*J. exp. Biol.* **103**, 205–223 (1983)
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# THE ROLE OF CARBONIC ANHYDRASE IN RESPIRATION, ION REGULATION AND ACID-BASE BALANCE IN THE AQUATIC CRAB *CALLINECTES SAPIDUS* AND THE TERRESTRIAL CRAB *GECARCINUS LATERALIS*

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(*Received 30 July 1981—Accepted 21 September 1982*)

SUMMARY

The enzyme carbonic anhydrase (CA), which is concentrated mainly in the osmoregulatory tissue of the gills, appears to be required for ion regulation but not for $CO_2$ excretion. An injection of the CA inhibitor acetazolamide produced an inhibition of between 90 and 100 %, which took 6 h to be fully effective, and 48–96 h to wear off. During the period of inhibition in *Callinectes sapidus* there was no change in either $O_2$ uptake or $CO_2$ excretion, nor was there any increase in blood $P_{CO_2}$. In blue crabs acclimated to 250 mosM salinity, at which the animals are ion regulators, inhibition of CA caused both $Na^+$ and $Cl^-$ concentrations in the blood to be lowered, with $Cl^-$ being lowered to a greater degree. As a result of an increase in the $Na^+ - Cl^-$ difference the animal experienced a 'metabolic' alkalosis: elevated blood pH and $HCO_3^-$ at constant $P_{CO_2}$. The data are consistent with the hypothesis that branchial CA functions in providing $H^+$ and $HCO_3^-$ as counterions for $Na^+$ and $Cl^-$ transport through the hydration of respiratory $CO_2$.

In the terrestrial *Gecarcinus lateralis*, inhibition of CA caused an increase in blood $P_{CO_2}$ but did not alter $O_2$ uptake or $CO_2$ excretion. After an initial acidosis, blood pH and $HCO_3^-$ increased and remained elevated. Blood osmolality, $Na^+$, $Cl^-$ and $Ca^{2+}$ concentrations all increased, and the animals experienced a high rate of mortality. These data suggest that CA in the land crab is also important in blood ion regulation, probably to combat desiccation.

## INTRODUCTION

The physiological functions of carbonic anhydrase in the vertebrates are well known. The erythrocyte enzyme is critical in the transport and excretion of metabolically produced $CO_2$, and carbonic anhydrase in the kidney is active in the resorption/excretion of bicarbonate ions and thus is involved in controlling the long-term acid-base status of the blood (Woodbury, 1965; Davenport, 1974).

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[redacted]y words: Carbonic anhydrase, acid-base regulation, crustaceans.

The overwhelming majority of the invertebrates which have been examined, including decapod crustaceans, however, lack red blood cells, and also lack any CA activity in the blood (Aldridge, 1977; Burnett, Woodson, Rietow & Vilicich, 1981; see also Henry & Cameron, 1982*a* for a more detailed treatment). Also the antennal gland of the crustaceans, which does not appear to play a role in blood acid-base regulation (Cameron & Batterton, 1978), does not have a high concentration of CA (Henry & Cameron, 1982*a*). Instead, the major concentration of CA is found in the gills (Aldridge, 1977; Burnett *et al.* 1981; Henry & Cameron, 1982*a*).

Crustacean gills perform a number of physiological functions. Primarily respiratory organs of $O_2$ and $CO_2$ exchange in both air- and water-breathing animals, the gills of the water breathers also function as organs of osmoregulation, ion regulation, and acid-base balance (Smith & Linton, 1971; Towle, Palmer & Harris, 1976; Mangum & Towle, 1977; Cameron, 1978*a*,*b*; Truchot, 1978, 1979). Gill carbonic anhydrase has been implicated in all of the above functions, but much of the evidence is circumstantial and some researchers have reported conflicting results.

Ever since it was first found in the gill tissue of invertebrates, carbonic anhydrase has been suggested as having an important role in $CO_2$ excretion (Ferguson, Lewis & Smith, 1937), but closer examination has revealed that the enzyme is primarily associated with the individual gills and the patches of salt transporting cells within those gills which are involved in osmo- and ion regulation (Henry & Cameron, 1982*a*). Very little enzyme activity is found in the respiratory lamellae.

Aldridge & Cameron (1979), and Cameron (1979*a*) reported no changes in either blood pH or total $CO_2$ ($C_T$) in freshwater-adapted *Callinectes sapidus* after an injection of the CA inhibitor acetazolamide (Diamox). They concluded on the basis of that experiment that the gill enzyme was not important in $CO_2$ excretion. They did not, however, confirm the assumption that the enzyme was indeed fully inhibited.

In contrast to this, Burnett *et al.* (1981) reported a significant disruption of blood pH, $C_T$ and $Cl^-$ ion concentration in the euryhaline marine crab, *Pachygrapsus crassipes*, after treatment with acetazolamide, with the most dramatic effect being on animals acclimated to salinities in which they regulated their haemolymph chloride ion concentrations significantly above those of the medium. The authors concluded that gill CA is important in both blood $Cl^-$ regulation and $CO_2$ excretion. The latter conclusion is rather tenuous and does not appear to be supported by their data as they reported an increase in blood pH, rather than the expected respiratory acidosis.

A respiratory acidosis was observed in the semi-terrestrial crab, *Cardisoma carnifex*, after an injection of acetazolamide (Randall & Wood, 1981), but $CO_2$ excretion was not significantly lowered. Also, only two animals were used in the pH and $P_{CO_2}$ determinations, and one of those died during the experiment. So despite the efforts of a number of workers, we are left without a clear picture of the physiological role of crustacean gill carbonic anhydrase. This report presents a study of gill CA function in respiration, ion regulation and acid-base balance in the aquatic crab, *Callinectes sapidus*, and the terrestrial crab, *Gecarcinus lateralis*. The two species used in this study are interesting because *C. sapidus* is an osmo- and ion conformer at high salinity (800–1000 mosM) but the animal regulates blood salts in low salinity; *G. lateralis*, which is fully terrestrial, maintains its blood hypo-osmotic and ionic to full-strength sea water (see Henry & Cameron, 1982*a*,*b* for details).

## MATERIALS AND METHODS

### *Experimental handling of animals*

*Callinectes sapidus* and *Gecarcinus lateralis* were collected and maintained as described previously (Henry & Cameron, 1982*a*). For experimentation, blue crabs were placed in large, flow-through aquaria of either 250 or 865 mosM salinity (details given by Henry & Cameron, 1982*b*). *G. lateralis* were put in individual Plexiglas chambers of approximately 400 ml volume. A layer of moist sand was placed in the bottom of each chamber and humidified air was pumped through an opening in the top. Each chamber was covered with black plastic to minimize disturbances to the animal.

Post-branchial (arterial) blood was sampled from the heart through rubber septa glued over small holes in the carapace using a 1 ml syringe and 23 ga needle (Henry & Cameron, 1982*b*). Injections of substances into the animal were performed through these septa. Prebranchial (venous) blood was sampled from the infrabranchial sinuses at the bases of the walking legs.

### In vivo *inhibition of gill CA by acetazolamide*

A 0·1 M solution of the soluble salt sodium acetazolamide was made in Cortland saline (Wolf, 1963) which had been adjusted to pH 9·0 by the addition of NaOH. The solution was then titrated down to pH 8·3 with 0·1 N-HCl without any loss of acetazolamide from precipitation. This was done in order to bring the acetazolamide solution as close to the pH of the crabs' blood (7·5–8·0) as possible while maintaining solubility of the drug.

The blood volume of the crab was estimated as roughly one third of the total fresh weight (Gleeson & Zubkoff, 1977) and the appropriate volume of inhibitor was injected to give a $10^{-4}$ M concentration in the blood.

Previous to and at various times after injection, animals were killed, the 7th gill pair in *C. sapidus* and the 9th in *G. lateralis* were dissected out, and the pH-stat assay procedure was carried out as described in detail elsewhere (Henry & Cameron, 1982*a*). Briefly, the assay involves measuring the catalysed rate of dehydration in a buffered solution of bicarbonate by holding the pH constant through the addition of HCl from an auto-titration apparatus (Radiometer-Copenhagen). The amount of titrant added per unit time is a reliable measure of the velocity of the reaction. Activity per g fresh weight of tissue and specific activity were reported as a percentage of the normal, pre-injection level in that gill pair.

