J. exp. Biol. (1977), **69**, 143–172
With 12 *figures*
Printed in Great Britain

THE IONIC AND WATER RELATIONS OF EMBRYOS OF *LYMNAEA STAGNALIS*, A FRESHWATER PULMONATE MOLLUSC

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(*Received* 9 *December* 1976)

SUMMARY

From cleavage to hatching *Lymnaea stagnalis* (L.) embryos exhibit net uptakes of radioactive sodium, potassium and calcium greater than the amounts of these ions initially present in the capsular fluid, implying the early development of mechanisms for ion uptake from the dilute external water. The rates of net uptake increase very rapidly during trochophore to veliger stages when the main volume increase and shell formation takes place.

^{22}Na influx increases dramatically at first cleavage from 0·03 to 0·04 pmol min^{-1} in the hour before cleavage to 0·23 pmol min^{-1}. A further increase occurs in early cleavage and for decapsulated morula to gastrula stages the influx is estimated to be 2·75 pmol min^{-1}. ^{22}Na efflux rates are also high in decapsulated morulae, blastulae and gastrulae (minimal estimates 1·40–3·35 pmol min^{-1}) indicating a rapid exchange of sodium between the embryos and the medium. In sodium-free medium the ^{22}Na efflux from decapsulated blastulae is 0·5 pmol min^{-1}, suggesting that exchange diffusion is a large component of the fluxes. The characteristics of ^{22}Na efflux indicate that sodium does not exchange with the medium as a single compartment.

Expansion of the 'recurrent' cleavage cavity of early stages is accompanied by a rise in exchangeable sodium content from 5 pmol in embryos without cavities to 11–19 pmol in embryos with normally expanded cavities. Experimental expansions of the blastocoel of morula–blastula stages produced by cyanide are also accompanied by great increases in embryo exchangeable sodium content.

A general model is proposed for the movements of sodium and water in the embryos and this provides a mechanism for hyperosmotic regulation by the embryos.

INTRODUCTION

During their direct development from single cell stage to small adult, freshwater pulmonates are individually enclosed within the capsules of an egg-mass and bathed by a nutrient fluid (capsular fluid). Thus to a large extent the developing embryos are protected from interaction with the environment. However, they do not seem to avoid the problems of maintaining osmotic and ionic balance in a dilute external medium. The capsular membranes of *Lymnaea stagnalis* and *Biomphalaria sudanica* are in fact

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very permeable to small ions and molecules (Beadle, 1969*a*; Taylor, 1973). Although the capsular membrane/capsular fluid system in these species maintains the internal cation concentrations at a somewhat higher level than in the external medium by a Donnan equilibrium and provides some buffering against changes in external cation concentration (Taylor, 1973), the ionic and osmotic concentrations inside the capsules are still considerably lower than their overall concentrations in the eggs and embryos (Raven & Klomp, 1946; Elbers, 1966). Beadle & Beadle (1969) showed that the sodium content of the embryos plus capsules of *B. sudanica* increased several-fold between laying and hatching, suggesting that a mechanism for taking up sodium from the external water must appear relatively early in development.

In the present study the development of such ion uptake mechanisms has been investigated further using *L. stagnalis*, whose egg masses contain larger numbers (usually > 100) of more or less synchronously developing embryos. In order to estimate the ionic composition of early stages more precisely, analyses were performed on decapsulated embryos and the study was extended to include calcium as well as sodium (with a few measurements of potassium content). It is concluded that mechanisms for the net uptake of exogeneous calcium and potassium must also appear at an early stage.

It is of particular interest to consider the mechanisms concerned with ionic and osmotic regulation in the early developmental stages before the appearance of any obvious organs or surfaces specialized for ion uptake or excretion. It might be supposed that the plasma membrane or vitelline membrane enclosing cleavage stages is relatively impermeable to ions and that the animal simply prevents any significant loss during this period. Thus the fluxes of sodium ions between the medium and embryos up to trochophore stage were investigated using the tracer ^{22}Na. It is shown that there is in fact a very rapid exchange of sodium with the medium from the onset of cleavage.

Before the appearance of protonephridia at the trochophore stage, the elimination of osmotically entering water presents a problem. Thus close attention has been paid to the function of the 'recurrent cleavage cavity' described by many authors in freshwater pulmonate embryos (reviewed by Raven, 1966). Early embryos of various stages have been equilibrated with ^{22}Na-labelled media and the sodium content of the cleavage cavity estimated by comparing the ^{22}Na content of embryos with collapsed, normally swollen, and experimentally induced greatly swollen cavities.

MATERIALS AND METHODS

Almost all of the egg masses used for this study were obtained from laboratory cultures of *Lymnaea stagnalis* kept at Newcastle upon Tyne, England. However, Fig. 8 also includes some data from experiments performed on embryos of *Lymnaea stagnalis* obtained in Canterbury, New Zealand (introduced from Europe). The individual egg capsules were separated from the outer envelope and gelatinous layers as described previously (Taylor, 1973), then placed in artificial lake water (0·50 mM-$NaHCO_3$, 0·10 mM-$KHCO_3$, 0·43 mM-$CaCl_2$, 0·25 mM-$MgSO_4$) until required.

The exchangeable sodium, calcium and potassium contents of embryos were determined from measurements of the radioactivity of embryos equilibrated within their capsules in artificial lake water labelled with ^{22}Na, ^{45}Ca or ^{42}K at known relative specif-

activity. They were then removed from their capsules by tearing the capsular membrane with fine forceps and the viscous, radioactive capsular fluid washed off with a slow stream of unlabelled lake water and transferred to planchettes for measurement of radioactivity (wash time 30–40 s unless otherwise stated).

Because of the difficulty in performing this operation in a rapid and standardized fashion and the very large amounts of sodium in the capsular fluid compared with that in early embryos, and also the possible contribution of the capsules as a diffusion barrier, flux experiments and some measurements of total sodium content were performed on embryos isolated from their capsules and from adhering capsular fluid before introducing them to radioactive media. For measurement of sodium efflux, groups of 50–100 embryos from the same egg mass were equilibrated in labelled medium for 5–6 h. They were then removed and washed for varying times in unlabelled medium and the ^{22}Na remaining in the embryos was measured. Essentially similar experiments in which unlabelled embryos were placed in labelled medium for varying periods were used to determine sodium influx. Medium volumes were large enough to allow changes in medium specific activity to be ignored during flux experiments.

