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HCO_3^- dehydration by the blood of an elasmobranch in the absence of a Haldane effect

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Abstract

We measured in vivo arterial P_{CO_2} and Ca_{CO_2} in *Scyliorhinus canicula* and found them to be very low (~ 1 Torr and 3 mmol l^{-1} respectively). In vitro, the Haldane effect was functionally absent, and there was no detectable β-adrenergic Na^+/H^+ exchange, in contrast to teleosts. The HCO_3^- dehydration rate of the blood, measured by a radioisotopic assay (Wood and Perry, *J. Exp. Biol. 157: 349–366, 1991*), was independent of steady-state deoxygenation or oxygenation, unaffected by rapid oxygenation, and insensitive to isoprenaline, amiloride, and removal of urea or TMAO. SITS and acetazolamide reduced the rate; HCO_3^-/Cl^- exchange rather than intracellular carbonic anhydrase (CA) was the rate-limiting factor. The rate was not altered by steady-state plasma [HCO_3^-], but increased linearly with P_{CO_2} and with RBC concentration, saturating at hematocrits $\geq 15\%$. The rate in separated plasma accounted for $\sim 50\%$ of the whole blood rate, was higher than in trout plasma or saline, and was inhibited by acetazolamide. The presence of CA in the normally circulating blood plasma of dogfish may contribute to highly efficient CO_2 excretion in vivo.

Keywords: Blood, CO_2 hydration/dehydration rate; Carbon dioxide, blood, HCO_3^- conversion rate, red cell exchange; Fish, dogfish (*Scyliorhinus canicula*); Protein, band 3; Red blood cell, CO_2 exchange

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1. Introduction

In many animals including teleost fish, Bohr protons are released from hemoglobin as the molecule becomes oxygenated. In consequence, the oxygenated blood holds less CO_2, and has a lower pH and total buffer capacity. This Haldane effect promotes CO_2 unloading from the blood at the gas exchange surface by increasing the supply of protons for HCO_3^- dehydration (Klocke, 1988). Using a modification of the radioisotopic assay of Wood and Perry (1991), Perry and Gilmour (1993) showed that rapid oxygenation elevates the HCO_3^- dehydration rate by about 40% in the blood of rainbow trout, a species with a well-defined Haldane effect (Jensen, 1989). In elasmobranchs, CO_2 excretion through the gills is highly efficient, resulting in Pa_{CO_2} (~ 1 Torr) and Ca_{CO_2} levels (~ 4 mmol·L^{-1}) often lower than in teleosts (e.g. Truchot et al., 1980; Heisler et al., 1988; Graham et al., 1990), yet the Haldane effect is reported to be either very small or absent (see Butler and Metcalfe, 1989 for a detailed review). This raises the question whether oxygenation has any effect on HCO_3^- dehydration by elasmobranch blood.

Many teleosts possess a β-adrenergically mediated Na^+/H^+ exchange mechanism (Nikinmaa, 1992) in the plasma membranes of the red blood cells (RBCs). The only study to date reports that such a mechanism is lacking in elasmobranch RBCs (Tufts and Randall, 1989). In trout blood, β-adrenergic activation of Na^+/H^+ exchange under acidotic conditions not only raises RBC pHi, but also reduces the rate of HCO_3^- dehydration (Wood and Perry, 1991; Perry et al., 1991; Wood and Simmons, 1994). The effect appears to be an indirect one, acting to lower the rate at which plasma HCO_3^- enters the RBC via the electroneutral "band 3" HCO_3^-/Cl^- exchanger. A similar HCO_3^-/Cl^- exchange mechanism is present in the RBCs of elasmobranchs and appears to be the rate-limiting step in the conversion of plasma HCO_3^- to molecular CO_2 (Obaid et al., 1979). Circulating catecholamine levels are much higher in elasmobranchs than teleosts (Butler et al., 1979; Butler and Metcalfe, 1989), but it is not known whether they have any influence on CO_2 excretion at the level of the RBC.

Another difference between teleosts and elasmobranchs is the very high levels of urea and trimethylamine oxide (TMAO) in the latter. Urea markedly influences the O_2 affinity of the hemoglobin (Weber et al., 1983; Tetens and Wells, 1984) whereas the TMAO/urea ratio is considered critical in protecting protein function in general against the destabilizing effects of high urea (Yancey and Somero, 1979). Nothing is known about the possible effects of urea and TMAO on HCO_3^- dehydration in elasmobranch blood.

The present study on *Scyliorhinus canicula* therefore has several objectives. The first is to critically reassess whether or not a Haldane effect and/or a β-adrenergic Na^+/H^+ exchange is present in the RBCs. The experimental difficulties in detecting a Haldane effect have been discussed by Cross et al. (1969) and Pleschka et al. (1970), and in detecting a β-adrenergic Na^+/H^+ exchange by Tufts and Randall (1989) and Nikinmaa (1992). Our second objective, employing the $[^{14}C]HCO_3^-$ assay of Wood and Perry (1991), is to examine the influence of oxygenation, β-adrenergic stimulation, and urea and TMAO levels on the rate of plasma HCO_3^- dehydration by the RBCs. The third, using this same approach, is to characterize the influence of several factors known to

be important in setting HCO_3^- dehydration rate in the blood of trout – the plasma rate, the plasma HCO_3^- level, and the effects of hematocrit, RBC lysis, and P_{CO_2} (Wood and Perry, 1991; Perry and Gilmour, 1993).

2. Materials and methods

Experimental animals Lesser spotted dogfish (*Scyliorhinus canicula*; 350–1100 g, $n = 33$) were netted off the coast of Brest, France in May and June, 1993, and held outdoors for up to one month in flowing seawater at 14 °C with daily feeding of herring. The fish were then moved to an indoor recirculating system (full strength seawater, 33 ppt, reconditioned by charcoal filtration and UV-irradiation) at 15 ± 1 °C and held without feeding for up to 1 week prior to experimentation.

In 27 dogfish, arterial catheters (PE50) were placed in the anterior mesenteric artery as described by Graham et al. (1990) while the fish were anaesthetized with MS-222 (0.05–0.125 $g \cdot L^{-1}$ as required). Catheters were filled with the basic saline (A) described below, containing 50 i.u. $\cdot ml^{-1}$ lithium heparin. The fish were allowed to recover for 24 h in individual, covered 20 or 40 L Perspex cylinders fed with a constant flow of air-equilibrated seawater. For comparative purposes, blood plasma was also obtained from 4 catheterized freshwater rainbow trout as described by Wood and Perry (1991).

Blood handling All blood was obtained from the 27 catheterized dogfish, unless otherwise stated. Blood was withdrawn slowly via the arterial catheter; sampling ceased as soon as any struggling commenced. For measurements of in vivo conditions in the blood, the first 0.7 ml was taken into a gas-tight Hamilton syringe. The remaining blood was immediately heparinized at 100 i.u. ml^{-1} by addition of 5% volume of a lithium heparin stock in basic saline (A), and stored on ice for 1–2 h before use. Depending on the particular experimental design, blood was either pooled from 3–4 different fish, or treated separately for each fish.

