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THE DISTRIBUTION OF BRANCHIAL CARBONIC ANHYDRASE AND THE EFFECTS OF GILL AND ERYTHROCYTE CARBONIC ANHYDRASE INHIBITION IN THE CHANNEL CATFISH *ICTALURUS PUNCTATUS*

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SUMMARY

Carbonic anhydrase (CA) activity was assayed in lysed erythrocytes and in branchial cytoplasm, mitochondria and microsomes of the channel catfish, *Ictalurus punctatus*. Branchial CA activity was highest in the cytoplasmic fraction, but activity was very low in mitochondria and microsomes. Erythrocyte CA activity was over four-fold greater than that in the gills.

Intact animals were injected with the CA inhibitors acetazolamide and benzolamide. Slow, intra-arterial injection of both inhibitors elicited transient side effects of apnoea, bradycardia and hypoxaemia. Acetazolamide and benzolamide induced a mixed but primarily respiratory acidosis. The onset and the time course of the acidosis were correlated with the inhibition of erythrocyte CA; acetazolamide acted faster because it is more freely diffusible than benzolamide. The acid–base disturbance in the blood reached its maximum after 2 h; compensation was delayed until 24 h, when CA inhibition began to disappear.

We conclude from these results that there is very little, if any, membrane-associated CA in the gill, and that the branchial enzyme is not quantitatively important in directly converting plasma HCO_3^- to CO_2 for excretion. Rather, CO_2 excretion is accomplished *via* the traditional chloride shift, followed by intracellular dehydration of HCO_3^- by erythrocyte CA. These results also suggest that branchial cytoplasmic CA inhibition might impair ion transport processes that are used to compensate blood acid–base disturbances and thus delay compensation of the respiratory acidosis.

Key words: carbonic anhydrase, gills, erythrocytes, respiration.

INTRODUCTION

The enzyme carbonic anhydrase (CA) was originally discovered in mammalian erythrocytes (Meldrum & Roughton, 1932); since then it has been found in red blood cells of every vertebrate species examined (reviewed by Maren, 1967*a*,*b*; Sanyal, 1984). The enzyme, which catalyses the reversible hydration of carbon dioxide, is known to be important in the transport and excretion of respiratory CO_2 both in animals that breath air and in those that breath water (Cameron, 1979; Swenson, 1984; Randall & Daxboeck, 1984; Perry, 1986). While erythrocyte CA appears to be most critical to CO_2 exchange, the major organs of respiration, the lung and the gill, also contain significant levels of CA activity (Effros, Shapiro & Silverman, 1980; Henry, Dodgson, Forster & Storey, 1986; Houston & McCarty, 1978; Houston & Mearow, 1982). Thus, respiratory tissue CA could also play a role in CO_2 excretion.

The respiratory role of pulmonary CA has been thoroughly investigated. Carbonic anhydrase is associated with the pulmonary endothelial membrane (Whitney & Briggle, 1982; Henry *et al.* 1986). As such, it is available to catalyse the dehydration of plasma HCO_3^-, facilitating CO_2 excretion and maintaining pulmonary capillary pH/P_{CO_2} equilibrium (Effros, Chang & Silverman, 1978; Bidani & Crandall, 1978; Bidani, Crandall & Forester, 1978; Klocke, 1978, 1980). However, the potential respiratory role of branchial CA is still uncertain.

Originally, CA activity in erythrocytes of fish was viewed as being sufficient for normal CO_2 transport and excretion (Maren, 1967*a*), and it was suggested that branchial CA functioned in hydrating respiratory CO_2 within the epithelial cells to H^+ and HCO_3^- for ion transport (Maetz, 1971; Kirschner, 1979). Conflicting evidence was presented by Haswell & Randall (1976, 1978) discounting the role of erythrocyte CA and assigning a respiratory function to branchial CA by default (Haswell, Randall & Perry, 1980). These ideas were shown to be incorrect (Cameron, 1978; Obaid, Critz & Crandall, 1979), and further experiments confirmed the respiratory function of erythrocyte CA (Wood, McDonald & McMahon, 1972; Perry, Davie, Daxboeck & Randall, 1982). The potential contribution of branchial CA to CO_2 excretion has never been completely disproved, and evidence is still being accumulated indicating a role for the enzyme in the elimination of respiratory CO_2 in some species (Swenson, Hildesley & Maren, 1982; Swenson, Maren & Hildesley, 1984; Swenson & Evans, 1984).

CA in fish gills has been histochemically localized to both the ion-transporting chloride cells and the respiratory lamellae (Dimberg, Hoglund, Knutsson & Ridderstrale, 1981), but the quantitative distribution of CA within the gill is unknown. Also, no attempt has been made to correlate branchial CA distribution, its availability to plasma HCO_3^-, and the respiratory and acid–base parameters of blood in the intact organism. The presence of CA on the basal membrane of the gill and its availability to plasma would allow for a respiratory function of the gill enzyme, but this possibility has never been investigated. The objectives of this study were to investigate the quantitative subcellular distribution of branchial CA, and to

investigate the comparative contributions of erythrocyte and branchial CA to respiratory CO_2 excretion.

MATERIALS AND METHODS

Maintenance and handling of animals

Channel catfish (*Ictalurus punctatus*) weighing between 500 and 1200 g were obtained from commercial ponds in Angleton, Texas. They were held in 2000-l fibreglass tanks equipped with recirculating biofilters at 23 ± 1 °C. Since animals were held for only a few days before use, they were not fed.

Before any experiment, individuals were anaesthetized in MS-222 (tricaine methanesulphonate, Sigma) and were fitted with two catheters while being ventilated artificially. A blood-sampling catheter (PE 60) was implanted in the dorsal aorta (DA) according to the method of Soivio, Nyholm & Westman (1975); a second catheter (PE 160), for pressure recording, was placed through the operculum and fastened with a sleeve (PE 200) against the outer surface. Catheters were filled with heparinized Cortland saline (Wolf, 1963).

Following surgery, catfish were transferred to darkened individual Plexiglas chambers with running dechlorinated tap water and allowed to recover for approximately 24 h.

Experimental protocol and blood acid–base measurements

Three control blood samples (approx. 1 ml) were taken from resting catfish *via* the DA catheter, before a treatment was administered. Blood pH was measured with a thermostatted glass microcapillary electrode (Radiometer G297/K497; Radiometer pHM 71 blood gas analyser); P_{O_2} was determined with a thermostatted cell and O_2 electrode (Radiometer E5046/D616). The remaining blood was then immediately centrifuged for about 15 s in an Eppendorf Microfuge. Total CO_2 (C_T) was measured on 20-μl samples of plasma with a Capni-Con total CO_2 analyser (Cameron Instruments Co.). The values of pH and C_T were used to calculate P_{CO_2}, with values for solubility calculated from Boutilier, Heming & Iwama (1984) and the pK′ calculated using the equation given by Heisler (1984).

