J. exp. Biol. **114**, 197–206 (1985)

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COMPENSATION OF HYPERCAPNIC ACIDOSIS IN THE AQUATIC BLUE CRAB, *CALLINECTES SAPIDUS*: THE PREDOMINANCE OF EXTERNAL SEA WATER OVER CARAPACE CARBONATE AS THE PROTON SINK

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Accepted 8 June 1984

SUMMARY

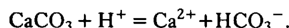
Radioactive labelling of the $CaCO_3$ in the crab's carapace was employed as a tool to study the contribution of the carapace carbonates to acute buffering of acid-base disturbances. Since Ca^{2+} uptake is extremely rapid during the post-moult period, crabs that moulted in the laboratory were incubated with ^{45}Ca for 5 days immediately following the moult in order thoroughly to load the carapace carbonate pool with radiolabel. After a subsequent 2-week interval for feeding and completion of the post-moult carapace mineralization phase, these ^{45}Ca-loaded crabs were subjected to a 24 h control period and a 24 h hypercapnic period (water equilibrated with 2 % CO_2 in air) to induce an internal acidosis. Compared with the control period, a compensatory increase in $[HCO_3^-]$ of 17 mequiv l^{-1} was observed in the blood, along with an apparent H^+ excretion to the external sea water of 10·8 mequiv kg^{-1}. A statistically significant increase in circulating $[Ca^{2+}]$ and in the specific radioactivity of the blood Ca^{2+} reached a maximum during the first 3 h of the compensatory phase. By measuring the radioactivity appearing in the water and the blood, and the specific radioactivity of the carapace carbonates, the contribution of the carapace carbonates was calculated to be 7·5 % of the total compensatory H^+ disposal. The rapid exchange of Ca^{2+} with the external medium, coupled with physiological regulation of blood $[Ca^{2+}]$ minimized changes in blood $[Ca^{2+}]$. The total dissolution of carapace $CaCO_3$ was approximately 0·9 mequiv kg^{-1}, less than 0·1 % of the quantity contained in the shell. The carapace carbonates, therefore, do contribute to acute buffering of hypercapnic acidosis, but their quantitative importance is small, with the gills serving to conduct most of the compensatory exchanges with the environment.

INTRODUCTION

The events associated with an externally imposed hypercapnia are now relatively well established for many aquatic animals. The internal P_{CO_2}, entrained by the elevated external P_{CO_2}, rises as the pH falls in a fairly short time. This initial acidosis

Key words: Crab, hypercapnia, buffering, carapace, carbonates.

is accompanied by a small increase in $[HCO_3^-]$ attributable to passive chemical buffering. During the next phase, active compensation produces a much increased $[HCO_3^-]$ with some return toward the initial pH (Cameron & Randall, 1972; Cameron, 1978; Heisler, 1980; Cameron, 1984). The active compensation may involve either outward transport of H^+ ions or inward transport of HCO_3^- ions, and is thought to be linked to exchanges for Na^+ and Cl^- ions, respectively (Krogh, 1938; Maetz & Garcia-Romeu, 1964; Cameron, 1976, 1978). It has been shown for two aquatic crabs that the primary organ of compensation is the gills, with the antennal glands having an insignificant role (Cameron, 1978; Truchot, 1979). In some crabs, however, the carbonate reservoirs of the carapace have been implicated in the compensation of acidosis, by the reaction:



$$CaCO_3 + H^+ = Ca^{2+} + HCO_3^-.$$

Participation of the carapace reservoirs in compensation of hypercapnic acidosis has only been shown for terrestrial crabs (Henry, Kormanik & Cameron, 1981; Wood & Randall, 1981) or for aquatic crabs when in air (DeFur, Wilkes & McMahon, 1980), and did not appear to be important in an aquatic crab (Henry *et al.* 1981). The evidence for participation of the carapace was an elevation in the circulating $[Ca^{2+}]$, which could not originate anywhere but the carapace with the animals in air. In the study of carapace buffering by the aquatic crab, however, there was no way to tell whether some dissolution of carapace carbonates may have occurred, followed by transport of Ca^{2+} to the external medium. The present study was designed to investigate the possibility of carapace buffering in hypercapnic acidosis by labelling the carapace with ^{45}Ca and measuring the rate of appearance of label during both control and hypercapnic acidosis treatments.

