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 tis-
sues but not in hemolymph. Crabs injected with acetazolamide, a specific CA inhibitor, demon-
strated a significant hemolymph acidosis. Hemol-
ymph CO2 tension (Pco2H) and CO2 content (Cco2H) also increased and remained significantly
raised for 96 h following treatment. No signifi-
cant changes could be detected in either hemo-
lymph oxygenation or ionic status (except for
HCO3-) as a result of acetazolamide treatment.
Crabs treated with acetazolamide, and also ex-
posed to air, exhibited a more pronounced hemo-
lymph acidosis with significantly increased respira-
tory (Pco2H) 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 CO2 load induced by air exposure. No signifi-
cant differences between experimental and control
groups during air exposure and recovery could be
detected in hemolymph oxygenation, ionic status,
NH3-NH4+ levels or respiratory and cardiac
pumping frequency and so the effects of acetazola-
amide treatment were apparently limited to CO2
removal across the gills. These results indicate that
branchial CA facilitates the removal of CO2 from
the hemolymph of SW adapted C. productus large-
ly by catalyzing the dehydrogenation of hemolymph
HCO3- to molecular CO2 at the gill. It is also rec-
ognized that gill CA may also serve to hydrate
molecular CO2 to H+ and HCO3-, for use as coun-
terions for ionic uptake mechanisms. Crab gill CA
thus appears to play an important role in CO2
excretion as well as hemolymph ionic regulation.

Introduction

The enzyme carbonic anhydrase (CA) is known to
 catalyze both the hydration and the dehydration
reactions of CO2 in biological carbonate systems.
The hydration reaction can be used to provide
counters (H+, HCO3-, OH-) for ion exchange
(Maren 1967) while the dehydration reaction facil-
itates CO2 mobilization and transport across aerial
(Klocke 1975; 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 func-
tion 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 cir-
culating red blood cells in addition to pulmonary
and branchial tissues. In vertebrates, circulating
CA is usually removed and the lung or gill is artifi-
cially perfused to study enzyme function in the re-
spiratory epithelia. The elucidation of the respira-
tory 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-
Iod (Ferguson et al. 1937; Van Goor 1937, 1940; Ebenfeldt 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 CO2 excretion. Burnett et al. (1981) had, however, demonstrated both significant ionic and acid-base changes resulting from acetazolamide treatment in another euryhaline crab Potamonastre crusoe, 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 CO2 to HCO3- 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 CO2 excretion is driven solely by the Pco2 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 CO2 excretion process. McManus and Burnett (1981) demonstrated that acetazolamide treatment causes significant CO2 retention in air exposed Potamonastrea crusoe and both significant CO2 retention and acidoses in a similar shore crab, Hemi-

trax radiata, 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 CO2 retention following acetazolamide treatment in the land crab Cardisoma gar-

nites.

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 other-

wise 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 (McManus and Wilkens 1977; deFur and McManus 1984a, b).

Materials and methods

Cancer productus of average mass 450±2.0 g (SEM) were collected using crab pots in Grapetree and Barnfield troughs on the west coast of Vancouver Island. In the Bamfield Marine Station where the 30% seawater was maintained, 30Lt tanks containing tanks were held in large 500 gallon aquaria in flowing sea water at 11±0.5°F, 31±3°C and main 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 Burn-
net et al. (1981). Briefly this method consists of following the rate of appearance of protons when saturating amounts of CO2 are added to tissue homogenates. 1 ml of homogenate was add-
ed to 24 ml of 20 mM Barbitol buffer and the rate of decline in pH between 8.1 and 8.0 was followed when 10ml of CO2 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 ho-

genate 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 (Dia-

mex, Leader) dissolved in millipore filtered sea water or an equal volume of sea water. The amounts were calculated to serve as controls. The amount of acetazolamide injected was not more than 0.05 mg kg-1 animal. 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 definitive statement concerning the level of suppression of CA activity in vivo, other than that the activity was inhibited to bring about the results outlined. Hemolymph was sampled from the animals at 1, 2, 4, 6, 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 syringes and homogenized. Aliquots for assay of oxygen tension (PaO2), oxygen content (CaO2), pH, carbon dioxide tension (PaCO2) and carbon dioxide content (CaCO2) for the final set of assays (0.3 ml) were taken from hemolymph, ionic, and metabolic analysis.