### *Blood osmotic, ionic and acid-base parameters*

Pre- and/or post-branchial blood samples (~200 $\mu$l) were taken before and after an acetazolamide injection ($10^{-3}$–$10^{-5}$ M final concentration in the blood), bracketing the time period in which the gill enzyme was found to be inhibited. Blood pH and total $CO_2$ ($C_T$) were measured immediately; pH was determined on a 50 $\mu$l sample using an acid-base analyser (Radiometer-Copenhagen PHM 71) and a water-jacketed capillary pH electrode which was thermostatted to the experimental temperature (20–22 °C). $C_T$ was determined on 20 $\mu$l of blood using a conductometric method

which depends on conversion of all combined forms (bicarbonates, etc.) to dissolved $CO_2$ gas by acidification, removal in a carrier gas stream, followed by absorption in alkali and detection by differential conductivity (Maffly, 1968; J. N. Cameron, unpublished).

The remaining blood was allowed to clot in the syringe; the clot was disrupted by expulsion into a centrifuge tube and separated from plasma via centrifugation (Eppendorf Model 5412). Blood osmolality, $Na^+$ and $Cl^-$ concentrations were measured by flame photometry and Ag-titration as described in detail by Henry & Cameron (1982*b*). Calcium was determined by atomic absorption (Perkin-Elmer 303).

A second set of animals was used to obtain blood $P_{CO_2}$ values, since in *C. sapidus* the blood clotted before all measurements could be made on one sample, and *G. lateralis* were too small to allow serial sampling of large enough volumes of blood. A water-jacketed $P_{CO_2}$ electrode and acid-base analyser (Radiometer-Copenhagen PHM 71), with the scale expanded four times, were used in the determinations (Henry & Cameron, 1982*b*). Control animals were injected with Cortland saline and sampled as above.

### *Whole-animal respiratory parameters*

Oxygen uptake ($\dot{M}_{O_2}$) and carbon dioxide excretion ($\dot{M}_{CO_2}$) were measured in both species previous to and after an acetazolamide injection ($10^{-3}$ M in blood) or an injection of Cortland saline (controls). Individual blue crabs were placed in sealed Plexiglas chambers through which air-equilibrated water flowed. Oxygen uptake was determined from the difference between the inflowing and outflowing water $P_{O_2}$ multiplied by the flow rate and expressed on a fresh-weight-specific basis for each crab. No auxiliary stirring in the chamber was used. Water $P_{O_2}$ was measured using a water-jacketed $P_{O_2}$ electrode (Radiometer-Copenhagen) and acid-base analyser (PHM 71). The $P_{O_2}$ electrode was calibrated using a sodium sulphite/sodium borate zero solution and air-equilibrated water. $\dot{M}_{CO_2}$ was determined in the same fashion, with total inspired and expired $CO_2$ being determined on 150 $\mu$l of water using the same procedure described above for $C_T$ in blood.

Individuals of *G. lateralis* were placed in sealed Plexiglas chambers through which humidified air was pumped. $P_{O_2}$ and total $CO_2$ in the open chambers were measured as described above, and then the chambers were sealed with a rubber stopper. At 1 h after the chambers had been sealed, the drop in $P_{O_2}$ (and increase in total $CO_2$) was measured on air samples that were withdrawn through a rubber septum using a 22 ga needle and 1 ml syringe. At the end of the experiment the animals were weighed and $\dot{M}_{O_2}$ and $\dot{M}_{CO_2}$ were expressed on a fresh-weight-specific basis.

## RESULTS

### *Inhibition of gill carbonic anhydrase*

The time course of gill CA inhibition by a $10^{-4}$ M-blood acetazolamide concentration in both species is shown in Fig. 1. At very low levels of activity it is difficult to measure the difference between the catalysed and uncatalysed rates of dehydration accurately. The natural variation in the uncatalysed and catalysed rates under id

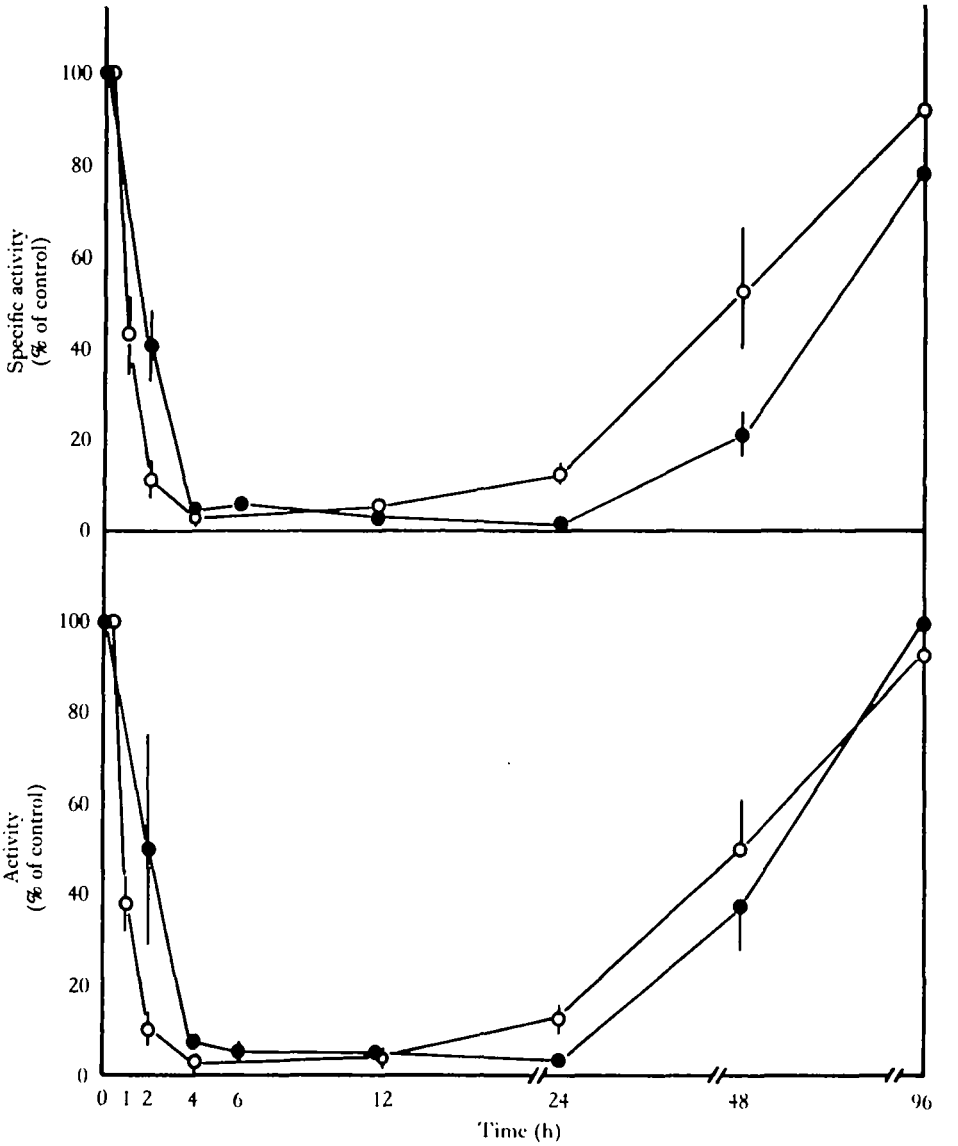


Fig. 1. The time course of inhibition of gill carbonic anhydrase activity by a $10^{-4}$ M blood concentration of acetazolamide in *C. sapidus* (open circles) and *G. lateralis* (closed circles). Mean ± S.E. n = 6. T = 22 °C.

conditions (using pure bovine CA which causes no foaming) is 2 and 5 %, respectively (Henry & Cameron, 1982*a*), and this increases when foaming occurs through the use of a crude tissue homogenate. Therefore, for the maximum degree of inhibition, 95 ± 5 % at 6–24 h, it can only be said that between 90 and 100 % of the enzyme activity was inhibited. The difference may be due to actual residual activity or it may be due to the inability of the assay to distinguish accurately between 0 and 5 % activity. A higher concentration of the drug ($10^{-3}$ M), therefore, was also used in the physiological studies.

