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CALCIUM BALANCE AT THE PREMOULT STAGE OF THE FRESHWATER CRAYFISH *AUSTROPOTAMOBIUS PALLIPES* (LEREBOULLET)

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SUMMARY

The premoult stage in *Austropotamobius pallipes* is characterized by a net loss of calcium which increases from D_0 to a maximum of 0·83 μmoles/g/h at D_4. The concentration of ionized calcium in the haemolymph does not increase during the premoult stage although there is an increase in complexed calcium. The electrochemical gradient across the body surface is similar to that at the intermoult stage and favours calcium outflux. Possible routes for calcium net loss have been discussed and a mechanism for elimination of calcium has been proposed.

INTRODUCTION

The changes in calcium metabolism involved in preparation for the moult in aquatic reptantian crustacea have been summarized by Passano (1960), Carlisle & Knowles (1959) and Travis (1960*a*). *Austropotamobius pallipes* appears to conform to the general pattern described by Passano. During the premoult stage calcium is removed from the old exoskeleton but only a small amount of this is retained after the moult, largely in the gastroliths (Chaisemartin, 1962, 1964, 1967). Gastrolith structure and formation in another crayfish, *Orconectes virilis*, has already been described (Travis, 1960*b*) and is not investigated in this paper. Most of the calcium removed during the premoult period in *Austropotamobius* is lost to the medium in soluble form (Chaisemartin, 1967) as is the case in marine decapods (Robertson, 1937; Travis, 1955). In the latter part of the premoult period (D_{3-4}) the concentration of calcium in the haemolymph of *Austropotamobius* rises (Chaisemartin, 1967; Greenaway, 1974) but it is important to remember that the concentration of ionized calcium and not total calcium concentration is the significant physiological parameter and this has not yet been measured. In the marine decapod *Panulirus argus*, the concentration of calcium in the urine rises in the premoult stage, reaching a level 20% above normal (Travis, 1955), but no data are as yet available for freshwater decapods. Chaisemartin (1967) found the potential difference across the epithelium of isolated podobranchs of *Austropotamobius* was lower at the premoult than at the intermoult stage, thus favouring calcium loss to the medium. Potential-difference measurements performed on isolated gill preparations, however, cannot safely be applied to intact animals (Greenaway, 1972) and measurements of the potential difference across the

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body surface of intact animals have, therefore, been made in this investigation determine whether such a shift in potential occurs in the intact crayfish.

The object of this work was to provide a detailed quantitative picture of the changes in calcium balance in *Austropotamobius* during the premoult period. Particular attention has been paid to points on which knowledge is poor, i.e. the activity of calcium in the blood and the route and mechanism of calcium loss from the body.

MATERIALS AND METHODS

Animals were collected and maintained as described previously (Greenaway, 1972). Crayfish kept in the laboratory usually began moulting in March. In the field several distinct moulting periods were observed; in June (males and non-ovigerous females), in late July/early August (females which did not moult in June) and in late August/early September (males and females). Moulting was synchronized, all participants moulting within a two-week period.

Experiments were carried out at 10 ± 1°C, but between measurements experimental animals were kept at 15°C in order to avoid lengthening the premoult by low temperatures. Animals preparing to moult were identified by calcium-balance experiments (Greenaway, 1974). In addition early premoult stages D_0 and D_1 could be identified, in small animals, by examining the setae on the uropods and telson (Stevenson, Guckert & Cohen, 1968) and crayfish at the later stages D_{3-4} were recognizable by flexibility of the branchiostegites and the appearance of the ecdysial line along the branchiostegite margin.

Calcium concentrations were measured using an E.E.L. 240 atomic absorption spectrophotometer. Ionic calcium was measured with an Orion calcium electrode.

RESULTS

Calcium balance

At the late intermoult stage, crayfish show a persistent low rate of net calcium loss to the medium of about 0·046 μmoles/g/h (Greenaway, 1972). This rate of net loss was increased from D_0 onwards and reached a very high level in animals at later premoult stages (D_{3-4}) (Fig. 1). The maximum net loss rate was observed a few days before the moult, and frequently a small reduction in loss rate occurred in the remaining days before ecdysis. In Table 1 some values for maximal net calcium loss are presented. The mean value of 0·83 μmoles/g/h is about 36 times the calcium efflux in early C_4 animals and 20 times the net calcium loss rate in late C_4 crayfish (Greenaway, 1972). The maximum net loss value of 1·495 μmoles/g/h is 65 times the intermoult efflux. Chaisemartin (1967) has also measured net loss in premoult (D_{3-4}) crayfish and gives a value of 0·89 μmoles/g/h, which is in close agreement with the mean value given in Table 1.

