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Carbon Dioxide Excretion in the Land Crab *(Cardisoma carnifex)*

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ABSTRACT The hemolymph of *Cardisoma carnifex* shows a small Haldane effect and some CO_2 is probably bound to blood proteins. Carbonic anhydrase activity is located in the gill epithelium but is absent from the blood. Inhibition of carbonic anhydrase activity in vivo by injection of 50 mg/kg Diamox into the infrabranchial sinus causes a prolonged increase in blood P_{CO_2} and the P_{CO_2} gradient across the gills. Both M_{O_2} and M_{CO_2}, however, are maintained at normal rates during rest and severe exercise. The gill diffusing capacity for CO_2 is reduced by Diamox treatment, and it is concluded that the catalysed bicarbonate dehydration reaction within the gill epithelium contributes to the pool of excreted CO_2. During exercise the CO_2 content of the blood is decreased, and bicarbonate is probably mobilized from other sources in the body. It is possible that Diamox interferes with this process.

The gills of crabs have been reported to contain carbonic anhydrase activity (Maren, '67; Aldridge, '77), and this activity has been coupled to Na^+/H^+ and HCO_3^-/Cl^- exchange mechanisms (Cameron, '79) considered to be present in crab gills (Cameron, '78a, b). There is no evidence for carbonic anhydrase activity in the hemolymph but hemocyanin has both a Bohr shift and a Haldane effect (Truchot, '76a, b). The blood occupies about 30% of body volume in crabs (Gleeson and Zubkoff, '77) and contains large CO_2 stores. There are large changes in the rate of CO_2 excretion, the gas exchange ratio and blood CO_2 content during severe exercise in the land crab, *Cardisoma* (Wood and Randall, '81a, b). In order to understand these changes we decided to attempt to elucidate patterns of CO_2 excretion in these crabs. Our principal experimental tool was to use Diamox (acetazolamide), a carbonic anhydrase inhibitor, and follow changes in CO_2 levels and excretion during rest and exercise in the crab.

METHODS

Unless otherwise stated, the methods used were as described in Wood and Randall ('81a). Experiments were carried out on 16 crabs weighing between 100 and 350 g at 25 ± 1°C. Arterial and venous hemolymph pH, P_{CO_2} and total CO_2 (C_{CO_2}) were measured before and 12–14 hours after the injection of 50 mg/kg Diamox (acetazolamide, Lederle Pharmaceutical) in 2.5 ml/kg of land crab saline (Skinner et al., '65) into the infrabranchial sinus. The crabs were exercised for 10 minutes at 0.5 body length per second (severe exercise) on the treadmill 12–14 hours after Diamox treatment. Blood samples were taken before and immediately after exercise. Resting and 0–0.25 hours postexercise oxygen uptake ($\dot{M}_{O_2}$) and carbon dioxide excretion ($\dot{M}_{CO_2}$) were measured as described in Wood and Randall ('80a) in these Diamox-treated crabs.

Carbonic anhydrase activity was determined in various crab tissues, including the gills, using a manometric method as described by Haswell and Randall ('76). The tissues (0.31–3.57 g) were frozen in 6 ml of crab saline and transported from Palau to Vancouver. Each sample was thawed, 5 ml of 300 mM sucrose was added, and the total was homogenized and then centrifuged at 20,000g for 35 minutes at 4°C. The supernatant was then centrifuged at 100,000g for 20 minutes at 4°C, and the microsomal pellet obtained was resuspended in 500 μl of Cortland saline (Wolf, '63). The supernatant and the resuspended microsomal fraction were analysed for carbonic anhydrase activity. Cortland saline was used as an enzyme-free control. Total protein was determined using a modified biuret method (Accu-Stat, Clay Adams, Parsippany, New Jersey) with albumin as a standard.

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Carbon dioxide blood curves were constructed in vitro by equilibrating blood in a tonometer with humidified gas mixtures supplied using Wosthöff gas mixing pumps. The total CO_2 content (C_{CO_2}) and pH of the blood were measured in vitro at 7.3, 14.6, 29.2, and 58.4 torr in the presence (P_{O_2} = 153 mm Hg) and absence of oxygen.

All data, unless otherwise indicated, are reported as means ± 1 standard error and "n" represents the number of different animals contributing to the mean. The significance ($P \leq 0.05$) of changes within an experimental group was determined by the Student's paired two-tailed t-test, using each crab as its own control. Differences between groups were assessed by the unpaired Student's two-tailed t-test.