<table>
<thead>
<tr>
<th>Na⁺</th>
<th>K⁺</th>
<th>Ca²⁺</th>
<th>Mg²⁺</th>
<th>Cl⁻</th>
<th>HCO₃⁻</th>
<th>mOsm/kg*</th>
</tr>
</thead>
<tbody>
<tr>
<td>474</td>
<td>6.8</td>
<td>10.9</td>
<td>61.8</td>
<td>N.D.</td>
<td>507</td>
<td>2.30</td>
</tr>
<tr>
<td>65</td>
<td>0.2</td>
<td>1.9</td>
<td>1.0</td>
<td>N.D.</td>
<td>2</td>
<td>0.84</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>N.D.</td>
<td>7</td>
<td>2</td>
</tr>
</tbody>
</table>

* Determined immediately, remainder stored frozen for winter samples

A third series of experiments (Series III), performed using a subgroup of 15 animals of mass 368±36 g (SEM), was immersed at 4 h air exposure period to induce additional hemolymph CO₂ 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 arthrobranchial membrane of the proximal joint of the first peripodite. 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 30 h after 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.

cpH, pCO₂, and pO₂ were measured using a Radiometer electrode thermomitted to 11.5±0.1°C. The pH electrode was calibrated with Radiometer huffers 1500 and 3150. The oxygen electrode was calibrated using nitrogen and argon equilibrated sea water. The pCO₂ electrode was calibrated using millipore filtered sea water equilibrated with 0.2 and 1.0% CO₂ mixed with air by Wösthoff gas mixing pumps. Full details of the methods used for calibrating the pCO₂ electrode and measuring pCO₂ are given in deFur et al. (1998). Electrode output was amplified and displayed on a Radiometer PHM71 or PHM72 acid-base analyzer system. For measuring pCO₂, a 30 s scale expansion was used to ensure accuracy of measurement of the low pCO₂ found in these cold water species. Oxygen content was measured using a Lea-Oxson oxygen analyzer (Lexington Instruments) with a modified technique as described by McMuhol et al. (1978). CO₂ content was measured on 40 μl hemolymph samples using the microchamber of Cameron (1979). Total CO₂ content is taken to mean measured CO₂, plus CO₂ (Cameron 1979) and also including carbonate CO₂ since the tissues used in these measurements are made (pH 1-2) is below that of the Pk for carbonate formation (pK = 4.85; Roughin and Ross-Bernardi 1966; Roughin 1979) effecting the release and concentration of CO₂ bound in the carbonate. Hemolymph samples were blanked with samples of each animal's individual sea water. Hemolymph samples were processed with the use of sea water, 80 μl samples and standards of 1.5 mM biacarbonate were substituted. Even so, increased variability was encountered and S-10 replicates were measured to improve accuracy of the final value.

**Table I. Ionic content of Barnfield sea water mus 1**

<table>
<thead>
<tr>
<th>Na⁺</th>
<th>K⁺</th>
<th>Ca²⁺</th>
<th>Mg²⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>474</td>
<td>6.8</td>
<td>10.9</td>
<td>61.8</td>
</tr>
<tr>
<td>65</td>
<td>0.2</td>
<td>1.9</td>
<td>1.0</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>4</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cl⁻</th>
<th>HCO₃⁻</th>
<th>mOsm/kg*</th>
</tr>
</thead>
<tbody>
<tr>
<td>507</td>
<td>2.30</td>
<td>930</td>
</tr>
<tr>
<td>2</td>
<td>0.84</td>
<td>6</td>
</tr>
<tr>
<td>7</td>
<td>2</td>
<td>7</td>
</tr>
</tbody>
</table>

Results

**Series I - carbonic anhydrase activity in various tissues**

Significant CA activity was found in hepatopancreas, male gonads, heart muscle and all gills, while epididymis (taken from underneath the carapace) and tissues from the chelae, the exoskeleton of the walking legs and the thoracic sternite 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. Carboxic anhydrase activity of Cancer productus tissues. Enzyme activity is expressed as mg⁻¹ according to the formula:

\[
\text{activity} = \frac{\text{rate of reaction}}{\text{enzyme concentration}}
\]

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Enzyme activity</th>
<th>ϵ</th>
<th>SEM</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatoepithelial</td>
<td>0.702</td>
<td>0.151</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Gonad (male)</td>
<td>0.196</td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Gill*</td>
<td>0.204</td>
<td>0.094</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.182</td>
<td>0.079</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.320</td>
<td>0.090</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.163</td>
<td>0.033</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.256</td>
<td>0.056</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.201</td>
<td>0.065</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>0.293</td>
<td>0.094</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>0.205</td>
<td>0.062</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>0.367</td>
<td>0.139</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Branchial cavity epithelium</td>
<td>0.037</td>
<td>0.023</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Muscle: Heart</td>
<td>0.070</td>
<td>0.070</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Cheliped</td>
<td>0.014</td>
<td>0.014</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Walking leg (mesosoma)</td>
<td>0.014</td>
<td>0.014</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Thoracic sterna</td>
<td>0.022</td>
<td>0.017</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Hemolymph</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

No measurable enzyme activity could be detected following incubation of tissue homogenates in 4 x 10⁻⁷ M acetazolamide.

