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POST-MOULT CALCIFICATION IN THE BLUE CRAB, *CALLINECTES SAPIDUS*: TIMING AND MECHANISM

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Summary

A series of experiments was conducted to elucidate the events immediately preceding and following moulting (ecdysis) in the blue crab, *Callinectes sapidus* Rathbun. The crabs gain weight (mostly water) to a level more than twice the pre-moult value excluding the shed carapace. The gain begins about 4 h before ecdysis, accelerates rapidly to a maximum rate at about the time of ecdysis, and is essentially complete by 2 h after ecdysis. Both calcification and net H^+ excretion remain at control (intermoult) levels until 1–2 h post-moult, whereupon a very rapid increase in both begins, with the same time course for both processes. The ratio of internal to external calcium concentration drops from 1·4:1 during intermoult to 0·85:1 by 2 days after the moult, reversing the electrochemical gradient during the post-moult period. Calcification is strongly inhibited by the protein synthesis inhibitors actinomycin D and cycloheximide. Isolated whole gills and gill slices do not show significant changes in rates of calcium uptake related to moult stage, indicating that the uptake of calcium across the gills may be largely passive. A low-affinity Ca^{2+}-activated ATPase is present in both gills and epithelium, but only the epithelial activity shows a substantial (fivefold) increase in the hours after ecdysis. The control mechanisms for stimulation of the transport processes remain elusive. Eyestalkless crabs progress normally through moult and post-moult calcification. Peptide profiles from blood and from suspected neuro-hormone-producing tissues differ markedly with moult stage, but several different approaches to developing a bioassay using peptides from crab tissues and vertebrate sources have been unsuccessful. The physiological events in the hours surrounding ecdysis suggest a complex and precisely timed series of control signals.

Introduction

The events associated with moulting (ecdysis) in crabs have been generally appreciated and described for a long time (e.g. Churchill, 1921). Around the time of ecdysis, there is a rapid weight gain and size increase, which is completed quickly after ecdysis (Gray & Newcombe, 1939). Hardening of the shell, which involves both protein tanning (Dendinger & Alterman, 1983; Andersen, 1985;

Key words: crab, moult, calcification, crustacean, ecdysis.

Reynolds, 1985) and calcification (Travis, 1957, 1963; Drach, 1939; Vigh & Dendinger, 1982; Roer, 1980; Cameron & Wood, 1985; Cameron, 1985), begins some time in the 12 h following ecdysis and continues for several days.

The rapid weight gain and changes in ion transport occurring within hours of the moult (Towle & Mangum, 1985; Cameron & Wood, 1985) suggest that it may be important to have an hour-by-hour description of the various components of ecdysis and post-ecdysial calcification. This is particularly important as a basis for investigating control of the sequence: parallel work on metamorphic moults of insects has shown that some hormonal signals may be present for as little as 10 min (Truman, 1985). The objective of the present work was to examine several processes associated with ecdysis on a shorter time scale, and to conduct some preliminary investigations into possible control mechanisms.

Materials and methods

Blue crabs (*Callinectes sapidus*) were either collected in the vicinity of Port Aransas, Texas by a variety of methods including pots, push nets and trawls or were purchased from local crabbers. The animals were transferred to holding tanks in the laboratory and were kept at temperatures ranging from 18 to 28 °C and supplied with running sea water. The salinity of holding tanks and for all experiments averaged $25{\cdot}2 \pm 1{\cdot}3$ ‰, and the ambient calcium concentration was $9{\cdot}3 \pm 0{\cdot}3$ mmol l^{-1}. All animals were fed daily *ad libitum* with chopped fish and shrimp. Crabs showing premoult 'signs' were transferred to individual holding compartments constructed of blackened Plexiglas.

Crabs showing the earliest stages of ecdysis were placed in preweighed wire-mesh baskets which were in turn placed in small, darkened aquaria supplied with running sea water. At various times from 10 h before until 14 h after moulting the animals were quickly removed in the wire-mesh basket, weighed, and returned to the aquarium. At the completion of ecdysis, the shed exoskeleton was removed, blotted free of excess water, and weighed.

Blood samples were all taken from and injections made into the pericardial space through a neoprene foam pad that had been glued over a thinned area of the shell just lateral to the heart. Samples for calcium analysis were either diluted with equal volumes of $0{\cdot}1$ mol l^{-1} HNO_3, for later analysis by atomic absorption spectrophotometry, or frozen for later analysis by ion chromatography. Samples for pH measurement were placed immediately in a thermostatted capillary-type pH electrode (Radiometer) calibrated with phosphate buffers.

Potential measurements were made between a catheter filled with 3 mol l^{-1} KCl/1 % agar glued in the pericardial space and another placed in the external bath. The opposite ends of the catheters were placed in well-type calomel electrodes which were connected to a millivoltmeter with >100 MΩ input impedance. All measurements were corrected for electrode and tip asymmetry potentials.

The eyestalk removal was performed on animals made torpid by cold. Both eyestalks were cut off at the base, and the wounds were cauterized. The animals were held individually and fed as usual until they moulted.

Net acidification rates were measured in closed-circuit systems described previously (Cameron, 1985; Heisler, 1984). Briefly, these contained the animal chamber, a re-aeration column, a particle filter and recirculating pump. A second circuit directed a small flow of water through three thermostatted chambers bubbled vigorously with 1% CO_2, a fourth chamber containing a pH electrode, and back to the system. A change in pH of the water at constant P_{CO_2} could be translated to a net H^+ excretion rate by calibrating the system with known additions of acid or base. Ammonia excretion was a negligible fraction of the net H^+ excretion and was not measured in most of these experiments. For experiments in which the calcium uptake rate was measured, the system was the same except that ^{45}Ca was added to the water and samples of both blood and water were taken for measurement of $[Ca^{2+}]$ and ^{45}Ca radioactivity (by liquid scintillation counting).

Gill assays

For the studies of calcium uptake by isolated gill slices, the sixth and seventh gills were quickly dissected free from freshly killed crabs and immediately placed in a shallow dish of chilled crab Ringer. Gills 6 and 7 were used because they are large and contain the thick, presumably osmoregulatory, epithelium on all lamellae (Copeland & Fitzjarrell, 1968). A short length of silicone rubber tubing was inserted into the cut end of the afferent vessel, and blood was gently rinsed out of the gill with a syringe attached to the other end of the tubing. By adding a small drop of Methylene Blue to the Ringer, gills could be checked for complete flushing; any that showed signs of clotting were discarded.

