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Fish Red Blood Cell Carbon Dioxide Transport *In Vitro*: A Comparative Study

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ABSTRACT. Red blood cell (rbc) carbon dioxide transport was examined *in vitro* in three teleosts (*Oncorhynchus mykiss, Anguilla anguilla, Scophthalmus maximus*) and an elasmobranch (*Scyliorhinus canicula*) using a radioisotopic assay that measures the net conversion of plasma HCO_3^- to CO_2. The experiments were designed to compare the intrinsic rates of rbc CO_2 excretion and the impact of haemoglobin oxygenation/deoxygenation among the species.

Under conditions simulating *in vivo* levels of plasma HCO_3^- and natural haematocrits, the rate of whole blood CO_2 excretion varied between 14.0 μmol ml^{-1} h^{-1} (*S. canicula*) and 17.6 μmol ml^{-1} h^{-1} (*O. mykiss*). The rate of CO_2 excretion in separated plasma was significantly greater in the dogfish, *S. canicula*. The contribution of the rbc to overall whole blood CO_2 excretion was low in the dogfish (46 ± 6%) compared to the teleosts (trout, 71 ± 4%; turbot, 64 ± 5%; eel, 55 ± 3%).

To eliminate the naturally occurring differences in haematocrit and plasma [HCO_3^-] as inter-specific variables, the rates of whole blood CO_2 excretion were determined in blood that had been resuspended to constant [HCO_3^-] (5 $mmol^{-1}$) and haematocrit (20%) in appropriate teleost and elasmobranch Ringer solutions. Under such normalized conditions, the rate of whole blood CO_2 excretion was significantly higher in the turbot (22.4 ± 1.3 μmol ml^{-1} h^{-1}) in comparison to the other species (16.4–18.4 μmol ml^{-1} h^{-1}) and thus revealed a greater intrinsic rate of rbc CO_2 excretion in the turbot.

To study the contribution of Bohr protons, the rates of whole blood CO_2 excretion were assessed in blood subjected to rapid oxygenation during the initial phase of the 3 min assay period. Rapid oxygenation significantly enhanced the rate of CO_2 excretion in the teleosts but not in the elasmobranch. The extent of the increase provided by the rapid oxygenation of haemoglobin was a linear function of the extent of the Haldane effect, as quantified in each species from *in vitro* CO_2 dissociation (combining) curves. Under steady-state conditions, deoxygenated blood exhibited greater rates of CO_2 excretion than oxygenated blood in the teleosts but not in the elasmobranch. As a consequence of the Haldane effect, rbc intracellular pH was increased in the teleosts by deoxygenation but was unaltered in the elasmobranch.

The results, by extrapolation, suggest that the rates of CO_2 excretion *in vivo* are influenced by the magnitude of the Haldane effect and the extent of haemoglobin oxygenation during gill transit in addition to the intrinsic rate at which the rbc converts plasma HCO_3^- to CO_2. COMP BIOCHEM PHYSIOL 113;2:121–130, 1996.

KEY WORDS. *Oncorhynchus mykiss, Anguilla anguilla, Scyliorhinus canicula, Scophthalmus maximus*, Haldane effect, red blood cell, carbon dioxide excretion, haemoglobin, Band-3

INTRODUCTION

Carbon dioxide (CO_2) excretion in fish (21) follows the classical pattern that has been established for most vertebrates in which the red blood cell (rbc) plays a pivotal role. Briefly, as blood flows through the branchial vasculature, a portion of the plasma HCO_3^- pool enters the rbc via the Cl^-/HCO_3^- exchanger also known as the band-3 protein (4,8,20,26). Within the rbc, the HCO_3^- is rapidly dehydrated in the presence of carbonic anhydrase to form molecular CO_2 (15). The CO_2 thus formed diffuses from the rbc into the plasma and then across the gill epithelium into the ventilatory water. Owing to the relatively slow rate of rbc Cl^-/HCO_3^- exchange (4) in comparison to the rapidity of catalyzed HCO_3^- dehydration or CO_2 diffusion, the rate-limiting step in CO_2 excretion is generally considered to be the entry of HCO_3^- into the rbc (21,22).

Recent studies utilizing rainbow trout rbc's *in vitro* have demonstrated that the oxygenation state of haemoglobin is an important variable influencing CO_2 excretion (22,35). First,

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the release of Bohr protons from haemoglobin during the rapid oxygenation of the blood (the Haldane effect) increases CO_2 excretion owing to the sudden increase in the availability of H^+ for the HCO_3^- dehydration reaction. Second, under steady-state conditions, oxygenation of trout blood lowers the rate of rbc CO_2 excretion. This latter effect may reflect the influence of oxygenation on rbc intracellular pH (22) or an O_2-mediated change in the interaction of haemoglobin with the band-3 protein (35). Thus, the rapid oxygenation of the blood as it flows through the gill is thought to enhance CO_2 excretion in accordance with the prevailing Haldane effect although the overall boost to CO_2 excretion is partially offset by the inhibitory effect of oxygenation, itself, on CO_2 excretion.

The current model for CO_2 excretion is based almost exclusively on studies performed on the rainbow trout and consequently little is known about the comparative aspects of CO_2 excretion in fish. There may be considerable differences among the species given the enormous inter-specific diversity in lifestyle, habitat and physiology of fishes. For example, the magnitude of the Haldane effect is large but variable among the teleost species (10,11,12) and is weak or absent altogether in most elasmobranchs that have been examined (2). It is possible, therefore, that a smaller or absent oxylabile component of CO_2 excretion in some fish species is compensated for by intrinscially greater rates of rbc CO_2 excretion. Owing to the importance of the rbc in CO_2 excretion, it is possible that species displaying low natural haematocrits have also developed intrinsically greater rates of rbc CO_2 excretion.

Thus, the goal, of the present study was to assess rbc CO_2 excretion *in vitro* in four species that were chosen to represent a wide range of Haldane effects and natural haematocrits. Three teleost species (*Oncorhynchus mykiss*, rainbow trout; *Anguilla anguilla*, European eel; *Scophthalmus maximus*, turbot) and an elasmobranch (*Scyliorhinus canicula*, lesser-spotted dogfish) were selected. The basic experimental design was to assess rbc CO_2 excretion a) under natural conditions of haematocrit and plasma $[HCO_3^-]$, b) under normalized conditions of constant haematocrit and $[HCO_3^-]$, c) during rapid oxygenation of the blood, and d) under steady-state conditions of oxygenation or deoxygenation.

