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Carbon dioxide excretion and carbonic anhydrase function in the Red Rock Crab *Cancer productus*

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Summary. The function of carbonic anhydrase (CA) in the Red Rock Crab, *Cancer productus* Randall, was investigated. CA activity was found to varying degrees in the gills and many other tissues but not in hemolymph. Crabs injected with acetazolamide, a specific CA inhibitor, demonstrated a significant hemolymph acidosis. Hemolymph CO_2 tension (Pa_{CO_2}) and CO_2 content (Ca_{CO_2}) also increased and remained significantly elevated for 96 h following treatment. No significant changes could be detected in either hemolymph oxygenation or ionic status (except for HCO_3^-) as a result of acetazolamide treatment. Crabs treated with acetazolamide, and also exposed to air, exhibited a more pronounced hemolymph acidosis with significantly increased respiratory (P_{CO_2}) and metabolic (lactate) components compared with the control group. Upon reimmersion acetazolamide treated crabs showed a slower recovery of hemolymph pH compared with the control group and no significant removal of the total CO_2 load induced by air exposure. No significant differences between experimental and control groups during air exposure and recovery could be detected in hemolymph oxygenation, ionic status, $NH_3+NH_4^+$ levels or respiratory and cardiac pumping frequency and so the effects of acetazolamide treatment were apparently limited to CO_2 removal across the gills. These results indicate that branchial CA facilitates the removal of CO_2 from the hemolymph of SW adapted *C. productus* largely by catalyzing the dehydration of hemolymph HCO_3^- to molecular CO_2 at the gill. It is also recognized that gill CA may also serve to hydrate molecular CO_2 to H^+ and HCO_3^- for use as counterions for ionic uptake mechanisms. Crab gill CA thus appears to play an important role in CO_2 excretion as well as hemolymph ionic regulation.

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Introduction

The enzyme carbonic anhydrase (CA) is known to catalyze both the hydration and the dehydration reactions of CO_2 in biological carbonate systems. The hydration reaction can be used to provide counterions (H^+, HCO_3^-, OH^-) for ion exchange (Maren 1967) while the dehydration reaction facilitates CO_2 mobilization and transport across aerial (Klocke 1978; Effros et al. 1978, 1980) and aquatic respiratory surfaces (Haswell and Randall 1978; Wood et al. 1982). The function of CA in ion and gas exchange has been well studied in mammals and fishes. The decapod crustaceans, however, are proving to be of interest for the study of CA function because of the high activity of the enzyme in gill tissues and the absence of activity in the hemolymph. This situation contrasts with that found in most vertebrates where CA occurs in circulating red blood cells in addition to pulmonary and branchial tissues. In vertebrates, circulating CA is usually removed and the lung or gill is artificially perfused to study enzyme function in the respiratory epithelia. The elucidation of the respiratory and ionoregulatory functions of branchial CA in decapods is therefore made easier by the natural lack of the enzyme in the hemolymph.

Carbonic anhydrase is known to be active in the gills of the several species of crustaceans stud-

ied (Ferguson et al. 1937; van Goor 1937, 1940; Ehrenfeld 1974; Aldridge and Cameron 1979; Randall and Wood 1981; Burnett et al. 1981; Henry and Cameron 1982). However, some confusion exists as to its function. Aldridge and Cameron (1979) and Henry and Cameron (1983) reported that although CA is present in gill tissue of fresh water acclimated *Callinectes sapidus*, injection of acetazolamide, a known inhibitor of CA activity, had no effects on acid-base balance and concluded that CA was not involved in CO_2 excretion. Burnett et al. (1981) had, however, demonstrated both significant ionic and acid-base changes resulting from acetazolamide treatment in another euryhaline crab *Pachygrapsus crassipes*, also acclimated to dilute media. Henry and Cameron (1983) were able to demonstrate acid-base and ionic effects in low salinity acclimated *C. sapidus* upon acetazolamide injection. These authors (Henry and Cameron 1983) concluded that gill CA functions in hydrating CO_2 to HCO_3^- and H^+ to supply counterions for Cl^- and Na^+ uptake in low salinity, a process which is potentially important in regulation of acid-base status by changing the strong ion difference. Henry and Cameron (1983) also maintained that CO_2 excretion is driven solely by the P_{CO_2} gradient from hemolymph to water and that excretion is unaided by CA.

Other recent evidence, however, does not exclude a possible role for branchial CA in the normal CO_2 excretion process. McMahon and Burnett (1981) demonstrated that acetazolamide treatment causes significant CO_2 retention in air exposed *Pachygrapsus crassipes* and both significant CO_2 retention and acidosis in a similar shore crab, *Hemigrapsus nudus*, during air exposure. These animals were acclimated to 30–32‰ sea water and no significant ion effects could be detected following treatment. Randall and Wood (1981) also demonstrated significant CO_2 retention following acetazolamide treatment in the land crab *Cardisoma carnifex*.

These results are difficult to integrate with the conclusions of Aldridge and Cameron (1979) and Henry and Cameron (1983) and thus provided the rationale for the present study. The Red Rock Crab *Cancer productus* was a particularly appropriate species for this study for a number of reasons. Firstly, it is a relatively stenohaline marine species and thus provides an interesting animal for comparison with the essentially euryhaline or otherwise modified species utilized above. Secondly, it is a relatively large animal and thus able to provide multiple samples of hemolymph without serious depletion of the hemolymph pool. Lastly, it is an animal whose basic respiratory physiology has been studied for several years in this laboratory and thus is relatively well understood (McMahon and Wilkens 1977; deFur and McMahon 1984a, b).

Materials and methods

27 *Cancer productus* of average mass 411±23 (SEM) g were collected using crab pots in Grappler and Bamfield Inlets on the west coast of Vancouver Island. In the Bamfield Marine Station where these experiments were conducted, the animals were held in large 500 gallon aquaria in flowing sea water at 11.5±0.5 °C, salinity 31–32‰ and mean ionic composition as shown in Table 1. Animals were fed fish every 2 days except during the experimental measurement periods. All animals were allowed 3–4 days to recover from capture procedures before use in experiments. At least 24 h prior to sampling, animals were removed from water and a small (1 mm) hole was drilled in each animal above the pericardium posterior to the heart. The hole did not penetrate the epidermis and was covered with a small piece of dental dam. This hole allowed easy access to pericardial hemolymph (McDonald 1977).

Three series of experiments were undertaken. The first (Series I) was to ascertain the presence and relative activity of CA in various tissues. Tissues were dissected from *C. productus* and assayed individually according to the method outlined by Burnett et al. (1981). Briefly this method consists of following the rate of appearance of protons when saturating amounts of CO_2 are added to tissue homogenates. 1 ml of homogenate was added to 24 ml of 20 mM Barbital buffer and the rate of decline in pH between 8.1 and 8.0 was followed when 10 ml of CO_2 saturated water was rapidly injected into the mixture. Inhibition of CA activity in tissue homogenates by acetazolamide was demonstrated by adding acetazolamide to the buffer and homogenate mixture at least 10 min prior to assay. All assays were carried out at 10±0.2 °C.

