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THE REGULATION OF HAEMOLYMPH CALCIUM CONCENTRATION OF THE CRAB *CARCINUS MAENAS* (L.)

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SUMMARY

After acclimation, *Carcinus* can maintain calcium balance in dilute (35–100 %) but not in low calcium sea water. 71 % of total haemolymph calcium (9·54 ± 0·42 mM) was in ionic form as compared with 90·9 % (9·9 mM) in sea water. On acclimation to dilute sea water the calcium activity of the haemolymph was greater than that of the medium, the difference being maintained by active calcium uptake. *Carcinus* is highly permeable to Ca^{2+}, influx from sea water being 0·513 ± 0·07 μmoles g^{-1} h^{-1} and the time constant for calcium influx 4·3 ± 0·48 h. Calcium space represented *ca.* 25 % wet body weight independent of body size or salinity of acclimation medium.

INTRODUCTION

There is considerable evidence available that the total haemolymph calcium concentration of marine crustacea is maintained at a level above that of sea water (e.g. Robertson, 1957, 1960*b*). In addition, there is evidence that haemolymph calcium can be maintained hyperionic to the medium when acclimated to dilute sea water (*Pachygrapsus crassipes*, Prosser, Green & Chow, 1955; *Hemigrapsus nudus*, Dehnel, 1967; *Cancer magister*, Englehardt & Dehnel, 1973) and in sea water of altered calcium content (*Carcinus maenas*, Robertson, 1937). As the integument (or parts of it) is permeable to calcium (Bethe, 1928; Robertson, 1937) the presence of some mechanism maintaining hyperionic calcium levels in the haemolymph is indicated. However, most measurements have been based on total haemolymph calcium concentrations. Only Robertson (1937, 1949, 1953) allowed for bound calcium but his values for diffusible calcium in the haemolymph of several decapods again indicate maintenance of haemolymph calcium concentration above that of full strength sea water. No measurements of ionized calcium/calcium activity in the haemolymph of marine crustacea have yet been published, however, and such measurements are necessary to establish the existence of hyperionic calcium regulation. This investigation provides such data and, in addition, presents experimental evidence concerning the nature of the regulatory process.

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MATERIALS AND METHODS

Carcinus maenas was collected between tide marks at Boulmer and Cresswell in Northumberland. Only animals in excess of 20 g body weight were used. Females with eggs were rejected but non-berried females were used. Calcium-free, artificial, sea water contained 29·9 g NaCl, 0·73 gKCl, 4·3 g $MgCl.6H_2O$, 7·49 g $MgSO_4.7H_2O$ and 0·1 g $NaHCO_3$ per l distilled water. The calcium concentration was varied by suitably mixing the calcium-free medium with normal sea water. Sea water was obtained from Cullercoats Bay, Northumberland, and had a concentration of 1000 m-osmoles l^{-1}. ^{45}Ca was obtained as isotonic $CaCl_2$ from the Radiochemical Centre Amersham.

Crabs were maintained in sea-water aquaria at 10 °C and fed on fish. Experiments were carried out at 10 ± 1 °C in polystyrene boxes containing aerated experimental media.

Haemolymph was taken from the base of the pereiopods in a pyrex glass pipette and blown out under mineral oil, quantitative samples being taken in 'Drummond microcaps'. Total calcium measurements were made using an EEL 240 atomic absorption spectrophotometer, samples and standards containing 2·35 mM $LaCl_3$. Ionised calcium was measured as before (Greenaway, 1972) using an Orion calcium electrode. Standards contained cations (as chlorides) at the concentrations expected in samples.

Potential difference was measured with a Vibron electrometer model 33 B-2 and two calomel KCl saturated electrodes which made contact with haemolymph and external medium via Ringer filled bridges. The crabs were held in seawater by a clamp with only the dorsal surface of the carapace above water level and the tip of one bridge inserted into the pericardium through a small hole in the carapace.