MATERIALS AND METHODS

The experiments were carried out on five blue crabs, between 179 and 290 g, collected in various pre-moult stages from Redfish Bay, Texas during October and November, 1983. The pre-moult individuals were captured in shaded, unbaited pots and the stage assessed according to signs described by Perry, Ogle & Nicholson (1979). The crabs were then transferred to individual aquaria supplied with running sea water between 20 and 26 °C. All experiments after moulting were conducted at 22 ± 1 °C. Food was offered *ad libitum*, but the crabs ceased feeding during the 24–48 h preceding moult.

Immediately after moulting, the crabs were transferred to individual 24-litre aquaria with vigorous aeration, but no exchange of water. To each aquarium, approximately 50 μCi of ^{45}Ca was added as the chloride salt in water. For the next 5 days, samples were taken twice daily for measurement of ^{45}Ca radioactivity, $[Ca^{2+}]$, total ammonia concentration and titratable alkalinity (TA). At each sample time, the titratable alkalinity of the aquarium was restored to the initial value by the addition of $NaHCO_3$ in the proper amount calculated from the change in TA and the aquarium volume. In two of the experiments, some additional Ca^{2+} was added to restore the amount taken up by the animal.

At the end of the 5-day carapace loading period, the crabs were transferred to individual 8-litre aquaria with aeration and running sea water, and feeding was resumed. The animals were fed daily for 2 additional weeks, by which time our earlier studies indicated that carapace mineralization would be essentially complete (Cameron & Wood, 1985).

For the hypercapnia experiments, the crabs were placed in 8-litre closed, recirculating aquarium systems, in which it was possible to measure changes in TA, ammonia, [Ca^{2+}] and ^{45}Ca radioactivity. For a 22-h control period, the water in the recirculation reservoir was vigorously bubbled with air. Blood samples were taken at 1, 3, 6 and 22 h after introduction into the chambers through rubber septa glued over the left side of the pericardial sinus. From each blood sample, measurements of pH, total CO_2, ^{45}Ca radioactivity and [Ca^{2+}] were made. Water samples, taken initially and at the same times, allowed calculations of apparent H^+ flux, ammonia excretion, net Ca^{2+} flux and ^{45}Ca efflux.

After the 22-h control period, the air was replaced with a mixture of 2% CO_2 in air, and measurements resumed in the same fashion. Water and blood samples were taken at 1, 3, 5, 8 and 24 h, with an additional blood sample at 25 h. The animals were then quickly killed and six samples of carapace taken. These samples were weighed wet, then dried to constant weight at 65°C and re-weighed. The mineral portion of the carapace samples was then extracted for several days in 2 N-HCl, and portions of the extract analysed for [Ca^{2+}] and ^{45}Ca radioactivity.

Analyses and calculations

Titratable alkalinity (TA) of the sea water samples was measured by titration of 10·0 ml aliquots with 0·02 N-HCl, standardized against dried and weighed Na_2CO_3. The titrations were performed with a micrometer syringe burette, and were reproducible to ±1 μl (0·1%). Ca^{2+} analyses were made with an atomic absorption spectrophotometer (Perkin-Elmer 360). ^{45}Ca radioactivity was measured with a liquid scintillation counter with window settings optimized. All the samples were counted with the same total volume of water and cocktail; blood samples of 200 μl, for example, were diluted with 4·8 ml of sea water. The acid extracts were partially neutralized with $NaHCO_3$, and counted in a final volume of 5 ml water plus cocktail. The blood pH was measured with a capillary microelectrode (Radiometer-Copenhagen) and total CO_2 with a conductometric apparatus (Capni-Con, Cameron Instrument Co.). The blood P_{CO_2} values were calculated from total CO_2 and pK′ values found for this species (Wood & Cameron, 1985). Total apparent H^+ excretion was calculated as the sum of decreases in TA and total ammonia excretion. All concentration data are given as mmol l^{-1} or mequiv l^{-1} as appropriate, and all means as ±1 S.E., unless otherwise stated.