MATERIALS AND METHODS

Experimental Animals

Freshwater rainbow trout (*Oncorhynchus mykiss*; mean weight = 533 ± 39 g; experimental N = 11) were obtained from a local hatchery and transported to the University of Brest where they were maintained indoors in large rectangular tanks supplied with flowing and aerated tap water (temperature = 15°C). They were fed daily to satiation with commercial salmonid pellets. Photoperiod was kept constant at 12 hr light : 12 hr dark. The fish were acclimated to these conditions for approximately 4 weeks prior to experimentation.

Seawater turbot (*Scophthalmus maximus*; mean weight = 482 ± 50 g; experimental N = 18) were obtained from outdoor tanks where they were kept under conditions of natural photoperiod in full strength (33 ppt) seawater at 15°C. They were fed with commercial pellets. After transportation to the University of Brest, the turbot were maintained without feeding at 15°C in recirculating full strength seawater for no longer than one week.

European eels (*Anguilla anguilla*; mean weight = 180 ± 23 g; experimental N = 13) were caught by local fishermen in brackish water off the coast of Brittany. They were transported to IFREMER, kept in full strength seawater at 15°C, and fed mussels for several months prior to being moved to University of Brest. At University of Brest they were maintained without feeding at 15°C in recirculating full strength seawater for no longer than 2 weeks.

Lesser-spotted dogfish (*Scyliorhinus canicula*; mean weight = 651 ± 30 g; experimental N = 15) were caught by net by local fishermen and then transported to a local public aquarium (Oceanapolis) where they were held outdoors under conditions of natural photoperiod in large circular tanks provided with flowing full strength seawater at 14°C. The fish were fed herring daily. Dogfish were transferred to University of Brest as required where they were maintained unfed in the recirculating seawater system (15°C) for no longer than 4 days.

Surgical Procedures

All fish were anaesthetized using ethyl-*m*-aminobenzoate (Sigma Chemical Company). For trout, turbot, and dogfish, the concentration of anesthetic used was 0.1 g l^{-1} whereas for the eel a much higher concentration (2.0 g l^{-1}) was required. Different surgical procedures were used for each species. In rainbow trout, the dorsal aorta was cannulated using polyethylene tubing (Clay Adams PE 50) according to the method of Soivio *et al.* (27). In turbot, the caudal artery was cannulated at the level of the caudal peduncle using PE 50 tubing according to the method of Wood *et al.* (32). In eels, the pneumogastric artery was cannulated using PE 50 tubing according to Hyde *et al.* (9). In dogfish, the anterior mesenteric artery was cannulated with stretched PE 50 tubing according to Graham *et al.* (7).

After surgery, all fish were transferred to customized Perspex chambers that were covered with opaque plastic and supplied with aerated flow-through freshwater (trout) or recirculating seawater (turbot, eels, dogfish) at 15°C. The fish were allowed to recover for 24–48 hr prior to experimentation. The cannulae were flushed daily with heparinized (50 units ml^{-1}) Cortland saline [teleosts (31)] or elasmobranch Ringer (in mmol l^{-1}; 250 NaCl; 7 Na_2SO_4; 3 $MgSO_4$; 4 KCl; 2 $CaCl_2 \cdot 2H_2O$; 5 $NaHCO_3$; 0.1 Na_2HPO_4; 4 glucose; 450 urea; 100 trimethylamineoxide; pH 7.8).

In Vivo *Experiments*

In order to obtain resting *in vivo* values for arterial blood acid-base and respiratory variables, a blood sample (0.7 ml)

was withdrawn anaerobically from the cannula into a heparinized syringe and subsequently analyzed for whole blood pH (pHe), rbc intracellular pH (pHi), total CO_2 ($CaCO_2$), PO_2 (PaO_2), total O_2 content (CaO_2), haematocrit (hct) and haemoglobin concentration ([Hb]) using conventional techniques (see below). From these values, arterial PCO_2 ($PaCO_2$) and plasma bicarbonate concentration ($[HCO_3^-]$) were calculated (see below).

In Vitro *Experiments*

I. CO_2 DISSOCIATION CURVES. To quantify the Haldane effect in each species, CO_2 dissociation (combining) curves were constructed *in vitro* using oxygenated and deoxygenated blood samples. Approximately 6 ml of blood was required to construct a pair (oxygenated and deoxygenated) of CO_2 dissociation curves. In most cases, this amount of blood could be obtained easily from a single animal although it was occasionally necessary to pool blood from two fish; a 6 ml blood sample was considered as a single *N* number for statistical purposes regardless of whether it was obtained from one or more fish. Blood sampling was terminated at the first signs of agitation or struggling. After sampling, the blood was maintained on ice (final heparin concentration = 100 units ml^{-1}) until commencing the experiment.

The basic experimental protocol involved equilibrating 0.5 ml blood samples with one of six different PCO_2's (nominally 0.033, 0.067, 0.13, 0.27, 0.53, and 1.07 kPa) with either air or nitrogen using Eschweiller tonometers immersed in 15°C water baths. In order to simultaneously equilibrate the oxygenated (air) and deoxygenated (nitrogen) blood, it was necessary to use two different sets of Wösthoff gas mixing pumps and this may partly explain the slightly different PCO_2's in the deoxygenated and oxygenated blood samples. The blood samples were equilibrated with humidifed gas for 30 min and then analyzed for CCO_2, pHe, pHi, Hct and [Hb]; from these values, PCO_2 and $[HCO_3^-]$ were calculated (see below).

In each species the magnitude of the Haldane effect was quantified by fitting hyperbolic curves to the data obtained from individual fish using iterative curve-fitting software (Sigmaplot; Jandel Scientific Inc.). From these curves, the values of $[HCO_3^-]$ in the oxygenated and deoxygenated blood were determined for a single PCO_2 of 0.53 kPa. This PCO_2 was chosen because it is probably representative of pre-branchial PCO_2 in the four species and the *in vitro* CO_2 excretion studies were conducted at this PCO_2.

