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CRAB GILL INTRA-EPITHELIAL CARBONIC ANHYDRASE PLAYS A MAJOR ROLE IN HAEMOLYMPH CO_2 AND CHLORIDE ION REGULATION

BY LOUIS E. BURNETT, PAUL B. J. WOODSON, MARTHA G. RIETOW* AND VICTORIA C. VILICICH

Department of Biology, University of San Diego, Alcala Park, San Diego, California 92110, *U.S.A.*

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SUMMARY

The distribution and function of the enzyme carbonic anhydrase in the crab *Pachygrapsus crassipes* was investigated. Carbonic anhydrase was found in gill epithelial tissue but not in the haemolymph, muscle, heart, hepatopancreas or gonads of male crabs.

Enzyme activity was completely inhibited *in vitro* by 2×10^{-4}M sodium acetazolamide (Diamox). Radiolabelled Diamox (2×10^{-4}M) in sea water was found to equilibrate with the intact crab's haemolymph within one hour.

Haemolymph CO_2 content, pH and chloride ion concentration were measured in crabs acclimated to different salinities and exposed to 2×10^{-4}M Diamox. Haemolymph CO_2 content increased at all salinities, especially low salinities, while pH remained unchanged, except at low salinities where it increased. Diamox impaired the regulation of haemolymph chloride at low salinities, but had no effect on chloride regulation at high salinities. Measurement of O_2 uptake (estimating CO_2 production) in crabs before and after Diamox exposure confirmed that elevated haemolymph CO_2 was not due to increases in tissue CO_2 production.

INTRODUCTION

Considerable evidence points to an important role for gill intra-epithelial carbonic anhydrase in CO_2 excretion by fish (cf. recent reviews by Cameron, 1979 and by Haswell, Randall & Perry, 1980). There is also evidence that lung intra-epithelial carbonic anhydrase may be important in mammalian CO_2 excretion (Klocke, 1977; Effros, Chang & Silverman, 1978; Crandall & O'Brasky, 1978). By way of contrast, Aldridge & Cameron (1979) report that in *Callinectes sapidus*, a crab which is known to possess gill intra-epithelial carbonic anhydrase (Aldridge, 1977), this enzyme is unimportant to CO_2 excretion. Therefore, we decided to investigate the role of gill intra-epithelial carbonic anhydrase in another crab, *Pachygrapsus crassipes* (Randall).

In water-breathing species, at least part of the CO_2 excreted (presumably HCO_3^-)

* Present address: Department of Physiology and Pharmacology, University of California, San Diego, La Jolla, California 92037.

is thought to be implicated in the movement of chloride ions between the animal and its environment (Cameron, 1979; Haswell *et al.*, 1980). Since mammalian erythrocyte carbonic anhydrase plays a major role in mobilizing intracellular CO_2 to HCO_3^-, which in turn bidirectionally exchanges with plasma chloride (Roughton, 1964), we were interested in establishing the relation between haemolymph chloride ion regulation and gill carbonic anhydrase function in a crab which regulated haemolymph chloride concentration in both hypo- and hypersaline environments. Therefore, the effects of carbonic anhydrase inhibition by sodium acetazolamide (generic name 'Diamox') were studied over a range of external salinities where we had established that the animal could regulate its haemolymph chlorinity. The effects of carbonic anhydrase inhibition on chloride regulation, haemolymph CO_2 content and haemolymph pH were investigated in crabs at different salinities.

MATERIALS AND METHODS

Pachygrapsus crassipes were collected along the rock jetties of Mission Bay, San Diego, California, and held in ten-gallon glass aquariums thermostated to 23 ± 1 °C. Only male crabs weighing between 10 and 35 g were used. Crabs were fed frozen smelt three times each week, but were starved for 24 h before experimentation.

