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# CALCIUM REGULATION IN THE FRESHWATER CRAYFISH *AUSTROPOTAMOBIUS PALLIPES* (LEREBOULLET)

## I. CALCIUM BALANCE IN THE INTERMOULT ANIMAL

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### INTRODUCTION

Calcium balance has been studied throughout the moulting cycle in several aquatic crustacea, namely the marine species *Carcinus maenas* (Robertson, 1937, 1960; Drach, 1939) and *Panulirus argus* (Travis, 1954, 1955*a*, *b*) and the freshwater crayfish *Austropotamobius* (Chaisemartin, 1962, 1964, 1965*a*, *b*, 1966, 1967; Bernard and Chaisemartin, 1965), *Procambarus* (Maluf, 1940) and *Orconectes* (McWhinnie, 1962). This paper is confined to a study of calcium regulation by *Austropotamobius pallipes* in the intermoult condition, i.e. stage $C_4$ in the moulting classification used by Drach (1939) and Travis (1960).

Although calcium uptake from the medium by postmoult aquatic crustacea is well known (e.g. Chaisemartin, 1967; Robertson, 1937, 1941, 1960) little information is available concerning the situation in the intermoult stage. It is not unreasonable to suppose that calcium regulation in the intermoult stage might be analogous to the situation for sodium regulation (Shaw, 1959, 1964; Bryan, 1960), i.e. that a continual loss of calcium in the urine and across the body wall (gills) be made good by calcium uptake from the medium – a steady-state situation. Available evidence supports this suggestion. Maluf (1940) found that hard-shelled specimens of *Procambarus clarkii* generally showed no net calcium movements when placed in calcium chloride solutions, although one animal showed a small net loss of calcium. Similarly, Chaisemartin (1967) using $^{45}Ca$ found no net calcium flux in intermoult ($C_4$) *Austropotamobius*. The flux in fact was low at about 0·1–0·14 $\mu$moles/g fresh wt./h. Intermoult *Carcinus* show a small net loss of calcium to the medium, most of this occurring through the antennary glands (Robertson, 1937). The calcium flux in *Austropotamobius* has not been split into passive and urinary components. In summary, aquatic crustacea in the intermoult stage either maintain calcium equilibrium with the medium or show a net loss of calcium to the medium.

Several attempts have been made to determine whether or not the absorption of calcium from the medium by freshwater crustacea is an active process (against an electrochemical gradient). Croghan, Curra & Lockwood (1965) measured the potential difference across the epithelium of isolated, perfused gills of *Austropotamobius* and recorded a mean potential of −60 mV (blood side negative) when the gill was bathed in a 1% dilution of crayfish Ringer's solution (0·135 mM-Ca). They concluded that calcium was in approximate electrochemical equilibrium across the gill epithelium.

Chaisemartin (1966, 1967) also recorded potentials across isolated, perfused gills of *Austropotamobius*. He obtained values of $-45$ mV for animals from soft water (0·13 mM-Ca) and $-37$ mV (both blood negative) for animals from hard water (2·1 mM-Ca). Both groups of animals were in their natural media. Chaisemartin concluded that the resultant electrochemical potentials favoured passive entry of calcium into the blood. Potential difference values across the body surface of intact crayfish, however, reveal a rather different picture. Bryan (1960) obtained mean potential difference values for *Astacus fluviatilis* (= *Austropotamobius pallipes*) of $+6·6$ and $+4·1$ mV (blood positive) for animals in Bristol tapwater (*c.* 2·0 mM-Ca) and artificial tapwater (1·82 mM-Ca) respectively. Clearly there is considerable discrepancy between results for isolated, perfused gill preparations and for intact animals. Amongst marine crustacea active calcium transport has been suggested in the crab *Carcinus* (Robertson, 1960) and the shrimp *Metapenaeus* (Dall, 1965). No potential difference or calcium activity measurements are available for these animals and in their absence active calcium transport remains to be demonstrated.

Depletion of body calcium caused by treating *Austropotamobius* with calcium-free or low-calcium waters apparently caused increased calcium uptake when the animal was returned to normal media (Chaisemartin, 1967; Bernard & Chaisemartin, 1965). Similarly, loss of calcium by the marine shrimp *Metapenaeus* was rapidly made good on return to full-strength sea water (Dall, 1965). During calcium depletion of *Austropotamobius* and *Metapenaeus* a fall in the concentration of calcium in the blood occurs. Depletion of *Austropotamobius* (Chaisemartin, 1967) and *Carcinus* (Robertson, 1937) caused the loss of greater amounts of calcium than were contained in the blood. The movement of calcium from tissues to blood under conditions of calcium depletion has been proposed in *Carcinus* (Robertson 1937) and demonstrated in *Austropotamobius*. In the freshwater snail *Limnaea stagnalis* net calcium movement from the shell to the blood, during depletion, has been demonstrated. *Limnaea*, however, does not show an increased rate of calcium transport after depletion of calcium (Greenaway 1971 *a*, *b*).

The object of the present investigation was to obtain a picture of the pattern of calcium regulation during the intermoult stage of *Austropotamobius*. In addition, electrochemical potential measurements across the body surface of intact crayfish have been made to determine whether calcium uptake is an active or a passive process. Finally, it was felt that further experiments were required on the effects of calcium depletion on calcium uptake in *Austropotamobius* as the data of Chaisemartin (1967) and Bernard & Chaisemartin (1965) were rather different from those obtained in similar experiments with *Limnaea* (Greenaway, 1971 *a*, *b*).