To assess whether arterial catheterization was a confounding factor (see Results), blood was obtained from 6 uncannulated dogfish by cardiac puncture. Particular care was taken to avoid any chance of hemolysis. The fish was rapidly anaesthetized with a high dose of MS-222 (0.2 $g \cdot L^{-1}$), the heart directly exposed, and the ventricle punctured with a wide bore 18 gauge needle attached to the open barrel of a 5 ml plastic syringe so that the blood would enter under its own pressure. The first 0.5 ml was discarded, the syringe barrel was changed, and then a 3–5 ml sample was taken in the same manner.

For the determination of CO_2 dissociation curves, blood was aliquotted into Eschweiler tonometers (15 ± 0.5 °C) gassed with various humidified mixtures ($P_{CO_2} = 0.5$–9 Torr) of either CO_2 in air or CO_2 in N_2 from Wosthoff 301a-F mixing pumps for 30 min at each P_{CO_2}. For other tests, blood was aliquotted into either round-bottom 100 ml tonometer flasks (if pre-equilibration was required) or directly into 20 ml glass scintillation vials for assays of HCO_3^- dehydration rate; both were mounted in a constant temperature (15 ± 0.5 °C) shaker bath and continually gassed with humidified mixtures from the Wösthoff pumps. Once the blood was placed in the assay vials, it

was equilibrated on open-circuit with the appropriate P_{CO_2} for at least 60 min prior to test. The standard P_{CO_2} used in most HCO_3^- dehydration tests was 3.75 Torr, close to the reported Pv_{CO_2} of 3.5 ± 0.3 Torr for this species (Butler et al., 1979).

Some experiments involved manipulation of hematocrit, and others resuspension of the blood in various salines. In the former, pooled blood was pre-equilibrated to $P_{CO_2} = 3.75$ Torr, and then centrifuged at $500 \times g$ for 5 min to separate plasma from RBCs. Appropriate amounts of homologous plasma and RBCs were remixed to achieve the desired hematocrits. In saline experiments, RBCs were washed three times with appropriate saline (see below) before final resuspension at the desired hematocrit. In experiments requiring lysis of the RBCs, the sealed assay vial containing the pre-equilibrated saline/RBC mixture was alternately dipped into liquid air and 15 °C water 3 times for rapid (~ 5 min) and complete freeze-thaw lysis.

Salines and drugs The basic saline (A) contained the following, in $mmol \cdot L^{-1}$: 250 NaCl; 7 Na_2SO_4; 3 $MgSO_4$; 4 KCl; 2 $CaCl_2.2H_2O$; 5 $NaHCO_3$; 0.1 Na_2HPO_4; 4 glucose; 450 urea; 100 trimethylamine oxide; pH 7.8. In TMAO-free saline (B), TMAO was omitted and NaCl was raised to 300 $mmol \cdot L^{-1}$; in urea-free saline (C), urea was omitted, and NaCl was raised to 475 $mmol \cdot L^{-1}$.

The following drugs were obtained from Sigma: lithium heparin, dimethyl sulfoxide (DMSO), l-adrenaline bitartrate, l-isoprenaline bitartrate; 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS), amiloride hydrochloride, acetazolamide, bovine erythrocytic carbonic anhydrase (10000 Wilbur-Anderson units per mg protein). Drugs were dissolved in either basic saline (adrenaline, isoprenaline, carbonic anhydrase) or saline containing 2% DMSO (SITS, acetazolamide, amiloride, and in some tests isoprenaline), and added to blood in 5% volume; final DMSO concentration did not exceed 0.1%. Similar additions of saline or saline-2% DMSO served as controls.

The HCO_3^- dehydration assay The assay was performed as described by Wood and Perry (1991; see their Fig. 1), with minor modification. The assay measures the *net* rate at which HCO_3^- originating in the blood plasma is dehydrated and evolved as CO_2 during a 3 min exposure to an external P_{CO_2} close to 0 Torr. As the label is placed in the blood plasma, and specific activity is measured in the blood plasma, it does not include the dehydration of HCO_3^- already inside the RBCs at the time of assay. In brief, 1.0 ml units of blood, plasma, or RBC/saline mixture as appropriate were aliquotted into separate 20 ml glass scintillation vials placed in a shaking water bath at 15 ± 0.5 °C. Each vial was sealed with a rubber septum containing an entry and exit port for gas equilibration on open-circuit. Samples were gassed continuously with humidified P_{CO_2} mixtures in either N_2 (for steady-state deoxygenation and rapid oxygenation tests) or air (for steady-state oxygenation tests) for at least 60 min prior to assay. At the start of an assay, 2 μCi (20 μl of 100 $\mu Ci \cdot ml^{-1}$ stock) of sodium [^{14}C]bicarbonate (Amersham) in basic saline (A) was added to the sample, the overlying gas phase was changed to either pure N_2 (for steady-state deoxygenation tests) or pure O_2 (for steady-state oxygenation and rapid oxygenation tests), the vial was sealed with a rubber stopper containing a $^{14}CO_2$ trap, and shaking was continued for exactly 3 min. Preliminary tests demonstrated that deoxygenated blood became fully oxygenated within 45 sec when the

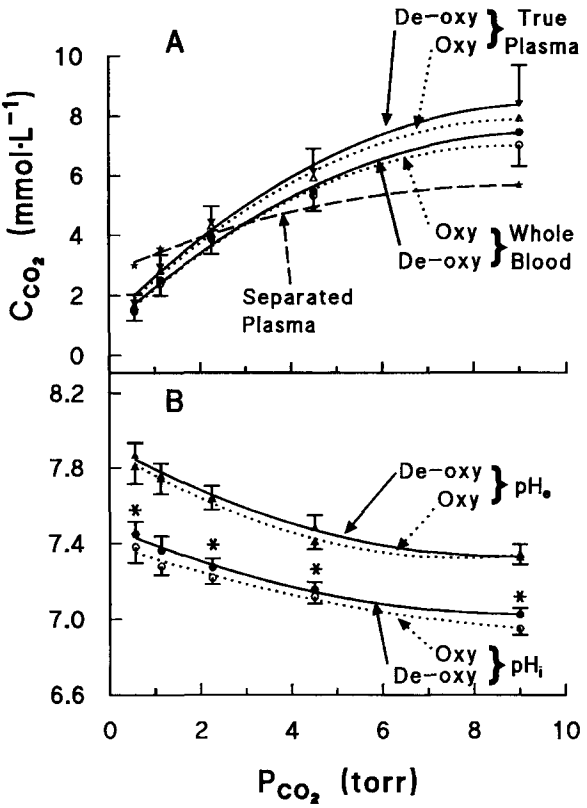

Fig. 1. (A) Mean CO_2 dissociation curves for whole blood and true plasma of the dogfish *Scyliorhinus canicula* under oxygenated and deoxygenated conditions. Data from paired comparisons for 3 separate animals (means ± 1 SEM) are shown; hematocrit = 14.2 ± 0.6%, hemoglobin = 3.77 ± 0.20 g.100 ml^{-1}. At all P_{CO_2} values, the C_{CO_2} of true plasma was greater than that of whole blood ($P<0.05$), but there were no significant differences between comparable oxygenated and deoxygenated values. The CO_2 dissociation curve for the separated plasma of one dogfish is also shown. (B) Relationships between P_{CO_2} and extracellular pHe and RBC intracellular pHi from the same equilibrations as in (A). Asterisks indicate significant differences ($P<0.05$) between oxygenated and deoxygenated values at the same P_{CO_2}.

