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CALCIUM REGULATION IN THE FRESHWATER MOLLUSC, *LIMNAEA STAGNALIS* (L.) (GASTROPODA: PULMONATA)

I. THE EFFECT OF INTERNAL AND EXTERNAL CALCIUM CONCENTRATION

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INTRODUCTION

It has been established that aquatic molluscs can fulfil their calcium requirements by absorption of calcium from the external medium (Schoffeniels, 1951; Jodrey 1953; Kado, 1960; van der Borght, 1962, 1963) although calcium present in the food is also utilized (van der Borght & van Puymbroeck, 1966). Little is known, however, concerning the nature of the calcium absorption process in either marine or freshwater molluscs. Van der Borght (1962) found that the absorption of calcium by *Limnaea stagnalis* was reduced in external solutions containing less than 0·25 mM Ca/l and that calcium equilibrium was reached in media containing 0·05 mM Ca/l. Van der Borght and van Puymbroeck (1964) claim that calcium is absorbed actively from the medium in *L. stagnalis*, *L. auricularia* and *Planorbis corneus*. Data for the urinary calcium loss is available for *Viviparus viviparus* (Little, 1965*b*) and *Margaritana margaritifera* (Chaisemartin, 1968).

Calcium uptake has been studied in aquatic crustacea but again little information is available concerning the dynamics of calcium transport. Calcium uptake in freshwater crustacea is cyclical, reaching a maximum immediately after the moult and falling to a low level in intermoult stages (Vincent, 1963; Bernard & Chaisemartin, 1965). This also appears true of marine crustacea (Robertson, 1937, 1960). Net uptake of calcium by *Astacus pallipes* is possible from external concentrations greater than 0·09 mM Ca/l and reaches a near maximum in media containing more than 0·2 mM Ca/l (Bernard & Chaisemartin, 1965; Chaisemartin, 1965). In *Gammarus pulex pulex* net calcium upake is possible from 0·025 mM Ca/l (Vincent, 1969). Depletion of calcium in *Astacus* results in an increased rate of calcium influx (Bernard & Chaisemartin, 1965) but calcium transport is not apparently against an electrochemical gradient (Chaisemartin, 1966). Robertson (1960) suggests that uptake of calcium by *Carcinus* is an active process under certain conditions but measurement of potential differences and of activities in blood and seawater are required to substantiate this.

In the present investigation the calcium fluxes and net movements between the blood of *L. stagnalis* and the external medium have been studied with the aid of the radioisotope ^{45}Ca. The effects of low external calcium concentrations on these fluxes and on tissue calcium concentration have also been considered. Potential difference

measurements between the blood and external calcium-containing solutions have been made in conjunction with activity measurements to determine whether or not calcium uptake is an active process against an electrochemical gradient.

MATERIALS AND METHODS

Materials and methods, apart from those described below were as detailed previously (Greenaway, 1970). The shells of experimental snails were coated with Paraplast wax in order to prevent loss of calcium from the outer surface of the shell interfering with the measurement of net calcium movements between blood and external medium.

Calcium concentrations of appropriately diluted blood, tissue and medium samples were measured with a Unicam SP 900 flame spectrophotometer operated at a wavelength of 622 mμ. This wavelength was selected from the other peaks in the calcium emission spectrum as it combined high sensitivity to calcium with low interference effects from the other ions present in the solutions. Interference from other cations was negligible when measuring diluted blood samples and artificial tap-water solutions containing more than 0·1 mM Ca/l. When samples of medium contained less, one or more of the other cations present caused enhancement of the calcium readings. To correct for this, the interfering ions (Na^+, K^+, Mg^{2+}) were added to the calcium standards in the concentrations in which they were present in the samples.

For measurement of calcium influx groups of snails, adapted to artificial tap water containing 1·0 mM Ca/l, were placed in ^{45}Ca-labelled artificial tap-water solutions. Calcium influx was calculated from the decline of radioactivity of the external medium by the method of Shaw (1959). Conditions during influx measurements were such that the total internal exchangeable calcium exceeded the amount of calcium in the external medium thus ensuring that errors due to back-diffusion of tracer were negligible. Influx measurements were made over periods of up to 10 h during which time the logarithm of the activity of the medium declined in a linear fashion (Fig. 1). This indicates that *L. stagnalis* was behaving as a single compartment with respect to calcium over this period.

Snails used in experiments on calcium efflux were loaded with ^{45}Ca in labelled artificial tap water (0·5 mM Ca/l) for several weeks prior to the experiment. The snails were then washed briefly in distilled water and divided into several groups. Blood samples were taken from one group in order to measure the specific activity of the blood. The other groups were placed in non-radioactive media and the efflux of ^{45}Ca was followed by measuring the radioactivity of the external medium. For this purpose 50 or 100 μl samples of the medium were dried on planchettes and counted on an I.D.L. low-background counter. Calcium efflux was calculated from the initial specific activity of the blood and the amount of radioactivity appearing in the medium.