II. RBC CO_2 EXCRETION. Red blood cell CO_2 excretion was monitored using a radioisotopic assay that has been previously described in detail (34). Briefly, the basic assay measures the net conversion of $[^{14}C]$-HCO_3^- to $[^{14}C]$-CO_2 in whole blood or separated plasma during a 3 min assay period. The contribution of the rbc's to the whole blood CO_2 excretion rate is determined by subtracting the rate achieved in the separated plasma. The assay was performed by adding 74 kBq (20 μl of 3700 kBq ml^{-1}) of sodium $[^{14}C]$bicarbonate (in teleost or elasmobranch saline) to 0.7 ml aliquots of blood or plasma that had been withdrawn from cannulated fish and pre-equilibrated (see below); it was not necessary to pool blood from different fish. The vial was then immediately gassed for 5 sec with either O_2 or N_2 (see below) and then sealed with a rubber stopper from which was suspended a plastic well containing a filter paper trap for CO_2 (soaked in 150 μl of hyamine hydroxide). After 3 min of shaking in a temperature-controlled (15°C) water bath, the filter was removed and assayed for ^{14}C activity. Whole blood or plasma pH was determined and the remaining blood was centrifuged (12,000 *g* for 2 min). The pellet was used to determine rbc pHi according to the freeze-thaw lysate method (36). Samples of true or separated plasma (50 μl) were assayed for ^{14}C activity and CCO_2 to determine the specific activity of HCO_3^- in the plasma (disints min^{-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. Two series of experiments were performed.

Series 1. In this series, CO_2 excretion was evaluated in whole blood or separated plasma under natural conditions of hct and $[HCO_3^-]$. Blood (about 3.5 ml) was withdrawn from cannulated fish, heparinized (50 units ml^{-1} final concentration), and then distributed into 3 × 0.7 ml aliquots in 20 ml glass scintillation vials. The remaining blood was centrifuged to yield a final 0.7 ml aliquot of separated plasma. The whole blood and plasma aliquots were then placed into a shaking water bath (15°C) and equilibrated with 0.5% CO_2 but under different conditions of oxygenation. The plasma sample and 1 blood sample were equilibrated with 0.5% CO_2 in air (oxygenated) while the remaining 2 blood samples were equilibrated with 0.5% CO_2 in N_2 (deoxygenated). After 1 hr of equilibration, the CO_2 excretion assay was performed under the conditions of a) continuing oxygenation, b) continuing deoxygenation, or c) rapid oxygenation of the deoxygenated blood. These conditions were achieved by gassing the vial just prior to the assay period (see above) with either N_2 (continuing deoxygenation) or O_2 (continuing oxygenation or rapid oxygenation of deoxygenated blood). Preliminary experiments revealed that 5 sec of gassing with O_2 was sufficient to totally oxygenate the deoxygenated blood sample within 45 sec of having sealed the assay vessel.

Series 2. In order to reveal potential differences among species in the intrinsic capacities of the rbc's to excrete CO_2, the CO_2 excretion assay was performed on rbc's which had been resuspended to a constant hct of 20% and $[HCO_3^-]$ of 5 mmol l^{-1}. In the teleosts, the rbc's were re-suspended in Cortland saline (modified for the seawater fish to contain 160 mmol l^{-1} NaCl) whereas the elasmobranch rbc's were resuspended in elasmobranch Ringer. The four species were examined simultaneously in a single assay to reduce variation. The basic experimental design was to assess the rate of CO_2 production in the appropriate saline and resuspended blood under conditions of oxygenation. Thus, the washed blood/saline was pre-equilibrated with 0.5% CO_2 in air and then gassed for 5 sec

with O_2 immediately prior to beginning the assay. CO_2 was excluded during oxygenation in order to initiate a gradient that promotes CO_2 excretion.

Analytical Procedures

Plasma CCO_2 was measured on 50 μl samples using the method of Cameron (3) in which the sample or standard (5 mmol l^{-1} $NaHCO_3$) was injected into an acid solution (0.01 M HCl) within a thermostated (37°C) cuvette housing a Radiometer PCO_2 electrode connected to a Radiometer PHM-73 analyzer. Whole blood or plasma pH and rbc pHi were measured with a Radiometer micro-capillary electrode (G299A) maintained at the experimental temperature (15°C) in a Radiometer BMS3 Mk2 blood micro system. Plasma HCO_3^- levels and PCO_2 were calculated using the Henderson-Hasselbalch equation and the appropriate constants for trout (teleosts) and dogfish listed in Boutilier *et al.* (1).

Haematocrit was determined by centrifuging approximately 80 μl of blood in heparinized capillary tubes for 5 min at 10,000 **g**. The concentration of haemoglobin in the blood or plasma was determined colorimetrically on 20 μl (blood) or 80 μl (plasma) samples using a commercial assay kit (Sigma chemical company).

PaO_2 was determined using a Radiometer electrode (E5046) that was housed in a thermostatted chamber and connected to a PHM-73 analyzer. Total O_2 content of the blood was measured according to the method of Tucker (29) using 20 μl samples.

Plasma and filter paper ^{14}C activities were determined by liquid scintillation counting (Packard 1600 TR Tri Carb LSC) and automatically corrected for quenching. The plasma or filter papers were counted in 10 ml of ACS II (Amersham) scintillation cocktail.

Statistical Analysis

The values shown in tables and figures are means ± 1 standard error of the mean (SEM). For multiple comparisons, the results have been statistically analyzed using one way analysis of variance followed by Fishers LSD multiple-comparison test. For pairwise comparisons, an unpaired two-tailed Students *t*-test was used. In all cases the fiducial limit of significance was set at 5%.

RESULTS

In Vivo *Experiments*

Arterial blood respiratory and acid-base variables from the four species are shown in Table 1. There was considerable variability among the species with respect to most variables, but particularly noteworty with respect to CO_2 excretion was the wide range of hct and [Hb] as well as the low $PaCO_2$ and [HCO_3^-] in the dogfish. The haematocrit was low in the turbot (9.6%), intermediate in the eel and dogfish (15.0–16.7%), and high in the trout (26.2%). These differences in hct were reflected by similar differences in [Hb]. The $PaCO_2$ in the teleosts ranged between 0.32 and 0.39 kPa while in the dogfish the $PaCO_2$ was only 0.13 kPa. Interestingly, the $PaCO_2$ in the turbot was similar to the other teleosts despite its low PaO_2. The [HCO_3^-] in the teleosts varied between 5.1 and 7.7 mmol l^{-1}; the [HCO_3^-] of 2.8 mmol l^{-1} in the dogfish was statistically lower than in the teleosts.