Catfish were given an injection of one of the following carbonic anhydrase inhibitors: quaternary ammonium sulphanilamide (QAS-chloride or QAS-bromide; Ferrosan), acetazolamide (Sigma) or benzolamide (the gift of Dr Thomas H. Maren, the University of Florida), through the DA catheter. The inhibitors were dissolved in Cortland saline; the pH values of the stock solutions were between 7·40 and 8·00, depending on the inhibitor and the concentration (dilute NaOH was added to dissolve acetazolamide and benzolamide fully). Initial measurements on QAS indicated that the inhibitor was rapidly distributed throughout the total extracellular fluid (ECF) pool. Thus, the inhibitor dosages were calculated to yield an initial ECF concentration of $1\ \text{mmol}\,l^{-1}$ for QAS and $10\ \mu\text{mol}\,l^{-1}$ for acetazolamide and benzolamide after an approximate 1:100 dilution from stock solutions. ECF volume was estimated at approximately 20 % of total body weight (Cameron, 1980); volume

of injectate was between 1 and 3 ml, depending on the weight of the fish. Blood samples were taken at regular intervals between 10 min and 24 h after injection, and blood O_2 and acid–base variables were determined as described above. Plasma and packed red cells were saved for subsequent measurements (see below).

Ventilatory and cardiovascular measurements

The opercular catheter was connected to a pressure transducer (Micron MP15D) and strain gauge preamplifier (Coulbourn Instruments, Type A) to record gill ventilation frequency (f_G) and opercular pressure amplitude (P_{OP}); these data were displayed on a thermal recorder (Watanabe). Heart rate (f_H) and blood pressure in the DA (P_{DA}) were also analysed *via* a pressure transducer (Cobe) connected to the DA catheter. These variables were analysed immediately before injection of a CA inhibitor and at intervals between 1 and 30 min post-injection. Opercular pressure was analysed as the mean of 10–15 individual pressure excursions, while mean systolic pressure was taken from the average of 10 pressure measurements taken over a 1-min period. Changes in these values with time were statistically analysed using a two-way analysis of variance (ANOVA), no replication, with the fiducial limit of significance at $P < 0{\cdot}05$. The ventilatory and cardiovascular responses to benzolamide were measured on only three animals and were not statistically analysed.

Determination of CA activity and inhibitor concentration

Carbonic anhydrase activity was measured in both plasma and haemolysates of packed red cells (lysed by the addition of distilled water). The catalysed rate of CO_2 hydration was determined according to the electrometric method of Maren & Couto (1979) as modified by Henry & Kormanik (1985). Protein concentrations were determined using the Coomassie brilliant blue dye-binding technique (Bio Rad Laboratories), and CA activity was reported as $\mu mol\ CO_2\ mg\ protein^{-1}\ min^{-1}$.

The concentration of injected CA inhibitor in plasma was measured by the method of Maren (1960). Briefly, a standard amount of CA (purified bovine erythrocyte CA, Sigma) was titrated with increasing amounts of a known concentration of inhibitor, thus producing a standard curve for CA inhibition *vs* inhibitor concentration. The CA standard was then titrated with plasma, and the inhibitor concentration was determined from a comparison of the amount of the resultant CA inhibition with that from the standard curve. Similar concentrations and volumes of inhibitor and plasma were used to avoid any dilution artefacts.

In one group of fish, total gill CA activity was also measured after treatment with acetazolamide. In these fish, the inhibitor was given *via* the caudal artery while the animal was under anaesthesia. The gills were ventilated with oxygenated water and the animals were kept under anaesthesia for the duration of the experiment. At 30 min post-injection, the heart was exposed and a catheter was inserted into the bulbus arteriosus. The gills were perfused with calcium-free, heparinized saline made hypoxic by bubbling with nitrogen to promote vasodilation. The heart was cut to allow for drainage, and the gills were perfused for 5–10 min until they appeared clear. The gill arches were cut out and the filaments were removed, placed into four

volumes of cold buffer and homogenized (see below). Only filaments that were completely free of red cells or clots were used. The homogenate was centrifuged for 20 min at 10 000 ***g*** at 5 °C (Sorvall RC-2B), and the supernatant was assayed for CA activity as described above.

Fractionation of branchial tissue

Catfish were anaesthetized, and the gills were perfused free of erythrocytes as described above. Filaments were dissected free of the gill arch and placed into approximately four volumes of cold buffer (225 mmol l^{-1} mannitol, 75 mmol l^{-1} sucrose, 10 mmol l^{-1} Trizma base, adjusted to pH 7·40 with phosphoric acid) (Henry & Kormanik, 1985; Henry *et al.* 1986). Branchial tissue was homogenized with a motor-driven Teflon/glass homogenizer. The crude homogenate was then subjected to differential centrifugation at 4 °C to produce three pellet fractions and a final supernatant: (1) cellular debris (whole cells, nuclei and large cell fragments) (270 ***g*** for 15 min), (2) a mitochondrial pellet (7500 ***g*** for 20 min), and (3) a microsomal pellet (110 000 ***g*** for 90 min). The remaining supernatant represented the cytoplasm (Henry *et al.* 1986).

Each fraction was then assayed for CA activity as described above. To differentiate between loosely bound CA and more integrally associated CA, each fraction was also resuspended in buffer and vigorously agitated by vortexing. The pellet was then recentrifuged, the supernatant was drawn off, and the pellet was resuspended in fresh buffer. CA activities in the resuspended pellet and the supernatant were determined. This allowed for a rough estimate of integral *vs* peripheral particulate CA to be made, and it also gave a measure of total recovery after the pellet was washed.

Plasma ion measurements

Plasma concentrations of sodium and potassium were measured by flame photometry (Radiometer FLM 3). Chloride concentrations were determined by coulometric titration (Buchler–Cotlove).