In the second series of experiments (Series II) animals were sampled for postbranchial hemolymph prior to experimental treatment, then were injected into the pericardium (i.e. immediately upstream from the heart) with either acetazolamide (Diamox, Lederle) dissolved in millipore filtered sea water or an equivalent volume (0.15–0.45 ml) of millipore filtered sea water to serve as controls. The amount of acetazolamide injected was sufficient to achieve, after equilibration, a final circulating concentration of 4×10^{-4} M (an amount which suppresses activity below measurable levels in vitro) assuming a hemolymph volume of 30% of animal body weight (Gleeson and Zubkoff 1977). It is likely, however, that the actual final circulating concentration was less than this because acetazolamide has been shown to permeate the general body surface in another species of crab (Burnett et al. 1981). Loss of inhibitor by this route may also have been accompanied by non-specific binding to tissues, urine clearance and chemical breakdown of acetazolamide. We, therefore, cannot make any definite statement concerning the level of suppression of CA activity in vivo, other than that enough enzyme activity was inhibited to bring about the results outlined. Hemolymph was sampled from the animals at 1–2, 4–6, 10–13, 21–24, 48 and in 12 animals 100–120 h following injection. Hemolymph (0.6 ml) was taken from the pericardial cavity from the sampling port described above into glass 1 ml syringes and stored on ice (max. 20 min) for analysis of oxygen tension (Pa_{O_2}), oxygen content (Ca_{O_2}), pHa, carbon dioxide tension (Pa_{CO_2}) and carbon dioxide content (Ca_{CO_2}). Separate samples (0.3 ml) were taken for hemolymph ion, osmotic and lactate analysis.

Table 1. Ionic content of Bamfield sea water $meq \cdot l^{-1}$

	Na^+	K^+	Ca^{++}	Mg^{++}	Cu^{++}	Cl^-	HCO_3^- *	mOsm/kg*
x̄	474	6.8	10.9	61.8	N.D.	507	2.30	930
SEM	65	0.2	1.9	1.0	N.D.	2	0.04	6
N	4	4	4	4	N.D.	7	21	7

Ionic content of resting *Cancer productus* Hemolymph $meq \cdot l^{-1}$

	Na^+	K^+	Ca^{++}	Mg^{++}	Cu^{++}	Cl^- *	HCO_3^- *	mOsm/kg*	Lactate*
x̄	464	7.0	15.4	45.7	0.70	459	8.97	900	0.13
SEM	33	0.02	1.5	2.3	0.06	3	0.46	6	0.02
N	10	12	12	12	19	24	18	24	24

* Determined immediately, remainder subsequently from frozen samples

A third series of experiments (Series III), performed using a subgroup of 13 animals of mass 368±36 (SEM) g, incorporated at 4 h air exposure period to induce additional hemolymph CO_2 loading. The protocol used for this series involved prebranchial hemolymph sampling in addition to postbranchial sampling as described above. Prebranchial hemolymph was taken from the infrabranchial sinus penetrated via the arthrodial membrane of the proximal joint of the last pereiopod. Post- and prebranchial samples were taken consecutively with postbranchial samples always taken first. The animals were then air exposed (air temperature maintained at sea water levels) for 4 h and returned to water for recovery. Hemolymph was sampled just prior to injecting the crabs with acetazolamide or millipore filtered sea water. Crabs were injected 15 min before emersion. In addition, hemolymph was sampled 0.25, 2, 6 and 20 h following reimmersion in sea water. Crabs were air exposed by draining sea water from the small experimental holding tanks in which they were kept. Hemolymph and sea water samples were analyzed as in Series II.

pH, P_{O_2} and P_{CO_2} were measured using Radiometer electrodes thermostatted to 11.5±0.1 °C. The pH electrode was calibrated with Radiometer buffers S1500 and S1510. The oxygen electrode was calibrated using nitrogen and air equilibrated sea water. The P_{CO_2} electrode was calibrated using millipore filtered sea water equilibrated with 0.2 and 1.0% CO_2 mixed with air by Wösthoff gas mixing pumps. Full details of the methods used for calibrating the P_{CO_2} electrode and measuring P_{CO_2} are given in deFur et al. (1980). Electrode output was amplified and displayed on Radiometer PHM71 or PHM72 acid-base analyzer systems. For measuring P_{CO_2} a 10× scale expansion was used to ensure accuracy of measurement of the low P_{CO_2} found in these cold water species. Oxygen content was measured using a Lex-O_2-Con oxygen analyzer (Lexington Instruments) with a modified technique as described by McMahon et al. (1978). CO_2 content was measured on 40 µl hemolymph samples using the micromethod of Cameron (1971). Total CO_2 content is taken to mean dissolved CO_2, HCO_3^- and CO_3^- (Cameron 1971) and also including carbamate CO_2, since the pH of the reaction vessel in which measurements are made (pH = 1–2) is below that of the pK for carbamate formation (pK = 4.85; Roughton and Rossi-Bernardi 1966; Roughton 1970) effecting the release and detection of CO_2 bound as carbamate. Hemolymph samples were bracketed with samples of standard 15 mM bicarbonate. For the lower levels of bicarbonate in sea water, 80 µl samples and standards of 1.5 mM bicarbonate were substituted. Even so, increased variability was encountered and 5–10 replicates were measured to improve accuracy of the final value.

$NH_3 + NH_4^+$ concentrations were measured on whole serum using a macromodification of the method of Solorzano (1969). Hemolymph and sea water ion concentrations were measured later in Calgary on separate samples frozen and sealed within 10 min of sampling and stored frozen until dilution immediately prior to analytical procedures. Na^+, K^+, Ca^{++}, Mg^{++} and Cu^{++} concentrations were measured using a Jarrel Ash 850 Atomic Absorption Spectrophotometer. Detailed methodology for ion analysis is similar to that used by Wilkes and McMahon (1982). Chloride, osmotic and lactate concentrations were measured immediately after sampling. Cl^- concentration was measured using a Radiometer CMT10 chloride titrator. Osmotic concentration, expressed as mOsm/kg, was measured with a Wescor 5100B vapor pressure osmometer. Hemolymph lactate concentration was measured using a Sigma assay No. 826-UV modified by adding 10 mM EDTA to the glycine buffer (Mangum, personal communication).

To assess the effects of acetazolamide on ventilation and perfusion, left and right scaphognathite frequency and heart beat frequency were measured using a modified impedance technique (Ansell 1973). These frequencies can be used to estimate ventilation volume using the relationship between body mass and scaphognathite pump stroke volume calculated for the related *Cancer magister* by McDonald (1977).

