Division of Comparative Physiology and Biochemistry, Society for Integrative and Comparative Biology

Extracellular Carbonic Anhydrase Activity and Carbonic Anhydrase Inhibitors in the Circulatory System of Fish

Author(s): Raymond P. Henry, Kathleen M. Gilmour, Chris M. Wood and Steve F. Perry

Source: *Physiological Zoology*, Vol. 70, No. 6 (November/December 1997), pp. 650-659
Published by: The University of Chicago Press. Sponsored by the Division of Comparative Physiology and Biochemistry, Society for Integrative and Comparative Biology
Stable URL: http://www.jstor.org/stable/10.1086/515878
Accessed: 17-09-2016 10:16 UTC

Extracellular Carbonic Anhydrase Activity and Carbonic Anhydrase Inhibitors in the Circulatory System of Fish

Raymond P. Henry[1,2,*]
Kathleen M. Gilmour[1,3]
Chris M. Wood[1,4]
Steve F. Perry[1,5]
[1]Bamfield Marine Station, Bamfield, British Columbia V0R 1B0, Canada; [2]Department of Zoology and Wildlife Science, 101 Cary Hall, Auburn University, Auburn, Alabama 36849-5414; [3]Division of Environmental and Evolutionary Biology, Graham Kerr Building, University of Glasgow, Glasgow G12 8QQ, Scotland, United Kingdom; [4]Department of Biology, McMaster University, Hamilton, Ontario L8S 4K1, Canada; [5]Department of Biology, University of Ottawa, Ottawa, Ontario K1N 6N5, Canada

Accepted by C.P.M. 4/30/97

ABSTRACT

Carbonic anhydrase activity in the extracellular fluid of lower vertebrates is considered to be minimal, either because of the absence of carbonic anhydrase or because of the presence of naturally occurring inhibitors. The presence of carbonic anhydrase activity and circulating inhibitors was measured in plasma and subcellular fractions of gill tissue in elasmobranchs and teleosts. Plasma carbonic anhydrase activity was confirmed in the former but in extremely low amounts, especially compared with activity in red cells. The activity was correlated with plasma iron concentration and red cell hemolysis, which suggests that it is a by-product of endogenous hemolysis during red cell turnover. A subcellular fraction of dogfish gills rich in microsomes contained significantly higher carbonic anhydrase activity than previously found in teleosts, making elasmobranchs the only aquatic lower vertebrates to possess putative basolateral membrane-associated carbonic anhydrase in the gill vasculature. It is suggested that branchial membrane-associated carbonic anhydrase is correlated more with a pH and/or CO_2-sensitive ventilatory drive than with the maintenance of resting CO_2 excretion. The occurrence and effectiveness of plasma carbonic anhydrase inhibitors were highly species-specific, with the salmonids having the most potent inhibitor. Cross-reactivity of inhibitor to red cell carbonic anhydrase appeared to be related to phylogenetic proximity. Selection for the presence of carbonic anhydrase inhibitors in fish plasma appears to be the result of multiple physiological pressures, including preservation of red cell intracellular pH, ventilatory control, and red cell fragility.

*To whom correspondence should be addressed at Department of Zoology and Wildlife Science, 101 Cary Hall, Auburn University, Auburn, Alabama 36849-5414; E-mail: henryrp@mail.auburn.edu.

Physiological Zoology 70(6):650–659. 1997. 0031-935X/97/7006-96136$03.00

Introduction

The traditional view of the distribution of the enzyme carbonic anhydrase (CA) in the circulatory system of vertebrates has undergone numerous modifications over the years. Originally discovered in mammalian erythrocytes (Meldrum and Roughton 1933), where the type I and II isozymes are found, CA was believed to be absent from the plasma of both mammals and lower vertebrates (e.g., Maren 1967). The idea that erythrocytes were the sole source of CA activity in the circulatory system changed with the discovery of the type IV isozyme localized to the endothelial membrane of the lung in both mammals and some lower vertebrates (Crandall and O'Brasky 1978; Effros et al. 1978; Klocke 1980; Whitney and Briggle 1982; Stabenau et al. 1996). In these organisms, while CA is absent from plasma, CA activity is available to catalyse the hydration/dehydration reactions in the plasma of certain tissue capillary beds. This did not appear to be the case among aquatic vertebrates (fish), however, as both plasma CA and basolateral membrane-associated CA in the gills are absent and/or physiologically insignificant, especially when compared with the high levels of erythrocyte CA (Perry et al. 1982; Swenson and Maren 1987; Henry et al. 1988, 1993).

Two recent reports on the distribution of CA in the circulatory system of elasmobranchs have raised new questions concerning the potential significance of extracellular CA. Measurable CA activity was found in the plasma of *Scyliorhinus canicula* (Wood et al. 1994). The rate of HCO_3^- dehydration in plasma is calculated as being as high as 50% of that for whole blood (i.e., with an in vitro assay, 50% of the CO_2 produced originated from HCO_3^- dehydration in the plasma, and 30% of that was inhibited by the CA inhibitor acetazolamide). However, the source of this particular pool of CA has not been identified. Furthermore, Swenson et al. (1995), using a large molecular weight CA inhibitor, report evidence for the presence and function of a membrane-associated CA in the gills of *Squalus acanthias*. These differences separate elasmobranchs

from teleosts with respect to the distribution of CA in the circulatory system, but the physiological importance of extracellular CA has not yet been established.

In some species, in addition to extracellular CA, there is also a CA inhibitor present in the plasma. This CA inhibitor appears to be fairly widespread in mammals, where it is believed to function in the inhibition of CA released from the normal cycle of erythrocyte lysis (Booth 1938; Van Goor 1948; Hill 1980; Roush and Fierke 1992). That a CA inhibitor should be selected for in the plasma of mammals is plausible, given the fragility and short life span of anucleate red blood cells. CA inhibitors, however, also occur in ectothermic lower vertebrates (i.e., fish), which do not have any apparent extracellular CA (Swenson and Maren 1987; Henry et al. 1988, 1993) and whose nucleated erythrocytes are more rugged and long-lived (see, e.g., Haswell and Randall 1976; Haswell et al. 1983; Dimberg 1994). It has been suggested that this inhibitor has multiple functions, such as dampening the Root effect in euryhaline fish adapting to seawater (Dimberg 1994) and preserving red cell intracellular pH regulation and volume regulation (Lessard et al. 1995). However, these conclusions are somewhat speculative, as they are based on work done primarily on one species, the rainbow trout (*Oncorhynchus mykiss).* Furthermore, the distribution of plasma CA inhibitors in fish is probably not uniform; for example, Heming and Watson (1986) report that plasma of a primitive holostean, *Amia calva,* does not contain a CA inhibitor.