overlying gas phase was changed to O_2. The CO_2 trap was a fluted filter paper (Whatman GF/A 2.4 cm glass microfibre filter) impregnated with 150 μl of 1 mol·L^{-1} of hyamine hydroxide in methanol. After 3 min, the filter was immediately removed and placed in fluor for assay of ^{14}C-radioactivity, and the blood was drawn into a gas-tight Hamilton syringe for immediate measurement of pHe, hemoglobin, hematocrit, and other parameters as required. The remaining blood was then centrifuged (12000 × *g* for 2 min) in a sealed tube for separation of true plasma and analysis of plasma C_{CO_2} and ^{14}C-radioactivity, and thereby plasma specific activity (dpm·μmol^{-1}). The RBC pellet was frozen in liquid air for later determination of RBC pHi. True plasma (2 × 50 μl) and filters were counted in 10 ml ACS fluor (Amersham) on a Packard 1600TR Tri

Carb liquid scintillation counter with automatic quench correction. The HCO_3^- dehydration rate for each sample was calculated by dividing the filter paper ^{14}C-radioactivity (dpm, corrected by the known trapping efficiency) by plasma specific activity ($dpm.\mu mol^{-1}$) and time (0.05 h).

Analytical techniques For determination of RBC water content (g/g dry weight), a RBC pellet was obtained by centrifugation at $12000 \times g$ for 2 min in a narrow plastic centrifuge tube (400 μl), cut out below the pellet surface, and dried to a constant weight at 65 °C. Plasma water content was similarly determined by drying. Standard techniques for hemoglobin (cyanmethemoglobin method), hematocrit ($10000 \times g$ for 5 min), total nucleoside triphosphate ("NTP"; phosphoglycerate phosphokinase glyceraldehyde phosphate dehydrogenase enzyme system from Sigma), urea (diacetyl monoxime system from Sigma), RBC intracellular pH (freeze-thaw lysate method on the pellet) and blood gas analyses were used (see Graham et al., 1990, Wood and Perry, 1991 for details). Mean cell hemoglobin concentration (MCHC; $g \cdot ml^{-1}$) was calculated from the hemoglobin/hematocrit ratio. NTP was expressed as umol/g hemoglobin concentration. For plasma and saline samples, the sensitivity of the hemoglobin assay was increased 4-fold to detect any possibility of hemolysis. Radiometer blood gas electrodes were thermostatted to 15 ± 0.5 °C for pH and P_{O_2} analyses, and 38 ± 0.5 °C for C_{CO_2} and C_{O_2} measurements in Cameron and Tucker chambers respectively. P_{CO_2} and HCO_3^- were calculated from pH and true plasma C_{CO_2} by rearrangement of the Henderson-Hasselbalch equation using values of pK′ and αCO_2 for *Scyliorhinus* plasma at 15 °C from Albers and Pleschka (1967).

Data are expressed as means $\pm$ 1 SEM (N) where N is the number of fish in individual experiments or the number of independent replicates in experiments based on a common blood pool. For paired comparisons, statistical significance ($P < 0.05$) of differences was assessed by means of Student's two-tailed paired t-test; the t-value was adjusted by the Bonferroni procedure for repeated paired comparisons. For independent comparisons, the data were subjected to one-way analysis of variance and specific differences were detected by means of Duncan's new multiple range test in cases where the F-value indicated significance. A 5% significance level was used throughout.

3. Results

In vivo conditions In comparison to most teleost fish, the arterial blood of *Scyliorhinus canicula* exhibited very low levels of Pa_{CO_2} and Ca_{CO_2} (and therefore plasma HCO_3^-), relatively low hematocrit, hemoglobin, and Ca_{O_2} levels, and very high plasma urea concentrations (Table 1). Whole blood C_{CO_2} levels were marginally lower than plasma values, but when individually measured hematocrit values (Table 1), mean RBC water content ($71.3 \pm 1.8\%$, $n = 5$), and mean plasma water content ($93.2 \pm 0.3\%$, $n = 3$) were taken into account, RBC intracellular C_{CO_2} (2.97 $mmol \cdot L\ H_2O^{-1}$) became identical to extracellular C_{CO_2} (2.96 $mmol \cdot L\ H_2O^{-1}$).

CO_2 dissociation curves and the Haldane effect Paired CO_2 dissociation curves (oxygenated/deoxygenated) were determined for the bloods of 3 different dogfish. The

Table 1
Measurements of arterial blood parameters *in vivo* in resting dogfish *Scyliorhinus canicula* at 15°C; means ± 1 SEM ($n = 9$)

Pa_{O_2} (torr)	98.1 ± 9.0	Whole blood Ca_{O_2} (mmol·L^{-1})	1.91 ± 0.15
Pa_{CO_2} (torr)	1.02 ± 0.11	Hematocrit (%)	16.9 ± 1.1
Whole blood Ca_{CO_2} (mmol·L^{-1})	2.66 ± 0.32	Hemoglobin (g·100 ml^{-1})	4.79 ± 0.15
True plasma Ca_{CO_2} (mmol·L^{-1})	2.76 ± 0.31	MCHC (g·ml^{-1})	0.295 ± 0.024
pHe	7.777 ± 0.017	RBC pHi	7.317 ± 0.019
Plasma urea (mmol·L^{-1})	465.9 ± 20.5		

oxygenated and deoxygenated points were determined simultaneously at each P_{CO_2}, but in practice the gas mixing pump cascades used for the oxygenated and deoxygenated equilibrations produced slightly different P_{CO_2} values. Therefore individual curves were plotted against measured P_{CO_2} values and interpolated to common nominal P_{CO_2} values prior to averaging for Fig. 1. For one fish, a separated plasma curve (oxygenated) was also measured.

The CO_2 dissociation curves of whole blood and true plasma were steep in the normal arterial – venous range (0.5–4 torr) and tended to flatten at higher P_{CO_2} (Fig. 1A). The slope for separated plasma was much lower in the physiological range. C_{CO_2} levels of true plasma were significantly greater than those of whole blood at all P_{CO_2} values. However, as with the in vivo observations, there was no discernable difference between intracellular and extracellar CO_2 concentrations once the hematocrit and respective H_2O contents were taken into account. Extracellular pH was about 0.46 units above RBC pHi at low P_{CO_2} both in vitro (Fig. 1B) and in vivo (Table 1). Both pHe and RBC pHi declined at higher P_{CO_2} values, as did the difference between the two (0.36 units at 9 Torr).

Oxygenation vs deoxygenation had no significant effect on CO_2 binding by either whole blood or true plasma, indicating the absence of a Haldane effect (Fig. 1A). Extracellular pH was similarly unaffected (Fig. 1B) and there was no significant difference in non-HCO_3^- buffer capacity for true plasma between oxygenated (10.1 ± 0.9 Slykes) and deoxygenated blood (10.8 ± 1.8 Slykes, $n = 3$). The buffer capacity of separated plasma was only 2.6 Slykes. Curiously, RBC pHi was slightly but significantly higher in deoxygenated blood at most P_{CO_2} values (Fig. 1B), which could suggest the presence of a small Haldane effect not seen in the other parameters.