In Vitro *Experiments*

The three teleost species displayed significant Haldane effects as demonstrated by comparing the CO_2 dissociation curves of deoxygenated and oxygenated blood (Fig. 1). There was no apparent Haldane effect in the dogfish. The magnitude of the Haldane effect in each species was quantified by comparing the plasma [HCO_3^-] in the oxygenated and deoxygenated blood at a PCO_2 of 0.53 kPa (see above) and then normalizing this Δ[HCO_3^-] for differences in [Hb]. As shown in Table 2, the turbot displayed the largest Haldane effect (0.89 mmol $mmol^{-1}$ Hb) followed by trout (0.60 mmol $mmol^{-1}$ Hb), eel (0.58 mmol $mmol^{-1}$ Hb) and dogfish (0.07 mmol $mmol^{-1}$

TABLE 1. In vivo arterial blood respiratory and acid-base variables in the four species utilized in this study; rainbow trout (*Oncorhynchus mykiss*), turbot (*Scophthalmus maximus*), European eel (*Anguilla anguilla*) and lesser-spotted dogfish (*Scyliorhinus canicula*).

	Rainbow trout (6)	Turbot (6)	Eel (6)	Dogfish (8)
Hct (%)	26.2 ± 0.8*	9.6 ± 0.9*	14.0 ± 2.1	16.7 ± 1.2
[Hb] (mmol l^{-1})	1.13 ± 0.04*	0.39 ± 0.03*	0.69 ± 0.10	0.72 ± 0.0.03
CaO_2 (mmol l^{-1})	3.26 ± 0.3*	0.76 ± 0.1*	1.61 ± 0.0.3	1.92 ± 0.2
PaO_2 (kPa)	13.1 ± 0.6	5.9 ± 0.5	9.3 ± 2.5	12.4 ± 1.1
pHe	7.83 ± 0.04	7.76 ± 0.05	7.94 ± 0.07	7.78 ± 0.02
RBC pHi	7.36 ± 0.05	7.40 ± 0.04	7.48 ± 0.02	7.31 ± 0.02
$PaCO_2$ (kPa)	0.39 ± 0.07	0.33 ± 0.03	0.32 ± 0.08	0.14 ± 0.02*
[HCO_3^-] (mmol l^{-1})	6.8 ± 0.8	5.1 ± 0.7	7.7 ± 0.9	2.79 ± 0.3*

The values shown are means ± 1 standard error; the numbers of animals used are indicated in parentheses; *indicates values that are statistically different from all other species.

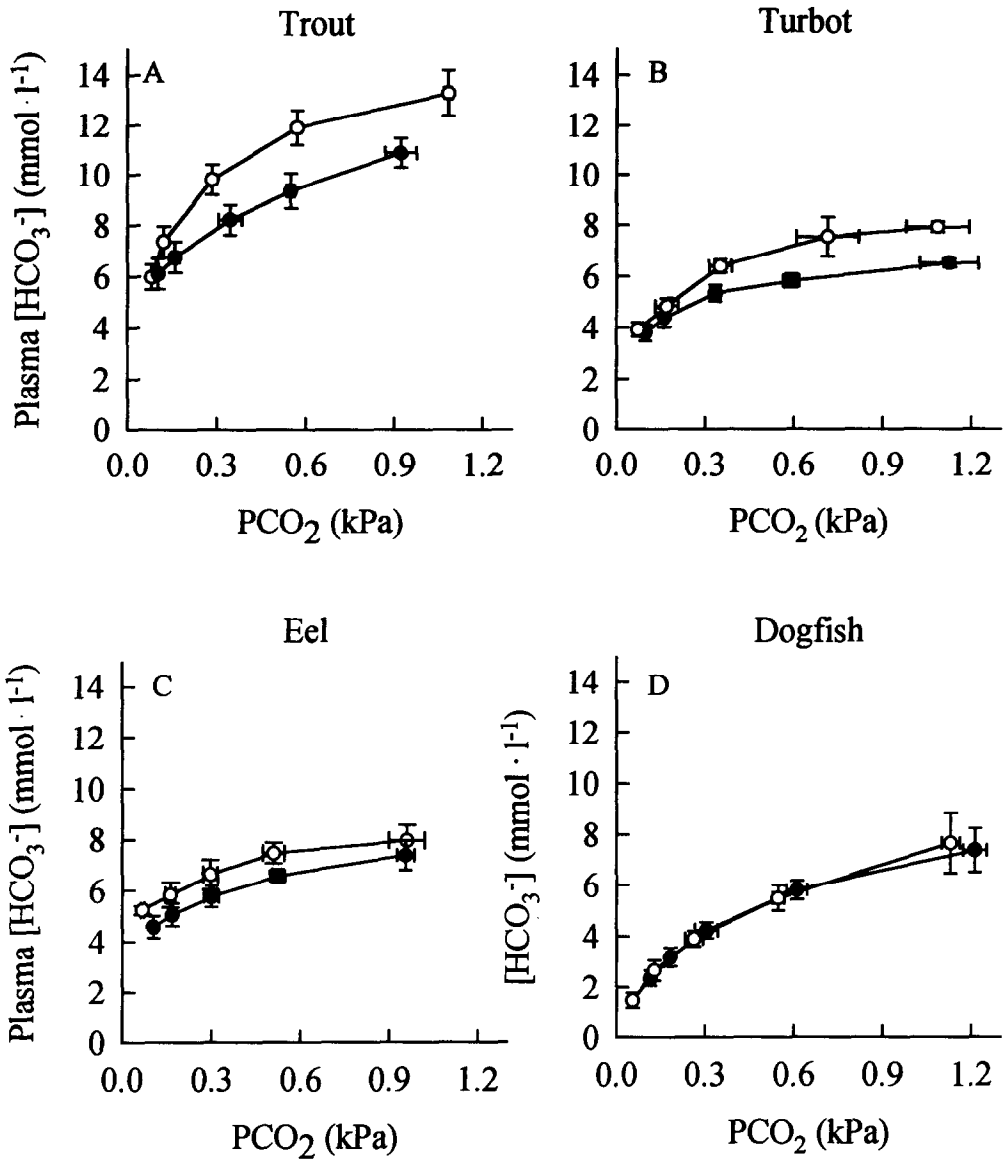

FIG. 1. ***In vitro*** **CO_2 dissociation (combining) curves in (A) rainbow trout (*O. mykiss*), (B) turbot (*S. maximus*), (C) European eel (*A. anguilla*), and (D) lesser-spotted dogfish (*S. canicula*) under oxygenated (filled symbols) and deoxygenated (empty symbols) conditions. Data shown are means ± 1 SE; *N* = 3 for each species. Note the absence of a measurable Haldane effect in the elasmobranch, *S. canicula*.**

Hb). The Haldane effect in the dogfish was not significantly different from zero.

