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# ACID-BASE BALANCE IN *CALLINECTES SAPIDUS* DURING ACCLIMATION FROM HIGH TO LOW SALINITY

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SUMMARY

When transferred from 865 to 250 m-osmol salinity, the blue crab *C. sapidus* maintains its blood $Na^+$ and $Cl^-$ concentrations significantly above those in the medium. When branchial carbonic anhydrase is inhibited by acetazolamide, ion regulation fails and the animals do not survive the transfer. An alkalosis occurs in the blood at low salinity, indicated by an increase in $HCO_3^-$ and pH at constant $P_{CO_2}$. The alkalosis is closely correlated with an increase in the $Na^+$–$Cl^-$ difference, a convenient indicator of the overall strong ion difference. The contribution of changes in $P_{CO_2}$ to acid-base changes was negligible, but the change in the total weak acid (proteins) may be important. It is suggested that the change in blood acid-base status with salinity is related to an increase in the strong ion difference, which changes during the transition from osmoconformity to osmoregulation in the blue crab, and which is related to both carbonic anhydrase and ion-activated ATPases.

## INTRODUCTION

The blue crab *Callinectes sapidus* is widely known for its ability to osmoregulate and thus survive in environments that range from hypersaline bays to freshwater bayous (Lynch, Webb & van Engel, 1973; Engel *et al.* 1974; Cameron, 1978). At salinities below about 800 m-osmol the blue crab actively regulates its blood osmolarity, maintaining the $Na^+$ and $Cl^-$ concentrations significantly above those in the ambient medium, whereas between 800 and 1000 m-osmol the osmotic and ionic concentrations in its blood conform to those in the medium.

The transition from osmoconformity to osmoregulation involves the activation of mechanisms which take up ions ($Na^+$ and $Cl^-$, for example) from the medium against an electrochemical gradient. The ion regulatory process has been linked to branchial $Na^+/K^+$ ATPases (Mangum & Towle, 1977; Towle, 1981). During the acclimation to low salinity both the activity of the enzyme and its concentration within the gills increase (Towle, Palmer & Harris, 1976; Neufeld, Holiday & Pritchard, 1980) and this is believed to be responsible for ion regulation in the blood. It also appears that

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branchial carbonic anhydrase plays an important role in this process (Henry Cameron, 1982*a*, *b*).

In blue crabs, blood pH rises along with the onset of osmotic and ion regulation as salinity is decreased (Weiland & Mangum, 1975). This inverse relationship between salinity and blood pH is also found in another euryhaline crab, *Carcinus maenas* (Truchot, 1981) and in euryhaline fish (Fontaine & Boucher-Firly, 1933*a*, *b*; Smatresk & Cameron, 1981). Despite its widespread occurrence, however, the cause of the alkalosis is not known. Based on the observation that both the blood ammonia concentration and ammonia excretion increase at low salinity, Mangum *et al.* (1976) suggested that the increases in blood ammonia and ammonia excretion resulting from the deamination of intracellular free amino acids used in cell volume regulation were the cause. Truchot (1981), on the other hand, attributed the alkalosis to some unknown basic compound which is produced intracellularly as a result of the volume regulatory process, but presented no evidence for this idea.

A different explanation was offered by Smatresk & Cameron (1981) for a euryhaline fish, based on the dependence of pH on the strong ion difference (Stewart, 1978). The strong ion difference (SID) is defined as the sum of the strong cations minus that of the strong anions in solution, and is one of the independent variables of acid-base chemistry. Smatresk & Cameron (1981) observed that in the gar, *Lepisosteus osseus*, the relative alkalosis at low salinity was correlated with an increased $Na^+$–$Cl^-$ difference, an indicator of an increased SID. The other independent variables of acid-base status are the partial pressure of $CO_2$ ($P_{CO_2}$) and the total weak acid (mostly protein). Since the blood $P_{CO_2}$ does not change as a function of salinity in crabs (Truchot, 1973, 1981; Taylor, 1977), we conducted the present study in order to see if there were changes in the SID in blue crab blood related to external salinity that would explain the alkalosis at low salinity. This report attempts to define further the relationship between blood acid-base balance, osmoregulation, and the functions of branchial carbonic anhydrase and ion-activated ATPases.