In light of this equivocal result, bloods from 6 additional dogfish were assessed at a single $P_{CO_2} = 4.13$ Torr. The same cascade of gas mixing pumps was used sequentially to ensure identical P_{CO_2} values for deoxygenated and oxygenated blood. These paired comparisons demonstrated that true plasma C_{CO_2}, pHe, and RBC pHi were identical under oxygenated and deoxygenated conditions (Table 2), confirming the functional absence of a Haldane effect.

Table 2
A paired comparison of true plasma CO_2 content, pHe, and RBC pHi in the bloods of 6 dogfish *Scyliorhinus canicula* equilibrated to an identical P_{CO_2} = 4.13 Torr under oxygenated or deoxygenated conditions; means ± 1 SEM (n = 6)

	Oxygenated	Deoxygenated
C_{CO_2} (mmol l^{-1})	6.34 ± 0.22	6.43 ± 0.25
pHe	7.550 ± 0.022	7.562 ± 0.013
RBC pHi	7.161 ± 0.020	7.162 ± 0.019

There are no significant differences.

Oxygenation status and HCO_3^- dehydration rate The absence of a Haldane effect was correlated with the absence of a significant stimulatory effect on HCO_3^- dehydration when deoxygenated blood was rapidly oxygenated during the course of the [^{14}C]HCO_3^- assay (Table 3; paired comparisons in individual fish). There were also no significant effects of rapid oxygenation on either pHe or RBC pHi (Table 3). A paired comparison for the same fish of HCO_3^- dehydration rates, pHe, and RBC pHi under steady-state oxygenation versus steady-state deoxygenation conditions similarly revealed no significant differences. However, when steady-state oxygenated blood was compared with rapidly oxygenated blood, there was a slightly higher HCO_3^- dehydration rate and lower pHe in the latter, but no difference in RBC pHi (Table 3). Surprisingly, the rate for separated plasma accounted for more than 50% of the rate for whole blood, at least under steady-state oxygenation (Table 3).

Adrenergic responsiveness Tests for the presence of a β-adrenergic Na^+/H^+ exchange in the RBCs of *Scyliorhinus canicula* were uniformly negative. These included the use of both adrenaline and isoprenaline in concentrations up to 10^{-4} M, various time

Table 3
A paired comparison of HCO_3^- dehydration rates, pHe, and RBC pHi in the plasma and whole blood of 6 dogfish *Scyliorhinus canicula* pre-equilibrated to P_{CO_2} = 3.75 torr. Whole blood was assayed under conditions of steady-state oxygenation, steady-state deoxygenation, and rapid oxygenation from the deoxygenated condition; means ± 1 SEM (n = 6)

	Plasma	Whole blood		
	Oxy	Oxy	Deoxy	Deoxy→Oxy
HCO_3^- dehydration rate (μmol·ml^{-1} h^{-1})	7.60^a ± 0.66	14.14^b ± 0.62	15.53^{b,c} ± 0.53	16.91^c ± 0.88
pHe	7.637^a ± 0.025	7.686^b ± 0.029	7.667^{a,b} ± 0.025	7.638^a ± 0.028
pHi	–	7.218^a ± 0.020	7.240^a ± 0.040	7.220^a ± 0.027

Means sharing the same letter are not significantly different from one another ($P > 0.05$).
Mean hematocrit = 16.3 ± 1.3%, mean hemoglobin = 4.20 ± 0.30 g·100 ml^{-1}.

courses, deoxygenated conditions, elevated P_{CO_2} (7.5 Torr), washing the RBCs in saline, and storing them overnight at 4 °C to remove endogenous catecholamines or persistent desensitization. In addition to pHe and RBC pHi, NTP per unit hemoglobin, MCHC, and RBC H_2O content were all evaluated as possible indicators of RBC responses to adrenergic stimulation (Fig. 2). All approaches proved negative.

Pharmacological characterization of HCO_3^- dehydration In view of the high HCO_3^- dehydration rate of separated plasma (Table 3), these tests were performed on a blood pool with a hematocrit enriched to 19% to ensure the maximum contribution of the RBCs (see Fig. 5). All drugs were allowed to act for 2 h, except isoprenaline which was added 5 min prior to test (see Wood and Perry, 1991). In accord with the absence of a β-adrenergic Na^+/H^+ exchange response, neither isoprenaline (10^{-4} M) nor

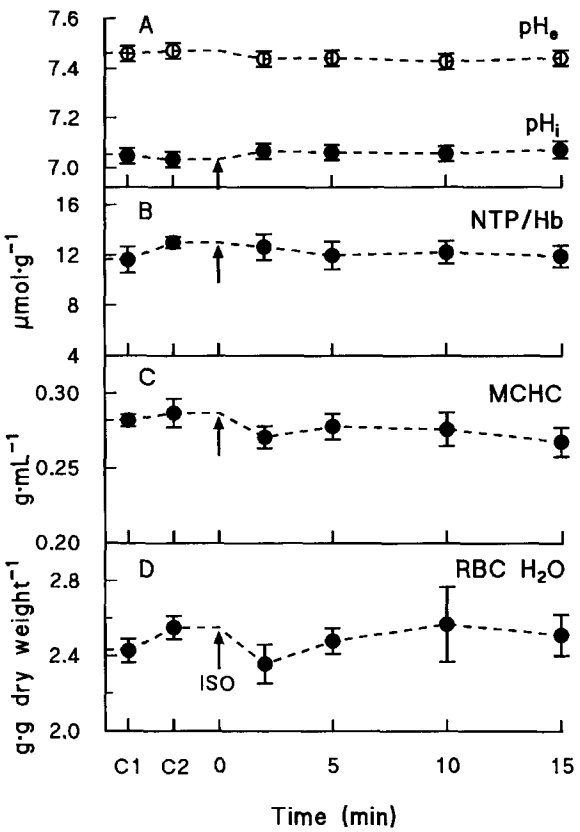

Fig. 2. The lack of influence of isoprenaline addition (ISO, 10^{-4} M at arrow) on RBC intracellular pHi, extracellular pHe, NTP/g hemoglobin (NTP/Hb), mean cell hemoglobin concentration (MCHC), and RBC H_2O content in the blood of the dogfish *Scyliorhinus canicula*. Data from separate experiments with 5 different blood pools (3 oxygenated, 2 deoxygenated, all at P_{CO_2} = 7.5 Torr) are shown. Means ± 1 SEM (n = 5). There were no significant differences ($P > 0.05$) relative to the pre-isoprenaline control values, C1 and C2.

amiloride (10^{-4} M) had any effect on the HCO_3^- dehydration rate (Fig. 3A). However, SITS (10^{-4} M) reduced the whole blood rate by 33%, equivalent to about a 50% inhibition of the RBC rate, indicating the involvement of a Cl^-/HCO_3^- exchange mechanism. Acetazolamide (10^{-4} M), a potent antagonist of carbonic anhydrase, reduced the whole blood rate by 70%, which was about 110% inhibition of the the RBC rate. Only acetazolamide caused a blood acidosis (Fig. 3B), presumably a reflection of the inhibited CO_2 excretion; pHe and RBC pHi were unaffected by the other drugs (Fig. 3B).

