SALT AND WATER REGULATION IN THE EMBRYOS OF FRESHWATER PULMONATE MOLLUSCS

I. THE EMBRYONIC ENVIRONMENT OF BIOMPHALARIA SUDANICA AND LYMNAEA STAGNALIS

By L. C. Beadle

Departments of Zoology, Makerere University College, Kampala, Uganda, and the University of Newcastle upon Tyne

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INTRODUCTION

Very little is known of osmotic and ionic regulation in the eggs and embryos of freshwater animals but reviews which have dealt with this subject have generally concluded that they must be protected by membranes almost impermeable to water and salts from the moment of laying until such time as they have developed mechanisms for maintaining their greatly hypertonic internal fluids. This would involve the storage of enough inorganic ions to carry them through to this point (Krogh, 1939, p. 180; Prosser & Brown, 1961, p. 27; Potts & Parry, 1964, p. 222). The general suppression of larval stages in fresh water as compared with marine animals has been explained as a device for delaying hatching to allow for the development of regulatory mechanisms by the time that they are fully exposed to the very dilute environment (Needham, 1930; Hutchinson, 1967, p. 190). There are, however, some exceptions such as the nauplius larvae of copepod crustacea and particularly the minute planula larvae of the freshwater medusae which certainly have no special regulatory organs and whose outer ciliated cells are fully exposed to the water. The adults, too, of the freshwater coelenterates are devoid of specialized organs for this purpose, but nevertheless Pelmatohydra has been shown to possess an ionic regulating mechanism involving the active uptake from the water of at least sodium and potassium (Lilly, 1955). The animal could obviously not exist without it. From weighing and analyses for ions in the eggs and embryos of freshwater salmonid fishes it has been shown that there is some exchange of water as well as of sodium and calcium with the environment prior to hatching (Hayes, 1949; Potts & Parry, 1964, p. 223).

It will be clear from this and the following papers that the fluid in which the eggs of these molluscs are immersed is bounded by a membrane which permits a very rapid exchange of water and inorganic ions with the outside medium and that the eggs and very early embryos are actively engaged in osmotic and ionic regulation.

MATERIAL AND METHODS

Biomphalaria sudanica, one of the vectors of intestinal bilharzia (Schistosoma mansoni), is a planorbid snail very common in tropical Africa and is found in a variety of habitats including swamps. It is easily cultured in either natural pond water or in
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Capsular fluid. The egg is embedded in this, and it corresponds to the perivitelline fluid of fish and frog. In freshwater molluscs this fluid contains large organic molecules which are taken up as the main food supply after gastrulation. In *Lymnaea stagnalis* it has a dry weight of about 15%, of which 3–6% is the polysaccharide galactogen and 6–8% protein (Horstmann, 1956).

Capsular membrane. Sometimes called the chorion (e.g. of the trout egg); in *Biomphalaria sudanica* about 0.1 μ thick, as seen in electron micrographs.

Capsule. This comprises all the foregoing items. In *B. sudanica* there may be 8–20 capsules per mass; in *L. stagnalis* up to 200.

Outer jelly. This surrounds the capsules and varies greatly in bulk, very small in *B. sudanica* and very large in *L. stagnalis*.

Envelope. The layer surrounding the entire mass. In *B. sudanica* it has a similar consistency to the capsular membrane but is thicker (about 1 μ). In *L. stagnalis* it is a very thick fibrous coat (Fig. 1).

The capsules of *Biomphalaria*, though spherical when isolated in water, are somewhat compressed between the upper and lower layers of the envelope from which they are difficult to separate. They are often compressed against one another. To isolate a capsule it is usually necessary to destroy those in contact with it. To estimate their volume the area and mean depth of 30 capsules in three masses were measured by micrometer. The calculated volumes ranged from 0.29 to 0.47 mm.³ with a mean of 0.34 mm.³.

The capsules of *Lymnaea* can be easily separated from the outer jelly and, owing to their regular oval shape, the volume can reasonably be estimated from the formula \( \frac{1}{6} \pi ab^2 \), where \( a \) is the length and \( b \) the greatest diameter (Raven & Klomp, 1946). The volumes of about 50 capsules from two masses ranged from 0.64 to 0.77 mm.³, giving a mean of 0.70 mm.³.

Fig. 1. Egg-masses of: (a) *Biomphalaria sudanica* of about average size attached to a flat surface; the capsules are pressed between the upper and lower envelope and the volume of the jelly is very small. (b) *Lymnaea stagnalis*, a portion of a very small mass which is cylindrical and the volume of the jelly is very large. The apparent variation in shape of the capsules is due to differences in orientation. They are in fact very regularly oval. Drawn to same scale from photographs of living masses.
Some properties of the capsular membrane and the colloid osmotic pressure of the capsular fluid.

