the Cl-/HCO3- exchanger itself (the band 3 membrane protein; Romanow and
Passow 1984, Hubner et al. 1992) as the cause of the phenomenon. Instead, the results indicated that the inhibition was linked to the $\beta_1$-activation of the well-known Na+/H+ antiporter in the red cell membrane (see Nikinmaa and Tufts 1989; Motais et al. 1992; Nikinmaa 1992 for reviews). For example, the extent of inhibition increased in proportion to
the change in RBC transmembrane pH gradient (pHe-pHi), and amiloride blockade of Na+/H+ exchange eliminated the inhibition.

At present, the explanation of the phenomenon remains elusive. Perry et al. (1991) proposed two possibilities which are outlined in Fig. 1. The first (1) was that activation of Na+/H+ exchange would cause an immediate decrease or reversal in the electrochemical gradient for the inward movement of HCO3- from plasma to RBC. This would result from a rise in intracellular [HCO3-] which would accompany the increase in RBC pHi owing to physicochemical buffering and the presence of intrarythrocytic carbonic anhydrase. The second (2), somewhat related explanation, was based on the observation of Motais et al. (1989) that significant "CO2 recycling" could occur from plasma to RBC after activation of Na+/H+ exchange. By this explanation (see Thomas and Perry 1992), intracellular P CO2 would fall due to carbonic anhydrase-mediated formation of intracellular HCO3-, some of which would leave by Cl-/HCO3- exchange. The normal P CO2 gradient from RBC to plasma would be reversed for some period, during which CO2 would tend to enter rather than leave the RBC. A portion of the CO2 formed at the uncatalysed rate in the plasma by the reaction of HCO3- with extruded H+ would also diffuse back into the RBC along the local P CO2 gradient. In both scenarios, a reduced net dehydration rate of plasma HCO3- would occur.

However, an entirely separate third possibility (3) is raised by the proposal of Randall and Brauner (1991) that Na+/H+ exchange and the intracellular HCO3- dehydration reaction may compete for the same pool of H+s. In this case, activation of Na+/H+ exchange would inhibit the HCO3- dehydration reaction itself by "starving" it of H+s. Based on work with mammalian RBCs, the explanation would appear unlikely because the rate of HCO3- dehydration catalyzed by carbonic anhydrase appears to be limited by the availability of buffers that can donate H+ ions to the active site of the enzyme, rather than by the availability of free H+ ions (Jonsson et al. 1976; Silverman and Vincent 1983). However, it is possible that the situation in teleost RBCs may be very different; teleost
hemoglobins have much lower buffer capacities than mammalian hemoglobins (Jensen 1989), and of course mammalian red cells lack the Na⁺/H⁺ antiporter. Therefore, the primary goal of the present study was to directly evaluate this explanation (3) by changing the oxygenation state of the hemoglobin. Hypoxia is well known to increase the intensity of the RBC Na⁺/H⁺ activation for any given plasma catecholamine level (Motais et al. 1987; Salama and Nikinmaa 1988; Nikinmaa and Jensen 1992; Reid and Perry 1991; Reid et al. 1993). At the same time, hypoxia raises RBC pHi by the Haldane effect because deoxygenated hemoglobin is a more effective binder of H⁺s than oxygenated hemoglobin (Jensen 1989). We reasoned that if competition for intracellular H⁺s were the dominant factor, then this competition would be more severe and catecholamine-induced inhibition of net HCO₃⁻ dehydration would be much greater in hypoxic blood as measured by the assay of Wood and Perry (1991).

Materials and methods

Experimental animals

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

Sampling and handling of blood

In order to avoid endogenously elevated catecholamine and lactate levels, blood was obtained from the arterial catheters without disturbance to the fish, exactly as described by Wood and Perry (1991). For each test, blood was pooled from 5–8 fish, heparinized at 150 i.u.ml⁻¹ (ammonium heparin; Sigma), and stored on ice for 2h prior to use. At this point, the pooled blood was routinely assayed for whole blood pH (pHe = 7.9–8.1) true plasma total CO₂ content (C CO₂ = 6.6–7.4 mmol.l⁻¹), and hematocrit. The latter was adjusted to 18–20% by gentle centrifugation (500 × g for 1 min), addition or removal of homologous plasma as necessary, and resuspension.

