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

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SUMMARY

Net uptake of calcium by *Austropotamobius* begins 15–30 min after the moult and rapidly reaches a maximum level (2 μmoles/g/h at 10°C) which is maintained throughout stages A and B. At stage C_1 the rate of net uptake falls sharply to a lower level which is gradually reduced until equilibrium is reached at C_4. The uptake mechanism is near-saturated at 0·4 mM-Ca/l and half-saturated at 0·13 mM-Ca/l. In the absence of external HCO_3^- net uptake is reduced. Calcium uptake is against an electrochemical gradient. The concentration of ionised calcium in the haemolymph remains unchanged during the intermoult cycle.

INTRODUCTION

The general pattern of calcium metabolism after the moult is well known for aquatic crustacea (Passano, 1960; Travis, 1960) and much data are available for the freshwater species. Immediately after the moult the total calcium content of *Austropotamobius pallipes* is only 10% of the intermoult level, and the only heavily calcified areas are the gastroliths (Chaisemartin, 1967). The calcium balance switches from negative in the premoult stage (D) to strongly positive in the postmoult. At stage A_1 calcium net uptake is maximal and declines thereafter to zero by stage C_4 (Chaisemartin, 1967). Chaisemartin also found that the potential difference across the isolated perfused gills of *Austropotamobius* changed in such a way during postmoult that calcium uptake was a passive process. However, in view of previous criticism of potential measurements across isolated gill preparations (Greenaway, 1972, 1974*b*) it is necessary to view this data with caution. The exact times at which calcium net uptake begins and at which maximum rates of net transport are achieved remain unknown but are generally agreed to occur shortly after the moult (Travis, 1955; Turpen & Angell, 1971) although *Daphnia magna* is thought to commence net uptake shortly before the moult (Porcella, Rixford & Slater, 1969). The primary stimulus triggering calcium net uptake has not been identified. Vincent (1963, 1969), investigating the relationship between calcification and external calcium in *Gammarus pulex pulex*, from hard water (2·5–2·75 mM-Ca/l) and soft water (0·0375–0·045 mM-Ca/l) populations, found that a reduction in the external calcium concentration increased the length of time necessary for calcification. In addition, a threshold calcium concentration (0·075 mM/l in hard water and 0·025 mM-Ca/l in soft water) was found below

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which completion of calcification was impossible. *G. pulex* from soft water habitats therefore, had a greater affinity for calcium than gammarids from hard-water populations. A similar situation exists for *Austropotamobius* (Chaisemartin, 1967).

The object of this work was to complete the quantitative analysis of calcium metabolism in *Austropotamobius* presented in previous papers (Greenaway, 1972, 1974*a*, *b*). In particular, it was felt necessary to clarify the situation regarding the energy requirements of calcium uptake and to elucidate the mechanism involved in calcium transport.

MATERIALS AND METHODS

Materials and methods not detailed below were as described previously (Greenaway, 1972, 1974*b*).

The experimental animals were all postmoult crayfish (stages A_1 to C_3), individual stages being separated using the criteria laid down by Chaisemartin (1967). Stages C_2 and C_3 were distinguished from C_4 by measuring calcium balance in small volumes of water. Postmoult crayfish showed a net calcium uptake under such circumstances, whilst intermoult animals were in approximate calcium equilibrium. *Austropotamobius* over-wintered in the laboratory began moulting in early March. Crayfish were collected from March onwards and generally moulted within several weeks of collection owing to the higher laboratory temperatures. A steady supply of moulting crayfish was thus maintained for experimental purposes until the first moult in the field took place in June.

RESULTS

Calcium uptake

The rate of calcium net uptake was measured throughout the postmoult period, the results for a typical animal being shown in Fig. 1. The rate of net uptake was very rapid during stages A and B. At the end of B_2 a sharp reduction in the rate of absorption occurred, although net uptake continued at a gradually decreasing rate until C_4 was reached. Calcification was accelerated when the temperature was raised above the normal 10 °C. The pattern of uptake was essentially similar to that described by Chaisemartin (1967) in *Austropotamobius*. Clearly, the bulk of net calcium uptake is complete by Stage C_1, the high rate in stages A and B ensuring a rapid initial calcification of the new exoskeleton.

