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THE IONIC PROPERTIES OF THE CAPSULAR FLUID BATHING EMBRYOS OF *LYMNAEA STAGNALIS* AND *BIOMPHALARIA SUDANICA* (MOLLUSCA: PULMONATA)

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INTRODUCTION

The embryos of freshwater pulmonate molluscs undergo direct development enclosed within individual egg capsules containing a viscous 'capsular fluid' (perivitelline fluid or albumen).

Considerable attention has been paid to the organic constituents of the capsular fluid and their secretion by the reproductive system of pulmonates (literature surveyed by Morrill, 1963, 1964; Morrill, Norris & Smith, 1964; Bayne, 1967, 1968*a*, *b*; Plesch, De Jong-Brink & Boer, 1971). In *Lymnaea stagnalis* the capsular fluid has a dry weight of 15% of which about a third is the polysaccharide galactogen (Horstmann, 1956*a*) and the remainder proteins. Both constituents are heterogeneous and have species-specific components (McMahon, von Brand & Nolan, 1957; Horstmann & Geldmacher-Mallinkrodt, 1961; Morrill *et al.* 1964; Wright & Ross, 1965). They exert a colloid osmotic pressure of about 1·5 mOsm in *Biomphalaria* and 4–5 mOsm in *Lymnaea* (Beadle, 1969).

The importance of the capsular fluid as a nutrient reserve for the developing embryo is demonstrated by the decline in colloid osmotic pressure, in galactogen content and in protein content during development and in the corresponding increase in lipid and protein content of the embryos (Beadle, 1969; Horstmann, 1956*a*, *b*; Morrill, 1964). The conditions in the capsular fluid are also important in that they constitute the main environmental factors in the development of the embryo from oviposition to hatching. Thus it is of interest to know the extent to which these conditions are affected by changes in the conditions in the surrounding water. In particular a knowledge of the ionic conditions in the capsular fluid in relation to the composition of the water is clearly fundamental to any study of the osmotic and ionic regulation of the embryos and would assist the interpretation of experiments concerning the effects of ions on morphogenesis (reviewed Raven, 1964), on protein uptake (Morrill, 1964) and on freshwater molluscan ecology (Boycott, 1936; Macan, 1950).

The ionic problems facing freshwater pulmonates were emphasized by the experiments of Beadle (1969), which indicated that the capsular membrane (membrana interna or chorion) and outer layers of the egg masses of *Biomphalaria* and *Lymnaea* (see Plesch *et al.* 1971, for a complete description of the numerous outer layers) allowed a free exchange of water, ions and other solutes of low molecular weight between the water and capsular fluid. It was further demonstrated that *Biomphalaria*

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embryos show a net uptake of sodium from the water via the capsular fluid (Beadle & Beadle, 1969).

Analyses of the inorganic constituents of the capsular fluid have been few. Beadle & Beadle (1969) showed that the sodium concentration in the capsules of *Biomphalaria* is of the same order as in the external water. In the very large capsules of the freshwater prosobranch *Marisa cornuarietis* the sodium and calcium concentrations are somewhat higher than in the water (Bartelt, 1970). There have been a number of histochemical demonstrations of high calcium levels in the capsular fluid of gastropods (George & Jura, 1958; Morrill *et al.* 1964; Bayne, 1968*a*).

In this paper a quantitative analysis of the ionic conditions in the capsular fluid and the exchanges with the medium is presented. Capsules from *Lymnaea* and *Biomphalaria* have been used, and the study has been extended to four cations – sodium, potassium, magnesium and calcium. In general, Beadle's observations have been confirmed although it now appears that all cations are maintained by a Donnan equilibrium at a somewhat higher concentration in the capsular fluid than in the surrounding water. When the medium is diluted they may reach many times the external concentration. The importance of this system to the developing embryo is discussed.

MATERIALS AND METHODS

Lymnaea stagnalis and *Biomphalaria sudanica* were cultured in the laboratory in plastic tanks and fed on cabbage, lettuce and wheat germ. Egg masses were obtained from the sides of the tanks and from pieces of floating polyethylene or expanded polystyrene.

Owing to their larger and more uniform volume and easier manipulation the majority of work was performed on the capsules of *Lymnaea* and results refer to this unless specifically stated to refer to *Biomphalaria sudanica*. Unless stated otherwise, analyses and experiments refer to capsules dissected out of the tunica capsulis and freed from the gelatinous tunica interna and membrana externa (terminology of Plesch *et al.* 1971) by carefully rolling them on dry filter paper. Capsules were equilibrated with the experimental media for at least 1 day at 22–25° C before making measurements at the same temperature. Only capsules containing pre-gastrula embryos were used. These were normally killed by exposure to a temperature of 50° C for several minutes. This procedure produced no visible change in the capsular fluid and was accompanied by no measurable change in capsular sodium content or in the potential difference across the membrane. The presence of a dead pre-gastula embryo probably introduces negligible contamination in the analyses involving whole capsules (capsule volume is about 1 μl, embryo volume about 1 nl).

Measurements of capsule volume

Isolated capsules of *Lymnaea* are regular prolate spheroids. Their volumes were calculated from the formula $\frac{1}{6}\pi ld^2$, where l and d are the longest and shortest diameters respectively, measured using an eyepiece micrometer. Capsules of *Biomphalaria* are rather irregular even when isolated. Their volumes were estimated from the formula $\frac{1}{6}\pi ldh$ where l, d and h are the longest diameter and diameters mutually at right angles to this.

Media

The standard medium used for most experiments was an artificial lake water (LW 1) with the following composition: $NaHCO_3$, 0·50 m-equiv./l; $KHCO_3$, 0·10 m-equiv./l; $MgSO_4$, 0·50 m-equiv./l; $CaCl_2$, 0·85 m-equiv./l; pH 7·4. Two other media, LW 2 and LW 3, contained the same salts in slightly different proportions (Table 3). The compositions of other experimental media are given in the appropriate sections of the results.

Capsular fluid samples

Samples of the capsular fluid of known volume for determination of the concentrations of ions or radioactivity were obtained in two ways.

(1) Capsules were removed from the experimental medium, blotted dry on filter paper and quickly transferred to a hydrofuge surface (a disposable plastic Petri dish) under liquid paraffin. Capsular fluid was drawn off with a Pyrex micropipette and pooled with samples from the other capsules to provide sufficient to fill a washed 'Drummond microcap' (1–5 μl).

(2) For the potassium determinations and some of the sodium determinations by the specific activity method, a whole capsule was used as the sample. Its volume was determined, as above, by measuring the capsule before removal from the experimental medium or (in the case of potassium determinations) by taking the mean volume of ten capsules from the same egg mass.

The two sampling methods give results for sodium concentration which agree to within 5% when used on the same egg mass.

Analysis of ions

Flame spectrophotometry. Sodium, potassium and some of the calcium concentration determinations of diluted samples were made using a Unicam SP 900 or EEL 240 flame spectrophotometer in the emission mode, operating at 589, 766 and 622 nm respectively. Interference effects in analyses of media were eliminated by employing calibration standards containing all ions in the same proportions as the samples. In capsular fluid samples the spectral interference was determined by scanning the base line on either side of the emission peak, and the enhancement or depression of sensitivity by other ions was estimated by the method of standard addition. The appropriate corrections were made where necessary.

Magnesium determinations and most of the calcium determinations were made by atomic absorption spectroscopy using the EEL 240 at wavelengths 285 and 422 nm respectively. 0·033% $LaCl_3$ was added to the samples and standards.

Specific-activity method. Capsules were equilibrated with solutions containing $^{22}Na^+$, $^{42}K^+$ or $^{45}Ca^{2+}$. Samples were then taken to measure the concentration of the ion in the medium, the medium radioactivity and capsular fluid radioactivity. The concentration of the ion in the capsular fluid was then calculated assuming its specific activity was the same as in the medium.

Samples were counted on planchettes using an I.D.L. low-background counter. Differential self-absorption was masked by spreading the samples with 0·5 ml of distilled water, 50 μl of M glucose and a trace of detergent, then drying at 60° C.