Carbonic anhydrase assay

Carbonic anhydrase activity (c.a.) was determined by following the rate of appearance of protons as the following reaction sequence proceeded to the right :

$$CO_2 + H_2O \underset{\text{c.a.}}{\overset{\text{c.a.}}{\rightleftharpoons}} H_2CO_3 \rightleftharpoons H^+ + HCO_3^-.$$

Since the uncatalysed hydration of CO_2 is quite rapid at room temperature, the reaction is most easily followed at 0 °C and in the presence of considerable proton buffer capacity (Maren, 1967). Under these conditions, the pH of a reaction mixture falls at experimentally practicable rates after addition of a saturating amount of substrate (CO_2). Any acceleration of this uncatalysed rate following addition of a sample may be attributed to the presence of a catalyst in the sample. We assume that if such catalytic activity can be abolished by addition of sodium acetazolamide (Diamox), a specific inhibitor of all carbonic anhydrases so far studied (Maren, 1967), then the catalyst in the sample is carbonic anhydrase.

Our assay buffer was 20 mM barbital (Sigma), adjusted with H_2SO_4 or NaOH to pH 8·31 at 25 °C. One hundred (100) μl of either sample (prepared as indicated below), or assay buffer (for determining the uncatalysed rate) was added to 25 ml of assay buffer stirred in a reaction vessel thermostated to 0 ± 0·1 °C. When the temperature and pH of the reaction mixture were steady, 5 ml of 0 °C distilled water, saturated with CO_2 at atmospheric pressure, was rapidly injected into the reaction vessel and the drop in pH subsequent to the reaction described above was followed using an Orion Research 801 pH meter with a Sargent-Welch S-30070-10 combination pH electrode. The output of the pH meter was recorded on a Brush-Gould

220 pen recorder. The reaction rate was calculated from the time required for the pH to fall one unit. Data are expressed as:

$$\frac{\left(\frac{\text{catalysed rate}}{\text{uncatalysed rate}}\right) - 1{\cdot}0}{\text{mg protein in sample}} = \text{mg}^{-1}.$$

Protein concentrations were determined using the Bio-Rad Protein Assay Kit (Bio-Rad), an adaptation based on Bradford (1976). Diamox (Lederle), was added to 25 ml of assay buffer in the reaction vessel at a concentration which provided the desired final concentration after addition of substrate. In inhibition experiments, tissue samples were allowed to equilibrate with the Diamox for 15 min before addition of substrate.

Tissue preparation for enzyme assays

Crabs acclimated to 34‰ sea water (530 mM chloride) for at least two days were killed quickly and various tissues were dissected from them. Prior to killing, haemolymph samples were taken as described below. Solid tissues were washed with cold assay buffer. All samples were then placed in 20 vol ice-cold assay buffer and disrupted using either ground glass homogenizers or sonication. In no case were the assay results different using these two types of disruption. Homogenates were then centrifuged at 100000*g* for one hour at 4 °C. The supernatants were decanted for assay and the pellets were resuspended by vortexing in 20 vol ice-cold assay buffer which was 1% v/v in Triton X-100 (Sigma; a non-ionic detergent). The suspension was centrifuged as before and the supernatant (presumably containing any enzyme which was tightly lipid-associated) decanted for assay. The pellet was resuspended in ice-cold assay buffer and kept for assays. Triplicate 100 μl samples of the supernatants and the pellet suspension were assayed as described above.

Penetration of (^3H)*-Diamox* in vivo

To achieve a specific circulating concentration of Diamox in crab haemolymph by injection, the haemolymph volume must be accurately known. Since the haemolymph volume of *P. crassipes* is unknown, the possibility of Diamox penetrating crab tissues from the sea water was investigated using tritium-labelled Diamox.

Tritium-labelled Diamox was prepared according to the method of Roblin & Clapp (1950) using ^{3}H-acetic anhydride (New England Nuclear). Our product melted from 260 to 261 °C (reported: 258–259 °C; Roblin & Clapp, 1950). The physiological activity of this product was verified by showing that it was equipotent with commercial Diamox (Lederle) as an inhibitor of carbonic anhydrase (from *P. crassipes* gills) in our assay procedure. Assuming that it was 100% Diamox, the specific activity of our product was 19×10^9 cpm/mole. Radio-activities were determined by liquid scintillation counting in Aquasol-2 (New England Nuclear). All samples were counted until a statistical sampling error of 3% was achieved.