*Blood acid-base parameters:* Callinectes *acclimated to low salinity*

In blue crabs acclimated to 250 mosM water for at least 2 weeks, there was a profound effect of CA inhibition on the blood acid-base status, which was directly related to the dose of acetazolamide given to the animal (Fig. 2). The transient depression in pH at 2 h for the lower concentrations of acetazolamide was probably related to stress brought about from handling the animals twice during that time period and not an effect of the inhibition. At 2 h the dose given did not completely inhibit the gill enzyme (Fig. 1) and blood pH was restored to near control values by 12–24 h, during which time gill CA is still inhibited by the drug. A $10^{-3}$ M concentration of the inhibitor, which according to Maren (1977) is enough to give a $10^{-4}$ M concentration intracellularly and inhibit 99·99% of the enzyme activity, caused a

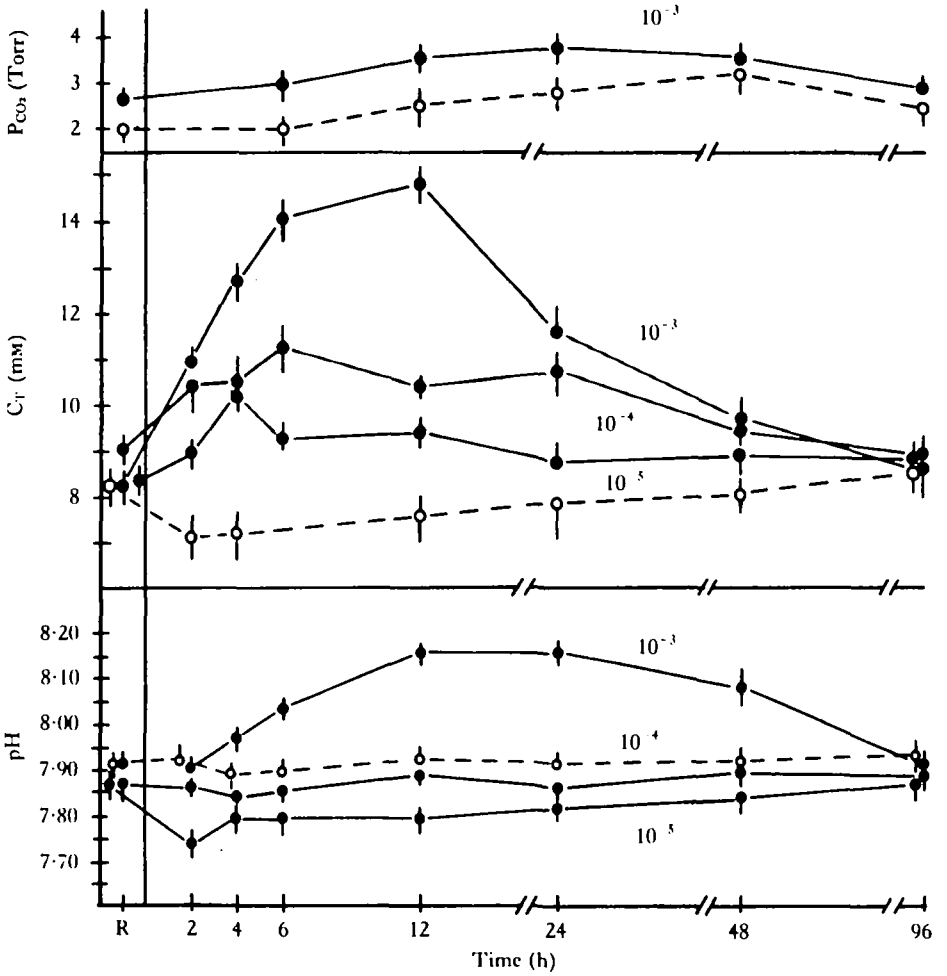


Fig. 2. Blood (prebranchial) pH, total $CO_2$ ($C_T$) and $P_{CO_2}$ in *C. sapidus* acclimated to 250 mosM prior to and after an acetazolamide injection. Open circles/dashed lines represent saline injected controls. Solid circles and lines represent injections of various concentrations of the drug; the molar concentrations are indicated on the figure. Mean ± S.E., n = 6. T = 22 °C.

gnificant elevation of pH (0·25 units) between 4 and 48 h ($P < 0{\cdot}01$, Student's $t$ test). The gradual return to the resting value of 7·90 by 96 h (Fig. 2) paralleled the time course for gill CA inhibition and recovery (Fig. 1).

All concentrations of acetazolamide used were effective in altering blood bicarbonate concentrations, but the degree of change was dose-dependent (Fig. 2). The

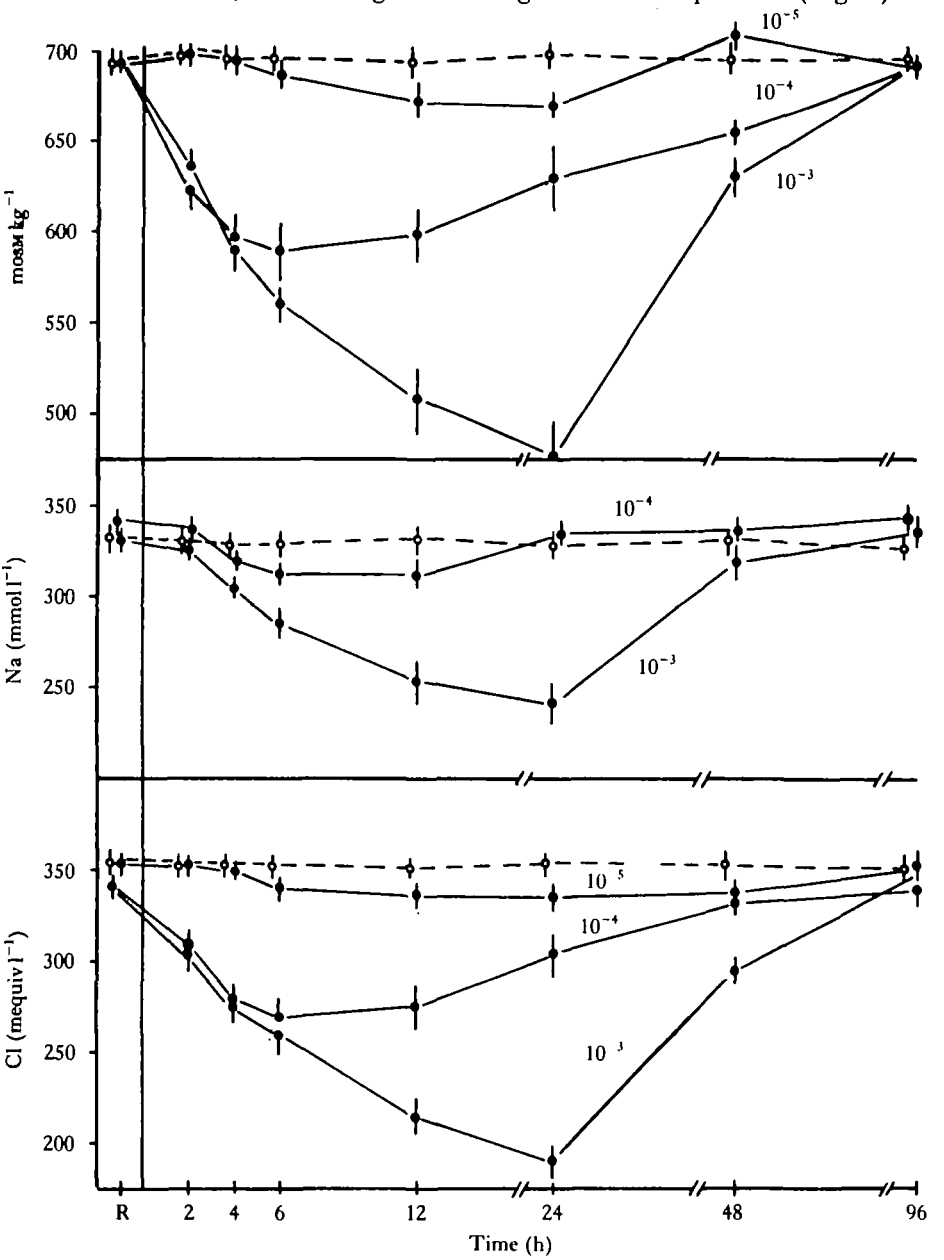


Fig. 3. Prebranchial blood osmolality, $Na^+$, and $Cl^-$ concentrations in *C. sapidus* acclimated to 250 mosm prior to and after injections of acetazolamide at various concentrations. Open circles/dashed lines represent controls. Closed circles/solid lines represent drug-treated animals, with concentrations shown in the figure. Mean ± S.E., n = 6. T = 22 °C.

lower concentrations, $10^{-5}$ and $10^{-4}$ M, caused an elevation of only about 2 mM, b[illegible] this was significant ($P < 0{\cdot}05$, $t$ test). A $10^{-3}$ M concentration of acetazolamide in the blood almost doubled the resting $HCO_3^-$ concentration, but pre-injection levels were also restored by 96 h (Fig. 2).