RESULTS

Radioactive calcium loading

During the 5 days of ^{45}Ca loading, the mean rate of Ca^{2+} uptake peaked at 4·07 ± 0·98 mmol kg^{-1} h^{-1} (8·14 mequiv kg^{-1} h^{-1}) during the first day, and the

corresponding apparent H^+ flux rate peaked at $9{\cdot}48 \pm 1{\cdot}35$ mequiv kg^{-1} h^{-1} also during the first day. The time course of Ca^{2+} influx and apparent H^+ efflux is given by the data in Fig. 1. This pattern is similar to that observed in a more extensive series of experiments with this same species (Cameron & Wood, 1985).

The total ^{45}Ca radioactivity taken up was calculated from the total disappearance of ^{45}Ca from the water during the 5-day uptake period, and averaged $12{\cdot}1$ μCi per crab. Since the mean weight was 243 g, the mean uptake per kg was $49{\cdot}6$ $\mu Ci\,kg^{-1}$. From carapace samples taken at the end of the experimental period, the total carapace Ca^{2+} was calculated as: (total ^{45}Ca activity, c.p.m.)/(S.R., c.p.m. $mmol^{-1}$) = total Ca^{2+}, and was $0{\cdot}55$ mol kg^{-1}. The mean specific activity of the carapace samples was 198 800 c.p.m. $mmol^{-1}$.

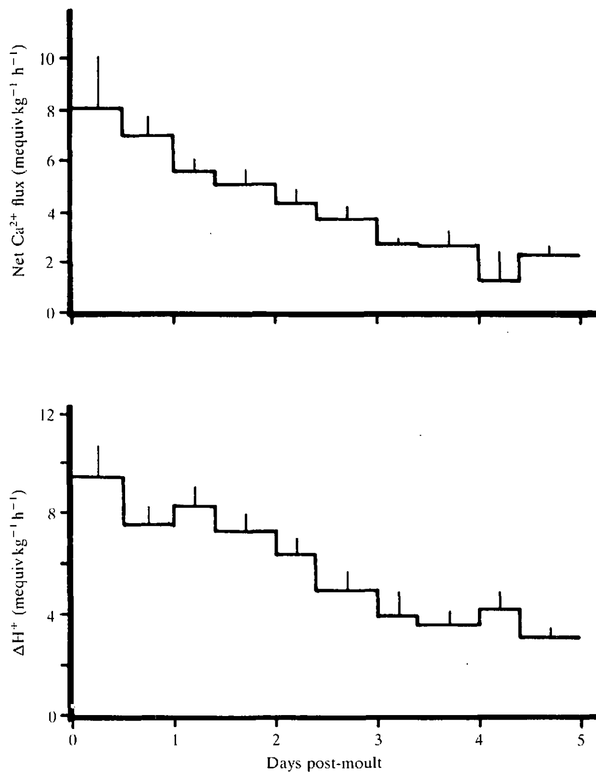


Fig. 1. Calcium uptake (upper panel) and apparent H^+ excretion (lower panel) for five crabs for a 5-day period following moulting.

Compensation of the hypercapnia

The blood acid-base responses to external hypercapnia are shown in Fig. 2, and were similar to what has been described for this species (Cameron, 1978) and other aquatic animals. The resting blood pH of 7·777 ± 0·010 was depressed in the first hour to 7·283 ± 0·070, then gradually restored to 7·581 ± 0·003 by 25 h. This pH compensation was accomplished by an increase in $[HCO_3^-]$ from 9·32 ± 1·72 $mmol\,l^{-1}$ at 1 h to 26·35 ± 1·14 $mmol\,l^{-1}$ at 25 h. The P_{CO_2}, calculated from pH, total CO_2 and pK′ values from an earlier study (Wood & Cameron, 1985), rose from <2 Torr during the control period to about 15 Torr by 1·5 h, and then changed little for the duration of the experiment.