The effect of external calcium concentration

The influence of the calcium concentration of the medium on the rate of net calcium uptake by crayfish at stage A_1 is shown in Fig. 2. The uptake mechanism was saturated at external concentrations greater than 0·4 mM-Ca/l and half-saturated at 0·13 mM-Ca/l. Some individuals had calcium affinities which were considerably higher than those given in Fig. 2. As the calcium concentration of the streams from which the crayfish were collected was always well above the near-saturation value of the uptake mechanism at the time of the moult the calcium uptake by postmoult crayfish in the field would consequently have been maximal. The maximum rate of net uptake averaged approximately 2 μmoles/g/h although individual animals showed rates as high as 3·17 μmoles/g/h, two to three times lower than those measured by Chaisemartin (1967). This discrepancy can be only partially explained by temperature differen

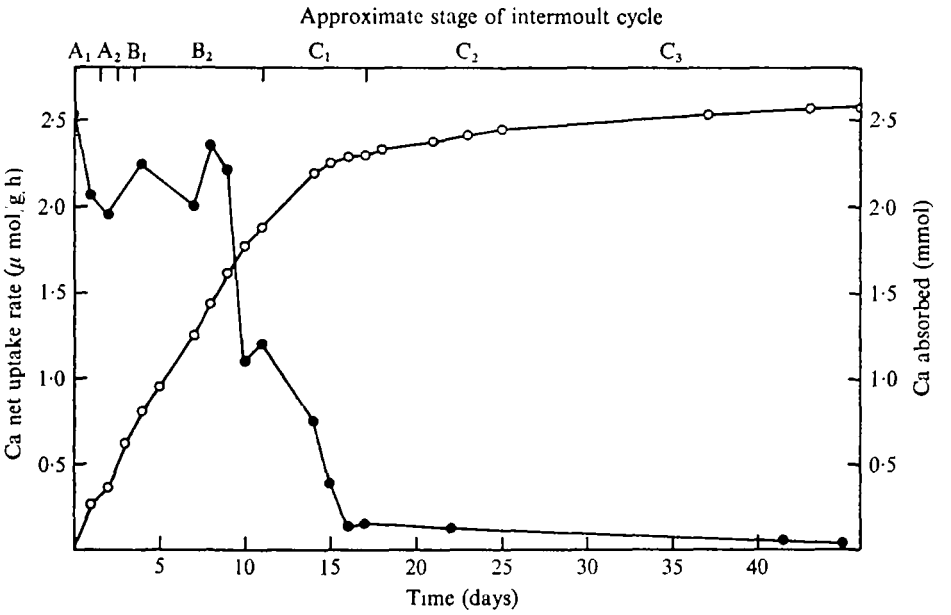

Fig. 1. The relationship between the rate of calcium net uptake and calcium recovery during the postmoult stages A, B and C_{1-3} for a single crayfish. ● represent net uptake values; ○ represent the amount of calcium absorbed.

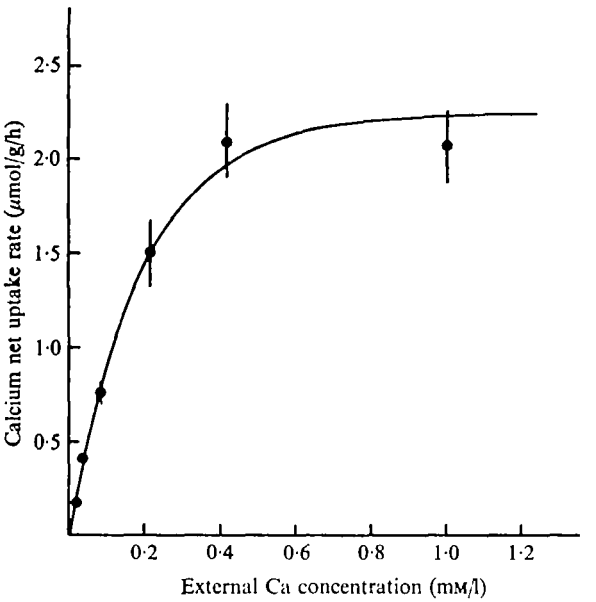

Fig. 2. The effect of increasing external calcium concentration on the rate of net uptake of calcium in early postmoult crayfish (stage A_1). ● represent mean values for net uptake of calcium. The vertical lines show the standard errors of the means.