Haemolymph pH, CO_2 content and chloride measurements

The function of gill carbonic anhydrase was investigated by inhibiting the gill carbonic anhydrase *in vivo* with Diamox and measuring the changes in haemolymph

pH, CO_2 content and chloride ion concentration. Enzyme function was studied at different salinities.

Crabs held in sea water of different salinities and 23 ± 1 °C for a minimum of two days (Prosser, Green & Chow, 1955) were transferred to 6 l glass aquaria which were completely covered with opaque plastic. A maximum of three crabs were placed in each aquarium containing 2 l of vigorously aerated sea water of the appropriate salinity. In some tanks, Diamox was added to a final concentration of 2×10^{-4} M. Crabs were held in these containers for 12 h, removed individually and their haemolymph sampled from the infrabranchial sinus at the base of the third or fourth walking leg. Glass syringes containing the haemolymph samples were kept on ice and the haemolymph pH determined using a Radiometer Acid-Base Analyzer (PHM71) and pH electrode (G297/G2) thermostated to $23 \pm 0{\cdot}1$ °C. Total haemolymph CO_2 was measured using the method of Cameron (1971). The chloride ion concentration of haemolymph was determined in triplicate, using the titrametric method of Schales & Schales (1952) with 100 μl samples. In all cases only one haemolymph sample ($\sim 0{\cdot}6$ ml) was taken from each crab.

To determine if there was any specific effect of Diamox on CO_2 production, oxygen uptake (which estimates CO_2 production) was measured in crabs at three different salinities before and after 12 h exposure to 2×10^{-4} M Diamox. Oxygen uptake was determined using a method described by Burnett, Chambers & Coster (1980*a*).

RESULTS

Distribution of carbonic anhydrase in P. crassipes

The following tissues contained no detectable carbonic anhydrase activity: haemolymph, muscle, heart, hepatopancreas and male gonads. The possibility that Triton X-100 in the second supernatant and resuspended pellets was inhibiting any carbonic anhydrase present was ruled out. When a sample of gill supernatant, which exhibited carbonic anhydrase activity (described below), was made 1% v/v in Triton X-100 and then assayed, it was evident that Triton X-100 at that concentration did not inhibit *P. crassipes* carbonic anhydrase. The possibility that carbonic anhydrase in non-gill tissues was present at very low concentrations or was relatively inactive due to some endogenous inhibitor released by disruption, was made unlikely by our finding that concentrated preparations, made by pooling materials from five to ten crabs in a volume of buffer equal to what we would normally use for tissue from one crab, still exhibited no detectable carbonic anhydrase activity. Suspensions of large gill fragments exhibited no activity, suggesting that the enzyme is not located extracellularly.

Carbonic anhydrase activity in the Triton X-100 free supernatants of individual crab's gills (all gill filaments were used from both sides of each crab) ranged from $4{\cdot}74$ mg^{-1} to $12{\cdot}82$ mg^{-1}. The average of 18 animals was $7{\cdot}75$ mg^{-1}. There appears to be no carbonic anhydrase with which membrane is tightly associated, since neither the Triton X-100 supernatant nor the resuspended pellet from the gill homogenates exhibited detectable activity. All these crabs had been acclimated to 34‰ sea water.

Table 1. *Inhibition of crab gill carbonic anhydrase activity by Diamox*

Carbonic anhydrase activity*	Diamox concentration
8·1	0
3·24	5×10^{-5} M
0	2×10^{-4} M

* Expressed as mg^{-1} of supernatant protein. These values were obtained from a homogenate of six sets of pooled crab gills, sonicated in 120 vol buffer.