RESULTS

Distribution of branchial CA

Carbonic anhydrase activity was found in all subcellular fractions of gill tissue (Fig. 1). The final supernatant (cytoplasm) contained by far the greatest level of CA activity, making up approximately 56 % of the total gill enzyme activity by weight and 67 % of the specific activity (Table 1). Spectrophotometric determination of haemoglobin (at 570 nm) in the supernatant fraction indicated that potential contamination from erythrocyte CA was negligible. Of the three pellet fractions, the cell debris fraction contained the highest levels of CA activity: over 10-fold greater than either mitochondrial or microsomal CA activity (Fig. 1). Upon resuspension and vigorous washing of the cell debris pellet, approximately 60 % of the initial particulate-associated CA activity was transferred to the supernatant (Fig. 2). Thus,

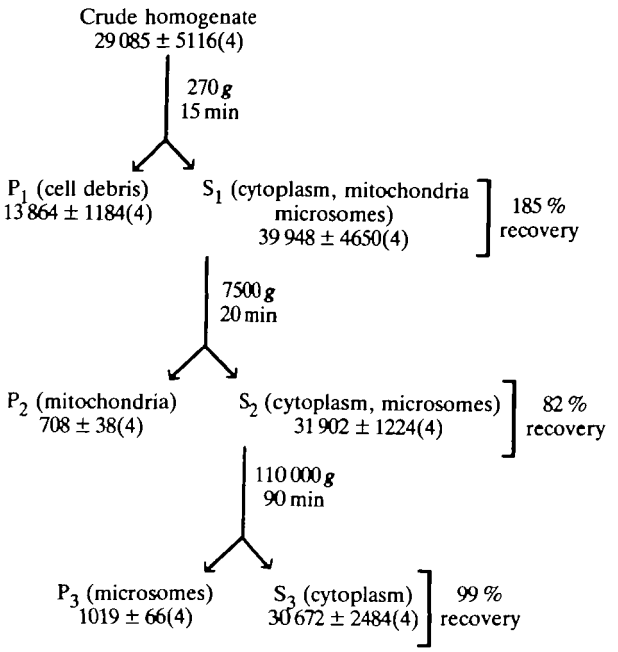

Fig. 1. Schematic representation of the fractionation of the gills of *Ictalurus punctatus* (4·02 ± 0·4 g in 10 ml of buffer, $N = 4$) *via* differential centrifugation. Total CA activity (mean ± S.E.M., $N = 4$) for each fraction is reported as (μmol CO_2 min^{-1} ml^{-1}) × (fraction volume in ml). Percentage recovery for each step in the procedure is also reported.

it appears that most of the CA activity in the cell debris pellet is not true particulate-associated CA, but rather it is soluble CA of the gill fragments and whole cells that is liberated after disruption of the pellet. The same pattern is seen for mitochondrial CA (Fig. 2). The microsomal CA, however, is more characteristic of true membrane-associated CA, as fully 95 % of the particulate CA remains in the pellet fraction after agitation and washing (Fig. 2). This membrane-associated CA made up about 9 % of the total branchial CA activity by weight and nearly 11 % of the specific activity (Table 1). Throughout the fractionation and washing procedures, percentage recovery was at least 80 % or better (Figs 1, 2).

Carbonic anhydrase activity in crude lysates of the erythrocytes was much higher than in any gill fraction. Specific activity of the lysate was over four-fold greater than branchial cytoplasmic CA activity and over 20 times greater than CA activity in the gill microsomes (Table 1).

Erythrocyte and gill CA inhibition

The two CA inhibitors, acetazolamide and benzolamide, have a residence time in the catfish plasma of not more than 24 h. Both compounds appear to wash out of the plasma slowly and at approximately the same rate (Fig. 3A). Erythrocyte CA activity was virtually 100 % inhibited 10 min after injection of acetazolamide; this level of inhibition persisted over the initial 4 h and then dropped to about 80 % by 24 h

Table 1. *The distribution of CA activity among crude subcellular fractions of gill homogenates of* Ictalurus punctatus

Subcellular fraction	min	*g*	μmol CO_2 g^{-1} min^{-1}	% total	μmol CO_2 mg protein^{-1} min^{-1}	% total
Cell debris	15	270	6048 ± 1131 (4)	26·6	111·7 ± 23 (4)	16·2
Mitochondria	20	7500	1875 ± 264 (4)	8·2	37·4 ± 4 (4)	5·4
Microsomes	90	110000	2038 ± 128 (4)	8·9	74·4 ± 4 (4)	10·8
Cytoplasm	(supernatant)		12817 ± 504 (4)	56·3	467·2 ± 20 (4)	67·6
Red blood cell lysate					1926 ± 81 (4)	

CA activity is reported as mean ± S.E.M. (N) based on both wet mass of gill tissue and on protein concentration.

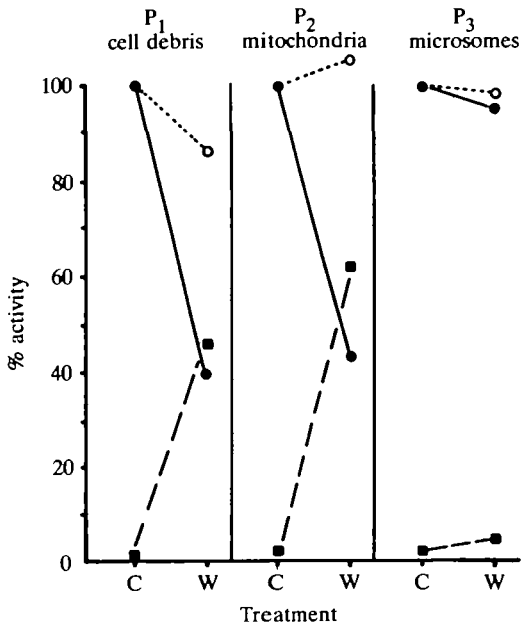

Fig. 2. The effect of washing and agitation on the CA activity initially associated with each branchial pellet fraction. Symbols are as follows: C, CA activity before treatment; W, CA activity after the washing procedure. Solid circles represent CA activity in the pellet, solid squares represent CA activity in the supernatant resulting from the washing procedure, and open circles represent percentage recovery.

(Fig. 3B). For benzolamide, CA inhibition at 10 min was only 80 % and did not reach 100 % until 30 min after injection. Benzolamide-induced CA inhibition was also present (80 %) at 24 h.

For branchial tissue perfused with acetazolamide, CA inhibition was complete by 30 min after injection; the time course of branchial enzyme inhibition was not followed past that point.

Quaternary ammonium sulphanilamide (QAS) was difficult to use in the catfish. The compound was cleared from the plasma rapidly; an initial injection of 1 mmol l^{-1} produced a circulating concentration of less than 10 μmol l^{-1} after 10 min. At a concentration high enough to produce a circulating level of 400 μmol l^{-1} QAS, the compound was fatal.