Tracer fluxes

To study the efflux of $^{22}Na^{+}$ and $^{45}Ca^{2+}$, tracer-equilibrated capsules were removed from the labelled medium, blotted on filter paper and placed in a large volume (> 10 ml per capsule, changed several times) of continuously stirred, unlabelled medium. They were then removed after a known interval of time and sampled for radioactivity measurement as described. Removal of the washing medium from the capsules was performed by dropping them on to a thick layer of filter paper and was completed in less than 3 sec.

The influx experiment was performed in an essentially similar manner except that the labelled and unlabelled media were reversed (sodium in the capsules < 1% of total in system).

Potential difference

The procedure for measuring the potential difference across the capsular membrane was as follows. The experimental medium bathing equilibrated capsules was absorbed with filter paper and the capsules were placed on a hydrofuge surface under liquid paraffin. They were then pierced at one end. A few nl of capsular fluid emerged from the hole but did not show any tendency to run over the surface of the capsule. The capsules were then moved against a drop (about 10 μl) of the original medium secured on the bottom with a glass rod, so that about a third of the capsule at the other end was immersed. The medium readily re-wetted the surface of the capsule but similarly did not spread over the surface. After allowing a few minutes for re-equilibration, the potential difference between the two droplets was measured using a pair of 2·5 M-KCl-filled microelectrodes (tip resistance 5–15 MΩ) connected via KCl bridges and calomel electrodes to a Vibron Electrometer, Model 33B-2.

Measurement of the potential difference between the jelly layers and the external medium was performed on whole egg masses immersed in LW 1.

RESULTS

General observations

The individual isolated capsules of *Lymnaea* (bounded by the membrana interna) are prolate spheroids about 1 × 1·5 mm. The individual capsules in a single egg mass have very uniform shapes (i.e. the eccentricity of the spheroids) and volumes (S.D. about 5–6% of mean volume and sometimes considerably less; Table 1). The variation in shape and volume between the constituent capsules of different egg masses is greater (S.D. 14·4% of the overall mean volume of 0·852 μl). This is consistent with the results of Horstmann (1956*a*) that the galactogen content is very constant in capsules from a single egg mass but varies widely between egg masses.

The volumes of a few isolated *Biomphalaria* capsules were measured (Table 2). The mean of the mean capsular volumes for each egg mass was 0·435 μl.

Ionic composition of the capsular fluid in relation to the composition of the external medium (artificial lake water)

The sodium, potassium, calcium and magnesium concentrations were determined in the capsular fluid of isolated *Lymnaea* capsules equilibrated (for at least 1 day) in

Table 1. *Mean volume of egg capsules in* 22 Lymnaea *egg masses*

Mean capsular volume (μl)	S.D. (%)	No. of capsules measured	Mean capsular volume (μl)	S.D. (%)	No. of capsules measured
1·081	5·9	5	0·815	3·9	5
1·045	12·3	12	0·798	4·5	38
1·025	4·4	23	0·771	5·1	10
0·995	6·6	10	0·760	7·6	25
0·987	2·8	10	0·753	3·9	37
0·946	6·7	15	0·742	5·5	45
0·904	5·6	5	0·728	12·2	10
0·885	4·1	10	0·694	1·9	5
0·865	6·7	38	0·688	7·3	5
0·860	3·5	10	0·639	3·6	10
0·827	4·4	5			

S.D. = standard deviation expressed as percentage of mean.

Table 2. *Mean volume of egg capsules in four* Biomphalaria *egg masses*

Mean capsular volume (μl)	S.D. (%)	No. of capsules measured
0·452	2·6	3
0·450	8·4	4
0·445	3·8	3
0·391	4·1	7

Table 3. *Concentrations of cations in the capsular fluid of* Lymnaea *in relation to concentration in the water* (*m-equiv./l*)

		Concentration in capsular fluid	
	Ion	S.D.	*n*
Medium LW 1 (Na, 0·50; K, 0·10; Ca, 0·85; Mg, 0·50)	Na	1·21 ± 0·07	8*
	Ca	15·22 ± 2·29	7
		15·23 ± 2·60	10*
	Mg	7·12 ± 0·16	4
Medium LW 2 (Na, 0·57; K, 0·10; Ca, 0·30; Mg, 0·25)	Na	2·09 ± 0·08	3
		2·02 ± 0·11	14*
	Ca	12·15 ± 1·27	6
Medium LW 3 (Na, 0·55; K, 0·10; Ca, 0·21; Mg, 0·25)	Na	2·76 ± 0·28	13*
	K	0·52	2*

* By specific activity method; others by atomic absorption or emission spectroscopy. S.D. = standard deviation of mean of values for *n* different egg masses. At least ten samples or capsules were averaged for each egg mass value.

artificial lake water, and the results are summarized in Table 3. Results obtained from flame photometry and specific activity methods are indicated separately, although it can be seen that where both methods have been used they agree quite closely (thus there is no evidence to suggest that any of the sodium or calcium in the capsular fluid is 'bound' in the sense that it is non-exchangeable).

No significant difference was found between the sodium concentrations in capsules containing the remains of heat-killed pre-gastrula embryos and capsules containing live embryos up to early trochophore stage. There was no correlation between capsular volume and capsular sodium concentration.

Table 4. *Concentrations of sodium and calcium in the capsular fluid of* Biomphalaria (*m-equiv./l*)

	Ion	Concentration in capsular fluid S.D.	*n*
Medium LW 1	Ca	6·7 —	1
		5·6 —	1*
Medium LW 3	Na	2·83 ± 0·21	3
		2·39 ± 0·33	4*

Conventions as in Table 3.

Three different artificial lake waters (LW 1, LW 2 and LW 3) have been prepared (all support normal development of *Lymnaea* embryos). They differ principally in the concentrations of Ca^{2+} and Mg^{2+}. The results from the three media have been tabulated separately.

All four cations (Na^+, K^+, Mg^{2+}, Ca^{2+}) are always present at higher concentrations in the capsular fluid than in the medium. However, the concentration of divalent ions in the medium affects the concentration of monovalent ions in the capsular fluid. In LW 3, Na^+ and K^+ are about five times as concentrated in the capsular fluid as in the medium. Raising the Ca^{2+} and Mg^{2+} concentrations in the medium progressively displaces these ions and in LW 1 the ratio $[Na^+]_{int}/[Na^+]_{ext}$ is only about 2·4. In this medium Ca^{2+} and Mg^{2+} are respectively about 18 and 14 times as concentrated in the capsular fluid as in the medium. Reduction of the Ca^{2+} concentration in the medium has a relatively small effect on the Ca^{2+} concentration in the capsular fluid so that the ratio for this ion rises to about 40 in LW 2.

The similar ratios for sodium and potassium (4·9 and 5·2 respectively in LW 3) are consistent with a Donnan equilibrium across the capsular membrane. The calcium and magnesium ratios should equal the monovalent ion ratios squared in this case. They are always much higher than this. However, the Donnan ratios strictly refer to the activities of the ions. This is discussed further later.

A few measurements of the Na^+ and Ca^{2+} concentrations in the capsular fluid of *Biomphalaria* were made (Table 4). Sodium concentrations† are similar to those of *Lymnaea* but the capsular concentration of calcium is only about 40% of the *Lymnaea* value.

Effect of dilution of the artificial lake water

A series of dilutions of LW 1 containing $^{22}Na^+$ or $^{45}Ca^{2+}$ were made with distilled water, the component ions remaining in the same proportions. *Lymnaea* capsules were equilibrated in the media for 1 week, then external sodium and calcium concentrations were measured by flame photometry, and the capsular concentrations determined by the specific activity method. Fig. 1 shows that as the external concentrations are reduced the internal concentrations of sodium and calcium are also

† Beadle and Beadle (1969) reported that the Na^+ concentration of *Biomphalaria* capsular fluid was identical to that of the medium. It now seems likely that these values were too low, perhaps due to insufficient account being taken of sample geometry effects. Although this means that the absolute values given in that paper are too low, the main conclusions, which were based on changes in Na^+ content, are of course still valid.