Solutions to be tested for calcium activity were then introduced into the blood space of the gills with a second syringe, and the gills were allowed to incubate whilst immersed in Ringer's solution for 20 min. At the end of the pre-incubation period, slices were cut from the gills with a fresh scalpel or razor such that each slice contained 8–20 opposing pairs of lamellae and a short length of the supporting arch and blood vessels. The slices were placed randomly into shallow wells of tissue culture plates which had been prepared with 0·5 ml of 0·2 μm-filtered sea water, $^{45}CaCl_2$ and 0·1 ml of the test solution. The tissue plates were agitated continuously for the first 5 min and every 30 s thereafter. After sample intervals of 2, 5, 10, 20 and 30 min, triplicate slices were quickly removed, dipped rapidly in three successive seawater (SW) rinses, and placed into scintillation vials containing 1 ml of 3 mmol l^{-1} EDTA solution. The slices were periodically crushed with a glass rod and allowed to sit for 2–4 days to extract all calcium. At the end of this period, a sample of the EDTA was removed for analysis of total calcium (by atomic absorption), and scintillation cocktail was added to the remainder. All results were expressed as specific radioactivity, i.e. count min^{-1} $mmol^{-1}$ Ca^{2+}.

During the period of isotope uptake, the blood space was open to the medium

via the cut vessel ends, so in theory calcium entering across the lamellar surfaces could diffuse back into the bath. The distance across the lamellar surface, however, was about 5–8 μm, whereas the average distance to an open vessel end was approximately 2 mm, nearly 1000 times farther. It is, therefore, unlikely that diffusion laterally through the blood space was significant.

Ca^{2+}-ATPase assays

Tissues for assay of Ca^{2+}-activated ATPase were dissected free as quickly as possible, blotted, and placed on ice. All subsequent steps were performed at 4°C with as little delay as possible. The tissues were homogenized in four volumes of a homogenizing medium with the following composition: 0·25 mol l^{-1} sucrose, 6 mmol l^{-1} EDTA, 20 mmol l^{-1} imidazole, adjusted to pH 6·8 with acetic acid. Just before use, 0·1 % deoxycholate (DOC) was added. The tissues were homogenized at 4°C in Teflon/glass, then centrifuged for 15 min at 300 *g*. This first pellet, containing nuclei and cell fragments, was discarded. The supernatant was then centrifuged for 20 min at 7500 *g* to isolate a mitochondrial pellet, which was also discarded in most experiments. The supernatant from this step was then centrifuged at 105 000 *g* for 90 min to isolate the microsomal (pellet) and cytosolic (supernatant) fractions. Most assays were conducted with the microsomal pellets, although in some early assays all fractions including the crude homogenate were used. The microsomal pellets were resuspended with 1 ml of the tissue homogenizing buffer by briefly homogenizing them (three strokes) in a small-volume Teflon/glass homogenizer. At the conclusion of the isolation procedure, the various fractions were either assayed immediately or quick-frozen in liquid nitrogen and stored at −70°C for later analysis. No samples were stored for more than 3 weeks.

The assays were conducted by adding 50 μl of fresh or freshly thawed fractions (containing 100–500 μg of protein, by Coomassie Blue dye assay) to 1 ml of an assay medium. The medium composition was: 20 mmol l^{-1} imidazole adjusted to pH 7·8 with acetic acid, 2·5 mmol l^{-1} ATP (metal-free, Sigma) and variable concentrations of $CaCl_2$. The assay timing was begun with the addition of the ATP, and samples were usually incubated for 20 min with agitation. After 20 min, 1 ml of 10 % (w/v) trichloroacetic acid (TCA) was added and the samples mixed vigorously and allowed to stand for 5 min. The samples were then centrifuged for 5 min at 5000 *g* to remove the precipitated protein, and the supernatant was decanted and saved for assay of the inorganic phosphate produced by hydrolysis of ATP during the incubation period. The inorganic phosphate was measured using a modified Fiske & Subbarow procedure (Sigma), based on colorimetric assays of the blue phosphomolybdate complex.

The usual procedure was to assay duplicate samples of each fraction in the presence of Ca^{2+}, and duplicate samples with no Ca^{2+} present. The water used for all reagents was double-distilled in glass and passed through ion-exchange purifiers (Milli-Q system), but even so the effect of addition of EGTA to the Ca^{2+}-free samples was tested to make sure no Ca^{2+} was present. All glassware was acid

washed and rinsed in ultrapure water to ensure a low blank. The Ca^{2+}-activated ATPase specific activity was taken as the difference in Pi formation with and without Ca^{2+} present, and was expressed as nmol phosphate μg^{-1} protein min^{-1}.

Peptide isolation

Peptide fractions of blood and various tissues were prepared by adding either declotted blood or freshly dissected, blotted tissue to an equal volume of 1 mol l^{-1} cold glacial acetic acid. The tissues were homogenized in glass, and then all samples were centrifuged for 5 min at 5000 ***g*** to remove precipitated protein. The pellet was resuspended in 1 mol l^{-1} acetic acid, recentrifuged, and this supernatant added to the first. The combined supernatant was then frozen and lyophilized, then dissolved in 5 ml of ultrapure water at 4°C. Preparative C-18 cartridges (Waters Sep-Pak) were then activated by a rinse with 5 ml of *n*-propanol: water (1:1) and washed three times with 5 ml of water. The redissolved supernatants were then introduced onto the cartridges, followed by five rinses with 3 ml of water. The peptide fractions were then eluted with 3 ml of *n*-propanol: water (1:1), collected over 200 μl of 0·1 mol l^{-1} sodium borate, pH 9·3. These samples were frozen and evaporated to near dryness in a vacuum centrifuge (Speed-Vac). The nearly dry samples were stored at −70°C until analysed.