## MATERIALS AND METHODS

### *Experimental handling of animals*

*Callinectes sapidus* were collected and maintained as described previously (Henry & Cameron, 1982*a*). For experimentation, blue crabs were placed in a large (50 l) aquarium that was divided into thirds by coarse mesh rubber netting, thus allowing three animals to be sampled at a time. Seawater (865 m-osmol) was equilibrated with ambient atmospheric partial pressure of oxygen ($P_{O_2}$) in a gas equilibration column and run through the aquarium in a continuous flow. The sides and top of the aquarium were covered with black plastic to minimize disturbances.

### *Injection and blood sampling*

Small diameter holes (~ 1 mm) were drilled through the carapace above the heart and pericardial cavity without puncturing the underlying membranes. Rubber septa were glued over the holes using cyanoacrylate glue (Krazy Glue). Post-branchial (arterial) blood was sampled through these septa using 1 ml syringes and 23 gauge

needles. All injections of substances into the animals were done through these septa. Pre-branchial (venous) blood was sampled from infrabranchial blood sinuses at the bases of the walking legs.

*Blood osmotic, ionic and acid-base parameters*

Pre- and/or post-branchial blood samples (~200 $\mu$l) were taken in the seawater-acclimated blue crabs and at various times after transfer to low salinity (250 m-osmol). Blood pH and total $CO_2$ ($C_T$) were measured immediately; pH was determined on a 50 $\mu$l sample using an acid-base analyser (Radiometer–Copenhagen PHM 71) and a water-jacketed capillary pH electrode which was thermostatted to the experimental temperature (20–22 °C). $C_T$ was determined on 20 $\mu$l of blood using a conductometric method which depends on conversion of all combined forms (bicarbonates, etc.) to dissolved $CO_2$ gas by acidification, removal in a carrier gas stream, followed by absorption in alkali and detection by differential conductivity (Maffly, 1968; Cameron, unpublished).

The remaining blood was allowed to clot in the syringe; the clot was disrupted by expulsion into a centrifuge tube and separated from plasma via centrifugation (Eppendorf Model 5412). Blood osmolality was determined on a vapour pressure osmometer (Wescor 5130B), chloride concentrations were measured by Ag titration on a chloridometer (Buchler–Cotlove), and sodium was measured by flame photometry (Radiometer–Copenhagen Model FLM 3).

A second set of animals was used to obtain blood $P_{CO_2}$ values since in *C. sapidus* the blood clotted before all measurements could be made on one sample. A water-jacketed $P_{CO_2}$ electrode and acid-base analyser (Radiometer–Copenhagen PHM 71) with the scale expanded four times were used in the determination. Calibration was done using humidified mixtures of 2 and 0·2% $CO_2$ in air delivered by gas mixing pumps (Wosthoff).

Resting values were obtained after the animals had been taken from the seawater holding tanks and placed in SW aquaria for 24 h; they were then moved to aquaria of 250 m-osmol salinity. At intervals after that transfer, the animals were sampled and the blood acid-base and osmotic parameters were measured.

Blue crabs acclimated to 865 m-osmol were given an injection of the carbonic anhydrase inhibitor acetazolamide ($10^{-4}$ M final concentration in blood), transferred to low salinity, and sampled as above.

*Branchial carbonic anhydrase activity*

In a separate group of animals, the seventh gill pair was dissected out and assayed for carbonic anhydrase (CA) activity previous to and following the low salinity transfer. The gills were blotted dry and homogenized in 4 vol. of cold phosphate buffer as described by Henry & Cameron (1982*a*). The determination of the catalysed dehydration of $HCO_3^-$ was carried out using the pH-stat method described in detail by Henry & Cameron (1982*a*). Briefly, the assay involved measuring the catalysed dehydration in a buffered solution of $HCO_3^-$ by holding the pH constant through the addition of HCl from an auto-titration apparatus (Radiometer–Copenhagen). The amount of titrant added per unit time is a reliable measurement of the velocity of the reaction.