Embryos would not develop normally when isolated in the artificial lake water described above. Though single-cell stages or early cleavage stages would usually undergo one or two further divisions after placing in this medium, they did not form a normal cleavage cavity and the blastomeres swelled before finally disintegrating in a few hours. However, development from single cell stage up to gastrulation was apparently normal in embryos isolated in simple saline media containing higher ionic concentrations. Thus, four times normal strength artificial lake water was used as the isolation medium. The cation concentrations in this medium are closer to the concentrations normally found in the capsular fluid (Taylor, 1973). However, this medium is by no means an ideal substitute for capsular fluid. Although morphological development was normal, some developmental stages were delayed in this medium. For example, it was found that the interval between 2nd and 3rd cleavage was about twice that in encapsulated embryos. In addition there was no development past gastrulation. If post-gastrula stages were placed in this medium they underwent further morphological changes but no size increase was observed. Encapsulated post-gastrula embryos normally ingest the capsular fluid and large vacuoles accumulate in the endoderm cells. This was not observed in saline-isolated embryos, and already formed vacuoles gradually disappeared. Similar observations were also reported for *B. sudanica* embryos in saline media (Beadle, 1969*a*).

The embryos were transferred between experimental media using braking pipettes (Kirk, 1950). In order to assure rapid transference with minimal damage it was necessary to carry over about 0·1–0·5 μl of fluid with each embryo, i.e. several hundred times the embryo volume (1–2 nl). During washout the unlabelled medium in contact with the embryo was continuously renewed by allowing them to fall through four 3 ml aliquots of medium contained in solid watch-glasses as quickly as possible and then changing the medium every 30 s up to about 5 min and every minute thereafter. Between changes a stream of unlabelled medium was directed over the embryos with the braking pipette. Samples of a known volume of the final washing medium from adjacent to the embryo were frequently taken to estimate ^{22}Na contamination of the

medium. Normally this was undetectable but in some very quickly washed (10–30 s) embryos a small correction had to be made.

A large component of the sodium efflux for early stages reported in this paper has a very high rate (a few minutes half time). Hence it is necessary to consider the possibility that this represents loss of extracellular surface contamination rather than loss of sodium from the embryo itself. The reasons for believing that this is not the case and that the above described rinse procedure is adequate are as follows:

1. The quantity of medium (of relatively low sodium concentration) which could be trapped under the vitelline membrane (pre-gastrula stages only) or within the relatively sparse surface microvilli (mean length about 0·5 μm – from unpublished electron microscopical observations) would represent an insignificant sodium pool ($\sim 1{\cdot}6 \times 10^{-2}$ pmol).

2. An 'unstirred layer' could exist at the surface of the embryo as it falls through the washing medium. However, under the conditions of stirring described above it seems unlikely that the layer would exceed a few tens of micrometers thickness and at the most, say, 100 μm (see House (1974) for estimations of unstirred layer thickness in various tissues). Note that the diameter of the embryo itself is only about 100 μm. The sodium in such layers would be expected to equilibrate with the medium within a few seconds (see for example Davson, 1970 p. 397).

3. When the embryos are washed in sodium-free saline the fast component disappears (see Fig. 11*a*).

In all experiments, unless stated to the contrary, groups of three embryos were washed together and placed on the same planchette for determination of radioactivity. For measurement of ^{42}K activity embryos were pooled in groups of five. Embryos and medium samples were placed on planchettes containing 0·5 ml of distilled water, 50 μl of M glucose and a trace of detergent. They were then mixed with an air jet, dried at 60 °C and counted using an I.D.L. low-background counter. The samples from experiments performed in New Zealand were dried on filter paper strips placed in 15 ml g/l 2,5-diphenyloxazole (P.P.O.) in toluene using a Nuclear Chicago liquid scintillation counter. Appropriate corrections were made for radioactive decay of ^{45}Ca and ^{42}K during the course of the experiment.

All operations with embryos were performed under a binocular dissecting microscope with viewing conditions suitable for estimation of developmental stage and state of expansion of the cleavage cavity. Care was taken that groups of embryos counted together on a single planchette were similar in these respects and any damaged embryos were discarded. To measure the diameters of normal and experimentally swollen embryos they were transferred to a cell mounted on the stage of a compound microscope (×10 objective) fitted with a micrometer eyepiece. Diameters were measured to the nearest 5 μm and used to estimate embryo volume on the assumption that they were spherical. Thus the precision of volume measurements was approximately ±0·1 nl for normal embryos of volume about 1·5 nl, ±0·15 nl for swollen embryos of about 2·5 nl, and ±0·3 nl for greatly swollen embryos of about 7 nl.

All embryos were kept in an incubator at 25 °C, except actually during observations or operations, which were carried out at room temperature (20–25 °C). Embryos were equilibrated in small volumes of radioactive or cyanide-containing solutio

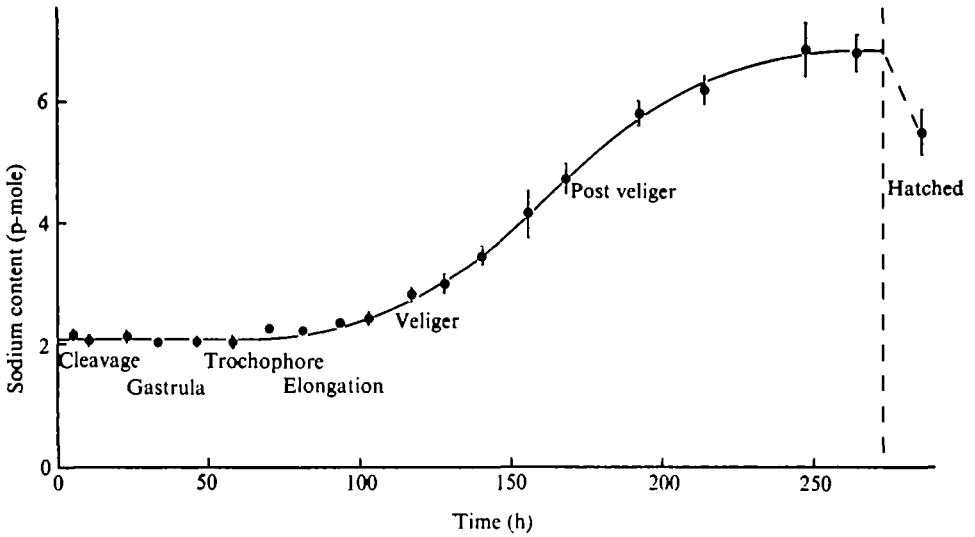


Fig. 1. Exchangeable sodium content of embryos plus capsules during development. Points represent the mean ± S.E. of six measurements (three egg masses used).

contained in solid watch glasses with lids sealed with petroleum jelly to prevent evaporation or loss of HCN.

Embryos are referred to as morulae from about 6 h from first cleavage (end of 16-cell stage) to about 16 h, blastulae from about 16–24 h, and gastrulae from about 24–35 h, unless more precise ages are given. Further details of experimental procedure and modifications to the isolation medium are given in the results section.

Embryo ion contents are stated in pmol (mol $\times 10^{-12}$) or nmol (mol $\times 10^{-9}$) and unless stated otherwise vertical bars on graphs represent ± S.E. of the mean.