For analysis of the peptide fractions, the samples were redissolved in 0·5 ml of water and filtered (0·45 μm, Gelman Acro-disc). The various peptides were separated by HPLC on a 3 μm C-18 reverse-phase column (Waters Nova-Pak) using the following solvent protocol: solvent A, 0·1 % (w/v) trifluoroacetic acid (TFA) in water; solvent B, 0·1 % TFA in *n*-propanol: water (3·5:1); gradient, 5 to 45 % B in 45 min at 0·5 ml min^{-1}. Simultaneous detection was by absorbance at 280 nm and native fluorescence with excitation at 254 nm and emission at 340 nm. The weak native fluorescence helped to confirm that absorbance peaks were, in fact, peptide material. The total peptide content was assayed by a fluorescamine method modified from Gruber *et al.* (1976) using commercially available bradykinin and insulin fragments as standards (Sigma). From some of the HPLC runs, 1 ml fractions were collected and assayed for confirmation that detected peaks were indeed peptides.

The same redissolved peptide fractions were also employed for various animal injection experiments. All reagents were HPLC grade and were filtered before use. Blank analyses showed virtually no contaminating material.

Results

Post-moult events

Weight gain

Significant weight gain was already evident 4 h before ecdysis, and the rate of gain accelerated rapidly through ecdysis. By 2 h after the moult the weight gain was essentially complete (Fig. 1). During the hour after the moult, the change is readily visible, as the new carapace is distinctly puckered and wrinkly immediately

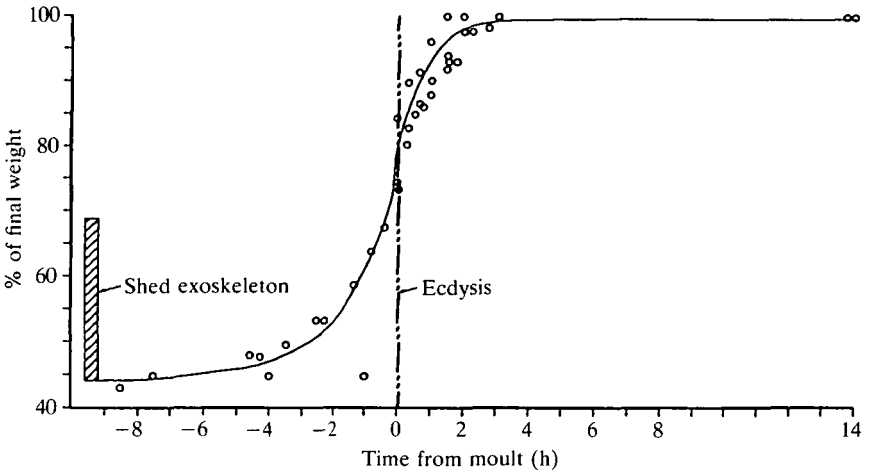

Fig. 1. The time course of weight gain during the hours before and after ecdysis in six crabs. The hatched bar on the left indicates the weight of the exoskeleton which is shed during ecdysis. All values calculated as percentage of weight reached 24 h after ecdysis; line fitted by eye.

afterwards and becomes smooth and fully extended by 1 h post-moult. The final weight was 2·27 times the initial weight after subtracting the weight of the shed carapace. Assuming that the extracellular fluid (ECF) volume 12 h before moult was 30 % of body weight (BW) and that all the weight gain at +2 h had entered the ECF, there would have been a dilution of the blood by a ratio of 5·2:1.

Calcium ratios

In the intermoult animals, the internal-to-external calcium concentration ratio was approximately 1·4:1 (Fig. 2), higher than the ion activity ratio of about 1·05:1 reported by Towle & Mangum (1985) using calcium ion electrodes. The calcium concentration dropped precipitously in the 12 h preceding ecdysis, increased somewhat for a few hours afterwards, then declined for a period lasting a week or more. The greatest reversal of the internal/external ratio, occurring at 2 days post-moult, coincided with the period of greatest calcification (Fig. 2; Cameron, 1985).

Transgill potentials

The mean electrical potential between the blood and the external sea water in four intermoult crabs was 2·4 ± 1·1 mV (±S.D.), inside positive, and for four post-moult crabs (0·5–2 days) it was 2·1 ± 0·3 mV. The difference was not significant.

Onset of acidification

In earlier work the post-moult acidification had been examined in 12- to 24-h increments, but it was of interest to measure the rate at which acidification began in the hours immediately following ecdysis. The pre-ecdysial crabs are characterized by a net negative H^+ excretion (i.e. net base excretion) of abou

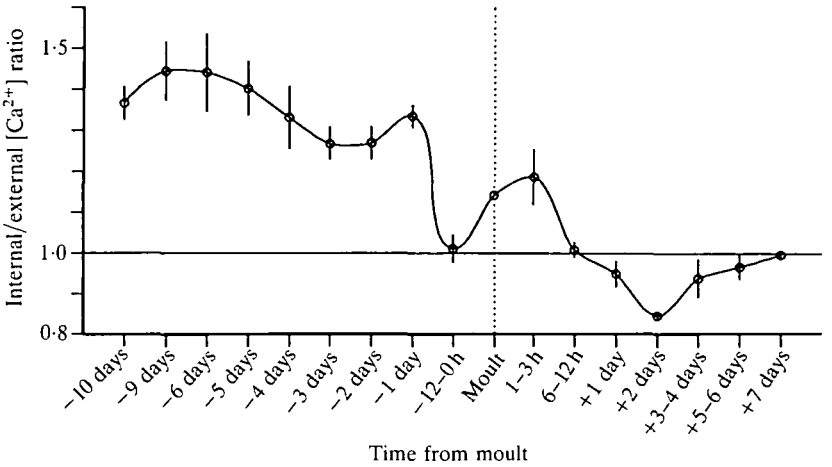

Fig. 2. The ratio of internal to external $[Ca^{2+}]$ as a function of time with respect to ecdysis. Data gathered from 22 crabs; vertical bars indicate ±1 S.E. and the horizontal line at equal concentration is for reference. Line fitted by eye.