Measurements of calcium activity were made using an Orion calcium-ion activity electrode in conjunction with a saturated KCl calomel reference electrode and Orion model 401 specific ion meter. The calcium electrode was calibrated with $CaCl_2$ activity standards buffered to pH 7 with KOH. Considerable difficulty was experienced when measuring the calcium activity of artificial tap water solutions as the high concentration of bicarbonate ion in these media caused prolonged drifting of readings not encountered with solutions containing little or no bicarbonate. The change

of scale reading when the electrode was placed in artificial tap water was separable into two components. For the first 1½ min. the change was rapid but then showed a slow drift lasting for the next 10–15 min. This slow drift component appeared to be an interference effect as on return to the $CaCl_2$ activity standard an incorrect value was obtained, although after about 30 min the reading for the standard had returned to the expected level. If the electrode was left in artificial tap water only for the duration of the period of rapid change the correct activity value for the calcium standard was rapidly attained on replacement of the electrode in the standard solution. In view of

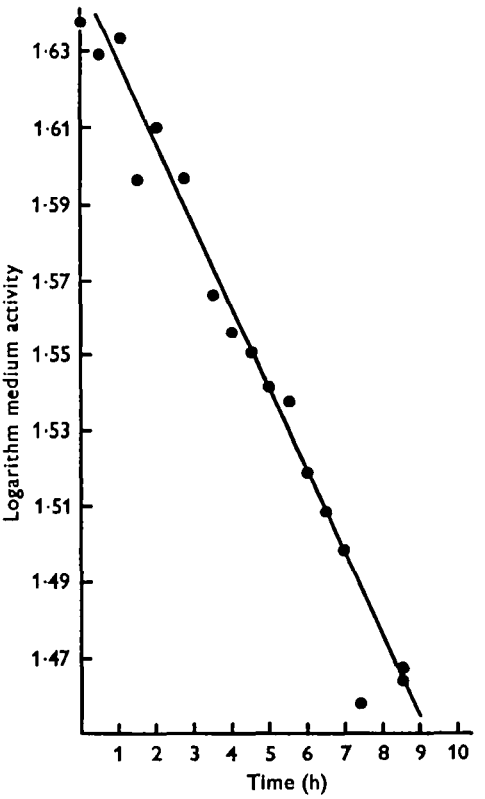

Fig. 1. The time course of the fall in radioactivity of the external medium during calcium influx from artificial tap water containing 1·0 mM Ca/l.

this, readings of calcium activity for artificial tap-water solutions were made 1½ min after immersion of the electrode in these media. No difficulty was experienced in the measurement of calcium activity in the blood. A snail was washed in distilled water, blotted dry with filter paper and caused to expel blood from the haemal pore (Greenaway, 1970). The blood was collected in an Orion microsample dish and a 5 μl sample was removed and diluted suitably for measurement of calcium concentration. The activity of the blood remaining in the microsample dish was then measured in an empirical manner using $CaCl_2$ standards containing sodium and potassium (as chlorides) in the concentrations at which they occur in the blood. No drift was encountered whilst making blood activity measurements.

RESULTS

Calcium influx and net uptake

Individual snails taken from the aquarium and placed in artificial tap water (1 mM Ca/l) generally showed a sustained net uptake of calcium although the rate of uptake by different snails, or by the same snail examined at different times, was extremely variable. Fig. 2 shows the decline in calcium concentration of 100 ml. of artificial tap water effected by single snails. Van der Borght (1962) demonstrated that *L. stagnalis* normally shows a net uptake of calcium from media containing more than 2 p.p.m. calcium (0·05 mM Ca/l). In the present work it was observed that growing snails, which were laying down new shell, showed rather more uniformity in the rate of calcium uptake than either starved snails or snails not obviously laying down new shell. Wagge (1951) has demonstrated that both protein and calcium are necessary dietary requirements for the formation of calcium deposits in *Helix aspersa*. It appears likely, therefore, that starvation in *L. stagnalis* might depress the rate of shell growth and hence affect the rate of calcium uptake. In view of these facts snails showing signs of recent shell growth were selected for measurements of influx and net uptake and were fed until just prior to the actual experiment.

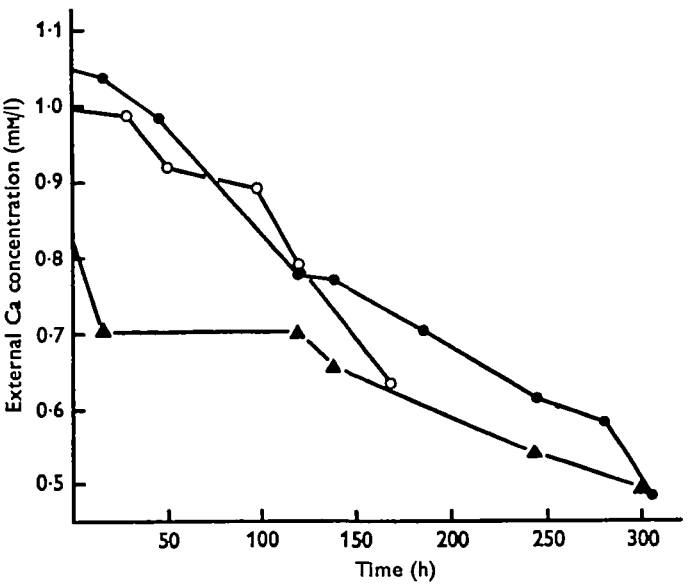

Fig. 2. The decline in calcium concentration of the external medium caused by net calcium uptake by *L. stagnalis*. Data is presented for three snails each in 100 ml of artificial tap water.