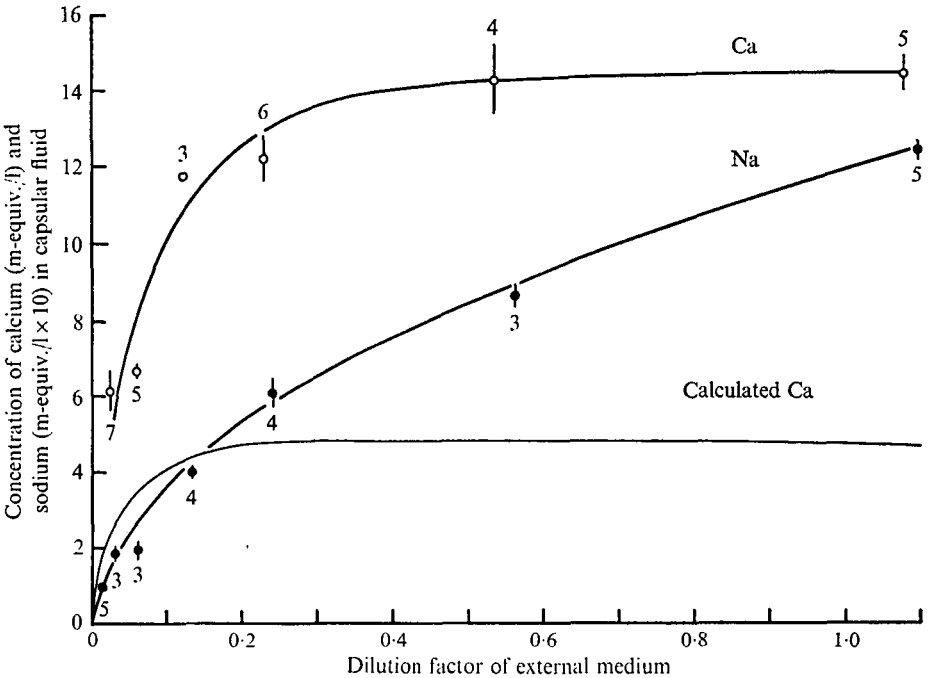

Fig. 1. Concentration of calcium (○) and sodium (●) in capsular fluid on dilution of external medium, keeping proportions of ions constant. Medium is LW 1 (Ca^{2+}, 0·85; Na^+, 0·50 m-equiv./l). Vertical lines represent ± S.E. Numbers of measurements given beside each mean value. Same three egg masses used for calcium and sodium measurements. Calculated line represents the expression $[Ca^{2+}]_o$. $[Na^+]_i^2/[Na^+]_o^2$, i.e. the maximum capsular calcium activity if the system is in Donnan equilibrium (see Appendix).

reduced. However, the internal concentrations do not change in proportion to the external concentration; e.g. the internal sodium concentration is only reduced by a factor of 10 for a 50-fold dilution of the medium. This 'cation buffering' effect of the capsular fluid/membrane system is even more marked in the case of calcium. The internal calcium concentration did not fall appreciably until the medium was diluted more than tenfold, and at 50 times dilution the capsules still retained a third to a half of their calcium, representing a mean concentration ratio of about 350 and over 400 in some individual capsules.

The value of the expression $[Ca^{2+}]_o[Na^+]_i^2/[Na^+]_o^2$ (where $[Ca^{2+}]_o$ and $[Na^+]_o$ are the concentrations of calcium and sodium ions in the medium and $[Na^+]_i$ is the corresponding concentration of sodium ions in the capsule) is also shown in Fig. 1. If the distribution of cations is determined by a Donnan equilibrium, then this expression will be nearly proportional to the calcium ion concentration in the capsular fluid (see Appendix). Although the variability of the measurements of total calcium concentration in the capsules is high, the measured and calculated calcium curves clearly have a rather similar form, and this is consistent with the existence of a Donnan equilibrium for sodium and calcium. The 'calcium-buffering' effect is very marked in the calculated curve, the calculated value of $[Ca^{2+}]_o[Na^+]_i^2/[Na^+]_o^2$ being within ± 1% of 4·8 m-equiv/l over the range of dilution 0·2–1·1. This is somewhat lower than the measured calcium concentration (about 14 m-equiv/l) and suggests some of

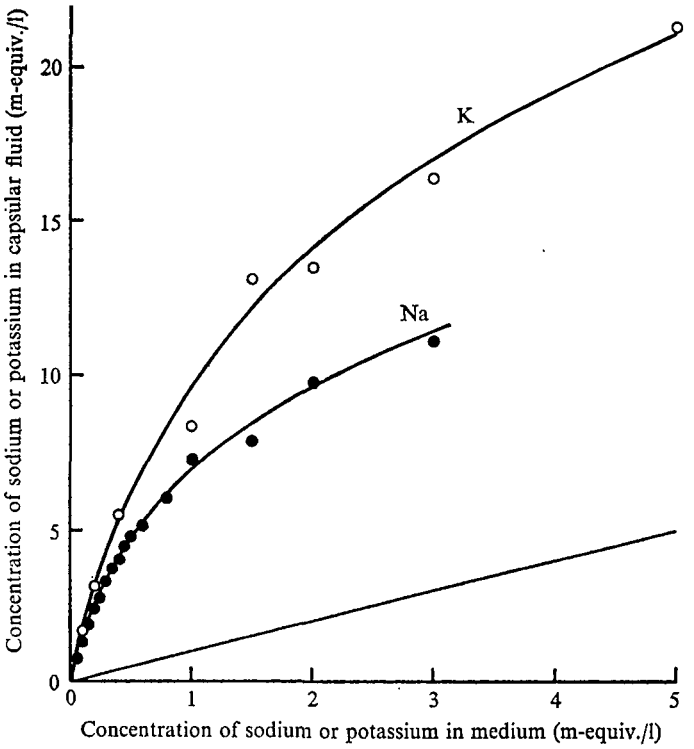

Fig. 2. Concentrations of Na^+ (●) and K^+ (○) in the capsular fluid of capsules equilibrated with pure NaCl or KCl solutions. Each point represents the mean of three capsules from the same egg mass.

the calcium in the capsules is bound. As shown in the Appendix, the calculated expression has a value which is somewhat greater than the calcium activity but less than the concentration of free calcium in the capsular fluid.

Relationship between internal and external ionic concentrations in media containing a single cation

As the concentration of an ion in the capsular fluid was found to be influenced by the concentrations of all other ions in the medium, the relationship between internal and external cation concentrations was examined with pure NaCl and KCl solutions as the bathing media. Internal sodium and potassium concentrations were determined by the specific activity method after equilibration for 1–2 days. Prior to this treatment they had been equilibrated in LW 1. Fig. 2 shows that in the absence of other cations the sodium and potassium ratios across the capsular membrane are much higher than they were in the corresponding mixed cation solution. Under these conditions sodium and potassium evidently replace the divalent cations initially present in the capsular fluid. At the highest concentrations used (Na^+ = 3 m-equiv./l; K^+ = 5 m-equiv./l) neither ion has 'saturated' the capsular fluid as the difference between the capsular and medium concentrations is still increasing. This is probably partly due to the fact that more of the 'bound' calcium is displaced at high concentrations of monovalent cations. Apparently potassium has a greater affinity for these sites than sodium.

Table 5. *Concentration of calcium in the capsular fluid after washing in various calcium-free media*

Medium	Washing time (days)	$[Ca^{2+}]$ of capsular fluid (m-equiv./l)
LW 1	6	17·3
Distilled water	1	10·5
	6	7·4
	14	4·4
Ca-free LW*	6	2·0
1 m-equiv./l NaCl	6	2·8
1 m-equiv./l KCl	6	1·9
1 m-equiv./l $MgCl_2$	6	< 0·5†
5 m-equiv./l NaCl	6	< 0·5†
5 m-equiv./l KCl	6	< 0·5†
5 m-equiv./l $MgCl_2$	6	< 0·5†

* $NaHCO_3$, 0·5; $KHCO_3$, 0·1; $MgCl_2$, 0·5 m-equiv./l.
† detection limit in this experiment.
Each mean of two measurements from each of at least two capsules.

It is clear that the relationship between internal and external cation concentration is markedly different from that expected from an 'ideal' Donnan equilibrium involving a single cation and a fixed concentration of impermeant anions. (In such a system the internal cation concentration, instead of approaching zero with external dilution, should approach a constant value equal to the internal anion concentration.) This is not unreasonable since (1) hydrogen ions also take part in the equilibrium; as their external concentration is not reduced by dilution, when the external sodium concentration drops below the hydrogen ion concentration, hydrogen ions replace sodium ions as the main internal cations; and (2) changes in internal pH resulting from the Donnan equilibrium affect the dissociation of the proteins which behave as weak acids (or Zwitter-ions) so that the concentration of impermeant anion is reduced by external dilution.