Ventilatory and cardiovascular responses

At rest, fish exhibited breathing patterns characterized by periods of apnoea in which gill ventilation ceased (e.g. Fig. 4). During the apnoeic periods, heart rate and blood pressure also fell. Injection of either acetazolamide or benzolamide caused a transient apnoea and bradycardia immediately following the injection (Fig. 5). The time courses of the responses to both inhibitors were similar; gill ventilatory frequency was significantly depressed immediately after injection but returned to

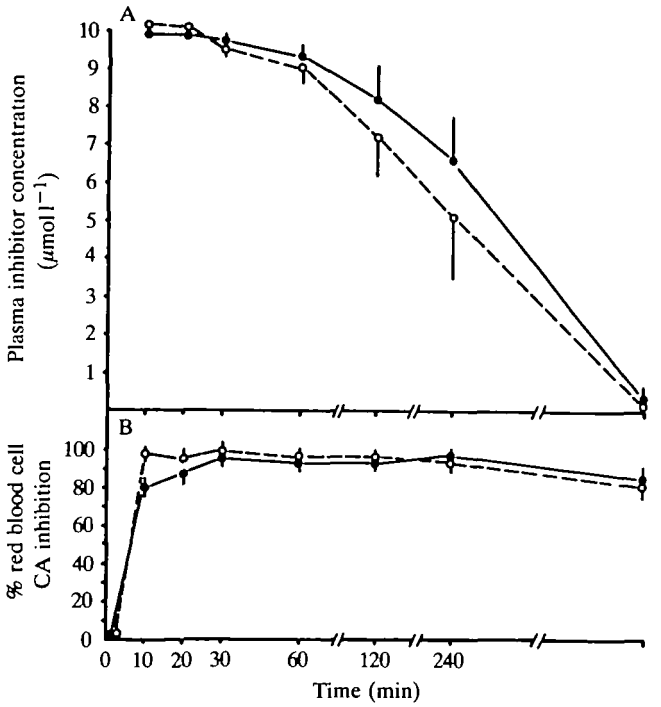

Fig. 3. (A) The measured concentrations of acetazolamide (○) and benzolamide (●) in the plasma of *Ictalurus punctatus* after an injection calculated to produce an extracellular fluid concentration of 10 μmol l^{-1} (mean ± S.E.M., $N = 4$–5). (B) The percentage inhibition of erythrocyte CA activity by acetazolamide and benzolamide (mean ± S.E.M., $N = 4$–5). Symbols as in A.

control values within 5 min (Fig. 5). The depression in fG was strongly correlated with a transient reduction in dorsal arterial P_{O_2} (Fig. 6). Opercular pressure was not affected in the acetazolamide-treated fish but was stimulated by benzolamide. Heart rate was depressed significantly by acetazolamide, but it recovered within 5 min and actually increased over the subsequent 25 min (Fig. 5). Dorsal aortic pressure increased transiently, but significantly, following an injection of acetazolamide, but it decreased after administration of benzolamide (Fig. 5). The transient changes in ventilatory and cardiovascular parameters were often accompanied by a loss of equilibrium in the animal.

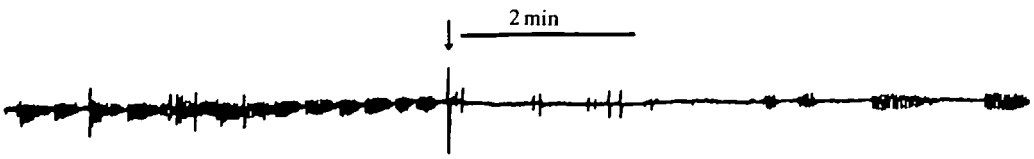

Fig. 4. Representative recording of opercular ventilatory pressure showing normal periods of apnoea and initial prolonged apnoea after an injection of acetazolamide (arrow).

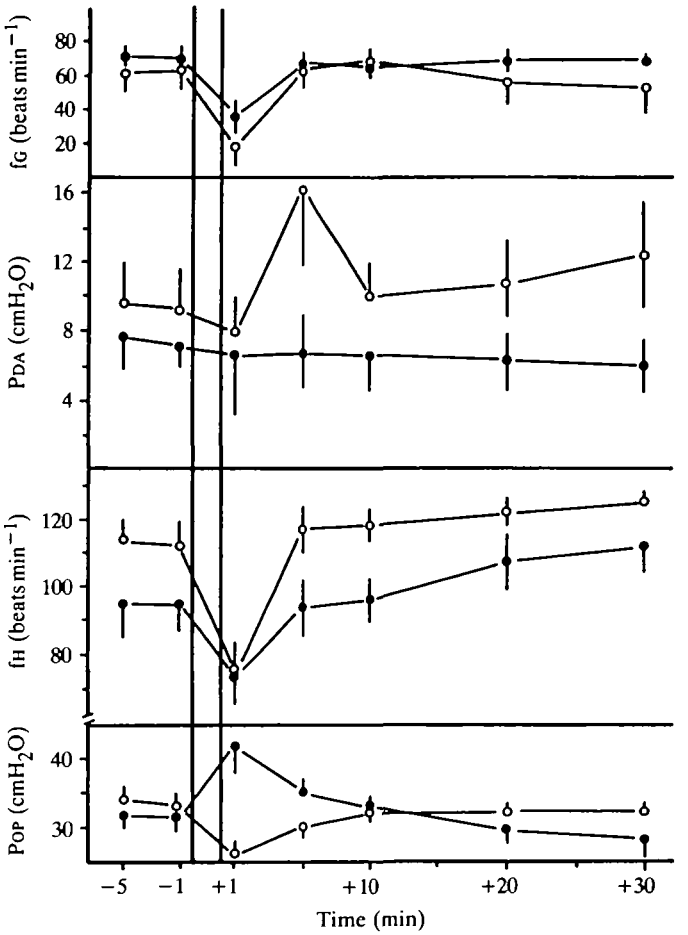

Fig. 5. Cardiovascular and ventilatory responses of *Ictalurus punctatus* prior to and following an intra-arterial injection of either acetazolamide (●, mean ± S.E.M., $N = 5$) or benzolamide (○, mean ± S.E.M., $N = 3$). Parallel vertical lines represent the 2-min period in which the injection was given. 1 cmH$_2$O = 98·1 Pa.

Blood acid–base responses

Injection of acetazolamide or benzolamide resulted in the development of an acidosis (Figs 7 and 8, respectively). The predominant component of the acidosis was respiratory: P_{CO_2} increased approximately three-fold, from 3 to 9 mmHg (1 mmHg = 133·3 Pa), and pH fell by 0·3 units following treatment with both inhibitors. Virtually all of the increase in P_{CO_2} took place within the initial 2-h period following inhibitor injection. The respiratory acidosis was still maximal at 24 h after treatment. Compensation of the acidosis was slow, with no appreciable [HCO_3^-] increase until 4 h post-treatment, and only partial compensation was achieved at 24 h. A small, non-respiratory component accompanied the respiratory acidosis for about 2 h following treatment with either inhibitor. This is probably a result of the

disruption of normal ventilatory patterns and the concomitant drop in arterial P_{O_2}. QAS ($<10\,\mu mol\,l^{-1}$) had no effect on acid–base status.