Measurements of influx and net uptake of calcium, from external solutions containing 0–3·0 mM Ca/l, have been made. The results are shown in Fig. 3. Influx and net uptake are related to the external calcium concentration in a non-linear manner, the uptake mechanism being half-saturated in 0·3 mM Ca/l and approaching saturation in external calcium concentrations greater than 1·0–1·5 mM Ca/l. The relationship between external calcium concentration and calcium influx may be described approxi-

mately by the Michaelis–Menten equation. A similar situation exists regarding sodium uptake in *L. stagnalis* (Greenaway, 1970). Van der Borght (1962) stated that the rate of calcium net uptake by *L. stagnalis* from external solutions containing more than 10 p.p.m. calcium (0·25 mM Ca/l) was fairly constant. Recalculation of his data, however, reveals that calcium net uptake is related to the external calcium concentration in a manner similar to that described in the present work. Furthermore, half maximum and maximum net uptake values occurred at external calcium concentrations of 0·35 and 1·5 mM/l respectively, very close to the values found here.

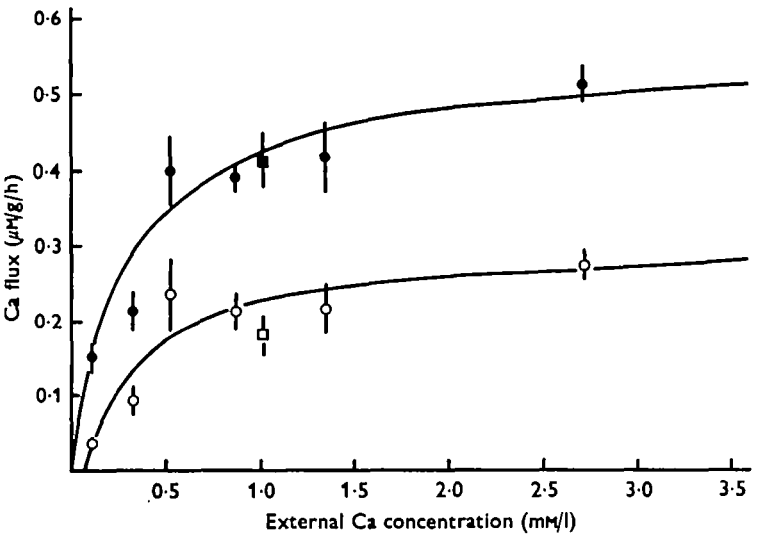

Fig. 3. The relationship between calcium influx and net uptake and the external calcium concentration. Each point represents the mean of at least five measurements on groups of snails adapted to artificial tap water (1·0 mM Ca/l). ●, Mean values for calcium influx; ○, mean values for net uptake of calcium; ■, mean calcium influx into depleted snails; □, mean net uptake of calcium by depleted snails. Vertical lines indicate standard errors.

Calcium activity in the blood and artificial tap water

The calcium activity of artificial tap-water solutions was consistently lower (30–40%) than the corresponding activity values calculated from the Debye–Huckel theory for solutions of ionic strength less than 0·1 M/l. Measurement of calcium activity of artificial tap-water solutions, in which chloride was substituted for bicarbonate, gave values close to the calculated activities. The bicarbonate in artificial tap water, therefore, reduced the expected calcium activity in the experimental media. Thomson & Ross (1966) found that the activity of calcium in sea water was lower than expected and attributed the difference between measured and calculated values to formation of complexes and ion pairs. Nakayama (1968) discovered that in saturated $CaCO_3$ solutions under partial pressure conditions resembling those of atmospheric CO_2 about 80% of the total calcium in solution was ionized. The remaining 20% was made up of $CaCO_3$ and $CaHCO_3^+$. It appears likely therefore, that the observed lowering of calcium activity in artificial tap-water solutions was due to the formation of complexes and ion pairs involving bicarbonate.

The calcium activity of the blood of normal snails is given in Table 1. The mean measured activity value was lower than the calculated value (1·765 mM/l) by almost 30%. This discrepancy could be explained by the presence in the blood of calcium 'bound' to blood proteins. Alternatively a portion of the calcium may form complexes with bicarbonate or organic anions in the blood. Van der Borght & van Puymbroeck (1964) found 0–6% of blood calcium in *L. stagnalis* to be bound. This is insufficient

Table 1. *The activity of calcium in the blood of* Limnaea stagnalis

Ca concentration (mM/l)	Ca activity (mM/l)
5·60	1·278
3·82	1·260
3·72	1·260
4·26	1·332
4·76	1·260
5·12	1·278
3·68	1·250
4·96	1·250
3·34	1·232
4·78	1·332
Mean 4·40 ± 0·24 S.E.	1·273 + 0·011 S.E.

to account for the observed discrepancy between measured and calculated activity values and a large proportion of blood calcium must be complexed. The bicarbonate concentration of the blood is high, Chaisemartin, Mouzat & Sourie (1967) giving a value of 15·8 mM/l, and this may be expected to reduce calcium activity in the blood by the formation of complexes and ion pairs. In addition Greenaway (1970) estimated the blood of *L. stagnalis* to contain about 10 m-equiv./l of organic anions. Small organic molecules may well form complexes with calcium yet still pass through dialysis tubing and thus avoid detection by the normal technique used to measure 'bound' calcium in invertebrate blood.