Loss of capsular fluid calcium in calcium-free media

Capsules initially equilibrated in LW 1 were washed in a series of calcium-free media. After 6 days the calcium concentration in the capsular fluid was measured by flame photometry. Measurements were also made on capsules washed in distilled water for 1 and 14 days. Washing was performed in a relatively large volume of the medium changed frequently (every few hours initially, extending to every few days in the longest washed capsules). The results are shown in Table 5. It is seen that the capsular calcium leaves very slowly in distilled water, a significant amount being retained after 2 weeks. Washing in dilute calcium-free salines displaces most of the calcium in 6 days. However, the 1 m-equiv./l potassium and sodium solutions do not displace all of it, 2–3 m-equiv./l remaining. Potassium again appears to displace the calcium more effectively than sodium.

After prolonged washing in frequently changed distilled water it was observed that the capsular fluid in a number of capsules became white and opaque (none of those in Table 5, however). This is probably caused by the capsular proteins approaching their iso-electric point and coagulating as the Donnan potential rises and the internal

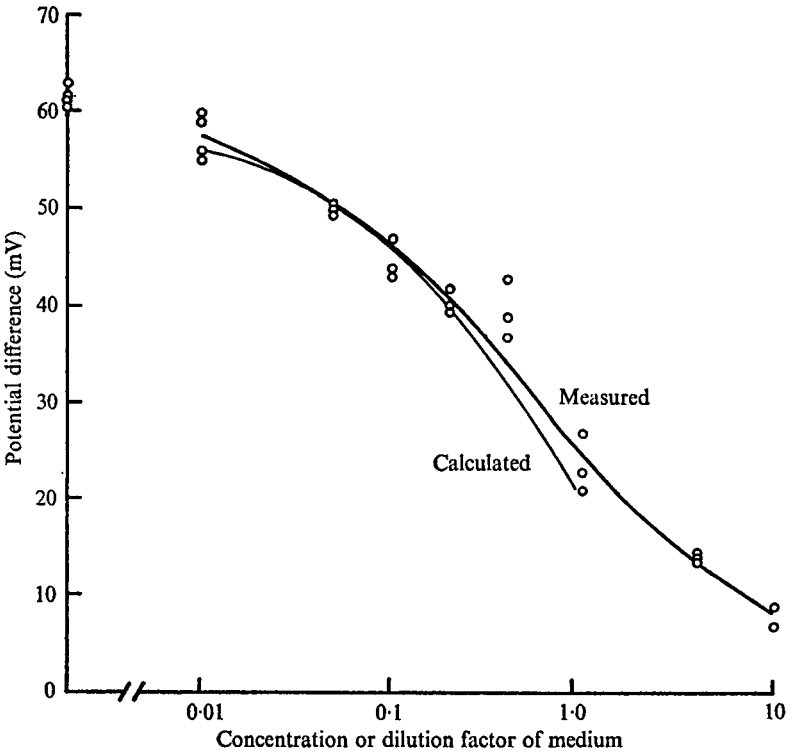

Fig. 3. Potential difference across the capsular membrane (inside negative) in capsules in equilibrium with media of varying strength containing ions in the proportions of LW 1. Calculated line, equilibrium potential for sodium calculated from Fig. 1. Each point is a single measurement. Capsules from a single egg mass.

pH falls. The precipitation is associated with a further loss of calcium. In a group of opaque capsules the mean calcium concentration of the capsular fluid was 2·3 m-equiv./l. The capsules quickly assume their normal transparent appearance if placed in lake water or pure NaCl solutions.

Potential difference across the capsular membrane

If there is a Donnan equilibrium across the capsular membrane then a potential should exist between the capsular fluid and the medium. The potential was measured in eight capsules in equilibrium with LW 1 and found to be $23{\cdot}0 \pm 0{\cdot}4$ (S.E.) mV, inside negative (capsules containing both live and dead pre-gastrula embryos). The equilibrium potential for sodium in LW 1, calculated from the mean sodium concentration given in Table 1 ($59 \log_{10} \cdot [Na^+]_i/[Na^+]_o$) is 22·6 mV and therefore agrees quite well.

The potential across the capsular membrane was also measured in capsules equilibrated with a series of solutions of varying strength containing ions in the proportions of LW 1 (Fig. 3) and in capsules equilibrated with a series of pure NaCl solutions (Fig. 4). The sodium equilibrium potentials calculated from the sodium concentration ratios given in Figs. 1 and 2 are also shown. The two sets of results are in reasonable agreement over the parts of the curves which coincide. The agreement between the

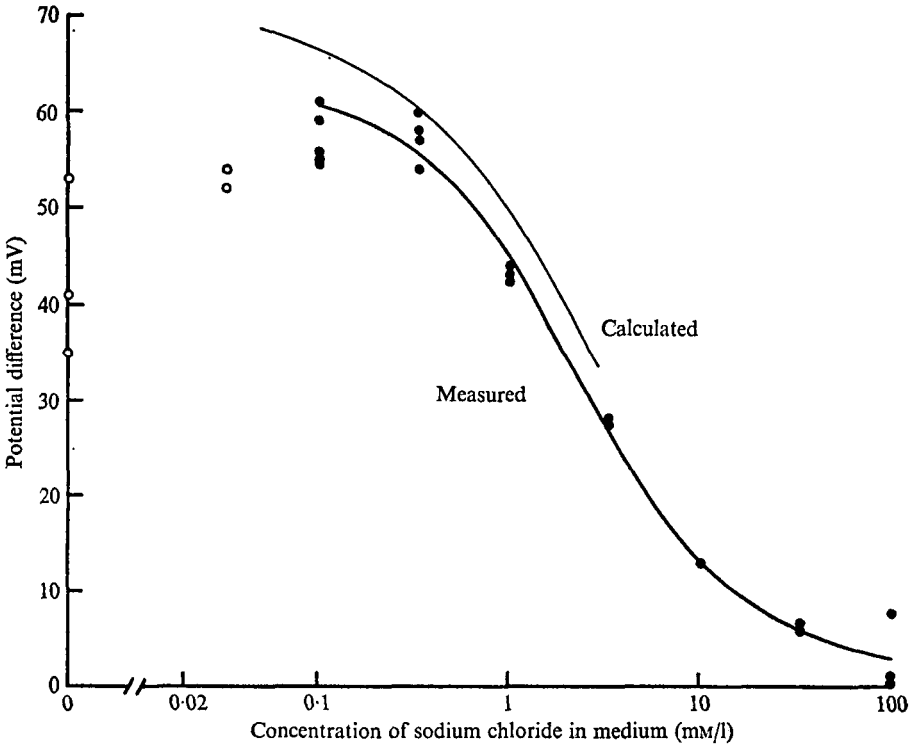

Fig. 4. Potential difference across capsular membrane (inside negative) in capsules in equilibrium with NaCl solutions of varying concentration. Calculated line, equilibrium potential for sodium calculated from Fig. 2. ○, See text. Each point is a single measurement. Capsules from a single egg mass.

calculated and measured potentials in the three pairs of experiments is consistent with the theory of a Donnan equilibrium across the capsular membrane and provides a comforting cross check on the sodium analyses.

In Fig. 4 it is seen that in the lowest NaCl concentration and in distilled water (open circles) the potentials are rather lower than expected (and also lower than the distilled water value in Fig. 3). These values are probably not directly comparable with the others since it was observed that in all of the capsules used for these particular potential measurements the capsular fluid had begun to change to the 'opaque' state reported in the previous section. This was not the case in any of the other capsules in these experiments.

Potential in the outer and inner jelly of the egg mass

In vivo the capsules are embedded in a gelatinous matrix (tunica interna and membrana externa), contained within a relatively tough envelope tunica capsulis). An additional layer of gelatinous material (pallium gelatinosum) is present on the external surface of the egg mass. Potentials were recorded between a reference microelectrode in the external solution and a measuring microelectrode inserted first into the pallium gelatinosum and then through the tunica capsulis into the tunica interna. The mean potential inside the pallium gelatinosum relative to the medium was $-33{\cdot}5$ mV and between tunica interna and medium -13 mV.