## MATERIALS AND METHODS

### *Materials*

Crayfish were collected from streams in Northumberland, principally the Hartburn, a tributary of the river Wansbeck, and Whittle Burn, a tributary of the Tyne. Specimens were identified by Dr R. W. Ingle of the Natural History Museum, London. The experimental animals were maintained in large aerated tanks in a cool basement and fed on minced beef and occasional animal scraps. The average size of crayfish

Table 1. *The composition of the calcium activity standards used to measure haemolymph calcium activity*

| Salt | Concentration (mM) |
|---|---|
| $CaCl_2$ | 1 or 10 |
| NaCl | 200 |
| KCl | 4·5 |
| $MgCl_2$ | 1·5 |

used was about 10 g but varied from 8 to 14 g. Winter intermoult stages ($C_4$–$D_1$) were used and animals were not fed during the experiments.

### *Methods*

Experimental animals were kept in polystyrene boxes in artificial tapwater solutions. The composition of the water was as used previously (Greenaway, 1970). The concentration of calcium was varied from 0 to 3 mM but the concentrations of the other cations were not altered. In order to ascertain that the experimental crayfish were in the intermoult stage ($C_4$–$D_1$) they were placed in 250 ml of artificial tapwater (1·0 mM-Ca) and the external concentration of calcium was followed. Any animals showing net calcium uptake or an unusually high rate of net loss were discarded. This was important in September when a few animals were still moulting. It is likely that some very early premoult stages were used as it is difficult to distinguish between stages $C_4$, $D_1$ and $D_2$ without resorting to histological techniques. No signs of formation of the new exoskeleton were observed in any animal when holes were bored in the carapace for blood sampling and potential measurements. Experiments were carried out at 10 ± 1 °C.

Measurements of the concentration of calcium in blood, medium and urine samples were made either with a Unicam SP 900 flame spectrophotometer or an EEL 240 atomic absorption spectrophotometer. Samples and standards contained lanthanum chloride at a concentration of 2·35 mM. This concentration of $LaCl_3$ was found sufficient to eliminate depression of calcium readings caused by bicarbonate in the samples.

Measurements of calcium activity in small samples of blood (0·3 ml.) were made using Orion calcium and reference electrodes, an Orion model 401 specific ion meter and Orion microsample dishes. The activity was measured in an empirical manner using $CaCl_2$ standards containing sodium, potassium and magnesium (as chlorides) in the concentrations at which they are found in the blood. Table 1 shows the composition of these standards. Blood samples for activity measurements were withdrawn from the heart in a Pyrex glass pipette and transferred directly to the microsample dish.

Measurements of the potential difference between the blood and the external medium were made using a Vibron electrometer model 33B-2 and two calomel-saturated KCl electrodes. The crayfish was held in a clamp and lowered into the experimental medium so that the lower parts of body were submerged and the gill chambers were ventilated. The dorsal surface of the carapace remained dry above the water and was smeared with petroleum jelly to prevent current leakage. One electrode was placed in the external solution whilst the other was in contact with the blood

via a bridge filled with crayfish Ringer's solution. The fine tip of the bridge was inserted into the pericardial cavity via a small hole made in the carapace with a dental drill. Potential measurements were made at room temperature (*c.* 20 °C), each measurement lasting about 15 min and being recorded on a chart recorder. The external medium was altered by siphoning out one solution and running in another.

Blood samples for determination of calcium concentrations or for radioactivity measurements were removed from the pericardial cavity via a small hole bored in the carapace. After sampling, the hole was sealed with petroleum jelly and loss of blood was negligible. The blood sample was collected in a fine Pyrex glass pipette and blown out under liquid paraffin. Small volumes were then taken and diluted suitably for analysis.

The rate of urine production was measured using (hydroxymethyl-$^{14}$C)-inulin obtained from the Radiochemical Centre, Amersham. Between 30 and 50 $\mu$l of crayfish Ringer's solution containing labelled inulin were injected into the pericardial cavity through a small hole bored in the carapace. The injection was carried out over a period of about 1 min using an Agla syringe, the crayfish being held in a clamp stand. After injection the hole in the carapace was sealed with petroleum jelly and the animal left for at least 5 h in artificial tapwater (1·0 mM-Ca). After this period the animal was washed in fresh artificial tapwater for a few minutes, to remove traces of inulin from the outer body surface, and placed in 100 ml artificial tapwater. Samples of this solution were removed at regular intervals for up to 2 days and assayed for radioactivity. When the rise in radioactivity became linear with respect to time, first urine and then blood samples were taken and assayed for calcium and radioactivity (inulin concentration). Knowing the radioactivity of the final urine and the rise in external radioactivity it was possible to calculate the rate of urine flow. The method of urine collection was as follows. The animal was removed from its experimental solution and washed for several minutes in distilled water. The body surface was then gently blotted dry and the apertures of the branchial chambers were plugged with tissue paper. Urine flow could be stimulated by tapping the base of the second antenna, near the nephropore, with the collecting pipette and sucking the urine released into the pipette. The total sample taken was generally about 5 $\mu$l and urine from the right and left nephropores was pooled.

Radioactive counting techniques and calcium flux measurements were as described previously (Greenaway, 1971*a*).