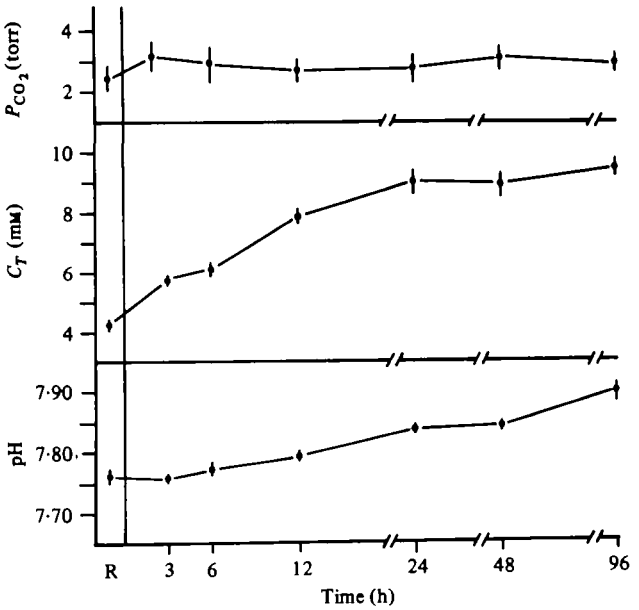


Fig. 1. Blood (prebranchial) $P_{CO_2}$, $C_T$ and pH in *C. sapidus* acclimated to 865 m-osmol (426 mM-Na and 489 mM-Cl) salinity and transferred to 250 m-osmol (116 mM-Na and 131 mM-Cl). Mean ± S.E., $n = 6$. $T = 25$ °C.

## RESULTS

### *Blood acid-base parameters*

An alkalosis began within 3 h after transfer to low salinity, with an increase in both pH and $HCO_3^-$ (Fig. 1). Throughout the course of the experiment both these parameters continued to increase. By 96 h the blood pH had increased approximately 0·15 units to a value of 7·90, and $HCO_3^-$ had risen about 5 mM to a value of 9·5 mM, both values being typical of blue crabs that had been acclimated to low salinity for 2–4 weeks (Henry & Cameron, 1982*b*). The blood $P_{CO_2}$, on the other hand, did not change significantly over the duration of the transfer ($P > 0{\cdot}05$, F test; Fig. 1).

### *Blood osmotic and ionic parameters*

Immediately after transfer from high to low salinity, there were steep drops in the blood osmolality, and $Na^+$ and $Cl^-$ concentrations (Fig. 2). Between 12 and 24 h blood values stabilized and there were no further significant changes over the course of the experiment ($P > 0{\cdot}05$, *t* test).

During the readjustment of blood osmotic and ionic concentrations there was a change in the $Na^+$–$Cl^-$ difference. In animals acclimated to 865 m-osmol (426 mM-$Na^+$ and 489 mM-$Cl^-$) the blood $Na^+$ concentration was 443 ± 2 mM and $Cl^-$ was 449 ± 4 mM. The $Na^+$ values are similar to those reported by Colvocoresses, Lynch & Webb (1974) for acute measurements on blue crabs taken from similar salinities (415 and 364 mM-$Na^+$ at 870 and 270 m-osmol, respectively). Our $Cl^-$ concentrations were higher at the high salinity (449 *v.* 368 mM) but agree for crabs at approximately the same low salinity (338 *v.* 327 mM).

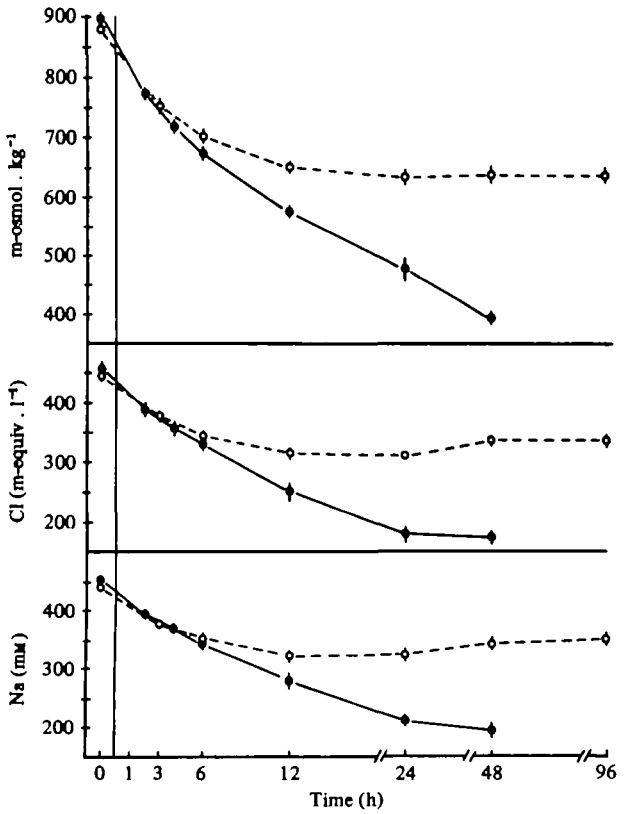


Fig. 2. Blood (prebranchial) osmolality, Na and Cl concentrations for *C. sapidus* acclimated to 865 m-osmol (426 mM-Na and 489 mM-Cl) salinity and then transferred to 250 m-osmol (116 mM-Na and 131 mM-Cl) (○ — — ○). ●—● Animals given an injection ($10^{-4}$ M in blood) of acetazolamide prior to transfer. Mean ± S.E., $n = 6$. $T = 25$ °C.