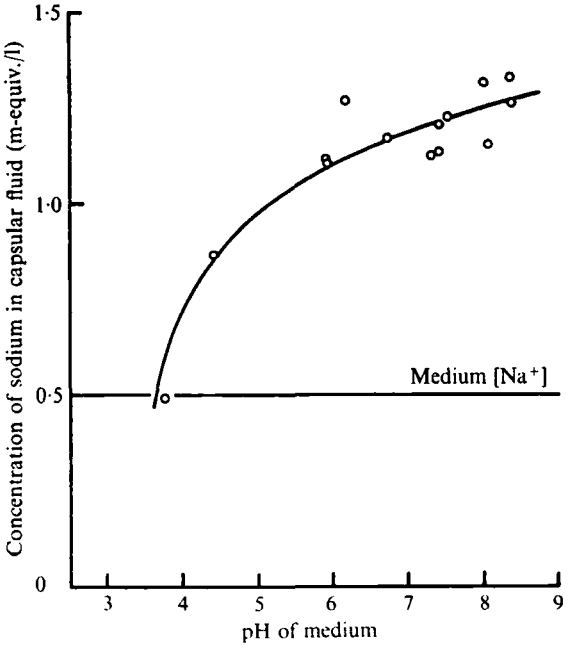

Fig. 5

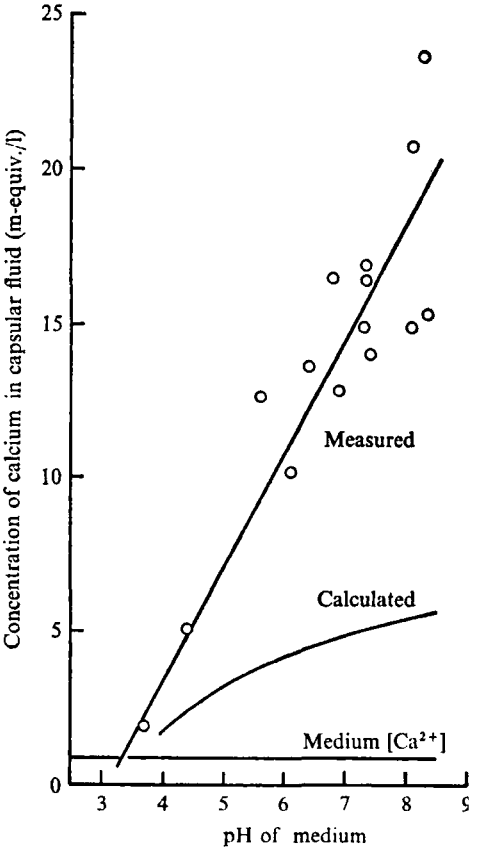

Fig. 6

Fig. 5. Relationship between external pH and sodium concentration in capsules. Capsules from three egg masses, each point a single measurement.

Fig. 6. Relationship between external pH and calcium concentration in the capsules. Capsules from three egg masses, each point a single measurement. Calculated curve $[Ca^{2+}]_o.[Na^+]_i^2/[Na^+]_o^2$ calculated from Fig. 5 represents the maximum calcium activity in the capsules (see Appendix).

The potentials are presumably associated with the polyanionic nature of these acid mucopolysaccharide layers (Plesch *et al.* 1971) and are thus analogous to Donnan potentials (Scott, 1968).

Effect of pH of the medium on composition of capsular fluid

A series of artificial lake waters containing $^{22}Na^+$ or $^{45}Ca^{2+}$ and having a range of pH values were prepared by adding small quantities of citric acid or 1 mM/l tris-HCl to LW 1 or by replacing bicarbonate with carbonate in LW 1. Capsules were equilibrated in these solutions for 1 day. The pH of the solutions was measured immediately after removal of the capsules for estimation of capsular sodium and calcium concentrations by the specific activity method. In Figs. 5 and 6 it is seen that the concentrations of these ions in the capsular fluid, especially that of calcium, are markedly dependent on the pH. Lowering the pH of the medium from 8·5 to 4·0 reduces the sodium concentration by about 45% and the calcium concentration by about 85%.

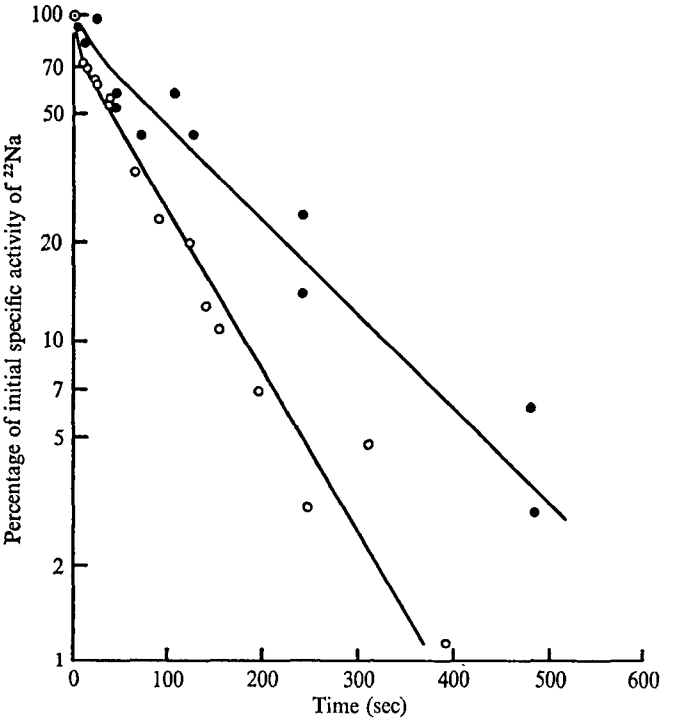

Fig. 7. Time course of loss in radioactivity from $^{22}Na^+$-LW 2-equilibrated capsules, during washout in unlabelled LW 2. ●, Single measurements on two egg masses containing large capsules (mean capsular volume 0·95 ± 0·02 μl [S.E. of 15] and 1·08 ± 0·03 μl [S.E. of 5]. ○, Mean of two measurements on an egg mass containing smaller capsules (0·73 ± 0·01 μl [S.E. of 10]).

This would be expected if the impermeant anions in the capsular fluid are weak acids or zwitterions. Lowering the pH would reduce the total charge on the impermeant anions and therefore the number of ions which could be held by a Donnan equilibrium. In Figs. 5 and 6 the sodium and calcium concentrations are the same as in the medium at about pH 3·5. At this pH there is thus no net charge on the capsular colloids; i.e. pH 3·5 is their mean isoelectric point.

In Fig. 6 the curve representing the maximum value of capsular calcium activity ($[Ca^{2+}]_o[Na^+]_i^2/[Na^+]_o^2$, see Appendix) derived from the curve in Fig. 5 is plotted. As in Fig. 1, the calcium activity is always much lower than the measured concentration.

Sodium and calcium fluxes across the capsular membrane

Beadle (1969*a*) demonstrated that the capsular membrane is highly permeable to solutes of low molecular weight by observing the osmotic effects of various external solutes on the turgor of capsules. It is of interest to place these observations on a more quantitative basis. Figs. 7 and 8 show the steady-state washout and uptake curves for $^{22}Na^+$ in *Lymnaea* capsules in equilibrium with LW 1, where the medium volume is sufficiently large to act as a constant reservoir. It is seen that the half-time for equilibrium of the capsules with $^{22}Na^+$ is very fast indeed, being 40–100 sec, the shorter half-times being given by the smaller capsules. These values can be compared with a half-time of about 10 sec, which would be expected if the capsules behaved