* Gill samples 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 differences in oxygenation, acid-base or ionic status between groups or tissues. Mean levels (Table 1) of O₂ were not significantly different from values previously published for this species (McMahon and Wilkins 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 effects on hemolymph oxygenation. Slight increases in both oxygen tension (Pₒ₂) and oxygen content (Cₒ₂) of postbranchial hemolymph were observed following either injection (Fig. 1A) and were probably associated with disturbance resulting from handling and injection procedures.

Values for concentration of Na⁺, K⁺, Mg⁺⁺, Ca⁺⁺, Cu⁺², Cl⁻, HCO₃⁻, lactate and nonvolatile in Table 1. Hemolymph ion levels have not been reported previously for C. productus but are essentially similar to those reported for C. rugifer 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⁺² and HCO₃⁻ levels are higher. Injection of either acetazolamide or saline induced no detectable change in any ion but HCO₃⁻ (see below).

Injection of saline caused a significant decrease in hemolymph pH in each of the series (Pₚ < 0.05). A slight depression of Pₒ₂ in the first 2 h but no further significant change in acid-base status (Fig. 2). 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 (Pₒ₂) is significantly elevated above preinjection values for 96 h following injection. Control group Pₒ₂ levels rose slowly after 10-12 h. Treatment group Pₒ₂ levels were significantly elevated over equivalent control

Table 3. Respiring oxygenation and acid-base status of Cancer productus hemolymph

<table>
<thead>
<tr>
<th>Treatment</th>
<th>pH</th>
<th>Pₒ₂ (Tor)</th>
<th>Cₒ₂ (mmol -1 l⁻1)</th>
<th>NH₄⁺ (mmol -1 l⁻1)</th>
</tr>
</thead>
</table>

| Postbranchial | 7.962  | +0.015    | 0.740             | 11                 |
| Prebranchial  | 0.20   | 0.017     | 0.02              | 0.016              |

* Includes controls for Series II plus Series III

Figures 1–4: Changes in hemolymph pH, Pₒ₂, Cₒ₂, and NH₄⁺ following injection of saline or acetazolamide (Cₐ) in Cancer productus.
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 $C_{\text{CO}_2}$ (Fig. 2). $C_{\text{CO}_2}$ 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 $C_{\text{CO}_2}$ 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 of air exposure showed significant and severe depletion of both pre- and postbranchial oxygen tensions and oxygen content (P$_{\text{O}_2}$, C$_{\text{O}_2}$, 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
postbranchial hemolymph which substantially overshoot initial values from 2 to at least 6 h following immersion 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 acidic at 2 h but with full recovery occurring by 6 h post reimmersion (Fig. 3).
Table 4. Differences between mean hemolymph Pco_{2} and calculated ambient water (air) Pco_{2} (at 0.3 Torr) in control and acetazolamide treated crusts

<table>
<thead>
<tr>
<th>Control</th>
<th>Acetazolamide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pretreatment</td>
<td>2.0</td>
</tr>
<tr>
<td>1-2</td>
<td>1.6</td>
</tr>
<tr>
<td>5-6</td>
<td>2.0</td>
</tr>
<tr>
<td>10-12</td>
<td>1.1</td>
</tr>
<tr>
<td>21-24</td>
<td>2.5</td>
</tr>
<tr>
<td>48</td>
<td>1.9</td>
</tr>
<tr>
<td>94</td>
<td>1.7</td>
</tr>
</tbody>
</table>

b) Emerged - reimmersed

Pre-emersion A

| | 1.3 | 1.8 |
| 3.75 m (emission air) | 2.7 | 6.0 |
| Hours reimmersion | 0.25 | 2.1 |
| 2 | 0.4 | 4.5 |
| 6 | 0.4 | 2.8 |
| 24 | 0.6 | 2.3 |

Associated with the more rapid restoration of \( \text{pH} \) in the controls, \( \text{Pco}_{2} \) levels of both pre- and postbrachial 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 \( \text{Pco}_{2} \) and \( \text{Pco}_{2} \) had fallen to levels significantly below initial values (Fig. 3) where they remained for the rest of the experimental period. The time course for \( \text{Pco}_{2} \) decrease was similar in the acetazolamide treated group but these did not decrease to initial levels until 6 h and remained significantly elevated over equivalent controls for at least 24 h. These elevated \( \text{Pco}_{2} \) levels greatly increased the gradient between \( \text{Pco}_{2} \) and ambient water (Table 4).