Blood ionic responses

The concentrations of the major ions in the plasma, Na^+, Cl^- and K^+, were not significantly altered ($P > 0{\cdot}05$, F-test) over the time course of the treatment by either acetazolamide or benzolamide. The strong ion difference (SID), which in this case was estimated as $([Na^+]+[K^+]-[Cl^-])$, did not exhibit a clear pattern over the initial period of the experiment for either inhibitor (Fig. 9). There was virtually no net change in the SID over the first 4 h of treatment, the time when erythrocyte and branchial CA inhibition was maximal and during which the respiratory acidosis was uncompensated. Between 4 and 24 h, however, there was a slight increase in the SID which corresponded to the small degree of compensation. Throughout the experiment, however, the changes in the SID and the changes in plasma HCO_3^- were well matched.

DISCUSSION

The overwhelming majority of CA activity in the gills of the catfish is cytoplasmic, rendering it unavailable to plasma HCO_3^-. Bicarbonate has been shown not to

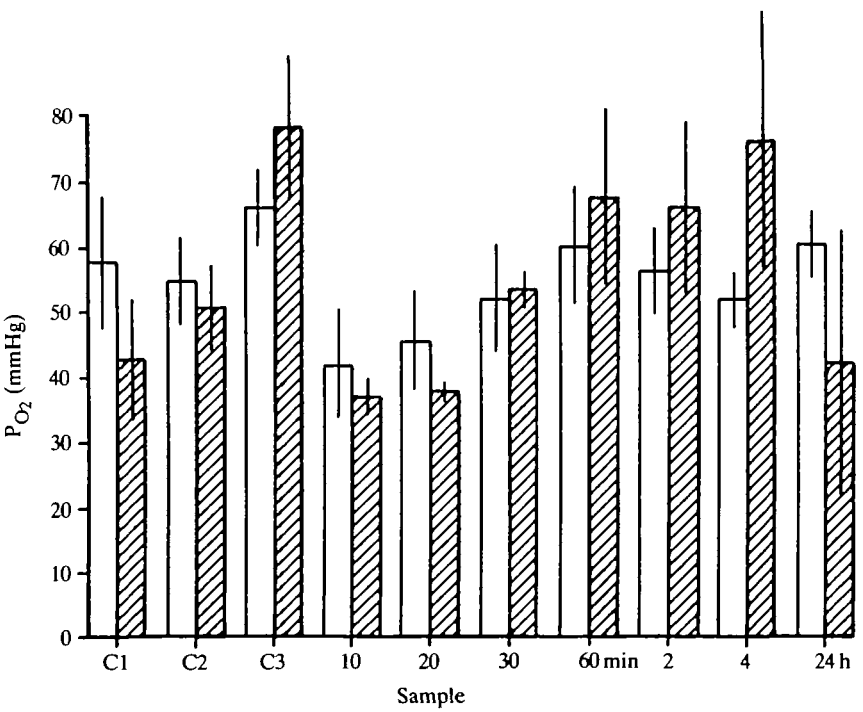

Fig. 6. Dorsal aortic P_{O_2} before and after an injection of the CA inhibitors acetazolamide (open bars, mean ± S.E.M., $N = 5$) and benzolamide (cross-hatched bars, mean ± S.E.M., $N = 3$–4). 1 mmHg = 133·3 Pa.

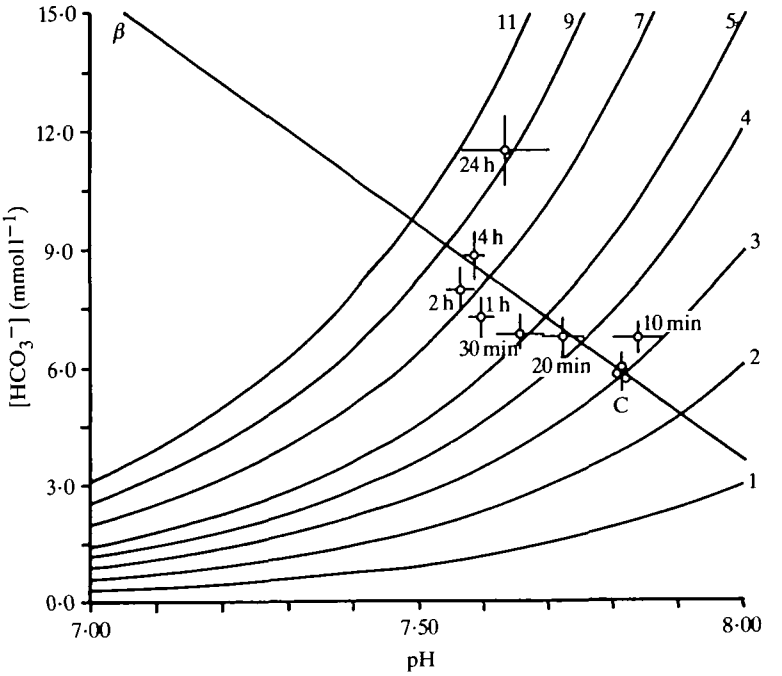

Fig. 7. Acid–base status of catfish under control (C) conditions and at various times following acetazolamide infusion. The vertical and horizontal bars show ±1 S.E. and the diagonal line has a slope equal to the *in vitro* non-bicarbonate buffer value (β) of −12·1.

permeate artificial membranes (Gutknecht, Bisson & Tosteson, 1977), the endothelial membrane of the lung (Effros, Mason & Silverman, 1981) or the basal branchial membrane of fish (Perry *et al.* 1982). It is highly unlikely that HCO_3^- enters the gill, and thus the major fraction of branchial CA can be excluded from having a role in respiratory CO_2 excretion. Cytoplasmic CA has been localized primarily to the chloride cells of the teleost gill (Dimberg *et al.* 1981), where it undoubtedly functions in branchial ion transport processes (Kirchner, 1979). The level of CA activity in the cytoplasm of catfish gills is remarkably similar to that of the gills of other euryhaline organisms (mostly arthropods) that also probably use similar mechanisms of branchial ion transport and regulation (Henry, 1984).