Under conditions of "natural" hct and [HCO_3^-] (see Table 1), the three teleosts displayed similar rates of whole blood CO_2 excretion (14.5–17.6 μmol ml^{-1} h^{-1}) when assayed under steady state conditions for oxygenation (Fig. 2). The dogfish blood displayed the lowest rate of CO_2 excretion (statistically lower than trout and turbot). Conversely, the rate of CO_2 excretion in separated plasma was highest in the dogfish. Consequently, the contribution of the rbc to whole blood CO_2 excretion was lower (47%) in the dogfish compared to the teleosts (55–71%). Within the teleosts, the contribution of the rbc to whole blood CO_2 excretion was highest in the trout (71%), intermediate in the turbot (64%) and lowest in the eel (55%).

Under conditions of constant hct (20%) and [HCO_3^-] (5 mmol l^{-1}), the resuspended blood of the turbot displayed the highest rate of "whole blood" (22.4 μmol ml^{-1} h^{-1}) or rbc CO_2 excretion (Fig. 3).The rates of CO_2 excretion in the other species were not different from each other (16.4–18.4 μmol ml^{-1} h^{-1}).

The rapid oxygenation of deoxygenated blood during the 3 min assay caused a significant increase in the whole blood CO_2 excretion in the teleosts but not in the dogfish (Fig. 4A).

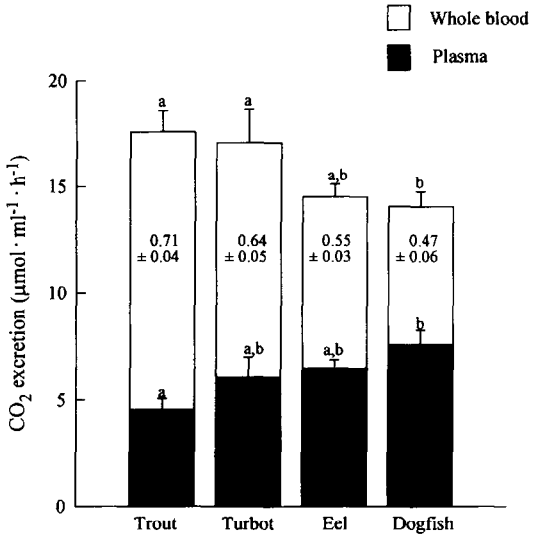

FIG. 2. A comparison of CO_2 excretion rates in whole blood (natural haematocrit and plasma [HCO_3^-]) and separated plasma (filled portion of bar) among three teleosts trout, turbot, eel) and an elasmobranch (dogfish) assayed under conditions of steady-state oxygenation. The % contribution of the red blood cell to the whole blood CO_2 excretion rate is indicated by the top portion of each bar and is also shown numerically. Data are shown as means ± 1 SE; N = 6 for each species. Statistical differences are illustrated by dissimilar letters.

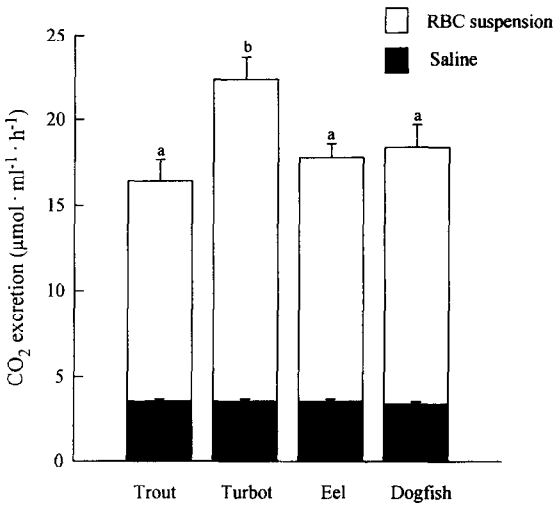

FIG. 3. A comparison of CO_2 excretion rates in blood that was re-suspended in Ringer to a constant haematocrit (20%) and plasma [HCO_3^-] (5 mmol l^{-1}) among three teleosts (trout, turbot, eel) and an elasmobranch (dogfish) assayed under conditions of steady-state oxygenation. The rate of CO_2 excretion in Ringer, alone, is indicated by the filled portion of each bar. Data are shown as means ± 1 SE; N = 6 for each species. Statistical differences are illustrated by dissimilar letters.

Normalization of the data to take into account the variable Hb levels in each species revealed that the effect of oxygenation was greatest in the turbot and absent altogether in the dogfish (Fig. 4B). In the teleosts, the rapid oxygenation of the blood was associated with a significant decrease in rbc pHi (Table 3) which was not evident in the dogfish. The extent of the increase in CO_2 excretion caused by oxygenation was a linear function of the magnitude of the Haldane effect. (Fig. 5). Thus, turbot displayed the largest O_2-mediated increase in CO_2 excretion followed by trout, eel and dogfish.

Under steady-state conditions in the teleost blood, the rate of whole blood CO_2 excretion was significantly greater in the deoxygenated blood when compared to the oxygenated blood (Fig. 6); this effect was not observed in the dogfish. The deoxygenated blood of the teleosts, but not the dogfish, displayed a significantly higher rbc pHi than the oxygenated blood (Table 3).