Table 2. *Measurements of potential difference between the blood and the external medium*

Ext. Ca conc. (mM/l)	Ext. Ca activity (mM/l)	Measured P.D. (mV) ± S.E.	Equil. potential (mV)
0·054	0·025	−38·5 ± 0·91(16)	−49·5
0·107	0·053	−32·4 ± 1·09(17)	−40·0
0·255	0·132	−24·5 ± 0·99(16)	−28·5
0·530	0·280	−19·1 ± 0·78(17)	−19·1
0·913	0·475	−13·5 ± 0·90(17)	−12·4
1·90	0·96	−5·2 ± 0·70(20)	−3·6
2·71	1·31	−1·4 ± 0·63(13)	+0·36

Brackets indicate no. of observations.

Potential difference

The potential difference between the blood of *L. stagnalis* and the external medium has been measured. The blood was negatively charged with respect to the medium and the size of the potential difference between the two increased with decreasing concentration in the medium (Table 2). The activity of calcium ion in the blood and external

medium was measured and used with the Nernst equation, $E = (RT/ZF) \ln (a_i/a_0)$ where a_i and a_0 are the measured internal and external calcium activities, to calculate the equilibrium potential at each external calcium concentration. Values are shown in Table 2. Fig. 4 shows the relationship between the equilibrium potential and the logarithm of the external calcium activity, together with the measured potentials. It can be seen that at external activities above 0·28 mM Ca/l there is a good agreement between observed and calculated equilibrium potentials. Below this a significant departure is apparent. The snails behave in artificial tap water with a calcium activity above 0·28 mM/l in a manner approximating to that of a calcium electrode, indicating that the surface epithelium must be highly and selectively permeable to calcium ions. Under such conditions an electrical potential would be set up across the body wall

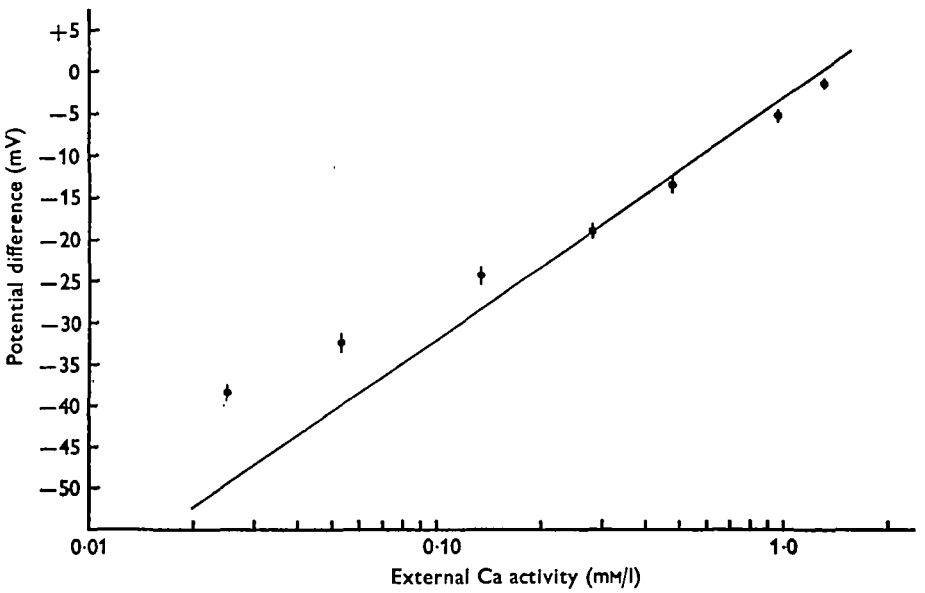

Fig. 4. A comparison between the mean potential difference, measured between the blood and the external medium, and the calculated equilibrium potential for calcium over a range of external calcium activity. ●, Mean values of the measured potential difference. Vertical lines represent standard errors. The unbroken line represents the calculated equilibrium potential for calcium over the range of external calcium activity studied.

as calcium ions would tend to diffuse out of the animal but be prevented by the impermeability of the epithelium to anions. The magnitude of this potential difference would be dependent on the external calcium activity (assuming the calcium activity of the blood to be constant). The body surface could well be dynamically impermeable to anions rather than impermeable in an absolute sense. At lower external calcium activities the apparent selective permeability to calcium is less marked, possibly due to permeability to hydrogen ions which tend to stabilize the potential as the external calcium activity is reduced. This would appear to be the simplest explanation of the data (Fig. 4) but other explanations are also possible.