This investigation reports on the nature and probable source of extracellular CA in elasmobranchs, on the distribution and inhibitory properties of the plasma CA inhibitor in teleosts, and on the relationship between extracellular CA activity and inhibition.

Material and Methods

Collection and Maintenance of Animals

Pacific spiny dogfish (*Squalus acanthias*), chinook salmon (*Oncorhynchus tshawytsha*), flounder (*Platichthys stellatus*), lingcod (*Ophiodon elongatus*), and black rockfish (*Sebastes melanops*) were collected off the coast of Vancouver Island, Canada, in May and June of 1995 by trawl. They were maintained at Bamfield Marine Station without feeding and under natural photoperiod in large circular tanks provided with flowing seawater at 12°C. Rainbow trout (*Oncorhynchus mykiss*) were obtained from West Creek Trout Farms (Mission, British Columbia) and transported to Bamfield Marine Station, where they were acclimated to seawater for a minimum of 2 wk before use (Gilmour et al. 1997). American eel (*Anguilla rostrata*) and bullhead catfish (*Ictalurus nebulosus*) were obtained by local fishermen or captured by seine net from the St. Lawrence River and held in 500-L round tanks supplied with flowing dechlorinated City of Ottawa tap water at 10°C (eel) or 20°C (bullhead).

Blood and Tissue Sampling

Animal handling, anesthesia, and surgery were performed as described in detail by Gilmour et al (1997). All fish were anesthetized using 0.1 g L^{-1} MS-222 (ethyl-m-aminobenzoate) in seawater and allowed to recover overnight before sampling. For dogfish, blood was drawn either from animals completely recovered from anesthesia via a catheter implanted in the coeliac artery or from lightly anesthetized animals via cardiac or caudal puncture, depending on the experiment. Blood from trout was obtained in a similar fashion from either a dorsal aortic cannula or via cardiac puncture. For all other species, blood was drawn via cardiac or caudal puncture into heparinized syringes. In order to avoid hemolysis, blood used in the CA analyses was taken from the initial sample withdrawn from the animal.

Blood was placed in microfuge tubes and centrifuged at 10,000 *g* for 1 min to separate plasma and erythrocytes. All species were assayed at Bamfield Marine Station except for eel and bullhead, whose plasma and erythrocytes were frozen in liquid nitrogen and shipped on dry ice to Auburn, Alabama, for analysis.

Gills from dogfish and trout were perfused with heparinized saline in order to obtain erythrocyte-free tissue. The fish were anesthetized as above, the heart was exposed, and a cannula was inserted into the ventricle and advanced into the ventral aorta. The cannula was secured with a ligature around the atrium. Saline (500 mmol L^{-1} NaCl plus 100 units mL^{-1} ammonium heparin for dogfish, and Cortland saline adjusted to 170 mmol L^{-1} NaCl plus 100 units mL^{-1} heparin for trout) was infused with a 50-mL syringe; a total of 150 mL was used to perfuse the gills of each animal. The gill arches were dissected out of the fish and visually inspected; any clots or lamellae appearing pink from residual blood were removed. The gills were then frozen in liquid nitrogen, wrapped in aluminum foil, and stored at −80°C. They were shipped on dry ice to Auburn, Alabama, where they were again stored at −80°C until they were analysed.

Gill tissue was homogenized and fractionated according to the procedure of Henry et al. (1986, 1988, 1993). Tissue was thawed briefly to 0°C on ice, weighed, and added to five volumes of buffer (225 mmol L^{-1} mannitol, 75 mmol L^{-1} sucrose, and 10 mmol L^{-1} Tris-PO_4 adjusted to pH 7.40 with 10% H_3PO_4). Lamellae were homogenized by a minimum of 16 passes in a motor-driven Teflon/glass homogenizer. The homogenate was then subjected to differential centrifugation: 750 *g* for 20 min to produce a pellet containing whole cells, nuclei, and large cell fragments; 8,500 *g* for 20 min to produce a mitochondrial pellet (both on a Sorvall RC-5B Superspeed centrifuge at 4°C); and 100,000 *g* for 60 min at 4°C to produce

a microsomal pellet and cytoplasmic supernatant (Beckman L8-M Ultracentrifuge). Each pellet was resuspended in buffer, and samples of the crude homogenate, pellet, and supernatant from each step were saved for CA analysis. CA was assayed electrometrically by the delta pH method of Henry (1991).

Experimental Protocol

CA activity was measured in both plasma and erythrocyte lysates of dogfish and trout. For plasma, aliquots ranging from 50 to 200 μL were added to the assay buffer. Erythrocyte pellets were lysed by adding one volume of 5 mmol L^{-1} EDTA in deionized water followed by mild sonication on ice (25 W for 30 s; Microson 50, Heat Systems Ultrasonics). The lysate was then diluted with the CA assay buffer (composition given above) by a factor of 400–500; 10 μL of the dilute lysate was then used in the CA assay. Trimethylamine oxide is a major osmotic constituent of elasmobranch blood, being present in concentrations as high as 70 mmol L^{-1}. It has been suggested that trimethylamine oxide alone may alter the CA assay, giving artificially high results (E. R. Swenson, personal communication). To test for this, aliquots of 50 to 500 μL of 70 mmol L^{-1} trimethylamine oxide were analysed in the CA assay.

In order to test the nature and occurrence of endogenous CA inhibitors in fish plasma (see, e.g., Dimberg 1994), 10 μL of dilute lysate of each species was assayed in the presence of 100 μL of its own plasma and plasma from other species. Furthermore, plasma from each species was tested for effectiveness as an inhibitor against purified CA (10 μL of 0.1 mg mL^{-1}) from bovine erythrocytes (Sigma). For dogfish, plasma samples with minimal detectable CA activity were chosen to test against the lysates of other species.

A series of experiments was performed to test whether the CA inhibitor in trout plasma acts as a scavenger for erythrocyte CA released during the course of normal cell lysis. Whole blood from trout and dogfish (2 mL) was progressively lysed by adding a series of aliquots of deionized H_2O of increasing volume. The blood was gently mixed and centrifuged. Plasma was then assayed for CA activity and hemoglobin (Hb) concentration.