Sodium balance

Measurements of the sodium loss rate to sodium-free artificial tap water have been made for premoult crayfish at stage D_4 (Table 2). The normal loss rate for animals at the intermoult stage is approximately 0·15 μmoles/g/h (Shaw, 1959), so clearly permeability to sodium is doubled in the late premoult stage. This increased sodiu

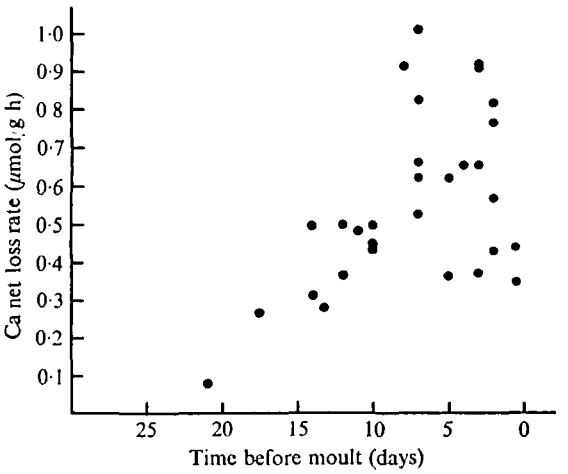

Fig. 1. The rate of net loss of calcium from crayfish during the premoult period. Values for several animals are included.

Table 1. *Maximum values for calcium net loss (μmoles/g/h) from crayfish at the late premoult stage*

0·653 0·914 1·470 1·016 0·915 0·661 0·621 0·703 0·904 0·618 0·659 0·823

Mean 0·830 ± 0·068 S.E.

Table 2. *Sodium loss rates from late premoult stage crayfish*

Stage	Time before moult (days)	Na loss rate (μ moles/g/h)
D_4	2	0·329
D_4	2	0·274
D_4	2	0·389
D_4	2	0·422
D_4	3	0·351
D_4	3	0·330
D_4	3	0·191
D_4	3	0·328
D_4	3	0·297
Mean		0·323 ± 0·02 S.E.

turnover may perhaps be indicative of a slight increase in permeability of the exoskeleton at this stage, although the increase in calcium net loss was ten times greater than that of sodium.

Total calcium loss in premoult

From Table 3 it is apparent that 83% of the total calcium content was lost during stages D (34%) and E (66%). The newly moulted crayfish contained only 17% of its calcium content at the previous intermoult stage. Of this calcium 60% is in the gastroliths and has been shown to originate in the old exoskeleton (Chaisemartin, 1967). Thus, much of the calcium removed from the old exoskeleton was passed into the body and 34% was stored. Values given in Table 3 agree well with the data of Chaisemartin (1967) and the total calcium loss during stages D and E is also similar that in marine decapods (Passano, 1960).

Table 3. *Calcium net loss during stages D and E*

Wet wt (g)	Estimated total Ca at C_4 (mmoles)	Total Ca in exuvium (mmoles)	Total Ca at A_1 (mmoles)	Ca lost in solution at stage D (mmoles)	Total Ca loss stages D and E (mmoles)	Total loss as % C_4 Ca content
7·5	7·1	5·05	0·714	1·336	6·386	89·9
9·1	8·8	4·71	1·396	2·694	7·404	84·1
4·6	4·1	2·22	0·806	1·074	3·294	80·3
4·6	4·1	2·23	0·756	1·114	3·344	81·6
4·9	4·4	1·58	0·940	1·880	3·460	78·6
3·8	3·3	2·05	0·715	0·535	2·585	78·3
4·6	4·1	2·29	0·740	1·070	3·360	82·0
Mean						82·1

Total calcium content at the intermoult stage was estimated from the data of Greenaway (1974).

Table 4. *Calcium concentration in the urine of premoult crayfish*

Stage	Time before moult (days)	Urine Ca conc. (mM/l)
D_3	8	1·8
D_3	10	1·3
D_3	11	2·1
D_4	3	3·1
D_4	6	1·0
D_4	2	3·4
D_4	3	3·0
D_4	4	2·0
D_4	2h	3·9

All values were for animals kept in artificial tap water (1 mM/l−Ca), which was renewed regularly.