## RESULTS

### *Normal animals*

#### *Calcium balance*

Calcium balance in winter intermoult animals (September–December) was studied by placing the crayfish individually in small volumes (250 or 500 ml) of artificial tapwater containing 1 mM-Ca and following the external calcium concentration over a period of about 1 week Although a few of the animals examined maintained a near steady state most crayfish showed a net loss of calcium to the medium. Further experiments revealed that the crayfish pass from a state of net uptake following the moult (stages A–$C_3$) to a state of calcium balance with the medium (early $C_4$) which may last several weeks. This state was followed by a period of net calcium loss (late

Table 2. *The net loss of calcium from late stage $C_4$ crayfish in artificial tapwater* (1 mM-*Ca*)

| No. | Calcium net loss rate (μmoles/g/h) | Date of measurement |
|---|---|---|
| 44 | 0·064 | 20. i. 71 |
| 45 | 0·036 | |
| 46 | 0·033 | |
| 47 | 0·040 | |
| 48 | 0·058 | |
| 49 | 0·049 | |
| 51 | 0·016 | |
| 52 | 0·045 | 2. ii. 71 |
| 53 | 0·062 | |
| 54 | 0·036 | |
| 55 | 0·069 | |
| 56 | 0·055 | |
| 57 | 0·029 | |
| Mean | 0·046 ± 0·004 S.E. | |

$C_4$ and $D_1$) which continued at a fairly steady rate throughout the winter until the late premoult stages ($D_3$–$D_4$), when the calcium loss rate rose dramatically (Greenaway, unpublished; Chaisemartin, 1967). Data given by Chaisemartin (1967) shows that his intermoult crayfish were in calcium balance when examined. Values for the net calcium loss rate from late $C_4$ animals are given in Table 2. The net calcium loss in these late intermoult animals seems unlikely to have been from the outer surface of the exoskeleton as this has been shown in *Austropotamobius* to be impermeable to calcium ions (Chaisemartin, 1965*b*, 1967). Similarly Maloeuf (1937) found the exoskeleton of *Cambarus bertoni* to be impermeable to electrolytes. The observed calcium loss must therefore occur via the urine or perhaps passively across the gills. This point is examined in more detail later.

*Calcium influx*

Calcium influx measurements were made on individual crayfish adapted to 1 mM-Ca in artificial tapwater. Each crayfish was placed in a small volume (100 ml) of approximately 0·5 mM-Ca artificial tapwater labelled with $^{45}Ca$. The calcium uptake mechanism was saturated at this external concentration (Bernard & Chaisemartin, 1965; Chaisemartin, 1967; Greenaway, unpublished) so the influx values given in Table 3 therefore represent the maximum rate of calcium uptake. The calcium influx was very low and each measurement took several days. Errors due to backflux of tracer, however, were negligible as the specific activity of the blood calcium never reached significant levels with respect to the external specific activity. Chaisemartin (1967) gave values for maximum calcium influx in intermoult animals of 0·1–0·14 μmoles/g/h, about ten times higher than found in the present investigation. This discrepancy can be largely accounted for by the higher temperature at which Chaisemartin's experiments were carried out (15–17 °C) and the size differences of the animals used. Chaisemartin used crayfish of 2–4 g fresh weight and has demonstrated (Chaisemartin, 1967) that the calcium flux per unit weight decreases with increasing body size.

Table 3. *Calcium fluxes in intermoult crayfish in artificial tapwater* (0·6 mM-*Ca*)

| External Ca conc. (mM) | Ca influx (μmoles/g/h) | Ca efflux (μmoles/g/h) | Weight (g) |
|---|---|---|---|
| 0·615 | 0·022 | 0·031 | 7·2 |
| 0·606 | 0·006 | 0·015 | 7·3 |
| 0·618 | 0·014 | 0·018 | 10·5 |
| 0·596 | 0·029 | 0·034 | 8·2 |
| 0·535 | 0 009 | 0·028 | 9·1 |
| 0·510 | 0·009 | 0·014 | 12·5 |
| 0·550 | 0·006 | 0·023 | 10·9 |
| Mean 0·576 | 0·014 ±0·003 S.E. | 0·023 ±0·003 S.E. | 9·4 |

The values for calcium influx were measured, whilst the calcium efflux has been taken as equal to calcium influx plus net calcium loss during the experiment. Animals were in approximate calcium equilibrium initially but showed a small net calcium loss during the experiment.

Table 4. *Urine production and calcium loss in the urine by intermoult crayfish in artificial tapwater* (1·0 mM-*Ca*)

| Weight (g) | U/B inulin | Urine flow rate (μl/g/h) | Urine Ca conc. (mM) | Urine Ca loss (μmoles/g/h) |
|---|---|---|---|---|
| 12·4* | 2·02 | 3·84 | 0·80 | 0·0030 |
| 14·7* | 2·13 | 2·22 | 1·15 | 0·0026 |
| 12·6 | — | 1·11 | 1·90 | 0·0021 |
| 13·9 | 2·11 | 2·01 | 1·35 | 0·0027 |
| 22·6 | 2·13 | 2·59 | 1·45 | 0·0038 |
| 9·7 | 2·11 | 1·83 | 2·80 | 0·0051 |
| 8·7 | 2·06 | 2·24 | 0·73 | 0·0016 |
| 10·7* | 2·19 | 0·81 | 1·03 | 0·0008 |
| Mean 13·2 | 2·11 ±0·02 | 2·08 ±0·31 | 1·40 ±0·22 | 0·0027 ±0·0004 |

Mean values are ±S.E. Animals marked with * were in steady-state (early $C_4$) whilst the other animals showed a small net calcium loss (late $C_4$). The difference between values for calcium concentration and loss in the urine was not significant ($P > 0·2$ and $P > 0·5$ respectively).