TABLE 2. Quantification of the Haldane effect in the four species utilized in this study

	$\Delta[HCO_3^-]$ (mmol l^{-1})	[Hb) (mmol^{-1})	$\Delta[HCO_3^-]/[Hb]$ (mmol mmol^{-1})
Trout	2.07 ± 0.20	3.45 ± 0.14	0.60 ± 0.02
Turbot	1.18 ± 0.09	1.32 ± 0.09	0.89 ± 0.03
Eel	0.80 ± 0.07	1.38 ± 0.07	0.58 ± 0.02
Dogfish	0.11 ± 0.04	1.56 ± 0.08	0.07 ± 0.01

The Haldane effect was quantified as the difference in true plasma [HCO_3^-] (Δ[HCO_3^-]) between deoxygenated and oxygenated blood at a PCO_2 of 0.53 kPa and then normalized for differences in haemoglobin concentration ([Hb]) among the various *in vitro* experiments. See text for each further details. Values shown are means ± 1 standard error; for species, three pairs of CO_2 dissociation curves were constructed (N = 3).

DISCUSSION

Interspecific Comparison of rbc CO_2 Excretion

This is the first study to compare the rates of rbc CO_2 excretion among several fish species under similar assay conditions. The radioisotopic procedure that was utilized (34) essentially measures the net rate of conversion of plasma HCO_3^- to gaseous CO_2. This process, in turn, is the summation of several steps including the entry of plasma HCO_3^- into the rbc in exchange for intracellular Cl^- (the so-called Cl^- shift; 4,20), its catalyzed dehydration to CO_2 in the presence of carbonic anhydrase, and finally the outward diffusive movement of CO_2 into the plasma and its evolution into the gas phase. Thus, any differences in the rates of rbc CO_2 excretion among the species could conceivably reflect differences in one or more of these steps. However, the catalyzed dehydration of HCO_3^- and the diffusion of CO_2 across the rbc membrane are generally not considered to be rate-limiting processes and are unlikely to vary amongst the species (13,21,24). Indeed, Perry and Gilmour (22) recently demonstrated that intracellular carbonic anhydrase activity was not a limiting factor governing the rate of whole blood CO_2 excretion in rainbow trout except under conditions of exceptionally low haematocrit (<5.0%). This observation is consistent with the generally accepted view that the activity of intracellular carbonic anhy-

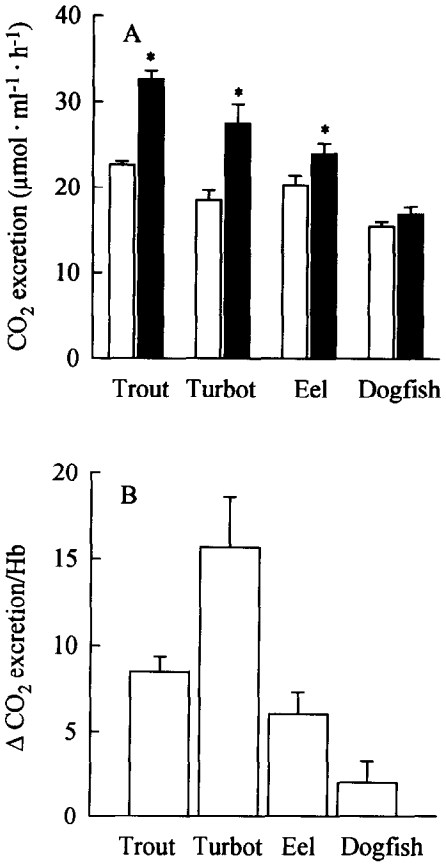

FIG. 4. (A) A comparison of the effects of rapid oxygenation on *in vitro* CO_2 excretion in the whole blood of three teleosts (trout, turbot, eel) and an elasmobranch (dogfish). Empty bars represent deoxygenated blood, filled bars represent rapidly oxygenated blood. Data are shown as means ± 1 SE; $N = 6$ for each species except turbot ($N = 4$). Statistical differences from the corresponding deoxygenated values are illustrated by asterisks. (B) The differences in the rates of CO_2 excretion between the deoxygenated and oxygenated blood when normalized for differences in haemoglobin among the species.

drase is well in excess of that required for catalysis of HCO_3^- dehydration. In the present study, therefore, it is likely that interspecific differences in the rate of whole blood CO_2 excretion reflected differences in the activity of rbc Cl^-/HCO_3^- exchange. Such differences might arise from variations in the numbers, affinities or activities of the Cl^-/HCO_3^- exchangers on the rbc membrane. Although the time course of the assay (3 min) is well in excess of gill transit time and the half time of rbc Cl^-/HCO_3^- exchange, it can nevertheless be used to detect inter- and intra-specific differences in rbc CO_2 excretion. For example, it is sensitive to treatments that slow the rate of excretion (e.g., application of the anion exchange inhibitor SITS) and treatments that accelerate the rate of excretion (e.g., increasing the haematocrit; 34).

It is important to emphasize that the radioisotopic assay employed estimates the rate of rbc CO_2 production from HCO_3^- of extracellular origin only and does not include the dehydration of intracellular unlabelled HCO_3^-. Thus, in this study any differences in the rates of rbc CO_2 excretion amongst the species must reflect differences in the dehydration of extracellular HCO_3^-.

TABLE 3. The effects of steady-state haemoglobin deoxygenation, steady-state oxygenation, or rapid oxygenation on rbc intracellular pH (pHi) in the four species utilized in this study.

	Steady-state deoxygenated-blood	Steady-state oxygenated blood	Rapid oxygenation
Trout	7.64 ± 0.01	7.46 ± 0.01*	7.33 ± 0.04*
Turbot	7.50 ± 0.06	7.37 ± 0.03*	7.38 ± 0.04*
Eel	7.48 ± 0.03	7.23 ± 0.04*	7.20 ± 0.04*
Dogfish	7.24 ± 0.04	7.22 ± 0.02	7.22 ± 0.03

The values shown are means ± 1 standard error; *indicates a significant difference from the deoxygenated blood.