500 μequiv kg^{-1} h^{-1} (Fig. 3). There is little change in this basal excretion until between 0·5 and 1 h post-moult, when acidification begins. By 2 h, quite high rates are reached compared with the usual acid–base response of crabs (cf. Cameron,

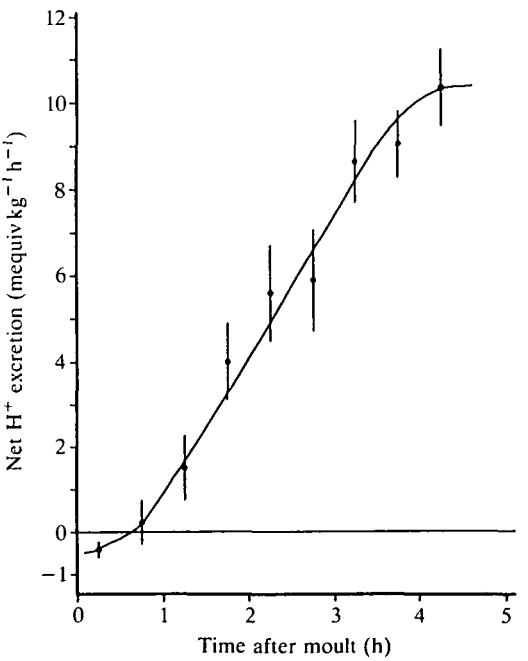

Fig. 3. Net H^+ excretion in eight crabs as a function of hours after ecdysis. Vertical bars indicate ±1 S.E.; line fitted by eye.

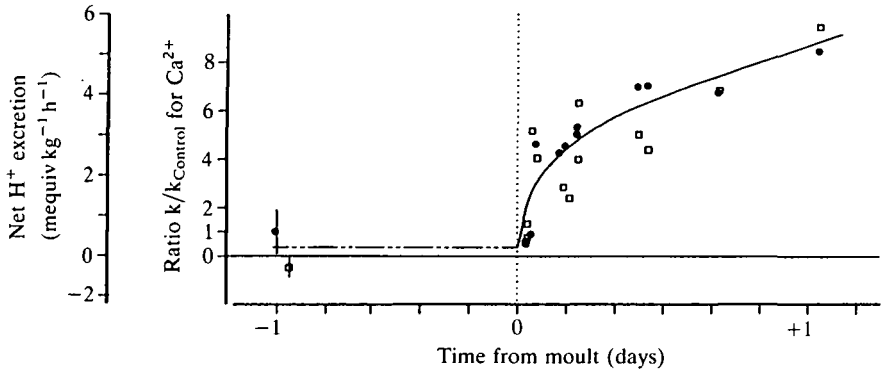

Fig. 4. Correlation between the onset of acidification (open squares) and calcium uptake (filled circles) in 14 crabs as a function of time from ecdysis. Vertical bars indicate ±1 S.E.; line fitted by eye. $k/k_{control}$ is the ratio of the rate constant for calcium uptake after moulting to the mean control (pre-moult) rate.

1986), and the rate continues to accelerate, reaching peak values between 12 and 48 h post-moult (Cameron, 1985; Cameron & Wood, 1985).

Onset of calcification

The onset of calcification was more difficult to measure accurately than the onset of acidification, owing to higher intrinsic variability among crabs and to the technical problems of measuring calcium fluxes. There was, however, a good correlation ($r = 0{\cdot}81$, $P < 0{\cdot}001$) between acidification and net calcium uptake during the period from 12 h prior to 12 h after ecdysis (Fig. 4) and there was no apparent difference in the timing of the increases. On a longer time scale this correlation has been noted previously (Cameron, 1985; Vigh & Dendinger, 1982).

Calcium-activated ATPase

The Ca^{2+}-activated ATPase from muscle was characterized by a higher affinity and higher V_{max} than that from the epithelium underlying the dorsal carapace (Fig. 5). The K_m for muscle enzyme was 0·96 mmol l^{-1} compared to 2·53 mmol l^{-1} for the epithelial enzyme; V_{max} was 12·1 nmol μg^{-1} min^{-1} for the muscle enzyme and 8·95 nmol μg^{-1} min^{-1} for the epithelial enzyme. The pH/activity profiles for Ca^{2+}-ATPases from muscle, epithelium and gill were also different (Fig. 6). The muscle enzyme had an optimum at pH 6·8, and the curve suggests the presence of two different enzymes. Both gill and epithelium showed maximal activity at pH values above 8 and dropped off sharply below pH 7·5.

The specific activity of Ca^{2+}-activated ATPase from gills and epithelium showed no significant change from moult stages C (intermoult) to E (ecdysis), but the epithelial activity increased significantly between 6 h and 7 days after moulting (Fig. 7). There was roughly a fivefold increase in epithelial activity, but no significant increase in gill activity. In all the preparations, activity in the cytosolic fraction was negligible, the majority of activity residing in the microsomal fraction

Inhibition of H^+ excretion

The impressive increases in transport rates following moulting suggested that new transport proteins might be synthesized during the immediate post-moult period. The effects of protein synthesis inhibitors were therefore assessed by injecting them into crabs immediately after the moult and then following the onset of acidification. Both actinomycin D ($16\,\mu g\,100\,g^{-1}$ BW) and cycloheximide ($50\,\mu g\,100\,g^{-1}$ BW) had dramatic effects on the onset of net H^+ excretion, as shown in Fig. 8. There was about a 2-h lag before actinomycin D took effect, whereupon the rate of acidification, which was just beginning to increase substantially, dropped to negative values. Measurement of acidification over longer periods in actinomycin-treated animals showed that by 24 h post-moult (and post-injection), rates of acidification had returned to normal, i.e. to the very high rates expected at that stage. The effects of cycloheximide were a little less dramatic, but nonetheless significant. By about 2 h post-injection, the increase in acidification stopped, and did not resume for about 24 h.

All treated crabs were held for several days without mortality, and all eventually completed normal calcification of their new exoskeletons. In addition, the effects of protein synthesis inhibitors on metabolic rate were assessed by measuring oxygen consumption rates for 3 h before and 6 h after injection. There were no significant changes with either drug ($N = 4$ for each).

Calcium kinetics in isolated gills

The rates of calcium uptake under various experimental conditions were assessed both in whole isolated gills and in gill slices, as described in Materials and methods. The gill slices showed a typical exponential approach to the specific radioactivity of the external bath, so log–linear regression slopes were used as an

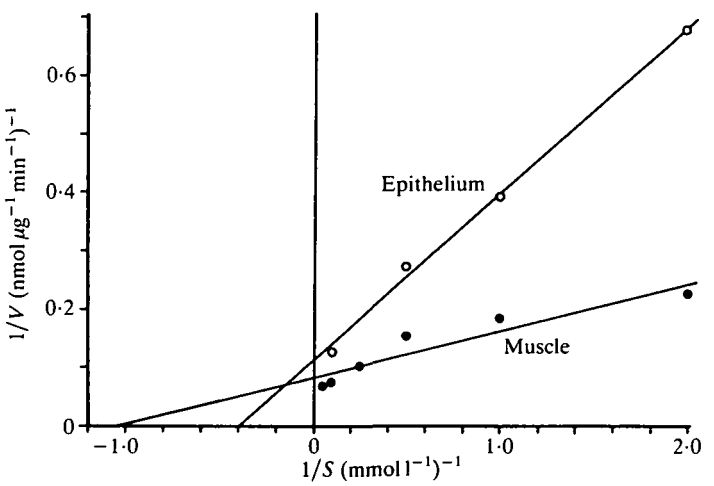

Fig. 5. Lineweaver–Burke plots for Ca^{2+}-activated ATPase from muscle and from the epithelium underlying the dorsal carapace (pH 7·8).