Table 2. *Demonstration that there is no haemolymph pH effect upon the addition of Diamox at a final concentration of* 2×10^{-4} M

(Haemolymph samples from three crabs were pooled, gently homogenized to break up the clot and centrifuged at 2000 ***g*** for 5 min. The supernatant was air-equilibrated at 23 °C and the pH measured at 5 min intervals.)

Time (min)	0	5	10	15	20
Control					
9 parts haemolymph / 1 part sea water	7·730	7·745	7·733	7·757	7·741
Experimental					
9 parts haemolymph / 1 part sea water with Diamox	7·739	7·733	7·770	7·747	7·749

Inhibition of gill intra-epithelial carbonic anhydrase by Diamox in vitro

When Diamox was added to a final concentration of 2×10^{-4} M in the assay reaction mixture, we detected no carbonic anhydrase activity in supernatants of gill tissue (Table 1). At a concentration of 5×10^{-5} M, Diamox partially inhibited the carbonic anhydrase activity.

Lack of a direct effect of 2×10^{-4} M *Diamox on haemolymph pH*

Since Diamox is a basic compound we thought it necessary to investigate the direct effect of Diamox at 2×10^{-4} M on haemolymph pH *in vitro*. When Diamox was added to haemolymph and the mixture air-equilibrated for 20 min, no change in pH was observed (Table 2).

Penetration of Diamox in vivo

To determine whether Diamox would penetrate *P. crassipes in vivo* from the external medium, and to determine how long it would take for a given haemolymph concentration to be reached in the presence of a specified water concentration, six crabs were placed in 34‰ sea water containing 2×10^{-4} M radioactive Diamox (3800 cpm/ml). Three crabs were removed and their haemolymph sampled at one hour and another three crabs at three hours after introduction of radioactive Diamox. Under these conditions, radiolabel in the external medium and crab haemolymph equilibrated within one hour (Table 3).

Table 3. *Uptake in individual crabs of radioactive Diamox from sea water*

Time (h) in 2×10^{-4} M radioactive Diamox (3800 cpm/ml)	cpm/ml haemolymph
1	4320
	3760
	3940
3	3600
	4008
	3840

Diamox effects on haemolymph chloride ion concentration, CO_2 content, pH and crab O_2 uptake

Pachygrapsus crassipes regulates its haemolymph chloride ion concentration over a wide range of salinities (Fig. 1). Twelve hours exposure to 2×10^{-4} M Diamox severely impairs the crab's ability to regulate haemolymph chloride under hypo-osmotic ($P \ll 0{\cdot}001$; analysis of covariance) but not under hyperosmotic conditions ($P \sim 0{\cdot}055$; analysis of covariance).

Diamox decreases O_2 uptake ($P < 0{\cdot}05$ according to a paired t test; Fig. 2), and by