Based on the measured pHe, C CO₂, and the known relationship between true plasma non-bicarbonate buffer capacity (β) and hematocrit (Wood et al. 1982), sufficient 140 mmol.l⁻¹ HCl was added very gradually to lower pHe to approximately 7.3. This value was chosen because it is typical of post-exercise acidosis (Milligan and Wood 1986) and because it yields maximum activation of the Na⁺/H⁺ antiporter under adrenergic stimulation (Borgese et al. 1987; Salama and Nikinmaa 1989). Plasma for “plasma only” assays was obtained by centrifugation (500 × g for 5 min) at this point. The blood was then divided into 1 ml aliquots in 20 ml glass scintillation vials which were placed in a shaking water bath at 15 ± 1°C. The scintillation vials were sealed with rubber stoppers fitted with entry and exit ports, the former served with a continual flow of a humidified gas mixture from either a normoxic or hypoxic gas source. Samples were gassed and shaken for at least 1h prior to experiment to ensure complete equilibration.

Twelve complete runs were performed in total. Each run consisted of 4 treatments on the same blood pool: normoxic + saline; hypoxic + saline; normoxic + noradrenaline (10⁻⁶M); hypoxic + noradrenaline (10⁻⁶M), facilitating the use of paired statistical tests. A few additional data points from incomplete runs were included in the correlation analyses. Normoxic (P O₂ = 155 torr, P CO₂ = 2 torr, balance N₂) and hypoxic gases (P O₂ = 15 torr, P CO₂ = 2 torr, balance N₂) were provided by gas mixing pumps (Wosthoff 301/a–f) and analyzed precision gas mixtures. Hypoxaemia (typical of venous blood or arterial blood during environmental hypoxia) rather than total anoxia was employed as we wished to avoid the possibility of an additional H⁺ source from RBC glycolysis. Blood lactate measured in two runs was stable at less than
I mmol.l⁻¹. Noradrenaline rather than adrenaline was used because it is the more potent natural agonist on this β₁-adrenergic response. The assay was started 5 min after the addition of noradrenaline (or saline vehicle) because the greatest inhibition of RBC CO₂ excretion rate was seen at this time (Wood and Perry 1991).

The assay

The assay measuring the conversion of plasma HCO₃⁻ to CO₂ via RBCs was performed as described by Wood and Perry (1991; see their Fig. 1) with minor modification. In brief, 50 µl of either 140 mmol.l⁻¹ NaCl or 2 × 10⁻⁵ M L-noradrenaline bitartrate (Sigma) in 140 mmol.l⁻¹ NaCl was added to an individual blood sample exactly 5 min prior to the start of the assay. Shaking and appropriate normoxic or hypoxic gassing was maintained until the start. At this point, 2 µCi (50 µl of 40 µCi.ml⁻¹) sodium [¹⁴C]bicarbonate (ICN Biomedicals Inc.) in 5 mmol.l⁻¹ HCO₃⁻ Cortland saline (Wolf 1963) was added to the blood sample. The vial was immediately ressealed with a new rubber stopper containing a ¹⁴CO₂ trap so as to form a closed system (no gassing), and shaking was continued for exactly 3 min. The CO₂ trap was a fluted filter paper (Whatman GF/A 2.4 cm glass microfibre filter) impregnated with 150 µl of 1 mol.l⁻¹ hyamine hydroxide in methanol.

At the termination of the assay, the filter was immediately removed and assayed for [¹⁴C] radioactivity, and the blood was drawn into a gas-tight Hamilton syringe for immediate measurement of pHe. The remaining blood was then centrifuged (12,000 × g for 2 min). The red cell pellet was frozen in liquid N₂ for later determination of RBC pH by the freeze-thaw lysate method (Zeidler and Kim 1977). The plasma was decanted anaerobically for measurements of C₃CO₂ (2 × 50 µl) and [¹⁴C] radioactivity (2 × 50 µl), and thereby plasma specific activity (dpm.umol⁻¹). The net rate of conversion of plasma HCO₃⁻ to CO₂ (i.e. dehydration rate in umol.ml⁻¹.h⁻¹) for each 1 ml sample assayed was calculated by dividing filter paper [¹⁴C] radioactivity (dpm, corrected for [¹⁴CO₂ trapping efficiency) by plasma specific activity (dpm. umol⁻¹) and time (0.05h).