In contrast to our original hypothesis, the blood of the dogfish did not exhibit higher rates of CO_2 excretion to compensate for the absence of a Haldane effect (see below). Indeed, under conditions of natural haematocrit and plasma $[HCO_3^-]$, the dogfish blood displayed the lowest rate of rbc CO_2 excretion. Based on the dependency of CO_2 excretion on haematocrit and plasma $[HCO_3^-]$ that was previously established for rainbow trout (34,22), the lower rate of whole blood CO_2 excretion in dogfish may reflect the combined impact of low plasma $[HCO_3^-]$ (2.8 mmol l^{-1}; Table 1) and intermediate haematocrit (16.7%; Table 1) in addition to the possibility of intrinsically lower activity of rbc Cl^-/HCO_3^- exchange. Despite the low haematocrit in the turbot (9.6%), the rate of whole blood CO_2 excretion was not statistically different than in trout (haematocrit = 26.2%) or eel (haematocrit = 14.0%) thus suggesting a greater activity of Cl^-/HCO_3^- exchange on a per rbc basis in the turbot (see below).

In addition to its lower rate of CO_2 excretion, the dogfish blood displayed a greater rate of CO_2 excretion by the plasma and consequently the contribution of the plasma to whole blood CO_2 excretion was high in this species (Fig. 2). The high rate of CO_2 excretion by the separated plasma of dogfish resulted from the catalysis of HCO_3^- dehydration by carbonic anhydrase because the rate of CO_2 excretion was lowered to typical teleost levels after the addition of the carbonic anhydrase inhibitor, acetazolamide (33). In contrast, the addition of acetazolamide to trout plasma was without effect (33). The presence of extracellular carbonic anhydrase did not arise from rbc lysis since there was no detectable haemoglobin in the plasma (33). The presence of extracellular carbonic anhydrase in the plasma of *S. canicula* and perhaps other species of elasmobranchs may explain the exceptionally low arterial PCO_2's in these fish. The rbc's of elasmobranchs are generally considered to lack an adrenergically activated Na^+/H^+ exchanger (30,33). In the absence of rbc Na^+/H^+ exchange, the presence of extracellular carbonic anhydrase in dogfish will assist CO_2 excretion without compromising normal physiological

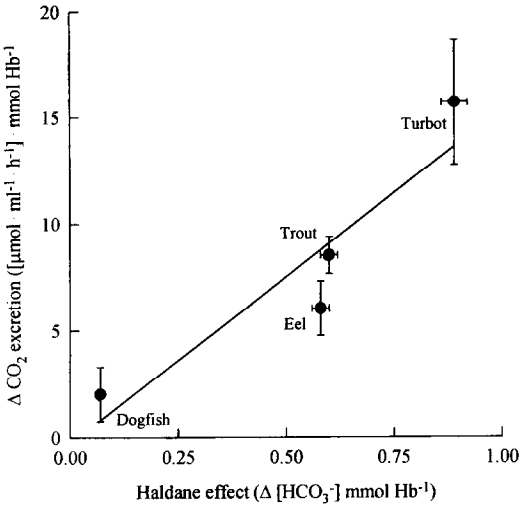

FIG. 5. The relationship between the magnitude of the Haldane effect (expressed as the difference in true plasma [HCO_3^-] between deoxygenated and oxygenated blood when normalized for differences in haemoglobin (Hb) levels among the species; Δ[HCO_3^-] mnol Hb^{-1}) and the effect of rapid oxygenation on whole blood CO_2 excretion (expressed as the difference in the rate of CO_2 excretion between deoxygenated and rapidly oxygenated blood; ΔCO_2 excretion). Data shown are means ± 1 SE; the linear regression equation is y = 53.0x − 0.04, r^2 = 0.90.

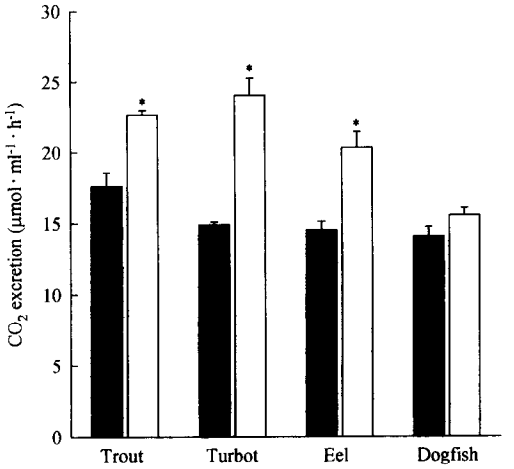

FIG. 6. The effect of steady-state oxygenation status on the rate of whole blood CO_2 excretion in three teleosts (trout, turbot, eel) and an elasmobranch (dogfish); filled bars represent oxygenated blood, empty bars represent deoxygenated blood. Data shown are means ± 1 SE. Asterisks indicate significant differences from the oxygenated blood.

function as it otherwise might in the teleosts that possess rbc Na^+/H^+ exchange (6,17,19; see also reviews by 16,28).

In order to reveal potential intrinsic differences in the rates of rbc CO_2 excretion among the species it was necessary to re-suspend the blood in saline at a constant haematocrit (20%) and plasma [HCO_3^-] (5 mmol l^{-1}). This demonstrated a high rate of rbc CO_2 excretion in the turbot blood in comparison to the other three species (trout, eel, dogfish). The simplest explanation for this result is that the turbot rbc exhibits a greater rate of Cl^-/HCO_3^- exchange owing to greater numbers of band-3 exchangers, activities and/or a higher affinity for the substrate, HCO_3^-. The greater intrinsic rate of rbc CO_2 excretion in the turbot clearly is beneficial to CO_2 excretion and may be viewed as an adaptive strategy to offset the particularly low haematocrit in this species, which would otherwise lower CO_2 exchange. If similar to other benthic flatfishes (e.g., *Platichthys stellatus*; 32; see 23 for review), the turbot is likely to have a high cardiac output. The high cardiac output is believed to help maintain normal rates of gas transfer in the face of low haematocrit. High cardiac output, on the other hand, will reduce the transit (residence) time of blood in the gill and clearly would only benefit CO_2 excretion if rbc Cl^-/HCO_3^- exchange activity is completed within this transit period. In trout, the half-time of rbc Cl^-/HCO_3^- exchange is about 400 msec (4,5) while the lower estimate of gill transit time is 500 msec. In turbot, the transit time is almost certainly less than in trout and thus a greater activity of rbc Cl^-/HCO_3^- exchange may be required to ensure completion of Cl^-/HCO_3^- exchange.