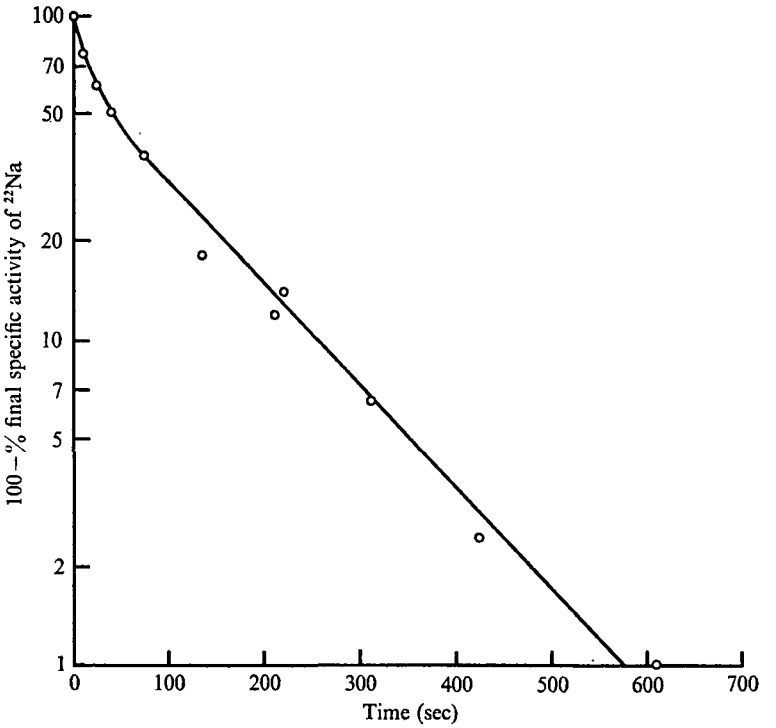

Fig. 8. Increase in radioactivity by LW2-equilibrated capsules in $^{22}Na^{+}$-LW2, expressed as difference between final, fully equilibrated activity and actual activity. Each point mean of two measurements.

simply as small unstirred volumes of aqueous solution without any external diffusion barrier (estimated from equilibration curves for spheres and cylinders calculated by E. J. Harris, 1956; assuming $D_{Na^+} = 1{\cdot}4 \times 10^{-5}$ cm^2.sec^{-1}; $r = 0{\cdot}5$ mm). Evidently the capsular membrane offers little resistance to the diffusion of sodium ions. This suggests that it has a very open porous structure. However, the membrane is very 'tight' towards substances of M.W. higher than 3300 (Beadle, 1969). Electron micrographs indicating that the capsular membrane has a fibrous nature in *Biomphalaria* (Beadle, unpublished) are consistent with these properties.

The washout curve for $^{45}Ca^{2+}$ in capsules in equilibrium with LW 1 is shown in Fig. 9. It has a half-time of 300 sec. Simple graphical compartmental analysis (Fig. 9) resolves the capsular calcium into two pools: 88·7% of the calcium exchanges with the medium with a half-time of 250 sec and 11·3% exchanges with a half-time of 1680 sec. (If the two compartments are in series then this method of analysis is not strictly valid. The error would, however, be quite small in this case.) The fast component presumably represents diffusion of calcium ions across the capsular fluid and capsular membrane. The relatively slower half-time of calcium ions compared with sodium ions may be attributed to two factors. Firstly, calcium ions have a slightly lower diffusion coefficient ($0{\cdot}8 \times 10^{-5}$ cm^2 sec^{-1}, calculated from equivalent conductance). This would increase the half-time proportionately. Secondly, calcium ions would be expected to be retarded to a slightly greater extent by collisions with the

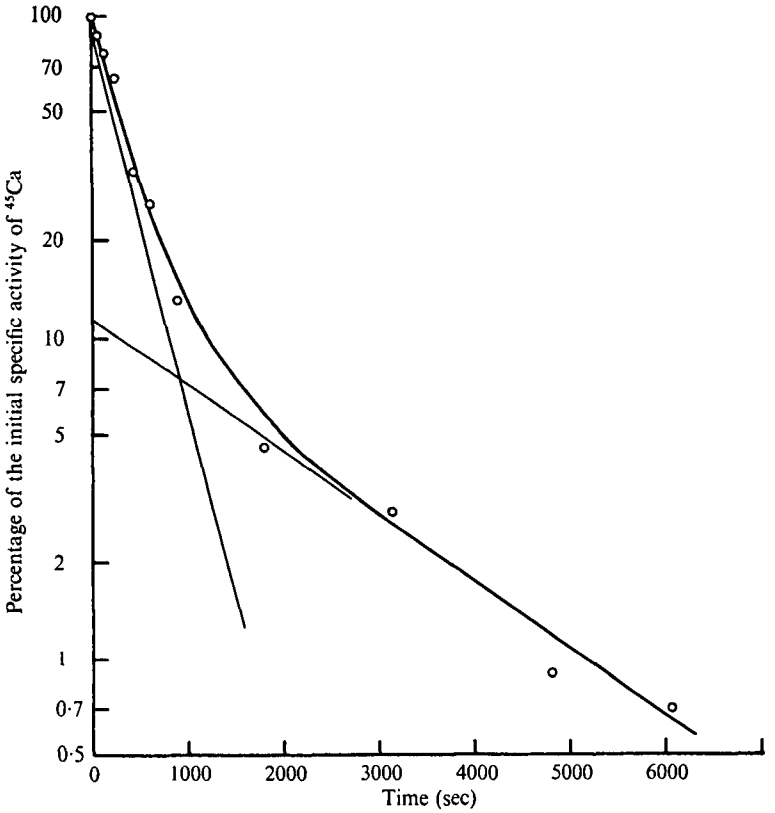

Fig. 9. Time course of loss in radioactivity by $^{45}Ca^{2+}$-LW 1-equilibrated capsules during washout in unlabelled LW 1. Each point is the mean of two measurements. The curve drawn is the sum of the two straight lines shown which represent compartments of 88·7 % and 11·3 % of the total Ca^{2+} declining with half times of 250 sec and 1680 sec respectively.

matrix of the capsular membrane as they have a larger hydrated ionic radius (Na^+, 5·6 Å; Ca^{2+}, 9·6 Å; Conway, 1954).

The slow component presumably represents a bound fraction of the calcium exchanging with free calcium. This fraction may not represent the whole of the non-diffusible calcium in the capsule since in general one would expect adsorbed or chelated calcium to be exchanged so rapidly as to be indistinguishable from the diffusible calcium.

Fig. 10 shows the washout of $^{22}Na^+$ from capsules equilibrated in lake water and washed in distilled water. In this case loss of activity represents a net loss of capsular sodium. About half of the sodium is lost in 500 sec. The curve is difficult to interpret precisely because the system is not in steady state, several ions are being lost simultaneously at varying rates and no doubt pH changes are occurring in the capsular fluid. It has been shown already that these factors will interact in a complex way. However, it is clear that as far as sodium is concerned, the capsular membrane and capsular fluid provide some protection against temporary, drastic dilution of the medium over a period of a few minutes, but little protection against dilutions lasting more than $\frac{1}{2}$ h. Under normal conditions the outer layers of the egg mass may provide an additional diffusion barrier.

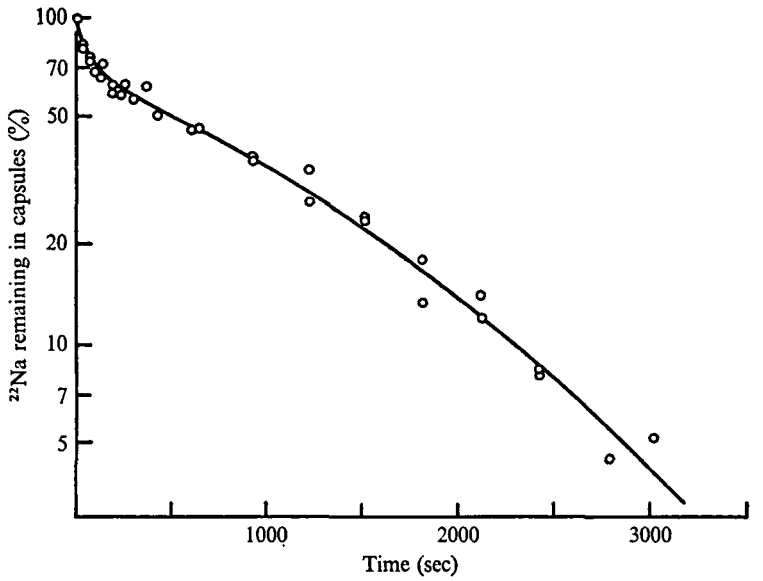

Fig. 10. Time course of loss in radioactivity by $^{22}Na^{+}$-LW 1-equilibrated capsules during washout in distilled water. Each point is the mean of two measurements. Two egg masses used.