Results are presented as means ±SEM except where otherwise stated. Levels of significance were assessed using Students *t*-test unpaired with a fiducial limit of $P = 0.05$.

Results

Series I – carbonic anhydrase activity in various tissues

Significant CA activity was found in hepatopancreas, male gonads, heart muscle and all gills, while epidermis (taken from underneath the carapace) and muscle tissues from the cheliped, the merus of the walking legs and the thoracic sterna contained only a little enzyme activity (Table 2). There was no detectable activity in hemolymph. No measurable enzyme activity could be detected following incubation of tissue homogenates in 4×10^{-4} M acetazolamide.

Table 2. Carbonic anhydrase activity of *Cancer productus* tissues. Enzyme activity is expressed as mg^{-1} according to the formula

$$\left(\frac{\text{catalyzed rate}}{\text{uncatalyzed rate}} - 1\right)/\text{mg protein}$$

Tissue		Enzyme activity		
		x̄	SEM	*N*
Hepatopancreas		0.702	0.151	3
Gonad (male)		0.196		1
Gill[a]	1	0.204	0.094	3
	2	0.182	0.079	3
	3	0.320	0.090	3
	4	0.163	0.033	3
	5	0.256	0.056	3
	6	0.201	0.065	3
	7	0.293	0.094	3
	8	0.205	0.062	3
	9	0.367	0.139	3
Branchial cavity epithelium		0.037	0.023	3
Muscle:	Heart	0.070	0.070	2
	Cheliped	0.014	0.014	3
	Walking leg (merus)	0.014	0.014	3
	Thoracic sterna	0.022	0.011	3
Hemolymph		0	0	3

No measurable enzyme activity could be detected following incubation of tissue homogenates in 4×10^{-4} M acetazolamide.

[a] Gills are numbered according to Pearson (1908)

Series II – oxygenation status, ionic, and acid-base status

Postbranchial hemolymph samples taken immediately prior to injection revealed no significant difference in oxygenation, acid-base or ionic status between groups or series. Mean levels (Table 1) of O_2 were not significantly different from values previously published for this species (McMahon and Wilkens 1977). The initial mean values for each group served as a control against which the effects of injection were compared (Figs. 1, 2, 3, 4). Series III animals yielded both pre- and postbranchial hemolymph values (Table 3).

Injection of either acetazolamide or saline had no significant effect on hemolymph oxygenation. Slight increases in both oxygen tension (Pa_{O_2}) and oxygen content (Ca_{O_2}) of postbranchial hemolymph were observed following either injection (Fig. 1 A) and were probably associated with disturbance resulting from handling and injection procedures.

Values for concentration of Na^+, K^+, Mg^{++}, Ca^{++}, Cu^{++}, Cl^-, HCO_3^-, lactate and osmolality in hemolymph and sea water are provided in Table 1. Hemolymph ion levels have not been reported previously for *C. productus* but are essentially similar to those reported for *C. magister* from water of equivalent composition (Hunter and Rudy 1975). In *C. productus* hemolymph, ionic composition does not differ significantly from that of sea water except that Mg^{++} levels are lower and Cu^{++} and HCO_3^- levels are higher. Injection of either acetazolamide or saline induced no detectable change in any ion but HCO_3^- (see below).

Injection of saline caused a significant decrease in hemolymph bicarbonate, a slight depression of P_{CO_2} within 1–2 h but no further significant change in acid-base status (Fig. 2). Such effects are commonly observed following handling, injection and/or hemolymph sampling procedures (McMahon et al. 1978). Markedly different results follow the injection of acetazolamide (Fig. 2). Hemolymph pH was significantly depressed for 2–10 h following injection but recovered within 24 h. The acidosis is partially respiratory since (Pa_{CO_2} is significantly elevated above preinjection values for 96 h following injection. Control group P_{CO_2} levels rose slowly after 10–12 h. Treatment group P_{CO_2} levels were significantly elevated over equivalent control

Table 3. Resting oxygenation and acid-base status of *Cancer productus* hemolymph[a]

		P_{O_2} (Torr)	C_{O_2} ($mmol \cdot l^{-1}$)	pH	P_{CO_2} (Torr)	C_{CO_2} ($mmol \cdot l^{-1}$)	NH_4^+ ($mmol \cdot l^{-1}$)
Postbranchial	x̄	40	0.30	7.962	1.8	8.04	0.39
	SEM	7	0.03	+0.015 −0.015	0.2	0.44	0.05
Prebranchial	x̄	18	0.14	7.941	2.0	8.14	0.42
	SEM	6	0.02	+0.017 −0.016	0.2	0.48	0.05
	N	12	10	11	11	11	6
Postbranchial-prebranchial		22	0.17	0.021	−0.2	−0.10	0.03