The total blood compensation may be calculated as the active increase in $[HCO_3^-]$ (i.e. between 1 and 25 h) times the volume of the blood compartment (from Wood & Cameron, 1985), or (26·35−9·32 $mequiv\,l^{-1}$) (0·266 $l\,kg^{-1}$) = 4·53 mequiv. The tissue compensation was undoubtedly larger, but could not be calculated directly, since the buffer values for the various tissue compartments are not known.

A direct measurement of the total H^+ disposal to the external sea water was obtained from the apparent H^+ fluxes, which are shown in Fig. 3. The variability in the first few hours of the control period is probably a reaction to the handling

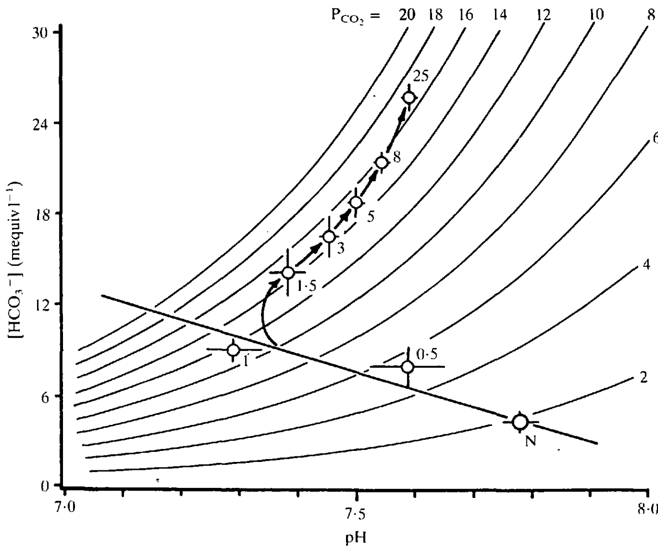


Fig. 2. A pH-HCO_3^- diagram of the acid-base parameters for five normocapnic crabs (N) and at various times (in parentheses) after the onset of hypercapnia (2 % CO_2 in air). The points shown are means ± S.E. for both pH (horizontal bars) and HCO_3^- (vertical bars).

involved in placing the animals in the experimental chambers, but a relatively low control rate of apparent H^+ excretion (cf. Fig. 1) was rapidly increased following the initiation of the hypercapnic period. The peak rate during the first hour was $2{\cdot}26 \pm 0{\cdot}51$ mequiv kg^{-1} h^{-1}, falling gradually over the duration of the hypercapnic period. By summing the area under the curve of Fig. 3 for the hypercapnic period, and subtracting for the control rate, a net apparent H^+ excretion due to compensation of 10·78 mequiv kg^{-1} is obtained. The net intracellular compensation, then, was the total minus the extracellular figure calculated above, or (10·78−4·53) = 6·25 mequiv kg^{-1}.

The responses of calcium to hypercapnia

The time course of $[Ca^{2+}]$ and ^{45}Ca specific radioactivity (S.R.) is shown in Fig. 4 for the 24 h control period and the 24 h hypercapnic period. The means for all data from the control and hypercapnic periods were significantly different (unpaired *t*-tests, $P < 0{\cdot}01$ in both cases), although the changes were fairly small. The total increase in blood Ca^{2+} was calculated using the control mean and the 24 h hypercapnic value, plus the blood volume, as: (9·94−8·73 mmol l^{-1}) (0·266 l kg^{-1}) = 0·32 mmol kg^{-1}. If all of this increase had come from the carapace (S.R. = 198 800 c.p.m. $mmol^{-1}$), the increase in ^{45}Ca radioactivity would have been (0·32) (198 800) = 63 459 c.p.m., or about 238 c.p.m. ml^{-1} of blood. The actual increase in blood radioactivity was 31 c.p.m. ml^{-1}, so assuming that no ^{45}Ca was transported to the external sea water, about 13·0% of the increased $[Ca^{2+}]$ could be attributed to carapace carbonate reservoirs.