The high intrinsic rate of rbc Cl^-/HCO_3^- exchange in the turbot may, in part, explain the low arterial PCO_2 in relation to arterial PO_2. The low arterial PO_2 in turbot is a characteristic of species with high-affinity haemoglobin and results primarily from low ventilation volumes. Generally, low ventilation volumes are associated with high arterial PCO_2 values. The apparent disagreement between $PaCO_2$ and PaO_2 in the turbot can thus be explained by the elevated rate of rbc Cl^-/HCO_3^- exchange which may serve to increase the "effective" diffusing capacity (overall diffusive conductance; 24) of the gill for CO_2.

Unlike the situation in turbot, alteration of the haematocrit in the eel, trout, or dogfish probably had little impact on the rate of whole blood CO_2 excretion. In trout (22) and dogfish (33), the rate of whole blood CO_2 excretion is positively related to haematocrit between 0–15%, but insensitive to further changes in haematocrit above 15%. The equalization of the rates of whole blood CO_2 excretion in the trout, eel and dogfish after re-suspension of blood to constant haematocrit and plasma [HCO_3^-] may have reflected the small changes in plasma [HCO_3^-] (22) although other studies (34,35) failed to demonstrate large effects of plasma [HCO_3^-]. Regardless of the underlying mechanisms, the results clearly demonstrated a high intrinsic rate of rbc CO_2 excretion in the turbot, but did not reveal a low intrinsic activity in the dogfish as the results of series 1 appeared to indicate (Fig. 2).

Effects of Oxygenation/Deoxygenation

The Haldane effect can be quantified in numerous ways. In the present study, we chose to quantify the Haldane effect as the difference in true plasma [HCO_3^-] between the deoxygenated and oxygenated blood because it is this difference which is important for CO_2 excretion. The suitability of this tech-

nique to detect a Haldane effect relies on the presence of a rbc Cl^-/HCO_3^- exchanger. Red cell Cl^-/HCO_3^- exchange has been documented in trout and dogfish (20,33) and seems a reasonable assumption for turbot and eel. The results demonstrated significant Haldane effects in the teleosts but not in the dogfish. The absence of a Haldane effect in *Scyliorhinus canicula* is in agreement with most previous studies on this (25) and other elasmobranch species (summarized in 2). Nevertheless, the lack of a Haldane effect in *S. canicula* and other elasmobranchs is surprising because, with a single exception (*Squalus acanthias*; 14), they are known to possess significant Bohr effects (see 2). It has been suggested that a large Pasteur effect in elasmobranch blood may counteract the effects of oxygenation/deoxygenation on pH or $[HCO_3^-]$ and thus obscure any Haldane effect (25) that might be occurring simultaneously. This explanation, however, is only plausible for long-term incubation of blood and is unlikely to explain the total absence of a Haldane effect (as measured by rbc pHi changes; Table 3) during rapid (3 min) oxygenation of deoxygenated blood.

It is generally accepted that the buffering capacity of elasmobranch haemoglobin is significantly higher than that of teleost haemoglobin (11,12). It has been argued that the higher buffering capacity of elasmobranch haemoglobin may partially offset the negative consequences of the absence of a Haldane effect for CO_2 excretion by increasing the availability of H^+ for the dehydration of HCO_3^- (12). It is also possible, however, that the high buffering capacity masks an existing Haldane effect by effective bufering of oxylabile protons. Regardless, if protons are being released from haemoglobin during oxygenation, as the Bohr effect implies, they do not contribute to a change in rbc pHi or true plasma $[HCO_3^-]$ and thus appear to have no role in CO_2 excretion (see below).

The rapid oxygenation of the deoxygenated teleost blood increased the rate of whole blood CO_2 excretion and thus confirmed and further extended the previous empirical observations of Perry and Gilmour (22) on rainbow trout. These findings support the notion that H^+ availability is an important factor limiting rbc HCO_3^- dehydration in the teleosts. Thus, the release of H^+ from haemoglobin during oxygenation of the blood (via the Haldane effect) is an important factor governing branchial CO_2 excretion. This study has clearly shown, as theory predicts, that the extent of the benefit of oxygenation on CO_2 excretion in a given species is dependent on the magnitude of the Haldane effect. Thus, the turbot, by virtue of its large Haldane effect, displayed the greatest increase in CO_2 excretion upon rapid oxygenation. This can be viewed as a further adaptive mechanism (in addition to high intrinsic rates of rbc CO_2 excretion) to aid CO_2 excretion in species such as turbot that exhibit low haematocrits. In agreement with the absence of a measurable Haldane effect in the dogfish, rapid oxygenation of the blood did not increase the rate of CO_2 excretion. It would appear therefore, that the oxygenation of haemoglobin, in itself, does not influence any of the steps in rbc CO_2 excretion.

Two previous studies (22,35) demonstrated that under steady-state conditions, oxygenated blood of rainbow trout displayed lower rates of CO_2 excretion than did deoxygenated blood. Perry and Gilmour (22) suggested that the underlying mechanism was related to effects of changing rbc pHi on the activity of the Cl^-/HCO_3^- exchanger whereas Wood and Simmons (35) suggested that conformational changes in haemoglobin linked to the cytoplasmic domain of the band-3 Cl^-/HCO_3^- exchanger were the basis of the effect. The results of the present study support the former hypothesis because oxygenation of the dogfish blood was without effect on both rbc pHi and CO_2 excretion; an effect was clearly evident in the teleost species that also displayed significant effects of oxygenation on rbc pHi.

During transit through the gill, oxygenation of the blood will affect CO_2 excretion in opposing ways. The Haldane effect will enhance CO_2 excretion whereas oxygenation of haemoglobin by an unknown effect (perhaps related to rbc pHi changes) will impede CO_2 excretion. Under conditions of full oxygenation, as in the present *in vitro* study, the positive influence of the Haldane effect predominates and there is a net benefit to CO_2 excretion. The contribution of final oxygenation status to rbc CO_2 excretion may become more important under conditions of environmental hypoxia. As haemoglobin oxygenation is reduced during hypoxia, the contribution of the Haldane effect to CO_2 excretion would be less and thus at such times it would be beneficial for deoxygenated blood to exhibit a greater rate of CO_2 excretion than oxygenated blood.

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