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

II. CALCIUM MOVEMENTS BETWEEN INTERNAL CALCIUM COMPARTMENTS

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INTRODUCTION

The existence of calcium deposits in the tissues of freshwater molluscs is well established (Numanoi, 1939; Carriker, 1946; Carriker & Bilstad, 1946; Little, 1965*a*; Kapur & Gibson, 1968*a*, *b*). Greenaway (1970) gave values for the Ca content of the fresh tissues of *Limnaea stagnalis*. In a histological investigation of the alimentary canal of *L. stagnalis appressa* Carriker & Bilstad (1946) described cells containing calcium granules in the digestive gland. Although appreciable amounts of calcium may be present in the fresh tissues, the shell of freshwater molluscs contains a very much larger amount and may be regarded as the major calcium-containing compartment. The blood forms a third calcium compartment, containing a relatively small amount of calcium the majority of which is in diffusible form and the remainder bound to blood proteins (Schoffeniels, 1951*c*; van der Borght & van Puymbroeck, 1964; Little, 1965*a*, *b*). There is evidence to suggest that movement of calcium occurs between the three calcium compartments described above (blood, fresh tissue deposits and shell). Calcium absorbed from the medium appears in the blood, fresh tissues and shells of certain freshwater molluscs (Schoffeniels, 1951*a*; Horiguchi *et al.* 1954; van der Borght, 1963; van der Borght & van Puymbroeck, 1967). Furthermore, Schoffeniels (1951*b*) found that radiocalcium injected into the blood of *Anodonta* appeared in the shell and fresh tissues, suggesting the movement of calcium from the blood to the other two calcium compartments. In support of this Horiguchi (1958) claimed that calcium absorbed by the freshwater bivalve *Hyriopsis schlegeli* was transferred from the blood to the shell and fresh tissues. The reverse movement of calcium from the shell to the blood has been observed in several marine bivalves (Dugal, 1939; Collip, 1921) and in *Anodonta* (Dotterweich & Elssner, 1935), calcium from the shell acting to buffer the blood against pH changes caused by carbon dioxide build-up. Greenaway (1971) has provided evidence indicating movement of calcium in both directions between blood and shell in *L. stagnalis*. Wagge (1952) obtained evidence for the transfer of calcium from shell to tissues in the terrestrial pulmonate *Helix aspersa*. Calcium movements between internal calcium compartments also occur in aquatic crustacea. Dall (1965) demonstrated that calcium absorbed from sea water by the shrimp *Metapenaeus* became distributed throughout the fresh tissues but was mainly concentrated in the exoskeleton. The reverse movement from exoskeleton to blood occurred when *Metapenaeus* was placed in calcium-free sea water. Similarly, in *Astacus pallipes* a

movement of calcium from exoskeleton to blood occurred during depletion in calcium-free water (Chaisemartin, 1965).

In this investigation measurements of the calcium content of the blood, of certain other fresh tissues and of the shell of *L. stagnalis* have been made to ascertain the relative magnitude of these calcium compartments. In addition ^{45}Ca has been used to examine the fate of calcium absorbed from the medium by *Limnaea* and to investigate the movements of calcium between the internal calcium compartments described above.

MATERIALS AND METHODS

Materials and methods used in this investigation, apart from those detailed below, were as described previously (Greenaway, 1970, 1971). All snails used in this series of experiments were of 1–2 g total weight.

In experiments involving measurements of specific activity the following procedure was used. In experiments on uptake of ^{45}Ca snails were removed at intervals from their experimental media containing radiocalcium and washed in distilled water. Two 5 μl blood samples were removed from each snail and one diluted for calcium determination. The other sample was diluted appropriately on a planchet for radioactive assay. Each snail was then removed from its shell and the mantle and digestive gland were dissected off; each part was placed in a silica crucible and dried at 100 °C for 24 h. The remaining fresh tissues plus washings from the shell interior and from dissecting instruments were treated similarly. The dry tissues were then ashed, dissolved in HCl and diluted to suitable volumes for determinations of calcium and radioactivity. Shells were dissolved in HCl and diluted with distilled water for analysis. One-ml volumes of the tissue samples plus a standard amount of 1 M dextrose, as a spreading medium, were dried on planchets and counted with an I.D.L. low-background counter.