After 96 h in 250 m-osmol salinity (116 mM-$Na^+$ and 131 mM-$Cl^-$), blood $Na^+$ dropped 90 mM to a value of 353 ± 5 mM while $Cl^-$ was lowered 111 mM to 338 ± 5 mM. $K^+$ concentrations also decreased, but only by about 1–2 mM. The difference in the two major ions ($Na^+$–$Cl^-$), which was negative at high salinity, changed significantly ($P < 0.05$, $t$ test) to positive after transfer to low salinity (Table 1). During that time the blood $HCO_3^-$ concentration also increased, as did the blood pH (Fig. 1, Table 1). It appears that the $HCO_3^-$ concentration (and also pH) was correlated with the $Na^+$–$Cl^-$ difference, an indicator of the overall strong ion difference. In a separate study on the blue crab, Engel *et al.* (1974) also presented data showing that the $Na^+$–$Cl^-$ difference increased upon acclimation to low salinity. In this instance the $Na^+$–$Cl^-$ difference at 20 °C changed by 15 mM from −20 (at 1050 m-osmol) to −5 (at 150 m-osmol) (values calculated from Figures 1 and 3, Engel *et al.* 1974).

*The effect of acetazolamide on low salinity adaptation*

A $10^{-4}$ M concentration of acetazolamide in the blood of the blue crabs caused a significant ($P < 0.05$, $t$ test) reduction of blood $Na^+$ and $Cl^-$ concentrations by 12 h, compared to values in the non-injected (control) animals (Fig. 2). Blood $Cl^-$ con-

Table 1. *The sodium/chloride difference, pH, bicarbonate and ammonia concentrations in the blood of* C. sapidus *previous to and following a transfer from* 865 *to* 250 *m-osmol salinity.*

(Data summarized from Figs. 1 and 2. Ammonia data from Mangum *et al.* 1976.)

| Time | [Na–Cl] (mM) | pH | $HCO_3^-$ (mM) | Ammonia (mM) |
|---|---|---|---|---|
| 0 | −6·5 | 7·763 | 4·29 | 0·033 |
| 3 | −0·8 | 7·761 | 5·75 | — |
| 6 | 7·6 | 7·774 | 6·13 | — |
| 12 | 3·6 | 7·795 | 7·97 | — |
| 24 | 11·6 | 7·838 | 9·04 | — |
| 48 | 6·0 | 7·842 | 8·97 | 0·124 |
| 96 | 14·3 | 7·910 | 9·57 | 0·141 |

centrations were depressed by $66 \pm 8$, $131 \pm 6$, and $162 \pm 9$ mM at 12, 24, and 48 h, respectively, and the blood $Na^+$ concentrations were lowered by $42 \pm 4$, $111 \pm 6$, and $147 \pm 5$ mM for those same time periods. By 48 h there was about 80% mortality among the drug-treated animals, and none survived through 96 h. In contrast, the same dose of acetazolamide was not fatal to blue crabs which had been fully acclimated to either 865 or 250 m-osmol. Also, for crabs at 865 m-osmol the inhibitor had only a slight effect on blood osmolality; blood $Na^+$ and $Cl^-$ concentrations were lowered by only about 5%, while $C_T$ was slightly elevated (see Henry & Cameron, 1982*b* for details).

*Carbonic anhydrase activity*

There was no change in either the activity per fresh weight of tissue or the specific activity of gill carbonic anhydrase over the first 4 days of low salinity adaptation (Fig. 3). Between 4 and 7 days, however, there was approximately a doubling in activity and specific activity, and by 14 days the CA activity had reached the same level of activity found in blue crabs collected from a low salinity habitat (see Henry & Cameron, 1982*a*, Figures 4 and 5). The increase in enzyme activity occurred several days after the blood osmotic and ionic concentrations had stabilized (Fig. 2 *v.* 3).

