

POST-MOULT CALCIFICATION IN THE BLUE CRAB (*CALLINECTES SAPIDUS*): RELATIONSHIPS BETWEEN APPARENT NET H⁺ EXCRETION, CALCIUM AND BICARBONATE

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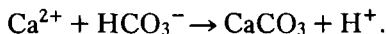
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SUMMARY

In the days immediately after moulting, manipulations of external pH, [HCO₃⁻], and [Ca²⁺] were used to determine the nature of the rapid net Ca²⁺ influx and attendant apparent net H⁺ efflux in the blue crab (*Callinectes sapidus* Rathbun). Both fluxes were strongly inhibited by reductions in external [Ca²⁺], [HCO₃⁻], or pH. The net Ca²⁺ influx was reversed at an external concentration of 2.5 mmol l⁻¹, and both fluxes were reversed by reducing the external [HCO₃⁻] to 0.2 mmol l⁻¹. The correlation between net Ca²⁺ flux and apparent net H⁺ flux was 0.61 ($P < 0.01$), but the variability and the time course of most experiments indicated that the link was indirect, rather than a direct coupling or co-transport. This conclusion was also borne out by acid-base disturbances that occurred in the low-[Ca²⁺] treatment. The results are consistent with the hypothesis that inward calcium transport is accompanied by both inward HCO₃⁻ transport and outward H⁺ transport, probably by separate exchanges with ions of like charge such as Na⁺ and Cl⁻. Crustecdysone (β -ecdysone) does not appear to be involved in control of these post-moult fluxes and calcification.

INTRODUCTION

The principle mineral component of the crustacean carapace is calcium carbonate, which in seawater species is almost completely lost after each moult (Passano, 1960; Cameron & Wood, 1985). Formation of a new shell in the days following the moult requires rapid inward transport of calcium, which must be electrically balanced. As carbonate forms and precipitates, there must also be a net outward transport of H⁺ ions on an equivalent basis, according to the reaction:



A number of transport combinations which achieve both electrical and acid-base balance are possible, including: (1) inward transport of 1 Ca²⁺ coupled with outward transport of 2 H⁺; in which case the HCO₃⁻ must be generated by reaction from

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CO₂, or (2) inward transport of 1Ca²⁺ coupled with inward transport of 1HCO₃⁻ and outward transport of 1H⁺. In earlier work the preliminary evidence favoured the second alternative, since the rate of calcification exceeded the rate of metabolic CO₂ generation, and the rate of inward calcium transport appeared to be sensitive to sea water [HCO₃⁻] (Cameron & Wood, 1985). The purpose of the present series of experiments was to test the relationship between calcium fluxes, apparent net H⁺ fluxes, and the external sea water conditions, including [Ca²⁺], [HCO₃⁻] and pH in an attempt to elucidate further the mechanisms of calcification following the moult.

METHODS AND MATERIALS

Male and female blue crabs (*Callinectes sapidus* Rathbun), ranging from 64 to 211 g, were collected with unbaited, shaded pots from Redfish Bay, Texas. Pre-moult crabs, which were recognized by various external signs (Perry, Ogle & Nicholson, 1979), were maintained in individual aquaria in the laboratory until they moulted. Food was offered *ad libitum*, but feeding usually ceased at 1–2 days prior to the moult. Temperatures in the laboratory ranged from 18 to 22°C, but all experiments were performed between 19.6 and 20.4°C (average 20).

Several hours prior to the beginning of each experiment, the freshly-moulted crabs were gently transferred to darkened acrylic chambers which formed part of a small-volume, recirculating system. The system was provided with vigorous aeration and temperature control. Animals from which blood samples were required were fitted with small neoprene foam septa, which were glued to the mid-dorsal carapace with cyanoacrylate cement. The experimental protocol consisted of periods of up to 8 h during which water and blood samples could be taken and various changes of media performed with minimal disturbance to the animals.