RESULTS

Figure 1 is a CO_2 content curve obtained in vitro for the hemolymph of *Cardisoma*. There is a small Haldane effect; oxygenation of hemocyanin causes the dissociation of protons from hemocyanin, reducing blood pH. The result is the dehydration of bicarbonate and therefore a reduction in total CO_2 content of the blood in this open in vitro system. The regression lines relating pH and total CO_2 for oxygenated and deoxygenated hemolymph were not significantly different. Oxygenated blood, however, generally contained less CO_2 than deoxygenated blood at the same pH, indicating some CO_2 binding to hemocyanin (Table 1). Thus oxygenation of hemocyanin probably results in the release of both protons and CO_2 from hemocyanin. The importance of this in vivo is not known, but in both cases the effect on CO_2 excretion is likely to be small.

The gill epithelium contains some carbonic anhydrase activity, whereas no activity was detected in any other tissue examined (Table 2). In fact, tissue samples other than the gills inhibited CO_2 release into the gas phase. The addition of protein to the assay chamber can cause foaming and this may retard the release of CO_2 into the gas space, thus reducing the apparent rate of CO_2 production (Motais, personal communication), and this may explain

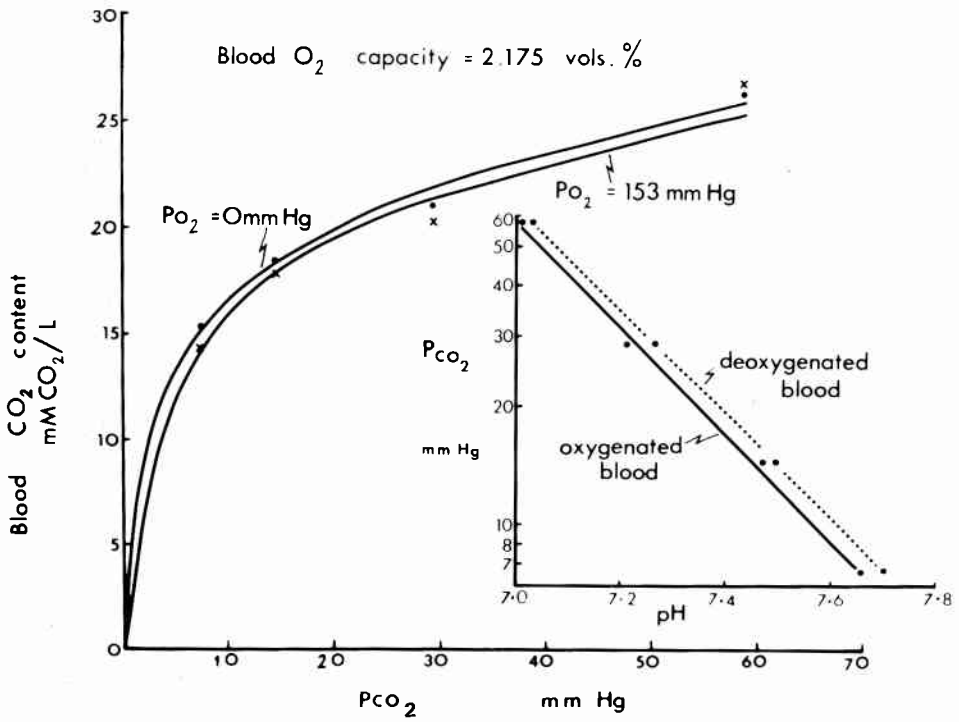

Fig. 1. The effects of oxygenation of hemocyanin on the CO_2 content of in vitro hemolymph of the land crab. Blood obtained from three individuals. (T, 25°C.) Hemolymph was equilibrated in vitro with gases containing from 1% to 8% CO_2 mixed with either air (oxygenated blood) or nitrogen (deoxygenated blood). Each point is the mean of duplicate values obtained at each CO_2 level.

the negative results obtained with samples other than the gills in comparison with saline. Thus the values reported for the gills are probably underestimates of the actual carbonic anhydrase activity.

Preliminary experiments indicated that the injection of Diamox (acetazolamide), a carbonic anhydrase inhibitor, into crabs caused a prolonged rise in blood P_{CO_2} and fall in blood pH (Fig. 2). As a result of these initial observations, it was decided to make measurements 12–14 hours after the injection of Diamox in order to permit recovery by the crab from the procedure and allow time for wide distribution of the drug.