The specific activity of blood calcium was also followed during ^{45}Ca efflux, from ^{45}Ca-loaded snails, to non-radioactive media. Snails were loaded for 2 weeks in ^{45}Ca-labelled artificial tap water containing 0·5 mM-Ca. After loading, the animals were divided into several groups and from one of these groups blood samples were taken for measurements of specific activity. The other groups were placed in unlabelled media for measurements of ^{45}Ca efflux. After 2–3 h half the snails from each group were removed and blood samples were taken for measurements of specific activity. At the end of the experiment the specific activity of the blood of the remaining animals was measured.

The term 'fresh tissues' in the following experiments refers to the soft parts of the snail exclusive of the blood, whilst the term 'total fresh tissues' includes both the soft parts and the blood.

RESULTS

Calcium compartments in Limnaea stagnalis

The body of *L. stagnalis* may be regarded as comprising three major calcium compartments: the shell, the blood and the fresh tissues. The calcium contents of the shell, blood, total fresh tissues and certain organs of the fresh tissues are shown in Table 1. The snails used in these experiments, of total weight 1–2 g, contained approximately 1500 μmole of calcium in the shell and 30 μmole calcium in the total fresh tissues

of which 3–4 μmole would normally be in the blood. From Table 1 it is apparent that specific calcium concentration of the mantle and digestive gland tissues is greater than that of the total fresh tissue. However, these two organs, together accounting for 20% of the total fresh tissue weight, contain only 25–30% of the total fresh tissue calcium. Appreciable amounts of calcium, therefore, must be stored in the remaining fresh tissues. Carriker (1946) found cells containing calcium deposits lining the outer walls of arteries and capillaries in *L. stagnalis appressa* suggesting calcium stores may be distributed throughout the fresh tissues.

Table 1. *Tissue calcium contents in* Limnaea stagnalis

Tissue	Ca content (μmole/g wet wt)
Shell	9543·3 ± 156 S.E. (11)
Blood	4·9 ± 0·1 S.E. (57)
Total wet tissues	22·7 ± 0·6 S.E. (33)
Mantle	49·6 ± 4·6 S.E. (27)
Digestive gland	37·8 ± 3·4 S.E. (26)
Reproductive organs	15·9 (2)

Brackets indicate the number of observations.

Distribution of ^{45}Ca absorbed from the medium

Batches of snails were placed in 500 ml volumes of artificial tap water containing 0·25 mM-Ca and labelled with ^{45}Ca. Snails were removed at intervals and the distribution of absorbed calcium was studied by measuring the specific activity of the calcium in the shell, blood, total fresh tissues, mantle and digestive gland of each animal. In this way the time course of the increase in specific activity was determined for each tissue and the relative specific activities of the tissues at any given time, after the start of the experiment, could be estimated. The increase in the specific activity of the shell calcium with time is shown in Fig. 1. The increase is seen to proceed in a roughly linear manner. Nevertheless, the specific activity of the shell calcium remained low, in comparison with that of the other tissues examined, throughout the experimental period. The time course of the appearance of ^{45}Ca in the soft parts is shown in Fig. 2. The slow increase in specific activity of the shell is to be expected in view of the large reservoir of unlabelled calcium present in the shell. Van der Borght (1963) and van der Borght & van Puymbroeck (1967) found that calcium absorbed from the medium by *L. stagnalis* was mainly located in the shell margin and to a lesser extent in the old shell. In these experiments no attempt was made to examine the shell margin and old shell separately.

Fig. 2 shows that the specific activity of the blood and tissue calcium rose rapidly during the first hours of immersion in the ^{45}Ca-labelled media and reached a constant level within 24 h. In the case of the digestive gland calcium, however, the specific activity continued to rise slowly. Half-labelling of the blood took 2–3 h whilst half-labelling of the fresh tissue calcium was less rapid, taking 9–10 h. The final specific activity of the fresh tissue calcium remained much lower than that of the blood, mantle calcium reaching 20%, digestive gland calcium 40% and total fresh tissue calcium 30% of the final specific activity of blood calcium. Inevitably the samples of mantle and digestive gland tissue were contaminated with blood. However, the calcium concentration

of the blood is low in comparison with the calcium concentration of these tissues (Table 1) and the amount of blood calcium of high specific activity in these tissues must therefore have been small. The total fresh tissue samples included the blood which contributed substantially to the final specific activity.