Because plasma CA activity among the 35 dogfish measured was highly variable, naturally occurring low levels of endogenous hemolysis could not be excluded as a source of the plasma CA. Previous work has indicated that Hb concentrations in the plasma are below the limit of detectability, even when the assay sensitivity is expanded fourfold (Wood et al. 1994). To examine the potential relationship between hemolysis and CA activity, 12 plasma samples were chosen, six at the high end of CA activity and six with little or no activity. These samples were also analysed for iron concentration via atomic absorption spectroscopy (Perkin Elmer) as a potentially more sensitive indicator of hemolysis.

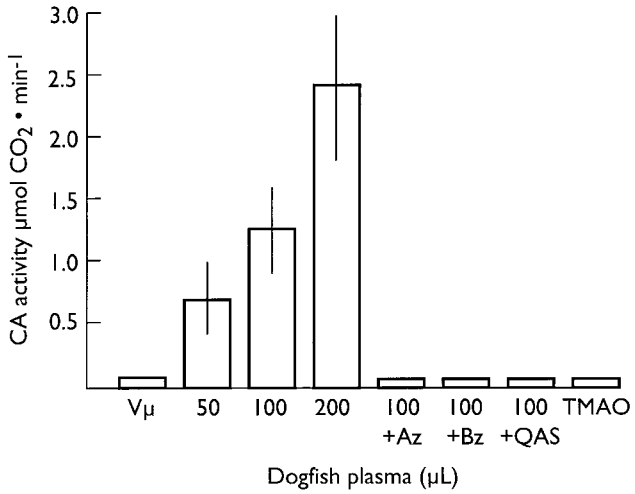

Figure 1. CA activity in dogfish plasma. The volume of plasma represents the amount added to 6 mL of assay buffer. Mean ± SEM ($N = 8$ for all values except trimethylamine oxide (TMAO), where $N = 5$). *Vμ*, uncatalysed rate of CO_2 hydration; *Az*, 5 μmol L^{-1} acetazolamide; *Bz*, 5 μmol L^{-1} benzolamide; *QAS*, 5 mmol L^{-1} quaternary ammonium sulfanilamde; *TMAO*, 500 μL of 70 mmol L^{-1} trimethylamine oxide added to the assay buffer.

Results

Extracellular CA Activity

Low but measurable CA activity was detected in the plasma of dogfish (Fig. 1). The increase in activity was linear with increased plasma volume, and acetazolamide (10 μmol L^{-1}), benzolamide (10 μmol L^{-1}), and quaternary ammonium sulfanilamide (5 mmol L^{-1}) reduced the activity to the uncatalysed rate. It is interesting that Wood et al. (1994) reported that, using the isotopic assay of Wood and Perry (1991), 100 μmol L^{-1} acetazolamide reduced plasma HCO_3^- dehydration by only 30%. The addition of up to 500 μL of 70 mmol L^{-1} trimethylamine oxide had no effect on the uncatalysed rate. Compared with CA activity in the erythrocytes, plasma CA activity was minuscule. Based on a standard volume of 1 mL, plasma CA activity was only 0.02% of that found in erythrocytes (Table 1). Furthermore, the amount of CA in 1 mL of plasma was enough to enhance the uncatalysed rate by a factor of 12, while the amount of CA within the equivalent volume of red cells was capable of enhancing the uncatalysed rate by a factor of nearly 19,000. Also, sampling protocol (i.e., blood sampled via catheter vs. cardiac puncture, and blood sampled in anesthetized vs. awake fish) had no effect on measured plasma CA activity, as long as the samples measured were those that were taken initially. Repeated sampling from the same individual resulted in increased plasma CA activity.

As reported previously (Henry et al. 1993), there was no CA activity in trout plasma. Also, CA activity in trout red cells was about 14-fold higher than that in dogfish, typical of the re-

Table 1: CA activity and enhancement factor in dogfish and trout plasma and erythrocytes

	Plasma	Erythrocytes
CA activity (μmol CO_2 mL^{-1} min^{-1}):		
Dogfish	5.97 ± 1.12 (34)	30,776 ± 9,403 (7)
Trout	1.81 ± .12 (17)	512,667 ± 28,103 (4)
Enhancement:[a]		
Dogfish	12.1 ± .9 (33)	18,926 ± 1,056 (7)
Trout	0	291,548 ± 2,198 (4)

Note. The uncatalysed rate of hydration is equal to 1.91 μmol CO_2 mL^{-1} min^{-1}. Values are mean ± SEM; sample sizes are given in parentheses.

[a] The factor by which 1 mL of either plasma or packed erythrocytes will increase the rate of the uncatalysed reaction.

ported differences in erythrocyte CA activity between teleosts and elasmobranchs (Maren et al. 1980).

High plasma CA activity in dogfish was associated with high iron concentration (Fig. 2). The slope of a regression analysis between the two measured variables, 6.1, was significantly different from 0, and the correlation coefficient, 0.71, was also significant ($P < 0.01$, df = 10). And while other factors, such as nutritional state, can also affect plasma iron concentrations, animals were starved for 72 h before each experiment in order to keep these variables at a minimum.

Progressive lysis of dogfish erythrocytes in vitro resulted in a linear increase in both CA activity and Hb concentration (Fig. 3). In response to serial additions of deionized water from 0 to 1,000 μL, CA activity increased from near 0 to 500 μmol CO_2 mL^{-1} min^{-1}, and Hb increased from 0 to 1.8 g 100 mL^{-1}.

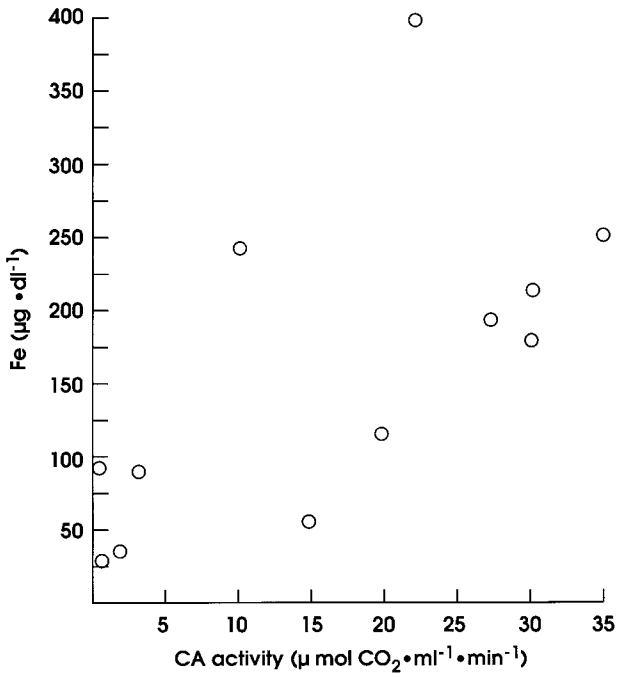

Figure 2. CA activity in dogfish plasma versus iron concentration from the same samples. Regression equation: $y = 6.1x + 39.9$; $r = 0.71$.