Diamox treatment results in a significant increase in both Pa_{CO_2} and Ca_{CO_2}, but little change in pH (Table 3) in resting crabs. The ability of the crab to maintain or increase oxygen uptake (M_{O_2}) and carbon dioxide excretion during rest and severe exercise was not significantly affected (Table 4). There was, however, a significant reduction in the gas exchange ratio, R, immediately following exercise. There was also a more marked increase in arterial and venous P_{CO_2} in crabs injected with Diamox compared with noninjected but exercising crabs (Fig. 3). There was, however, no significant difference in blood C_{CO_2} between these two groups of crabs, and, as expected, blood pH was also reduced; the change, however, was not statistically significant (Table 5). The time to fatigue during severe exercise was not significantly different in Diamox-treated and -untreated crabs, being 4.07 ± 0.7(8) minutes and 4.62 ± 0.68(7) minutes, respectively.

There were marked increases in the mean P_{CO_2} gradient across the gills during both rest and following exercise in crabs treated with Diamox (Table 6). This increased gradient maintained CO_2 excretion following carbonic anhydrase inhibition; that is, there was no dif-

TABLE 1. The relationship between pH and total CO_2 (C_{CO_2}) in hemolymph equilibrated in vitro with gases containing from 1% to 8% CO_2 mixed with either air (oxygenated hemolymph) or nitrogen (deoxygenated hemolymph)

Oxygenated hemolymph		Deoxygenated hemolymph	
pH	C_{CO_2}	pH	C_{CO_2}
7.652	14.33	7.699	15.21
7.471	17.71	7.491	18.18
7.214	20.14	7.265	20.85
7.011	26.64	7.036	26.22
$pH = 8.375 - 0.053\ C_{CO_2}$ ($r = 0.971$)		$pH = 8.588 - 0.060\ C_{CO_2}$ ($r = 0.988$)	

The hemolymph was obtained and mixed from three crabs. Each point is the mean of duplicate values obtained at each CO_2 level. The same data are plotted in Figure 1.

TABLE 2. Carbonic anhydrase activity in various tissues of the land crab, Cardisoma carnifex

	Carbonic anhydrase activity $E = \frac{K_C - K_O}{K_O}$ 100 µl samples		Protein µg/ml		CA activity per mg protein	
Sample	Microsomal	Supernatant	Microsomal	Supernatant	Microsomal	Supernatant
Gills	+0.054	+0.038	711.8	1416.5	+0.759	+0.268
Lateral branchial epithelium	−0.024	−0.014	1062.4	1422.1	−0.226	−0.098
Central branchial epithelium	−0.147	−0.043	738.5	1440	−1.991	−0.299
Hemolymph	−0.049	−0.033	1395.8	1423.3	−0.351	−0.232

K_O = rate of control sample; K_C = rate of experimental sample. Duplicate measurements were made on four samples of each tissue; means of all measurements for each tissue are presented.