Of the three particulate fractions, the cell debris pellet contained the highest initial level of CA activity. The removal of most of the CA activity after resuspension and a single washing, however, suggests that the cell debris pellet itself does not represent a significant independent fraction of gill CA activity. Rather, most of the CA activity is probably soluble CA, associated with intact cells and cell fragments, and liberated by the washing procedure. The mitochondrial pellet also possesses extremely low levels of CA activity, the bulk of which does not appear to be integrally associated with that fraction. Microsomal CA activity, although relatively low, appears to be integral as virtually none was lost from the fraction after washing. Although this fraction of the total gill CA activity is potentially available to mobilize plasma HCO_3^- for CO_2

excretion, it is extremely small. Gill microsomal CA specific activity is only about 4% of that in the erythrocyte; this difference must be very conservative, since erythrocyte CA specific activity is minimized by the high haemoglobin concentration and microsomal CA specific activity is magnified by the low protein concentration in that fraction. Thus it is doubtful that gill CA could contribute significantly to CO_2 excretion.

Acetazolamide, benzolamide and QAS were originally used in the hope that they would selectively inhibit intracellular and extracellular CA, and thus help to elucidate the role of membrane-associated (branchial) *vs* intracellular (erythrocytic) CA. The use of these inhibitors in conscious fish was, however, unexpectedly complicated. Injection of all three inhibitors elicited potent systemic responses. The loss of equilibrium and, in particular, the altered ventilatory patterns suggest that the initially high inhibitor concentration in the plasma had toxic side effects on the central nervous system. The depressed gill ventilation and subsequent hypoxaemia probably stimulated the bradycardia and pressor response, which are typical teleost reflex responses to hypoxia (see Shelton, Jones & Milsom, 1986, for a recent review). These effects were minimized, but not entirely eliminated, by giving the injections over a 2-min period. It is likely that the impaired branchial gas transport contributed to the initial acidosis by raising arterial P_{CO_2} levels. The prolonged hypoxaemia may

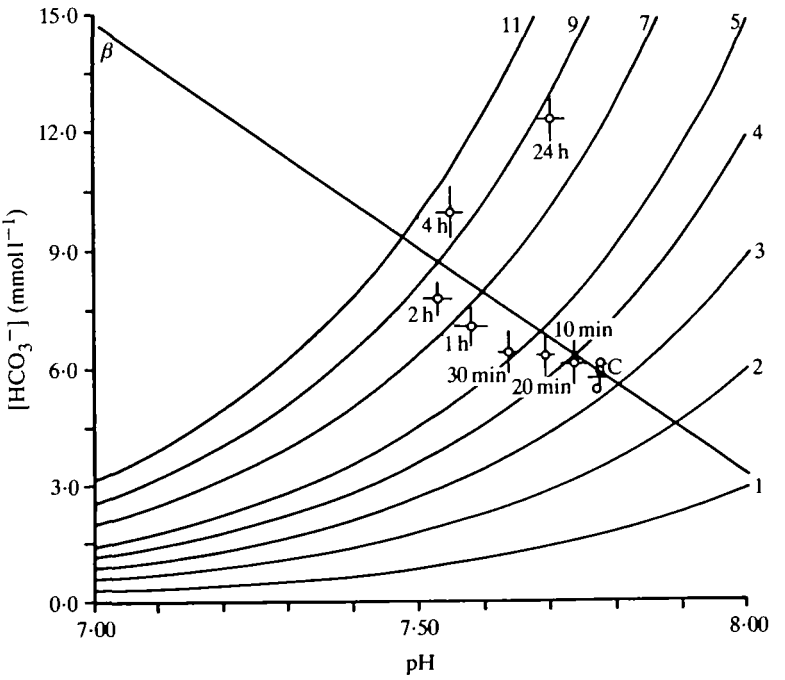

Fig. 8. Acid–base status of catfish under control (C) conditions and at various times after benzolamide infusion. The vertical and horizontal bars show ±1 S.E. and the diagonal line has a slope equal to the *in vitro* non-bicarbonate buffer value (β) of −11·5.

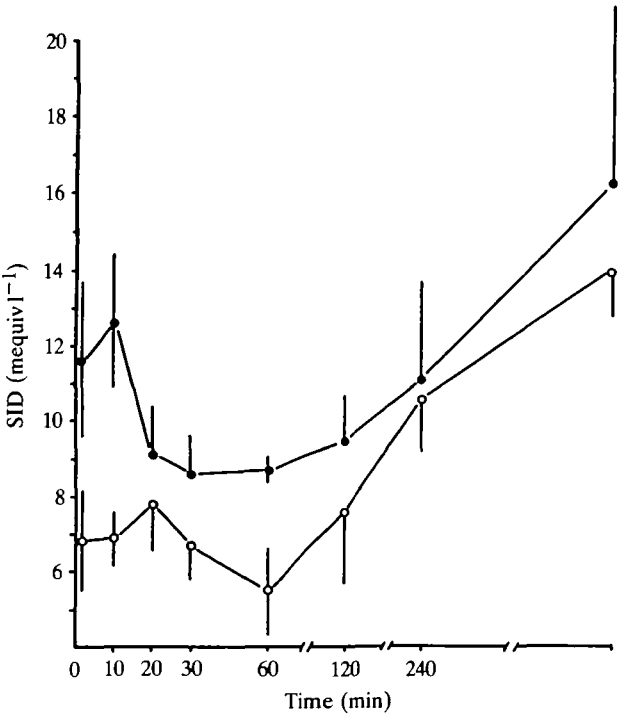

Fig. 9. Plasma strong ion difference (SID) in *Ictalurus punctatus* before and after an injection of acetazolamide (●) or benzolamide (○). Mean ± S.E.M., $N = 4–5$.

have additionally resulted in increased plasma lactate levels, thus contributing to, or accounting for, the observed transient metabolic acidosis.

QAS in the ECF at concentrations below $10\,\mu mol\,l^{-1}$ had no effect on blood acid–base status. This compound was probably restricted to the ECF (Henry, 1987), and the lack of an effect suggests that there is no significant fraction of CA (i.e. branchial membrane-associated CA) available to ECF HCO_3^-. The concentrations that were sub-lethal to the animal were also probably close to or below the K_i (inhibitor constant) of the inhibitor (Henry, 1987); thus, it is also possible that some putative membrane-associated CA remained uninhibited. The other two inhibitors used, acetazolamide and benzolamide, are much more potent, and the concentrations used were between 100 and 1000 times their K_i values, respectively (Sanyal, 1984). Both compounds permeated the erythrocytes, although benzolamide moved more slowly. This difference in permeability allowed for an initial separation of the putative effects of extracellular *vs* intracellular CA inhibition (i.e. 20 min after injection), but that difference was much less than previously reported for other systems (Maren, 1967*a*).