Hemolymph lactate levels were significantly elevated above controls both during and after 15 min following 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 \( \text{CO}_{2} \) levels following reimmersion (Fig. 3 and 4). In control crabs \( \text{CO}_{2} \) levels dropped rapidly reaching values not significantly above initial values within 15 min and, as with \( \text{Pco}_{2} \), declined significantly below these initial values by 2 h. Some recovery occurred slowly but by 24 h \( \text{CO}_{2} \) levels were still significantly depressed. A quite different picture resulted in the acetazolamide-treated animals. Little or no decrease in \( \text{CO}_{2} \) occurred in either pre- or postbrachial hemolymph. \( \text{CO}_{2} \) levels remained significantly elevated above both initial and equivalent control values for at least 24 h. To further test the hypothesis that the prolonged \( \text{CO}_{2} \) retention observed following air exposure resulted from acetazolamide treatment rather than from 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. \( \text{Ca}^{++} \) 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 \( \text{NH}_{3} + \text{NH}_{4}^{+} \) levels increased during emersion and decreased slowly upon reimmersion in both groups (Table 5). The apparent \( \text{NH}_{3} + \text{NH}_{4}^{+} \) excretion (i.e. prebranchial minus postbranchial ammonia concentration) also increased during emersion suggesting that some excretion can occur across the air exposed gills. 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 Wilken 1977; de Fur and McMahon 1984a, b). The data are essentially similar to the record for most other aquatic crabs (see McMahon and Wilken 1983, for review). With the exception of \( \text{Ca}^{++} \) (de Fur and
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 \( \text{NH}_3 + \text{NH}_4^+ \) levels allowing preliminary assessment of the differential need for branchial elimination of ammonia have previously been reported only for the terrestrial species Cardisoma carnifex (Wood and Rastall 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 multi-pore 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 \( \text{CO}_2 \) (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-
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 aceatalomide 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 aceatalomide-induced changes in acid-base status in immersed (Series II) *C. pro-


ductor (Fig. 4A, and B) shows the development of an initial slight acidosis followed by a rapid compensatory increase in base excess. Since a small and barely significant increase in hemolymph lactate occurs at this time, the initial acidosis is of mixed origin. *P*<0.05; 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 and CO2 relationships for aceatalomide-treated *C. productus* (present study) and hypercapnic exposed *C. sapidus* (Tru-

chot 1975b). In Truchot's study, the difference between water and hemolymph CO2 remained constant (PO2=PO2=1.8 Torr while in the present study, the difference is similar (2.0 Torr) prior to aceatalomide treatment but rises to 4.3 Torr 12 h after treatment (Table 4). Despite the obvious difference in CO2 retention above, measurements of CO2 excre-

tion using the method reported by Henry and Cam-

eron (1983) failed to demonstrate significant de-

pression. CO2 accumulates slowly in the hemo-
lymph and given the difficulty of accurately quan-
tifying very small increments of CO2 in the flow-
through system used by Henry and Cameron (1983), the small depression of MCO2 needed to pro-

duce the necessary elevation of Pco2 (Table 4) were below the limits of the technique used. Recent ex-

periments using a more accurate technique (McMahon and Barlett, unpublished results) do demonstrate a significant, but transitory decrease in MCO2 shortly after aceatalomide treatment in SW acclimated *C. sapidus*. A similar time course for inhibition is evident from data obtained from perfused gill beds (Burnett 1983). Clearly the nor-
mal mechanisms of CO2 transport across the gill are impeded by aceatalomide treatment, but we will return to this important point below.

The results utilize an emersion-reimmersion sequence which allowed separa-
tion of the effects of aceatalomide 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 was evidenced by seriously reduced cir-
culating oxygen tensions (Fig. 1B) and oxygen consumption (de Fur and McMahon 1984a). Simi-

larly, Pco2 rises during emersion but the resulting increase in MCO2 is not associated with emersion alone (compare Fig. 4B and C) for several reasons. Firstly, reduced oxygen uptake ef-

fects a switch to anaerobic metabolism and the lactate produced (Fig. 3) adds a substantial meta-
bolic component. Secondly, branchial ion ex-

change mechanisms which could normally contrib-

Table 5. Effect of aceatalomide on hemolymph [NH3·+H+] levels during emersion (Em) and reimmersion (Rim) in Cancer productus