Normal sea water (SW, salinity 28–32‰) was used as the control treatment. Acidified sea water (low [HCO₃⁻]) was prepared by gradual titration of sea water to pH 6.7–6.8 with HCl under vigorous aeration. A sea water with the same pH but with high [HCO₃⁻] was prepared by equilibrating normal sea water with 1% CO₂ (P_{CO₂} = 7.5 Torr) in air at least overnight. For manipulations of [Ca²⁺] an artificial sea water was prepared from distilled water, containing (g l⁻¹): NaCl, 25.12; KCl, 0.70; MgCl₂·6H₂O, 4.67; NaHCO₃, 0.176; MgSO₄, 3.13; and CaCl₂·4H₂O, 1.72. Calcium was reduced appropriately in some solutions without corresponding replacement of chloride. More information on the composition of the various solutions used is given in Table 1.

Table 1. *Salient features of the various solutions used*

Solution	Salinity	pH	P _{CO₂}	[HCO ₃ ⁻]	[Ca ²⁺]
Normal sea water (SW)	28–32	7.76	<0.1	1.94	9.49
Artificial SW	30	7.54	<0.1	1.77	7.23
Acidified SW	28–32	6.70	<0.1	0.22	9.45
High-CO ₂ SW	28–32	6.84	7.8	2.38	7.19
High-[HCO ₃ ⁻] SW	28–32	8.16	<0.1	4.83	7.78
High-[Ca ²⁺] SW	28–32	7.31	<0.1	1.74	17.93
Low-[Ca ²⁺] SW	30	7.92	<0.1	1.82	2.45

Concentrations given in mmol l⁻¹, P_{CO₂} in Torr and salinity in parts per thousand.

The 'apparent net H^+ excretion' is an operational term which includes excretion of H^+ or NH_4^+ ions and/or uptake of OH^- or HCO_3^- ions. It was measured at each time interval by titration of a 10.0-ml water sample to pH 4.000 with 0.020 mol l^{-1} in a micrometer burette, and by adding the total ammonia excreted to the difference in titration values over the time interval (cf. Cameron & Kormanik, 1982; Cameron & Wood, 1985). Absolute accuracy to the third decimal point is not claimed, but with low-drift electrodes (less than $0.005 \text{ pH day}^{-1}$), the difference between successive samples could be measured with a reproducibility of $\pm 1\text{--}2 \mu\text{l}$, which corresponded to an overall sensitivity of approximately $10 \mu\text{equiv net } H^+$ change in the system. Calcium and magnesium concentrations in the sea water were measured by atomic absorption spectrophotometry, and calcium in blood samples by a colorimetric cresolphthalein complexone method (Sigma 586). The colorimetric assay required less sample volume; a series of standards and samples was analysed with both methods, giving essentially identical results. Total ammonia concentration in water was measured using the phenolphthorite method (Solorzano, 1969), and in blood samples with an enzymatic method (Sigma 170-UV). Total CO_2 in water and blood were measured with a conductometric apparatus (Capni-Con 3, Cameron Instrument Co.), and blood pH with a capillary type microelectrode (Radiometer). Results were analysed with standard statistical methods, and are presented as $\pm 1 \text{ s.e.}$ unless otherwise stated.

RESULTS

Effects of low pH and $[HCO_3^-]$

The effects of the acidified sea water and high CO_2 sea water treatments on six crabs are shown in Fig. 1. In acidified sea water ($\text{pH} = 6.7$, $[HCO_3^-] = 0.22 \text{ mmol l}^{-1}$),