*Calcium loss*

The concentration of calcium in the urine and the rate of urine production have been measured and values are given in Table 4. The mean value for urine calcium concentration of 1·4 mM may be compared with those for *Cambarus clarkii* of 2·2 mM (Lieneman, 1938) and *Potamobius* 2·7 mM (Scholles, 1933). These values fall within the range of variation found in this study. The mean value for urine flow rate was 2·08 μl/g/h, equivalent to 5·1 % body wt./day. Bryan (1960) found the rate of urine production in *Astacus fluviatilis* (= *Austropotamobius pallipes*) to be 8·2% body wt./day at 20 °C, a value almost double that given in Table 4. However, Werntz (1963) demonstrated a temperature effect on the rate of urine flow in freshwater *Gammarus fasciatus*, the flow rate at 25 °C being double that at 15 °C. If a similar situation exists in *Austropotamobius* between 10 and 20 °C Bryan's values, obtained by a weight increase method, become compatible with those in the present study. The mean rate of calcium loss in the urine (0·0027 μmoles/g/h) is considerably lower than the efflux rate (Table 3). Presumably therefore much of the normal calcium loss by the animal (about 88%) occurs by a route other than the excretory organs, i.e. the gills. Simi-

Table 5. *Calcium loss to calcium-free water from crayfish adapted to a range of external calcium concentration*

| Adaptation conc (mM) | Calcium loss rate μmoles/g/h | | | | | |
|---|---|---|---|---|---|---|
| | 1·0 | 0·5 | 0·25 | 0·1 | 0·05 | 0·025 |
| | 0·017 | 0·017 | 0·028 | — | — | — |
| | 0·015 | 0·012 | 0·014 | 0·018 | 0·012 | 0·013 |
| | 0·028 | 0·024 | 0·030 | 0·010 | — | — |
| | 0·032 | 0·032 | 0·063 | 0·026 | 0·032 | 0·022 |
| | — | 0·020 | 0·022 | 0·018 | 0·024 | 0·031 |
| | 0·024 | 0·020 | 0·068 | 0·024 | 0·038 | 0·038 |
| Mean | 0·023 | 0·021 | 0·038 | 0·019 | 0·027 | 0·026 |
| | ±0·003 | ±0·003 | ±0·008 | ±0·003 | ±0·005 | ±0·005 |

All mean values ±S.E. Animals were in calcium balance in artificial tapwater (1·0 mM-Ca) initially.

larly, urinary sodium loss represents only a small fraction (6%) of the total sodium loss (Bryan, 1960). The values in Table 4 marked with an asterisk were for animals previously found to be in calcium balance in artificial tapwater (1 mM-Ca) and the remaining animals showed a small net loss to the medium. The steady-state animals showed a rather lower calcium concentration and calcium loss in their urine than did the animals in negative calcium balance. However, the difference between the two sets of data was not significant. In the marine crab *Carcinus* intermoult stages generally show a net loss of calcium to the medium which Robertson (1937, 1960) considered to be largely via the excretory organ. This appears reasonable as the net passive loss across the gills would be low, the calcium concentration gradient between gills and sea water being small.

The effects of external calcium concentration on the rate of calcium loss were examined. The net calcium loss from crayfish which were in calcium balance with artificial tapwater (1 mM-Ca) was measured to calcium-free water. The animals were than adapted to artificial tapwater of lower calcium concentration for a few days and the new loss rates were measured. Although in balance in artificial tapwater (1 mM-Ca) the crayfish showed a small net loss in artificial tapwater containing less than 0·5 mM-Ca. The loss rates are shown in Table 5. The loss rate over the first hour of measurement was high and values shown in Table 5 are for the rather lower steady loss rate found to occur after this period. No significant difference was found between mean loss rate of animals adapted to high and low calcium concentrations. As a net loss of calcium occurred when the animals were placed in low-calcium solutions it appears that adaptation to low external calcium concentrations does not decrease calcium loss from the blood.

The *U/B* inulin ratio was always greater than 2 in these experiments, thus confirming that water is reabsorbed from the primary urine, as shown previously by Riegel & Kirschner (1960) and Kirschner & Wagner (1965). In addition it would appear that considerable reabsorption of calcium took place. The ionized calcium in the blood of *Austropotamobius* was approximately 6·3 mM (Table 6). In a kidney forming urine by filtration the primary urine or filtrate would be expected to show at least this concentration of calcium. The fact that the final urine has a very much lower calcium concentration (1·4 mM) indicates that considerable reabsorption must have occurred.

Table 6. *Values for the activity of calcium in the haemolymph*

| Haemolymph Ca conc. (mM) | Haemolymph Ca activity (mM) | Haemolymph ionized Ca (mM) | Date |
|---|---|---|---|
| 11·6 | 1·142 | 5·2 | 9. ix. 70 |
| 12·5 | 1·153 | 5·0 | |
| 9·8 | 0·965 | 4·6 | |
| 12·2 | 1·153 | 5·0 | |
| 12·2 | 1·270 | 6·0 | 27. i 71 |
| 11·9 | 1·020 | 4·8 | |
| 12·0 | 1·398 | 6·6 | |
| 12·2 | 1·312 | 6·2 | |
| 12·8 | 1·355 | 6·4 | |
| 13·5 | 1·355 | 6·4 | |
| 12·2 | 1·272 | 6·0 | 10. x. 71 |
| 11·7 | 1·379 | 6·5 | |
| 11·0 | 1·447 | 7·0 | |
| 13·8 | 1·540 | 7·3 | |
| 13·2 | 1·870 | 8·8 | |
| 10·8 | 1·680 | 7·9 | |
| 13·3 | 1·630 | 7·7 | |
| Mean 12·2 ± 0·24 S.E. | 1·349 ± 0·056 S.E. | 6·3 ± 0·23 S.E. | |