During both the control and the hypercapnic periods, there was also an increase in ^{45}Ca radioactivity in the external sea water over the 24-h measurement period. The

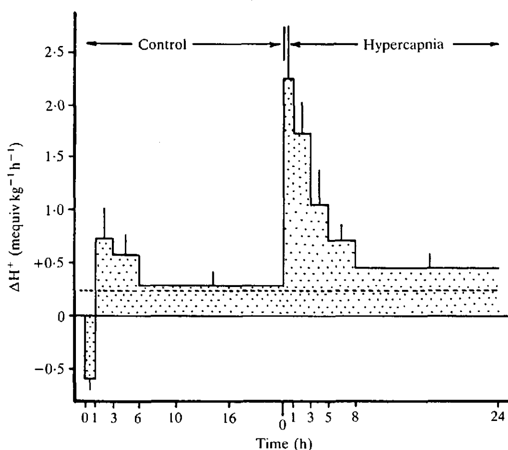


Fig. 3. Apparent H^+ excretion during a 24-h control period and a 24-h hypercapnic period. Vertical bars represent 1 S.E. The dashed horizontal line represents the control mean, which was used as the baseline for assessing the hypercapnic response.

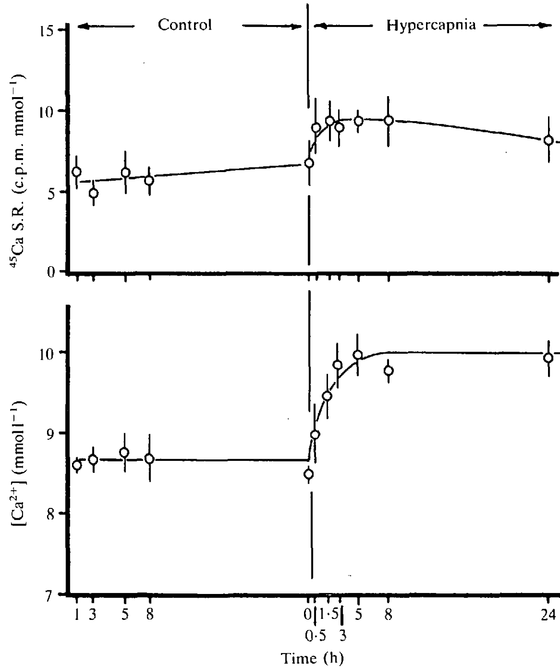


Fig. 4. The time course of circulating haemolymph Ca^{2+} (lower panel) and ^{45}Ca radioactivity (S.R., upper panel), defined as c.p.m. $mmol^{-1}$. The vertical bars represent ±1 S.E.

mean values were $136\,900 \pm 22\,600$ c.p.m. kg^{-1} for the 24-h control period, and $304\,200 \pm 27\,980$ c.p.m. kg^{-1} during the hypercapnic period. An independent calculation of carapace carbonate dissolution was obtained by assuming that the greater rate of ^{45}Ca appearance during the hypercapnic period was due only to carapace buffering, and that the control value represented normal exchange, either *via* the blood or directly with the external parts of the carapace. The difference then, was (304 200–136 900), or 167 300 c.p.m. The total ^{45}Ca radioactivity appearing during the hypercapnic period and attributable to buffering is the sum of the water plus the blood increases. The blood value was (31 c.p.m. ml^{-1}) (266 ml kg^{-1}) = 8246 c.p.m. kg^{-1}, so the sum, (167 300+8246) = 175 546 c.p.m. kg^{-1}, corresponds to the neutralization of 0·88 mequiv kg^{-1} of H^+ ions by the shell carbonate reservoirs (calculated from the S.R. of calcium in the carapace). The total H^+ disposal is (10·78+0·88) = 11·66 mequiv kg^{-1}, and the carapace portion of that is 7·6% of the total. If all of the H^+ disposal had been in the carapace carbonate reservoir, an increase in ^{45}Ca activity of (11·66) (198 800) = $2{\cdot}32 \times 10^6$ c.p.m. kg^{-1} would have been observed in the blood and external sea water.