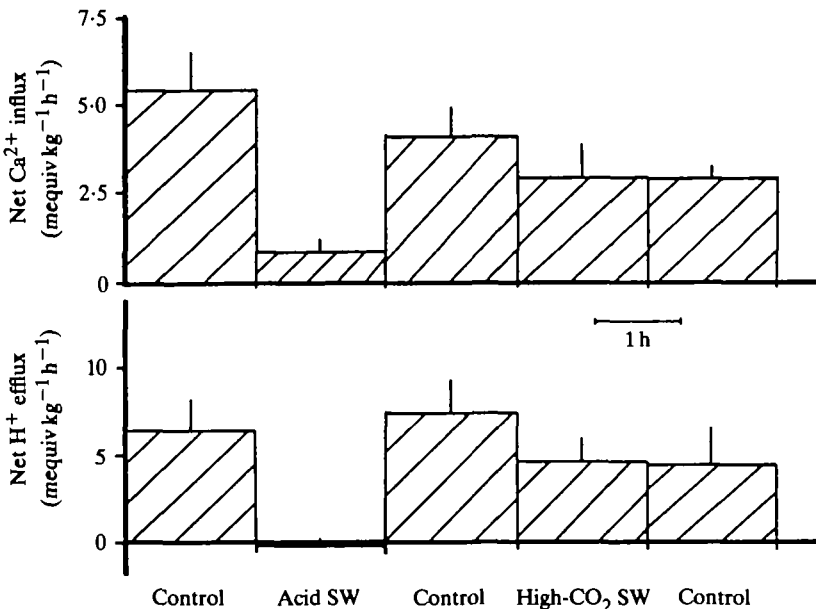


Fig. 1. Net Ca^{2+} influx and apparent net H^+ efflux for six crabs during sea water (SW) control, acidified sea water (Acid SW), a second SW control, high CO_2 sea water, and a final SW control treatment. See Table 1 for the composition of the media. The vertical bars represent 1 s.e.

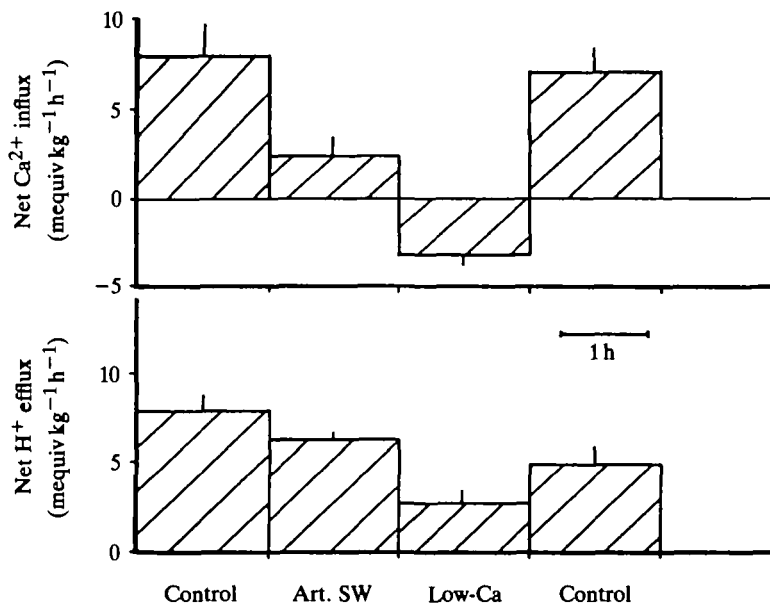


Fig. 2. Net Ca^{2+} influx and apparent net H^{+} efflux for seven crabs in normal sea water (Control), artificial sea water (Art. SW), low- $[\text{Ca}^{2+}]$ artificial sea water (Low-Ca), and a final control. See Table 1 for the composition of the media. The vertical bars represent 1 s.e.

both net Ca^{2+} influx and apparent net H^{+} efflux were strongly inhibited ($P < 0.01$, analysis of variance, ANOVA). The high- CO_2 sea water treatment, which lowered pH by the same amount and increased $[\text{HCO}_3^-]$, did not, however, significantly (by ANOVA) inhibit either net Ca^{2+} influx or apparent net H^{+} efflux (Fig. 1).

Effects of altered sea water $[\text{Ca}^{2+}]$

The effects on seven crabs of reducing external $[\text{Ca}^{2+}]$ to 2.45 mmol l^{-1} are shown in Fig. 2. Originally the artificial sea water was intended as an additional control, but due to excess hydration of the reagent and attendant weighing errors, the $[\text{Ca}^{2+}]$ was reduced from 9.49 to 7.23 mmol l^{-1} (Table 1), which significantly ($P < 0.01$, ANOVA) affected both net Ca^{2+} influx and apparent net H^{+} efflux (Fig. 2). Treatment with $2.45 \text{ mmol l}^{-1} \text{ Ca}^{2+}$ resulted in a reversed Ca^{2+} net flux (i.e. a net efflux), and a 65 % reduction in the apparent net H^{+} efflux.