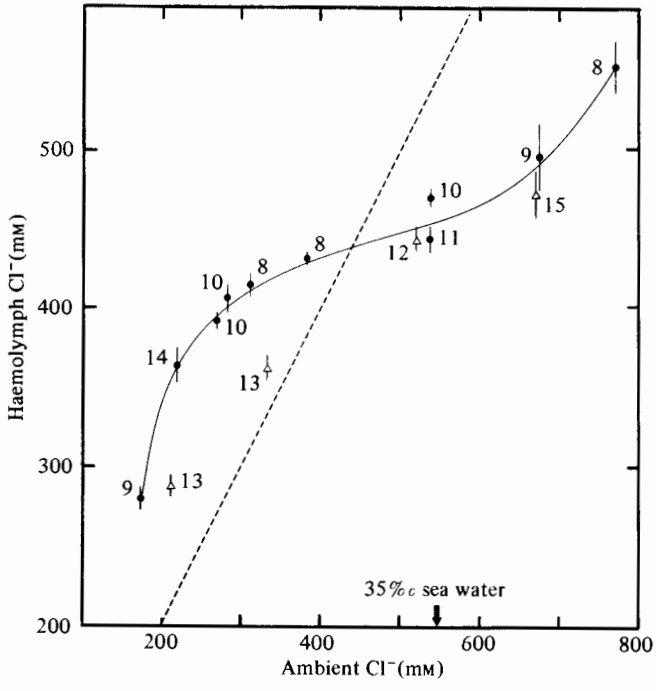

Fig. 1. Haemolymph chloride ion concentration in *Pachygrapsus crassipes* is regulated at low and high salinities and 23 °C (closed circles). Exposure to 2×10^{-4} M sodium acetazolamide impairs chloride regulation at low salinities but has no effect on chloride regulation at high salinities (open triangles). Data are expressed as the mean ± standard error. The number of observations is indicated next to each data set. The dashed line is the line of identity.

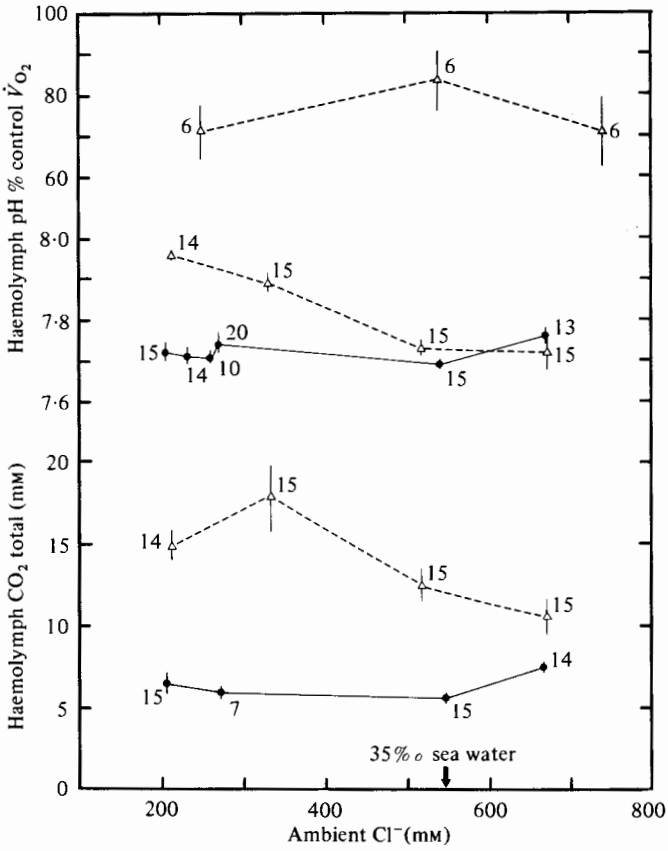

Fig. 2. Exposure to 2×10^{-4} M sodium acetazolamide (open triangles) depresses O_2 uptake ($\dot{V}_{O_2}$), elevates haemolymph pH at low salinities and elevates haemolymph CO_2 content at all salinities investigated in *Pachygrapsus crassipes* at 23 °C. Control values are depicted by closed circles. Data are expressed as the mean ± standard error. The number of observations is indicated next to each data set.

inference decreases CO_2 production assuming RQ remains constant. On the other hand, Diamox produces an elevation in haemolymph CO_2 content at all salinities studied (Fig. 2); the increase is greatest at low salinities, the same salinities at which haemolymph chloride regulation was most impaired by Diamox (Fig. 1). Furthermore, haemolymph CO_2 content at the highest salinity is significantly different from haemolymph CO_2 content at the lowest salinities ($P < 0.005$; Newman–Keuls test),

While haemolymph pH changes little in control crabs over the range of salinities studied, it is raised by Diamox exposure at low, but not high, salinities (Fig. 2).