Apart from acting as a container for the fluid, the capsular membrane has an important function in protecting the early embryo from the attacks of micro-organisms. As soon as it is damaged bacteria, ciliates and rotifers are quick to make an entrance.

The size of molecules which can pass the membrane was determined by immersing isolated capsules in aqueous solutions of substances of increasing molecular weight and observing the point of shrinkage. The eggs of newly laid masses were first destroyed by exposing to about 2° C. in a refrigerator for 24 hr. Complications arising from the subsequent uptake or alteration of the capsular fluid by the embryo were thus avoided.

Treatment with lake water of 10 times standard concentration had no apparent effect. In 100 times standard, however, the capsules of both species began to shrink by wrinkling within 5 min. After 3 hr. re-swelling was well under way and the normal turgor was regained in about 5 hr. The capsular membranes are thus more permeable to water than to inorganic ions, but are in fact very permeable to both. It follows from this that the concentration of diffusible ions should be equal on both sides of the membrane, apart from a possible Donnan effect due to large indiffusible ions in the capsular fluid. That the sodium was approximately equally distributed on the two sides was shown by immersing masses of Biomphalaria in normal lake water containing $^{22}$Na. The final activity per capsule was compared with that of an equal volume of the water (capsular volume assumed to be 0.34 mm$^3$). It will be seen from Table 1 that the concentration on the two sides was approximately equal. This was an important point to settle in relation to experiments on sodium uptake by embryos described in the next paper (Beadle & Beadle, 1969), where details of the technique are to be found. Counts were taken after 8 hr. and once per day for the following 4 days.

Table 1. Egg-masses of Biomphalaria sudanica immersed (after destruction of eggs by cooling) in standard lake water containing two concentrations of $^{22}$Na

(Counts taken after 8 hr. and once daily for next 4 days.)

<table>
<thead>
<tr>
<th>No. of capsules in mass</th>
<th>Per capsule (mean of 5 counts)</th>
<th>From 0.34 mm$^3$ (mean vol. of capsules) of water</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>52 (44–59)</td>
<td>50</td>
</tr>
<tr>
<td>9</td>
<td>53 (48–60)</td>
<td>—</td>
</tr>
<tr>
<td>9</td>
<td>99 (94–108)</td>
<td>120</td>
</tr>
<tr>
<td>11</td>
<td>113 (112–116)</td>
<td>—</td>
</tr>
<tr>
<td>14</td>
<td>122 (120–127)</td>
<td>—</td>
</tr>
<tr>
<td>18</td>
<td>100 (96–108)</td>
<td>—</td>
</tr>
</tbody>
</table>

Some indication of the pore-size of the capsular membrane was obtained from immersion of isolated capsules in solutions of non-electrolytes in standard lake water. With Biomphalaria no permanent shrinkage could be induced by solutions of sugars of molecular weight up to that of sucrose (342). Ten mM sucrose caused immediate...
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Shrinkage, but within 7 hr. normal volume had been regained. But raffinose (M.W. 504) induced permanent shrinkage in concentrations of 1.5 mM and over. This demonstrated both the impermeability to raffinose and an internal colloid osmotic pressure equivalent to that of about 1.5 mM non-electrolyte. The latter of course relates to the capsular fluid before development has started. Experiments on capsules containing live embryos showed, as expected, a diminution in colloid osmotic pressure towards the end of development when the embryo had ingested much of the organic contents of the fluid.

The pore-size of the capsular membrane of *Lymnaea stagnalis* is, however, very different from that of *Biomphalaria*. Forty mM raffinose caused no shrinkage even temporarily. In 50 and 100 mM raffinose there was some shrinking within a minute followed by re-swelling during the next few hours. The membrane is therefore permeable to raffinose (M.W. 504). It is difficult to find a non-electrolyte of molecular weight much higher than 500 which is sufficiently soluble, but two of the Carbowaxes (polyethylene glycols), no. 1500, M.W. 500–600, and no. 4000, M.W. 3300, were tried. The molecular weight of no. 1500 is close to that of raffinose and the same result was obtained; that is, it passed readily through the capsular membrane. The membrane was, however, impermeable to no. 4000 and, by using a range of concentrations, the initial colloid osmotic pressure of the capsular fluid was found to be equivalent to 4–5 mM non-electrolyte, which is considerably higher than in *Biomphalaria* (c. 1.5 mM). Within about 2 days of hatching the colloid osmotic pressure had fallen to about 1 mM.