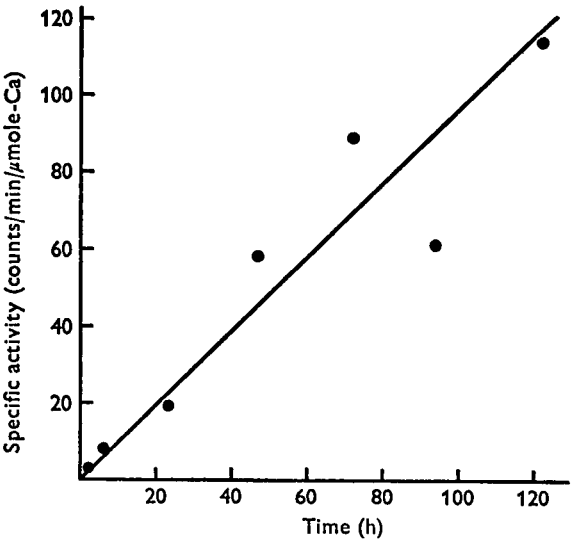

Fig. 1. The increase in specific activity of shell calcium of snails kept in labelled artificial tap water containing 0·25 mM-Ca Each point represents the mean for six animals.

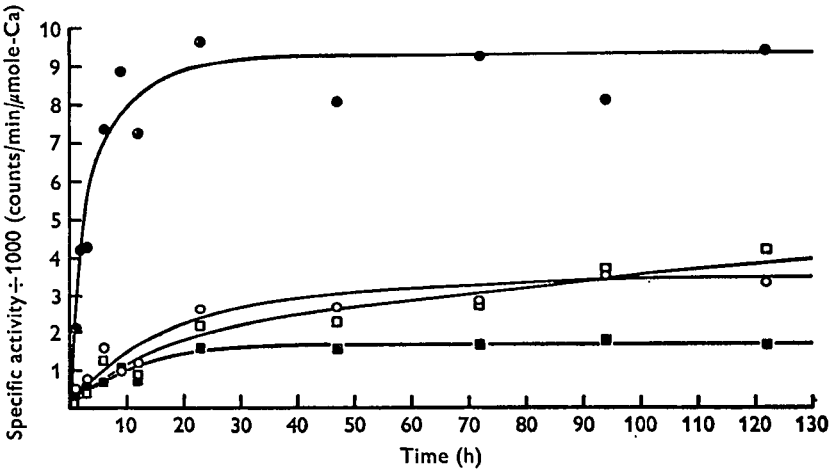

Fig. 2. The increase in specific activity of calcium in fresh tissue of snails kept in labelled artificial tap water containing 0·25 mM-Ca. ●, Mean values for blood; ○, mean values for total fresh tissue; ■, mean values for mantle; □, mean values for digestive gland. Each point represents the mean for 4–6 animals.

Table 2 shows values for the total radioactivity of the shell and total fresh tissues at time intervals of up to 122 h after immersion in ^{45}Ca-labelled media. The radioactivity of the total fresh tissues rose rapidly at first but between 22 and 47 h reached a fairly constant level. The shell radioactivity rose more slowly but continued to increase

Table 2. *The radioactivity of the shell and total wet tissues during absorption of* ^{45}Ca

Time (h)	Total radioactivity (counts/min)	
	Shell	Wet tissues
6·5	14967	45951
22	24258	79444
47	73725	74661
72	106422	96888
94	56350	87441
122	116660	81623

Each value represents the mean for six snails.

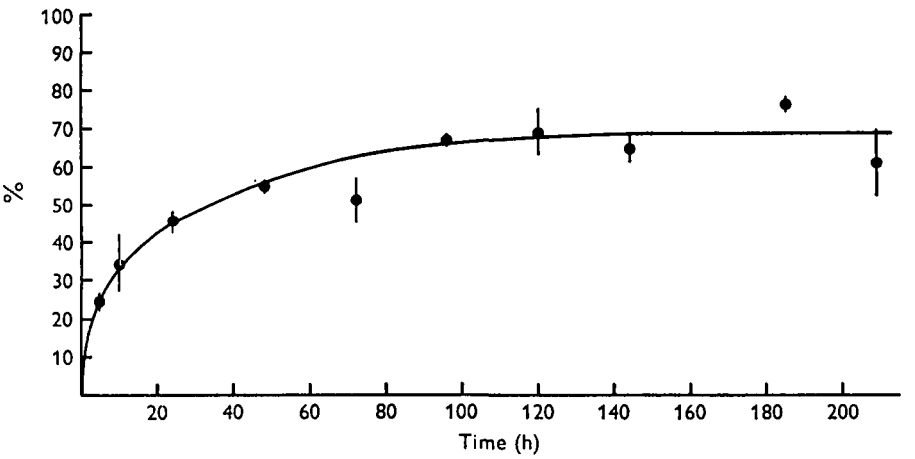

Fig. 3. Specific activity of calcium in the blood, as a percentage of the specific activity of calcium in the medium, plotted against time. Each point is the mean for 5–6 animals. Vertical lines represent standard errors.