The results of these inhibitor studies also confirm the conclusions concerning the relative contributions of erythrocyte and branchial CA to CO_2 excretion. The onset and duration of the respiratory acidosis was correlated with the inhibition of

erythrocyte CA. A significant acidosis did not develop until 20 min after administration of either inhibitor; at that time erythrocyte CA was between 80 and 100% inhibited. If branchial microsomal CA were involved in CO_2 excretion it would have been immediately inhibited by either acetazolamide or benzolamide circulating in the plasma, and a respiratory acidosis would have developed much sooner. This was not the case. Also, the respiratory acidosis persisted for 24 h after injection, by which time both inhibitors had been cleared from the plasma. Thus, any branchial CA involved in CO_2 excretion should have been functioning at that time, and the respiratory acidosis should have been at least partially ameliorated. Again, this was not the case. Also, at this time erythrocyte CA was still largely inhibited. These results strongly suggest that branchial CA is not quantitatively important in CO_2 excretion. Similar conclusions were obtained using an isolated, perfused fish head preparation (Perry *et al.* 1982).

It is not entirely clear why CA in teleost branchial tissue should lack a role in CO_2 excretion, whereas CA in the mammalian lung is known to facilitate the process. One speculation might be that branchial endothelial CA is not necessary for aquatic CO_2 excretion in fish. The high capacitance of water for CO_2, the relatively long residence time of branchial capillary blood (compared to mammalian pulmonary capillary residence time), and the presence of counter-current exchange may make the gill efficient enough at CO_2 exchange so that an additional source of CA other than that found in the erythrocyte is not required.

Aquatic organisms compensate blood acid–base disturbances by adjusting ion transport mechanisms in both gills and kidneys; this alters the blood strong ion difference (SID) and thus blood pH is readjusted (Cameron, 1976; Stewart, 1978). Branchial cytoplasmic CA becomes fully inhibited after 30 min of perfusion with acetazolamide and, although the time course of inhibition and recovery was not directly measured, presumably it is similar to that in the red blood cells. If so, gill CA would remain inhibited for 24 h, and its normal role in ion transport of providing H^+ and HCO_3^- to be used as counterions for active Na^+ and Cl^- uptake would be disrupted (Kirschner, 1979). Assuming there are ion transport processes involved in branchial acid–base regulation, we would expect an impairment of the ability to compensate acid–base disturbances. This prediction is supported by the data (Figs 7, 8). A comparable level of hypercapnia in normal channel catfish leads to a 5 mequiv l^{-1} increase in HCO_3^- in 2 h (from 6 to 11 mequiv l^{-1}; Cameron, 1980), compared to reduction at 2 h after treatment with acetazolamide or benzolamide. The relatively small compensation achieved after 24 h probably reflected gradual recovery of CA activity. At that time plasma SID and HCO_3^- were beginning to offset the decreased pH; erythrocyte CA was also beginning to recover from inhibition, and so presumably was branchial CA.

In summary, CA in the catfish gill does not appear to be important in respiratory CO_2 excretion. The fraction of branchial CA available to plasma HCO_3^- is very small compared to both the gill cytoplasmic fraction and CA in the erythrocyte. A respiratory acidosis that is induced by CA inhibition correlates with the inhibition of

erythrocyte CA, and the impaired ability to compensate this acidosis correlates with the apparent disruption of branchial ion transport processes as a result of the inhibition of gill cytoplasmic CA.

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REFERENCES

BIDANI, A. & CRANDALL, E. D. (1978). Slow postcapillary pH changes in blood in anesthetized animals. *J. appl. Physiol.* **45**, 674–680.

BIDANI, A., CRANDALL, E. D. & FORSTER, R. E. (1978). Analysis of postcapillary pH changes in blood *in vivo* after gas exchange. *J. appl. Physiol.* **44**, 770–781.

BOUTILIER, R. G., HEMING, T. A. & IWAMA, G. K. (1984). Physicochemical parameters for use in fish respiratory physiology. In *Fish Physiology*, vol. 10A (ed. W. S. Hoar & D. J. Randall), pp. 403–430. New York: Academic Press.

CAMERON, J. N. (1976). Branchial ion uptake in the Arctic grayling: resting values and effects of acid–base disturbances. *J. exp. Biol.* **64**, 711–725.

CAMERON, J. N. (1978). Chloride shift in fish blood. *J. exp. Zool.* **206**, 289–295.

CAMERON, J. N. (1979). Excretion of CO_2 in water-breathing animals, a short review. *Mar. biol. Letts* **1**, 1–13.

CAMERON, J. N. (1980). Body fluid pools, kidney function, and acid–base regulation in the freshwater catfish, *Ictalurus punctatus*. *J. exp. Biol.* **86**, 171–185.

DIMBERG, K., HOGLUND, L. B., KNUTSSON, P. G. & RIDDERSTRALE, Y. (1981). Histochemical localization of carbonic anhydrase from young salmon (*Salmo salar* L.) adapted to fresh and salt water. *Acta physiol. scand.* **112**, 218–220.

EFFROS, R. M., CHANG, R. S. Y. & SILVERMAN, P. (1978). Acceleration of plasma bicarbonate conversion to carbon dioxide by pulmonary carbonic anhydrase. *Science* **199**, 427–429.

EFFROS, R. M., MASON, G. & SILVERMAN, P. (1981). Role of perfusion and diffusion in $^{14}CO_2$ exchange in the rabbit lung. *J. appl. Physiol.: Respirat. Environ. Exercise Physiol.* **51**, 1136–1144.

EFFROS, R. M., SHAPIRO, L. & SILVERMAN, P. (1980). Carbonic anhydrase activity of rabbit lungs. *J. appl. Physiol.* **49**, 589–600.

GUTKNECHT, J., BISSON, M. A. & TOSTESON, F. C. (1977). Diffusion of carbon dioxide through lipid bilayer membranes: effects of carbonic anhydrase, bicarbonate and unstirred layers. *J. gen. Physiol.* **69**, 779–794.

HASWELL, M. S. & RANDALL, D. J. (1976). Carbonic anhydrase inhibitor in trout plasma. *Respir. Physiol.* **28**, 17–27.

HASWELL, M. S. & RANDALL, D. J. (1978). The pattern of carbon dioxide excretion in the rainbow trout, *Salmo gairdneri*. *J. exp. Biol.* **72**, 17–24.