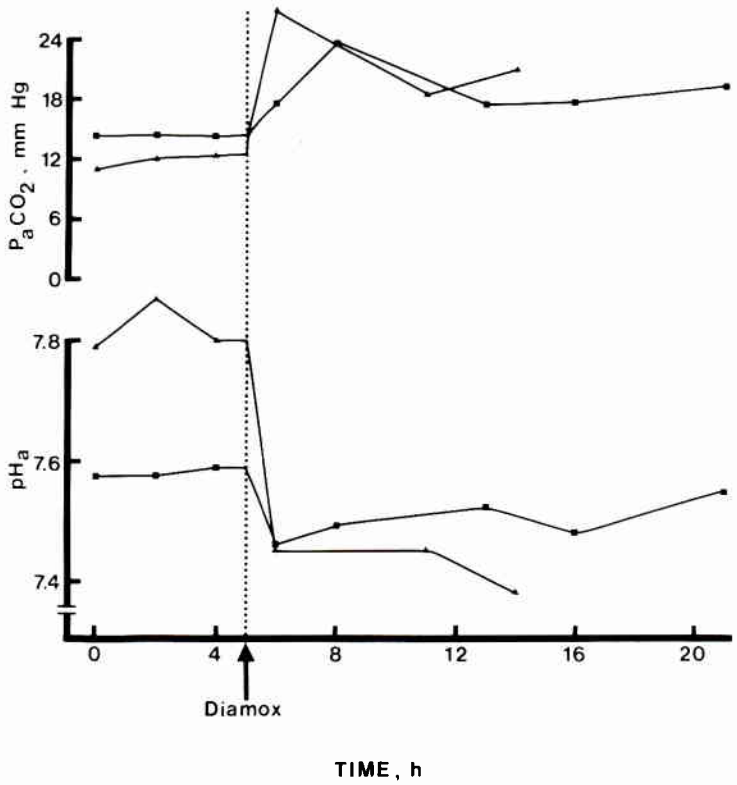

Fig. 2. The effects of an intravascular injection of Diamox (50 mg/kg) on blood pH and $PaCO_2$ on two individual crabs.

TABLE 3. The effect of Diamox injections on arterial blood pH (pHa); total CO_2 (Ca_{CO_2}) and carbon dioxide tension (Pa_{CO_2}) in resting crabs

	pHa	Ca_{CO_2} mM/L	Pa_{CO_2} mm Hg
Control (n = 8)	7.540 ±0.028	17.62 ± 0.78	14.5 ± 1.15
12–14 hours after injecting 50 mg/kg Diamox (n = 8)	7.508 ±0.018	21.39 ± 1.79	17.7 ± 1.67
P =	n.s.	0.02	0.01

Control and experimental values were compared using a two-tailed t-test; n.s. = nonsignificant, P > 0.05.

ference in the rate of CO_2 excretion between noninjected and Diamox-treated crabs. The gill diffusing capacity for carbon dioxide, defined as the amount of CO_2 excreted per unit P_{CO_2} different across the gills, was reduced, therefore, following Diamox injections into crabs (Table 6).

DISCUSSION

Carbonic anhydrase activity has been reported to exist in the gills of many crabs (Maren, '67; Aldridge, '77), but it has been concluded that the enzyme is involved in ion transfer (Cameron, '79) rather than CO_2 excretion because blood pH and C_{CO_2} were unchanged by Diamox treatment (Aldridge and Cameron, '79; Cameron, '79). Our results are in agreement with those of Aldridge and Cameron in that Diamox injections had little effect on blood pH in the land crab, *Cardisoma;* however, our conclusions are different because our investigations were more extensive and show

TABLE 4. The effect of Diamox injections on oxygen uptake, $\dot{M}_{O_2}$; carbon dioxide excretion, $\dot{M}_{CO_2}$; and the gas exchange ratio, R, during rest and immediately following severe exercise

	Rest			Severe exercise		
	$\dot{M}_{O_2}$	$\dot{M}_{CO_2}$	R	$\dot{M}_{O_2}$	$\dot{M}_{CO_2}$	R
Control (n = 5)	53.28 ± 4.65	32.19 ± 3.13	0.60 ±0.01	137.71 ± 17.06	164.87 ± 26.17	1.17 ±0.05
12–14 hours after injecting 50 mg/kg Diamox (n = 5)	48.11 ± 2.65	28.26 ± 2.48	0.61 ±0.03	167.77 ± 10.31	136.57 ± 13.44	0.81 ±0.05
P =	n.s.	n.s.	n.s.	n.s.	n.s.	0.001

Data expressed in ml/kg/hr at STP. Control and experimental values were compared using a paired two-tailed t-test; n.s. = nonsignificant, P > 0.05.

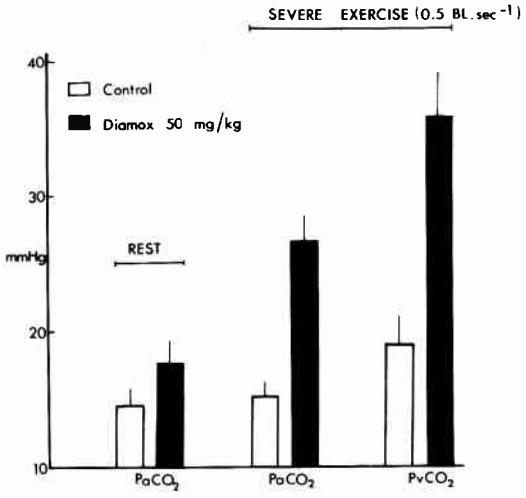

Fig. 3. The effects of an intravascular injection of Diamox (50 mg/kg) on mean $PaCO_2$ and $PvCO_2$ in crabs during rest and immediately following severe exercise. Vertical bars represent + 1 S.E.M.