throughout the experiment, and after 47 h exceeded the total fresh tissue activity. At the end of the experiment 41 % of the activity absorbed from the medium was located in the total fresh tissues and 59 % in the shell. Thus there was a continuing transfer of Ca from the medium to the shell which was not related to tissue calcium exchange.

In the experiments described above a marked fall in the specific activity of calcium in the medium occurred making the relationship between the specific activities of calcium in blood and medium rather difficult to interpret. In order to obtain a clearer picture of this relationship a second experiment was carried out under conditions where changes in the specific activity of external calcium would be minimal. A number of snails were placed in a polystyrene vessel, holding 10 l of ^{45}Ca-labelled artificial tap water containing 0·3 mM-Ca and groups of animals were removed at known time intervals for measurement of the specific activity of calcium in the blood. The specific activity of calcium in the medium was measured at the same time. During the experiment the external concentration and specific activity of calcium remained constant. Fig. 3 shows the specific activity of calcium in the blood expressed as a percentage of the specific activity of calcium in the medium. The specific activity of calcium in the

blood rose rapidly over the first 20 h and slowly thereafter until a constant level was reached after about 100 h at approximately 70 % of the specific activity of calcium in the medium.

Loss of radioactivity from loaded snails

Measurements of the specific activity of calcium in the blood have been made during the efflux of ^{45}Ca, from loaded snails, to non-radioactive media. In both artificial tap water (1 mM-Ca and calcium-free artificial tap water a fall of specific activity of calcium in the blood was recorded during the experimental period (Table 3). In the

Table 3. *Changes in specific activity of blood calcium during ^{45}Ca efflux to unlabelled media*

Medium	Time (h)	Specific activity (counts/s/μmole-Ca) of blood calcium	Concentration (mM) of blood calcium
Artificial tap water (1 mM Ca)	0	449·9	5·38
	2·5	354·2	5·68
	6·5	298·8	5·87
Ca-free artificial tap water	0	524·1	5·09
	3	461·2	5·16
	7	365·3	5·66

calcium-free medium specific activity of calcium in the blood fell by 13 % during the first 3 h of efflux and by 30 % over the whole experimental period (7 h). The mean blood concentration of the experimental snails rose slightly during the experiment although a net loss of calcium, equal to the calcium efflux, occurred (Greenaway, 1971). During efflux to artificial tap water, containing 1 mM-Ca the specific activity of calcium in the blood fell by 21 % in the first 2½ h of the experiment and by 35 % over the whole 6 h period. During this experiment there was a net uptake of calcium from the medium at a rate of 0·135 μmole/g/h and a small rise in blood concentration was again observed. The significance of these results is discussed below.

DISCUSSION

The data presented on the distribution of ^{45}Ca in the tissues, following absorption from the medium, provides a considerable amount of information concerning calcium movement between internal compartments. ^{45}Ca absorbed from the medium was distributed throughout the snail within 1 h of immersion in radioactive medium. Clearly the calcium absorbed from the medium must have been transported rapidly around the body, presumably in the blood. Certainly the blood became labelled more rapidly and reached a higher specific activity than the other tissues. Horiguchi (1958) demonstrated that ^{45}Ca absorbed from the medium by the freshwater bivalve *Hyriopsis schlegeli*, was transported by the blood. In the marine shrimp *Metapenaeus* calcium absorbed from sea water by the gills passed rapidly into the blood (Dall, 1965). As the total ^{45}Ca found in the mantle and digestive gland of *L. stagnalis* was greater than could be accounted for by contamination from labelled blood contained in the tissue samples it follows that an exchange of blood calcium and tissue calcium had occurred. The specific activity of the total fresh tissues reached only 30 % and the mantle