DISCUSSION

Calcium has been qualitatively demonstrated in the capsular fluid of a number of species of terrestrial and freshwater gastropods, including *Lymnaea stagnalis* (George & Jura, 1958; Morrill *et al.* 1964; Bayne, 1968*a*; Bartelt, 1970). For *Lymnaea stagnalis* and *Biomphalaria sudanica* these observations have now been quantified and extended to both sodium and calcium in the latter species and to the four cations, sodium, potassium, calcium and magnesium in the former. In both species all of these cations were at a higher concentration in the capsular fluid than in the medium under all conditions.

The activities of cations, particularly divalent ones, in the capsular fluid will not be as high as their chemical concentrations, partly as a result of the high ionic strength of the polyvalent capsular colloids, and partly because some of the calcium may not be in freely diffusible form. However, the potential measurements clearly indicate that in *Lymnaea* the internal activity of cations is always higher than their activity in the medium and that this situation is maintained by a Donnan equilibrium across the capsular membrane. These measurements indicate that in LW 1 the ratios of internal to external activity are about 3 and 10 for monovalent and divalent cations respectively, and that on dilution of the medium these ratios may rise to over 10 and over 100 respectively. A Donnan equilibrium of this kind probably occurs in other freshwater gastropods since proteins are invariably present in the capsular fluid (Morrill, 1963; Morrill *et al.* 1964; Bayne, 1968*a*; Wright & Ross, 1965, 1966). A potential of about 20 mV has been recorded between the capsular fluid and the medium (LW 3) in *Biomphalaria* (L. C. Beadle, personal communication).

The capsular membrane/capsular fluid system thus appears to act as a 'cation buffer' maintaining the internal cation activities at a higher level than in the external

medium and varying in a non-linear manner with the external concentration. As the impermeant anions in the capsular fluid behave as weak acids, the fluid also acts as a pH buffer although at the expense of changes in the total internal cation concentration.

The tracer-flux experiments indicate that the capsular membrane allows a very rapid diffusional exchange of ions and small molecules with the medium. This has obvious importance for gas exchange, nitrogenous excretion and uptake of ions from the medium but indicates that the capsular membrane is not providing much protection from the dilute external medium.

The importance of the 'cation buffer' for the ionic relations of the embryo will now be considered. The perivitelline fluid could serve as a store, albeit a dynamic store, of cations which are incorporated into the embryo during development. Indeed it is known that the capsular fluid is taken up, by pinocytosis or by ingestion, at all stages of development (Raven, 1946; Bluemink, 1967, 1970) and metabolized by the embryo (Horstmann, 1956*a*, *b*; Morrill, 1964).

However, it is probable that ions taken up in this way do not contribute greatly to the total influx into the embryo at any stage. *Biomphalaria* embryos must accumulate exogenous sodium since the total sodium content of embryos *plus* capsules increases during development (Beadle & Beadle, 1969). *Lymnaea* embryos can take up exogenous sodium and calcium (Taylor, 1973). Furthermore, from the two-cell stage onwards the embryos rapidly exchange water and sodium with the capsular fluid or an artificial bathing medium (Raven, 1946; Taylor, 1973). Since the embryos are both hyperosmotic and hyperionic with respect to sodium from the single-cell stage onwards (Raven & Klomp, 1946; Taylor, 1973) it appears that the embryos are exhibiting active, hyperosmotic regulation from the two-cell stage at the latest.

The importance of the elevated cation activities in the capsular fluid is not obvious. The embryo cannot make any saving in the theoretical minimum energy requirements for ion uptake by this arrangement. The presence of the Donnan equilibrium across the capsular membrane means that the activity ratios of permeant anions are the reciprocals of the cation ratios. The energy requirements for neutral salt uptake are thus exactly the same as if it were bathed by the external medium.

It is worth pointing out that the embryo probably does not need to take up as many anions as cations. R.Q. measurements on *Lymnaea* embryos indicate that they retain a considerable proportion of the respiratory carbon dioxide (Baldwin, 1935). This is probably incorporated into the shell as calcium carbonate, a mechanism also suggested for the adult (Greenaway, 1971*b*). Embryos will develop normally in media of very low chloride concentrations (Taylor, 1973) and it may be that bicarbonate is the main inorganic anion in the embryonic tissues. However, cations must be exchanged for hydrogen ions in this scheme, and since the hydrogen ion concentration in the capsular fluid is raised by the Donnan equilibrium, the embryos still make no theoretical overall economy in minimum energy requirements by possession of the capsule (unless the hydrogen ions are lost down their concentration gradient but this is rather unlikely under normal conditions).

Another way in which the capsular fluid could be important for the ionic relations of the embryos is as follows. In other freshwater animals the rate of ion uptake depends on the external concentration and the 'affinity' of the transporting mechanism, the relationship being described by an equation similar to the Michaelis–Menten

equation (Shaw, 1964). At low external concentrations the rate of uptake is proportional to the external concentration but at higher concentrations the pump is 'saturated' and the influx becomes independent of concentration. Ion uptake mechanisms with these characteristics have been demonstrated in adult *Lymnaea* for both sodium (external concentration for half max. influx 0·25 m-equiv./l; near max. influx 1·5–2·0 m-equiv./l) and calcium (half max. influx 0·6 m-equiv./l; near max. influx 2–3 m-equiv./l) (Greenaway, 1970, 1971*a*). If pumps of similar affinity were present in *Lymnaea* embryos, neither would be saturated when bathed directly by the medium LW 1. However, for the encapsulated embryo the sodium pump would be working at about half its maximum rate and the calcium pump at near maximum rate even at 20 times dilution of the medium (Fig. 1). Greenaway's figures actually refer to concentrations. In terms of activities saturation would be achieved at an even lower level.

It is therefore tentatively suggested as a basis for further experiments that an important function of the capsular membrane/capsular fluid system is to regulate the rate of ion uptake by the embryos, by keeping their ion-uptake mechanisms near saturation. Shaw (1964) demonstrated that sodium regulation in the crayfish is a negative feedback mechanism dependent on the stimulation of the uptake rate consequent on depletion of blood sodium. A similar stimulation of sodium uptake occurs on sodium depletion of adult *Lymnaea* although this could not be shown for calcium uptake (Greenaway, 1970, 1971*a*). However, a feedback regulatory mechanism of this kind would probably be unsuitable for small embryos since there is an inevitable time lag before it can come into operation, and embryos of large surface area to volume ratio would no doubt be depleted very quickly.

The cation buffer is probably important in other ways, particularly with respect to calcium. Calcium ions reduce the permeability to ions and water of the membranes in a variety of cells and tissues (see Davson, 1970, for literature). This property is particularly important in freshwater animals. The classical case of the flatworm, *Procerodes ulvae* may be quoted. This animal can survive long periods in soft water to which 2–4 m-equiv./l Ca^{2+} has been added but at low calcium concentrations the animal quickly disintegrates as a result of increased osmotic water uptake and salt loss (Pantin, 1931; Weil & Pantin, 1931). That this might also apply to *Lymnaea* embryos is suggested by some experiments performed by Raven & Klomp (1946). Between oviposition and first cleavage the embryos at the single-celled stage swell due to osmotic uptake of water. However, the rate of water uptake is considerably higher in embryos isolated in distilled water than in the encapsulated embryos. If more than 2·5 m-equiv./l calcium ions are added, the swelling is normal. In calcium-free media the cleavage of isolated embryos has an abnormal course, the blastomeres remaining spherical and separating instead of adhering, before forming a cleavage cavity. This effect is also relieved by the presence of 2·5 m-equiv./l calcium ions. Raven and Klomp interpreted this as an effect on the vitelline membrane, though it seems also possible that calcium ions are needed for the formation of the septate desmosomes which are present between the blastomeres (Bluemink, 1967; Taylor, unpublished observations). Calcium ions are known to affect the properties of septate desmosomes (Loewenstein, Nakas & Socolar, 1967).