RESULTS

Ionic composition of embryos during development

Sodium content of embryos plus capsules

Capsules from three egg masses were immersed in ^{22}Na-labelled lake water and at twice daily intervals two capsules from each egg mass were removed for measurement of ^{22}Na content. Fig. 1 shows the calculated total exchangeable sodium content of the embryos plus capsules at various stages up to hatching.

In pretrochophore stages the component due to the embryo itself is very small indeed (see next section) and is undetectable against the high sodium content of the capsular fluid. Since the exchange time for capsular sodium is very short and as there is no bound sodium in the capsules (Taylor, 1973), these values may be taken to represent total sodium content. The mean value of the sodium content for encapsulated embryos up to trochophore stage is 2·09 ± 0·03 nmol (S.E., first 7 points in Fig. 1) and is consistent with previously reported values for the sodium content of the capsular fluid (Taylor, 1973).

After the trochophore stage the embryo grows rapidly and the total sodium content

of the encapsulated embryos increases until at hatching it has a mean value of 6·8 nmol. The mean sodium content of the just hatched embryos is also portrayed in Fig. 1 (5·5 nmol). Thus, approximately 1·3 nmol or about 60% of the original capsular sodium remains in the capsules at hatching.

The sodium content of embryos without capsules

In order to measure the small quantities of sodium present in early developmental stages, embryos developing within their capsules in ^{22}Na-labelled lake water were periodically removed and individually counted on planchettes after careful removal of the capsular membrane and capsular fluid (Fig. 2: note that the units on the ordinate are 3 orders of magnitude smaller than in Fig. 1 and are presented on a logarithmic scale).

The exchangeable sodium content of cleavage stages up to gastrula is very low (10–20 pmol) but increases at an exponential rate between early trochophore and veliger stage. From Figs. 1 and 2 it is seen that up to hatching the sodium content increases by a factor of about 350. It is possible that the exchangeable sodium content of early stages does not represent their total sodium content. The possibility is considered further in the discussion. As the animals grow their radioactivity must approximate to total sodium content as they only have access to ^{22}Na-labelled sodium. The values in Fig. 2 for exchangeable sodium content, at least up to trochophore stage, probably underestimate their true values by 5–10%. As is shown in subsequent sections the rate of exchange for sodium is very rapid indeed and approximately this proportion of sodium is lost even during the 30–40 s washing period.

Calcium content of embryos without capsules

A similar experiment to the last was performed, this time culturing the encapsulated embryos in ^{45}Ca-labelled lake water and at intervals measuring the radioactivity of the decapsulated embryos (wash time in this experiment 60–75 s) (Fig. 3).

In post-gastrula stages the rate of calcium accumulation is very high indeed and increases exponentially up to veliger stage (about 1·3 pmol in blastula to gastrula stages to about 130 nmol at hatching). As discussed for sodium, it is possible that the exchangeable calcium underestimates total calcium content but this is unlikely to be the case for post trochophore stages.

Potassium content of decapsulated embryos

^{42}K-labelled lake water was used to demonstrate potassium uptake by early embryos. As the decay time of ^{42}K is very short ($T_{\frac{1}{2}}$ = 12·4 h) it was not possible to follow the potassium content of embryos from a single egg mass with adequate sensitivity for more than about 30 h. Thus capsules from three egg masses at blastula, early gastrula and late trochophore stage were immersed in labelled medium as soon as possible after arrival of the isotope. After freeing from the capsular membranes and washing, embryos were counted in groups of five. Fig. 4 shows the time course of ^{42}K uptake by these embryos. It is difficult to distinguish the uptake due to initial equilibration with the medium from net uptake caused by changes in total potassium content. However, it can be inferred that a rapid net uptake of potassium parallels the uptakes of sodium and calcium during trochophore to elongation stage. In embryos whic

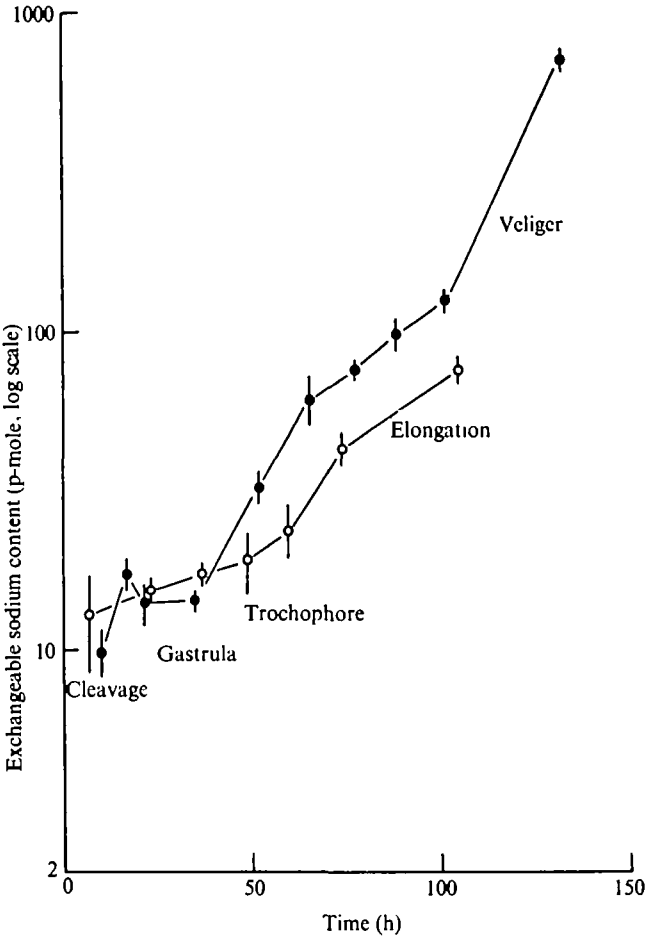


Fig. 2. Exchangeable sodium content of decapsulated embryos (developing within capsules). Points represent means ± S.E. of five measurements. Different symbols represent different egg masses.

have equilibrated for longer than 10 h, the potassium content is several times higher than the sodium content at the same stage.

Sodium content and the cleavage cavity

The values of exchangeable sodium content of cleavage stages up to gastrula (Fig. 2) show a high degree of individual variability (total range about 5–30 pmol). This was probably not entirely due to experimental imprecision but may have been associated with the changes in the size of the cleavage cavity (or blastocoel) which occur periodically during cleavage in pulmonates (Raven, 1966). Experiments were carried out to test this idea.