HCO_3^- dehydration by blood plasma The relatively high HCO_3^- dehydration rate of separated plasma (Table 3) and the large inhibitory effect of acetazolamide in whole blood (Fig. 3) suggested that carbonic anhydrase activity might be present in blood plasma. In confirmation, acetazolamide (10^{-4} M) caused a significant 30% inhibition of the dehydration rate in plasma separated from blood freshly drawn from the arterial catheters of individual dogfish (Table 4; paired comparisons). Plasma from rainbow trout obtained in the same manner exhibited a significantly lower rate which was

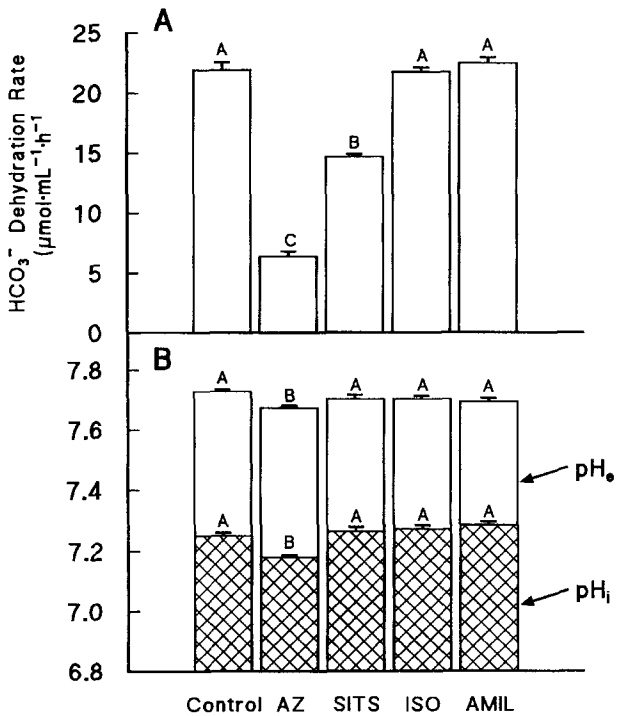

Fig. 3. The effects of acetazolamide (AZ), 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS), isoprenaline (ISO), amiloride (AMIL), all at 10^{-4}M in 0.1% DMSO, and control treatment (0.1% DMSO alone) on (A) HCO_3 dehydration rate and (B) extracellular pHe and RBC pHi in the blood of the dogfish *Scyliorhinus canicula* pre-equilibrated to $P_{CO_2} = 3.75$ Torr. A common pool of blood (hematocrit = 18.9%, hemoglobin = 4.81 g. 100 ml^{-1}) was used for all tests. Means ± 1 SEM ($n = 5–6$). Means sharing the same letter are not significantly different from one another ($P > 0.05$).

Table 4
A paired comparison of HCO_3^- dehydration rates ($\mu mol \cdot ml^{-1} \cdot h^{-1}$) in plasma pre-equilibrated to $P_{CO_2} = 3.75$ Torr in the presence or absence of 10^{-4} M acetazolamide. Plasma was obtained from dogfish *Scyliorhinus canicula* by arterial catheter or cardiac puncture (see text), and from rainbow trout *Oncorhynchus mykiss* by arterial catheter; means ± 1 SEM (*n*)

	Plasma	Plasma and acetazolamide (10^{-4} M)
Catheter		
Dogfish	5.40 ± 0.36 (10)	3.72 ± 0.17*
Trout	3.49 ± 0.11 (4)	4.05 ± 0.33 (4)
Cardiac puncture		
Dogfish	6.10 ± 0.39 (6)	4.63 ± 0.26* (6)

* significant differences ($P < 0.05$) by paired comparison.
Rates in dogfish and trout plasma were significantly different ($P < 0.05$) in the absence of acetazolamide only.

unaffected by acetazolamide (Table 4). Although the sensitivity of the hemoglobin assay was increased 4-fold (detection limit ~0.1 g. 100 ml^{-1}), there was no evidence of hemolysis in the dogfish plasma samples. Nevertheless, it remained possible that the stress of anaesthesia, surgery, and/or catheter sampling may have caused undetectable hemolysis sufficient to release significant amounts of RBC carbonic anhydrase into the blood plasma. However a similar inhibitory effect of acetazolamide was seen in blood plasma taken by cardiac puncture with methods (see Materials and methods) specifically designed to avoid any chance of hemolysis (Table 4). Thus carbonic anhydrase activity appears to a normal component of circulating blood plasma in *Scyliorhinus canicula*.

Urea and TMAO effects on HCO_3^- dehydration rate RBCs from a common pool were washed and resuspended at identical hematocrit (15%) for 8 h prior to test in three different salines. The control urea concentration (450 $mmol \cdot L^{-1}$) used in these tests was close to that measured in vivo (Table 1). HCO_3^- dehydration rates were identical in control saline (urea = 450, TMAO = 100 $mmol \cdot L^{-1}$), TMAO-free saline, and urea-free saline (Fig. 4A). The absence of TMAO caused a very slight decrease in pHe and no change in RBC pHi (Fig. 4B). However the absence of urea caused a marked change in H^+ distribution, with a large fall in pHe and a large rise in RBC pHi.

The influence of hematocrit and RBC lysis on HCO_3^- dehydration rate A common blood pool pre-equilibrated to $P_{CO_2} = 3.75$ Torr was constituted to various hematocrits with homologous plasma. The HCO_3^- dehydration rate increased more or less linearly between 0% (i.e. separated plasma) and 15% but saturated at higher hematocrits (Fig. 5). A similar pattern was seen in a separate experiment at $P_{CO_2} = 1.88$ Torr (not shown).

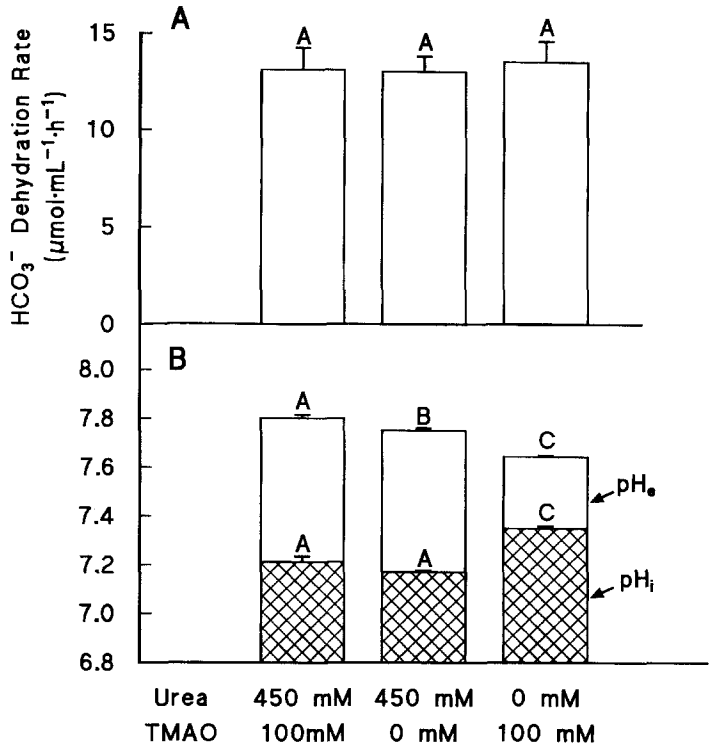

Fig. 4. The effects of TMAO and urea on (A) HCO_3^- dehydration rate and (B) extracellular pHe and RBC pHi in RBCs of the dogfish *Scyliorhinus canicula*. RBCs from a common pool were resuspended at hematocrit = 14.8%, hemoglobin = 4.28 g. 100 ml^{-1} in basic saline (urea = 450, TMAO = 100 mmol·L^{-1}), TMAO-free saline, or urea-free saline for 8 h prior to test. Means ± 1 SEM (n = 6–7). Means sharing the same letter are not significantly different from one another ($P > 0.05$).