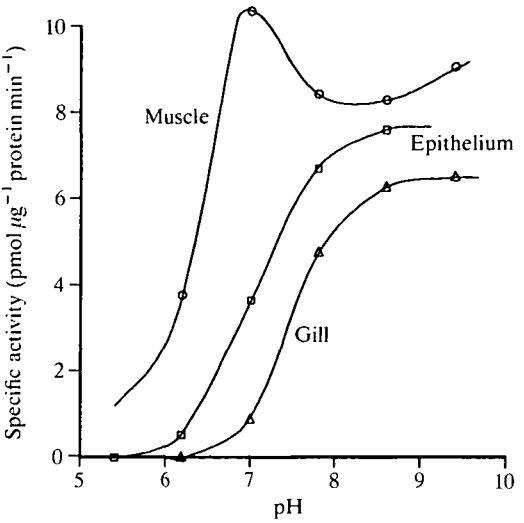

Fig. 6. pH/specific activity profiles for microsomal Ca^{2+}-activated ATPase from muscle, gills and the epithelium underlying the dorsal carapace. All samples taken from 1 day post-moult animals.

index of the rate of calcium uptake. The results (Table 1) showed statistically significant differences between intermoult and post-moult gills, but the increase was only 25%, much less than the increase in whole-animal uptake. Pre-incubation of the intermoult gills with the peptide fraction isolated from post-moult blood had no effect, but pre-incubation with a peptide extract from post-moult thoracic ganglia did stimulate Ca^{2+} uptake.

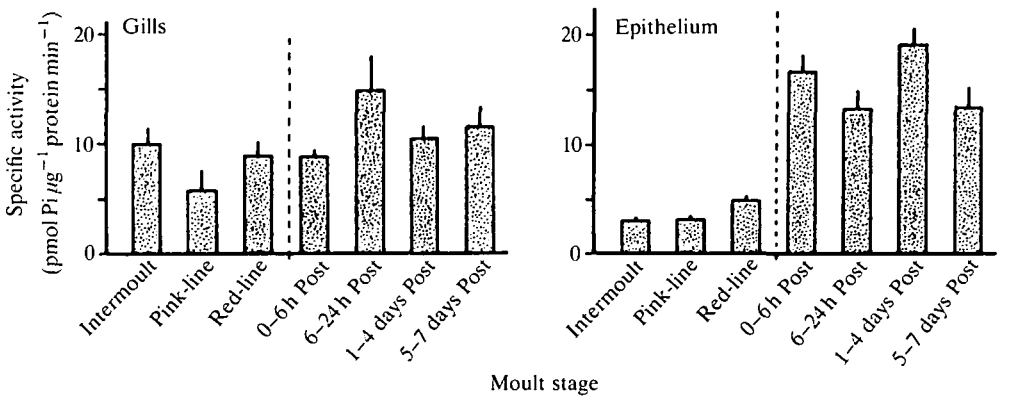

Fig. 7. Changes in the specific activity of Ca^{2+}-activated ATPase from gills and epithelium as a function of moult stage. A 'pink-line' crab is approximately 2 days and a 'red-line' crab approximately 1 day from moulting. The dotted line indicates ecdysis, and the vertical bars indicate 1 S.E. $N = 5$ for gills at each stage and $N = 7$ for epithelium at each stage.

Effects of various peptides

A number of vertebrate hormones affect the concentration of Ca^{2+} in the blood, so on the chance that some of these might also affect the blue crab, several trials were conducted. Substances tested were: calcitonin ($2{\cdot}5\,\mu g\,100\,g^{-1}$ BW), parathyroid hormone (in a dose to bring the blood to $0{\cdot}5$ i.u. ml^{-1}), vitamin D3

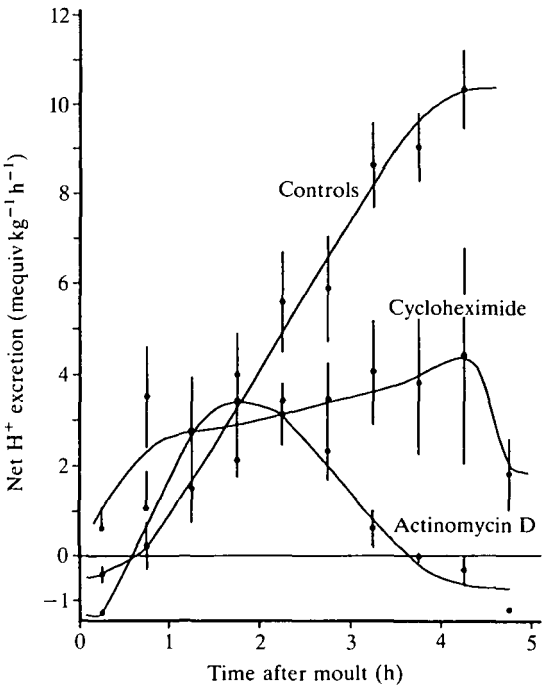

Fig. 8. The effects of protein synthesis inhibitors cycloheximide and actinomycin D on the onset of acidification after ecdysis. The control data are duplicated from Fig. 3. Vertical bars indicate ±1 S.E.; lines fitted by eye.

Table 1. *The rates of ^{45}Ca uptake by gill slices*

Treatment	Slope	S.D.	*P*
Intermoult gills			
Controls	0·0668	0·0062	
+PM blood peptide extract	0·0587	0·0077	NS
+PM thoracic ganglion extract	0·0942	0·0096	$0{\cdot}02 < P < 0{\cdot}05$
Post-moult gills			
Controls	0·0837	0·0232	0·06.