In contrast to pH and $C_T$, blood $P_{CO_2}$ was virtually unaffected by $10^{-3}$ M acetazolamide. $P_{CO_2}$ in control animals was elevated slightly, probably being attributable to handling stress, and the $P_{CO_2}$ in the drug-treated crabs were also raised only a small degree (1 Torr) (Fig. 2). In both cases there was no significant difference among the means ($P > 0{\cdot}05$, F test).

*Blood osmotic and ionic parameters:* Callinectes *acclimated to low salinity*

Inhibition of carbonic anhydrase clearly disrupted the blue crab's ability to maintain its blood osmolality above that of the ambient medium (250 mosM) (Fig. 3). Also, the effect appeared to be dose-dependent and more prolonged at the higher drug concentrations, with blood osmolality being depressed between about 5 and 35 %. In all cases, however, blood osmolality was restored to normal, resting values by 96 h.

The response of the two major ions in the blood, $Na^+$ and $Cl^-$, which make up over 90 % of the blood osmolality, was also dose-dependent and followed the same pattern (Fig. 3). At any particular time during the course of CA inhibition, however, blood $Cl^-$ concentrations were lowered more than those of $Na^+$ (Fig. 3). For example, for the $10^{-3}$ M-acetazolamide treatment, blood $Na^+$ was lowered by $70 \pm 9$, $100 \pm 12$, and $115 \pm 15$ mM at 6, 12 and 24 h, respectively, while blood $Cl^-$ was depressed by $81 \pm 8$, $126 \pm 12$, and $152 \pm 14$ mM respectively for those same times (mean $\pm$ S.E., $n = 6$). The differences at 12 and 24 h were significant ($P < 0{\cdot}05$, $t$ test), and corresponded to the largest changes in blood acid-base status (Figs 2, 3).

*Blood acid-base, osmotic and ionic parameters:* Callinectes *acclimated to high salinity*

In *C. sapidus* acclimated to 865 mosM, blood osmolality and ion concentrations conform to those of the ambient medium, and $10^{-3}$ M-acetazolamide had only slight effects on the blood acid-base status and blood ion concentrations (Fig. 4). There was no significant difference among mean osmolality values over the time course ($P > 0{\cdot}10$, F test). Sodium and chloride concentrations were depressed by less than 5 % of resting values, but again, the concentrations of the two ions were affected differently. At 6 and 12 h, blood $Cl^-$ was lowered $5 \pm 1$ and $20 \pm 4$ mM, while the differences for $Na^+$ were only $2 \pm 0{\cdot}5$ and $12 \pm 3$ mM.

Blood pH in the acetazolamide-injected animals did not differ from that in the controls over the initial 6 h, but it was slightly below control values at 12 and 24 h (Fig. 4). Total $CO_2$ increased significantly ($P < 0{\cdot}05$, $t$ test) by almost 3 mM over 12 h, but then returned to control values between 24 and 48 h. The perturbation in blood acid-base status occurred concomitantly with the disruption in the pre-injection $Na^+$–$Cl^-$ difference, as was the case in the low salinity acclimated animals, but the effects of an acetazolamide injection in the seawater-acclimated crabs were slight compared to those in blue crabs acclimated to low salinity (Figs 2, 3, 4).

*Blood acid-base parameters:* Gecarcinus

A more typical respiratory acidosis took place in *G. lateralis* during the ini[illegible]

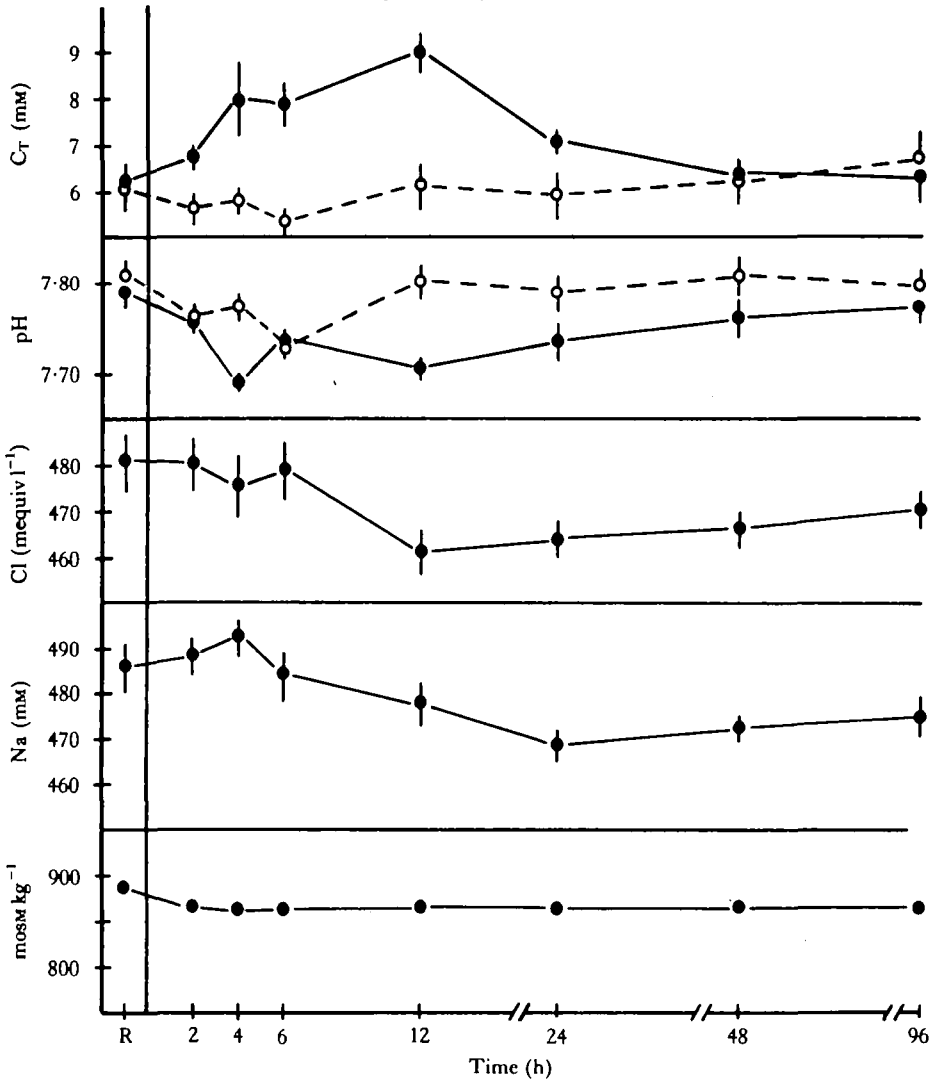


Fig. 4. Prebranchial blood acid-base and osmotic parameters for *C. sapidus* acclimated to 865 mosM prior to and after injections of acetazolamide ($10^{-3}$ M in blood). Open circles/dashed lines represent controls. Mean ± S.E., n = 6. T = 22 °C. Where error bars are not shown, the S.E. was smaller than the circle used.

period after a $10^{-4}$ or $10^{-3}$ M-acetazolamide injection, with blood $P_{CO_2}$ increasing 2–7 Torr, respectively, over the first 6 h (Fig. 5). Blood pH fell approximately 0·1 units and $C_T$ rose about 3 mM over the first 3 h. By 12 h, $P_{CO_2}$ dropped about 2 Torr, but $C_T$ had increased another 2 mM and blood pH was restored to resting values regardless of drug dosage (Fig. 5). By 96 h, $C_T$ had more than doubled, and blood pH also increased to greater than resting values despite the fact that $P_{CO_2}$ also remained elevated.