[a] Includes controls for Series II plus Series III

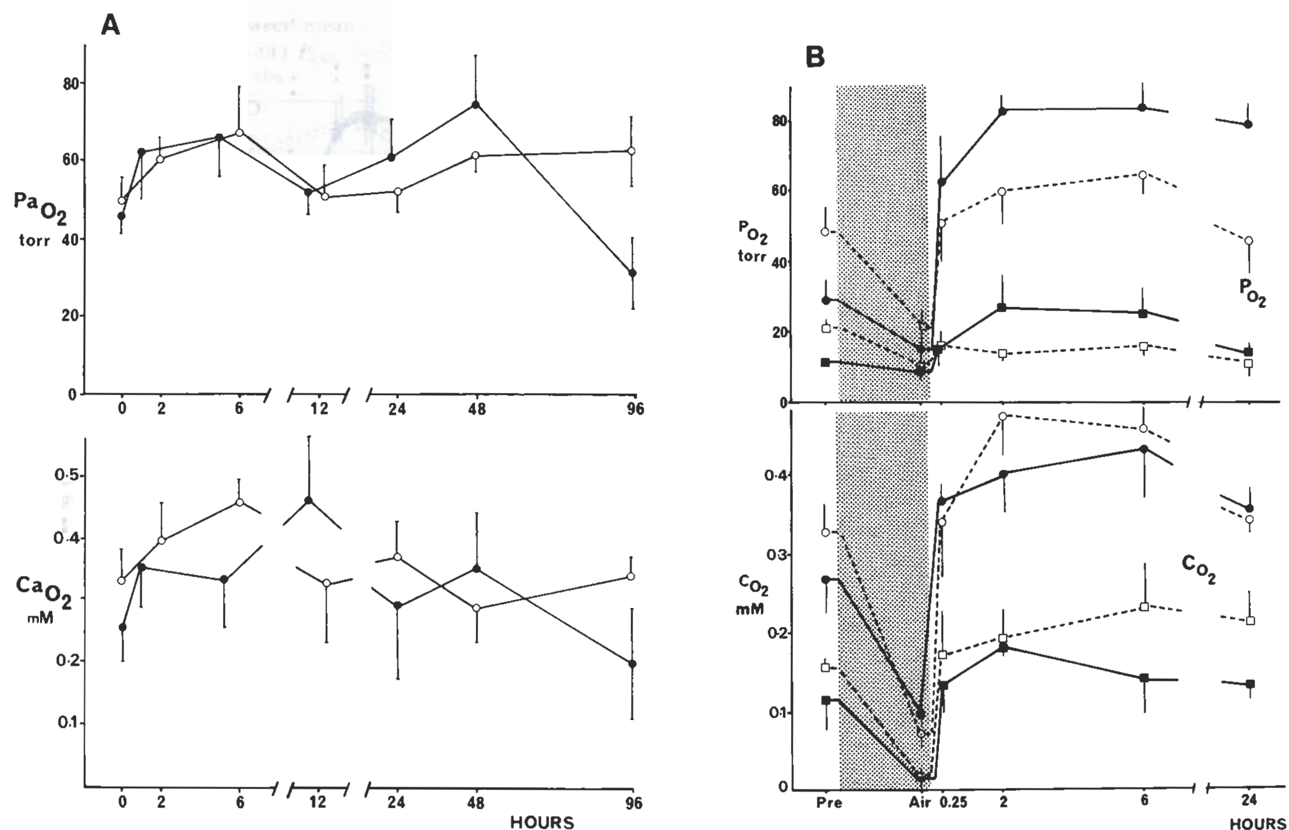


Fig. 1. A Postbranchial hemolymph P_{O_2} and C_{O_2} in Series II. **B** Postbranchial (●, ○) and prebranchial (■, □) hemolymph P_{O_2} and C_{O_2} in Series III, emersed saline injected (closed symbols) and acetazolamide injected (open symbols) crabs. The shaded area represents the period of air exposure. Data are expressed as mean ±SEM

values (i.e. values taken at similar times following saline injection) only for 12 h. Hemolymph lactate concentrations rose slightly following injection of both acetazolamide and saline but the only significant elevation occurred after 1.5 h in the acetazolamide group. More striking divergence from either initial or control injected animals was seen in hemolymph C_{CO_2} (Fig. 2). C_{CO_2} rose progressively for 24 h following acetazolamide injection and remained significantly elevated over initial and equivalent control values for 96 h. By 120 h following acetazolamide treatment C_{CO_2} was still elevated but control values were not available for comparison.

The extreme longevity of the acetazolamide induced responses was interesting but did not allow effective study of the rate of release of the CO_2 load. Thus, in another series (Series III) of animals we induced an additional CO_2 load utilizing a 4 h period of air exposure. Similar periods of air exposure occur naturally in this animal's littoral habitat.

Series III – effects of hemolymph CO_2 loading

Initial resting oxygenation and acid-base status of pre- and postbranchial hemolymph did not differ from that of Series II animals and the two data sets are combined in Table 3.

Oxygenation status. Immediately following injection of either saline or acetazolamide, animals were air exposed for 4 h. Hemolymph samples taken after 3.75 h air exposure showed significant and severe depletion of both pre- and postbranchial oxygen tensions and oxygen content (P_{O_2}, C_{O_2}, Fig. 1 B) compared with samples taken prior to emersion indicating seriously limited oxygen uptake at the gill surface. No significant differences could be ascertained in the extent of oxygen depletion in the two groups. Upon reimmersion oxygen levels increased dramatically, reaching levels equivalent to or above pre-emersion values within 15 min. The most dramatic changes occurred in

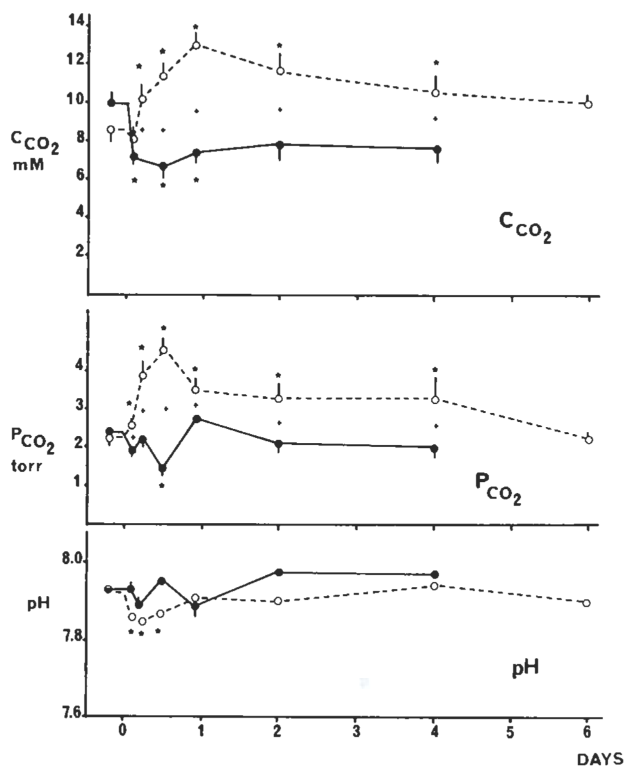


Fig. 2. Postbranchial hemolymph C_{CO_2}, P_{CO_2} and pH in Series II, immersed saline injected (closed circles) and acetazolamide injected (open circles) crabs. Data are expressed as mean ±SEM. ⋆ denotes significant difference from initial values within each group: + denotes significant difference between treatments