The slopes were calculated by linear regression of time in min (x) *vs* $-\ln(1 - SR_t/SR_f)$, where SR_t is the specific radioactivity at time t, and SR_f is the final or external bath specific radioactivity.

P values from comparisons of intermoult control slope with others by Student's *t*-test using combined variance for regression through origin.

PM, pre-moult.

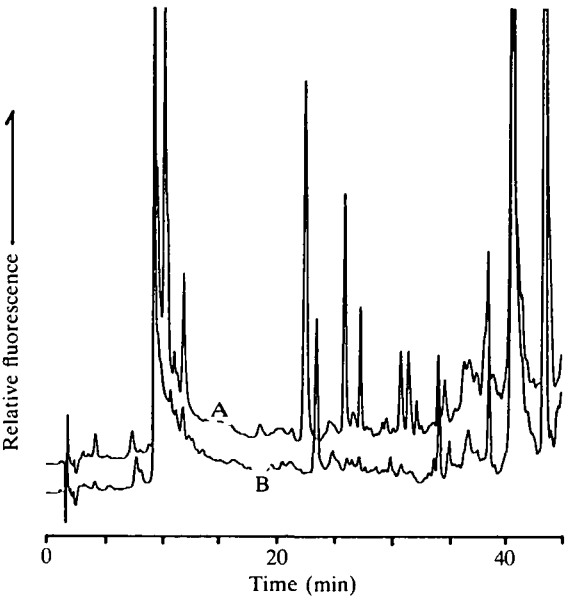

Fig. 9. HPLC profiles of the peptide fraction isolated from post-moult (A) and pre-moult (B) blood samples.

(30 nmol 100 g^{-1} BW) and sham saline injections. Variables measured included blood pH, blood [Ca^{2+}] and net H^+ excretion. All variables were measured for 3–4 control periods of 0·5–1 h after a suitable recovery period from handling and transfer to the experimental chambers. At the end of the control period the material to be assayed was injected pericardially. The experiment was then continued for 3–4 h. There was no significant response of any variable to any of these treatments (one-way analysis of variance).

Peptide extracts were also prepared from pooled intermoult blood samples, pooled post-moult (0–6 h) blood samples, pre- and post-moult thoracic ganglia, pre- and post-moult brains, and pre- and post-moult pericardial organs (see Materials and methods). Peptide profiles of these extracts were analysed by HPLC. The profiles of each were quite complex, and there were conspicuous and consistent differences from one moult stage to another (Fig. 9). That is, certain prominent peaks in post-moult extracts were not present in intermoult extracts, and *vice versa*.

Using a protocol similar to that used for the vertebrate peptides, the effects of injections of these materials on blood [Ca^{2+}], net H^+ excretion and blood pH were measured. The injections were contained in 100 μl of crab Ringer, and contained a total of 2–15 μmol l^{-1} of peptide as measured by fluorescamine assay. In one early series of experiments ($N = 4$) the post-moult peptide fraction appeared to depress the blood [Ca^{2+}] (paired t-tests, $P < 0{\cdot}05$), but this result could not be repeated in later series ($N = 6$). No other treatment had any significant effect on any parameter measured.

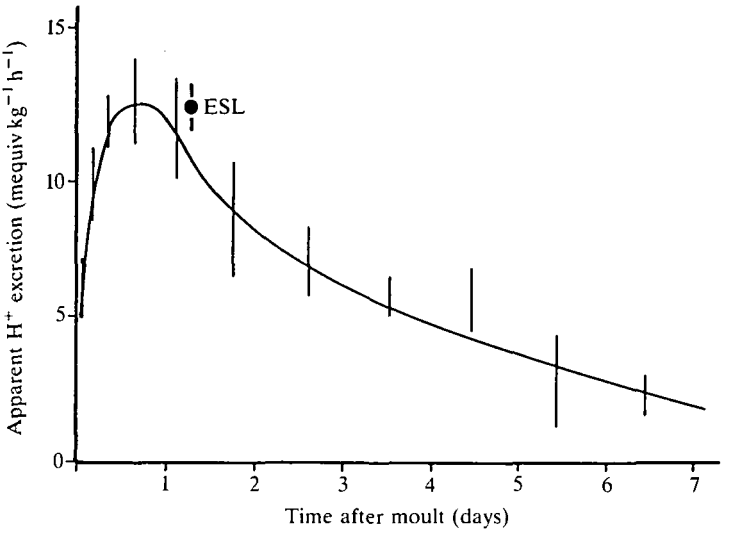

Fig. 10. Net H^+ excretion in seven eyestalkless crabs (ESL) at 30 h after ecdysis compared with normal crabs (line and vertical bars). Normal crab data are redrawn from Cameron & Wood (1985).

Effects of eyestalk removal

The various neurosecretory tissues of the eystalk are known to produce a suite of peptide hormones (Newcomb, 1983; Newcomb *et al.* 1985; Stuenkel, 1983), only a few of which have a known physiological function. The eyestalk complex is also known to be involved in the control of moulting *via* production of the moult-inhibiting hormone (MIH), a small neurosecretory peptide (Webster, 1986; Webster & Keller, 1986). Eyestalks were therefore surgically removed from seven crabs during the intermoult (C) stage. These crabs were held in individual aquaria and fed *ad libitum* until they moulted, a period ranging from 1 to 4 weeks. The rate of H^+ excretion was measured in these crabs, by methods described earlier (Cameron & Wood, 1985), for a period between 30 and 36 h after ecdysis. The net H^+ excretion rate was not significantly different from that of crabs with normal, intact eyestalks (Fig. 10), and the progress of calcification over the next few days was normal.

Discussion

Post-moult events

Gray & Newcombe (1939) measured the rate of increase in shell width in 26 blue crabs and concluded that the maximum size was reached by the majority of animals by 2 h after ecdysis. That agrees with the present data, except that they apparently did not appreciate how much of the weight gain had already occurred by the completion of ecdysis (Fig. 1). The total weight gained as a percentage of the pre-moult weight minus the exuvia was about twice as large in this study as in earlier

work on the lobster (Mykles, 1980) and the blue crab (Mangum *et al.* 1985). The reason for this is not clear; temperature and salinity were similar in the Mangum *et al.* study.