A summary of the morphological changes which occur during early cleavage, particularly in relation to the cleavage cavity may be helpful at this stage (Fig. 5*a–i*) (see Raven, 1946, 1966, for a fuller description). Constriction of the egg (*a*) by the cleavage furrow (*b*) leaves a dumbel-shaped embryo in which the blastomeres are

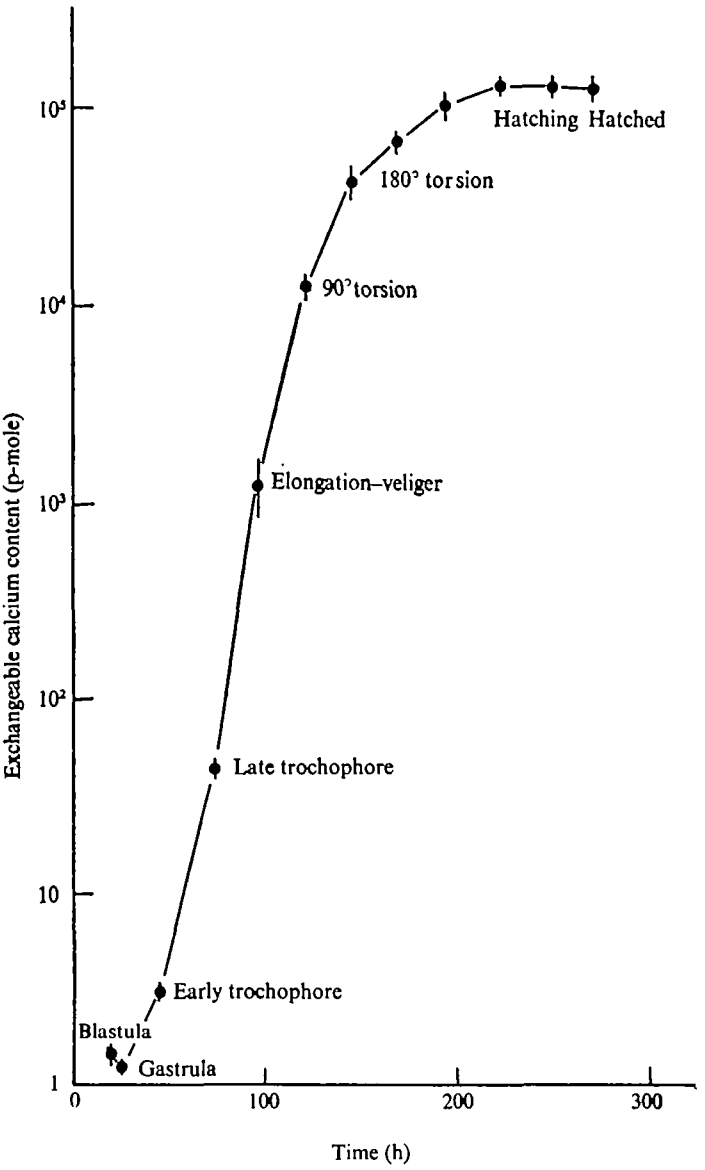


Fig. 3. Exchangeable calcium content of decapsulated embryos (developing within capsules). Points represent means ± S.E. of 8–12 embryos (five egg masses used).

only just touching (*c*). The cells then begin to press together until the embryo becomes nearly spherical again, the two blastomeres being closely applied to each other over a flat partition (*d*). The lens-shaped cleavage cavity then forms, pushing the blastomeres apart again (*e*, *f*), although they still adhere around the periphery of the cavity. Eventually the cell junction breaks at one point (at either animal or vegetal pole) and the cavity collapses quickly expelling its contents to the exterior (*g*). This process takes only a few seconds and is accompanied by the emission of a fluid of different refractive index to the capsular fluid. Expansion and contraction of the cleavage cavi

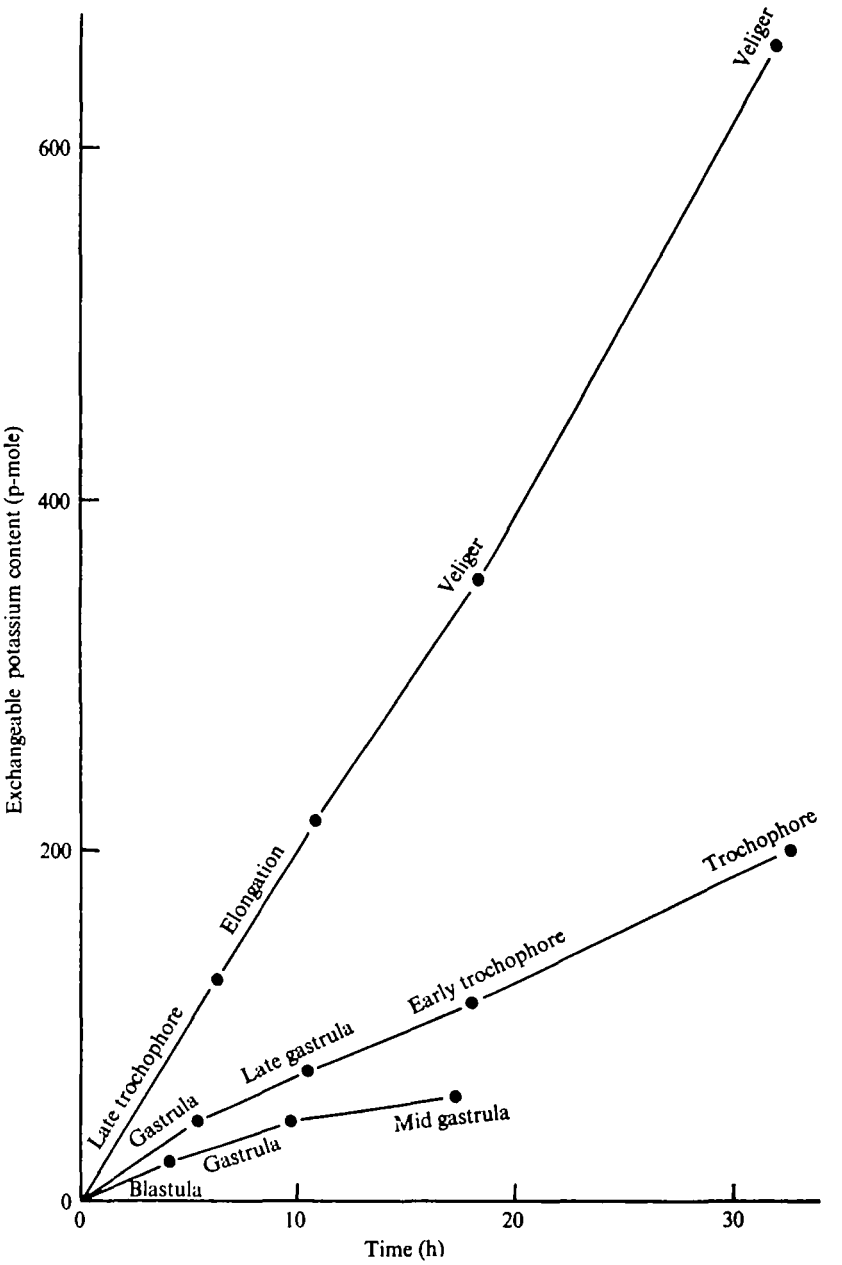


Fig. 4. Exchangeable potassium content of decapsulated embryos (developing within capsules). Time zero represents time of immersion in ^{42}K-labelled lake water for three different egg masses commenced at blastula, early gastrula and late trochophore respectively. Points are means of ten embryos pooled in two groups of 5.