Table 1. *Threshold calcium concentrations for net uptake of calcium by postmoult crayfish*

Animal no.	Minimum Ca conc. (μM/l)	Stage of moult
62	1·0	A_2
70	2·5	A_1
90	1·0	A_2
49	2·0	A_{1-2}
83	0·25	A_2

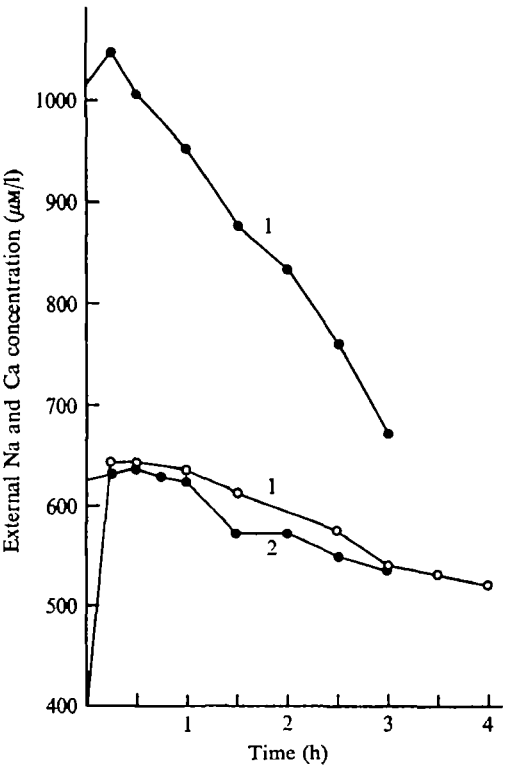

Fig. 3. The onset of net uptake of calcium and sodium after ecdysis. ● represent values for the calcium concentration of the medium, and ○ the sodium concentrations. Two animals were used and can be identified by their numbers 1 and 2.

(assuming a Q_{10} of 2 for calcium uptake) – Chaisemartin's measurements being made at 12–14°C and by the fact that Chaisemartin used smaller animals than were employed in the present study. A discrepancy also existed between values for calcium influx in intermoult animals (Greenaway, 1972).

Table 1 shows data for the threshold concentration for net uptake of calcium by postmoult crayfish. Net gain of calcium was possible down to an external concentration of 1·35 μmoles/l, indicating a very high affinity for calcium ions at this stage. Chaisemartin (1967) has recorded a very low value for calcium efflux at this stage, which would help achieve a low threshold.

Experiments were performed to determine the exact time after the moult w[illegible]

Table 2. *The concentration of ionized calcium in the haemolymph of postmoult crayfish*

Stage of moult	Weight (g)	Total Ca (mM/l)	Ionized Ca (mM/l)	Ca activity (mM/l)	% Ca ionized
A_1	6·0	15·2	8·0	2·80	52·6
A_1	4·7	12·3	5·3	1·84	42·5
A_1	5·1	11·4	5·4	1·87	47·1
A_1	10·2	13·5	6·2	2·17	45·9
A_1	9·1	15·0	6·4	2·25	42·8
A_1	5·0	14·5	7·2	2·52	49·7
A_1	14·4	13·7	6·9	2·42	50·4
A_1	8·1	14·4	7·8	2·73	54·2
A_1	11·5	14·4	7·2	2·52	50·0
Mean ± S.E.		13·8 ± 0·4	6·7 ± 0·3	2·35 ± 0·1	48·4 ± 1·28
B_2	8·0	10·8	8·2	2·85	75·5
B_2	6·5	10·0	8·4	2·94	84·0
B_2	6·0	11·1	8·0	2·80	72·1
B_2	13·6	12·9	7·6	2·66	59·1
B_2	7·4	13·2	6·8	2·38	51·5
B_2	4·7	13·3	6·6	2·31	49·6
B_2	4·8	12·7	6·4	2·24	50·4
B_2	25·5	12·2	5·8	2·03	47·5
B_2	7·0	13·7	7·5	2·63	54·9
Mean ± S.E.		12·0 ± 0·4	7·3 ± 0·28	2·54 ± 0·1	60·5 ± 4·2