DISCUSSION

Carbonic anhydrase activity in P. crassipes

Our results indicate that the gills of *P. crassipes*, like that of *C. sapidus* (Aldridge, 1977), contain a Diamox-sensitive catalyst of the hydration of CO_2. We assume this

catalytic activity to be due to a carbonic anhydrase. The tissue disruption and extraction experiments strongly suggest that this enzyme is located intracellularly, within the gill epithelial cells, and is in a soluble, or at most loosely membrane-associated form. Our experiments do not distinguish different intracellular pools of this enzyme. Given the extensive infolding of gill epithelial tissues (Copeland, 1968; Taylor & Butler, 1978), the possibility that the enzyme is distributed among functionally distinct subcellular compartments must be kept in mind when speculating on the function of the enzyme.

The lack of any detectable activity in haemolymph, muscle, heart, hepatopancreas and male gonads does not prove the absence of the enzyme in these tissues; it is in principle impossible to prove the absence of an *in vivo* functional enzyme using *in vitro* techniques. This caveat about the extension of *in vitro* results to *in vivo* reality also concerns the interpretation of our values for the specific activity of the gill carbonic anhydrase; all that is significant about these data are that the specific activities of gill extracts are well above zero, and that this *in vitro* activity is subject to a dose-dependent inhibition by Diamox.

We suggest that Diamox inhibits gill carbonic anhydrase *in vivo* as well as *in vitro*. This suggestion is supported by our experiments with radiolabelled Diamox, showing rapid equilibration of the label between sea water and haemolymph at Diamox concentrations which are effective in totally inhibiting carbonic anhydrase *in vitro* and by our observations that haemolymph CO_2 is elevated while O_2 consumption (and by inference CO_2 production) is depressed. The use of O_2 consumption in estimating CO_2 production does, however, present some difficulties. A recent study on a land crab indicates that the ratio of CO_2 production to O_2 consumption (RQ) increases from 0·7–0·9 to above 1·0 when the crabs exercise (Herreid, Lee & Shah, 1979). It is impossible to evaluate changes in RQ accompanying Diamox exposure from evidence presented here. However, in the present study, even a 25% increase in RQ in Diamox-exposed crabs would not lead to CO_2 production greater than in control crabs, since O_2 consumption declines in the presence of Diamox by 20–25%. This makes it unlikely that changes in tissue CO_2 production contribute to elevated haemolymph CO_2 contents (Fig. 2). The most parsimonious explanation of our observations of elevated haemolymph CO_2 is that the gill intracellular $CO_2 \rightleftharpoons HCO_3^-$ equilibrium is: (1) critical to the CO_2 excretion rate, and (2) strongly affected by intracellular carbonic anhydrase.

Another possible interpretation of the elevated haemolymph CO_2 is that Diamox mobilizes CO_2 from some unknown source within the crab into the haemolymph. For example, some recent evidence suggests that crabs can use internal stores of $CaCO_3$, presumably from the shell, to buffer the haemolymph against severe decreases in pH which may occur when a crab is exposed to air (deFur, Wilkes and McMahon, 1981). Mobilization of internal CO_2 stores during Diamox exposure cannot be completely ruled out in the present study. However, a release of $CaCO_3$ stores associated with a decrease in haemolymph pH similar to that observed by deFur *et al.* is unlikely, since pH did not decrease during exposure to Diamox in any of our experiments.

In support of our interpretation that carbonic anhydrase facilitates CO_2 excretion,

Table 4. Pachygrapsus crassipes *were injected with a physiological saline in which Diamox was dissolved to achieve a final circulating concentration of* $2{\cdot}7 \times 10^{-3}$ M

Control crabs received injections of physiological saline. Haemolymph was sampled three and six hours after injection. The haemolymph of each crab was sampled only once. Data are expressed as x ± S.E. (*N*). *P* values according to Student's *t* test are given comparing experimental to control data.

Three hours after injection

Control (Ambient Cl^- = 268 mM)			Experimental (Ambient Cl^- = 268 mM)		
pH	CO_2tot (mM)	Cl^- (mM)	pH	CO_2tot (mM)	Cl^- (mM)
$7{\cdot}709^{+0{\cdot}036}_{-0{\cdot}033}$ (6)	$4{\cdot}97 \pm 0{\cdot}29$ (5)	$378 \pm 24{\cdot}7$ (3)	$7{\cdot}809^{+0{\cdot}057}_{-0{\cdot}051}$ (6)	$7{\cdot}43 \pm 1{\cdot}15$ (6)	$316 \pm 29{\cdot}76$ (6)
			N.S.	$P < 0{\cdot}05$	N.S.