The above experiments were carried out with isolated capsules of *Lymnaea*. It is interesting to know what influence the envelope and massive outer jelly has on the diffusion of substances from the water to the capsules. Whole masses were immersed in 100 mM raffinose for 12 hr. In contrast to the temporary shrinkage caused in naked capsules, there was no visible effect at any time. On dissecting the capsules out into 100 mM raffinose, there was no shrinkage, showing that the raffinose had entered the capsules. This was confirmed by exposing them next to standard lake water for 1 hr. followed by re-immersion in 100 mM raffinose. Shrinkage occurred within 1 min. followed by re-swelling within 2 hr. The envelope and outer jelly therefore retard the inward diffusion of raffinose but do not prevent it. Similar experiments (using sucrose) with intact masses and isolated capsules of *Biomphalaria* showed that the very thin envelope and scanty outer jelly had no detectable influence on the rate of inward diffusion of the sugar.

If it is true that the function of the capsular fluid is to provide a sterile source of nourishment for the post-gastrulation stages, then extracted embryos should survive in standard lake water until their food supplies are exhausted. Stages prior to gastrulation should be the least viable and subsequently survival should depend on the amount of capsular fluid previously ingested, provided that the medium is kept sterile.

With the sterilizing technique described above (Methods) a high proportion of the extracted embryos of *Biomphalaria* could be kept free of infection for at least a week. Those that had been mechanically damaged by the dissecting needles disintegrated within 1 hr. and were discarded, as were those which subsequently became infected. Each batch of embryos was isolated in sterile standard lake water in covered hollow glass blocks. The embryonic stages will be fully listed in the next paper (Beadle & Beadle, 1969).
Starting stages

IVa: 9 early blastulae; 24 hr. normal, 48 hr. all disintegrated without apparent infection.

Ve: 8 early trochophores (post-gastrulation); 48 hr. actively moving but yolk vacuoles gone; 72 hr. all disintegrated.

VIIIa: 10 embryos with head and foot rudiments but no eyes or tentacles. In 2 days tentacles and eyes appeared, then progressive dying off to 6 days.

IXa. 25 embryos with tentacles and eye rudiments. Lived for 12-14 days, becoming progressively less active and exhausting yolk vacuoles.

Xb: 22 advanced embryos within 2 days of hatching. Behaved as normal hatched snails and were fed after 10 days.

So far as it goes this experiment supports the conclusion that the capsular fluid has no special osmotic or ionic function during normal development. The experiment with early blastulae is the most crucial. Whatever the cause of ultimate disintegration, the fact that this rather simple and undifferentiated organism about 0.1 mm. in diameter can maintain itself for more than 24 hr. in lake water suggests that it is capable of actively regulating its salt and water content.

Discussion

The capsular membrane is concerned with retaining a nutritive medium (capsular fluid) round each egg and with protecting them from the attacks of micro-organisms. It provides no significant barrier to the diffusion of water and ions, and metabolic breakdown products up to a M.w. of 350 could rapidly diffuse through it. The significance of the greater pore-size of the capsular membrane of *L. stagnalis* is not obvious though the capsules are surrounded by a larger volume of jelly which retards diffusion.

The vitelline membrane immediately surrounding the egg is therefore the only structure between the egg and the outside water which might control the exchange of water and ions. There is no evidence that it does so, and it will be shown in the next paper that sodium is taken up by the pregastrula embryo through this membrane.

According to Elbers & Bluemink (1960) electron-micrographs of embryos of *L. stagnalis* show that capsular fluid is taken up by pinocytosis into small vacuoles in the superficial cells of early cleavage stages by temporary rupture of the vitelline membrane. This, however, does not obviously relate to the main exchanges of salt and water.

Summary

1. The envelope and capsular membranes which separate the eggs from the external water are freely permeable to water and inorganic ions.

2. The capsular membrane of *B. sudanica* is permeable to sucrose (M.w. 342) but not to raffinose (M.w. 504), that of *L. stagnalis* is permeable to raffinose but not to Carbowax (polyethylene glycol, M.w. 3000–3300).

3. By means of $^{22}$Na it was shown that, as expected, the concentration of sodium in the capsular fluid of *B. sudanica* is approximately equal to that in the external water.
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4. The initial colloid osmotic pressure of the capsular fluid of *B. sudanica* is equivalent to that of approximately 1.5 mM/l. solution of non-electrolyte and of *L. stagnalis* to that of 4–5 mM/l.

5. Observations on embryos of *B. sudanica* extracted from the capsular fluid and kept in lake water support the contention that, at least from late cleavage stages onwards, they are capable of active salt and water regulation.

The work described in this and the following two papers in the series was done during the tenure of a Wellcome Research Professorship at Makerere and continued at Newcastle with the support of the Medical Research Council. I am therefore very much indebted to the Wellcome Trust and to the Medical Research Council. When writing these papers I have had some helpful discussion with Professor J. Shaw.

REFERENCES