calcium net uptake begins, results for two animals being shown in Fig. 3. The animals were kept in small volumes of artificial tap water and the calcium concentration (and in one case sodium) followed for a short time after the moult. The moult was complete within the first five minutes of the experiments shown in Fig. 3. Net uptake of calcium was evident after 15 and 30 min respectively, for the two animals studied. Sodium net uptake was also apparent within 30 min of the moult. Before the moult, and for a short period after, net loss of calcium occurred and was replaced rather suddenly by net uptake. The initial rate of net calcium uptake for animal 1 was 1·38 μmoles/g/h at room temperature (approx. 20°C). Little net loss of calcium from the moulting fluid occurred, although there was a significant loss of sodium at ecdysis.

Calcium activity in the haemolymph

The concentration of ionized calcium in the haemolymph of crayfish at stages A_1 and B_2 was measured and the calcium activity calculated (Table 2). Values for the concentration of ionized calcium in the haemolymph of animals at stage A_1 were not significantly different from those at any other stage of the intermoult cycle. Values for animals at stage B_2 were significantly higher than those for intermoult crayfish ($P < 0{\cdot}01$), but did not differ significantly from those at stages D_4 ($P = 0{\cdot}8$) or A_1 ($P > 0{\cdot}2$). Values for ionized calcium are, in fact, similar at all stages of the intermoult cycle which have been examined and although significant statistical differences exist between some of these values it is unlikely that they are biologically significant. The level of non-ionized calcium in the haemolymph is greater in animals at stages D_4 and A_1 than at other stages, and this reflects the higher total calcium of the haemolymph at these stages (Greenaway, 1974*a*).

Table 3. *Potential-difference values during net uptake of calcium by postmoult crayfish*

Animal no.	Stage of moult	Potential difference (mV)	Net uptake rate (μmoles−Ca/ Animal/h)
8	B_1	+4	10·0
93	B_1	+3	7·1
13	B_1	−1	3·3
14	B_1	−1	5·1
15	B_1	+1	3·1
10	B_1	−2	12·0
9	B_1	−4	1·0
Mean	B_1	0	5·9
25	C_1	+5	3·6
26	C_1	−7	5·75
27	C_1	+3	9·0
32	C_1	−8	3·6
37	C_1	−10	3·6
41	C_1	−4	8·9
Mean	C_1	−3·5	5·6