Six hours after injection

Control (Ambient Cl^- = 251 mM)			Experimental (Ambient Cl^- = 253 mM)		
$7{\cdot}844^{+0{\cdot}041}_{-0{\cdot}038}$ (7)	$5{\cdot}82 \pm 0{\cdot}64$ (7)	$332 \pm 2{\cdot}06$ (6)	$8{\cdot}22^{+0{\cdot}03}_{-0{\cdot}02}$ (9)	$16{\cdot}95 \pm 1{\cdot}72$ (9)	$231^{*} \pm 5{\cdot}34$ (8)
			$P < 0{\cdot}005$	$P < 0{\cdot}005$	$P < 0{\cdot}005$

* This set of Cl^- titrations was anomalous in that slight precipitates were observed early in the titration, which then disappeared on further addition of $HgNO_3$. The anomaly was due to the crab haemolymph under these conditions, and not the titration solutions, since the same solution worked normally with other crab haemolymph samples.

we have recently found that Diamox inhibits the excretion of $H^{14}CO_3^-$ added to the artificial internal perfusate of an isolated gill of *Cancer anthonyi* in organ culture (Burnett *et al.* 1980*b*; Woodson *et al.* 1980). The posterior gills used in these studies were found to contain Diamox-sensitive carbonic anhydrase activity.

Relation to prior work with Diamox in a crab

Our results with *P. crassipes* sharply contrast with those of Aldridge & Cameron (1979) who, using blue crabs (*Callinectes sapidus*) acclimated to fresh water, showed that Diamox injection had no effect on haemolymph pH or CO_2 content after six hours. In an effort to explain the discrepancies between the two studies, we conducted an experiment on *P. crassipes* using methods similar to those used by Aldridge & Cameron. We acclimated the crabs to 16‰ salinity water, the lowest salinity at which they can regulate haemolymph chloride, in order to approximate both the direction and degree of chloride regulation found in *C. sapidus*. Diamox was dissolved in a physiological saline (based on Prosser *et al.* 1955) approximating the ionic content of the crab's haemolymph at 16‰ salinity. Crabs were then injected with the solution to achieve a final circulating Diamox concentration in the haemolymph of $2{\cdot}7 \times 10^{-3}$M (after Aldridge & Cameron, 1979 and based on the assumption of haemolymph volume = 30% wet animal weight; Gleeson & Zubkoff, 1977). Haemolymph withdrawn after three hours in one group of crabs and after six hours in another group of crabs was analysed for pH, CO_2 and chloride content. The results (Table 4) are consistent with our findings of an elevation in haemolymph CO_2 content and inhibition of chloride regulation during Diamox exposure. Thus, the discrepancies between the results of the two studies are not likely to be due to the differences in dose or route of administration of Diamox.

We would like to point out, however, that Diamox, a basic compound, has been

observed to produce a non-specific effect on carbonic anhydrase activity when used at more than millimolar concentrations (Maren, 1977). In addition, repeated haemolymph sampling produces slight haemolymph acidosis and lowering of CO_2 content (McMahon, Butler & Taylor, 1978). Multiple haemolymph sampling (six times within 12 h) on *C. sapidus*, a crab which is normally very aggressive and excitable, may have shifted haemolymph pH and CO_2 content in directions opposite to that which we observed in *P. crassipes*. Thus, both the high Diamox concentration and multiple haemolymph sampling in the Aldridge & Cameron (1979) study may have masked differences in haemolymph pH and CO_2 content before and after treatment.