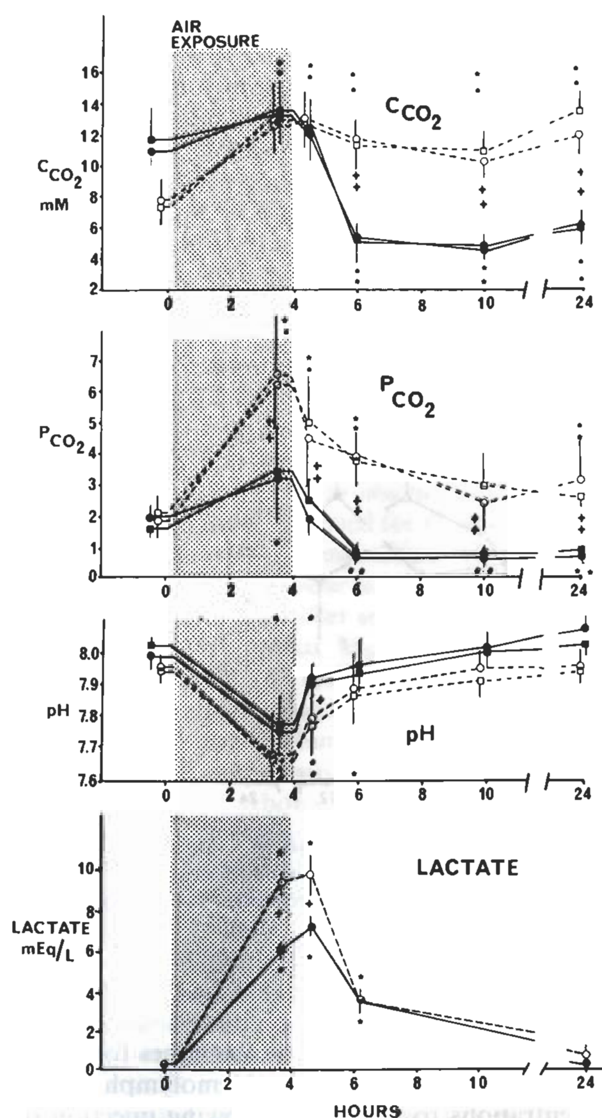


Fig. 3. Postbranchial (●, ○) and prebranchial (■, □) hemolymph C_{CO_2}, P_{CO_2}, pH and lactate in Series III, emersed saline injected (closed symbols) and acetazolamide injected (open symbols) crabs. The shaded area represents the period of air exposure. Data are expressed as mean ±SEM. ⋆ and + indicate significant differences as in Fig. 2

postbranchial hemolymph which substantially overshot initial values from 2 to at least 6 h following reimmersion and declined only slowly thereafter (Fig. 1B).

Acid-base and ionic status. Emersion resulted in a marked and significant acidosis in both treated and control groups (Fig. 3). Final mean pH was lower in the acetazolamide treated group but the difference was not significant.

The ensuing acidosis was at least partially respiratory in origin since P_{CO_2} increased significantly in both groups. P_{CO_2} rose to significantly greater levels in the acetazolamide-treated group. As expected, air exposure was associated with a marked increase in C_{CO_2} (Fig. 3). In the control group initial C_{CO_2} levels were very variable and significantly higher than those found (prior to injection) in the acetazolamide group. Due to this variability the increase in prebranchial C_{CO_2} during air exposure failed to reach significance ($P<0.1$), but by combining pre- and postbranchial samples, a significant difference overall could be demonstrated ($P<0.025$). Although the final hemolymph C_{CO_2} levels following air exposure were similar in the two groups, the increase in the acetazolamide treated group was correspondingly (significantly) greater.

Upon reimmersion hemolymph pH levels were reestablished in both groups but at different rates. In the controls, pH levels approached pre-emersion values within 15 min and were not significantly depressed after 2 h. Recovery occurred more slowly in the acetazolamide-treated group with prebranchial pH remaining significantly acidotic at 2 h but with full recovery occurring by 6 h post reimmersion (Fig. 3).

Table 4. Differences between mean hemolymph Pv_{CO_2} and calculated ambient water (air) P_{CO_2} (=0.3 Torr) in control and acetazolamide treated crabs

		Control	Acetazolamide
a) *Immersed*			
Pretreatment		2.0	2.0
Hours post-treatment	1–2	1.6	2.3
	5–6	2.0	3.7
	10–13	1.1	4.3
	21–24	2.5	3.2
	48	1.9	3.0
	94	1.7	3.0
b) *Emersed – reimmersed*			
Pre-emersion & treatment		1.3	1.8
3.75 h emersion (air)		2.7	6.0
Hours reimmersion	0.25	2.1	4.5
	2	0.4	3.5
	6	0.4	2.8
	24	0.6	2.5

Associated with the more rapid restoration of pH in the controls, P_{CO_2} levels of both pre- and postbranchial hemolymph fell rapidly reaching levels not significantly different from pre-emersion in 15 min. This rapid decline continued so that by 2 h following reimmersion both Pa_{CO_2} and Pv_{CO_2} had fallen to levels significantly below initial values (Fig. 3) where they remained for the rest of the experimental period. The time course for P_{CO_2} decrease was similar in the acetazolamide treated group but these did not decrease to initial values until 6 h and remained significantly elevated over equivalent controls for at least 24 h. These elevated P_{CO_2} levels greatly increased the gradient between Pv_{CO_2} and ambient water (Table 4).

Hemolymph lactate levels were significantly elevated above controls both during and 15 min after air exposure of acetazolamide-treated animals. Peak hemolymph lactate levels were detected 15 min following reimmersion in both treated and control groups. The rate of removal of lactate from hemolymph, however, seemed unaffected by acetazolamide treatment, since hemolymph levels were virtually identical in both control and treated groups 2 h after reimmersion.

An even more striking difference between the two groups was seen in C_{CO_2} levels following reimmersion (Fig. 3 and 4). In control crabs C_{CO_2} levels decreased rapidly reaching values not significantly above initial values within 15 min and, as with P_{CO_2}, declined significantly below these initial values by 2 h. Some recovery occurred slowly but by 24 h C_{CO_2} levels were still significantly depressed. A quite different picture resulted in the acetazolamide-treated animals. Little or no decrease in C_{CO_2} occurred in either pre- or postbranchial hemolymph C_{CO_2} upon reimmersion. In fact, C_{CO_2} levels remained significantly elevated above both initial and equivalent control values for at least 24 h. To further test the hypothesis that the prolonged CO_2 retention observed following air exposure resulted from acetazolamide treatment rather than from either a perfusion or ventilation limitation of branchial gas exchange, rates of scaphognathite and heart pumping were measured before and after air exposure (Fig. 5). Both rates were maintained or increased slightly 15–45 min following reimmersion and declined slowly thereafter. No significant differences could be detected between acetazolamide treated and control groups.

Despite these marked differences in acid-base status and bicarbonate ion concentrations, no significant change in osmolality, or in any other ion measured, could be demonstrated to result from air exposure or from either injection treatment. Emersion caused a slight (10%) but not significant hemoconcentration in both control and acetazolamide treated animals. Ca^{++} levels were elevated during air exposure and returned slowly on reimmersion in controls and remained stable during emersion. These changes, however, were slight and not significant. Hemolymph $NH_3+NH_4^+$ levels increased during emersion and decreased slowly upon reimmersion in both groups (Table 5). The apparent $NH_3+NH_4^+$ excretion (i.e. prebranchial minus postbranchial ammonia concentration) also increased during emersion suggesting that some excretion can occur across the air exposed gill. Decreased ammonia levels in serum and decreased prebranchial/postbranchial ammonia differences across the gills following reimmersion may reflect changes in nitrogen metabolism occurring in compensation for imbalance resulting from the air exposed period. Injection of acetazolamide had no effect on the level of ammonia in pre- or postbranchial hemolymph (Table 5).