RBC lysis was employed to evaluate the relative importance of HCO_3^-/Cl^- exchange and intracellular carbonic anhydrase activity in limiting the rate of HCO_3^- dehydration through dogfish RBCs. For these experiments, it was necessary to work with washed RBCs suspended in basic saline, because lysis of RBCs in plasma resulted in instantaneous clotting. Hematocrits were enriched to the plateau region of the curve (Fig. 5) to ensure maximum rates, and a pre-equilibration P_{CO_2} of 3.75 Torr was employed. A saturating level of bovine carbonic anhydrase (3 mg·ml^{-1}; see Perry and Gilmour, 1993) was used to define the maximum rate of the assay.

The addition of carbonic anhydrase to either separated plasma or RBCs suspended in saline elevated HCO_3^- dehydration to the same maximum rate, significantly above that of the RBCs in saline (Fig. 6). Note that the saline rate alone was significantly lower than the plasma rate, in accord with preceding results. Lysis of the RBCs in saline significantly elevated HCO_3^- dehydration to the same maximum rate, which could not be further increased by exogenous carbonic anhydrase (Fig. 6). These results demonstrate that access of extracellular HCO_3^- to intracellular carbonic anhydrase (i.e. the

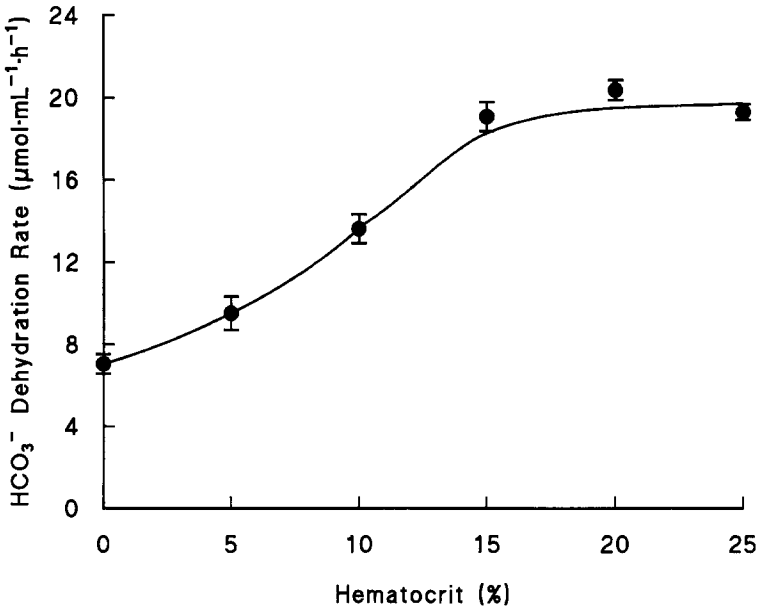

Fig. 5. The influence of hematocrit on HCO_3^- dehydration rate in the blood of the dogfish *Scyliorhinus canicula*. A common blood pool constituted to different hematocrits with homologous plasma and pre-equilibrated to P_{CO_2} = 3.75 Torr was used. Means ± 1 SEM (n = 6).

rate of HCO_3^-/Cl^- exchange), rather than intracellular carbonic anhydrase activity, is the rate-limiting factor.

P_{CO_2} and HCO_3^- effects on HCO_3^- dehydration Common blood pools with normal hematocrits (15–16%) were used in these experiments. In the first, blood and homologous plasma were pre-equilibrated to a range of P_{CO_2} values (0.38–7.5 Torr), a treatment which of course altered both P_{CO_2} and plasma HCO_3^- concentration. In the

Table 5
A comparison of HCO_3^- dehydration rates, pHe, and RBC pHi in a common pool of blood from *Scyliorhinus canicula* at two different levels of plasma HCO_3^-. The blood was pre-equilibrated to P_{CO_2} = 0.38 Torr at endogenous or elevated HCO_3^- levels for 6h prior to test; means ± 1 SEM (n = 6)

	Endogenous HCO_3^-	Elevated HCO_3^-
True plasma HCO_3^- (mmol·L^{-1})	2.21 ± 0.12	4.86 ± 0.05*
pHe	7.907 ± 0.009	8.293 ± 0.009*
RBC pHi	7.489 ± 0.005	7.747 ± 0.005*
HCO_3^- Dehydration rate (μmol·ml^{-1}·h^{-1})	5.65 ± 0.34	6.14 ± 0.30

* indicate significant differences ($P < 0.05$).
Hematocrit was 16.5% and hemoglobin was 4.70 g·100 ml^{-1}

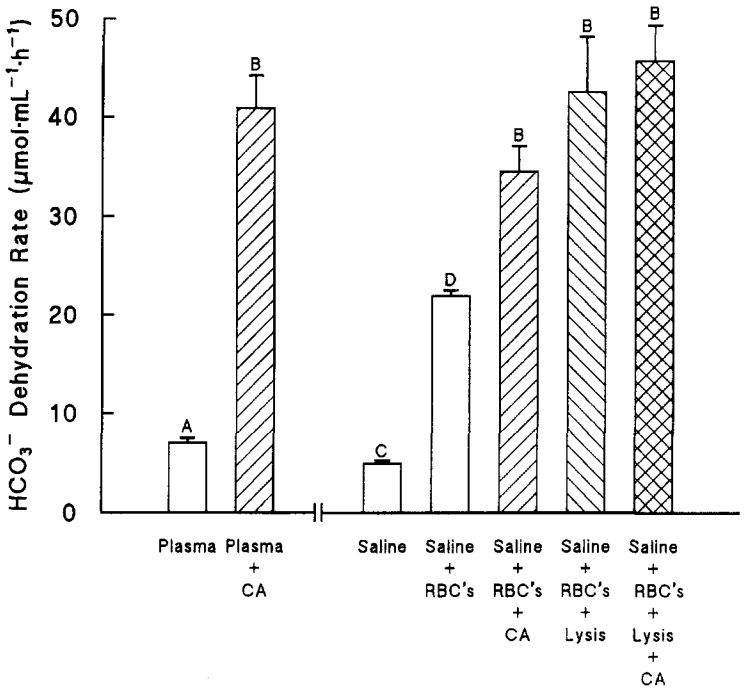

Fig. 6. The influence of bovine carbonic anhydrase (CA; 3 mg·ml^{-1}) and RBC lysis on HCO_3^- dehydration rates in dogfish (*Scyliorhinus canicula*) plasma and RBCs suspended in saline at hematocrit = 25.4%, hemoglobin = 6.82 g. 100 ml^{-1}. Means ± 1 SEM (n = 6–8). Means sharing the same letter are not significantly different from one another ($P > 0.05$).

second, blood at the lowest P_{CO_2} (0.38 Torr) was "spiked" with $NaHCO_3$ to raise the plasma HCO_3^- level. A pre-equilibration gassing period of 6 h was used to ensure equilibrium conditions in spiked and non-spiked blood, after which the actual plasma HCO_3^- concentration in spiked blood equilibrated to P_{CO_2} = 0.38 Torr was equal to that at P_{CO_2} = 3.75 Torr in non-spiked blood.