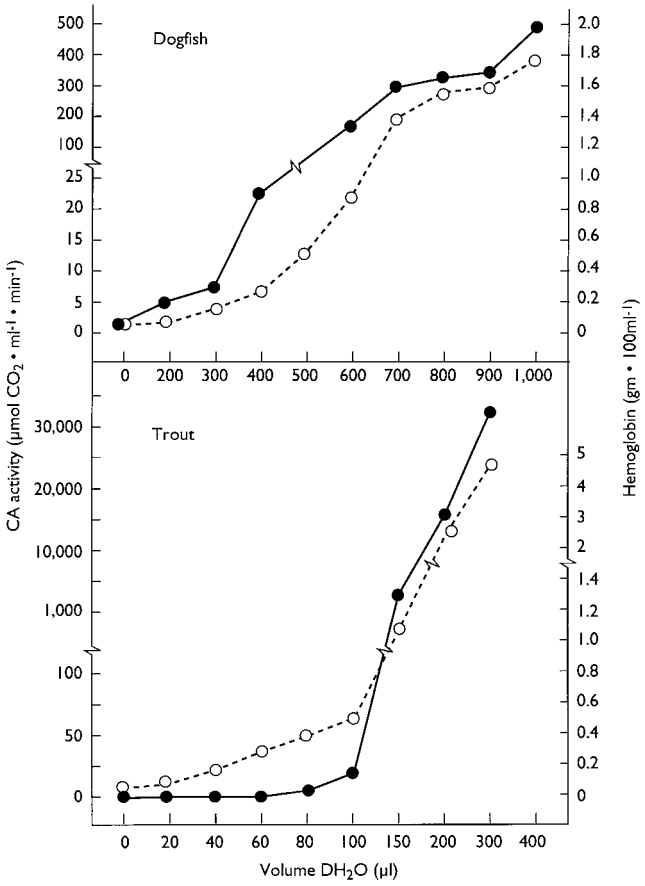

Figure 3. CA activity (*filled circles*) and Hb concentration (*open circles*) in the supernatant of whole blood in response to progressive erythrocyte lysis by the addition of increasing volumes of deionized water (DH_2O). Values represent the mean of duplicate experiments.

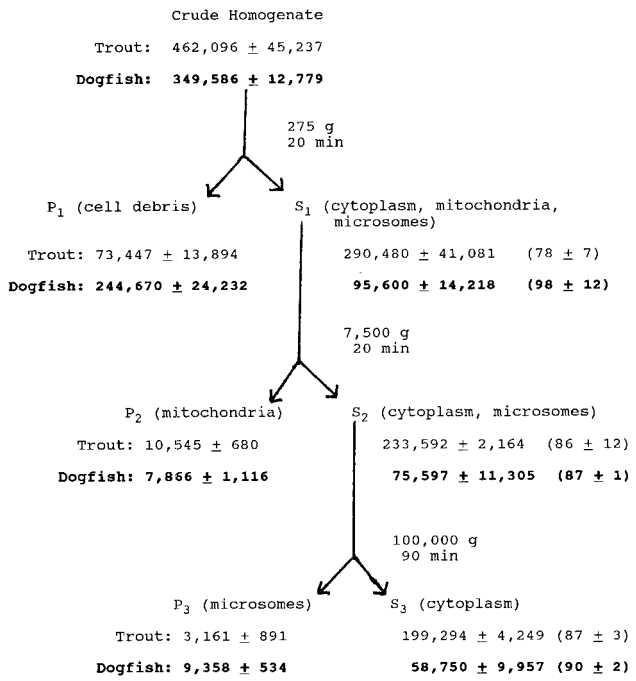

Figure 4. Subcellular distribution of CA activity in freeze-clamped gill tissue of trout and dogfish. Total CA activity for each fraction is reported as micromoles of CO_2 per minute per milliliter multiplied by the fraction volume in milliliters. Values are mean ± SEM ($N = 4$) for each species. Percent recovery for each step in the fractionation is reported in parentheses on the right. P_1, cellular debris; P_2, mitochondria; P_3, microsomes; S_1, cytoplasm, mitochondria, and microsomes; S_2, cytoplasm and microsomes; S_3, cytoplasm.

Trout blood, however, showed a different pattern. Over the stepwise addition of up to 100 μL of deionized water, there was a progressive increase in Hb concentration but no change in CA activity (Fig. 3). This indicated that lysis was taking place, as Hb was detected in the supernatant, but any increase in CA activity was being suppressed. At an apparent critical point, the addition of 150 μL of deionized water, the increase in both CA activity and Hb became linear.

These results indicate that in addition to having CA activity in the plasma, dogfish also lack a circulating CA inhibitor. The CA inhibitor in trout plasma was effective only up to a certain degree of hemolysis, above which it became saturated with excess CA.

The distribution of CA among subcellular fractions of the gills was different in dogfish than it was in trout in one important aspect. The microsomal pellet, which contained the external and internal membrane fraction of the gill homogenate, contained threefold higher CA activity in dogfish than it did in trout (Fig. 4). This could be a very conservative estimate of the difference, as the initial pellet fraction (whole cells and large cell fragments that also contain microsomal CA activity) in dogfish also had significantly higher CA activity than it had in trout. Fractionation of freeze-clamped gills did not seem to affect either the qualitative or quantitative measurement of the subcellular distribution of CA activity; the data reported here for trout were very similar to those for fresh gill tissue (Henry et al. 1993) and for teleosts in general (Henry et al. 1988). Gills of elasmobranchs appear to have significantly higher microsomal CA activity than do those of teleosts (Fig. 4; cf. Henry et al. 1988, 1993).

Endogenous Plasma CA Inhibitors

Naturally occurring CA inhibitors were found in the plasma of most of the fish species examined. The strongest degree of self-inhibition (plasma vs. red cells within a species) was found within the salmonids. For the current experimental conditions (100 μL plasma vs. 10–50 μL dilute lysate), chinook and trout plasma abolished 93% and 100% of the CA activity in the lysates, respectively (Table 2). Eel plasma also contained an effective CA inhibitor (90%). The CA inhibitors in plasma of the other fish examined were much less effective; they produced from 6% to 42% inhibition depending on the species. Dogfish plasma, which has low levels of CA activity to begin with, had no effect on CA activity of the lysate (Table 2).