Calcium influx was measured as follows. Crabs were acclimated to the relevant media for at least 3–4 days and then transferred to a large volume (7 l) of ^{45}Ca-labelled medium. The specific activity of calcium in the haemolymph was followed for several days and from this data the influx was calculated using the equation

$$SA_2 = SA_1 \frac{m_1}{m_1+m_3}\left(1-\exp\left(-\frac{m_1+m_3}{a_2}t\right)\right)$$

(Greenaway, 1971) where SA_1 and SA_2 are specific activities of the medium and haemolymph respectively, m_1 the influx from medium to haemolymph, m_3 the flux from haemolymph to exoskeleton, t the time constant for m_1 and a_2 the number of calcium ions in the haemolymph. The haemolymph volume was measured as the calcium space. A known amount of Ca-45 labelled crab Ringer's solution (usually 25 μl) was injected into the pericardium via a hole in the carapace. The crab was then left in moist air for 30 min to allow complete mixing of the injected fluid, and thereafter, haemolymph samples were removed at regular intervals and radioactivity measurements made. Haemolymph radioactivity declined rapidly and exponentially, as ^{45}Ca exchanged with ^{40}Ca in the exoskeleton. The radioactivity at zero time was calculated by regression analysis and was used to estimate the space into which injected calcium became distributed.

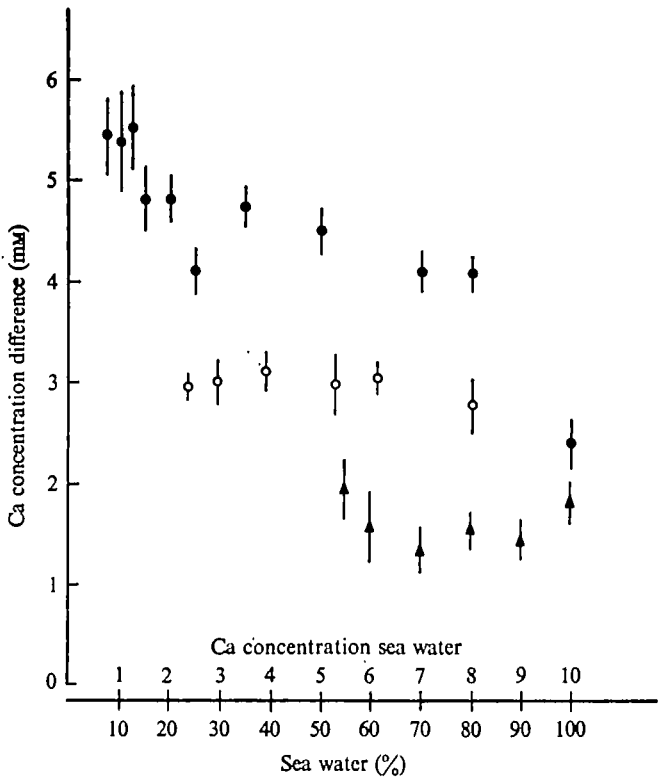

Fig. 1. Regulation of total haemolymph calcium concentration by crabs acclimated to dilute and low calcium sea water. ●, *Carcinus* acclimated to dilute sea water; ○, *Carcinus* acclimated to low calcium sea water; ▲, *Cancer* acclimated to dilute sea water. All values ± S.E. A single point represents the values for 100% and 10 mM-Ca^{2+} acclimated *Carcinus*.

Measurements of radioactivity were made using standard planchetting techniques and an IDL low background, Geiger-Müller counter.

RESULTS

Total haemolymph calcium concentration

Two groups of *Carcinus* were acclimated stepwise to dilute sea water and to low calcium media and their haemolymph calcium measured. Three to four days acclimation were allowed in each solution, new equilibria being reached in 24–48 h. Differences between haemolymph calcium and that of the experimental media have been calculated for the range of media used (Fig. 1). Data are also presented for *Cancer pagurus*. *Carcinus* acclimated to dilute sea water clearly maintains a haemolymph calcium concentration significantly above that of external media ($P < 0{\cdot}001$ for each point shown), the difference increasing with increasing dilution. *Carcinus* acclimated to low calcium sea water also regulated haemolymph calcium above that of the medium ($P < 0{\cdot}001$ for each point) but the difference between haemolymph and sea-water levels remained more or less constant over the range of calcium concentration examined. *Cancer* maintained a lower difference (although still significant at the

Table 1. *Ionized and total calcium concentration in the haemolymph of crabs acclimated to dilute sea water*

%	Sea-water Ca conc. (mM)			Haemolymph Ca conc. (mM)		
	Total	Ionized	% Ionized	Total	Ionized	% Ionized
100	9·90	9·00	90·9	13·41 ± 1·07	9·54 ± 0·42	71·3 ± 2·5 (12)
75	7·39	6·62	89·7	11·62 ± 0·18	7·94 ± 0·15	68·2 ± 1·6 (30)
50	5·10	4·55	89·3	9·60 ± 0·21	5·47 ± 0·18	57·1 ± 1·6 (23)
35	—	—	—	8·52 ± 0·20	5·75 ± 0·21	67·8 ± 2·4 (19)
25	2·66	2·28	85·6	6·11 ± 0·20	4·68 ± 0·17	76·7 ± 2·1 (11)
15	—	—	—	6·75 ± 0·47	3·67 ± 0·31	55·2 ± 3·1 (13)

Values are means ± S.E. Figures in parentheses indicate number of animals sampled.