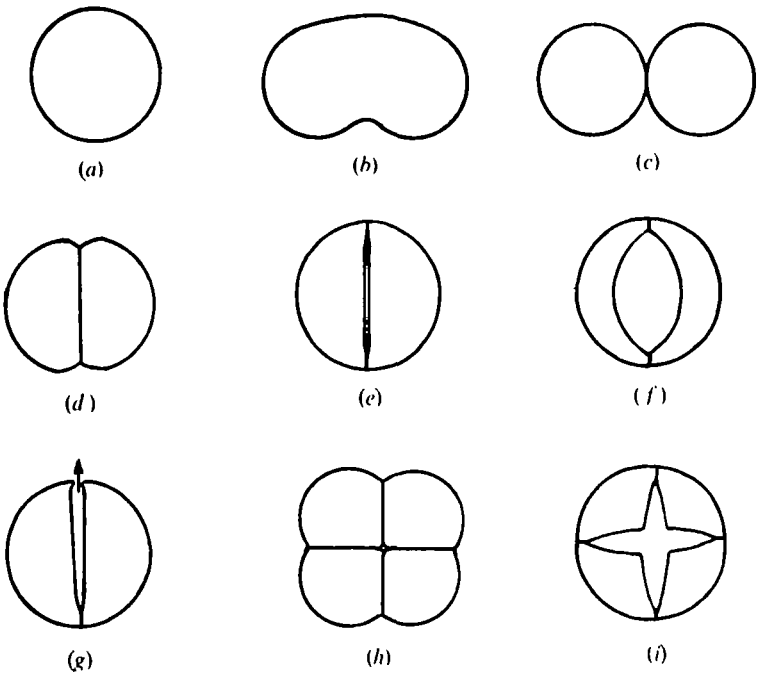


Fig. 5. Diagrammatic representation of the first two cell divisions in *Lymnaea stagnalis* embryos showing appearance of the cleavage cavity (see text for further explanation).

occurs once or twice more before the onset of the next division (*h*). Up to the 8–16 cell cleavage, collapse of the cleavage cavity always immediately precedes cell division and no cleavage cavity is present while the cells are dividing. During each cycle the cleavage cavity reswells (*i*) and collapses one or more times. The number varies between different embryos, suggesting that bursting is to some extent a fortuitous event rather than being rigidly controlled by the cleavage cycle. After the 16-cell stage, cell division is no longer synchronised throughout the embryo, and expansions and contractions of the cleavage cavity do not take place in such a regular fashion. However in early morulae both contracted (mulberry-like) and expanded, spherical forms with a clear central cavity are observed. During later stages the cleavage cavity never completely disappears but in the present study occasional emissions of relatively large quantities of clear fluid of different refractive index to the capsular fluid have been observed at all stages up to and including the blastula.

The sodium content of early cleavage stages of embryos developing within capsules

Capsules separated from a large egg-mass, containing uncleaved eggs about 1 h before the first cleavage, were placed in ^{22}Na-labelled lake water. They were then removed after varying intervals of time during early cleavage and the ^{22}Na content of the embryos measured. The time taken to remove the capsular membrane and wash off the viscous capsular fluid was standardized at 90 s (the minimum time to avoid accidentally collapsing the cleavage cavity). Careful observation was made of the cleavage stage reached, taking particular note of the presence or absence of a cleavage cavity at the time they were placed on the planchette. Three embryos showing the

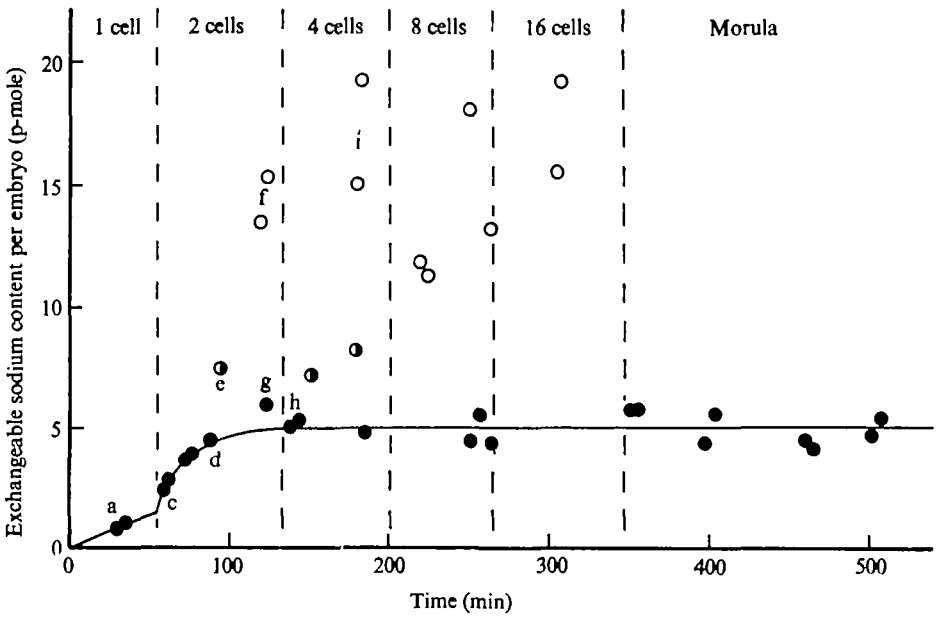


Fig. 6. Exchangeable sodium content (expressed in pmol at medium specific activity) of encapsulated early cleavage stages at successive times after placing in ^{22}Na-labelled lake water (embryos removed from capsules immediately before counting). Points represent three embryos pooled. Vertical lines are the approximate times that most embryos were undergoing cell division. ●, No cleavage cavity visible; ◑, small cleavage cavity; ○, large cleavage cavity. Letters *a–i* refer to cleavage stages shown in Fig. 5.

same state of division and appearance of cleavage cavity were pooled for each measurement (Fig. 6).