## DISCUSSION

*Blood acid-base status and the strong ion difference*

The changes in acid-base status in the blue crab during acclimation to low salinity were similar to those observed for *C. maenas* by Truchot (1981). Immediately after the transfer, there was a small decrease in blood pH and a small increase in the $P_{CO_2}$; these changes were significant for *C. maenas*, but not for *C. sapidus*. This may represent an attempt by the animals to reduce gill permeability and thus conserve blood ions, but it is more likely that it represents a transient acidosis brought about from handling stress, since Cameron (1978) showed that the blue crab does not reduce its gill permeability to ions in low salinity. During the next several days there was a progressive increase in both the blood pH and $HCO_3^-$ concentrations in both species, reaching new steady state values that indicate that the relative alkalosis is a permanent feature of low salinity acclimation. The pH and $HCO_3^-$ concentration remained

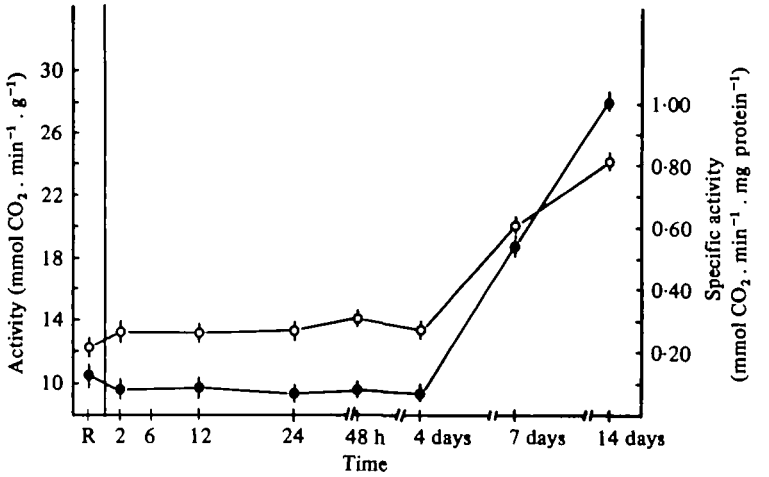


Fig. 3. Carbonic anhydrase activity of gill no. 7 of *C. sapidus* previous to and following a transfer from 865 to 250 m-osmol salinity. Mean ± S.E., $n = 6$. $T = 22$ °C. ○, Specific activity; ●, activity per g fresh wt tissue.

elevated in *C. maenas* after 7 days at low salinity, which would appear to contradict Truchot's suggestion that the alkalosis is only a transient event.

In both species the alkalosis is of non-respiratory, or 'metabolic', origin, since the $P_{CO_2}$ did not rise significantly over resting values (Fig. 1, present study; Truchot, 1973, 1981; Taylor, 1977). The conditions for 'metabolic' alkalosis, i.e. increased pH and $HCO_3^-$ at constant $P_{CO_2}$, were correlated with an increase in the $Na^+$–$Cl^-$ difference, taken as an indicator of the strong ion difference (cf. Table 1). Other cations in the blood, $K^+$, $Ca^{2+}$ and $Mg^{2+}$, total about 48 mmol.$l^{-1}$ at 870 m-osmol external salinity, and about 35 mmol.$l^{-1}$ at 270 m-osmol (Colvocoresses *et al.* 1974). The amounts of these other strong cations (and anions) were not measured in this study, but it seems highly unlikely that they could completely counteract the 20 mmol.$l^{-1}$ increase in the $Na^+$–$Cl^-$ difference reported in Table 1.

That there was not a precise relationship between the change in the strong ion difference (SID) and the $HCO_3^-$ concentration is not too distressing. Measurement of all of the strong ions, including their actual activity coefficients, would provide a different quantitative picture, but the qualitative conclusion, i.e. that the 'metabolic' alkalosis was related to changes in the SID, would not differ.

There is probably also some acid-base contribution of changes in total weak acid, as protein concentrations have been reported to increase in crabs at low salinity (Siebers *et al.* 1972; Lynch & Webb, 1973). Blood protein concentrations have previously been linked to amino acid metabolism and cell volume regulation (reviewed by Gilles & Pequeux, 1981), but since proteins carry a net negative charge an increase in their concentrations in blood would also help to offset an increase in the SID. In *C. sapidus* blood proteins more than double, increasing from around 45 mg.$ml^{-1}$ at 900 m-osmol ambient salinity to about 110 mg.$ml^{-1}$ at 300 m-osmol (Lynch & Webb, 1973).