Discussion

The initial (pretreatment) data provide a more complete picture of hemolymph oxygenation, acid-base and ionic status than was previously available for this species (McMahon and Wilkens 1977; de Fur and McMahon 1984a, b). The data are essentially similar to the record for most other aquatic crabs (see McMahon and Wilkens 1983, for review). With the exception of Ca^{++} (de Fur and

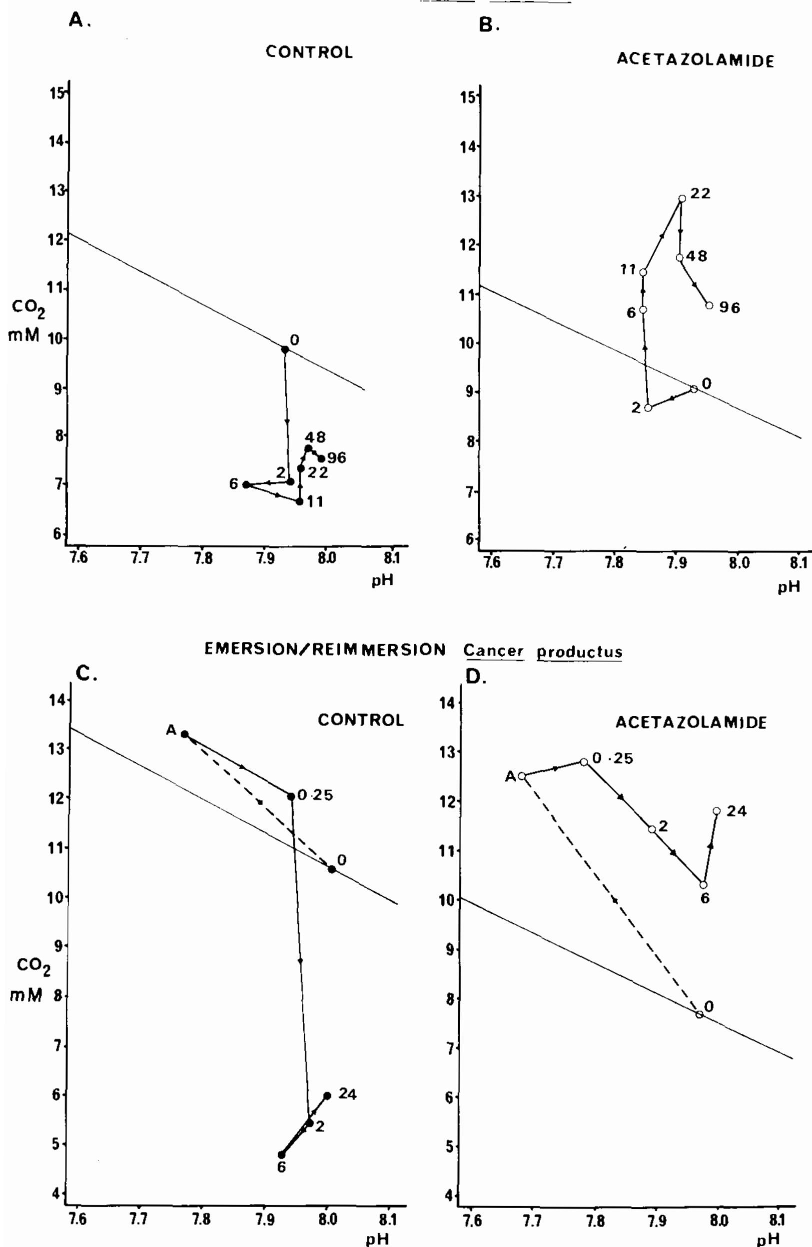


Fig. 4. pH-total CO_2 diagrams showing acid-base changes in **A** control, saline injected (Series II, immersed, closed circles); **B** acetazolamide injected (Series II, emersed, open circles); **C** control, saline injected (Series III, emersed, closed circles); **D** acetazolamide injected crabs (Series III, emersed, open circles). Values are expressed as means. Hours post-treatment (**A** and **B**) or hours post-reimmersion (**C** and **D**) are indicated beside each data point. A = 4 h air exposure, O = prior to injection

McMahon 1984b) hemolymph ion levels have not been reported for this species but are similar to those reported for *C. magister* (Hunter and Rudy 1975). Simultaneous measurements of pre- and postbranchial $NH_3 + NH_4^+$ levels allowing preliminary assessment of the differential needed for branchial elimination of ammonia have previously been reported only for the terrestrial species *Cardisoma carnifex* (Wood and Randall 1981).

The format of the present study provided double controls. The initial oxygenation, acid-base and ionic status of the animals was determined prior to any treatment. In each series of experiments animals received injections of acetazolamide or millipore filtered sea water (control). The control group served to monitor the effects of injection, sampling and other handling procedures. These effects include a slight but nonsignificant increase in oxygen tension and content (Fig. 1A), and a significant decrease in total CO_2 (Fig. 2). Such effects are characteristic of animals at rest but subjected to repetitive sampling (Truchot 1975a; McMahon et al. 1978). Examination of the relevant section of Fig. 4A indicates that this is a fully compen-

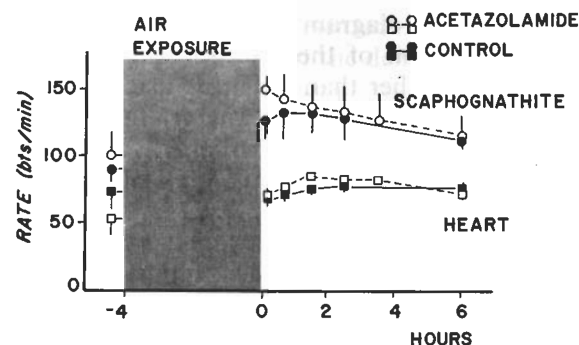


Fig. 5. Scaphognathite and heart rates prior to and following air exposure in control and acetazolamide treated animals. Data are expressed as mean ±SEM

Table 5. Effects of acetazolamide on hemolymph $[NH_3+NH_4^+]$ levels during emersion (Em) and reimmersion (Rim) in *Cancer productus*

	Pre	4 h Em	0.25 h Rim	2 h Rim	6 h Rim	20 h Rim
a) *Control*						
arterial						
x̄	0.40	0.45	0.48	0.38	0.35	0.33
SEM	0.09	0.10	0.11	0.10	0.09	0.09
Venous						
x̄	0.42	0.52	0.50	0.37	0.38	0.33
SEM	0.11	0.13	0.09	0.10	0.09	0.09
N	6	6	6	6	6	6
Δv-a	0.02	0.07	0.02	–	0.03	0
b) *Acetazolamide*						
Arterial						
x̄	0.39	0.41	0.47	0.38	0.39	0.34
SEM	0.05	0.05	0.05	0.04	0.03	0.03
Venous						
x̄	0.42	0.48	0.48	0.42	0.39	0.32
SEM	0.05	0.06	0.04	0.06	0.03	0.05
N	6	6	6	6	4	4
Δv-a	0.03	0.07	0.01	0.04	0	–

sated respiratory alkalosis probably resulting from disturbance and hyperventilation and possibly hyperperfusion of the gills. These effects are probably masked in the other groups by the opposing effects of emersion and/or acetazolamide treatment.