In the period up to the time when the cleavage cavity first appears (d) the embryos are still coming into equilibrium with the ^{22}Na in the medium. The embryos lacking a cleavage cavity equilibrate with a mean ^{22}Na content equivalent to an exchangeable sodium content of $5{\cdot}0 \pm 0{\cdot}2$ (S.E., $n = 16$) pmol. In this experiment equilibration with the medium is obviously not a simple process, as there is a sharp increase in the rate of ^{22}Na uptake which coincides with the onset of cleavage. This is shown more clearly in Fig. 7. From cleavage (*c*) up to the appearance of the cleavage cavity (*d*) the ^{22}Na content at time t mins can be represented by the simple exponential expression (least-squares fit, exponential regression):

$$5(1 - e^{-0{\cdot}0467t}) \text{ pmol},$$

i.e. a sodium influx of 0·23 pmol min^{-1}. The data are insufficient to make an accurate estimate of the sodium influx in uncleaved eggs but if the assumption is made that they also contain a single exchangeable sodium pool of about 5 pmol then a similar treatment reveals that the sodium influx is only of the order of 0·04 pmol min^{-1}, i.e. about one sixth of that after cleavage. (A further complication in this calculation is that it will take a few minutes for the capsules themselves to come into equilibrium with the medium so that the eggs are not immediately exposed to label at full specific activity. As a rough correction, time zero in Fig. 7 is put 4 min after immersion in the medium – about the time taken for the capsules to come into 90% equilibrium, see Taylor, 1973, Fig. 7).

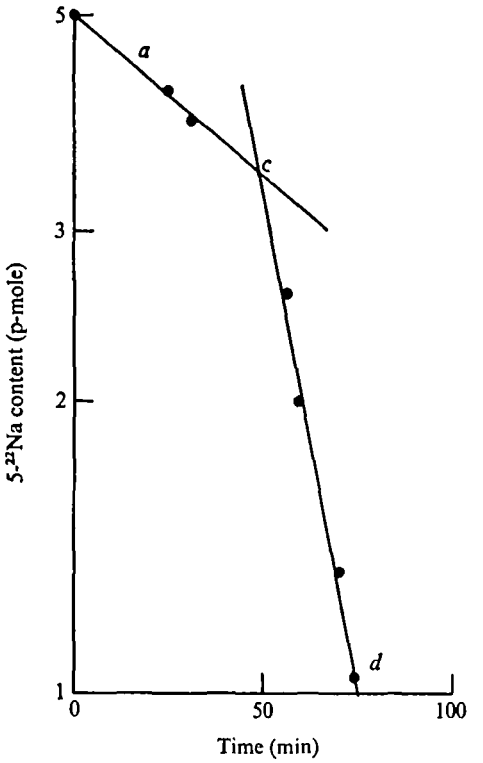


Fig. 7. Influx of ^{22}Na into 1–2 cell embryos. Calculated from the first 100 min of Fig. 6 on the assumption that their total exchangeable sodium content is 5 pmol. Letters *a*, *c* and *d* refer to cleavage stage as portrayed in Fig. 5. Time zero represents the time 4 min after immersion in ^{22}Na-labelled lake water (see text). Lines fitted by least squares method; exponential regression.

Fig. 6 demonstrates strikingly that the exchangeable sodium content of early cleavage stages varies with the state of expansion of the cleavage cavity. Embryos with no visible cleavage cavity have a low exchangeable sodium content (about 5 pmol). Embryos with clearly visible cavities (either mid-cycle or just prior to division) have much higher exchangeable sodium contents (11–19 pmol). Embryos with just discernible cavities (immediately after division or after a collapse of the cavity in mid-cycle) have intermediate values of 7–8 pmol.

The most plausible interpretation of these observations is that the cleavage cavity contains a fluid of high sodium concentration and that swelling and contraction of the cavity are accompanied by corresponding changes in embryo total sodium content. It is also worth pointing out that the above estimates of total sodium content may be approximately 10–20% low due to loss of ^{22}Na during the washing procedure if (as seems likely) early cleavage stages exhibit sodium efflux rates as high as those demonstrated below for later stages.

The sodium content of saline-isolated, decapsulated embryos during early cleavage

A similar experiment to the previous one was performed using embryos isolated from their capsules before placing in labelled medium (Fig. 8). The mean exchangeable sodium content of embryos without a cleavage cavity was $6{\cdot}5 \pm 0{\cdot}4$ (S.E., $n = 13$)

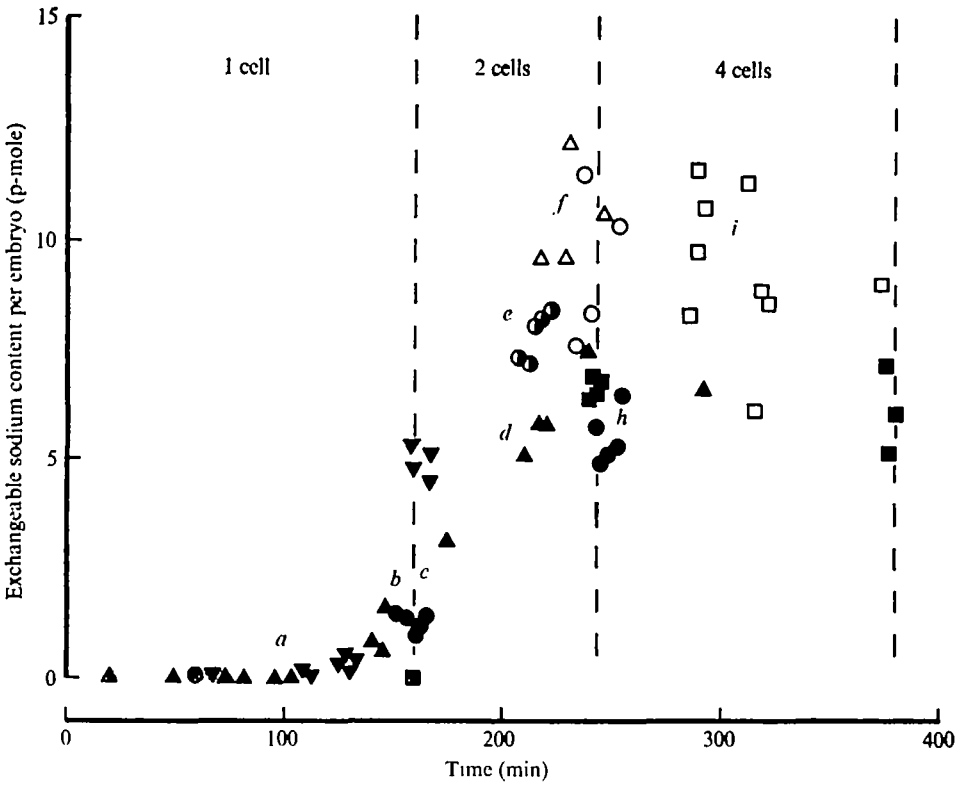


Fig. 8. Similar experiment to Fig. 6 except that embryos were isolated from capsules before placing in ^{22}Na-labelled saline. Symbols ○, □, △ and ▽ represent embryos from four different egg masses. Asterisks are at times of immersion of groups of embryos from each egg mass in labelled medium. Open and closed symbols represent appearance of cleavage cavity as in Fig. 6. Letters *a–i* refer to cleavage stages shown in Fig. 5.

pmol, slightly higher than the value obtained in encapsulated embryos, and here also expansion of the cleavage cavity was accompanied by a rise in embryo exchangeable sodium content. However, although the embryos were washed more rapidly (30 s), the exchangeable sodium contents of embryos with swollen cavities were less (7–12 pmol) than for encapsulated embryos. Possibly this is connected with some deficiency in the isolation medium. One-to-eight cell stages seem particularly sensitive to artificial media (see also comments in materials and methods section).