Mortality was a factor in this series of experiments, since with $10^{-4}$ or $10^{-3}$ M-acetazolamide 40 % of the crabs had died by 96 h, and 100 % by 7 days. Those animals

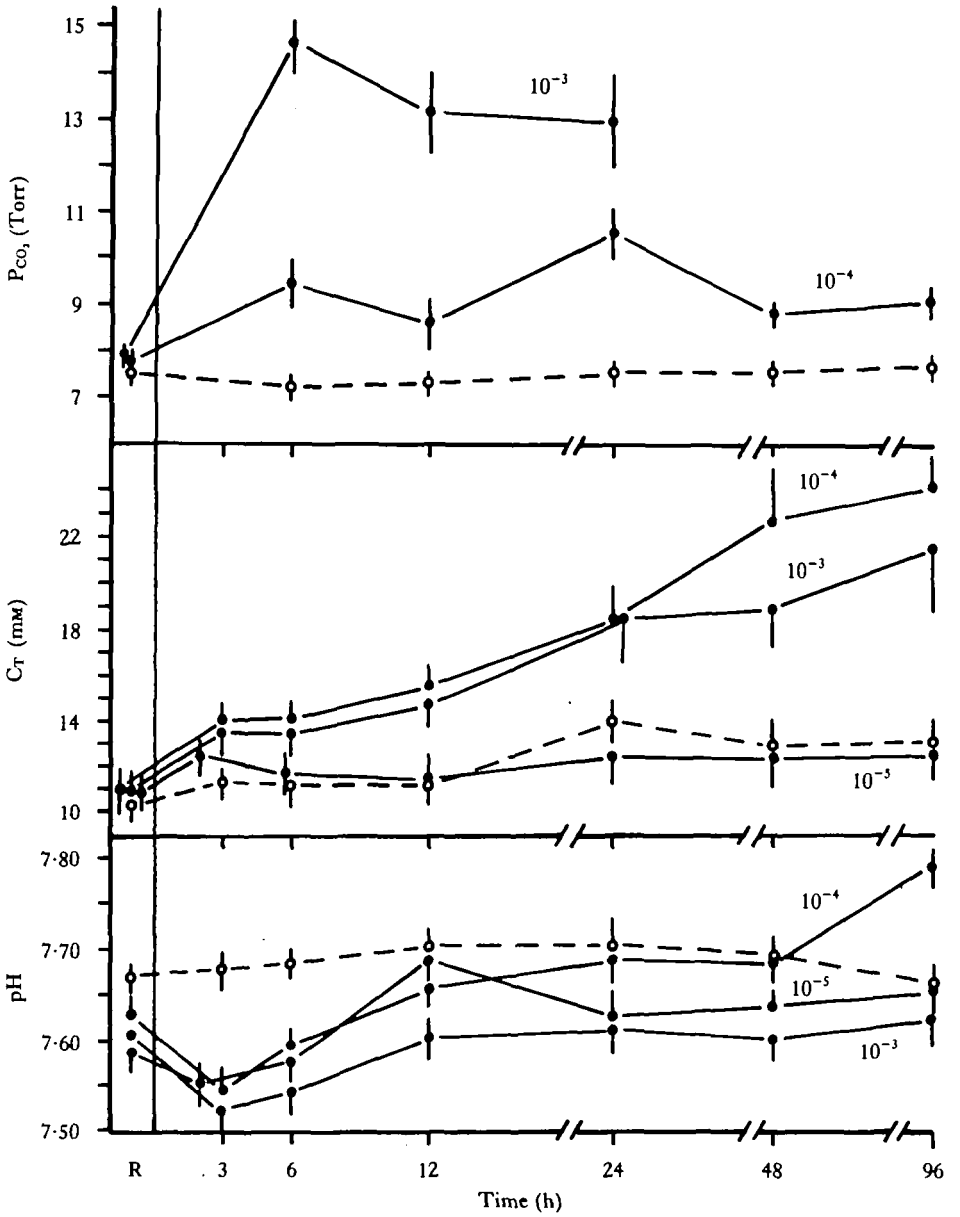


Fig. 5. Postbranchial blood acid-base parameters of *G. lateralis* previous to and following an injection of acetazolamide. Open circles/dashed lines represent saline-injected controls. Solid circles and lines represent acetazolamide-injected animals, with concentrations being shown on the figure. Mean ± S.E., n = 6. T = 22 °C.

used in the $P_{CO_2}$ determination for $10^{-3}$ M-acetazolamide all died by 48 h. The high rate of mortality was not seen in the animals treated with $10^{-5}$ M acetazolamide, but neither were the effects on pH and $C_T$.

*Blood osmotic and ionic parameters:* Gecarcinus

The effect of $10^{-4}$ M-acetazolamide on blood $Na^+$, $Cl^-$ and $Ca^{2+}$ was to increa