Urinary calcium loss

To determine the manner in which calcium is eliminated the calcium concentration in the urine of crayfish at the late premoult stage (D_{3-4}) was measured (Table 4). Assuming urine flow to be similar to that of intermoult crayfish (a not unreasonable assumption) calcium net loss in the urine at stage D_4 would be about 0·006 μmoles/g/h. Even if urine flow was increased 10 times, urinary net loss of calcium could only represent a small fraction of the observed loss.

The mean value for the concentration of calcium of the final urine at D_4 (2·7 mM/l) was considerably lower than the level of ionized calcium in the haemolymph (7·4 mM/l) given in Table 5. As with animals at the intermoult stage then, calcium reabsorption must occur from the primary urine.

Calcium activity in the haemolymph

The concentration of ionized calcium in the haemolymph of *Austropotamobius* at the late premoult stage (D_4) was measured as described previously (Greenaway, 1972) and the calcium activity calculated using an activity coefficient of 0·35 (Table 5). Ionized calcium represents 47% of the total calcium in the haemolymph at stage D_4 and the other 53% must, therefore, be bound or complexed as suggested previously (Greenaway, 1972). The earlier data for the activity of calcium in the haemolymph were in error, a lower activity coefficient having been used. The corrected mean valu

Table 5. *Ionized calcium and calcium activity in the haemolymph of premoult crayfish*

Stage	Weight (g)	Haemolymph calcium (mM/l) Total	Ionized	Complexed	Activity	% of Ca ionized
D_4	6·5	15·2	8·3	6·9	2·91	45·4
D_4	7·0	13·5	7·6	5·9	2·66	56·3
D_4	4·3	14·1	9·7	4·4	3·40	68·8
D_4	5·2	16·0	7·2	8·8	2·52	45·0
D_4	5·7	18·6	6·6	12·0	2·30	35·4
D_4	7·6	18·9	5·8	13·1	2·03	30·7
D_4	5·1	17·1	6·6	10·5	2·31	38·6
Mean values		16·2	7·4	8·8	2·56	47·1
±S.E.		0·73	1·559	1·13	0·159	4·58

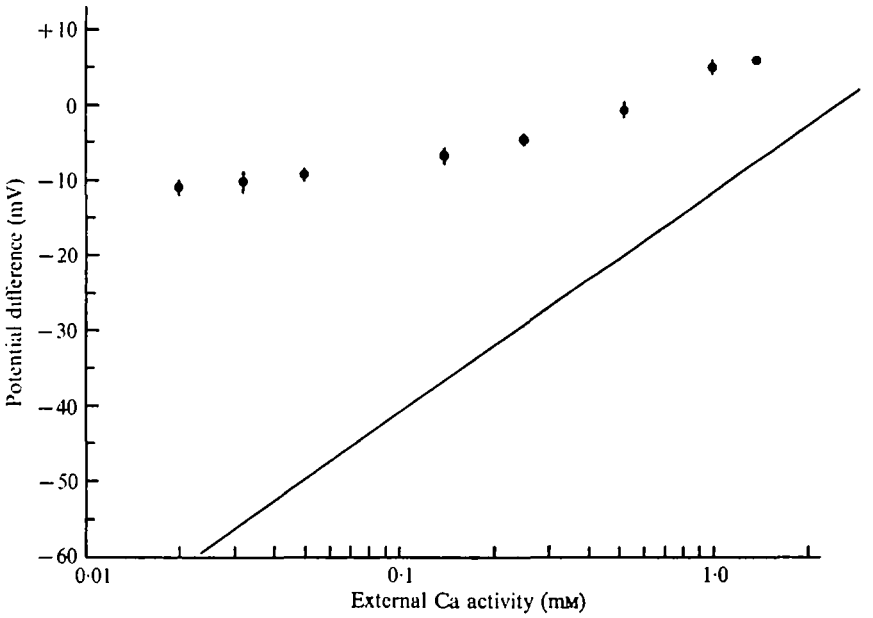

Fig. 2. A comparison between the mean potential difference (●), as measured between the haemolymph and the external medium, and the calculated equilibrium potential for calcium (unbroken line) over a range of external calcium activity. Vertical lines represent standard errors of the means.

using the more accurate coefficient (0·35), is 2·205 mM/l. This slightly higher value in no way alters conclusions reached in this previous work. The level of ionized calcium in the haemolymph of premoult animals was not significantly different from that in crayfish at the intermoult stage ($P > 0·05$) although the total calcium concentration was much greater. This fact is of importance when considering the mechanism of calcium net loss and will be discussed later. McWhinnie (1962) found that the concentration of the non-protein-bound fraction of the calcium in the haemolymph of *Orconectes virilis* did not alter markedly during the premoult period, supporting the measurements made in this study.