The preponderance of evidence is that the weight gain is driven by stimulation of monovalent ion uptake, either across the midgut (Mykles, 1980) or across the gills, followed by osmotic water influx. Towle & Mangum (1985) discussed the evidence, and found an increase of Na^+/K^+-ATPase activity in gills averaging about 37 % in the D4 stage. A stage D4 crab is identified as having open ecdysial sutures (see Mangum, 1985; Passano, 1960; Drach & Tchernigovtzeff, 1967). In our laboratory we have found that this stage averages 12–18 h, but may be as short as 1 h. If the weight gain shown in Fig. 1 were indeed correlated with increased Na^+/K^+-ATPase activity, one might expect that the activity would be highest between 4 h before and 2 h after ecdysis. Towle & Mangum's (1985) data actually show the highest activity in stages A1 to B2, after the weight gain has stopped. It would be interesting to remeasure the Na^+/K^+-ATPase activity on an hour-by-hour basis.

Data on the changes in internal Ca^{2+} show less agreement among investigators. Towle & Mangum (1985) show free Ca^{2+} activity declining from an intermoult ratio of about 1·05:1 to 0·77:1 in stage B1 (about 2–3 days after ecdysis). Their data do not show a drop in the immediate pre-ecdysis period like that reported here (Fig. 2); but their data were not gathered with the same time resolution and are otherwise in general agreement with the present study. Guderley (1977) reported a similar ratio of 1·2:1 for intermoult and 0·88:1 for 'freshly moulted' *Cancer magister*, and Robertson (1960) also noted a decrease in blood $[Ca^{2+}]$ after the moult. The majority of samples taken for the −12 to 0 h point in Fig. 1 were taken within 4 h of the moult, so the decline at that time is probably due to simple dilution of the blood by the salt and water uptake discussed above. The later decline (at 1–2 days post-moult) is more likely to be a result of the rapid calcification occurring at that time.

The timing of the onset of acidification is in general agreement with the course of events described in earlier work (Cameron & Wood, 1985; Cameron, 1985), but on a finer time scale. The change from essentially control rates, which usually represent a small net base excretion, to the very high rates of H^+ excretion characteristic of the post-moult period occurs in a short time interval – between 1 and 4 h post-ecdysis (Fig. 3). The timing seems quite consistent among crabs, although there is more variability in the peak values reached. Smaller crabs reach higher mass-specific rates; some individuals have exceeded 20 mequiv kg^{-1} h^{-1}.

Most other work has also shown that the rapid increase in calcification occurs in the immediate post-moult period. Both Welinder (1975) and Vigh & Dendinger (1982) reported virtually no calcium in various regions of epithelium at stage E (ecdysis), and that peak calcification was reached at 1–2 days after moult. Cameron & Wood (1985) found almost no calcium in whole animals immediately upon completion of the moult. This corresponds with direct observations of the appearance of calcium crystals in various crustaceans at times ranging from 4 t

12 h after moult (Drach, 1937). Henry & Kormanik (1985) reported increased Ca^{2+} uptake in three isolated pieces of epithelium at a stage just before the moult.

Since tanning of proteins is thought to occur in the post-moult period, it was of interest to see whether there might be a lag between the onset of acidification and the onset of calcification. Some of the reactions involved in tanning appear to produce excess H^+ (Andersen, 1985), and an early acidification without parallel calcification might be interpreted as having been caused by tanning. Data on calcium uptake rates are less precise, because of larger experimental errors, but the onset of calcification appeared to follow the same time course as did acidification (Fig. 4). Over longer time periods a correlation has been noted previously between net acidification and net calcium uptake (Cameron, 1985); evidently this relationship also holds on an hour-by-hour basis following ecdysis, since the correlation coefficient was highly significant.

Ca^{2+}-ATPase activity

Considerable Ca^{2+}-activated ATPase activity was found in both the gills and the epithelium underlying the dorsal carapace. In most tissues there is some Ca^{2+}-ATPase present whose function is apparently to help maintain the very low free intracellular Ca^{2+} activity required by cells. The affinity of this enzyme is usually very high, with K_m values in the micromolar range (Godfraind-Debecker & Godfraind, 1980). In the present work calcium was present in the assay at relatively high concentrations (usually 10 mmol l^{-1}), and the K_m values were in the low millimolar range, as might be expected of an enzyme whose function is transepithelial transport in a millimolar concentration environment. The shape of the pH/activity curves (Fig. 6) suggested that there might be two enzymes present in muscle, one with an optimum at about pH 7, and the other with an optimum similar to that of epithelium and gill at values above pH 8.

What is perhaps surprising about the Ca^{2+}-ATPase studies is that, like Towle & Mangum's (1985) studies of Na^+/K^+-ATPase, the increase in activity is small in relation to the increase in observed transport. There is about a fivefold increase in epithelial activity in the post-moult period (Fig. 7), but net calcium transport increases approximately 100-fold. Gills did not show any change in activity, whatever the moult stage. The lack of any increase during the period of rapid calcification provides further support for the idea that calcium entry into the animal is largely passive, driven by the reversed calcium gradient (Fig. 2).

Gill assays

The assays conducted both with whole isolated gills and with gill slices were well-behaved in the sense of showing predictable and reproducible calcium kinetics. The time-series samples showed a good fit to an exponential model of uptake, and specific activity remained low enough to obviate back-flux corrections. In terms of shedding light on the mechanisms of post-moult calcification, however, they were disappointing. There was a statistically significant increase in the calcium uptake of post-moult gills (Table 1), but the increase was so small in

relation to the changes in net calcium uptake rates of whole animals as to be physiologically insignificant. There was also a statistically significant stimulation of intermoult gills by a peptide extract from post-moult thoracic ganglia, but whether this is physiologically meaningful will require further investigation.

The lack of a large increase in Ca^{2+} uptake by post-moult gills, taken from a period in which net calcium uptake is very high, implies that calcium movement across the gills may be largely passive. During the post-moult period the chemical gradient is directed inwards (see above; Fig. 2). The equilibrium potential for Ca^{2+} in intermoult crabs, calculated from total concentration, is +8·9 mV (inside positive) and switches to −4·6 mV at 2 days post-moult. Given that part of the blood calcium is protein-bound, however, the true equilibrium potentials are probably always higher than the transgill potential, favouring inward movement. The relatively rapid equilibration between isolated gill slices and the bathing medium, as well as the rapid uptake by whole animals, points to a high calcium permeability. The possibility of limiting conditions in the isolated gill assays should be recognized, however. If, for example, there were a significant Ca^{2+}/H^{+} exchange component, the lack of gill perfusion might have led to a rapid alkalosis in the blood/tissue space of the isolated gills, and such an alkalosis could have limited any further exchange.