calcium only 20% of the final specific activity of calcium in the blood, these levels being attained within 30 h. The half-time of labelling was short (9 h) so the exchangeable fraction of tissue calcium must turn over rapidly. Exchange of the digestive gland calcium appeared to comprise a fast and a slow component. It is possible that the slow component may not represent exchange between blood calcium and digestive gland calcium but deposition of further calcium in the cells of this tissue. The shell became labelled at a fairly constant rate throughout the period of measurement indicating a continual gain of ^{45}Ca from the blood. Again this could have been due to a deposition of labelled calcium in the shell or to exchange with blood calcium. It has been shown that the specific activity of calcium in the blood reached a constant level 30% below that in the external medium. Two factors could account for this difference between the specific activities. In the first case, if a large proportion of blood calcium was bound, and also non-exchangeable, the specific activity of calcium in the blood would remain lower than that in the external medium by a percentage similar to that of bound calcium in the blood. In fact only a small amount of blood calcium is non-dialysable (0–6%) and the remainder is freely diffusible (van der Borght & van Puymbroeck, 1964). Bound calcium, therefore, can account for only a small part of the discrepancy between the specific activities of calcium in blood and in medium. Thus the second possibility, that the specific activity of calcium in the blood is kept at a lower level than that in the medium by exchange with a large reservoir of calcium of low specific activity (the shell) must account for the major part of the difference between final specific activities of calcium in the blood and in the medium. Using the equation

$$\text{S.A.}_2 = \text{S.A.}_1 \times \frac{m_1}{m_1+m_3}\left(1-\exp\left(-\frac{m_1+m_3}{m_2}t\right)\right),$$

(for derivation see Appendix), the calcium influx (m_1) into the blood, calculated from data given in Fig. 3, was 0·20 μ mole-Ca/g/h which agrees fairly closely with the calcium influx at the same external concentration found previously (Greenaway, 1971). From the same equation the rate of calcium exchange between the shell and the blood was 0·09 μ mole-Ca/g/h. Information regarding calcium movements between blood and shell can also be obtained from the data on changes in the specific activity and concentration of calcium in the blood during ^{45}Ca-efflux from loaded snails to calcium-free artificial tap water. Efflux to calcium-free media was accompanied by a fall in the specific activity and a slight rise in concentration of calcium in the blood. Efflux to calcium-free media was due to a net loss of calcium to the medium (Greenaway, 1971) so a transfer of calcium of low specific activity from the shell to the blood must have occurred at a rate similar to the rate of net loss to the medium. Calcium exchange between the shell and the blood has been calculated to occur at a rate of 0·09 μ mole-Ca/g/h, a value similar to the rate of net loss to the medium by snails in calcium-free artificial tap water. It appears likely therefore that the normal rate of calcium movement from shell to blood may account for the maintenance of blood calcium concentration during net loss. During ^{45}Ca efflux to artificial tap water (1 m-Ca) a fall in specific activity of calcium in the blood was also measured. However, since a mean net uptake rate of 0·135 μ mole-Ca/g/h was recorded during these experiments, a fall in the specific activity of calcium in the blood was not surprising.

It may be helpful at this point to summarize the calcium movements involved in

calcium regulation by *L. stagnalis*. A diagramatic summary is provided in Fig. 4. Calcium movements between the blood and the medium in normal fed snails may be separated into several components. First there is a 1 : 1 exchange, probably on a passive basis, and secondly a loss of calcium from the blood, probably largely in the urine (Greenaway, 1971). Calcium uptake is normally greater than loss, resulting in a net