0·001 level), haemolymph being 1–2 mM higher in calcium concentration than the medium over the range 50–100 % sea water.

Total calcium concentration in the hamolymph for crabs in 100 % sea water was 12·8 ± 0·28 mM, a value similar to that found by previous workers (Robertson, 1937; Webb, 1940; Riegel & Lockwood, 1961). Values given by Lockwood & Riegel (1969), however, are in error apparently being given in m-equiv.l^{-1} although expressed as mM. Even after conversion to mM units their values appear to be lower than normal, means in some cases being lower than sea-water calcium levels.

Ionized calcium concentration

Measurements of ionized calcium concentration in haemolymph and experimental sea-water media have been made for *Carcinus* (Table 1). Activity values for *Carcinus*, *Cancer* and sea water are shown in Fig. 2. Different groups of crabs were used in each dilution. Ionized calcium/calcium activity was maintained above that of the medium in all solutions to which crabs were acclimated although the difference was not significant in 100 % ($P > 0·35$), 75 % ($P > 0·05$) or 50 % ($P > 0·2$) sea water. The difference was highly significant at lower dilutions. Ionized calcium/calcium activity values for 50 % sea-water acclimated crabs were lower than expected from the other values. This was not an artifact caused by the use of different crabs at each sea-water dilution as further measurements on a single group of crabs, acclimated to 75 %, 50 % and 35 % sea water in turn, gave near identical results. Values for ionized calcium in the haemolymph are lower than those for diffusible calcium (Robertson, 1937), indicating that it is not only large protein molecules which are responsible for complexing calcium but also smaller ones which can penetrate dialysis membranes. No trends were observed in the level of non-ionized calcium in crabs adapted to dilute media and it is probable that it is regulated passively by the level of calcium complexing substances in the blood.

The concentration of ionized calcium in sea water was 91 % of total calcium so 9 % must have been complexed by inorganic anions. Experiments with standard solutions containing sulphate, as well as chloride ions, indicated that sulphate ions at sea-water concentrations would complex *ca.* 7 % of the total calcium. Thus the remaining 2 % is probably associated with bicarbonate, carbonate, phosphate and other inorganic

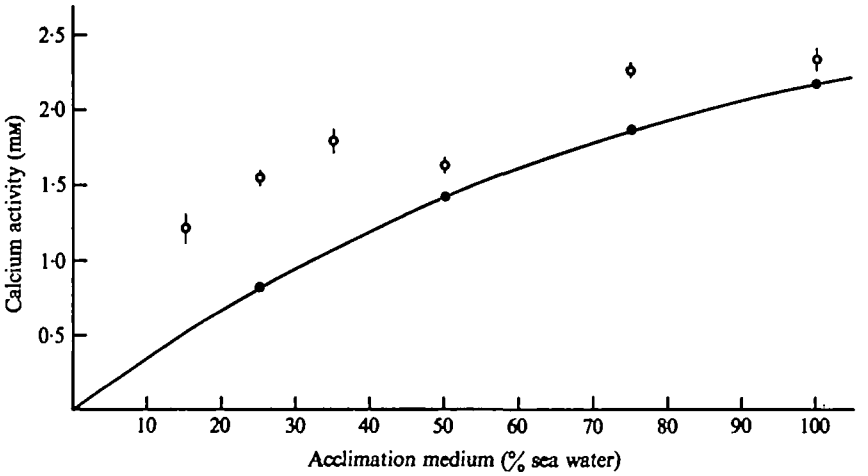

Fig. 2. Calcium activity in sea water and in the haemolymph of crabs acclimated to dilute sea water. ●, Sea-water calcium activity; ○, Calcium activity in *Carcinus* haemolymph ± S.E.