Analytical techniques

Whole blood (extracellular) pHe and RBC pH were determined with a micro-capillary electrode (G297/G2) thermostatted to the experimental temperature and connected to a PHM-71 Mk2 acid-base analyzer (Radiometer-Copenhagen). Plasma C₃CO₂ was measured with a Capni-con Total CO₂ Analyzer, Model II (Cameron Instruments) calibrated with NaHCO₃ standards in the range of interest (0–4 mmol.l⁻¹). Scintillation counting with quench correction on an LKB Rackbeta 1217 Counter was employed to measure [¹⁴C] radioactivity in plasma (50 µl in 10 ml Amersham ACS II) and filter papers (in 10 ml of customized cocktail containing 2.0 g PPO + 0.1 g POPOP in 0.8 l of toluene + 0.21 of 95% ethanol).

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

Results

There was no effect of any of the experimental treatments on the rate of conversion of HCO₃⁻ to CO₂ in separated plasma, which was 3.16 ± 0.08 umol.ml⁻¹.h⁻¹. Therefore, for each run, the respective plasma rates were subtracted from the whole blood rates to yield RBC conversion rates, which are the values reported.

Under normoxia, noradrenaline (10⁻⁶M) significantly inhibited the rate of conversion of plasma HCO₃⁻ to CO₂ via trout RBCs (10.21 ± 0.98 umol.ml⁻¹.h⁻¹) by 36.1 ± 5.7% (Fig. 2A). Under hypoxia, noradrenaline significantly reduced the rate of conversion by 30.0 ± 3.7%. Neither these relative reductions nor the absolute reductions (-4.03 ± 0.82 umol.ml⁻¹.h⁻¹ in normoxia vs. -3.78 ± 0.63 umol.ml⁻¹.h⁻¹ in hypoxia) were
The effects of normoxia + saline, hypoxia + saline, normoxia + 10^-6 M noradrenaline (NAD), and hypoxia + 10^-6 M NAD on (A) the rate of conversion of plasma HCO_3^- to CO_2 via trout RBCs ("RBC CO_2 excretion rate"), and (B) the pH gradient (pHe−pHi) across the RBC membrane. Means ± 1 SEM (N = 12). * indicates a significant difference (p < 0.05) due to the presence of NAD relative to the appropriate saline treatment. † indicates a significant difference due to the presence of hypoxia relative to the appropriate normoxic treatment.

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

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

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

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

Overall, there was a highly significant positive correlation (r = 0.71) between pHe and the conversion rate of plasma HCO_3^- to CO_2 via RBCs in individual blood samples (Fig. 4A). In contrast, there was no significant correlation between RBC pHi (r = −0.18) and the conversion rate (Fig. 4B). These data indicate that pHe rather than pHi was by far the more important influence contributing to the strong positive correlation (r = 0.65) between the transmembrane pH gradient (pHe−pHi) and the conversion rate (Fig. 5A). However, there was an equally strong positive correlation (r = 0.64) between plasma C_{CO_2} and the observed rate (Fig. 5B) even though noradrenaline had no effect on C_{CO_2} (Fig. 3C). Clearly, more than one factor was involved in determining the rates of conversion of
plasma HCO$_3^-$ to CO$_2$ by trout RBCs in these experiments.

**Discussion**

**Comparison with previous studies**

Measured rates of conversion of plasma HCO$_3^-$ to CO$_2$ in separated plasma and via RBCs in trout blood in the present study were similar in magnitude to those determined earlier by Wood and Perry (1991) and Perry et al. (1991) under similar conditions with the same assay system. As discussed by these authors, the observed rates lie in the general range of estimated values for rainbow trout in vivo, and are far below the greatly elevated values yielded by the unphysiological conditions present in the “boat” assay used by earlier workers (Heming and Randall 1982; Tufts et al. 1988). The 30–36% inhibition of HCO$_3^-$ dehydration induced by 10$^{-6}$ M noradrenaline (Fig. 2A) was lower than the 54% inhibition reported by Perry et al. (1991), but the change in the pHe–pHi gradient (Fig. 2B) was also lower by about the same proportion in the present study. Intra-specific differences in the extent of adrenergic activation of Na$^+$/H$^+$ exchange are well documented and may result from differences in season, previous oxygen regime, and hormonal status (Nikinmaa and Tufts 1989; Reid and Perry 1991; Thomas and Perry 1992).