<table>
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b. Aceatalomide

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to acid-base compensation (Truoch 1978; McMahon et al. 1978; Henry and Cameron 1985) 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 acids drive 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$^{2+}$-levels rise during emersion of C. prothetos and postulated that solution of shell carbonate could provide additional buffering under these conditions. No significant rise in Ca$^{2+}$-levels could be detected in the present study, but M.S. Haswell (personal communication) has shown that Ca$^{2+}$-removal 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$_{CO_2}$ changes 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 kinematics. The most dramatic effects of acetazolamide treatment are observed following reinmersion 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/CO$_2$ format popularized by Davenport (1974), since we measured total CO$_2$ including carbonate 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 that CA, we are unable to provide any additional explanation for this phenomenon.

The rapid loss of hemolymph 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 reinmersion resulting in a reduction of hemolymph CO$_2$. We presented and analyzed data that far show that injection of acetazolamide effects a compensated respiratory acidosis but provides no mechanism. There is no evidence for increased endogenous production of CO$_2$ and thus the effects presumably result from elimination of CO$_2$ excretion at the gills. Since neither NH$_4$+ nor lactate are excreted nor postbranchial hemolymph oxygenation 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 (Marden 1967), an enzyme which we have shown to be active in the gills of C. prothetos, we must assume inhibition of this enzyme to be in-
volvolved. The evidence presented in the present study supports involvement of the dehydration pathway

\[ \text{HCO}_3^- + \text{H}^+ \rightarrow \text{CO}_2 + \text{H}_2\text{O} \]

which is normally utilized to facilitate CO₂ 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 CO₂ within the epithelial cell to produce HCO₃⁻ thus providing substrate for a HCO₃⁻/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 CO₂ 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 CO₂ excretion across crab gills, maintaining that this process is driven solely by the F₀₋Fᵢ gradient from hemolymph to water. Aldridge and Cameron (1979) reached this conclusion with the aid of a computer simulation which modeled CO₂ excretion across the gills of the blue crab Callinectes sapidus. Using rates typical of the uncatalyzed CO₂ hydration and dehydration reactions, these authors calculated rates of CO₂ 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 CO₂ 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 CO₂/HCO₃⁻ system is in a state of chemical disequilibrium favoring molecular CO₂ which is continually being produced by the tissues. This situation could allow the actual concentration of dissolved CO₂ at the gill to be greater than that measured (see Aldridge and Cameron 1979, for discussion) and thus results in a CO₂ 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 CO₂ excretion as postulated in the present study.

In further support of the conclusion that branchial CO₂ excretion is unaided by CA, Henry and Cameron (1983) presented evidence that acetzolamide treatment did not significantly elevate F₀₋Fᵢ in C. sapidus but did report an increase in total CO₂ together with a decrease of pH, results which would normally be associated with elevated F₀₋Fᵢ 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 CO₂ excretion.

The results of the present study are consistent with those obtained by Burnett (1983) who measured a decrease in CO₂ efflux across artificially perfused crab gills following acetzolamide treatment. Both studies indicate that gill CA dehydrates HCO₃⁻ from the hemolymph pool to dissolved CO₂. The conversion of hemolymph HCO₃⁻ to CO₂ serves to maintain the dissolved CO₂ gradient between hemolymph and SW and thus to potentiate CO₂ efflux. Additional recruitment may also occur from the carbamate CO₂ pool, but the role of carbonates in CO₂ 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 CO₂ excretion is that the reaction may be limited by the slow movement of HCO₃⁻ across the basement 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 endoepithelial cell CA (Lomnserholm 1982; Ryan et al. 1982). Such a location could allow continuous depletion of HCO₃⁻ at the epithelial cell basal membrane and hence facilitate HCO₃⁻ (and H⁺) entry.

Whatever the route, molecular CO₂ 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 CO₂ or may become hydrated within the cell to
form H+ and HCO3-. In dilute media where active ion uptake occurs, sufficient quantities of H+ and HCO3- may be removed in exchange for Na+ and Cl-, such that HCO3- excreted may form a substantial or even major part of the animal's CO2 efflux. However, in animals in isosmotic environments, such as a crab in seawater, relatively little of the CO2 efflux may be diverted via this route. Since differential movements of Na+, Cl-, H+, and HCO3- may also be involved in other branchial functions such as regulation of acid-base status and astrogastonic excretion, the final portioning of CO2 efflux may represent a complex blending process which balances the animal's particular requirements at any 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 (McMahan et al. 1982 and present study) can be reexamined 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 gas apparatus of the portunid which may allow more efficient transfer of molecular CO2.

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