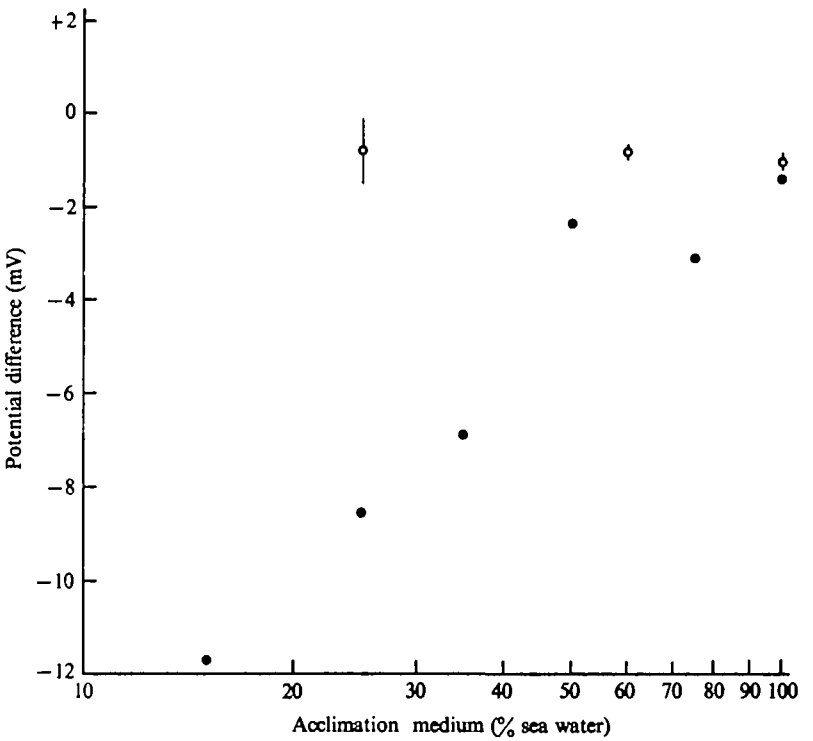

Fig. 3. Measured P.D. and calculated equilibrium potentials across the body surface of *Carcinus*. Equilibrium potentials were calculated using calcium activity values from Fig. 2 recalculated as m-moles kg^{-1} water. ●, Calculated equilibrium potentials; ○, measured potential differences ± S.E.

Table 2. *Calcium influx values*

Sea water (%)	Ca flux (μmoles g^{-1} h^{-1}) m_1	m_2	t (h)
100	0·513 ± 0·07	0·280 ± 0·06	4·3 ± 0·48 (8)
75	0·424 ± 0·10	0·472 ± 0·12	4·2 ± 0·53 (8)
50	0·122 ± 0·01	0·275 ± 0·04	6·3 ± 0·67 (8)
35	0·133 ± 0·02	0·341 ± 0·05	4·2 ± 0·41 (6)

Values are means ± S.E. Figures in parentheses represent number of measurements.

anions. At the lower sulphate concentrations found in haemolymph $CaSO_4$ complexes represent about 0·2 mM of the non-ionized calcium.

Electrochemical potential across the body wall

The potential difference across the body wall of *Carcinus* was measured and compared with calculated equilibrium potentials for calcium (Fig. 3). Crabs acclimated to 100 % sea water were in calcium equilibrium with the medium. In dilute sea water there was a small electrochemical gradient favouring calcium loss from the haemolymph and this was particularly marked in media of less than 50 % sea water. Thus, in dilute media maintenance of haemolymph calcium by uptake from the water would require active transport of calcium.

Calcium balance

Calcium balance in crabs acclimated to various sea-water dilutions (35–100 %) was followed using small volumes of water. In most cases crabs maintained calcium balance with the medium although a few crabs showed a small net loss. In crabs acclimated to low calcium sea water, balance was not maintained in media containing 7·5 mM-Ca or less, a continual net loss to the medium occurring. A similar lack of balance by *Carcinus* was demonstrated by Robertson (1937); crabs placed in calcium-free sea water continued to lose calcium to the medium even when the external calcium concentration reached 5 mM.

Calcium influx

Calcium influx was measured to determine whether calcium uptake increased after acclimation to dilute sea water. Variability of calcium influx between crabs was high (Table 2) and effectively obscured any increase in calcium transport that may have occurred in dilute media; this would in any case have been small in relation to the passive flux. With the method used for measuring calcium influx it was possible to make only one measurement per crab. This precluded the use of successively acclimated single crabs to eliminate individual variability. Measurements of calcium net loss also proved inconclusive. Values for the time constant for calcium influx, t, are included in Table 3 to emphasize the high permeability of the crabs to calcium ions suggested by the above data (Fig. 1) and previous measurements by other workers (Bethe, 1928; Robertson, 1937).