The importance of the potential measurements in relation to the uptake of calcium may now be considered. If active uptake is defined as movement against an existing electrochemical gradient, then the net uptake of calcium from external media contain-

ing less than 0·5 mM Ca/l must be an active process. In concentrations greater than 0·5 mM Ca/l, however, net uptake of calcium occurs in the absence of an electrochemical gradient. Nor is there a favourable gradient to provide the driving force for a passive entry large enough to explain the measured net uptake. It is, therefore, necessary to postulate the presence of an inwardly transporting calcium mechanism which is active at low calcium concentrations, but which at concentrations above 0·5 mM-Ca/l moves the ions with virtually no free energy change and, hence, with minimal energy requirements. Van der Borght & van Puymbroeck (1964) measured the potential difference between the blood of *L. stagnalis* and a solution containing 0·25 mM $CaCl_2$/l. Their value (−15 mV) was lower than the calculated equilibrium potential (−37·3 mV) and consequently they suggested that an active uptake of calcium must occur to maintain normal blood concentration. Their calculations, however, were based on the ratio of internal and external calcium concentrations which they considered equal to the calcium activity ratio. This assumption is invalid and introduces a considerable error into the calculation. Recalculation of the equilibrium potential using the calculated activity value for 0·25 mM $CaCl_2$/l (= 0·22 mM/l) and the measured value for calcium activity in the blood (Table 1) gives a potential of −22·2 mV. Calcium uptake at this external activity, therefore, was against an electrochemical gradient slightly greater than found in this investigation. Kirschner, Sorenson & Kriebel (1960) measured the potential difference across the isolated mantle of a freshwater clam and found that the size and sign of the potential obtained was related to the calcium concentration of the bathing Ringer's solution. Kirschner (1963) found that this potential difference across the isolated mantle was made up of a small potential difference between the bathing solution on the mantle cavity side and the mantle interior, and a much larger potential difference between the mantle interior and the bathing solution on the shell side of the mantle. No evidence for active calcium transport was obtained using this preparation.

Calcium loss

The mean rates of calcium loss from individual snails adapted to a range of calcium concentrations between 0·065 and 1 mM Ca/l are shown in Table 3. The value for calcium loss rate for snails adapted to 0·065 mM Ca/l has been taken as equal to calcium influx at the same concentration, any exchange component of calcium influx at this concentration being negligible. It appears from these loss data that little if any significant reduction in the calcium loss rate occurred when snails were adapted to low external calcium concentrations. The urinary and passive components of calcium loss have not been separated but an estimate of the possible rate of urinary calcium loss can be obtained from data on other species. The urine calcium concentration of *Viviparus viviparus* was 1·5 mM Ca/l and the flow rate 15–55 μl/g/h (Little, 1965*b*) giving a urinary loss rate of 0·023–0·082 μM Ca/g/h at 19 °C. Similar data for the bivalve *Margaritana margaritifera* sets calcium loss in the urine at about 0·04 μM-Ca/g/h at 12–15 °C (Chaisemartin, 1968). Data for the total calcium loss by *Viviparus* and *Margaritana* are not available and hence it is not possible to estimate that fraction of the total loss attributable to the urine. As both these animals have similar blood calcium concentrations to *L. stagnalis* it appears likely that a large fraction of the total calcium loss in *L. stagnalis* may be accounted for in the urine.

The sum of net uptake and loss rate for calcium in *L. stagnalis* is lower than the calcium influx at external calcium concentrations greater than 0·1 mM/l (Table 4, Fig. 3). This suggests that calcium influx over this range of external concentration is not a true measure of the actual uptake of calcium ions and an exchange component must, therefore, represent part of the calcium influx in external solutions containing more than 0·1 mM Ca/l. Below 0·1 mM Ca/l the exchange component appears to be

Table 3. *Calcium loss rates from snails adapted to the range of calcium concentration* 0·065–1·0 mM/l

Acclimatization conc. (mM/l)	Calcium loss rate (μM/g/h) ± S.E.
1·00	0·105 ± 0·010 (9)
0·50	0·096 ± 0·011 (14)
0·25	0·069 ± 0·006 (7)
0·10	0·102 ± 0·024 (4)
0·065	0·100 (Influx value)

Brackets indicate the number of observations.

Table 4. *The exchange component of calcium influx in snails adapted to artificial tap water* (1·0 mM Ca/l)

Ca loss rate	0·105
Ca influx	0·425
Ca net uptake	0·225
Net uptake + loss	0·330
Exchange component	0·095

Values as μM Ca/g/h.

less in evidence. With regard to the results given previously, the presence of an exchange component might be readily explained. If the highly selective permeability of the surface epithelium to calcium (deduced from the potential difference measurements) is true, then this would allow an exchange of labelled calcium in the medium with unlabelled calcium in the blood, at a rate proportional to the external calcium concentration. This process could occur independently of calcium movement through the proposed calcium transport system. If no potential difference changes occurred on altering the calcium concentration of the external medium one would expect a more or less linear relationship between the size of the exchange component and the external calcium concentration. Both calcium influx and net uptake appear to follow enzyme-saturation kinetics and it follows, therefore, that the exchange component will follow a similar pattern. As the measured potential differences increased with decreasing external calcium concentration this departure from linearity of the relationship between external calcium concentration and the size of the exchange component is to be expected. Clearly other alternatives, such as exchange diffusion or an exchange mechanism linked to the calcium transport system, could also explain either wholly or partially the observed exchange component. A passive exchange, however, remains the simplest hypothesis.