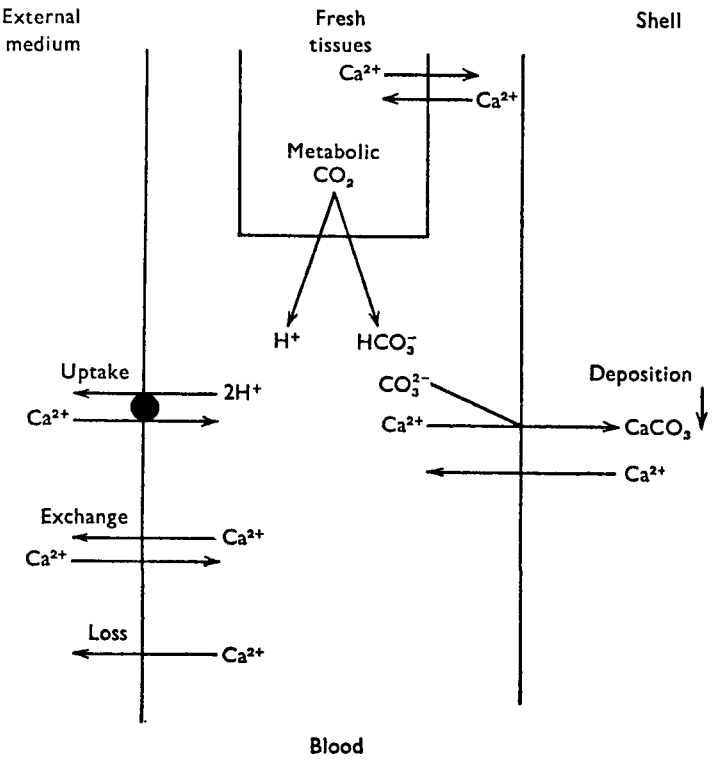

Fig. 4. Diagram illustrating the calcium movements between the internal calcium compartments and the external medium.

movement of calcium into the blood, calcium gained in this way being deposited in the shell as $CaCO_3$. A supply of metabolically produced CO_2 is available in the tissues and in *Crassostrea* is sufficient to render the uptake of HCO_3^- or CO_2 from the medium unnecessary. This CO_2 may be readily converted to the carbonate (Wilbur, 1964). It seems likely that the uptake of calcium ions may be in exchange for hydrogen ions or perhaps accompanied by HCO_3^-. The latter would seem unnecessary as metabolic HCO_3^- would then have to be excreted. An exchange of calcium between blood and shell and between blood and tissues has also been demonstrated. During net loss in calcium-free water the deposition of $CaCO_3$ ceases and a backflow of calcium from shell to blood compensates the loss of blood calcium to the medium.

It has been shown that the blood calcium concentration of *L. stagnalis* is maintained within fairly narrow limits during conditions of net calcium uptake from the medium and net calcium loss from the blood. Such a precise regulation of the blood calcium concentration under conditions varying from net uptake to net loss would suggest the

presence of a sensitive mechanism controlling the direction and magnitude of internal calcium movements. The simplest possible hypothesis to account for the situation described above is as follows. Potts (1954) found that the blood of the freshwater bivalve *Anodonta* was saturated or supersaturated with respect to aragonite. An increase in either pH or calcium concentration in the blood could, therefore, cause precipitation of calcium carbonate, provided other conditions remained unchanged. If it is assumed that the blood of *L. stagnalis* is also saturated with calcium carbonate, net uptake of calcium into the blood would cause precipitation of calcium carbonate in the shell. Net loss of calcium from the blood, however, would reduce the degree of saturation of the blood, causing a net movement of calcium from shell to blood. Such a mechanism would constantly compensate for changes in the direction of the net calcium flux between the snail and the outside medium and would also permit calcium exchange between the blood and the shell. It must be emphasized that the control of blood calcium concentration is unlikely to be as simple as this and must be co-ordinated with the mobilization of the organic constituents of the shell. Data given by Dall (1965) suggests that the blood of *Metapenaeus* may be supersaturated with respect to calcium carbonate in sea water of 20‰ salinity, calcium deposition thus being favoured and requiring only nucleation to initiate calcification. Dall also suggests that the enzyme carbonic anhydrase, present in the epidermis of *Metapenaeus*, may catalyse the solution of calcium deposits thus enabling resorption of calcium from the exoskeleton. No attempt has been made to find the site of this enzyme in the present study. Its presence has been demonstrated, however, in the mantles of several molluscs including aquatic species (Freeman & Wilbur, 1948; Tsujii & Machii, 1953; Kawai, 1954, 1955; Clark, 1957).

SUMMARY

1. Three major calcium compartments have been identified in *L. stagnalis*: the shell, the blood and the fresh tissues.

2. The distribution of ^{45}Ca, absorbed from the medium, in the tissues of *L. stagnalis* has been studied. Absorbed calcium appears first in the blood and then in the shell and other tissues.

3. 30% of the total fresh tissue calcium and about 20% of mantle calcium exchanges with blood calcium. A continual exchange between shell and blood calcium occurs.

4. During net calcium loss from *L. stagnalis* a net movement of calcium from shell to blood occurs at a rate similar to the rate of net loss. During net calcium uptake, the reverse movement from blood to shell at a rate similar to the rate of net uptake occurs.