To measure calcium influx it was necessary to know the space in which calcium was distributed within *Carcinus*. Calcium space was measured in crabs acclimated to both

Table 3. *Calcium space in crabs*

Animal no.	Weight (g)	Ca space (ml)	Ca space (% body wt.)	Ca efflux (μmoles g^{-1} h^{-1})
	(A) Acclimated to 100% sea water			
97	28·0 M	7·03	25·3	0·769
98	20·0 F	5·79	28·9	0·713
101	43·9 M	11·36	25·9	0·748
102	39·2 M	10·29	26·2	0·534
103	48·3 M	13·93	28·8	0·592
104	30·0 M	6·76	22·5	0·557
105	44·0 M	9·45	21·5	0·509
106	20·6 M	4·88	23·7	0·759
107	22·1 M	6·10	27·6	0·679
108	24·5 M	7·30	29·8	0·882
Mean	—	—	26·0 ± 0·9 S.E.	0·674 ± 0·04 S.E.
	(B) Acclimated to 50% sea water			
109	32·6 *F*	8·57	26·3	0·596
110	24·2 M	4·93	20·4	0·720
111	45·6 M	11·14	24·4	0·470
112	16·5 M	4·14	25·1	0·724
113	33·5 M	7·06	21·1	0·461
Mean	—	—	23·4 ± 1·16 S.E.	0·594 ± 0·06 S.E.

Ca efflux was calculated using the relation efflux $= (a_s/t)$. M and F indicate sex of crabs used.

100% and 50% sea water. The values (Table 3) indicate a calcium space equivalent to *ca.* 25% wet body weight, largely independent of both body size and salinity over the range used. These values compare with inulin space values of 19·4% (Binns, 1969), 18–20% (Siebers & Lucu, 1973); amaranth space 36% (Siebers & Lucu, 1973); sodium diatrizoate space 19·2% (Riegel *et al.* 1974). Injected ^{45}Ca, therefore, distributed itself in a larger volume than insulin or diatrizoate but in a smaller volume than amaranth.

DISCUSSION

The above data show that during intermoult *Carcinus* is in calcium equilibrium when acclimated to 100% sea water. Robertson (1937, 1960*a*) has made several suggestions concerning the mode of regulation of haemolymph calcium concentration in *Carcinus* in 100% sea water. The present evidence indicates that his earlier view, that diffusible calcium was in equilibrium and total calcium maintained above ambient by protein binding of calcium ions, was substantially correct.

When *Carcinus* is acclimated to dilute sea water the situation is not so clear. The evidence presented is strongly against a passive regulation of calcium ions due either to impermeability of the cuticle or to utilization of calcium from the exoskeleton. However, no direct evidence has been obtained to demonstrate that regulation is achieved by increased uptake from the medium. Nevertheless, this is the only other possibility and such uptake, being against an electrochemical gradient, would involve active transport of calcium.

In crabs acclimated to low-calcium sea-water media a calcium net loss was observed, the haemolymph level being maintained above that of the medium by only 2·5–3·0 mM (a difference probably representing the bound calcium fraction.) On the other hand,

crabs acclimated to dilute sea water regulated the ionized calcium in the haemolymph. It would seem likely, therefore, that regulation of haemolymph ionized calcium is a result of a drop in external salinity rather than of calcium concentration. *Cancer* acclimated to dilute sea water appears to behave in similar fashion to *Carcinus* in low calcium sea water.

A remarkable feature of calcium regulation in *Carcinus* is the very high permeability to calcium ions. The time constant for calcium influx of 4·3 h is considerably lower than that of 7·1 h for sodium (Shaw, 1961). *Carcinus*, therefore, exhibits a greater permeability to calcium ions than to sodium ions despite the larger hydrated ion of the former. As crabs in 100 % sea water are in calcium equilibrium it is probable that most of the measured influx is due to a 1:1 exchange process between internal and external calcium pools. A large exchange also occurs between tissue (exoskeleton) and haemolymph calcium pools (see m_3 values in Table 2).

Robertson (1937, 1960*b*) reported elevated and reduced total calcium levels in the haemolymph of premoult and postmoult *Carcinus* respectively. Data for diffusible calcium in soft crabs indicated that both diffusible and total calcium levels were reduced in postmoult. Measurements of ionized calcium at these stages are necessary to determine whether ionized as well as total calcium is altered, especially as the former has been shown to remain constant throughout the intermoult cycle in another decapod (Greenaway, 1974*a*, *b*). This information would have an important bearing both on the energetics of calcium absorption in postmoult crabs and on calcium elimination in premoult stages.

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