Calcium efflux

Ca efflux, from ^{45}Ca-loaded snails, has been measured to artificial tap water (1·0 mM Ca/l) and to calcium-free artificial tap water. Values are given in Table 5.

The efflux and net loss of calcium to calcium-free artificial tap water were not significantly different; calcium efflux, therefore, gives a reasonably accurate measure of the rate of net loss of calcium ions. Efflux to artificial tap water was more than three times that to calcium-free artificial tap water. Assuming that the rate of loss of calcium to the medium was similar in both cases, a large proportion (about 70%) of the efflux to artificial tap water (1·0 mM-Ca/l) must have been due to an exchange of ^{45}Ca in the blood for ^{40}Ca in the medium. This confirms the presence of an exchange component of the calcium flux described above. In these measurements the exchange component of calcium efflux (0·24 μM/g/h) was, in fact, somewhat higher than deduced from influx measurements made at the same external concentration (Table 4).

Table 5. *Calcium efflux from ^{45}Ca-loaded snails*

Ca efflux to artificial tap water (μM/g/h)	Ca efflux to calcium-free artificial tap water (μM/g/h)	Ca net loss to calcium-free artificial tap water (μM/g/h)
0·485	0·109	0·135
0·264	0·119	0·085
0·329	0·065	0·096
0·298	0·064	0·105
Mean 0·342 ± 0·038 S.E.	0·089 ± 0·014 S.E.	0·105 ± 0·012 S.E.

Table 6. *Some values of the minimum equilibrium concentration for calcium*

Ca concentration (mM/l)
0·036
0·031
0·095
0·075
0·065
0·052
0·085
0·050
Mean 0·062 ± 0·008 S.E.

Calcium-depleted snails

An attempt was made to measure the minimum equilibrium concentration of calcium for *L. stagnalis* using the technique described for sodium (Greenaway, 1970). The shells of the experimental snails were coated with wax to ensure that the equilibrium measured between the blood and outside medium was not affected by loss of calcium from the shell. Minimum balance concentrations of calcium were generally reached in either the first volume of calcium-free water or after the first change of medium. A small proportion of the snails examined failed to achieve calcium balance, showing a slow continuous net loss to the medium. The data for minimum equlibrium concentrations of calcium given in Table 6, therefore, excludes these snails. The mean value of 0·062 mM Ca/l agrees well with the value of 2 p.p.m. (0·05 mM/l) at which van der Borght (1962) found that *L. stagnalis* reached calcium balance with the medium. Little (1965*a*) measured the minimum equilibrium concentration of calcium for *Viviparus viviparus* and obtained a mean value of 0·2 mM Ca/l. No precautions were apparently taken to prevent loss of calcium from the shell, and the equilibrium

between tissues and medium alone may have been lower than this. *Astacus pallipes* shows a net calcium loss to media containing less than 0·09 mM Ca/l (Chaisemartin, 1965). Vincent (1963, 1969) has shown that *Gammarus pulex pulex* from hard-water streams can achieve a net gain of calcium from external calcium concentrations greater than 0·075 mM/l whilst animals from a soft-water stream showed a net gain from concentrations greater than 0·025 mM Ca/l.

The effect of calcium depletion on blood concentration

Snails were depleted of calcium individually in large volumes ot calcium-free artificial tap water. The medium was changed once daily and the loss of calcium from each snail recorded. After 2 weeks of depletion a blood sample was taken from each animal for calcium analysis, the snails were killed and their total calcium in fresh tissue was measured. The effects of calcium depletion on calcium concentrations in blood and tissues are shown in Table 7. Little change in calcium concentration of the

Table 7. *The effect of calcium depletion on concentrations of calcium in blood and tissues*

Estimated Ca in Total fresh tissues (μM)	Measured Ca loss (μM)	Ca in fresh tissues after depletion (μM)	Conc. of Ca in blood after depletion (mM/l)
31·5	29·5	34·0	2·1
35·7	28·2	30·0	5·2
26·5	20·0	37·0	5·1
43·8	49·0	18·5	4·5
55·5	52·6	38·0	5·3
51·0	77·5	99·5	6·0
25·0	60·6	30·3	9·4
Total 269·0	317·4	287·0	—
Mean 38·4 ± 4·5 S.E.	45·3 ± 7·7 S.E.	41 ± 10·0 S.E.	5·4 ± 0·8 S.E.

blood occurred despite the loss from the snails of greater amounts of calcium than the fresh tissues were estimated, from the normal calcium concentration in tissues (Greenaway, 1970), to contain. Similarly the total fresh-tissue calcium had not, apparently, been diminished. Calcium loss from the outside of the shell was prevented by coating the shell with wax. As both blood calcium and fresh-tissue calcium were normal after prolonged depletion, the calcium lost from the blood must have been derived from the shell. Little (1965*a*) depleted *Viviparus viviparus* of ions with de-ionized water and recorded a slight elevation of mean calcium concentration in the blood although two of the three snails examined had reduced calcium concentrations in their bloods.