In vitro analysis of several tissues demonstrates CA activity in gill tissue but not hemolymph of *C. productus*. Activity was slightly greater in the more posterior gills but the difference was less than that described for *Callinectes sapidus* (Henry and Cameron 1982).

Analysis of the acetazolamide-induced changes in acid-base status in immersed (Series II) *C. productus* (Fig. 4A and B) shows the development of an initial slight acidosis followed by a rapid compensatory increase in bicarbonate. Since a small and barely significant increase in hemolymph lactate occurs at this time, the initial acidosis is of mixed origin. P_{CO_2}, however, continues to rise and the overall picture emerging after 12 to 24 h is that of a fully compensated respiratory acidosis. Supporting evidence is provided by the very close similarity between the pH-total CO_2 relationships for acetazolamide-treated *C. productus* (present study) and hypercapnic exposed *Carcinus maenas* (Truchot 1975b). In Truchot's study, the difference between water and hemolymph CO_2 remained constant ($Pv_{CO_2}-Pw_{CO_2}=1.8$ Torr) while in the present study, the difference is similar (2.0 Torr) prior to acetazolamide treatment but rises to 4.3 Torr 12 h after treatment (Table 4). Despite the obvious CO_2 retention above, measurements of CO_2 excretion using the method reported by Henry and Cameron (1983) failed to demonstrate significant depression. CO_2 accumulates slowly in the hemolymph and given the difficulty of accurately quantifying very small increments of CO_2 in the flow-through system used by Henry and Cameron (1983), the small depression of M_{CO_2} needed to produce the necessary elevation of P_{CO_2} (Table 4) were below the limits of the technique used. Recent experiments using a more accurate technique (McMahon and Burnett, unpublished results) do demonstrate a significant, but transitory decrease in M_{CO_2} shortly after acetazolamide treatment in SW acclimated *C. sapidus*. A similar time course for inhibition is evident from data from isolated perfused crab gills (Burnett 1983). Clearly the normal mechanisms of CO_2 transport across the gill are impeded by acetazolamide treatment, but we will return to this important point below.

The final series of experiments utilized an emersion-reimmersion sequence which allowed separation of the effects of acetazolamide on acid-base balance in animals with circulatory systems either virtually closed off from or open to the environment. The isolation of the circulatory system during emersion is evidenced by seriously reduced circulating oxygen tensions (Fig. 1B) and oxygen consumption (de Fur and McMahon 1984a). Similarly, P_{CO_2} rises during emersion but the resulting acidosis is more severe than that resulting from hypercapnia alone (compare Fig. 4B and C) for several reasons. Firstly, reduced oxygen uptake effects a switch to anaerobic metabolism and the lactate produced (Fig. 3) adds a substantial metabolic component. Secondly, branchial ion exchange mechanisms which could normally contrib-

ute to acid-base compensation (Truchot 1978; McMahon et al. 1978; Henry and Cameron 1983) are virtually eliminated by air exposure. Several compensatory mechanisms including skeletal buffering (de Fur et al. 1980) and perhaps ionic exchange with the tissues remain.

In the closed system (air exposed crabs) several important differences in response arise from acetazolamide treatment. Total hemolymph CO_2 pools in both control and experimental groups at the end of 3.75 h of air exposure are identical. Differences between the control and the experimental groups are evident, however, in the relative distribution in the total CO_2 pool of dissolved and other species of CO_2. The large and significant elevation of P_{CO_2} in experimental over control groups (Fig. 3) may be related to the greater elevation of lactate during this period. In the acetazolamide-treated group the metabolic acidosis drives part of the bicarbonate pool into the direction of dissolved CO_2. In control animals the same reactions occur, but to a lesser extent, partially because less lactate is produced and perhaps also because acetazolamide inhibits interactions between hemolymph and shell carbonate systems (Giraud 1981). de Fur et al. (1980) first noted that Ca^{++} levels rise during emersion of *C. productus* and postulated that dissolution of shell carbonates could provide additional buffering under these conditions. No significant rise in Ca^{++} could be detected in the present study, but M.S. Haswell (personal communication) has shown that Ca^{++} removed from the shell of crayfish during acid-base disturbance may be taken up by the tissues and thus not necessarily build up in the hemolymph. We, therefore, hypothesize that the greater acidosis and the larger P_{CO_2} change in the treated group is due to a larger metabolic component and to the absence of shell buffering. At the present time we can offer no explanation for the significant elevation of hemolymph lactate effected by acetazolamide treatment. The situation is complex with many possibilities including both direct (i.e. acetazolamide) or indirect (i.e. resultant increases in circulating H^+ and CO_2 levels) effects on either the rate of production, release, or utilization of lactate. Unfortunately little is known of the relevant biochemical pathways in crustaceans and resolution must await much needed work on lactate kinetics.

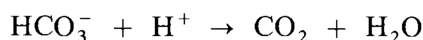
The most dramatic effects of acetazolamide treatment are observed following reimmersion in water, i.e. return to a circulatory system open to the environment. Despite the evident hyperventilation (Fig. 5) occurring in both groups, acid-base effects in the two groups are markedly different. The pH-total CO_2 diagram (Fig. 4C and D) allows some interpretation of the results. This figure is presented here rather than the more usual pH/bicarbonate/P_{CO_2} format popularized by Davenport (1974), since we measured total CO_2 (including carbamate CO_2) and also since a marked discrepancy between measured and calculated P_{CO_2} (see De Fur et al. 1980) occurs during air exposure and thus the P_{CO_2} isopleths of the Davenport diagram are not representative. Interestingly, this discrepancy is abolished by treatment with acetazolamide but apart from suggesting some involvement of CA, we are unable to provide any additional explanation for this phenomenon.

The rapid loss of hemolymph C_{CO_2} following reimmersion in control crabs incorporates two identifiable components, an initial respiratory compensation which is apparent within 15 min and a slower metabolic component which is complete within 2 h (Figs. 3, 4C and D). Acetazolamide virtually abolishes the metabolic component suggesting involvement of CA in this latter step. The fast component probably involves diffusion of molecular CO_2 across the gills down the increased P_{CO_2} gradient induced by air exposure. Examination of the P_{CO_2} data of Fig. 3 and Table 4 suggests that the rate of decline in molecular CO_2 is similar in both control and treated groups except that a substantially larger gradient is needed to power adequate CO_2 excretion in the treated group.