Inhibitor studies

Both actinomycin D and cycloheximide dramatically inhibited the onset of acidification after ecdysis (Fig. 8). Both drugs were employed at doses reported in other tissues and animals to be effective in blocking protein synthesis, actinomycin D at the transcriptional level and cycloheximide at the translational level (e.g. Bikle *et al.* 1978). There was no significant inhibition of general metabolic activity, as manifested by oxygen consumption rates, nor was there permanent interference with normal calcification. Although it is tempting to speculate that these protein synthesis inhibitors directly blocked production of new membrane transport proteins, certainly other interpretations are possible. For example, one could argue that they blocked the synthesis of a peptide neurohormone required for stimulation of the transport processes, or interfered with other tissue growth occurring after the moult. One would like to have more specific assays, perhaps using isolated tissues or cell fractions, to pinpoint the site and mode of inhibition by these inhibitor drugs.

Control and peptide studies

The focus on peptide neurohormones was based on circumstantial evidence. First, the principal steroids known in crabs, the ecdysones, appear to have more to do with control of the preparation for the moult than with the actual events of ecdysis and post-ecdysial hardening. Ecdysone titres reach peak levels some time before the moult, then fall sharply, reaching intermoult values by or just after ecdysis (Hopkins, 1983, 1986; Soumoff & Skinner, 1983). This is a pattern similar to that in insects, in which there are at least two important hormones that are

apparently triggered by the falling ecdysone titres: eclosion hormone and bursicon (Truman *et al.* 1981; Reynolds, 1985; Truman, 1985). Both these are peptide neurohormones, secreted by regions of the brain, corpora cardiaca and thoracic ganglia.

A suite of peptide neurohormones is also manufactured by the eyestalk X-organ/sinus gland complex (Newcomb, 1983; Stuenkel, 1983; Keller & Kegel, 1984), including the moult-inhibiting hormone (Webster, 1986; Webster & Keller, 1986; Mattson & Spaziani, 1985, 1986). A logical experiment, then, was to remove the eyestalks completely and see whether there was any effect on the post-moult calcification events. There was not. Evidently any hormonal signals involved in triggering the transport processes under study must originate somewhere else.

Peptide fractions were isolated at different moult stages from brain, thoracic ganglion and pericardial organs, all tissues known to contain neurosecretory cells (Johnson, 1980; Cooke & Sullivan, 1982). Peptide extracts were also prepared from blood and gills. The peptide profiles from all these sources were complex, and showed consistent differences related to moult stage. It is now necessary to develop some type of bioassay which will indicate which fractions, if any, have calcium or H^+ transport stimulating activity.

So far all attempts to develop such a bioassay have failed. The gill assays described were one such attempt. Whole animals were also employed, using injections of various materials and looking for changes in blood calcium concentration, net calcium flux or net H^+ excretion. Some further experiments were done using Ussing chambers and pieces of dorsal epithelium, but the tissues were simply too fragile to dissect free and mount properly. Various other experiments have also been tried, including injections of substances with calcium activity in vertebrates, such as calcitonin, PTH and vitamin D3. None of these substances has any effect on the crab, in spite of a report of a calcitonin-like peptide in the lobster (Fouchereau-Peron *et al.* 1987). Samples of peptide extracts from pre- and post-moult brains and thoracic ganglia were also assayed by J. W. Truman for eclosion hormone activity, all with negative results.

Conclusion

Our description of the timing and rates of various physiological processes associated with ecdysis is now reasonably complete. The earliest events are weight gain and, almost certainly, active uptake of monovalent ions across the gills or gut to drive the osmotic water influx and weight gain. A whole suite of behaviour is associated with ecdysis, including seeking shelter, particular postures, and finally the movements necessary to effect ecdysis. The weight gain continues for only 1–2 h after ecdysis, and by the time it is complete the calcification processes have accelerated to high rates. Calcification involves several transport processes, both at the gills and across the epithelium (Fig. 11). The activity of a microsomal Ca^{2+}-activated ATPase increases substantially in the epithelium, indicating an active Ca^{2+} transport at that site, but there is no corresponding increase in gills. As Ca^{2+} is transported into the new carapace, forming $CaCO_3$, there must be parallel

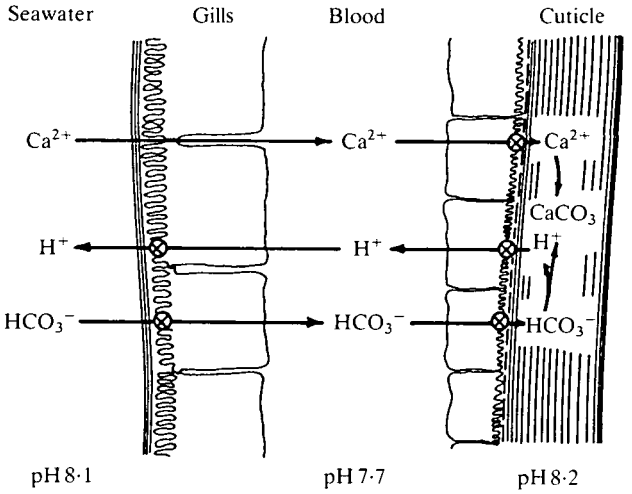

Fig. 11. Schematic model of the transport processes involved in post-moult calcification. Ca^{2+} crosses the gills passively, but is transported into the calcified layers of the cuticle. As $CaCO_3$ is formed, HCO_3^- must be transported both across the gills and across the endothelium, and H^+ must be transported across both tissues in the opposite direction. The calcified layers are maintained at an alkaline pH with respect to the blood. The symbol ⊗ indicates active transport, but the transport site may not be apical, as indicated in the diagram. See text for discussion.

transport of HCO_3^- across the gills from sea water and across the epithelium from the blood. H^+ is formed as a product of the reaction, and equivalent quantities of H^+ must be transported in the reverse direction to be excreted eventually to the sea water. All these transport processes change from near zero to extremely high rates in a matter of hours.

The control of all these processes is, however, much less clear. The timing of the various physiological and behavioural events suggests a series of hormonal signals, each with specific target processes. This is an area in which little is known, however, and much work remains to be done.

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