The low-calcium treatment also had marked effects on the blood. Blood $[\text{Ca}^{2+}]$ fell from 11.60 ± 0.20 to $5.30 \pm 0.34 \text{ mmol l}^{-1}$ between the initial control and the low calcium treatment, while the blood $[\text{Mg}^{2+}]$ remained unchanged at $16.8 \pm 0.7 \text{ mmol l}^{-1}$. There was also an acid-base disturbance (Fig. 3). During the low-calcium treatment, a non-respiratory ('metabolic') alkalosis developed, which was probably due to differential effects of the low $[\text{Ca}^{2+}]$ on Na^{+} and Cl^{-} permeability, resulting in changes in the strong ion difference (SID) (Stewart, 1978; Henry & Cameron, 1983).

Effects of elevated $[\text{HCO}_3^-]$ and $[\text{Ca}^{2+}]$

Increased external $[\text{Ca}^{2+}]$ did not have any measurable effect on either the net Ca^{2+} influx or the apparent net H^{+} efflux ($N = 5$ crabs). In successive 1.5-h treatments of control SW, high- $[\text{Ca}^{2+}]$ and control SW, net Ca^{2+} influx rates were 8.56 ± 0.87 ,

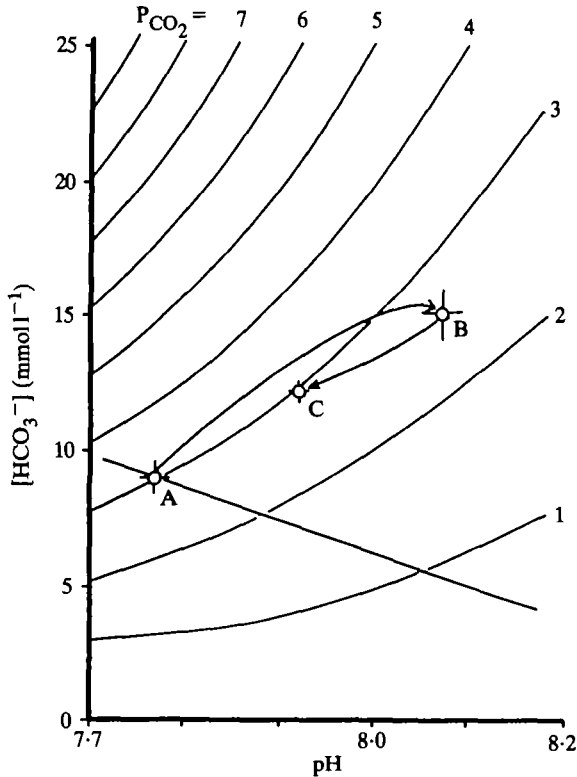


Fig. 3. A pH/ HCO_3^- diagram showing the time course of acid-base events during the experiments of Fig. 2. Point A represents the initial control period, point B the low- $[\text{Ca}^{2+}]$ period, and point C the partial recovery during the final control period. Horizontal and vertical bars represent ± 1 s.e., and the curved lines are the contours of constant CO_2 partial pressure (in Torr).