*Haemolymph calcium activity*

Values for the calcium activity in the haemolymph of *Austropotamobius* are given in Table 6. The activities of calcium in artificial tapwater solutions were as given previously (Greenaway, 1971*a*). The activity varied at different times of the year but further measurements over the whole of the intermoult period would be necessary to establish any cyclical pattern of variation. The mean value for the activity of 1·35 mM-Ca is about 45% lower than the calculated value (2·48 mM), indicating that a large proportion of haemolymph calcium was in non-ionic form. The actual concentrations of ionized calcium are also shown in Table 6. The non-ionized calcium in the haemolymph probably exists in three major components: calcium bound to blood protein, calcium complexed by small organic anions, and calcium complexed with inorganic anions such as sulphate, bicarbonate, carbonate and phosphate (Greenaway, 1971*a*; Moore 1969). In the freshwater crayfish *Orconectes limosus* about 18% of the haemolymph calcium is bound to the blood protein (Andrews, 1967). It seems likely that a similar amount of haemolymph calcium in *Austropotamobius* may be protein bound, although one might expect this to vary at different stages of the moult. This would mean that about 10 mM-Ca in the haemolymph would not be protein-bound. As 6·3 mM-Ca is ionized about 3·7 mM-Ca must be complexed by small organic and inorganic anions. In the lobster *Homarus americanus* 12–13% of the haemolymph calcium is non-diffusible and presumably protein-bound (Hayes, Singer & Armstrong, 1962).

*Potential difference measurements*

Measurements of the potential difference existing across the body wall of intermoult crayfish were made using artificial tapwater solutions with a range of calcium concentration (0–3 mM). Initial potential measurements were found to be some 10 mV greater (more negative) at any given external calcium concentration than those fo

Table 7. *The rate of calcium loss during measurement of potential differences*

| Initial Ca loss rate (μmoles/g/h) | Final Ca loss rate (μmoles/g/h) | Normal Ca loss rate (μmoles/g/h) |
|---|---|---|
| 0·197 | 0·044 | 0·045 |
| 0·284 | 0·058 | 0·062 |
| 0·287 | 0·096 | 0·036 |
| 0·520 | 0·230 | 0·055 |
| 0·389 | 0·056 | — |
| 0·261 | 0·098 | — |
| 0·704 | 0·123 | — |
| Mean 0·377 ± 0·062 S.E. | 0·101 ± 0·022 S.E. | 0·050 ± 0·005 S.E. |

The values for normal calcium loss rate were for net calcium loss to artificial tapwater (1 mM-Ca) before P.D. measurements were made.

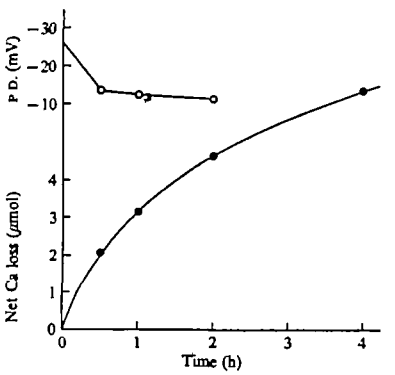


Fig. 1. Net calcium loss and potential difference measurements for a single crayfish (late $C_4$) in artificial tapwater (0·1 mM-Ca). For further explanation see text. ○, values for potential difference. ●, values for net calcium loss.

animals which had been in the apparatus for several hours. Over the first 1–2 h this potential decreased until steady values were obtained. The steady values were then maintained up to 4 h, the maximum period of measurement. It seemed possible that the initial potentials measured might have been due to an abnormally high rate of loss of calcium ions from the blood or to an inhibition of calcium uptake due to the handling necessary to set the animal up in the potential measuring apparatus. In order to test this hypothesis the net calcium loss rate to 0·1 mM-Ca in artificial tapwater and the potential difference were measured simultaneously over a 3 h period. As can be seen from these measurements (Table 7), the initial calcium loss rate (0–1 h) was very much higher than over the remaining period of measurement. This high loss rate coincided with the highest (most negative) potentials recorded. Results for a typical animal are shown in Fig. 1. Net calcium loss is markedly increased by the handling procedure but the animal appears to recover within 2 h and then maintains a steady potential difference. It is possible that the calcium uptake may be affected but this would be difficult to measure as the influx is very low (Table 3). As the influx is so low even its complete inhibition could not account for the large increase in net loss observed. In view of these findings the final steady potential values have been regarded as the actual potential existing in undisturbed crayfish. Table 8 shows mean

Table 8. *The effect of external calcium activity on the existing potential difference across the body wall*

| External Ca conc. (mM) | External Ca activity (mM) | Potential difference (mV ± S.E.) |
|---|---|---|
| 2·940 | 1·41 | +0·4 ± 1·0 (7) |
| 2·040 | 1·00 | +1·0 ± 1·7 (7) |
| 0·980 | 0·525 | −2·1 ± 1·0 (16) |
| 0·510 | 0·272 | −7·8 ± 1·8 (10) |
| 0·260 | 0·138 | −11·4 ± 1·2 (8) |
| 0·120 | 0·062 | −10·9 ± 1·2 (9) |
| 0·054 | 0·030 | −15·8 ± 2·2 (8) |
| 0·032 | 0·015 | −17·7 ± 1·5 (9) |

Figures in parentheses give no. of observations.