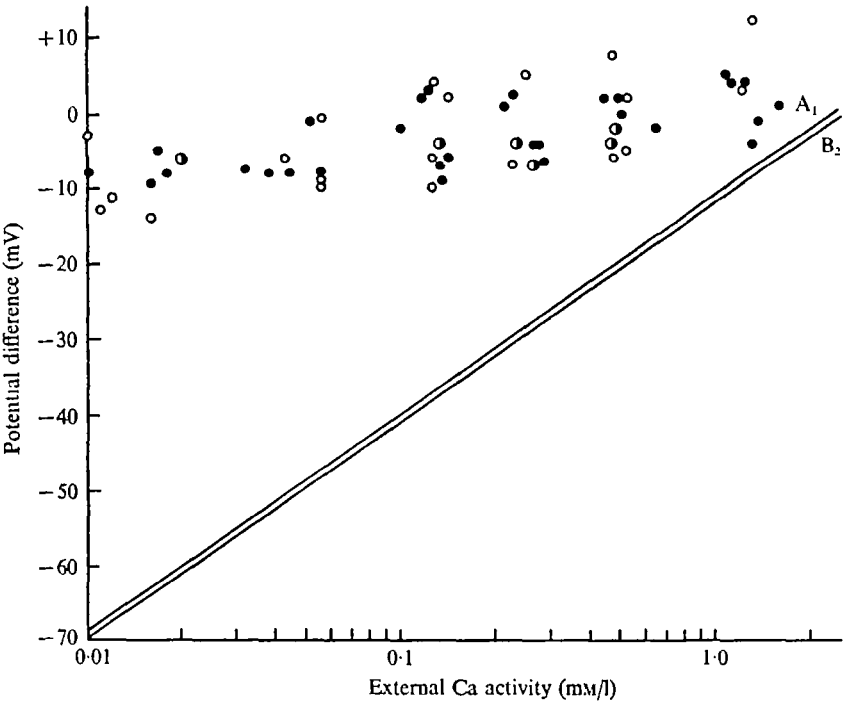

Fig. 4. The potential difference across the body surface of postmoult crayfish in relation to the external calcium activity. ◑, values for crayfish at stage A_1; ●, values for crayfish at stage B_1; ○, values for crayfish at stage C_1. The unbroken lines represent the calculated equilibrium potential for calcium in crayfish at stages A_1 and B_2.

Table 4. *The effect of Cl^- as the sole anion on the rate of net uptake of calcium by postmoult crayfish*

Animal no.	Rate of net uptake of Ca (μmoles/g/h)		
	ATW	Cl ATW	ATW
15	1·67	0·68	1·99
16	0·54	0·17	0·59
19	1·96	0·69	2·48
20	1·22	0·78	1·81
21	0·94	0·37	1·58
Mean	1·27	0·54	1·69

Table 5. *The effect of sulphate as the sole anion on the rate of net uptake of calcium by postmoult crayfish*

Animal no.	Rate of net uptake of Ca (μmoles/g/h)		
	ATW	SO_4 ATW	ATW
15	1·53	0·52	1·52
17	0·71	0·29	0·58
19	1·71	0·71	2·17
20	1·08	0·46	1·16
21	1·25	0·45	0·97
22	2·18	0·91	2·93
Mean	1·41	0·55	1·55

Potential-difference measurements

Potential-difference measurements across the body-wall of intact crayfish at various postmoult stages have been made as described previously (Greenaway, 1972). Animals which were too soft to be clamped firmly in the usual apparatus were placed in a V-shaped trough and immobilized with strips of parafilm. Two series of measurements were carried out. The first was designed to measure the potential difference when net uptake of calcium was occurring (Table 3). The potential-difference values shown are similar to those found for both intermoult and premoult crayfish. The values for the equilibrium potential for calcium in postmoult crayfish show that net calcium uptake occurred against an electrochemical gradient (Fig. 4).

The second series of experiments concerned the effect of external calcium concentration on the potential difference across the body-wall. Values for animals at three postmoult stages (A_1, B_1, C_1) are shown to be rather similar at any given activity of calcium (Fig. 4). At all external activities of calcium, uptake was against an electrochemical gradient, a conclusion which contrasts with that of Chaisemartin (1967) who proposed that net calcium uptake was a passive process.

The effect of the external anion on calcium net uptake

The effect of different external anions on the rate of net calcium uptake by postmoult crayfish was examined. The normal artificial tap water (ATW) used as an experimental medium contained chlorides and bicarbonates of sodium, potassium, calcium and magnesium (Greenaway, 1972). In the experiments described below two additional, artificial media were used having either chloride (Cl ATW) or sulphate (SO_4 ATW)

Table 6. *The effect of the addition of bicarbonate on the rate of net uptake of calcium from Cl ATW*

Animal no.	Rate of net uptake of Ca (μmoles/g/h)			
	ATW	Cl ATW	+$NaHCO_3$	ATW
15	1·56	1·04	1·56	2·19
16	0·54	0·34	0·68	0·63
19	1·60	0·54	0·99	2·36
14	0·96	0·50	2·00	1·16
78	2·56	1·05	2·02	—
96	0·76	0	0·63	—
Mean	1·33	0·58	0·98	1·58

as the sole anion. Cationic composition remained unchanged. All experimental solutions were buffered with 2 mM/l Tris (hydroxymethyl) methylamine. This concentration of Tris had no effect on the rate of net uptake of calcium, and pH was generally maintained at 7·0–7·3. In each experiment the rate of net calcium uptake was measured in ATW, then in an experimental solution and finally in ATW again. In Tables 4 and 5 data for the net uptake of calcium from ATW, Cl ATW and SO_4 ATW are compared. In each of the latter two solutions net uptake was decreased by approximately 60%. The large variability between individual net uptake rates was due to the use of crayfish at different postmoult stages, rates being high in animals at stages A and B and lower at stage C.