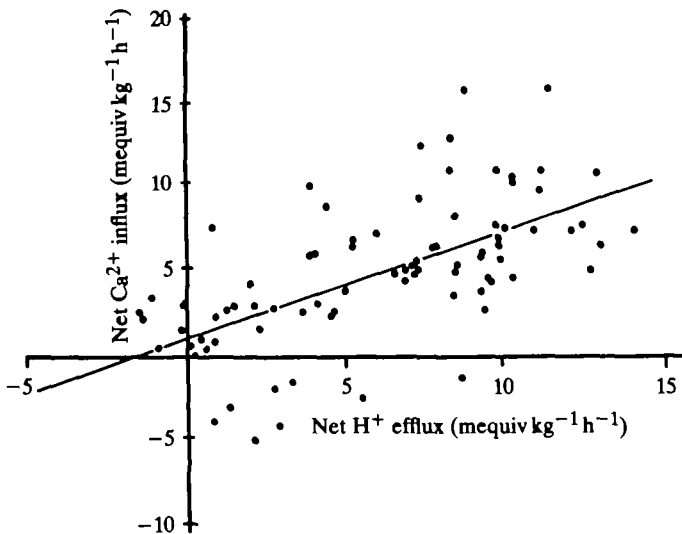


Fig. 4. Mean values of net Ca^{2+} influx and apparent net H^+ efflux for each crab averaged for each experimental treatment ($N = 87$). The regression line shown has the equation $y = 0.650x + 0.895$.