The results of the Aldridge & Cameron (1979) study can be explained if there was a decrease in both CO_2 production and CO_2 excretion. O_2 uptake (and thus CO_2 production) measured in two *P. crassipes* treated with Diamox in a manner similar to that used with *C. sapidus* showed no significant change after five hours (unpublished results of ours). However, the possibility of a Diamox-induced change in CO_2 production in *C. sapidus* was not explored. Finally, the contrasting results of the two studies may be related to (1) species-specific differences in the function of carbonic anhydrase in crab gills or (2) the possibility that six hours exposure to Diamox is insufficient time to measure a slowly accumulating effect on haemolymph CO_2 content or pH.

Effects of Diamox on chloride regulation

Diamox-sensitive chloride transport (Fig. 1) has been observed in other epithelia: gills of the goldfish (Maetz & Garcia-Romeu, 1964) and rainbow trout (Kerstetter & Kirschner, 1972), intact toadfish (Kormanik & Evans, 1979), the crayfish *Astacus leptodactylus* (Ehrenfeld, 1974), and frog skin (Ehrenfeld & Garcia-Romeu, 1978; Watlington, Jessee & Baldwin, 1977). A major observation of the present work is that Diamox affects haemolymph CO_2 content most strongly in hyposaline water (Fig. 2). Thus CO_2 excretion and regulation of haemolymph chlorinity under hyposaline conditions may be limited together by the rate at which *either* CO_2 or HCO_3^- can be supplied to (or removed from) certain intracellular transport sites within the gill. This suggestion is really a multipart theory, and we will now consider its various components.

First, it must be admitted that in addition to its known effect as an inhibitor of carbonic anhydrases from numerous different species, Diamox may be a specific inhibitory ligand of the chloride inward-transporting mechanism under hyposaline conditions. We ignore this possibility only because there is lack of evidence for it, there is plenty of evidence for the carbonic anhydrase inhibition role, and finally because we do not know how to settle the problem experimentally.

Given the assumption that the sole site of action of Diamox is carbonic anhydrase, we must then consider that the enzyme is known to catalyse both the hydration of CO_2 and the dehydration of HCO_3^- (H_2CO_3 is probably not the substrate for the dehydration reaction; DeVoe & Kistiakowsky, 1961; Khalifah, 1971). Furthermore Diamox appears to be a competitive inhibitor of the hydration reaction but a non-competitive inhibitor of the dehydration reaction (Maren, 1967). The present data say nothing about the *in vivo* substrate or prevailing direction of catalysis. The possibility that there are different subcellular pools of the enzyme, some catalysing one reaction

and some the other, cannot be ignored either (e.g. the enzyme favours the dehydration reaction in acid media; Khalifah, 1971). Thus, we must consider that Diamox may be making the rate of supply or removal of either (or both) CO_2 or HCO_3^- to intracellular sites limiting, and thereby impairing the inward transport of chloride. Clearly the number of possibilities is unpleasantly large. All we can assert, remembering our assumption about the sole site of action of Diamox, is that under hyposaline conditions the inward movement of chloride is coupled to the outward movement of CO_2 in such a way that the gill intracellular $CO_2 \rightleftharpoons HCO_3^-$ interconversion rate, as controlled by carbonic anhydrase, is critical to CO_2 and Cl^- translocation processes.

Diamox-induced haemolymph pH changes

The inhibition of chloride ion regulation during Diamox exposure at low salinity is associated with an increase in haemolymph pH (Fig. 2). The alkalosis is probably an effect of the breakdown of ion regulation (and thus osmotic regulation), and not a result of carbonic anhydrase inhibition *per se*. Mangum & Towle (1977) have attributed the haemolymph alkalosis associated with the migration of *Callinectes sapidus* to dilute waters to a cellular volume regulation mechanism, in which intracellular free amino acid pools are reduced to accommodate the dilution of the haemolymph. The deamination of the free amino acid pool produces ammonia which passes into the haemolymph and picks up a proton, raising the pH. A similar situation is likely to exist in *P. crassipes* when its haemolymph suddenly becomes dilute due to a Diamox-induced inhibition of Cl^- ion regulation.

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