Within the slower component at least three mechanisms involve participation of CA and thus can be blocked by acetazolamide. Firstly, CO_2 could be moved across the gills as HCO_3^-, i.e. an ionic exchange involving the hydration reaction of CO_2. Secondly, CO_2 may move across the gills by the dehydration of hemolymph HCO_3^- to the more readily diffusible CO_2. A third possibility is that $CaCO_3$ stores which had been utilized during air exposure are being replenished on reimmersion resulting in a reduction of hemolymph CO_2.

The data presented and analyzed thus far show that injection of acetazolamide effects a compensated respiratory acidosis but provides no mechanisms. There is no evidence for increased endogenous production of CO_2 and thus the effect presumably results from limitation of CO_2 excretion at the gills. Since neither $NH_3 + NH_4^+$ excretion nor postbranchial hemolymph oxygenation are affected by acetazolamide treatment, we must assume that this is a specific effect on branchial CO_2 excretion. Since acetazolamide is a specific inhibitor of CA (Maren 1967), an enzyme which we have shown to be active in the gills of *C. productus*, we must assume inhibition of this enzyme to be in-

volved. The evidence presented in the present study supports involvement of the dehydration pathway



$$HCO_3^- + H^+ \rightarrow CO_2 + H_2O$$

which is normally used to facilitate CO_2 excretion across respiratory exchange systems of both aquatic (Haswell and Randall 1978; Wood et al. 1982) and aerial (Klocke 1978; Effros et al. 1978; Effros et al. 1980) gas exchange surfaces.

The conclusion that branchial CA is important to the ionoregulatory function of crab gills is well established (Burnett et al. 1981; Henry and Cameron 1983). CA is presumed to catalyze the hydration of CO_2 within the epithelial cell to produce HCO_3^- thus providing substrate for a HCO_3^-/Cl^- ion exchange mechanism. But, as we suggest above, it is also probable that branchial CA (perhaps located on or near the basal membrane, see discussion below) also functions in catalyzing the dehydration of a portion of the hemolymph bicarbonate pool to CO_2 which can more easily diffuse across the respiratory surface (Gutknecht et al. 1977). Aldridge and Cameron (1979) and Henry and Cameron (1983) reject the hypothesis that branchial CA is involved in CO_2 excretion across crab gills, maintaining that this process is driven solely by the P_{CO_2} gradient from hemolymph to water. Aldridge and Cameron (1979) reached this conclusion with the aid of a computer simulation which modeled CO_2 excretion across the gills of the blue crab *Callinectes sapidus*. Using rates typical of the uncatalyzed CO_2 hydration and dehydration reactions, these authors calculated rates of CO_2 excretion similar to those assumed to occur in the animal and thus concluded that branchial CA was not involved. More recently, however, Henry and Cameron (1983) have measured CO_2 excretion directly in *C. sapidus* and obtained values twice those of Aldridge and Cameron's assumption, considerably reducing the accuracy of the model. It is still possible to explain the discrepancy by assuming that the hemolymph CO_2/HCO_3^- system is in a state of chemical disequilibrium favoring molecular CO_2 which is continually being produced by the tissues. This situation could allow the actual concentration of dissolved CO_2 at the gill to be greater than that measured (see Aldridge and Cameron 1979, for discussion) and thus results in a CO_2 efflux greater than predicted. The existence of such a chemical disequilibrium state has yet to be experimentally established but, in fact, its established presence would not preclude a function for CA in CO_2 excretion as postulated in the present study.

In further support of the conclusion that branchial CO_2 excretion is unaided by CA, Henry and Cameron (1983) presented evidence that acetazolamide treatment did not significantly elevate P_{CO_2} in *C. sapidus*. These data, however, were collected from animals acclimated to dilute SW, conditions in which the buildup of hemolymph P_{CO_2} may have been masked by a large ionic disturbance which resulted from the acetazolamide treatment. Henry and Cameron (1983) do not report P_{CO_2} changes following acetazolamide treatment of SW acclimated *C. sapidus* but do report an increase in total CO_2 together with a decrease of pH, results which would normally be associated with elevated P_{CO_2} levels. In fact the data for SW acclimated *C. sapidus* are qualitatively similar to those presented here for *C. productus* and could be construed as supporting, rather than against, a role of branchial CA in CO_2 excretion.

The results of the present study are consistent with those obtained by Burnett (1983) who measured a decrease in CO_2 efflux across artificially perfused crab gills following acetazolamide treatment. Both studies indicate that gill CA dehydrates HCO_3^- from the hemolymph pool to dissolved CO_2. The conversion of hemolymph HCO_3^- to CO_2 serves to maintain the dissolved CO_2 gradient between hemolymph and SW and thus to potentiate CO_2 efflux. Additional recruitment may also occur from the carbamate CO_2 pool, but the role of carbamates in CO_2 transport in crustaceans is poorly understood and cannot be assessed at this time (Truchot 1976; Randall and Wood 1981).

A criticism of the proposed role of branchial CA in CO_2 excretion is that the reaction may be limited by the slow movement of HCO_3^- across the basal membrane of the epithelial cell and its associated unstirred layers (Gutknecht et al. 1977). This problem may be reduced in respiratory epithelia (a) since the rapid and pulsatile hemolymph flow may reduce the effect of unstirred layers on the hemolymph side, and (b) if branchial epithelial CA is concentrated in a region on or directly beneath the basal membrane where it lies very close to the hemolymph as is the case with vertebrate pulmonary endothelial cell CA (Lonnerholm 1982; Ryan et al. 1982). Such a location could allow continuous depletion of HCO_3^- at the epithelial cell basal membrane and hence facilitate HCO_3^- (and H^+) entry.

Whatever the route, molecular CO_2 entering the gill epithelial cell from the hemolymph may follow either one of two pathways. It may simply diffuse directly to the ambient medium as dissolved CO_2 or may become hydrated within the cell to

form H^+ and HCO_3^-. In dilute media where active ion uptake occurs, sufficient quantities of H^+ and HCO_3^- may be removed in exchange for Na^+ and Cl^-, such that HCO_3^- excreted may form a substantial or even major part of the animal's CO_2 efflux. However, in animals in isoionic environments, such as a crab in seawater, relatively little of the CO_2 efflux may be diverted via this route. Since differential movements of Na^+, Cl^-, H^+ and HCO_3^- may also be involved in other branchial functions such as regulation of acid-base status and nitrogenous excretion, the final portioning of CO_2 efflux may represent a complex blending process which balances the animal's particular requirements at that time.

The apparent differences in response to acetazolamide between the euryhaline forms such as *Callinectes sapidus* (Henry and Cameron 1983) and the stenohaline forms such as *C. productus* (McMahon et al.1982 and present study) can be reduced by a reexamination of the previous data. The differences between low and high salinity acclimated animals can be explained by differential use of the hydration pathway to aid ion exchange. Differences between *C. sapidus* and *C. productus* in sea water are of small magnitude only and may stem from the larger and more efficient gill apparatus of the portunid which may allow more efficient transfer of molecular CO_2.

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