Fig. 8 also demonstrates the low sodium influx in the single cell stage. At times greater than 1 h before cleavage the influx is very low indeed and barely detectable in these experiments. The influx then increases to a similar value to that in encapsulated embryos (about 0·03 pmol min^{-1}) prior to cleavage and then dramatically increases again with the onset of cleavage.

Exchanges of sodium with the medium and total exchangeable sodium content of decapsulated embryos from morula to trochophore stage

Sodium efflux in morula to trochophore stages

Decapsulated embryos were equilibrated in ^{22}Na-labelled saline (in groups of 50–100 from the same egg mass). They were then washed in unlabelled medium for varying periods before measurement of ^{22}Na activity remaining. Fig. 9(*a–d*) shows the relationship between ^{22}Na content and time for morulae (5 egg-masses), blastulae (7 egg-masses), gastrulae (2 egg-masses) and trochophore-elongation stages (1 egg-mass) respectively. The blastulae were precisely age-standardized to between 21 and 22 h from first cleavage over the entire period of washing. The ages of morulae were more variable (6–16 h). The data for the two gastrula containing egg masses (just after gastrulation, 25 h and mid-gastrula, 35 h) are plotted separately as they obviously had greatly different exchangeable sodium contents. The embryos described as trochophore-elongation stage were placed in ^{22}Na-labelled medium at late trochophore stage and equilibrated for 24 h. By this time they had advanced to elongation stage morphologically but had undergone no further size increase, presumably associated with the deficiency of nutrients in the medium, as discussed in the materials and methods section.

In all of these stages ^{22}Na is lost to the medium at a very high rate, the half times for ^{22}Na loss being of the order of 4–8 min. Table 1 shows values of the mean total exchangeable sodium content and total sodium efflux for these embryos estimated by simple exponential regression (dashed lines in Fig. 9*a–d*). When examined for periods longer than six minutes, the semi-logarithmic plots of the efflux data for morula to gastrula stages (Fig. 9*a–c*) are obviously quite non-linear. Thus the above treatment will somewhat underestimate both the total efflux and the exchangeable sodium content. It is not a feasible proposition to compute a statistical 'best fit' to a two or more compartment model for these data. However, in the Discussion and Appendix it is shown that efflux curves which provide a reasonable 'eye fit' to the data (solid lines in Figs. 9*a–c*) are consistent with the two compartment model proposed for sodium in early embryos.

The calculated values of the exchangeable sodium contents of the saline-isolated decapsulated morula to gastrula stages (10·7–21·2 pmol) are in the same range as those of encapsulated embryos (Fig. 2). The sodium content of the decapsulated trochophore-elongation stages (55·3 pmol) is similar to the value found for normally developing late trochophore stages.

Sodium influx in decapsulated morula to gastrula stages

An experiment to measure ^{22}Na influx was performed using decapsulated embryos from late morula to early gastrula stage (2 egg masses) (Fig. 10). Each value of ^{22}Na content has been multiplied by a factor (1·094) calculated from the efflux curves to

Fig. 9. Efflux of ^{22}Na from decapsulated embryos in saline, (*a*) Morulae (6–16 h from first cleavage); (*b*) blastulae (21–22 h); (*c*) gastrulae (25 and 35 h); (*d*) trochophore-elongation. Dashed lines fitted by simple exponential regression (least squares) to first 6 mins in (*a–c*) and all data points in (*d*). See Table 1 for constants. For explanation of solid line see Discussion and Appendix. Points and bars represent antilog (mean log ^{22}Na content ± S.E.). Number of measurements used for each point indicated in brackets. (*a–c*) Three embryos pooled for each measurement, (*d*) embryos measured individually.