HCO_3^- dehydration rate was extremely sensitive to P_{CO_2}, increasing in an almost linear fashion over the P_{CO_2} range tested in both whole blood and separated plasma (Fig. 7). Increases in whole blood and plasma rates were proportional. However a 2.2-fold increase in steady-state plasma HCO_3^- concentration at P_{CO_2} = 0.38 Torr had no significant effect on the HCO_3^- dehydration rate, though it markedly elevated both pHe and RBC pHi (Table 5).

4. Discussion

The functional absence of a Haldane effect and the significance of plasma carbonic anhydrase Arterial blood gas and acid-base parameters in intact *Scyliorhinus canicula*

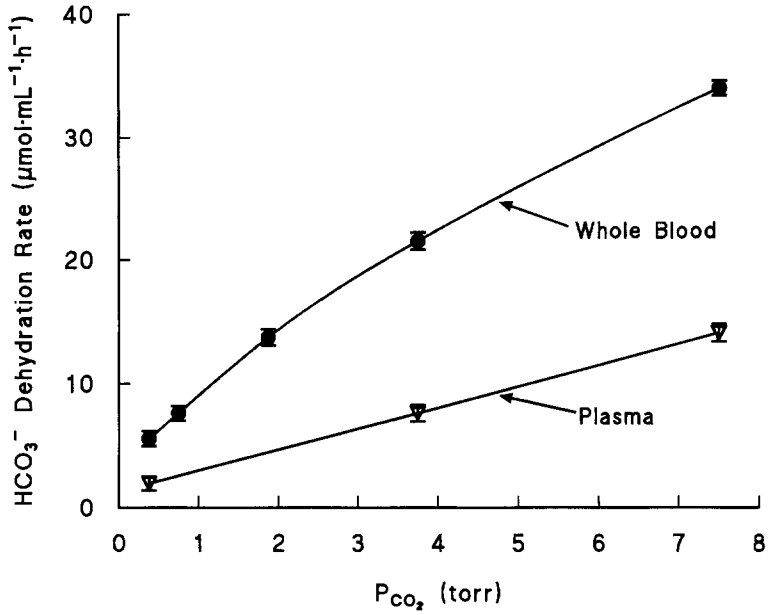

Fig. 7. The influence of pre-equilibration P_{CO_2} on HCO_3 dehydration rates in a common pool of whole blood (hematocrit = 15.4%, hemoglobin = 4.68 g·100 ml^{-1}) and separated plasma of the dogfish *Scyliorhinus canicula*. Means ± 1 SEM ($n = 6$).

(Table 1), most particularly the low Pa_{CO_2} and Ca_{CO_2} values, were very similar to those previously reported for this species (Truchot et al., 1980) and other elasmobranchs (Heisler et al., 1988; Graham et al., 1990). In vitro, CO_2 dissociation curves and buffer relationships (Fig. 1) were very similar to those established earlier for *Scyliorhinus canicula* and the congeneric *Scyliorhinus stellaris* (Albers et al., 1967; Piiper and Baumgarten-Schumann, 1968; Pleschka et al., 1970). In agreement with previous studies (reviewed by Butler and Metcalfe, 1989), the present results provide no convincing evidence for the presence of a Haldane effect (Fig. 1, Table 2). The problems in establishing a Haldane effect in elasmobranch blood include analytical errors associated with such low P_{CO_2} and C_{CO_2} levels and the presence of a vigorous Pasteur effect which may introduce a complicating acidification during deoxy incubations (Cross et al., 1969; Pleschka et al., 1970). The paired comparisons and short incubation times (30 min) were designed to overcome these difficulties. Nevertheless, in light of these problems, the equivocal results of Fig. 1B, the theoretical challenge that the small Bohr effect which is present in *Scyliorhinus* blood should be accompanied by a small Haldane effect (Pleschka et al., 1970), and the possible masking influence of the high buffer capacity of elasmobranch hemoglobin (Jensen, 1989), we are reluctant to conclude that the a Haldane effect is absolutely absent. We are however confident in concluding that if present, it plays a negligible role in CO_2 transport.

The most convincing evidence for this *functional* absence is the failure of HCO_3^- dehydration rate to respond to rapid oxygenation (Table 3). In rainbow trout blood, rapid oxygenation accelerates HCO_3^- dehydration rate by up to 40% (Perry and Gilmour, 1993). Amongst different teleost species, the magnitude of this response is directly correlated with the size of the Haldane effect seen in steady-state CO_2 dissociation curves (Perry et al., submitted). Teleosts also exhibit a curious phenomenon whereby the steady-state deoxygenated condition itself accelerates HCO_3^- dehydration rate relative to the steady-state oxygenated condition (Perry and Gilmour, 1993; Wood and Simmons, 1994; Perry et al., submitted). This phenomenon is also lacking in the blood of *Scyliorhinus canicula* (Table 2). The functional importance in teleosts may be to accelerate HCO_3^- dehydration in the early stages of oxygenation before the Bohr protons are released from hemoglobin.

The HCO_3^- dehydration rate of *Scyliorhinus canicula* blood at normal hematocrit tends to be lower than that of teleosts (Perry et al., submitted) and lacks the "boost" provided by both deoxygenation and rapid oxygenation. How then does the dogfish achieve such an efficient CO_2 excretion ($Pa_{CO_2} \sim 1$ Torr, $Ca_{CO_2} < 3$ mmol·L^{-1}) at the gills? The present finding of significant carbonic anhydrase activity in the normally circulating blood plasma of *Scyliorhinus canicula* (Tables 3, 4, Fig. 6) may provide the answer. To our knowledge, carbonic anhydrase in the plasma (in the absence of RBC hemolysis) has never been reported previously in fish. In light of the very short transit time of blood through the gills (1–3 seconds; Butler and Metcalfe, 1989), even small amounts of carbonic anhydrase in the plasma may be highly effective in accelerating the rate of HCO_3^- dehydration in vivo. The key point is that dehydration of HCO_3^- *within the plasma* avoids "band 3" HCO_3^-/Cl^- exchange, which is the rate-limiting step in RBC-mediated dehydration (Obaid et al., 1979), a fact confirmed by the present study (Figs. 3,6). Indeed the half-time for HCO_3^-/Cl^- exchange in dogfish blood (Obaid et al., 1979) may be comparable to blood transit time through the gills (Butler and Metcalfe, 1989). Direct HCO_3^- dehydration by plasma carbonic anhydrase, in combination with the shorter diffusion distance from plasma to water, the highly efficient countercurrent exchange in the gills, and the very low metabolic rates in elasmobranchs (Butler and Metcalfe, 1989) may more than compensate for the low endogenous rate and the absence of oxygenation effects on RBC-mediated HCO_3^- dehydration. It may also explain why elasmobranchs have much lower RBC carbonic anhydrase concentrations than teleosts (Maren, 1967).