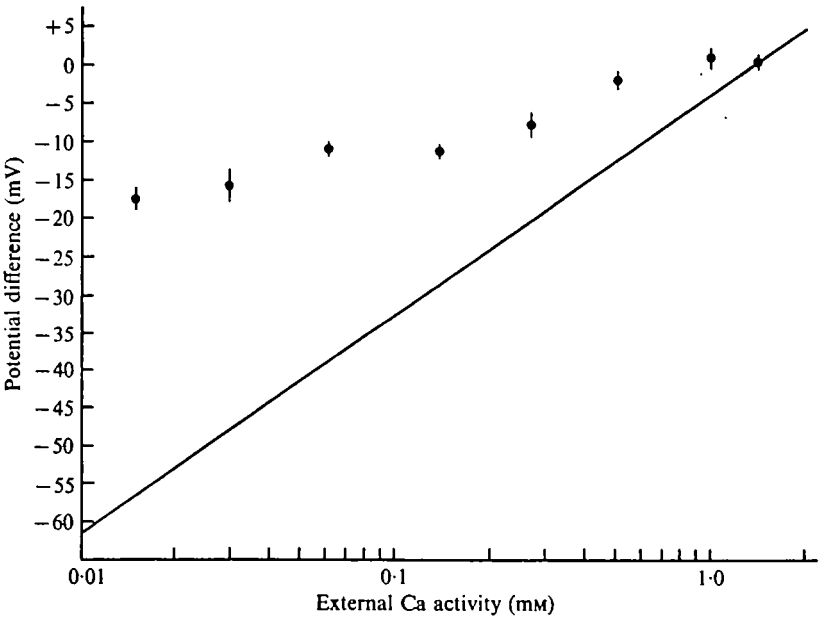


Fig. 2. A comparison between the mean potential difference, measured between the haemolymph and the external medium, and the calculated equilibrium potential for calcium over a range of external calcium activity. ●, Mean values of the measured potential difference. Vertical lines represent standard errors. The unbroken line represents the calculated equilibrium potential for calcium over the range of external calcium activity studied.

values for the electrical potential across the surface epithelium over a range of external calcium concentration. In Fig. 2 these values are compared with the calculated equilibrium potentials over the same range of external calcium activity. When in calcium balance the calcium uptake necessary to balance calcium loss is against an electrochemical gradient in external media having calcium activities less than 1·4 mM-Ca (3 mM-Ca concentration). This means that in most fresh waters calcium uptake would be an active process requiring the expenditure of energy. From Fig. 2 it can be seen that there is a change in potential difference of approximately 10 mV per tenfold concentration change in external calcium activity, suggesting that the body surface is selectively permeable to calcium to some degree. In *Limnaea* the body surface also

showed a selective permeability to calcium ions, in fact behaving much as a calcium electrode over a certain range of calcium concentration (Greenaway, 1971*a*). Bryan (1960) recorded a potential difference of $4{\cdot}1 \pm 3{\cdot}2$ mV (blood positive) across the body surface of *Astacus*, in an external medium containing 1·82 mM-Ca. This agrees quite closely with the results depicted in Fig. 2 for solutions of similar calcium concentration. By contrast, Croghan *et al.* (1965), using isolated perfused gills of *Austropotamobius*, measured much larger potentials (blood negative) and suggested that calcium was in equilibrium across the body surface in a 1% dilution of Ringer solution (0·135 mM-Ca). Chaisemartin (1966, 1967) used the same technique as Croghan *et al.* with rather similar results and concluded that there was a small electrochemical gradient favouring the inward movement of calcium ions. It is apparent that potential measurements made on isolated, perfused gills bear little relationship to the situation in the intact crayfish. Calcium concentrations instead of activity ratios were used to calculate the equilibrium potentials and this would have introduced an error into the calculation of equilibrium potentials. Furthermore, the calcium activity of the perfusing Ringer's solutions will have been very different from that in haemolymph for the reasons discussed earlier. For example, if the calculated equilibrium potential for calcium of $-58$ mV given by Croghan *et al.* is recalculated using the measured haemolymph calcium activity (Table 6) and calculated medium calcium activity the new value obtained is $-31{\cdot}6$ mV. Thus there is apparently an electrochemical gradient favouring calcium uptake, a situation not found in the intact animal. Quite clearly then, perfusing the isolated gill with crayfish Ringer's solution provides an internal calcium activity very different (about 100% higher) from that which normally exists in the haemolymph.

### *Depleted animals*

#### *Calcium balance experiments*

Animals were placed in calcium-free artificial tapwater and the external calcium concentration followed. The rise in external calcium concentration with time is shown in Fig. 3. Net calcium loss was initially at a high rate but this gradually declined to give a steady loss rate. A similar situation was observed during the measurement of the loss rates presented earlier (Table 5). None of the animals examined came into calcium balance with the medium but continued to show a steady net loss although they had maintained calcium balance earlier in 1 mM-Ca. Chaisemartin (1965*a*, 1967) obtained rather similar results on much smaller crayfish (2 g). The decline in loss rate occurred at quite low external calcium concentrations (10–15 $\mu$M), as can be seen in Fig. 3.

It is possible that this decline in loss rate may have been due to an activation of the calcium uptake mechanism. However, data presented later suggest that no such activation might be expected. The alternative is that the permeability of the body wall to calcium may be increased in calcium-free or very low-calcium media. If this was the case then as external concentration rose the calcium permeability and hence loss rate might be expected to decrease, causing the observed results. The fact that the mean initial loss rate of animals used in balance experiments was twice the maximum calcium efflux (Table 3) provides support for this permeability theory.