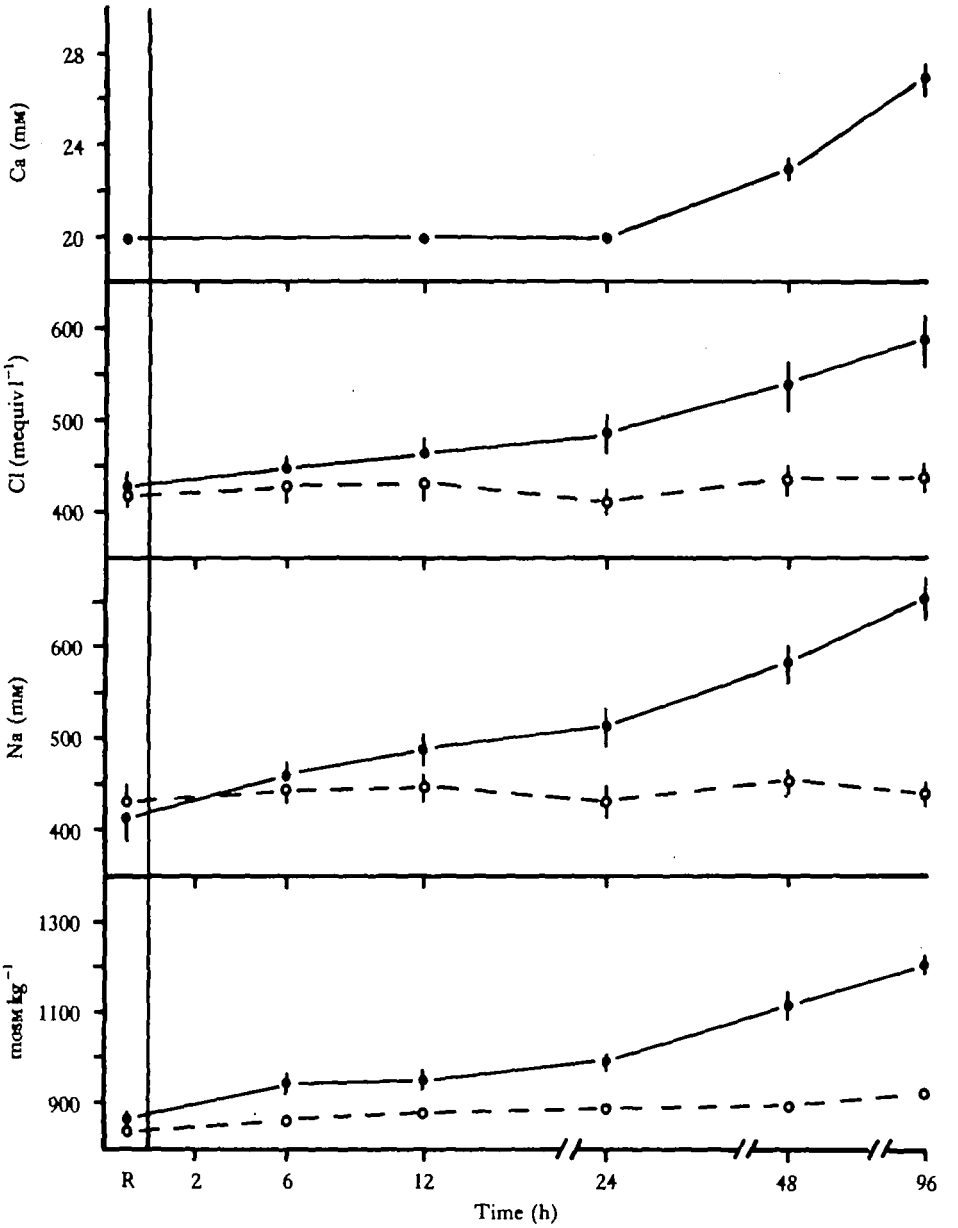


Fig. 6. Prebranchial blood osmolarity, $Na^+$, $Cl^-$ and $Ca^{2+}$ concentrations in *G. lateralis* prior to and following an injection of acetazolamide ($10^{-4}$ M in blood). Open circles/dashed lines represent controls. Mean ± S.E., n = 6. T = 22 °C. Where no error bars are shown, the S.E. was smaller than the circles used to show the point.

their concentrations and therefore also increase blood osmolality (Fig. 6). The increase in blood $Na^+$ from 515 ± 17 to 655 ± 14 mM was greater than that for $Cl^-$ (490 ± 12 to 585 ± 18 mM) between 24 and 96 h, while blood $Ca^{2+}$ rose from 20 ± 0·5 to 27 ± 0·8 mM (mean ± S.E. n = 6). The increase in the blood concentrations of the major ionic species in *G. lateralis* corresponded to the increase in the $HCO_3^-$ concentration and the

rise in pH (Fig. 5). Both the ionic and acid-base parameters failed to return to resti[illegible] values by 96 h, and the high rate of mortality made it impossible to follow the experiment beyond that time.

### *Whole-animal respiration*

For blue crabs acclimated to 250 mosM, the inhibition of CA by $10^{-4}$ M acetazolamide had no effect on either oxygen uptake ($\dot{M}_{O_2}$) or carbon dioxide excretion ($\dot{M}_{CO_2}$). Neither an injection of Cortland saline nor acetazolamide caused any significant change in the resting values of $\dot{M}_{O_2}$ or $\dot{M}_{CO_2}$ ($P > 0{\cdot}05$, $t$ test; Table 1). The values for RQ were high, being around 2 (Table 1), but they are typical of aquatic crustaceans (Bosworth, O'Brien & Amberson, 1940). The RQ did not change significantly over the time course of the experiment ($P > 0{\cdot}05$ $t$ test), with the exception of one high value of 2·62 at 48 h.

A similar pattern was seen in *G. lateralis*. An injection of $10^{-4}$ M-acetazolamide did not significantly alter $\dot{M}_{O_2}$, and also did not significantly lower $\dot{M}_{CO_2}$ ($P > 0{\cdot}05$, $t$ test; Table 1). RQ values of slightly less than 1 were typical of air-breathing animals in general, and with the exception of one high value at 24 h, they did not vary significantly throughout the experiment ($P > 0{\cdot}05$, $t$ test; Table 1).

## DISCUSSION

### *Inhibition of gill CA by acetazolamide*

There is a lag of about 4 h between the time of an injection of acetazolamide and the time of 95 % inhibition of the gill enzyme (Fig. 1), which is probably due to the time needed for the drug to cross the basal membrane of the gill and effect inhibition. The delay in action of acetazolamide has been until now undocumented. In humans an injection of acetazolamide takes only minutes to block the activity of the red cell enzyme (Maren, 1967). This difference probably explains the negative results reported in previous studies of the effects of acetazolamide. Cameron (1979*a*) found no change in blood pH, $C_T$ and $Cl^-$ ion flux in *C. sapidus* after a Diamox injection, but he only waited 1–2 h before taking the measurements. Similarly, Aldridge & Cameron (1979) reported no change in blood pH or $C_T$ at 3 and 6 h after injection; in neither study was CA activity monitored, thus making it impossible to confirm whether or not there was a significant degree of inhibition. In contrast, after 12 h of exposure to acetazolamide Burnett *et al.* (1981) did see an effect on pH, $C_T$ and $Cl^-$ ion concentration in the blood of *P. crassipes*.

The drug is also cleared rather slowly from the two decapod species, compared to mammals. The inhibition does not begin to wear off until between 24 and 48 h in the crabs, whereas the half time of the drug in rabbit plasma is only 100 min (Maren, 1967), with over 90 % being recovered in the animal's urine. The mechanism of clearance was not investigated, but it probably involves diffusive loss, excretion, and metabolic breakdown.

For the blue crab the correlation between the time courses of CA inhibition (Fig. 1) and those for the changes in blood pH, $C_T$, $Na^+$ and $Cl^-$ concentrations (Figs 2, 3) is very good, showing that when the enzyme is indeed inhibited there are speci[illegible]

Table 1. *Oxygen uptake ($\dot{M}O_2$) and carbon dioxide production ($\dot{M}CO_2$) in* C. sapidus *and* G. lateralis *previous to and following an injection of acetazolamide ($10^{-4}$ M in blood) or Cortland saline (controls)*

Mean ± S.E., n = 6–8. T = 22–25 °C.

| Sample: h after injection | $\dot{M}CO_2$ ($mlCO_2\,gm^{-1}\,h^{-1}$) Control | $\dot{M}CO_2$ ($mlCO_2\,gm^{-1}\,h^{-1}$) Acetazolamide | *G. lateralis* $\dot{M}O_2$ ($mlO_2\,gm^{-1}\,h^{-1}$) Control | $\dot{M}O_2$ ($mlO_2\,gm^{-1}\,h^{-1}$) Acetazolamide | RQ Control | RQ Acetazolamide |
|---|---|---|---|---|---|---|
| R | 0·0206 ± 0·002 | 0·0226 ± 0.002 | 0·0284 ± 0·002 | 0·0269 ± 0·002 | 0·73 | 0·84 |
| 6 | 0·0187 ± 0·001 | 0·0225 ± 0·002 | 0·0240 ± 0·002 | 0·0288 ± 0·001 | 0·78 | 0·78 |
| 12 | 0·0214 ± 0.002 | 0·0231 ± 0·002 | 0·0271 ± 0·002 | 0·0274 ± 0·002 | 0·79 | 0·84 |
| 24 | 0·0182 ± 0·002 | 0·0317 ± 0·003 | 0·0248 ± 0·002 | 0·0296 ± 0·002 | 0·73 | 1·07 |
| 48 | 0·0197 ± 0·002 | 0·0217 ± 0·002 | 0·0252 ± 0·002 | 0·0288 ± 0·001 | 0·78 | 0·75 |
| 96 | 0·0191 ± 0·004 | 0·0234 ± 0·002 | 0·0262 ± 0·003 | 0·0279 ± 0·002 | 0·73 | 0·84 |
| | ($mlCO_2\,kg^{-1}\,min^{-1}$) | | *C. sapidus* ($mlO_2\,kg^{-1}\,min^{-1}$) | | | |
| R | 1·91 ± 0·19 | 1·86 ± 0·22 | 0·88 ± 0·14 | 0·96 ± 0·12 | 2·17 | 1·94 |
| 6 | 1·80 ± 0·24 | 1·75 ± 0·17 | 0·91 ± 0·11 | 0·96 ± 0·14 | 1·98 | 1·82 |
| 12 | 1·94 ± 0·22 | 2·03 ± 0·22 | 0·81 ± 0·16 | 0·97 ± 0·12 | 2·40 | 2·09 |
| 24 | 2·02 ± 0·28 | 1·92 ± 0·19 | 0·86 ± 0·12 | 0·83 ± 0·06 | 2·35 | 2·31 |
| 48 | 1·88 ± 0·18 | 1·89 ± 0·06 | 0·92 ± 0·15 | 0·72 ± 0·08 | 2·04 | 2·62 |
| 96 | 1·97 ± 0·20 | 1·84 ± 0·17 | 0·84 ± 0·10 | 0·86 ± 0·14 | 2·35 | 2·14 |

effects. This emphasizes the importance of monitoring enzyme activity in order to ensure that the inhibitor is effective. It should be mentioned that caution should be used in comparing enzymological data obtained *in vitro* with *in vivo* blood measurements, but in this case the two appear to be compatible.