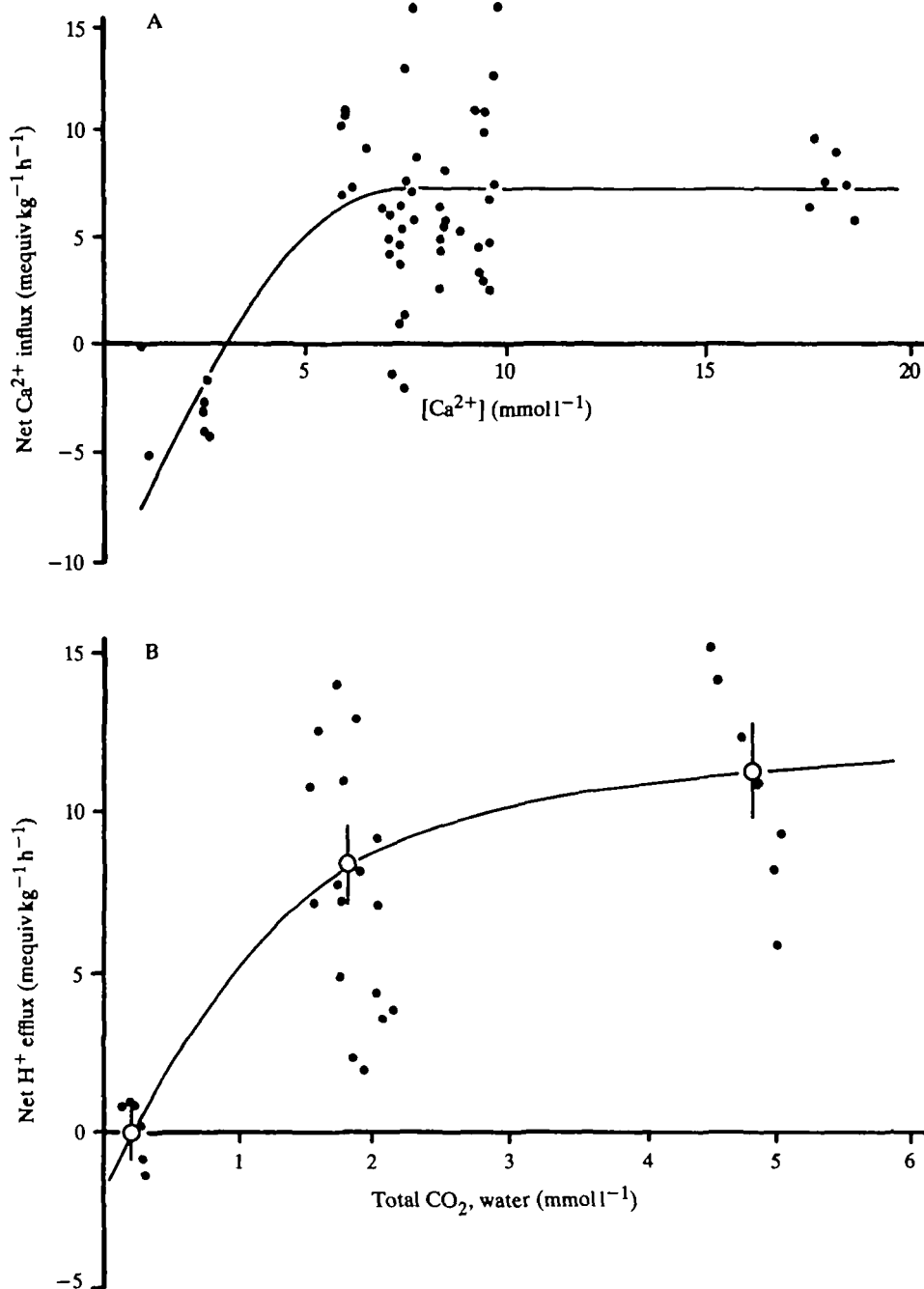


Fig. 5. (A) The relationship between net Ca^{2+} influx and external $[\text{Ca}^{2+}]$. (B) The relationship between apparent net H^{+} efflux and total CO_2 concentration in the sea water (approximately equal to $[\text{HCO}_3^-]$).

7.42 ± 0.67 and 6.25 ± 1.73 mequiv h^{-1} ; apparent net H^{+} efflux rates were 9.30 ± 1.21 , 8.82 ± 1.22 and 9.98 ± 0.97 mequiv h^{-1} . There were some significant changes in the blood, with $[\text{Ca}^{2+}]$ rising from 9.31 ± 0.35 to 10.94 ± 0.48 mmol l^{-1} ,

Table 2: Simple correlation coefficients for flux and external conditions for pooled experiments

	[HCO ₃ ⁻]	SW pH	[Ca ²⁺]	Net Ca ²⁺ influx
Apparent net H ⁺ efflux	0.22	0.21	0.29	0.62
<i>P</i>	0.039	0.052	0.006	<0.001
SW [HCO ₃ ⁻]		0.51	-0.02	0.11
<i>P</i>		<0.001	NS	NS
SW pH			-0.07	0.16
<i>P</i>			NS	NS
SW [Ca ²⁺]				0.45
<i>P</i>				<0.001

Each datum is the average of 3 to 5 determinations from a 1.5 to 2.5 h period.

Fluxes are given in $\mu\text{equiv kg}^{-1} \text{h}^{-1}$; concentrations in mmol l^{-1} .

NS, not significant.

and the blood pH falling slightly, but not significantly. These changes were opposite to those observed in the low-[Ca²⁺] treatment, but much less pronounced.

By increasing the [HCO₃⁻] from about 2 to nearly 5 mmol l^{-1} , a small but significant ($P < 0.05$, ANOVA) increase in the apparent net H⁺ efflux was observed (from 8.52 ± 1.65 to 11.05 ± 1.51 mequiv h^{-1}), but there was no change in the net Ca²⁺ influx ($N = 6$ crabs). The blood pH in the high-[HCO₃⁻] treatment rose from 7.798 ± 0.037 to 7.937 ± 0.035 ($P < 0.01$, paired *t*-test) and the blood [HCO₃⁻] from 9.81 ± 0.61 to 11.22 ± 0.47 mmol l^{-1} ($P < 0.05$), with no changes in [Ca²⁺] or [Mg²⁺].

Analysis of the combined data

Day-to-day fluctuations in natural sea water composition, plus the variability included in the various experimental solutions provided a wide range of external [Ca²⁺] and [HCO₃⁻] in the combined experimental series. Pooling all of the data resulted in the relationship between net Ca²⁺ influx and apparent net H⁺ efflux shown in Fig. 4. The slope of the line was 0.650 ± 0.089 (\pm s.d., $N = 87$), indicating a stoichiometry of approximately $3\text{H}^+ : 2\text{Ca}^{2+}$. The relationships between net Ca²⁺ influx and external [Ca²⁺], and between apparent net H⁺ efflux and external [HCO₃⁻] are shown in Fig. 5A and Fig. 5B, respectively.