The absence of adrenergic responsiveness In teleost RBCs, demonstration of a β-adrenergic Na^+/H^+ exchange may be elusive if blood incubation conditions are not appropriate (Perry et al., 1991; Nikinmaa, 1992). Therefore, a variety of conditions known to be effective in teleosts (deoxygenation, respiratory acidosis, storage, washing, high agonist levels) and a variety of indices (RBC pHi, pHe, NTP/Hb, MCHC, and RBC water content) were tested; all yielded negative results (Fig. 2). In agreement with Tufts and Randall (1989), we conclude that the response is lacking in the RBCs of elasmobranchs. Functionally, the absence of a β-adrenergic Na^+/H^+ exchange is in accord with the presence of carbonic anhydrase in the blood plasma, for the latter would tend to short-circuit RBC pHi adjustment (Nikinmaa, 1992), and with the

general lack of effect of catecholamines on gas exchange in elasmobranchs (Butler and Metcalfe, 1989). High circulating levels of adrenaline and noradrenaline may be more involved in cardiovascular regulation (Butler and Metcalfe, 1989).

The absence of a β-adrenergic Na^+/H^+ exchange is also in accord with the lack of influence of β-adrenergic stimulation on HCO_3^- dehydration (Fig. 3A). In teleosts, β-adrenergic inhibition of HCO_3^- dehydration appears to be an indirect effect of Na^+/H^+ exchange activation on the rate of HCO_3^-/Cl^- exchange, mediated through an acute change in the transmembrane HCO_3^- gradient (Wood and Perry, 1991; Perry et al., 1991; Wood and Simmons, 1994). As such, it provides a mechanism at the level of the RBC to modulate CO_2 excretion independent of ventilation and perfusion. At present, there is no evidence for such a mechanism in elasmobranchs; in any case, it is difficult to see how it would work in the presence of plasma carbonic anhydrase.

The influence of urea and TMAO HCO_3^- dehydration through the RBCs was surprisingly unresponsive to large manipulations of plasma urea and TMAO (Fig. 4A), in contrast to hemoglobin O_2 affinity (Weber et al., 1983; Tetens and Wells, 1984) and the function of many other proteins (Yancey and Somero, 1979). Non-penetration of the extracellular urea and TMAO changes is unlikely, in view of the 8 h incubation time and the rapidity (minutes) with which external urea passively equilibrates into the RBCs of this species (Walsh et al., 1994). The simplest explanation is that the rate-limiting step, the "band 3" HCO_3^-/Cl^- exchange protein, is protected from the destabilizing effects of urea by its intra-membrane location. Nevertheless, urea is clearly not without influence on the RBC, as revealed by the large decrease in pHe – pHi when urea was removed (Fig. 4B). The mechanism is unknown, but the unaltered HCO_3^- dehydration rate (Fig. 4A) indicates that HCO_3^- entry is insensitive to the steady-state pH gradient across the RBC membrane, the expected situation if HCO_3^- and H^+ are passively distributed according to a Donnan equilibrium (see below).

The influence of RBC concentration and lysis Qualitatively, the effects of hematocrit and RBC lysis were very similar to those reported earlier in rainbow trout blood (Wood and Perry, 1991; Perry and Gilmour, 1993). As in trout, and in agreement with the stopped-flow experiments of Obaid et al. (1979) on the blood of another dogfish *Mustelis canis*, the lysis experiments (Fig. 6) demonstrated that HCO_3^-/Cl^- exchange rather than intracellular carbonic anhydrase activity was clearly the rate-limiting factor in RBC-mediated dehydration. When this limitation was removed by lysis so that HCO_3^- had free access to intracellular carbonic anhydrase, the rate increased to the assay maximum. Increases in hematocrit in intact blood never produced the maximum rate. Furthermore, blockade of "band 3" by SITS caused a marked inhibition of the rate (Fig. 3) in all three species.

In intact dogfish blood, the HCO_3^- dehydration rate reaches a maximum at a hematocrit of 15% (Fig. 5), just as in rainbow trout blood (Wood and Perry, 1991; Perry and Gilmour, 1993). It is noteworthy that the normal hematocrit in *Scyliorhinus canicula* ($\sim$17%; Table 1) is just sufficient to provide the maximum rate, while the normal hematocrit in trout ($\sim$26%; Perry et al., submitted) provides excess capacity. In the

dogfish, extracellular carbonic anhydrase activity may provide the same safety margin as excess RBCs in the trout.

The influence of P_{CO_2} and HCO_3^- The data of Fig. 7 represent the first rigorous test of the effect of P_{CO_2} on HCO_3^- dehydration rate in fish blood, though Wood and Perry (1991) reported correlative evidence of a similar effect in trout blood. This marked dependence of the rate on the pre-equilibration P_{CO_2} is the expected result, since the P_{CO_2} gradient from the blood to the sink provides the net driving force for the whole CO_2 excretion process, just as in vivo. In the absence of other control mechanisms, the venous P_{CO_2} of blood entering the gills is likely the major local influence on the rate of CO_2 excretion across the branchial epithelium in dogfish in vivo.

The plasma HCO_3^- level itself is probably of only minor consequence to the rate under steady-state conditions (Table 5), similar to the situation in trout blood (Wood and Perry, 1991). A similar explanation probably applies, which requires that HCO_3^- and Cl^- are passively distributed across the RBC membrane according to a Gibbs-Donnan equilibrium. Albers et al. (1969) have presented evidence for a passive distribution of Cl^- in the blood of *Scyliorhinus canicula*. Thus there will be no driving force for net HCO_3^- entry, regardless of the plasma HCO_3^- concentration, until the blood is exposed to a lower P_{CO_2} as it enters the gills or the assay is started. At this point the catalyzed conversion of HCO_3^- to CO_2 by carbonic anhydrase in the RBCs will lower intracellular HCO_3^-. The HCO_3^- distribution ratio across the RBC membrane will no longer be dictated by the membrane potential, and net HCO_3^- entry/Cl^- exit will occur because of the electrochemical disequilibrium. In trout blood, this disequilibrium appears to increase slightly with higher extracellular HCO_3^- levels, yielding a shallow dependence of CO_2 excretion rate on plasma HCO_3^- concentration (Wood and Perry, 1991). In dogfish blood, we did not test a wide range of HCO_3^- levels. However based on the fact that HCO_3^- dehydration will be occurring simultaneously in the plasma compartment, we would predict even less dependence of absolute rate on plasma HCO_3^- concentration than in trout blood under steady-state conditions. However, this does not preclude potentially large effects on dehydration rate of acute changes in plasma HCO_3^-, as documented in trout blood under non-steady-state conditions (Wood and Perry, 1991; Perry and Gilmour, 1993).

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