The effect of calcium depletion on calcium uptake

Snails were depleted of calcium by treatment with calcium-free artificial tap water for 2 weeks. Groups of depleted snails were then placed in labelled artificial tap water (1 mM Ca/l) and calcium influx and net uptake were followed. Food was provided during depletion but in amounts insufficient to have a significant effect on calcium balance. Values for calcium influx and net uptake are presented in Table 8 and Fig. 3. Clearly, prolonged calcium depletion does not increase influx or net uptake of calcium

above the normal values. Krogh (1939) depleted *L. stagnalis*, and several other freshwater molluscs, of ions in distilled water for up to 1 month but could not detect a net uptake of calcium when they were placed in a 1·0 mM/l $CaCl_2$ solution. This result is difficult to explain, as normal fed animals show a net calcium uptake, and it may be due to the effects of prolonged starvation or treatment with distilled water. In *Astacus pallipes* calcium depletion increases the calcium influx (Bernard & Chaisemartin, 1965) and the same is apparently true of the marine shrimp *Metapenaeus* (Dall, 1965).

Table 8. *Values for influx and net uptake of calcium by calcium-depleted snails*

External conc. (mM Ca/l)	Influx (μM Ca/g/h)	Net uptake (μM Ca/g/h)
0·896	0·486	0·245
0·905	0·650	0·345
0·925	0·255	0·043
0·885	0·469	0·183
1·335	0·435	0·251
1·395	0·342	0·137
0·943	0·315	0·088
0·950	0·342	0·141
0·925	0·416	0·177
0·925	0·440	0·199
Mean 1·008	0·415 ± 0·035 S.E.	0·181 ± 0·027 S.E.

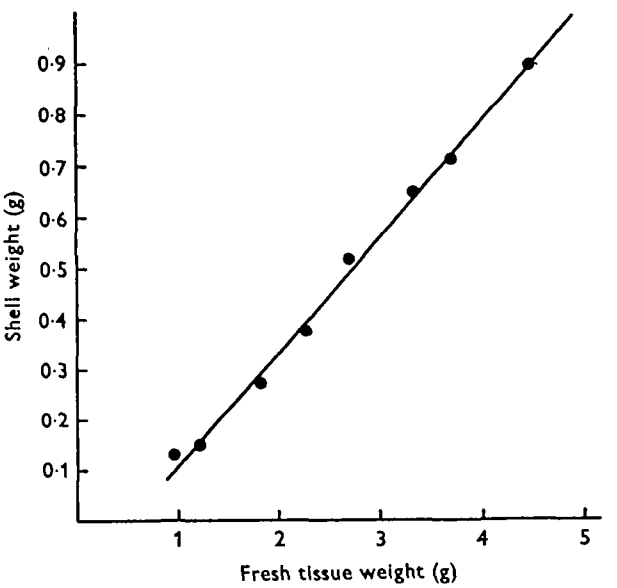

Fig. 5. The relative increase in weight of the shell and of total fresh tissues in *L. stagnalis*. Each point represents the mean for a large number of snails.

Long-term uptake of calcium

The weights of shell and of total fresh tissues have been measured in the normal size range of sexually mature snails (1–6 g total weight). The relationship between fresh tissue and shell weight is shown in Fig. 5. Over the range of size examined shell weight bears a linear relationship to fresh-tissue weight. This indicates that the rate of shell

growth and hence the rate of calcium deposition in the shell is proportional to the rate of weight increase of the fresh tissues. The rate of calcium deposition may be roughly equated with the rate of net calcium uptake from the medium.