5. A simple mechanism which might account for the control of blood calcium concentration has been proposed.

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APPENDIX

This appendix concerns the calculation of the increase in specific activity of calcium in the blood of animals in an external medium containing radiocalcium and from which there is a net transfer of calcium to the blood. In addition there is a flux between the blood and the shell and a net transfer of calcium to the shell equal to that into the blood.

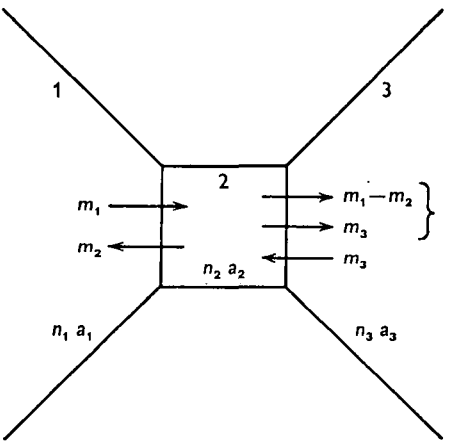

Fig. 5. Diagram illustrating movements of labelled and unlabelled calcium ions between calcium compartments. For explanation for symbols see text.

Consider a three-compartment system (1–3) representing the external solution, the blood and the shell. Let the outer compartment be considered sufficiently large for the total number of calcium ions (a_1) and the number of labelled ions (n_1) to remain constant. The influx from 1 to 2 is m_1 and the efflux is m_2, so that the net transfer is m_1-m_2. The number of calcium ions in the blood (compartment 2) is a_2 and the number of labelled ions is n_2. The shell compartment (calcium ions, a_3; and labelled ions, n_3) is also very large so that n_3 remains at zero. The influx to the shell is m_3 plus the net transfer (m_1-m_2) and the efflux, m_3. The accompanying diagram illustrates the movement of labelled and unlabelled calcium ions between the compartments (Fig. 5).

$$\frac{dn_2}{dt} = \frac{m_1}{a_1}n_1 - \frac{m_2}{a_2}n_2 - \frac{m_1-m_2}{a_2}n_2 - \frac{m_3}{a_2}n_2 + \frac{m_3}{a_3}n_3.$$

Since in the course of a short experiment $n_3 = 0$, the last term diasppears and a_1, a_2 and n_1 can be considered constant.

Therefore
$$\frac{dn_2}{dt} = \frac{m_1}{a_1}n_1 - \frac{m_1}{a_2}n_2 - \frac{m_3}{n_2}n_2.$$

Hence
$$\frac{dn_2}{dt} = \frac{m_1+m_3}{a_2}n_2 = \frac{m_1}{a_1}n_1.$$

Multiplying each side by

$$\exp\left(\frac{m_1+m_3}{a_2}t\right)$$

and integrating,

$$n_2 \exp\left(\frac{m_1+m_3}{a_2}t\right) = \int \frac{m_1}{a_1} n_1 \exp\left(\frac{m_1+m_3}{a_2}t\right)$$

$$= \frac{m_1}{a_1} n_1 \frac{a_2}{m_1+m_3} \exp\left(\frac{m_1+m_3}{a_2}t\right) + c$$

(c = integration constant).

When $t = 0$, $n_2 = 0$. Therefore

$$0 = \frac{a_2}{a_1}\frac{m_1}{m_1+m_3} n_1 + c.$$

Hence

$$c = -\frac{a_2}{a_2}\frac{m_1}{m_1+m_3} n_1.$$

Therefore

$$n_2 = \frac{m_1}{m_1+m_3}\frac{a_2}{a_1} n_1\left(1-\exp\left(-\frac{m_1+m_3}{a_2}t\right)\right).$$

Therefore

$$\frac{n_2}{a_2} = \frac{m_1}{m_1+m_3}\frac{n_1}{a_1}\left(1-\exp\left(-\frac{m_1+m_3}{a_2}t\right)\right).$$

But the specific activity of the blood, S.A.$_2$, is $\frac{n_2}{a_2}$, and that of the medium (S.A.$_1$) is $\frac{n_1}{a_1}$.

Therefore,

$$\text{S.A.}_2 = \text{S.A.}_1 \frac{m_1}{m_1+m_3}\left(1-\exp\left(-\frac{m_1+m_3}{a_2}t\right)\right).$$