TABLE 5. Total CO_2 (C_{CO_2}), pH, and P_{CO_2} in arterial (a) and venous (v) blood of crabs following ten minutes' severe exercise in control crabs and crabs injected with Diamox 12–14 hours before the exercise period

	pHa	pHv	ε_{CO_2a} mM/L	ε_{CO_2v} mM/L	Pa_{CO_2} mm Hg	Pv_{CO_2} mm Hg
Control (n = 8)	7.339 ±0.058	7.240 ±0.066	16.36 ± 2.44	16.88 ± 2.54	15.1 ± 1.0	19.0 ± 2.0
50 mg/kg Diamox	7.218 ±0.036	7.098 ±0.026	17.10 ± 1.48	17.74 ± 0.49	26.7 ± 1.9	35.9 ± 3.1
n	5	7	5	7	5	7
P	n.s.	n.s.	n.s.	n.s.	0.001	0.001

TABLE 6. The mean CO_2 gradient (A) and the gill diffusing capacity (B) during rest and immediately following exercise in untreated and Diamox injected (50 gm/kg) crabs

	Rest	Following severe exercise
A. Mean P_{CO_2} gradient across the gills (mm Hg)		
Control	8	14
Diamox	11	28
B. Gill diffusing capacity $ml \cdot kg^{-1} \cdot hr^{-1} \cdot mm\ Hg^{-1}$		
Control CO_2	4.024	11.776 (× 2.93)
Diamox CO_2	2.569	4.878 (× 1.90)
Control O_2	0.543	1.091 (× 2.00)

The mean gradient was determined by subtracting the observed level in the branchial chamber from the mean blood value (Pa_{CO_2} + $Pv_{CO_2}/2$). In the case of control CO_2 values only Pa_{CO_2} was measured and therefore used. The gill diffusing capacity was determined by dividing the CO_2 uptake (ml/kg/hr) by the mean CO_2 gradient (mm Hg).

TABLE 7. Carbon dioxide excretion in crabs corrected for changes in blood CO_2 content during and following severe exercise

Time after severe exercise (hr)	$\dot{V}_{CO_2}$ ml/kg/hr		Gas exchange ratio, R	
	a	b	a	b
0	164.87	127.70	1.17	0.93
½	105.46	105.90	0.96	0.96
1	60.22	61.56	0.68	0.70
2	43.74	53.55	0.52	0.65
4	40.68	47.55	0.62	0.74
6	36.42	40.05	0.67	0.73

a) Measured values (see Figs. 10 and 11 in Wood and Randall, 1981a). b) Values corrected for changes in blood CO_2 content (see Wood and Randall, 1981b), assuming a blood volume of 30% body volume (R. Harris, personal communication) and that 1 gram of crab is equivalent to 1 ml.

a clear effect of Diamox on CO_2 gradients across the gills. These changes in P_{CO_2} differences across the gills could be due to alterations in blood flow and/or distribution in the gills. This is unlikely, however, because oxygen uptake during rest and exercise, which would have been disturbed by such changes, was unaffected by Diamox treatment (Table 4). The change in gill diffusing capacity was larger for CO_2 than for oxygen between rest and exercise in intact crabs, whereas these changes were similar following Diamox injection (Table 6). Thus similar factors (such as improved ventilation–perfusion relationships or conditions for diffusion) were operating in increasing gill diffusing capacity for both oxygen and carbon dioxide, and this was unaffected by Diamox injections. In intact, untreated crabs, however, an additional factor results in a much larger increase in CO_2 compared with oxygen diffusing capacity during exercise. This factor is eliminated by Diamox treatment and could be the catalysed dehydration of bicarbonate within the gill epithelium. The gill epithelium contains carbonic anhydrase, and we thus conclude that Diamox inhibits carbonic anhydrase activity in the gill epithelium and that this enzyme is involved in CO_2 excretion. What, then, is the role of this emzyme in CO_2 excretion? The most probable explanation is that it catalyses the formation of CO_2 from bicarbonate, which enters the gill epithelium from the blood. A source of protons is required as well, and these also probably enter the gill epithelium from the blood. Thus there are similarities between crabs and fish in terms of the CO_2 and bicarbonate movements into the gill epithelium (Haswell et al., '80).