Both simple and multiple correlation analyses were carried out on the pooled data, and the simple correlation coefficients are given in Table 2. Because some of the variables were changed in a concerted manner, and thus internally correlated, certain partial correlation coefficients were examined. The apparent net H⁺ efflux, for example, was closely correlated with the net Ca²⁺ influx when all other variables were held constant ($r = 0.61$, $P < 0.001$), but not to an extent greater than shown by the simple correlation coefficient. The apparent net H⁺ efflux was also about equally correlated with water pH and water [HCO₃⁻], contrary to the apparently greater sensitivity to [HCO₃⁻] shown in Fig. 1. The higher [HCO₃⁻] and lower pH were offsetting effects in that treatment. Finally, the correlation of net Ca²⁺ influx with external [Ca²⁺] is only marginally improved by partial analysis.

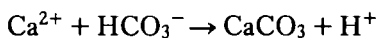
Possible endocrine involvement

Since the 'switching on' of calcium uptake and associated processes after the moult is quite dramatic, some simple experiments were performed to test the involvement of the eyestalk neuroendocrine system. In the first series, cross-transfusions of 1 ml of defibrinated blood were performed between an inter-moult and a post-moult crab. Despite double defibrination, the animals appeared to die from coagulation of the cross-injected blood in the pericardial cavity. Next, eyestalk extracts from 1 day post-moult crabs were prepared by homogenizing the soft tissues from both eyestalks in 0.5 ml saline and injecting them into intermoult crabs. There was no effect of this treatment on either net Ca^{2+} influx or apparent net H^+ efflux. Two further crabs were injected with 50 μg of β -ecdysone on day 3 post-moult, with no measurable effect on any parameter. Finally, measurements of net Ca^{2+} influx and apparent net H^+ efflux were made on two crabs at 1 day post moult, their eyestalks ligated, and on the next day the measurements repeated. Other than the normal slight decline in fluxes with time after moult, there was no detectable effect of the eyestalk ligation.

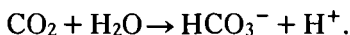
DISCUSSION

In our previous work we demonstrated that the source of CO_2 for newly deposited CaCO_3 in the shell had to be at least partly external HCO_3^- , since the rate of calcification exceeded the metabolic rate of CO_2 production, and there was a net uptake of CO_2 for several days after the moult (Cameron & Wood, 1985). The data in Fig. 3 actually show a normal partial pressure gradient for CO_2 from inside to out, so the metabolic CO_2 contribution to carbonate formation is probably very small. That is, if the new carapace were a significant metabolic CO_2 sink, the partial pressure of CO_2 in the blood would be depressed.

The rapid uptake of Ca^{2+} after the moult, which has been studied by several authors previously (Vigh & Dendinger, 1982; Roer, 1980; Roer & Dillaman, 1984; Greenaway, 1974, 1983; Cameron & Wood, 1985), must be balanced both from the electrical and the acid-base standpoint. The immediate reaction is:



and the HCO_3^- must be supplied either by transport from the external environment, or from metabolically produced CO_2 by the reaction:



This leads to the electrically and acid-base balanced scheme (Fig. 6) in which both external HCO_3^- and internally produced CO_2 react with incoming Ca^{2+} to form the CaCO_3 of the new carapace. The scheme also balances if both CO_3^{2-} ions come from external HCO_3^- . The model predicts a $4\text{H}^+:2\text{Ca}^{2+}$ stoichiometry, or a slope of 0.5 for the line in Fig. 4, and the difference between the calculated slope (0.65 ± 0.089) and 0.5 is not statistically significant.

The results in Figs 1 and 2 are consistent with the model of Fig. 6, in that they show