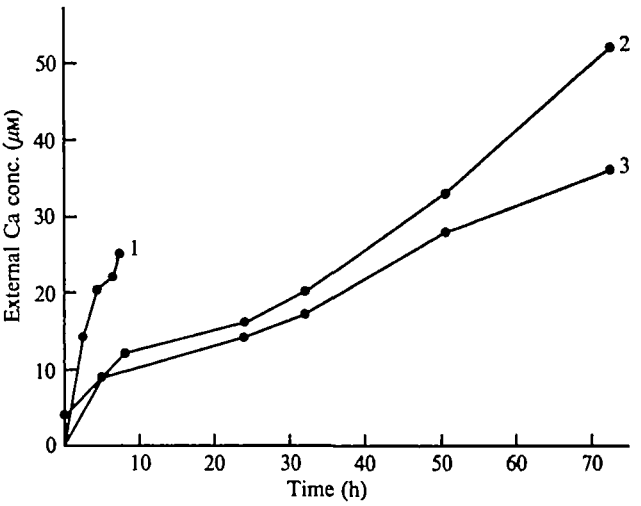


Fig. 3. The time course of calcium loss to calcium-free artificial tapwater for three crayfish. Values for animal 1 (early $C_4$) represent calcium loss to 150 ml of water whilst values for animals 2 and 3 (late $C_4$) represent calcium loss to 250 ml of water.

*The effect of calcium depletion on haemolymph calcium concentration*

Crayfish which were in steady state with respect to calcium in artificial tapwater (1 mM-Ca) were depleted of calcium by placing them individually in 1 l volumes of calcium-free artificial tapwater and changing the water regularly. Haemolymph samples were taken at intervals. The calcium loss was more or less linear with respect to time over the experimental period (35 days). The mean results for 5 animals are shown in Fig. 4. During the course of the experiments the haemolymph calcium concentration fell rapidly to a level about 15% below the initial value and then remained more or less constant for the remainder of the experiment. The mean net loss of calcium in this period was 157 μmoles. As the apparent loss of calcium from the haemolymph was only 4–5 μmoles/animal most of the lost calcium must have originated elsewhere, i.e. calcium moved from other tissues to the haemolymph at a rate similar to the rate of net calcium loss to the medium. Chaisemartin (1965*a*, 1967) determined the effect of calcium depletion on the haemolymph calcium concentration and total calcium content of 2 g crayfish. Depletion resulted in a rapid fall in haemolymph calcium concentration from 8 to 3 mM within 10 days. Total calcium loss in this period was much greater than could be supplied by the haemolymph and Chaisemartin (1967) demonstrated that the loss calcium originated largely in the exoskeleton. 10% of total body calcium was considered mobilizable, all parts of the exoskeleton contributing more or less equally.

*The effect of calcium depletion on the rate of uptake*

Animals were depleted by placing them in 1 l baths of calcium-free artificial tapwater renewed every other day. Calcium loss was again more or less linear with time. After varying periods of depletion the crayfish were transferred to small volumes of artificial tapwater (0·5–1·0 mM-Ca) in order to measure any net uptake of calcium. A few animals showed a very small net gain of calcium during the first few hours

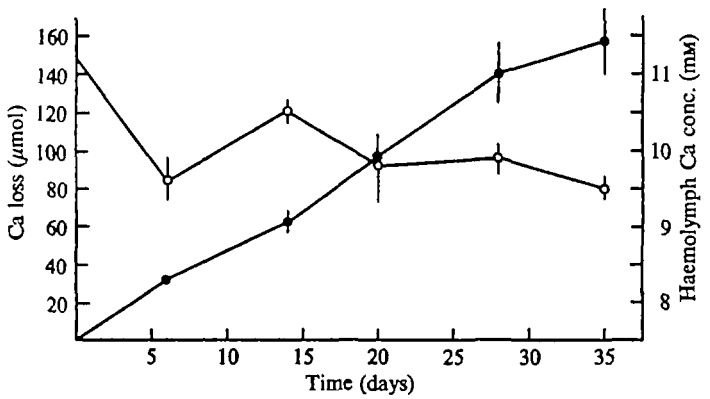


Fig. 4. The effect of calcium depletion on the concentration of calcium in the haemolymph. O, Values for the concentration of calcium in the haemolymph. ●, Values for net calcium loss. Vertical lines represent standard errors.

Table 9. *The net uptake of calcium, after depletion, by five small crayfish (nos. 65–69) placed in* 100 *ml artificial tapwater*

| | External calcium concentration (μM) | | | | |
|---|---|---|---|---|---|
| Time (h) | 65 | 66 | 67 | 68 | 69 |
| 0 | 1000 | 1010 | 1010 | 990 | 1010 |
| 2·5 | 950 | 1000 | 1020 | 980 | 990 |
| 6 | 970 | 980 | 1050 | 960 | 970 |
| 19 | 1000 | 1060 | 1220 | 960 | 960 |
| 46 | 1000 | 1120 | 1510 | 970 | 970 |
| Net loss during depletion (μmoles) | 90·5 | 108·5 | 257 | 44·5 | 32 |
| Maximum net uptake (μmoles) | 5 | 2 | 0 | 3 | 5 |
| Net uptake after 46 h (μmoles) | 0 | −11 | −50 | 2 | 4 |
| Weight (g) | 4·0 | 1·9 | 2·0 | 2·1 | 1·4 |

after transfer but this was gradually lost again within 24 h. The majority of animals showed no net uptake at all, despite loss of up to 10% of their total calcium, and either maintained calcium balance with the medium or showed the small net loss characteristic of late stage $C_4$ animals. These results differ markedly from the findings of Bernard & Chaisemartin (1965) and Chaisemartin (1967) on small crayfish (2–4 g) and isolated gill preparations. They found that a loss of more than 10% of total calcium was irreversible and eventually led to death, but that smaller losses resulted in an appreciable net uptake on return to calcium-containing media, thus making possible recovery of the lost calcium. In view of Chaisemartin's results the above experiments were repeated using small crayfish. It was calculated, using Chaisemartin's (1967) data for total body calcium, that less than 10% of the total calcium content was lost from these small animals during depletion. Following depletion the crayfish were transferred each into 100 ml of artificial tapwater (1·0 mM-Ca) and the external calcium concentration was followed. Results for five animals are presented in Table 9.