DISCUSSION

In two previous studies of aquatic crabs, an increase of circulating [Ca^{2+}] has been looked for as evidence that the carbonate reservoirs of the carapace participate in acute buffering of acid-base disturbances. In neither case has any such evidence been found (DeFur *et al.* 1980; Henry *et al.* 1981), although [Ca^{2+}] does increase in terrestrial crabs (Henry *et al.* 1981), aquatic crabs emersed (DeFur *et al.* 1980) and in molluscs during anoxic acidosis (Dugal, 1939). Previous experimental designs, however, were not adequate to address the possibility that Ca^{2+} was dissolved from the carbonate matrix, but that the regulation of blood [Ca^{2+}] was sufficiently rapid and precise as to prevent any increase in blood concentration by transporting the excess Ca^{2+} to the external medium. That the crab's gills are capable of a high rate of Ca^{2+} transport is not in doubt: very high rates of influx are observed following moulting (Roer, 1980; Greenaway, 1983; Cameron & Wood, 1985), and the half-time for turnover of Ca^{2+} in the normocapnic intermoult blue crab is only 4·3 h (J. N. Cameron & C. M. Wood, unpublished data). Labelling of the Ca contained in the carapace carbonates during the rapid uptake phase following the moult in this study gave me a way of 'tagging' the shell carbonates, in order to detect any increase in circulating Ca^{2+} originating from the carapace during an acute hypercapnic acidosis.

The results of the experiments make two points fairly clearly: (1) there is some participation in buffering by the carapace carbonates, but (2) the relative importance of this mechanism is small, only about 7·5 % of the total compensation originating from carapace carbonates. The physiological significance of this dissolution of the carapace is probably not very great. Approximately 0·9 mequiv kg^{-1} of HCO_3^- appears from the shell reservoirs, and this represents less than 0·1 % of the total carbonate content of the carapace (Cameron & Wood, 1985). The response also appears to be somewhat transient; the data of Fig. 4 show that most of the increase in both ^{45}Ca radioactivity and circulating [Ca^{2+}] occurs in the first 3 h, whereas the active compensation continues for at least 25 h (cf. Fig. 2). The rate of appearance of ^{45}Ca in the external sea water is also higher during the first 3 h, but was rather variable, preventing a more quantitative conclusion. Studies of the carapace fluid compartment have shown recently that the environment for calcification is actively regulated at a pH of 0·3–0·4 units above that of the blood (Cameron & Wood, 1985). It is possible that there is a transient acidosis in the carapace fluid compartment caused by the hypercapnia, but that subsequent regulation restores the high carapace fluid pH relatively quickly, halting the reaction of H^+ with $CaCO_3$, and the resultant efflux of Ca^{2+} from this compartment. A similar pattern of regulation has been observed in individual tissues of the crayfish (Gaillard & Malan, 1984) and in crustacean muscle fibres (Roos & Boron, 1981).

Even in the terrestrial crabs, where the increase in circulating Ca^{2+} is greater (DeFur *et al.* 1980; Henry *et al.* 1981; Wood & Randall, 1981), it may simply be that the longer time required for compensation of hypercapnic acidosis (cf. Truchot, 1975; Cameron, 1981) also causes the carapace fluid pH to remain depressed for a longer period, leading to a greater entry of carapace carbonates into the haemolymph. Henry *et al.* (1981) observed a maximum increase of 6 or 7 mmol l^{-1}

Ca^{2+} in *Gecarcinus lateralis* during hypercapnia, DeFur *et al.* (1980) a 12 mmol l^{-1} increase in *Cancer productus* during emersion. Assuming a haemolymph volume of 30% for each, the total amount of $CaCO_3$ dissolved was less than 4 mmol kg^{-1} in both studies, and assuming a carapace carbonate content similar to that for *Callinectes sapidus* (1·04 mol kg^{-1}, Cameron & Wood, 1985), the total dissolution would be less than 0·5% of the total reservoir for these terrestrial crabs.

In conclusion, part of the compensatory increase in $[HCO_3^-]$ that occurs during hypercapnic acidosis in the aquatic crab, *Callinectes sapidus*, originates from the carbonate reservoirs of the carapace, but this appears to be a phenomenon of little quantitative importance, either to the total compensatory response or to the maintenance of deposited carbonates.

The capable technical assistance of Anna T. Garcia is gratefully acknowledged. Supported by NSF Grant PCM80-20982 to the author.

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