HASWELL, M. S., RANDALL, D. J. & PERRY, S. F. (1980). Fish gill carbonic anhydrase: acid–base regulation or salt transport? *Am. J. Physiol.* **238**, R240–R245.

HEISLER, N. (1984). Acid–base regulation in fishes. In *Fish Physiology*, vol. 10A (ed. W. S. Hoar & D. J. Randall), pp. 315–401. New York: Academic Press.

HENRY, R. P. (1984). The role of carbonic anhydrase in blood ion and acid–base regulation. *Am. Zool.* **24**, 241–253.

HENRY, R. P. (1987). Quaternary ammonium sulfanilamide: a membrane-impermeant carbonic anhydrase inhibitors. *Am. J. Physiol.: Reg., comp. Int. Physiol.* **252**, R959–965.

HENRY, R. P., DODGSON, S. J., FORSTER, R. E. & STOREY, B. T. (1986). Rat lung carbonic anhydrase: activity, localization, and isozymes. *J. appl. Physiol.* **60**, 638–645.

HENRY, R. P. & KORMANIK, G. A. (1985). Carbonic anhydrase activity and calcium deposition during the molt cycle of the blue crab, *Callinectes sapidus*. *J. crust. Biol.* **5**, 234–241.

HOUSTON, A. H. & MCCARTY, L. S. (1978). Carbonic anhydrase (acetazolamide-sensitive esterase) activity in the blood, gill and kidney of the thermally acclimated rainbow trout, *Salmo gairdneri*. *J. exp. Biol.* **73**, 15–27.

HOUSTON, A. H. & MEAROW, K. M. (1982). Branchial and renal (Na^+/K^+) ATPase and carbonic anhydrase activities in a eurythermal freshwater teleost, *Carassius auratus* L. *Comp. Biochem. Physiol.* **71**A, 175–180.

KIRSCHNER, L. B. (1979). Control mechanisms in crustaceans and fishes. In *Mechanisms of Osmoregulation in Animals: Maintenance of Cell Volume* (ed. R. Gilles), pp. 157–222. New York: J. Wiley & Sons.

KLOCKE, R. A. (1978). Catalysis of CO_2 reactions by lung carbonic anhydrase. *J. appl. Physiol.* **44**, 882–888.

KLOCKE, R. A. (1980). Equilibrium of CO_2 reactions in the pulmonary capillary. *J. appl. Physiol.* **48**, 972–976.

MAETZ, J. (1971). Fish gills: mechanisms of salt transfer in fresh water and sea water. *Phil. Trans. R. Soc. Ser.* B **262**, 209–249.

MAREN, T. H. (1960). A simplified micro method for the determination of carbonic anhydrase and its inhibitors. *J. Pharmac. exp. Ther.* **130**, 26–29.

MAREN, T. H. (1967*a*). Carbonic anhydrase: chemistry, physiology and inhibition. *Physiol. Rev.* **47**, 595–781.

MAREN, T. H. (1967*b*). Carbonic anhydrase in the animal kingdom. *Fedn Proc. Fedn Am. Socs exp. Biol.* **26**, 1097–1103.

MAREN, T. H. & COUTO, E. O. (1979). The nature of anion inhibition of human red cell carbonic anhydrases. *Archs Biochem. Biophys.* **196**, 501–510.

MELDRUM, N. U. & ROUGHTON, F. J. W. (1932). The CO_2 catalyst present in blood. *J. Physiol., Lond.* **75**, 15.

OBAID, A. L., CRITZ, A. M. & CRANDALL, E. D. (1979). Kinetics of bicarbonate/chloride exchange in dogfish erythrocytes. *Am. J. Physiol.* **237**, 132–138.

PERRY, S. F. (1986). Carbon dioxide excretion in fishes. *Can. J. Zool.* **64**, 565–572.

PERRY, S. F., DAVIE, P. S., DAXBOECK, C. & RANDALL, D. J. (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.

RANDALL, D. J. & DAXBOECK, C. (1984). Oxygen and carbon dioxide transfer across fish gills. In *Fish Physiology*, vol. 10 (ed. W. S. Hoar & D. J. Randall), pp. 263–314. New York: Academic Press.

SANYAL, G. (1984). Comparative carbon dioxide hydration kinetics and inhibition of carbonic anhydrase isozymes in vertebrates. *Ann. N.Y. Acad. Sci.* **429**, 165–178.

SHELTON, G., JONES, D. R. & MILSOM, W. K. (1986). Control of breathing in ectothermic vertebrates. In *Handbook of Physiology; The Respiratory System*, vol. 2, part 2 (ed. A. P. Fishman), pp. 857–909. Bethesda, MD: American Physiological Society.

SOIVIO, A., NYHOLM, K. & WESTMAN, K. (1975). A technique for repeated sampling of the blood of individual resting fish. *J. exp. Biol.* **63**, 207–218.

STEWART, P. A. (1978). Independent and dependent variables of acid–base control. *Respir. Physiol.* **33**, 9–26.

SWENSON, E. R. (1984). The respiratory aspects of carbonic anhydrase. *Ann. N.Y. Acad. Sci.* **249**, 547–560.

SWENSON, E. R. & EVANS, D. H. (1984). Inhibition of gill carbonic anhydrase blocks CO_2 excretion in the isolated perfused head of the dogfish "pup", *Squalus acanthias*. *Bull. Mt. Desert Isl. biol. Lab.* **24**, 45–49.

SWENSON, E. R., HILDESLEY, M. A. & MAREN, T. H. (1982). The renal and branchial handling of CO_2/HCO_3^- excretion: roles of branchial and erythrocytic carbonic anhydrase in *Squalus acanthias*. *Bull. Mt. Desert Isl. biol. Lab.* **24**, 72–75.

SWENSON, E. R., MAREN, T. H. & HILDESLEY, M. A. (1984). Further studies on elasmobranch CO_2 and HCO_3^- excretion: roles of branchial and erthyrocyte carbonic anhydrase in *Squalus acanthias*. *Bull. Mt Desert Isl. biol. Lab.* **24**, 72–75.

WHITNEY, P. L. & BRIGGLE, T. V. (1982). Membrane-associated carbonic anhydrase purified from bovine lung. *J. biol. Chem.* **257**, 12 056–12 059.

WOLF, K. (1963). Physiological salines for freshwater teleosts. *Progve. Fish Cult.* **25**, 135–140.

WOOD, C. M., MCDONALD, D. G. & MCMAHON, B. R. (1982). The influence of experimental anaemia on blood acid–base regulation *in vivo* in the starry flounder (*Platichthys stellatus*) and the rainbow trout (*Salmo gairdneri*). *J. exp. Biol.* **96**, 221–237.