Mangum *et al.* (1976) have suggested that the cause of the pH increase in low

salinity waters is the increase in blood ammonia concentration. This cannot be true at least in any direct way. Their ammonia concentration data (Table 1) show an increase of only 0·15 mmol.$l^{-1}$, and even using a conservatively low buffer value for *C. sapidus*' blood of 6 mmol/pH, the change in blood pH would only be less than 0·02, rather than the 0·15 observed (Fig. 1). The further possibility, though not made explicit by Mangum *et al.* (1976), is that increased ammonia excretion occurs by increased $Na^+/NH_4^+$ exchange in the gills, thus causing an increased loss of $H^+$ ions in low salinity. This possibility cannot be ruled out, but there is no evidence that $Na^+/NH_4^+$ exchange increases at low salinity, and recent work by Kormanik & Cameron (1981*a*, *b*) indicates that ammonia excretion across blue crab gills occurs mostly by non-ionic $NH_3$ diffusion.

Even if altered patterns of ammonia excretion contribute to the alkalosis in crustaceans, it may not be important in other euryhaline animals. Euryhaline fish exhibit the same inverse relationship between salinity and blood pH, but their intracellular pool of free amino acids (FAA) may or may not be important in cell volume regulation: Cowey, Daisley & Parry (1962) reported no change in the muscle FAA pool in the Atlantic salmon in salt *v.* fresh water; but small changes have been found in the eel, mullet and flounder (Lasserre & Gilles, 1971; Huggins & Colley, 1971; Colley, Fox & Huggins, 1974). Nevertheless, ammonia excretion in fish does not appear to change over a wide range of environmental $Na^+$ (Maetz, 1973).

*Carbonic anhydrase and low salinity adaptation*

The increase in gill CA activity took place over several days (Fig. 3), probably reflecting the induction of new enzyme. Branchial CA from the blue crab does show higher levels of activity in low concentrations of NaCl (Henry & Cameron, 1982*a*), but in this experiment the NaCl concentration in the assay solution was kept constant at 35 mM, so the increase in the activity was more likely a result of an increase in the amount of enzyme present in the gill.

The pattern of the induction of CA during low salinity adaptation in the blue crab is similar to that for the $Na^+/K^+$ ATPase, an enzyme which is believed to be important in ion and osmoregulation (Neufeld *et al.* 1980). Both enzymes have been localized in the areas of the gill lamellae that contain dense populations of chloride cells (Neufeld *et al.* 1980; Henry & Cameron, 1982*a*). Thus the slow increase in the concentrations of these two enzymes in gill tissue most probably reflects the time of cell division and differentiation and the proliferation of the osmoregulatory patches of chloride cells. Although no work of this type has been done in crabs, Shirai & Utida (1970) reported a time course of 14 days for the increase in the chloride cell population in the gills of the euryhaline eel, *Anguilla japonica*, exposed to a salinity change. Evidence that ATPase activity is also rapidly modulated during a salinity change (Towle *et al.* 1976) indicates that other possible mechanisms of modulating CA activity cannot be ruled out at this time.

That branchial CA is critical to the blue crab's ability to adapt to low salinity is shown clearly by the animals' failure to survive a low salinity transfer when the enzyme is blocked by acetazolamide (Fig. 2). The high mortality is closely linked to a breakdown in the ion regulatory mechanisms which normally maintain blood $Na^+$

and $Cl^-$ concentrations significantly above those in the low salinity environment. At the time of death (48 h), blood $Na^+$ and $Cl^-$ concentrations were 147 and 162 mM lower, respectively, than in the untreated animals. A similar dose of acetazolamide has no effect, however, on either oxygen uptake or $CO_2$ excretion (Henry & Cameron, 1982*b*). Whereas animals fully acclimated to 250 m-osmol salinity experience the same depressed concentrations of $Na^+$ and $Cl^-$ but survive (Henry & Cameron, 1982*b*) blue crabs in the acute stage of low salinity acclimation do not.

The data presented here suggest that changes in the SID which occur when the blue crab makes the transition from osmoconformity to regulation are important to the observed changes in the blood acid-base status of the animal. The relative importance of various mechanisms of alteration of the SID are not yet known, but clearly both ion regulation and acid-base balance fall under the control of the same process of active uptake of $Na^+$ and $Cl^-$ against electrochemical gradients, presumably by the action of the $Na^+/K^+$ ATPase and possibly also a $Cl^-/HCO_3^-$ ATPase (Depew & Towle, 1979; Towle, 1981). Carbonic anhydrase, although not directly involved in the ion exchange process, probably maintains local supplies of $HCO_3^-$ and $H^+$ for counterions via the accelerated hydration of respiratory $CO_2$ within the gill tissue. This possibility was investigated in more detail and the results are given in a separate study (Henry & Cameron, 1982*b*).

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