DISCUSSION

Sodium regulation in *L. stagnalis* has been shown to conform, except in details, to the general pattern of sodium regulation of freshwater animals (Greenaway, 1970). Calcium regulation in this snail differs in several important aspects from sodium regulation, and a comparison between the two is therefore valuable. It has been demonstrated that sodium uptake is related to the external sodium concentration, the relationship being described approximately by the Michaelis–Menten equation (Greenaway, 1970). Calcium uptake over the range of concentrations examined shows similar characteristics, and both sodium transport and calcium transport are by high-affinity mechanisms. Although the two transport mechanisms are similar in this respect they clearly differ in others. The most clearly marked of these differences is the energy requirements of the two systems. Sodium uptake is against a considerable electrochemical gradient (Greenaway, 1970) whereas the gradient against calcium uptake is small at low external concentrations and absent or even slightly favourable at greater external concentrations. Calcium uptake, therefore, has a comparatively low energy requirement and this may represent an important economy as regards energy usage, the calcium throughput being large. It is not clear at present whether or not calcium uptake in other freshwater molluscs and in aquatic crustacea is an active process. This subject is discussed further below. It should be noted that the normal snail is in sodium balance with the medium (Greenaway, 1970) but not in calcium balance, generally showing a net calcium uptake from the medium. At least in the snails used in these experiments, calcium net uptake from the medium appears to be related on a long-term basis to the rate of growth of the fresh tissues. This may be a result of the fairly constant conditions under which the animals were reared in the laboratory. Certainly oysters continue to absorb calcium from sea water at a high rate even when not feeding and in a state of semihibernation (Galtsoff, 1934). Net movement of calcium from blood to shell (van der Borght, 1962) and from shell to blood effectively buffer against changes of calcium concentrations in the blood under conditions of net uptake and loss of calcium, respectively. Net uptake or loss of sodium, however, has a marked effect on the sodium concentration in the blood, no tissue stores being present. Depletion of blood sodium in *L. stagnalis* increased the rate of sodium influx two to three times. A similar increase has been observed in sodium-depleted *L. limosa* (Chaisemartin, 1970). No significant difference was found in this study between influx and net uptake of calcium in normal and in calcium-depleted *L. stagnalis*. It is possible that calcium loss from the blood goes undetected by the snail as no change in calcium concentration of the blood occurs. Certainly, lost calcium could rapidly be regained by a depleted snail, under favourable conditions, at little cost in terms of energy usage and with no increase in the rate of calcium transport. The rate of calcium loss from normal snails is similar to the sodium loss rate although an 11-fold concentration difference exists between sodium and calcium levels in the blood, the sodium concentration gradient between blood and medium, therefore, being much greater than for calcium. *L. stagnalis* must

be considerably more permeable to calcium than to sodium. This is supported by the potential difference measurements reported earlier from which it appears that *L. stagnalis* is selectively permeable to calcium ions, indeed behaving almost like a calcium electrode when the external calcium concentration is altered. No such behaviour was found with respect to sodium when the external sodium concentration was changed (Greenaway, 1970). Alternatively if the loss was mainly urinary, then much greater resorption of sodium than of calcium must occur.

It has been shown in the present study that calcium uptake by *L. stagnalis* requires minimal energy expenditure except at low external concentrations. At higher external concentrations uptake is not against an electrochemical gradient and may even be assisted by a small favourable gradient. A 'level transport' mechanism has been proposed under the latter conditions. Little data is available concerning calcium transport in other aquatic invertebrates. Chaisemartin (1966) found calcium uptake by *Austropotamobius* (=*Astacus*) *pallipes* to be a passive process but further evidence on this point is required. Active uptake of calcium by *Carcinus* (Robertson, 1960) and *Metapenaeus* (Dall, 1965) has been suggested on the basis of the difference in concentration of calcium in the blood and external medium. In the absence of measurements of calcium activity and of potential difference, however, active uptake of calcium by these animals remains unproven.

Boycott (1936) classed *L. stagnalis* as a calciphile species requiring a minimum of 0·5 mM Ca/l in the water. He did, however, record the species from a few localities having concentrations as low as 0·125 mM Ca/l. Similar conclusions regarding the calcium requirements of *L. stagnalis* in this country have been reached by Macan (1950, 1963). It is interesting to find, therefore, that calcium uptake is a process with low energy requirements at external concentrations greater than 0·5 mM Ca/l. Net calcium uptake by the snail is possible from concentrations greater than 0·062 mM Ca/l, however, although the energy requirements at such concentrations are greater. In Scandinavia *L. stagnalis* is found in lakes having calcium concentrations approaching the minimum equilibrium concentration found in this investigation (Hubendick, 1947; Macan, 1963). *L. stagnalis* can efficiently extract calcium from its food and under experimental conditions about 20% of the normal calcium intake of snails fed on lettuce was derived from this source (van der Borght & van Puymbroeck, 1966). It seems possible, therefore, that the Scandinavian strain of *L. stagnalis* might extract sufficient calcium from its food and from the water to provide a serviceable shell even in such low concentrations of calcium. The possibility remains, however, that the Scandinavian snails may be a separate physiological race with a calcium-uptake mechanism of higher affinity for calcium at low external concentrations than the British strain.

SUMMARY

1. Calcium regulation in normal and calcium-depleted snails has been investigated.

2. *L. stagnalis* has an uptake mechanism with a high affinity for calcium ions and shows a positive calcium balance in media containing more than 0·062 mM Ca/l.

3. Influx and net uptake of calcium are related to external calcium concentration in a non-linear manner. The uptake mechanism is half-saturated and near-saturated in external media containing 0·3 and 1·0–1·5 mM Ca/l respectively.

4. Calcium uptake from external concentrations of less than 0·5 mM Ca/l is against a small electrochemical gradient whereas from external concentrations greater than 0·5 mM Ca/l there is no adverse gradient.

5. Calcium depletion does not significantly alter the normal influx or net uptake rate of calcium from 1·0 mM Ca/l.

6. The calcium concentration in the blood remains constant during net uptake from, and net loss to, the medium.

7. A comparison is made between the mechanisms of sodium regulation and calcium regulation in *L. stagnalis*.

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