There was some degree of cross-reactivity between plasma and lysate of different species. Again, the strongest effects were seen using plasma from the two salmonids. Trout plasma produced 95% inhibition against chinook lysate, and chinook plasma was 90% effective against trout lysate (Table 2). Trout and chinook plasma were less effective against CA activity in lysate from nonsalmonid species, in which they produced from 54% to 74% inhibition. The CA inhibitor in eel plasma was also effective against lysate of other fish: it was most potent for trout and chinook lysate (79% and 81%, respectively) and less so for the other species (42%). The CA inhibitors from plasma of lingcod, rockfish, and flounder were generally weaker against lysate of other species (27%–56% for lingcod, 30%–75% for rockfish, and 8%–40% for flounder; Table 2). The volume of the lysates from each species was adjusted to give approximately equal levels of CA activity. While this may not reflect the relative total amounts of CA activity in the blood of each species, it is probably close, as teleosts have a high-turnover, type II CA isozyme in their red cells and total CA activity that is present in extreme excess (i.e., 10,000-fold) of what is necessary for normal CO_2 excretion (Maren et al. 1980; Henry et al. 1993). Small differences in total blood CA activity between teleost species are probably not significant against this large background. Plasma from bullhead had no inhibitory effect on lysate of any other species. Furthermore, no plasma samples were inhibitory toward lysate of dogfish or toward pure bovine erythrocyte CA.

Discussion

Extracellular Fluid CA Activity

The results presented here confirm the presence of circulating CA activity in elasmobranch plasma (Wood et al. 1994) but

Table 2: Percent inhibition of 100 μL of plasma on the CA activity of 10–50 μL of lysate

	Plasma							
	Dogfish	Trout	Cod	Rockfish	Flounder	Eel	Chinook	Bullhead
Lysate:								
Dogfish	0 ± 0[a]	0 ± 0[a]	. . .	. . .	0 ± 0[a]	0 ± 0[a]	0 ± 0[a]	0 ± 0[a]
Trout	0 ± 0[a]	100 ± 0[a]	. . .	. . .	8	79 ± 8[a]	90 ± 6[a]	0 ± 0[a]
Lingcod	0 ± 0[a]	. . .	27	. . .	. . .	. . .	. . .	. . .
Rockfish	0 ± 0[a]	. . .	. . .	42	. . .	. . .	. . .	. . .
Flounder	0 ± 0[a]	74	. . .	. . .	35	42	56	. . .
Eel	0 ± 0[a]	60	27	30	40	90 ± 6[a]	53 ± 4[a]	0 ± 0[a]
Chinook	0 ± 0[a]	95	56	. . .	25	81	93 ± 7[a]	0 ± 0[a]
Bullhead	0 ± 0[a]	71	52	75	26	42	67	6 ± 4[a]
BCA[b]	0 ± 0[a]	0 ± 0[a]	. . .	. . .	0	0	0	0

Note. The volume of lysate for each species was adjusted to give an approximate 10-fold increase in the uncatalysed rate of CO_2 hydration.

[a] Values are reported as mean ± SEM ($N = 4–6$). All other are values reported as the mean of duplicate assays.

[b] Bovine carbonic anhydrase from erythrocytes (Sigma).

indicate a different interpretation of plasma CA function. The plasma CA activity represents only 0.02% of the total activity, on the basis of equivalent volumes (1 mL) of plasma versus red cells. Given that the average hematocrit in elasmobranchs is about 20%, plasma CA activity would contribute a maximum of 0.1% of the total CA activity in the circulatory system. The high red cell CA activity is coupled with rapid anion (Cl^-/HCO_3^-) exchange that occurs at a rate similar to that found in mammalian erythrocytes (Obaid et al. 1979). Furthermore, a concentration of benzolamide that was low enough not to inhibit CA within the red cells but that was high enough to fully inhibit plasma CA activity did not alter arterial P_{CO_2} in dogfish (Gilmour et al. 1997), and a large-molecular-weight CA inhibitor that is restricted to the plasma also had no effect on arterial pH or P_{CO_2} (Swenson et al. 1996). Also, benzolamide treatment did not alter CO_2 excretion in dogfish after strenuous exercise (Swenson and Maren 1987). Taken together, this information strongly suggests that plasma CA activity does not play a significant role in resting CO_2 excretion in dogfish; rather, excretion of CO_2 occurs via the classic Jacobs-Stewart cycle, as is the case for other aquatic vertebrates (e.g., Cameron and Polhemus 1974). In a separate study in which methazolamide and benzolamide were used to selectively inhibit red cell and gill CA, respectively, Swenson and Maren (1987) also concluded that in *Squalus acanthias* CO_2 excretion took place via the red cells.

In mammals, the uncatalysed rate of HCO_3^- dehydration must be enhanced by 700 times in order for normal CO_2 excretion to occur during the short time in which blood resides in the pulmonary capillaries (Kernohan et al. 1963); plasma CA activity in dogfish enhances the uncatalysed rate by a factor of 12 at most, and usually much less. While the transit time of blood in elasmobranch gills is still fairly rapid (1–3 s; Butler and Metcalfe 1989), the metabolic rate, and therefore CO_2 production, are very low. Extracellular fluid CA activity (plasma and/or gill) may be more important under conditions of higher CO_2 production, such as exercise, but this remains to be shown.

It is possible that the contribution of plasma CA activity to HCO_3^- dehydration originally reported for in vitro conditions (50%; Wood et al. 1994) was overestimated as a result of the method used to measure CO_2 excretion (Wood and Perry 1991). Using that same method, Perry et al. (1996) reported that the red cell contribution to CO_2 excretion in teleosts, which have no extracellular fluid CA, ranged from 55% to 71%. As discussed by Gilmour et al. (1997), the radioisotopic assay used in these studies is probably limited by the rate of diffusion of CO_2 out of the reaction medium (Cameron 1979; Obaid et al. 1979), a limitation that becomes increasingly important as the rate of HCO_3^- dehydration increases (for a more detailed discussion of the limitations of this assay see Henry and Heming [1998]). This could have resulted in a disproportionate underestimation of the dehydration rate of whole blood relative to that for plasma and, therefore, an overestimation of the contribution of plasma to in vivo HCO_3^- dehydration.