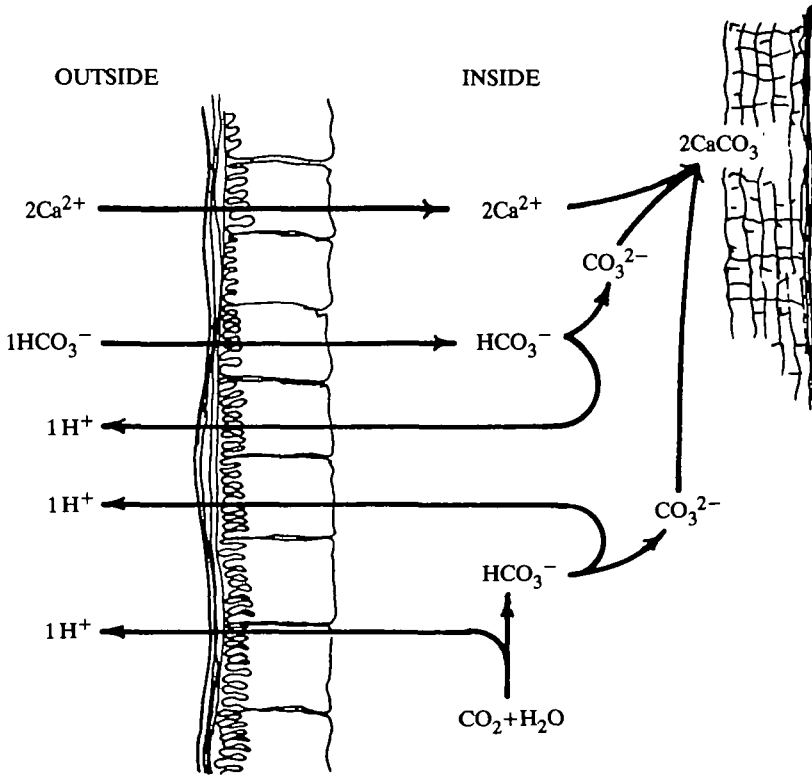


Fig. 6. A diagrammatic scheme of electrically and acid-base balanced net transport of Ca^{2+} , HCO_3^- and H^+ in the post-moult crab. See text for discussion.

that reductions of external $[\text{Ca}^{2+}]$ or $[\text{HCO}_3^-]$ reduce the influx of those ions. The data of Fig. 1 also indicate that a reduction of external pH or increase of external $[\text{H}^+]$, reduces the observed apparent net H^+ efflux, as would be predicted from the model in Fig. 6.

The coupling between the movements of Ca^{2+} , HCO_3^- and H^+ is not very tight, however, as indicated by various experiments. In Fig. 2, for example, the low- $[\text{Ca}^{2+}]$ treatment actually reversed the Ca^{2+} flux, but the apparent net H^+ efflux continued, albeit at a reduced rate. In the high external $[\text{HCO}_3^-]$ experiments, an increase in the apparent net H^+ efflux was shown without any increase in the net Ca^{2+} influx; and finally, temporary inequalities of the various fluxes produced acid-base disturbances in the blood, as shown in Fig. 3 and discussed in connection with the high external $[\text{HCO}_3^-]$ treatment (above). The link between the various fluxes appears to be indirect, possibly controlled by changes in either the blood acid-base status, or that of various intracellular compartments. Any further conjecture concerning the actual transport mechanisms seems premature on the basis of present evidence.

The data of Fig. 5 seem to show that the uptake mechanisms (for Ca^{2+} and HCO_3^-) are saturated at normal sea water concentrations, but the experimental design was not intended to give details information about the shape of the kinetic curve.

It seems quite likely that the post-moult fluxes are under neuroendocrine

control. Ecdysone was an early suspect, based on reports that it affects calcium deposition and mobilization in the crayfish (McWhinnie, Cahoon & Johanneck, 1967; McWhinnie, Kirchenberg, Urbanski & Schwartz, 1972). According to some early reports, ecdysone reaches a peak shortly *after* ecdysis (Adelung, 1967; Faux *et al.* 1969), but later studies have shown a peak before ecdysis (Hopkins, 1983; Soumoff & Skinner, 1983). Ecdysone has also been accorded a general role in epidermal events during the moult cycle (Highnam & Hill, 1977). Since neither eyestalk ligation, injection of eyestalk extracts, nor direct injection of ecdysone produced any measurable effects, I conclude that neither ecdysone nor the other distinguishable peptide hormones of the eyestalk complex is responsible for post-moult calcification in the blue crab. There is also considerable neurosecretory activity in the central nervous system, however, and it seems probable that a calcium neuro-hormone will eventually be discovered.

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