The above data suggest that net uptake was reduced in the absence of bicarbonate, and a second experiment was carried out to investigate this possibility. As before, the normal rate of net uptake was established for a number of animals. These were then transferred to Cl ATW and the new rate of net uptake of calcium recorded. Bicarbonate, as $NaHCO_3$, was then added to the Cl ATW in amounts sufficient to raise the bicarbonate concentration to 0·5 mM/l, and the rate of net uptake remeasured. Results are presented in Table 6. The addition of bicarbonate enhanced net uptake of calcium, suggesting that the low rates of uptake measured in Cl ATW and SO_4 ATW were due to the virtual absence of bicarbonate in these solutions. This possibility is discussed below.

DISCUSSION

The pattern of calcium metabolism in the postmoult crayfish may now be summarized. Net uptake of calcium begins 15–30 minutes after the moult and immediately reaches a very high rate. During stages A and B the net uptake of calcium is maximal (approximately 2 μmoles/g/h), but falls sharply between stages B_2 and C_1. It is interesting to note that the maximal rate of net calcium transport is more than twice that given by Shaw (1959) for sodium. In addition, the affinity of the net uptake mechanism for calcium is greater than that for sodium. Net uptake of calcium continues throughout the remaining postmoult stages, C_{1-3}, at a slowly declining rate until equilibrium is reached at C_4 when calcification is complete. Calcium absorption occurs via the gills (Chaisemartin, 1965) and has been shown to be an active process over the normal range of calcium concentration found in fresh water

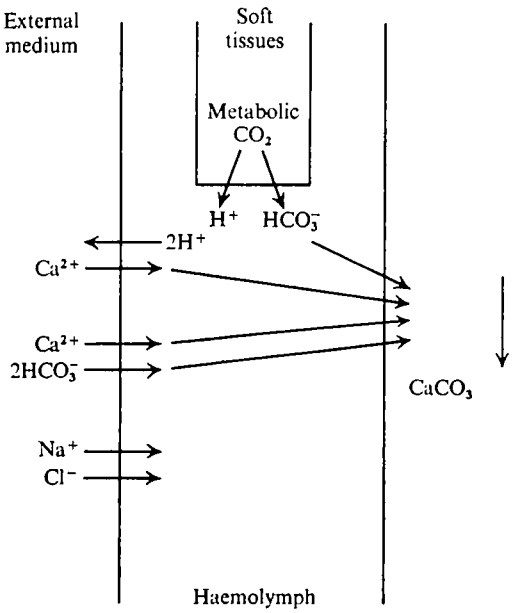

Fig. 5. Diagram to illustrate the proposed calcium movements in crayfish at the postmoult stage.

The level of ionized calcium in the haemolymph of *Austropotamobius* remains constant throughout the intermoult cycle (Greenaway, 1972, 1974*b*, present study).

The absence of bicarbonate from the external medium has been shown to reduce net uptake of calcium by postmoult crayfish by approximately 60%. It seems possible, therefore, that 60% of net calcium uptake was accompanied by bicarbonate, and that in the absence of bicarbonate calcification and consequently calcium net uptake were reduced. However, as net calcium uptake continued at 40% of the normal level even in the absence of external bicarbonate, an alternative source of bicarbonate is indicated, presumably metabolic CO_2. To test this hypothesis the rate of bicarbonate production by resting crayfish was calculated from data supplied by Sutcliffe (personal communication). Maximum bicarbonate production at 9°C was 3·75–5·0 μ-equiv./g/h but in practice availability will be lower than this as both CO_2 and HCO_3^- are rapidly lost across the gills. As the mean maximum rate of calcium net uptake (= calcification) was approximately 4·0 μ-equiv./g/h at 10°C, metabolic CO_2 could theoretically satisfy the entire bicarbonate requirement, but in practice will supply rather less. This fits well with the 40% contribution suggested above. The picture in *Austropotamobius* thus appears to be similar to that in molluscs, where both metabolic and environmental bicarbonate contribute to carbonate formation during calcification (Wilbur, 1972).