Circulating CA in the plasma of elasmobranchs is probably a byproduct of another physiological process: hemolysis resulting from the natural turnover and lysis of circulating red cells. Plasma CA activity in dogfish was low but variable, and it was entirely absent in some individuals. Hb concentrations in plasma were always very low, but the level of CA activity was correlated with plasma iron concentration, suggesting that endogenous red cell lysis, and not lysis as a result of sampling procedures, is the source of activity. The corpus cavernosum in elasmobranchs has been suggested, but not confirmed, as a potential site for endogenous red cell lysis, and therefore it

could be the source of both CA and Hb (Butler and Metcalfe 1989). It is possible that Hb is cleared more rapidly from the circulatory system than is CA and that the lack of an endogenous plasma CA inhibitor results in a residual level of CA activity. There is evidence suggesting that plasma CA inhibitors in mammals function to scavenge the zinc associated with the active site of CA that has been released by endogenous hemolysis and that the inhibitory nature of these proteins is a secondary function (reviewed by Henry and Heming [1998]). If this is the case, then elasmobranchs, which lack a plasma CA inhibitor, might have a slower turnover of plasma CA relative to the turnover of other proteins such as Hb. This would help explain why variable levels of CA activity, but not Hb, are found in the plasma in vivo. Again, while it is impossible to completely rule out lysis as a result of sampling, the available evidence here and reported previously (Wood et al. 1994) indicates that some low level of endogenous plasma CA activity does exist in elasmobranchs.

Plasma CA in elasmobranchs may be a result of erythrocyte lysis, but it is apparent that membrane-associated CA with an extracellular orientation in the branchial vasculature of the circulatory system has been selected for. The function of branchial membrane-associated CA in elasmobranchs, however, is not clear. Selective inhibition of extracellular fluid CA activity (plasma and membrane-associated) in dogfish results in the reduction of bicarbonate clearance from the plasma but without altering arterial pH or P_{CO_2} (Swenson et al. 1995, 1996). This is believed to be a consequence of the inhibition of the catalysed CO_2 reactions in the plasma within the branchial vasculature. The microsomal fraction of dogfish gills has three times the CA activity found in teleost gills (Fig. 4; Henry et al. 1988, 1993), and it is this fraction of branchial CA that could be available to catalyse the plasma CO_2 reactions.

The significance of branchial membrane-associated CA and the catalysis of the CO_2 reactions in plasma is not clear, but the absence of an increase in arterial P_{CO_2} when this fraction of CA is selectively inhibited argues against its role in resting CO_2 excretion. It is possible, however, that branchial membrane-associated and/or plasma CA is important in maintaining rapid chemical equilibration between CO_2 and H^+ for purposes of ventilatory control. Elasmobranchs possess a strong ventilatory response to hypercapnia that involves changes in both ventilatory frequency and amplitude (Heisler et al. 1988; Graham et al. 1990; Wood et al. 1990; Perry and Gilmour 1996). Ventilation in teleosts, which lack any source of extracellular fluid CA (Henry et al. 1988, 1993), is driven primarily by hypoxia, with hypercapnia producing changes in ventilatory amplitude only. Furthermore, CA injection alters the CO_2-sensitive component of the overall ventilatory response in rainbow trout following strenuous exercise (Wood and Munger 1994). This suggests that extracellular fluid CA is correlated with a CO_2-/H^+-sensitive ventilatory control.

The distribution and function of extracellular fluid CA (specifically pulmonary CA) among air-breathing vertebrates, whose ventilation is driven primarily by CO_2/H^+ (reviewed by Dejours [1975]), also supports this idea. Endothelial membrane-associated CA in the mammalian lung is also present in low quantity compared with activity in red cells. Pulmonary CA can enhance the rate of HCO_3^- dehydration by a factor of 130–150 over the uncatalysed rate versus a factor of about 13,000 over the uncatalysed rate for CA in the red cell (Bidani et al. 1983). Furthermore, selective inhibition of pulmonary membrane-bound CA does not alter pulmonary CO_2 excretion (Heming et al. 1986); rather, it was concluded that the primary function of extracellular fluid CA (i.e., vascular membrane-associated CA with an extracellular orientation) was in the maintenance of rapid chemical equilibrium among the CO_2 species in the pulmonary vasculature. This would provide chemoreceptors with rapid and accurate information on changes in the levels of CO_2 and H^+ ions in the blood and would therefore facilitate rapid ventilatory adjustments. In general, aquatic lower vertebrates (i.e., fish) possess an O_2-sensitive ventilatory drive while lacking extracellular fluid CA. Terrestrial lower vertebrates (e.g., turtles), in contrast, have a CO_2-sensitive ventilatory drive and also possess pulmonary endothelial CA (Stabenau et al. 1996). It is possible that membrane-bound CA in the gas exchange organ (gills and lungs) evolved in conjunction with a CO_2-/H^+-sensitive ventilatory drive (reviewed by Henry and Heming [1998]). If this is the case, then elasmobranchs would represent a unique branch in the evolution of both CA and ventilatory control. The question of membrane-bound CA and ventilatory control in elasmobranchs deserves more detailed investigation.

Endogenous Extracellular CA Inhibitors

The distribution and function of endogenous CA inhibitors in fish plasma are also not clear. There appears to be a correlation between the presence and effectiveness of the plasma CA inhibitor and the release of catecholamines in response to hypoxia. Trout and chinook have the most potent CA inhibitors, and both species release large amounts of catecholamines into the blood during hypoxic stress (Tufts and Randall 1989). Catecholamines stimulate proton transport out of the red cell via Na^+/H^+ exchange, thus preserving intracellular pH in the face of a hypoxic-induced blood acidosis. CA activity in plasma would effectively short-circuit this mechanism by catalysing the conversion of H^+ and HCO_3^- to CO_2, which rapidly equilibrates across the red cell membrane, regenerating protons via hydration by intracellular CA, as has been shown to occur in fish red cells in vitro (Motais et al. 1989; Nikinmaa 1990).

This correlation, however, does not hold true across all teleosts. Red cells from eel are only slightly sensitive (if at all) to catecholamines, and only small amounts of catecholamines are released in hypoxia (Hyde and Perry 1990; Perry and Reid 1992*a*, 1992*b*), but there is still a potent CA inhibitor in the

plasma. Existing evidence suggests that flounder red cells have limited sensitivity to catecholamines (Milligan and Wood 1987; Wood and Milligan 1987), yet flounder plasma contains a CA inhibitor. Red cells of the Atlantic cod have a pronounced adrenergic response (Berenbrink and Bridges 1994), but plasma of the Pacific lingcod has a very weak inhibitor. However, red cells of the two species may not be similar in their adrenergic response.