Bicarbonate is the major form of CO_2 in crab blood, with additional CO_2 bound to proteins and low concentrations of molecular CO_2 and carbonate making up the total. The relative contribution of these various pools to CO_2 excretion is difficult to determine. Crab hemolymph lacks carbonic anhydrase activity and so bicarbonate hydration–dehydration reactions in the hemolymph must occur at the uncatalysed rate. The importance of the contribution of the uncatalysed bicarbonate

dehydration reaction to CO_2 excretion will depend on the relative rates of entry of CO_2 and bicarbonate into the gill epithelium and the blood residence time in the gills. If the movement of bicarbonate into the gill epithelium is slow and blood residence time in the gills long, then as CO_2 diffuses from blood into the branchial chamber it can be replaced by uncatalysed bicarbonate dehydration within the blood, and this reaction will play an important role in CO_2 excretion. A decrease in blood residence time, as undoubtedly occurs during exercise, and/or more rapid entry of blood bicarbonate and protons into the gill epithelium will reduce the contribution of the uncatalysed reaction to CO_2 excretion. Thus it seems probable that bicarbonate dehydration within the gill epithelium will be more important during and following activity. First, blood residence time in the gills, although unknown, is undoubtedly reduced during exercise, decreasing the time available for the uncatalysed reaction within the hemolymph to contribute to the excreted CO_2 pool. Second, carbonic anhydrase inhibition has a much more marked effect on the gill diffusing capacity following exercise than during rest (Table 6).

It thus appears that CO_2 can cross the gills from blood as molecular CO_2 or enter the epithelium as bicarbonate and form CO_2, which then diffuses into the gas space. Diamox inhibition of gill epithelial carbonic anhydrase presumably reduces the importance of the latter in CO_2 excretion, which is maintained under these conditions by increasing the P_{CO_2} gradient across the gills and more molecular CO_2 is removed from the blood. Clearly the changes in CO_2 following carbonic anhydrase inhibition are not large, and the role of this enzyme in CO_2 excretion is limited, particularly during rest. This is probably a reflection of the fact that, compared with vertebrates, CO_2 excretion rates are low, and also blood residence times in the gills may be much longer, increasing the importance of the uncatalysed dehydration reaction in CO_2 excretion across the respiratory surface. Our experiments were carried out at 25°C, and crabs at lower temperatures will have much lower rates of CO_2 excretion. Under these circumstances the role of gill carbonic anhydrase may be negligible.

The changes in gas exchange ratio, R, with exercise in crabs are complex. The R values are low during rest and then increase rapidly following exercise (Herreid et al., '79; Wood and Randall, '80a). This large oscillation in R is partly due to decreases in the magnitude of the blood CO_2 pool associated with the production of metabolic acid (Wood and Randall, '81b). The blood volume of crabs is large (30% of body volume, R.J. Harris, personal communication; Gleeson and Zubkoff, '77), and there are significant changes in blood CO_2 content during and following exercise. If CO_2 excretion is adjusted for changes in the blood store, we have a better estimate of tissue CO_2 production by the crab (Table 7). There are still considerable variations in R during exercise even after M_{CO_2} has been corrected for changes in blood CO_2 content. These oscillations in R could be related either to changes in the tissue respiratory quotient or to changes in body CO_2 stores other than those in the blood. The exoskeleton is another large CO_2 store in crabs and perhaps CO_2 is lost from the shell during activity and then replaced during periods of rest. This would account for the low R value during rest and the high values observed following exercise. This mobilization of bicarbonate during exercise will reduce the oscillations in pH in the face of an acid load (Truchot, '79; Wood and Randall, '81b). The mobilization of calcium carbonate from the exoskeleton should result in a rise in blood calcium. In fact, we did observe such a rise when body CO_2 stores were mobilized during exercise (Wood and Randall, '81b).

Diamox reduced the elevation in the R value following exercise, and this could be a reflection of a decrease in the mobilization of bicarbonate from body stores. If, however, one estimates the difference in blood CO_2 content before (Table 3) and after (Table 5) exercise, there appears to be a larger change in those animals treated with Diamox. Thus the reduced R values are likely to be due to some action on either the tissue respiratory quotient or reduced mobilization of bicarbonate from sources such as the shell. The enzyme, carbonic anhydrase, may be involved in the mobilization and/or deposition of shell bicarbonate, and Diamox may interfere with this process as well as affect the pattern of CO_2 excretion across the gills of crabs. This may be related in some way to our observation that crabs treated with Diamox tended to autotomize their legs much more easily than untreated crabs, particularly following severe exercise.

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