To maintain electrical balance in the haemolymph calcium ions absorbed must either be accompanied by an anion or exchanged for another cation. From the above evidence it is clear that calcium is absorbed both with and without bicarbonate. Thus calcium absorption after the moult must utilize both mechanisms; uptake together with bicarbonate and exchange for another cation (presumably H^+). This seems reasonable the bicarbonate ions of metabolic origin utilized in calcification are balanced by

production of a similar amount of H^+. Exchange of these surplus H^+ for calcium i[illegible] and the combination of calcium and bicarbonate ions to form $CaCO_3$ would maintain electrical neutrality in the haemolymph. Sodium and chloride ions are presumably absorbed simultaneously in postmoult crayfish, to restore their concentration in the haemolymph reduced by water uptake at the moult. These suggestions are summarized in Fig. 5.

No attempt has been made in this study to investigate the mechanism of calcification. It is worth noting, however, that any theory concerning the mechanism of calcification in crustacea must fit the pattern of calcium metabolism shown in this series of papers. Thus, with the electrode theory of calcification (Digby, 1972) for example, it is necessary to explain why the new exoskeleton is not calcified before the moult and how the rate of calcification is matched to the observed rate of net uptake of calcium (Fig. 1).

One of the most striking features of calcium metabolism in *Austropotamobius* is the abruptness with which a high rate of net loss of calcium at D_4 is replaced by an even greater rate of net uptake after the moult. The switch from net loss to net uptake is presumably triggered by events taking place at, or shortly after, the moult, as net uptake begins very rapidly after completion of ecdysis. The trigger mechanism may be involved with the actual shedding of the old exoskeleton or the fall in osmotic pressure of the haemolymph due to water absorption (although this begins before the moult). Whatever the nature of the trigger mechanism it seems likely that it may act via a hormone which switches net calcium transport from the outward secretion suggested in premoult (Greenaway, 1974*b*) to an inward secretion.

The calcium requirements of aquatic crustacea are very high, as the exoskeleton has to be calcified not once but many times during the course of the life cycle. In addition, each period of calcification is of necessity rather short as the exoskeleton must be hardened as quickly as possible. This contrasts with the situation in molluscs where the shell is only formed once and the effort is spread over the whole of the adult life (although mainly in the young animal; Zischke, Watabe & Wilbur, 1970). In view of the urgency and magnitude of the calcium requirement in freshwater crustacea it is interesting to see how they cope with the problem. In *Austropotamobius* the rate of net calcium transport is very high and the uptake mechanism is saturated at low external calcium concentrations (0·4 mM/l). There is evidence that crayfish from soft waters may have an uptake mechanism with a slightly higher affinity than found in the present study (Chaisemartin, 1967), a situation found in *G. pulex* (Vincent, 1963, 1969). In *Echinogammarus berilloni* low water temperature as well as calcium concentration limits distribution (Vincent, 1972). The minimum calcium concentration for successful completion of calcification by *Austropotamobius* is not known, but French populations are known from water containing only 0·135 mM-Ca/l (Chaisemartin, 1967) and D. W. Sutcliffe (personal communication) reports that crayfish from hard Lake District streams had difficulty in completing the moult when kept in Lake Windermere water (0·16 mM-Ca/l). In marine environments calcium is available at much higher concentrations than in fresh water, but no information is available concerning the calcium affinity in marine crustacea apart from an observation by Robertson (1937) that net uptake by postmoult *Carcinus* was similar in both full-strength and 50% sea water. It would be interesting to examine the dynamics [illegible]

ium transport in a euryhaline crustacean such as *Gammarus duebeni* which can complete its life cycle in fresh water (Sutcliffe, 1970) and sea water.

I wish to thank Professor J. Shaw for his continuing interest in this work.

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