The more intense activation of Na$^+$/H$^+$ exchange in hypoxaemic blood (Fig. 2B) was in accord with previous observations and can be attributed to an increase in the number of surface-associated $\beta_1$-adrenergic receptors, resulting increased c-AMP production, and a greater responsiveness of Na$^+$/H$^+$ exchange to this c-AMP (Motais et al. 1987; Salama and Nikinmaa 1988; Reid and Perry 1991; Reid et al. 1993). It has been suggested that the oxygenation status of haemoglobin itself can serve as a signal transducer by
conformational interactions with membrane proteins (Motais et al. 1987; Nikinmaa and Jensen 1992). While RBC O2 content was not measured, the hypoxia (PO2 = 15 torr) applied under the acidic conditions of the present study likely lowered the O2 saturation of the hemoglobin from about 85% to 30% based on the O2 dissociation curves of Eddy (1971) for rainbow trout blood.

The small increase in pHi (Fig. 3B) associated with this partial deoxygenation was due to the Hal-dane effect as the deoxygenated hemoglobin bound more H+ ions (Salama and Nikinmaa 1988; Jensen 1989). It is interesting that in an open system where PCO2 but not pHe was directly controlled, the rise in pHe (Fig. 3A) associated with deoxygenation was considerably greater than that in pHJ (Fig. 3B). This reflects a Donnan equilibration of H+ ions (in the absence of noradrenaline) across the RBC membrane in combination with the different buffer capacities of the extracellular and intracellular com-

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**Fig. 4.** (A) The relationship between extracellular pHe and "RBC CO2 excretion rates" in trout blood. (B) The relationship between RBC intracellular pHi and the rate of conversion of plasma HCO3- to CO2 via trout RBCs ("RBC CO2 excretion rate"). ○ = normoxia + saline, ● = hypoxia + saline, □ = normoxia + 10^-6 M noradrenaline (NAD), and ■ = hypoxia + 10^-6 M NAD. The equations of the regression lines and the significance of the correlation coefficients are given.

**Fig. 5.** (A) The relationship between the pH gradient (pHe–pHi) across the RBC membrane and "RBC CO2 excretion rates" in trout blood. (B) The relationship between plasma total CO2 concentration (C CO2) and the rate of conversion of plasma HCO3- to CO2 via trout RBCs ("RBC CO2 excretion rate"). ○ = normoxia + saline, ● = hypoxia + saline, □ = normoxia + 10^-6 M noradrenaline (NAD), and ■ = hypoxia + 10^-6 M NAD. The equations of the regression lines and the significance of the correlation coefficients are given.
partments (Wood et al. 1982). The increased pHe at constant $P_{\text{CO}_2}$ is associated with an elevation of plasma $\text{HCO}_3^-$, thereby explaining the increased plasma $\text{C}_{\text{CO}_2}$ (Fig. 3C).

**The effects of hypoxia on the conversion of plasma $\text{HCO}_3^-$ to $\text{CO}_2$ via RBCs**

Despite the reduced availability of free intracellular $\text{H}^+$ associated with the Haldane effect and a more intense activation of $\text{Na}^+/\text{H}^+$ exchange, the noradrenaline-induced inhibition of $\text{HCO}_3^-$ dehydration was not intensified by hypoxia (Fig. 2A). This result, in combination with the general lack of correlation between pHi and the observed rate (Fig. 4B), would appear to eliminate explanation (3) of Fig. 1—that adrenergic inhibition of the conversion of plasma $\text{HCO}_3^-$ to $\text{CO}_2$ is due to a shortage of intracellular $\text{H}^+$ for the $\text{HCO}_3^-$ dehydration reaction (Randall and Brauner 1991). Likely, as in mammals (Jonsson et al. 1976; Silverman and Vincent 1983), the ability of intracellular buffers (i.e., hemoglobin) to transfer $\text{H}^+$ ions to the active site on carbonic anhydrase is more than adequate to prevent $\text{H}^+$ availability from becoming a limiting factor.

This therefore leaves the two original explanations offered by Perry et al. (1991) — (1) an alteration in the electrochemical gradient for $\text{HCO}_3^-$ entry and/or (2) recycling of $\text{CO}_2$ from plasma to RBC. Neither of these possibilities can be eliminated by the present results, and indeed there is no reason why they should be mutually exclusive. The strong positive relationship between pHe–pHi and the conversion rate (Fig. 5B) is exactly as expected from both explanations (1) and (2) and is in good agreement with the data of Wood and Perry (1991). Similarly, the strong positive relationship between plasma $\text{C}_{\text{CO}_2}$ and the conversion rate (Fig. 5B) suggests that external substrate ($\text{HCO}_3^-$) concentration is also important, but in the absence of intracellular $\text{C}_{\text{CO}_2}$ measurements and the likely presence of non-equilibrium $P_{\text{CO}_2}$ in both the RBC and plasma compartments after adrenergic stimulation, the $\text{HCO}_3^-$ gradients (1) cannot be calculated. From experimental manipulations of plasma $\text{HCO}_3^-$ concentrations, Wood and Perry (1991) concluded that the $\text{HCO}_3^-$ gradient had a small influence under resting (Donnan equilibrium) conditions, but potentially a very large influence under non-equilibrium conditions.