That a dose-dependent response of the physiological parameters to the drug was seen indicates that not all of the enzyme is inhibited by a $10^{-5}$ or $10^{-4}$ M concentration in blood (Figs 2, 3, 4). It is possible that in *C. sapidus*, regardless of the amount of drug used, some of the branchial enzyme (5–10 %, Fig. 1) remained functional. That would explain why, although blood ion concentrations were severely depressed, they always remained above those in the ambient medium. Carbonic anhydrase is one of the most efficient enzymes known and, at least in the vertebrates, as little as 0·1 % of normal activity is enough to maintain certain physiological functions (i.e., $CO_2$ excretion) (Maren, 1967). This may also be the case in the blue crab because, although ion- and osmoregulatory functions were severely cut back, they were never completely eliminated.

### *$CO_2$ excretion in* C. sapidus

The inhibition of branchial CA by acetazolamide had no significant effect on either blood $P_{CO_2}$ (Fig. 2) or whole-animal $CO_2$ excretion (Table 1). These results contradict the suggestion of Woodson, Burnett, Cullinan & Alberico (1980) and Burnett *et al.* (1981) that gill CA is important in $CO_2$ excretion in another aquatic crab, *P. crassipes*. The rise in blood pH is also opposite from what one would expect (i.e., a respiratory acidosis) if an inhibition of CA were retarding $CO_2$ excretion (Fig. 2).

Interestingly, Burnett *et al.* (1981) also saw a rise in pH along with elevated total $CO_2$ ($C_T$) and lowered blood $Cl^-$, as is the case in the blue crab. Without the data for $P_{CO_2}$ and $CO_2$ excretion, however, they interpreted the rise in $C_T$ ($= HCO_3^-$ at physiological pH) as resulting from the inability of the animal to excrete its $CO_2$. The same results can just as easily occur in a metabolic alkalosis (see below).

If CA is not required for $CO_2$ excretion, then the process must be a passive one, depending on the diffusion of $CO_2$ from blood to water along its $P_{CO_2}$ gradient. This is possible even though blood $P_{CO_2}$ is relatively low in aquatic crabs (2–3 Torr) because of the high solubility of $CO_2$ in water (Dejours, 1975). It has also been suggested that a $P_{CO_2}$ disequilibrium in the blood, resulting from the absence of blood CA, could increase the $P_{CO_2}$ gradient at the site of excretion, the gills (Cameron, 1979*b*; R. P. Henry & J. N. Cameron, unpublished observations).

Also, it is unlikely that blood $HCO_3^-$ is the major species of $CO_2$ which is excreted, as is the case in the vertebrates, since the basal membrane of the gill is generally considered to be impermeable to $HCO_3^-$, and in the hypothesis of Randall & Wood (1981), the transport of $HCO_3^-$ from blood into the gill is the critical step in the process. Crustacean blood contains no CA activity (Henry & Cameron, 1982*a*) thus limiting the conversion of plasma $HCO_3^-$ to $CO_2$ to the slow, uncatalysed rate which is much slower than the transit time of blood in the gill (Cameron, 1979*b*). It is possible that membrane-bound CA exposed to plasma would facilitate $CO_2$ excretion via the catalysed dehydration of plasma $HCO_3^-$ to $CO_2$, but this is unlikely since $Cl^-$ is a strong competitive inhibitor of CA (Maren, 1967; Henry & Cameron, 1982*a*), and blue crab blood has 250 mM-$Cl^-$ vs 10 mM-$HCO_3^-$.

The intracellular location of the enzyme also presents problems in $CO_2$ excretion. Gutknecht, Bisson & Tosteson (1977), using an *in vitro* system, have shown that $HCO_3^-$ diffusion across a membrane is limited by the diffusion of $HCO_3^-$ through the unstirred layer at the surface of the membrane, a process which is very slow. CA on the blood side of the membrane (the typical vertebrate condition) would facilitate $HCO_3^-$ diffusion by catalysing the dehydration reaction, thus keeping a high $P_{CO_2}$ in the unstirred layer; diffusion of $HCO_3^-$ would actually take place as $CO_2$. CA on the intracellular side of the membrane (the invertebrate condition) would have no effect on $HCO_3^-$ diffusion from the blood.

### *Acid-base balance and ion regulation in* C. sapidus

The acid-base disturbance that is caused by the inhibition of branchial CA by acetazolamide is typical of a metabolic alkalosis characterized by elevated blood pH and $HCO_3^-$ at constant $P_{CO_2}$ (Fig. 2). At the same time that acid-base status is disrupted, blood $Na^+$ and $Cl^-$ concentrations are significantly lowered (Fig. 3); $Cl^-$ is lowered proportionally more than $Na^+$ in every case, with the greatest difference between the concentrations of these two ions occurring together with the largest increase in pH and $HCO_3^-$. It appears that acid-base balance in the low salinity acclimated blue crab is linked to the ion regulatory process and, more specifically, to the difference in $Na^+$–$Cl^-$.

There is a large body of evidence supporting the hypothesis that $Na^+$ and $Cl^-$ regulation in both fish and crabs is accomplished by $Na^+/H^+$ (or $NH_4^+$) and $Cl^-/OH^-$ (or $HCO_3^-$) exchanges (see reviews by Kirschner, 1979; Heisler, 1980). The exchanges have been considered to be independent of each other (Garcia Romieu & Maetz, 1964), and the relative rates of $Na^+/H^+$ and $Cl^-/HCO_3^-$ exchanges which control the concentrations of the two major ions in the blood have been shown to be sensitive to blood acid-base disturbances (Truchot, 1975, 1979; Cameron, 1976, 1978*b*).

The inhibition of CA by acetazolamide has also been shown to depress both $Na^+$ and $Cl^-$ uptake in various animals (Payan & Maetz, 1973; Dietz, 1974; Garcia Romieu & Ehrenfeld, 1975; Alvarado, Dietz & Mullen, 1975), so it is not unreasonable to suggest that the depression in blood $Na^+$ and $Cl^-$ concentrations by acetazolamide in *C. sapidus* is a result of the inhibition of the $Na^+/H^+$ and $Cl^-/HCO_3^-$ exchanges. Payan & Maetz (1973) reported that, although $Na^+$ uptake was depressed by acetazolamide, $Na^+$ efflux remained unchanged. With only the uptake mechanisms impaired by the drug, it appears that the animal 'leaks' ions into the medium thus reducing their concentrations in the blood. Cameron (1978*a*) has shown that passive $Cl^-$ efflux in the blue crab is about 30 % greater than that for $Na^+$, so if the uptake of both ions is affected equally by CA inhibition the higher rate of passive $Cl^-$ efflux could explain why blood $Cl^-$ was lowered more than $Na^+$.

Regardless of the cause, the effect of acetazolamide is to increase the $Na^+$–$Cl^-$ difference in the blood. This may result in an increase in the strong ion difference (SID) in blood (Stewart, 1978). The observed change in the $Na^+$–$Cl^-$ difference is in the direction that would be expected to produce an alkalosis resulting from an increase in $HCO_3^-$ concentrations which would offset the relative increase in positive charge. The largest increase in blood $HCO_3^-$ occurs at the same time as the largest change in the $Na^+$–$Cl^-$ difference, but only makes up about 25 % of the change. The

complete SID was not measured; in fact, to do so is impossible. Since other strong ions were not measured, the change in the $Na^+$–$Cl^-$ difference may have overestimated the change in the total SID, and the rise in $HCO_3^-$ may have actually accounted for more of the charge difference. Presumably changes in other strong ions ($Ca^{2+}$, or $K^+$, e.g.), total weak acid (mostly protein), or ion activity account for the rest of the apparent charge difference.