While it is valid to conclude that plasma CA inhibitors were selected for scavenging CA released during hemolysis, this selection was probably a result of more fundamental reasons than the preservation of catecholamine-regulated proton extrusion during hypoxia, such as the conservation and metabolic recycling of zinc associated with the active site of CA (reviewed by Henry and Heming [1998]). Lessard et al. (1995) reported that an in vivo injection of CA into trout plasma does not abolish catecholamine-mediated intracellular pH regulation. Furthermore, Wood and Munger (1994) reported that an injection of CA into trout reduces the secondary pH-/P_{CO_2}-sensitive, postexercise ventilatory drive and thus actually prolongs the blood acidosis.

The data reported here (Table 2) indicate that there may not be a single unifying reason for the absence of plasma CA activity in teleosts. That absence is made possible only by the presence of the plasma CA inhibitor that neutralizes any CA released from either normal red cell turnover or acute hemolysis during conditions of stress (e.g., hypoxia or exercise). However, neither the distribution nor effectiveness of the inhibitors is uniform in teleosts. Bullhead plasma lacks a CA inhibitor entirely (Table 2), and therefore its red cells would presumably be unprotected from a blood acidosis, regardless of the source of protons. A CA inhibitor may not be necessary, however, if the degree of hemolysis, even under stress, is low enough to prevent a significant increase in the rate of bicarbonate dehydration. It is possible that the selective pressures for the evolution of plasma CA inhibitors included red blood cell fragility; if so, one would predict that salmonid red cells are quite fragile and susceptible to hemolysis under stress and that bullhead red cells are not. This prediction, and many of the above conclusions concerning the evolution and function of plasma CA inhibitors, are supported by incomplete experimental evidence. It is clear that a more thorough and systematic study of all fish groups is needed before we can fully understand the selective pressures that gave rise to both extracellular CA and CA inhibitors.

Acknowledgments

This work was supported by National Science Foundation grant IBN 93-04844 to R.P.H. and by National Science and Engineering Research Council of Canada operating grants to S.F.P. and C.M.W. We are grateful to the director, Dr. A. N. Spencer, and staff (particularly Dave Hutchinson and the late John Boom) of the Bamfield Marine Station for their generous hospitality and support.

Literature Cited

Berenbrink M. and C.R. Bridges. 1994. Catecholamine-activated sodium/proton exchange in the red blood cells of the marine teleost *Gadus morhua*. J. Exp. Biol. 192:253–267.

Bidani A., S.J. Mathew, and E.D. Crandall. 1983. Pulmonary vascular carbonic anhydrase activity. J. Appl. Physiol. 55:75–83.

Booth V.H. 1938. The carbonic anhydrase inhibitor in serum. J. Physiol. 91:474–489.

Butler P.J. and J.D. Metcalfe. 1989. Cardiovascular and respiratory systems. Pp. 1–47 in T.J. Shuttleworth, ed. Physiology of Elasmobranch Fishes. Springer, Berlin.

Cameron J.N. 1979. Chloride shift in fish blood. J. Exp. Zool. 206:289–295.

Cameron J.N. and J.A. Polhemus. 1974. Theory of CO_2 exchange in trout gills. J. Exp. Biol. 60:183–194.

Crandall E.D. and J.E. O'Brasky. 1978. Direct evidence for participation of rat lung carbonic anhydrase in CO_2 reactions. J. Clin. Invest. 62:618–622.

Dejours P. 1975. Principles of Comparative Respiratory Physiology. Elsevier, North Holland, N.Y.

Dimberg K. 1994. The carbonic anhydrase inhibitor in trout plasma: purification and its effect on carbonic anhydrase activity and the Root effect. Fish Physiol. Biochem. 12:381–386.

Effros R.M., R.S.Y. Chang, and P. Silverman. 1978. Acceleration of plasma bicarbonate conversion to carbon dioxide by pulmonary carbonic anhydrase. Science 199:427–429.

Gilmour K.M., R.P. Henry, C.M. Wood, and S.F. Perry. 1997. Extracellular carbonic anhydrase and an acid-base disequilibrium in the blood of the dogfish, *Squalus acanthias*. J. Exp. Biol. 200:173–183.

Graham M.S., J.D. Turner, and C.M. Wood. 1990. Control of ventilation in the hypercapnic skate *Raja ocella*. I. Blood and extracellular fluid. Respir. Physiol. 80:259–277.

Haswell M.S., J.-P. Raggin, and C. LeRay. 1983. An investigation of the carbonic anhydrase inhibitor in eel plasma. Comp. Biochem. Physiol. 74A:175–177.

Haswell M.S. and D.J. Randall. 1976. Carbonic anhydrase inhibitor in trout plasma. Respir. Physiol. 28:17–27.

Heisler N., D.P. Toews, and G.F. Holeton. 1988. Regulation of ventilation and acid-base status in the elasmobranch *Scyliorhinus stellaris* during hyperoxia induced hypercapnia. Respir. Physiol. 71:227–246.

Heming T.A., C. Geers, G. Gros, A. Bidani, and E.D. Crandall. 1986. Effects of dextran-bound inhibitors on carbonic anhydrase activity in isolated rat lungs. J. Appl. Physiol. 61:1849–1856.

Heming T.A. and T.A. Watson. 1986. Activity and inhibition

of carbonic anhydrase in *Amia calva,* a bimodal-breathing holostean fish. J. Fish Biol. 28:385–392.

Henry R.P. 1991. Techniques for measuring carbonic anhydrase activity in vitro: the electrometric delta pH and pH stat assays. Pp. 119–125 in S.J. Dodgson, R.E. Tashian, G. Gros, and N.D. Carter, eds. The Carbonic Anhydrases: Cellular Physiology and Molecular Genetics. Plenum, New York.

Henry R.P., S.J. Dodgson, R.E. Forster, and B.T. Storey. 1986. Rat lung carbonic anhydrase: activity, localization, and isozymes. J. Appl. Physiol. 60:638–645.

Henry R.P. and T.A. Heming. 1998. Carbonic anhydrase and respiratory gas exchange. In S.F. Perry and B.L. Tufts, eds. Fish Physiology. Vol. 17. Fish Hemoglobin and Respiration. Academic Press, New York (in press).

Henry R.P., N.J. Smatresk, and J.N. Cameron. 1988. The distribution of branchial carbonic anhydrase and the effects of gill and erythrocyte carbonic anhydrase inhibition in the channel catfish, *Ictalurus punctatus.* J. Exp. Biol. 134:201–218.