Potential-difference measurements

Measurements of the potential difference across the body surface of premoult crayfish (D_{3-4}) were made as described previously (Greenaway, 1972) and these values, together with calculated equilibrium potentials for calcium, are shown in Fig. 2. It is clear that the existing potential difference favoured loss of calcium to the medium. The values given in Fig. 2 are very similar to those for intermoult-stage crayfish (Greenaway, 1972). There has, therefore, been no shift in potential during premoult which might enhance calcium loss; a finding in contrast to that for isolated, perfused gill preparations of *Austropotamobius* (Chaisemartin, 1967).

DISCUSSION

The pattern of calcium metabolism in the premoult crayfish may now be summarized. Calcium net loss increases from late stage D_0 and reaches a very high level in stages D_{3-4} before falling slightly 1–2 days before the moult. Haemolymph calcium concentration has been shown to rise in stages D_{3-4} (Greenaway, 1974; Chaisemartin, 1967; Andrews, 1967) but there is no significant rise in ionized calcium and this increase must, therefore, be in the complexed fraction. The electrochemical gradient for calcium remained similar to that for intermoult crayfish.

In view of the very high rate of net calcium loss from premoult crayfish it is necessary to consider the manner in which calcium is eliminated. Firstly, it is essential to establish whether calcium removed from the old exoskeleton is lost directly to the medium or, as is more generally believed (Passano, 1960), reabsorbed into the blood and then eliminated. The balance of evidence, in fact, favours the latter mechanism. The normal impermeability of the exoskeleton to calcium (Chaisemartin, 1965, 1967) is maintained in premoult by the intact epicuticle and exocuticle (Travis, 1960*a*). Thus, the only direct route for the net loss of calcium to the medium would be from the median dorsal rupture in the old exoskeleton which opens 1–2 days before the moult (personal observation). However, most of the net loss of calcium has occurred by this time and in any case such a route is unlikely as it would entail the simultaneous loss of valuable organic materials from the moulting fluid. Some direct loss of calcium from the moulting fluid, however, probably occurs in the vicinity of the gills, where the cuticle is likely to be more permeable. In addition Chaisemartin (1967) has provided direct evidence that calcium removed from the old exoskeleton is absorbed into the body. Similarly, in the marine decapod *Panulirus* the rise in blood and urine calcium concentration at premoult (Travis, 1955) could only be sustained by reabsorption of calcium from the old exoskeleton. It is probable, therefore, that the bulk of calcium removed from the old exoskeleton in premoult passes into the body for storage or elimination. The next question, therefore, concerns the manner in which excess calcium is removed from the body.

Unlike marine decapods, in which calcium elimination occurs primarily through the urine (Robertson, 1937; Travis, 1955), there is no significant net loss of calcium through the urine in *Austropotamobius:* the concentration of calcium in the urine of late premoult crayfish being only doubled, whereas net calcium loss increased 20-fold. To account for the maximum net loss rate of calcium (0·83 μmoles/g/h) in the u[redacted]

alone, the flow would need to be increased to 150 times the intermoult level (i.e. 765% body weight per day). Chaisemartin (1967) reports no significant loss of calcium from the gut of *Austropotamobius* during premoult, so the only remaining route is across the gills, as suggested for intermoult crayfish (Greenaway, 1972).

The mechanism of calcium elimination must also be considered. The simplest method would be a passive calcium loss down the electrochemical gradient, which favours outward calcium movement during premoult. However, as the electrochemical gradient during premoult is similar to that at intermoult, passive calcium loss should also be similar. In fact, total loss increased 20 times and it is necessary, therefore, to postulate an outward transport of calcium. As the electrochemical gradient favours calcium loss there is no need to suggest active calcium secretion, and energy requirements would be low. Outward movement of calcium ions could be accompanied by an anion (HCO_3^-) or perhaps exchanged for $2H^+$.

Although much is known regarding hormonal control of the intermoult cycle in decapod crustacea, little information is available concerning the control of calcium metabolism. The role of crustecdysone in the regulation of calcium during premoult, however, has been the subject of several recent investigations. The evidence obtained suggests that the hormone does not control calcium reabsorption in premoult stages but does play a role in the formation of calcium stores (Graf, 1972*a*, *b*; McWhinnie, *et al.* 1972). Passano (1960) in a review of older work confirms this view. McWhinnie, Cahoon & Johanneck (1969) have provided some evidence for a second hormone in the eyestalks of *O. virilis* which apparently promotes calcium reabsorption from the exoskeleton.

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