An interesting variant on this idea is that the adrenergic inhibition of plasma $\text{HCO}_3^-$ conversion is in some way a direct consequence of the non-equilibrium acid load acutely added to the plasma by $\text{Na}^+/\text{H}^+$ exchange. Unfortunately, the idea is difficult to test experimentally. When the RBC's add acid to the plasma, then their intracellular pH and $\text{HCO}_3^-$ rise instantaneously (explanation 1) and intracellular $P_{\text{CO}_2}$ falls instantaneously (explanation 2). However when small amounts of exogenous acid are added to the plasma by the investigator, then both pHe, and to a lesser extent, pHi fall, and $P_{\text{CO}_2}$ increases throughout the system. We have found that the conversion of plasma $\text{HCO}_3^-$ to $\text{CO}_2$ measured by the assay actually increases rather than decreases in the first few minutes because the whole process is driven by the $P_{\text{CO}_2}$ gradient from RBC to atmosphere. Over the longer term, the measured rate decreases slightly, because of the change in the $\text{HCO}_3^-$ gradient.

The clear stimulation by hypoxia of the net conversion rate of plasma $\text{HCO}_3^-$ to $\text{CO}_2$ via RBCs in both the presence and absence of noradrenaline (Fig. 2A) was an unexpected finding, but one which has been observed independently by Perry and Gilmour (1993). There is obvious functional importance. In vivo, this phenomenon will help enhance $\text{CO}_2$ excretion at the gills during environmental hypoxia, thereby contributing to lowered $\text{Pa}_{\text{CO}_2}$, raised RBC pH, and improved conditions for $\text{O}_2$ loading (Tetens and Christensen 1987; Thomas and Motais 1990; Perry and Thomas 1991). We suggest two possible explanations for the mechanism involved. The first is that the higher plasma $\text{HCO}_3^-$ associated with the Haldane effect in hypoxaemic blood (Figs. 3C) increases the supply of external substrate and therefore possibly the electrochemical gradient for $\text{HCO}_3^-$ entry. The strong positive correlation between the $\text{HCO}_3^-$ conversion rate and both pHe (but not pHi) and extracellular $\text{C}_{\text{CO}_2}$ (Figs. 4,5) supports this idea. The second is the interesting possibility of a direct effect of hemo-
globin oxygenation status on the activity of the band 3 Cl\(^{-}\)/HCO\(_3\)\(^{-}\) exchanger. In mammalian RBCs it is well established that the cytoplasmic domain of band 3 preferentially binds to deoxygenated hemoglobin (Salhany et al. 1980; Walder et al. 1984; Chetrite and Cassoly 1985, but it remains unclear whether this interaction has any influence on the rate of anion exchange (Jennings 1989). The trout RBC may be a useful system for future studies on this question.

In the present study, we have addressed the cause behind the adrenergic inhibition of plasma HCO\(_3\)\(^{-}\) conversion seen in trout blood in vitro. In vivo, the situation is undoubtedly more complex, and two features deserve special attention in future studies. The first is the extent to which a redistribution of HCO\(_3\)\(^{-}\) transport occurs as a result of elevated pHi and RBC swelling associated with hypoxia and adrenergic stimulation. Both factors will ensure that a greater proportion of total blood HCO\(_3\)\(^{-}\) is inside the RBCs right from the point of loading at the tissues. Therefore at the gills (and in the assay), a greater proportion of total amount of HCO\(_3\)\(^{-}\) dehydrated to excreted CO\(_2\) could originate from intracellular HCO\(_3\)\(^{-}\) stores. This would not be detected by the present assay system where the label is added to the plasma compartment. The second is the fact that the release of Bohr protons by hemoglobin oxygenation should promote the overall HCO\(_3\)\(^{-}\) dehydration rate as blood passes through the gills (Jensen 1989). Support for this idea has recently been provided by Perry and Gilmour (1993) using a modified version of the present assay system, but it is unclear how adrenergic stimulation would interact with this process.

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