All crayfish but one (67) showed an initial net gain of calcium although this was only retained in two animals. In each case the calcium uptake was very small in relation to the amount lost during depletion.

## DISCUSSION

For much of the winter intermoult stage *Austropotamobius* shows a negative calcium balance. At first sight it appears surprising that the animal does not maintain calcium balance through the intermoult period. It is possible, however, that the calcium loss involved is not important to the animal. If one takes the net calcium loss rate to be of the order of 0·046 $\mu$moles/g/h given earlier (Table 2) a 10 g crayfish would lose approximately 2 m-moles of calcium during a 6-month winter intermoult. This figure represents a maximum value for calcium loss as the loss rate may be lower at the lower water temperatures experienced in the winter months. In addition, calcium in the food may also make a significant contribution to the calcium balance. However, even using the value of 2 m-mol for calcium loss, this represents a loss of only 15% of total calcium (using the data of Chaisemartin, 1967, for total calcium). Crayfish subjected to similar net loss during depletion in the laboratory appeared normal, so such a loss would appear to be tolerable. During the premoult and moult stages about 90% of the total calcium content at early $C_4$ stage is lost (Chaisemartin, 1967). It would therefore be of no advantage to the crayfish to conserve calcium by maintaining accurate calcium balance during the intermoult stages, always assuming that the rate of calcium loss could be tolerated. In fact it would be advantageous to allow a small net calcium loss as this would reduce the amount of energy required for calcium uptake.

The calcium influx was very low, but for animals in calcium balance calcium influx has been shown to be against a small electrochemical gradient at external calcium activities less than 1·4 mM. Thus at least part of the calcium influx must be facilitated by an active transport mechanism. In order to obtain further information on this active component the measured ratio of calcium influx ($M_1$) and efflux ($M_2$) (values given in Table 3) was compared with the flux ratio calculated from the equation derived by Ussing (1949):

$$M_2/M_1 = (a_2/a_1) \exp [-(zF/RT) E],$$

where $a_2$ and $a_1$ are the internal and external calcium activities respectively, $E$ is the measured potential difference and the other symbols have their usual meaning. The measured ratio (1·64) was lower than the calculated value (2·82). This could be explained in several ways, the two most likely explanations being as follows: first, that the influx consisted of a large exchange component plus a small passive component, and secondly, that the flux was composed of a small active component plus a larger passive component. It seems unlikely that an exchange component would be larger than the passive flux component and the second alternative seems the more likely. This is supported by the potential measurements, which suggested an active component of calcium uptake. However, small changes of $E$ would radically alter the calculated flux ratio and not too much emphasis can be placed on the above interpretation.

The importance of using calcium activity rather than concentration values in the calculation of Nernst potentials has already been mentioned. It is permissible to use

concentration values when there is a very large difference between concentration values on either side of the surface membrane, the error involved then being small, e.g. sodium regulation in most freshwater animals. However, if the concentration difference of an ion across the surface epithelium is small the activity of that ion rather than its concentration must be considered if large errors are not to be introduced into the calculation of equilibrium potentials. This will be particularly true if there are also large differences in ionic strength on either side of the surface membrane. Calcium, too, is especially prone to form complexes with organic and inorganic anions and molecules. The advent of specific ion electrodes permits direct measurement of ionized calcium in solution and it is important that such measurements be used.

Croghan *et al.* (1965) made potential-difference measurements across the epithelium of isolated, perfused gills of *Austropotamobius* and considered that conclusions drawn from their experiments were relevant to the situation in the normal gill. It has been shown earlier, however, that potential difference measurements made on isolated, perfused gills are very different from those made on the intact animal. In addition, the perfusate had a very different concentration of ionized calcium from that expected in normal haemolymph. It is apparent therefore that the isolated preparation is not normal in these respects and its behaviour cannot be taken to be indicative of the situation in the intact animal.

Crayfish depleted of calcium in calcium-free water showed a small fall in haemolymph concentration but did not show net calcium uptake on return to calcium containing media. Most of the calcium lost during depletion originated in the exoskeleton (Chaisemartin, 1967). This is very similar to the situation in the freshwater mollusc *Limnaea* (Greenaway, 1971*a*, *b*). The lack of stimulation of net calcium uptake after depletion in *Austropotamobius* is not surprising in view of the foregoing discussion concerning calcium balance. The reservoir of calcium in the exoskeleton is probably sufficiently great to enable the crayfish to tolerate the larger-than-usual net calcium loss, involved in calcium depletion, without loss of skeletal function. The results obtained for depleted animals in this investigation were very different from those of Chaisemartin (1965*a*, 1967) and Bernard & Chaisemartin (1965). It is difficult to reconcile the two sets of results. The only obvious difference in experimental conditions was the small size of crayfish used by Chaisemartin (2 g). Results for small animals in this work, however, revealed only a very slight tendency towards net calcium uptake after depletion, and recovery was very far from complete. Further experiments are required on the effects of depletion on calcium uptake in order to clarify this situation.

SUMMARY

1. Calcium regulation in normal and in calcium-depleted specimens of *Austropotamobius pallipes* in the intermoult condition has been investigated.

2. Calcium turnover was very low and the normal calcium balance was negative for much of the winter intermoult stage.

3. Calcium uptake was against a small electrochemical gradient, at least part of the influx occurring by active transport.

4. Most of the calcium loss occurred across the gills, and the urine contribution was small.

5. Calcium-depleted animals showed only a small fall in haemolymph calcium concentration and calcium uptake was not significantly increased by depletion.

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