The same general patterns of blood ion and acid-base disturbance in response to $10^{-3}$ M-acetazolamide are seen in the high salinity acclimated blue crab, but to a much lesser degree. At that salinity (865 mosM) *C. sapidus* is an osmoconformer, its blood ion concentrations, for the most part, passively reflecting those in the ambient medium. The very small change in the $Na^+$–$Cl^-$ difference (~8 mM) and the equally small rise in $HCO_3^-$ (~3 mM) most probably represent baseline levels of the active ion exchange processes which, although not needed for ion regulation, are used in controlling the acid-base status of the blood. Such a mechanism would be employed to restore normal acid-base status of the blood following a disturbance such as an acidosis resulting from strenuous exercise (e.g., by swimming) (Cameron, 1978*b*).

### *The role of branchial carbonic anhydrase*

It is unlikely that branchial CA plays a direct role in ion uptake in the blue crab, since the actual transport process is believed to be handled by membrane-bound $Na^+/K^+$ ($NH_4^+$) and $Cl^-/HCO_3^-$ ATPases (Kerstetter & Kirschner, 1974; Towle *et al.* 1976; Magnum & Towle, 1977; DePew & Towle, 1979). $Na^+$ and $Cl^-$ transport are considered to be independent of each other (reviewed by Kirschner, 1979) but both are affected by the CA inhibitor acetazolamide. The most plausible way for CA to play a role in ion regulation without being directly responsible for ion transport is for it to be involved in providing the counter-ions ($HCO_3^-$ and $H^+$) for the exchanges via the intracellular hydration of respiratory $CO_2$ (Fig. 7), an hypothesis, that is similar to the model of Kirschner (1979) for fish. This model shows the proposed mechanisms of $Na^+$ and $Cl^-$ uptake and movement into the blood and the involvement of branchial CA (for a detailed discussion of the transport mechanisms see Kirschner, 1979). $CO_2$ is shown as diffusing from blood to water down its $P_{CO_2}$ gradient as discussed above.

Since branchial CA is involved in supplying counter ions for the ion transporting enzymes which maintain $Na^+$ and $Cl^-$ concentrations in the blood, and since a change in the concentrations of these ions appears to change the SID and thus to alter the acid-base status of the blood, gill CA is also involved in blood acid-base regulation, although in an indirect manner. It appears, then, that the role of branchial CA is primarily non-respiratory, in the sense that the enzyme has little or no function in the respiratory process of $CO_2$ excretion.

### *Respiration and acid-base balance in* G. lateralis

The role of branchial CA in the terrestrial crab, *G. lateralis*, is less clear. Although there is a clear respiratory acidosis during the early stages of inhibition of the gill enzyme (Fig. 5), there is no significant lowering of $CO_2$ excretion (Table 1). $\dot{M}_{CO_2}$ is actually elevated at 24 h after an injection of the inhibitor. This is the same pattern as seen in the semi-terrestrial crab, *Cardisoma carnifex* by Randall & Wood (1981). Thus, although inhibition of CA causes an increase in $P_{CO_2}$, the actual process

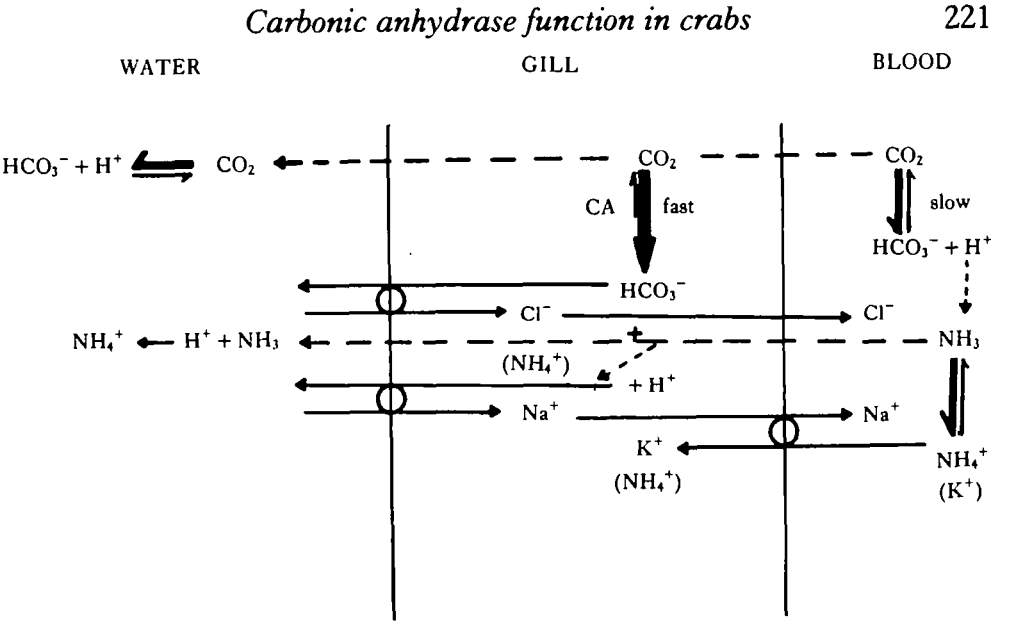


Fig. 7. A model of branchial CA function in the osmoregulating blue crab. The dashed lines represent movement by diffusion; solid lines represent some form of coupled transport. Large arrows show predominant direction of reactions. See text for detailed explanations.

$CO_2$ excretion appears to be occurring via diffusion down the $P_{CO_2}$ gradient from blood to air. The increase in blood $P_{CO_2}$ at 24 h might explain the increased $CO_2$ excretion value at that time, but this is highly speculative since $P_{CO_2}$ was also elevated at 6 and 12 h while $M_{CO_2}$ was not. The elevation of blood $P_{CO_2}$ may indicate a respiratory function for the branchial enzyme, but its activity does not seem to be critical to normal $CO_2$ excretion.

The apparent respiratory acidosis was virtually fully compensated after 12 h by a rise in blood $HCO_3^-$ (Fig. 5), but $HCO_3^-$ concentrations continued to rise through the duration of the experiment. By 96 h $HCO_3^-$ concentrations were almost three times resting values, and blood pH was also elevated for the $10^{-4}$ M-acetazolamide injection.

Concomitant with the rise in blood $HCO_3^-$ was an elevation in blood $Na^+$, $Cl^-$ and $Ca^{2+}$ concentrations (Fig. 6). The steepest rise, 24–96 h, coincided with the onset of mortality in these animals. $Na^+$ increased to a greater degree than did $Cl^-$, thus altering the resting $Na^+$–$Cl^-$ difference and presumably also the SID. The continued increase in blood $HCO_3^-$ may also involve the increased $Na^+$–$Cl^-$ difference, but even so, it did not entirely make up the charge difference.

The increase in the concentrations of the blood ions indicates that branchial CA is involved in ion regulation, in this case probably to combat desiccation. Without the functioning enzyme the crab appears to dry out: blood osmolality increases by over 300 mosM and the animal dies. If this is so, then the direction of ion transport in *G. lateralis* is outward, the opposite direction to that in *C. sapidus*. Very little is known about the mechanism of outward ion transport (reviewed by Kirschner, 1979), and there is not enough information either to propose a mechanism or to propose a role branchial CA in the process.

The increase in blood $Ca^{2+}$, which parallels that of $HCO_3^-$, is indicative of yet third potential role of Ca in the terrestrial crab. Integument CA in crabs is believed of function in laying down and calcifying the exoskeleton (Giraud, 1981), and with the enzyme inhibited, exoskeletal $CaCO_3$ could dissolve, elevating blood $Ca^{2+}$ and $HCO_3^-$ concentrations. This is plausible since the exoskeleton has been suggested to be an important source of blood buffers in crabs (deFur, Wilkes & McMahon, 1980; Henry, Kormanik, Smatresk & Cameron, 1981).

The function of CA in the terrestrial *G. lateralis* appears to be complex, in the respect that there is evidence that the gill enzyme is involved in the regulation of both blood $P_{CO_2}$ and ion concentrations and that CA in the integument may be needed for $CaCO_3$ deposition in the shell. An injection of acetazolamide inhibits CA throughout the entire animal, and the responses of these three areas are superimposed upon each other. More research is needed to clarify the function of the gill enzyme and to separate the responses of CA in other tissues in this species.

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