Henry R.P., B.L. Tufts, and R.G. Boutilier. 1993. The distribution of carbonic anhydrase type I and II isozymes in lamprey and trout: possible coevolution with erythrocyte chloride/bicarbonate exchange. J. Comp. Physiol. 163B:380–388.

Hill E.P. 1980. Carbonic anhydrase inhibitor in plasma of dogs and rabbits. Proc. Int. Union Physiol. Sci. 29:472.

Hyde D.A. and S.F. Perry. 1990. Absence of red cell pH and oxygen content regulation in American eel (*Anguilla rostrata*) during hypercapnic acidosis in vivo and in vitro. J. Comp. Physiol. 159B:687–693.

Kernohan J.C., W.W. Forrest, and F.J.W. Roughton. 1963. The activity of concentrated solutions of carbonic anhydrase. Biochim. Biophys. Acta 67:31–41.

Klocke R.A. 1980. Equilibrium of CO_2 reactions in the pulmonary capillary. J. Appl. Physiol. 48:972–976.

Lessard J., A.L. Val, S. Aota, and D.J. Randall. 1995. Why is there no carbonic anhydrase activity available to fish plasma? J. Exp. Biol. 198:31–38.

Maren T.H. 1967. Carbonic anhydrase: chemistry, physiology and inhibition. Physiol. Rev. 47:595–781.

Maren T.H., B.R. Friedland, and R.S. Rittmaster. 1980. Kinetic properties of primitive vertebrate carbonic anhydrases. Comp. Biochem. Physiol. 67B:69–74.

Meldrum N.U. and F.J.W. Roughton. 1933. Carbonic anhydrase: its preparation and properties. J. Physiol. (Lond.) 80:113–142.

Milligan C.L. and C.M. Wood. 1987. Regulation of blood oxygen transport and red cell pHi after exhaustive exercise in rainbow trout (*Salmo gairdneri*) and starry flounder (*Platichthys stellatus*). J. Exp. Biol. 133:263–282.

Motais R., B. Fievet, F. Garcia-Romeu, and F. Thomas. 1989. Na^+/H^+ exchange and pH regulation in red blood cells: role of uncatalyzed H_2CO_3 dehydration. Am. J. Physiol. 256:C728–C735.

Nikinmaa M., ed. 1990. Vertebrate Red Blood Cells. Vol. 28 of Zoophysiology. Springer, Heidelberg.

Obaid A.L., A.M. Critz, and E.D. Crandall. 1979. Kinetics of bicarbonate/chloride exchange in dogfish erythrocytes. Am. J. Physiol. 237:R132–R138.

Perry S.F., P.S. Davie, C. Daxboeck, and D.J. Randall. 1982. A comparison of CO_2 excretion in a spontaneously ventilating blood-perfused trout preparation and saline-perfused gill preparations: contribution of the branchial epithelium and red blood cell. J. Exp. Biol. 101:47–60.

Perry S.F. and K.M. Gilmour. 1996. Consequences of catecholamine release on ventilation and blood oxygen transport during hypoxia and hypercapnia in an elasmobranch (*Squalus acanthias*) and a teleost (*Oncorhynchus mykiss*). J. Exp. Biol. 199:2105–2118.

Perry S.F. and S.D. Reid. 1992*a.* The relationship between adrenoceptors and adrenergic responsiveness in trout (*Oncorhynchus mykiss*) and eel (*Anguilla rostrata*) erythrocytes. J. Exp. Biol. 167:235–250.

———. 1992*b.* Relationships between blood oxygen content and catecholamine levels during hypoxia in rainbow trout and American eel. Am. J. Physiol. 263:R240–R249.

Perry S.F., C.M. Wood, P.J. Walsh, and S. Thomas. 1996. Fish red blood cell carbon dioxide transport in vitro: a comparative study. Comp. Biochem. Physiol. 113A:121–130.

Roush. E.D. and C.A. Fierke. 1992. Purification and characterization of a carbonic anhydrase II inhibitor from porcine plasma. Biochemistry 31:12536–12542.

Stabenau E.K., A. Bidani, and T.A. Heming. 1996. Physiological characterization of pulmonary carbonic anhydrase in the turtle. Respir. Physiol. 104:187–196.

Swenson E.R., L. Lippincott, and T.H. Maren. 1995. Effect of gill membrane-bound carbonic anhydrase inhibition on branchial bicarbonate excretion in the dogfish shark, *Squalus acanthias.* Bull. Mt. Desert Isl. Biol. Lab. 34: 94–95.

Swenson E.R. and T.H. Maren. 1987. Roles of gill and red cell carbonic anhydrase in elasmobranch HCO^-_3 and CO_2 excretion. Am. J. Physiol. 253:R450–R458.

Swenson E.R., B.T. Taschner, and T.H. Maren. 1996. Effect of membrane-bound carbonic anhydrase (CA) inhibition on bicarbonate excretion in the shark, *Squalus acanthias.* Bull. Mt. Desert Isl. Biol. Lab. 35:17.

Tufts B.L. and D.J. Randall. 1989. The functional significance of adrenergic pH regulation in fish erythrocytes. Can. J. Zool. 67:235–238.

Van Goor H. 1948. Carbonic anhydrase—its properties, distribution and significance for CO_2 transport. Enzymologia 13:73–165.

Whitney P.L. and T.V. Briggle. 1982. Membrane-associated carbonic anhydrase purified from bovine lung. J. Biol. Chem. 257:12056–12059.

Wood C.M. and C.L. Milligan. 1987. Adrenergic analysis of extracellular and intracellular lactate and H^+ dynamics after strenuous exercise in the starry flounder *Platichthys stellatus*. Physiol. Zool. 60:69–81.

Wood C.M. and R.S. Munger. 1994. Carbonic anhydrase injection provides evidence for the role of blood acid-base status in stimulating ventilation after exhaustive exercise in rainbow trout. J. Exp. Biol. 194:225–253.

Wood C.M. and S.F. Perry. 1991. A new in vitro assay for carbon dioxide excretion by trout red blood cells: effects of catecholamines. J. Exp. Biol. 157:349–366.

Wood C.M., S.F. Perry, P.J. Walsh, and S. Thomas. 1994. HCO^-_3 dehydration by the blood of an elasmobranch in the absence of a Haldane effect. Respir. Physiol. 98:319–337.

Wood C.M., J.D. Turner, R.S. Munger, and M.S. Graham. 1990. Control of ventilation in the hypercapnic skate *Raja ocellata*. II. Cerebrospinal fluid and intracellular pH in the brain and other tissues. Respir. Physiol. 80:279–298.