There is an extensive literature dealing with the effects of cations (particularly

lithium) and other agents on morphogenesis in *Lymnaea* (reviewed by Raven, 1964). The main conclusion from this work is that there exists in the egg cortex (perhaps the plasmalemma itself) a gradient (the cortical field) which controls the distribution of morphogenic substances in the egg, and thus the patterns of later cellular differentiation. It was suggested that calcium ions are involved in controlling the stability of the field. The possession of a calcium activity buffer in the capsular fluid would obviously be very valuable in such a system.

A disadvantage of the cation buffer system in the capsular fluid is that, although it buffers well when all the external ions are varied in proportion, fluctuations in any one of the cations in the medium will produce an inverse change in all of the other cations in the capsule. For example, a small increase in the activities of divalent cations in the medium produces a large decrease in the activities of internal monovalent ions; dilution of the medium produces a fall in capsular pH, and a fall in external pH causes a marked decrease in the activities of internal cations, particularly of calcium. Mutual interactions of this sort obviously must be considered when studying the lower or upper limits of an ion in the medium for normal development (Beadle & Taylor, 1973) or when interpreting the results of the kinds of experiments mentioned above, concerning the effects of high concentrations of cations on morphogenesis.

The ability of the embryo to cope with ionic conditions in the environment and to perform normal morphogenesis and ionic regulation is one of the many factors limiting the distribution of freshwater molluscs. Clearly calcium is among the more important ions in this respect. However, it is now clear that, from the embryos' point of view, the calcium activity in the capsular fluid is likely to be the most important parameter and that simply measuring the calcium content of the water would give a very unrealistic estimate of this value. For example, a high magnesium content of the water or a low pH would seriously reduce the physiologically available calcium. Effects of this kind may contribute towards some of the difficulties encountered in trying to correlate the distribution of molluscs in general and *Lymnaea stagnalis* in particular with calcium content of natural waters (Boycott, 1936; Macan, 1950). With respect to pH perhaps one should consider the extreme pH reached during the diurnal cycle rather than the average value as the limiting factor. Morphogenetically disastrous effects can be induced very quickly at critical developmental stages.

Most of the experiments reported here have been performed on capsules which have been isolated from the outer layers of the egg mass. However, embryos in such capsules will develop perfectly normally when bathed in artificial lake water. Indeed, whatever the conditions in the outer layers themselves, if they are permeable to water and solutes, they cannot affect the equilibrium conditions in the capsular fluid.

The demonstration of Donnan potentials in the pallium gelatinosum and tunica interna confirm their polyanionic nature as suggested by the histochemical demonstrations of acid mucopolysaccharides in these layers (Plesch *et al.* 1971; Jura & George, 1958). This would account for the high calcium content observed by a number of workers (Jura & George, 1958; Bayne, 1968*a*).

It is probable that an important function of the outer layers of the egg mass is to provide an additional diffusion barrier affording better protection for the embryo against transient changes in medium composition and possibly also against brief exposure to the air (Bayne, 1968*b*). Jura & George (1958) noted that the jelly appeared

to have a protective function during early cleavage against experimentally applied sodium tauroglycocholate but that this did not seem to apply to lithium chloride. This was explained as the result of binding of tauroglycocholate with calcium in the jelly. Possibly a more important factor is that the polyanionic jelly layers would be expected to be much less permeable to anions (especially to anions of relatively high M.W. like tauroglycocholate) than to cations (Scott, 1968). It is probable that in the case of tauroglycocholate the capsular fluid does not reach diffusion equilibrium with the medium until after the embryos have passed their critical period. This property of the outer layers of the egg-mass may have some general significance in the protection of the embryo from the possible toxic effects of organic anions in the medium.

SUMMARY

1. The cations sodium, potassium, calcium and magnesium are always at a higher concentration in the capsular fluid (perivitelline fluid) of *Lymnaea stagnalis* and *Biomphalaria sudanica* than in the bathing medium. The concentration of each ion is a complex function of the concentrations of all ions in the medium and of pH.

2. Typical values for the ratio of capsular concentration to medium concentration for *Lymnaea* capsules isolated in slightly alkaline water of average hardness are 2·4–5 for monovalent and 18–40 for divalent cations.

3. Lowering the pH of the medium from 8·5 to 4·0 reduces the capsular concentrations of monovalent ions by about 45% and of divalent ions by about 85%.

4. Consideration of the potential difference between the capsular fluid and the medium, and comparison of the monovalent and divalent ion ratios, indicates that a Donnan equilibrium exists across the capsular membrane.

5. On dilution of the medium the above ratios may rise more than tenfold and the potential difference may rise from about 23 mV to above 60 mV (inside negative). It is concluded that the capsular fluid/membrane system buffers the internal cation activities against changes in external cation activities, particularly in the case of calcium.

6. It is suggested that the 'calcium-buffer' may be important in maintaining ion-uptake mechanisms near saturation, in reducing the permeability of the embryo to salts and water, in maintaining cellular adhesion and in morphogenesis.

7. The fluxes of $^{22}Na^{+}$ and $^{45}Ca^{2+}$ across the capsular membrane have half-times of 40–100 sec and about 300 sec respectively. The membrane offers little resistance to the free diffusion of these ions.

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APPENDIX

If the distribution of cations is determined by a Donnan equilibrium across the capsular membrane,

$$\sqrt{\frac{[Ca^{2+}]_i f_1}{[Ca^{2+}]_o f_2}} = \frac{[Na^+]_i f_3}{[Na^+]_o f_4},$$

where $[Ca^{2+}]$ and $[Na^+]$ are the concentrations of free (diffusible) calcium and sodium ions in the capsular fluid (i) and medium (o) and f_1–f_4 are the corresponding activity coefficients.

$$\therefore \quad [Ca^{2+}]_i = [Ca^{2+}]_o \left(\frac{[Na^+]_i}{[Na^+]_o}\right)^2 \frac{f_2 f_3^2}{f_1 f_4^2}.$$

From the Debye-Hückel theory, $f_1 = f_3^4$; $f_2 = f_4^4$.

$$\therefore \quad [Ca^{2+}]_i = [Ca^{2+}]_o \left(\frac{[Na^+]_i}{[Na^+]_o}\right)^2 \sqrt{\frac{f_2}{f_1}} \tag{1}$$

and

$$[Ca^{2+}]_i f_1 = [Ca^{2+}]_o \left(\frac{[Na^+]_i}{[Na^+]_o}\right)^2 \sqrt{f_2 f_1}. \tag{2}$$

Under the conditions of the experiment shown in Fig. 1, f_1 and f_2 will be reasonably constant so that the concentration $[Ca^{2+}]$ and the activity $[Ca^{2+}]_i f_1$ of free calcium ions in the capsules will be proportional to $[Ca^{2+}]_o[Na^+]_i^2/[Na^+]_o^2$ as a first approximation. Further, the calcium activity must be less than the value of this expression (as $f_2 f_1 < 1$) and the calcium concentration greater (as $f_2/f_1 > 1$).

It is not possible to calculate the actual values of either of these quantities since the bound fraction of the measured capsular calcium concentration is not known. However, one can estimate their possible range. Consider the capsules in LW 1.

$$f_2 = 0{\cdot}78 \text{ (from Debye-Hückel equation)},$$

$$[Ca^{2+}]_o[Na^+]_i^2/[Na^+]_o^2 = 4{\cdot}8 \text{ m-equiv./l} \quad \text{(from Fig. 1)}.$$

If it is assumed that there is no bound calcium at all in the capsules, $[Ca^{2+}]_i$ = 14·5 m-equiv./l (from Fig. 1). Substituting these values in equation (1) one obtains $f_1 = 0{\cdot}086$ and the internal calcium activity, $[Ca^{2+}]_i f_1 = 1{\cdot}24$ m-equiv./l.

These represent absolute minimum values and are rather improbable. The ionic strength of the capsular fluid would be very high indeed to produce such a low activity coefficient. Moreover, the agreement between the measured potentials and calculated sodium equilibrium potentials (Fig. 3) suggests that the ratio $f_3/f_4 = \sqrt[4]{f_1}/\sqrt[4]{f_2} \simeq 1$. If the more reasonable value of, say, $f_1 = 0{\cdot}5$ is substituted in equation (1) one obtains $[Ca^{2+}]_i$ = 6·0 m-equiv./l and $[Ca^{2+}]_i f_1$ = 3·0 m-equiv./l, implying that a considerable proportion of the capsular calcium is bound.