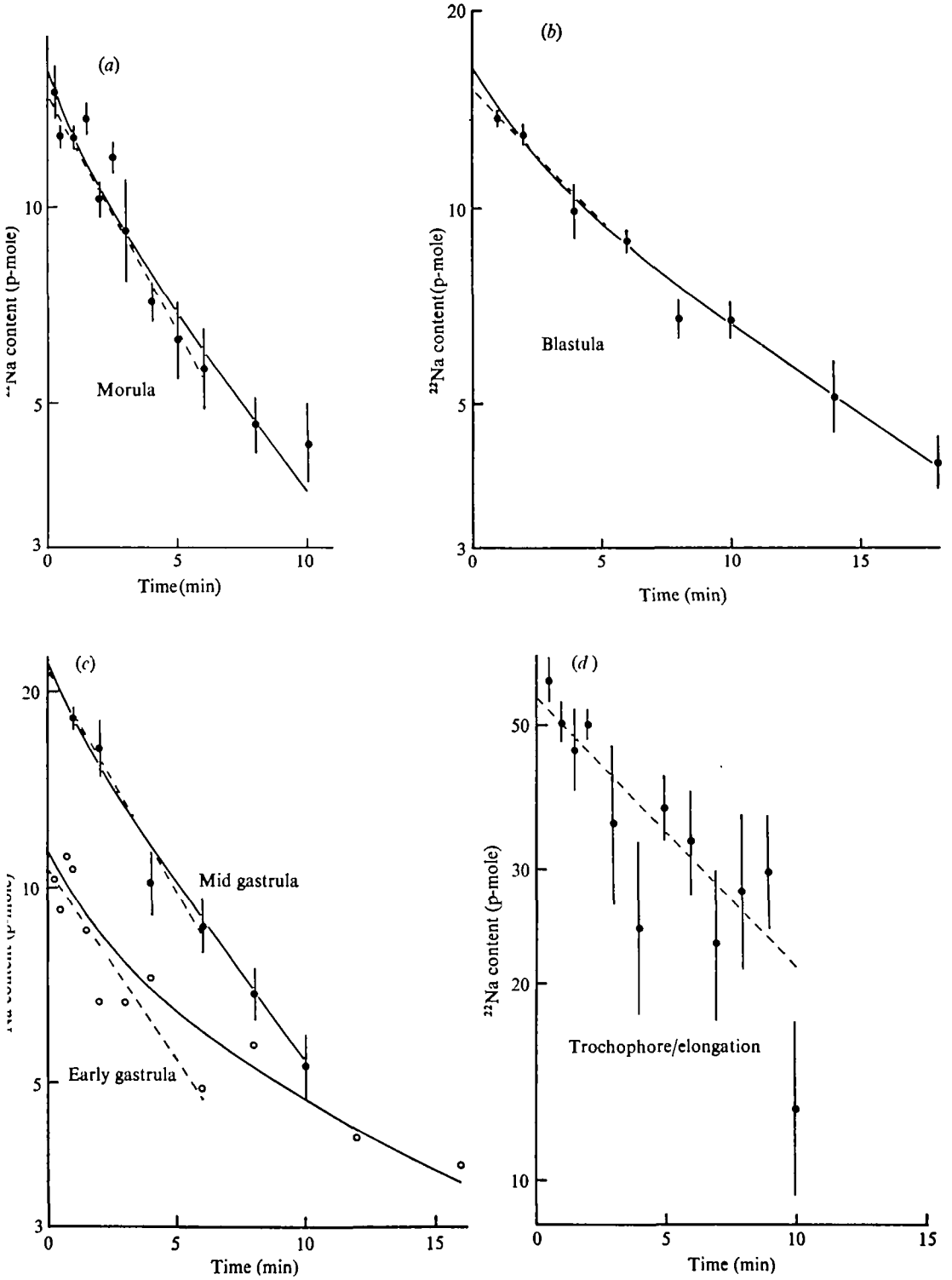


Fig. 9*a–d*. For legend see facing page.

Table 1. *Exchangeable sodium contents and efflux rates for decapsulated morula to late trochophore stages*

	Exchangeable sodium content (pmol)	^{22}Na efflux			
		Initial rate constant (min^{-1})	Total efflux (pmol min^{-1})	n	r^2
Morula	14·8	−0·165	2·45	69	0·66
Blastula	15·2	−0·091	1·40	40	0·60
Early gastrula	10·7	−0·134	1·44	17	0·71
Mid gastrula	21·2	−0·158	3·35	20	0·71
Trochophore-elongation	55·3	−0·095	5·27	68	0·29

Calculated from data in Fig. 9(*a–d*) by a least squares fit to a simple exponential expression from all the individual data points during the first 6 min (10 min for trochophores) of washout in normal saline. n = total number of observations, each observation representing the ^{22}Na content of three embryos pooled. r^2 = determination coefficient.

correct as far as possible for loss of ^{22}Na during the brief (30–40 s) wash to remove the labelled medium.

The solid line drawn represents the expression

$$^{22}\text{Na content} = 15(1 - e^{-0.183t}) \text{ pmol},$$

i.e. an influx of 2·75 pmol/min. (The values of 15 pmol for the equilibrium ^{22}Na content was chosen from data in Figs. 2, 9*a–c* in order to calculate the exponential regression by least squares but in fact does not influence the calculated value of the influx very greatly).

The influx rate is similar to the efflux rate for morula to gastrula stages (Table 1) and indicates that there is a very rapid exchange of the sodium in the embryo with that in the medium.

Sodium loss into sodium-free media

To investigate whether exchange diffusion contributed significantly to the high rate of exchange of sodium with the medium, the ^{22}Na loss into sodium-free media was measured. Blastulae 21–22 h from first cleavage, equilibrated with ^{22}Na-labelled normal medium as in previous experiments, were washed in either a sodium-free four times strength artificial lake water, with potassium, calcium and magnesium salts as before (8 egg-masses), or in distilled water (5 egg-masses). Their ^{22}Na contents at successive time intervals are shown in Fig. 11(*a*) and (*b*) respectively. The solid line is the calculated curve representing sodium efflux into normal medium for embryos at the same age as shown in Fig. 9(*b*).

Placing the embryos in sodium-free media clearly affects sodium loss, and in both cases, after washing for 18 min, approximately twice as much ^{22}Na remains in the sodium-free washed embryos as in those washed in the normal (2 mM) sodium medium. However, to assess the importance of exchange diffusion the initial sodium efflux in sodium-free medium must be examined and in this respect the two sets of conditions differ in their effect. In the sodium-free saline, as far as can be determined within the variability of the data, the sodium efflux is immediately reduced. The non-linearity of the semi-logarithmic plot is no longer apparent and the ^{22}Na content may be rep[illegible]

Table 2. *Changes in volume and exchangeable sodium content of morula to blastula stages treated with* 1 mM *cyanide*

Egg mass number	Normal medium: Embryo vol. nl	s.e.	^{22}Na/embryo (pmol)	3–5 h in 1 mM cyanide: Embryo vol. nl	s.e.	^{22}Na/embryo (pmol)	23–24 h in 1 mM cyanide: Embryo vol. nl	s.e.	^{22}Na/embryo (pmol)
1	1·5	0·05 (10)	12·5 (3) 18·8 (3) 15·1 (3)	2·4	0·17 (10)	22·6 (3) 54·4 (3) 27·6 (3) 56·7 (3)		—	—
2	1·7	0·07 (10)	7·8 (3) 8·3 (3) 12·1 (3)		—	—	7·1	0·00 (10)	78·8 (3)
3	1·5	0·05 (10)	17·4 (3) 22·0 (3) 18·1 (3)		—	—	6·6	0·22 (10)	74·0 (2) 81·2 (3) 77·5 (3)
4	1·9	0·06 (10)	22·2 (3) 17·6 (3) 18·0 (3)	2·6	0·15 (10)	47·0 (3) 54·0 (3) 39·6 (3)	4·1*	— (1)	102·2* (1)
5	1·3	0·04 (10)	22·2 (3) 19·6 (3) 19·8 (3)	2·1	0·07 (10)	126·2 (3) 37·5 (3) 52·2 (3)	5·0* 2·7*	— (1) — (2)	60·7* (1) 33·3* (2)

The numbers of embryos measured in calculation of the mean embryo volume and the numbers counted together on a planchette for ^{22}Na measurement are given in parentheses. In each egg mass/treatment set the same group of embryos were used for both measurements but, except for those indicated by asterisks, they were not individually related to each other.

sented by the simple exponential expression (computed from a least-squares fit of all points up to 18 min):

$$13{\cdot}76e^{-0{\cdot}036t},$$

i.e. a total influx of 0·50 pmol min^{-1}. This is about 35% of the estimated rate in normal saline. These results suggest that there is a fairly high exchange component in the sodium fluxes.

However, in the blastulae washed in distilled water, the initial rate of sodium loss was still very high, although after about 6 min sodium loss appeared to have virtually ceased. After 3–4 min in distilled water (but not in the sodium-free saline) a number of embryos showed signs of separation and swelling of the blastomeres, the proportion of such embryos and extent of the degeneration increasing with time. These embryos had very much lower ^{22}Na contents than others showing no visible signs of degeneration that had been washed for the same period. Such embryos were not included in the data shown in Fig. 11(*b*). However it is possible that invisible changes in cellular adhesion or other membrane properties take place almost immediately. The interpretation of these washout curves is considered further in the Discussion. Raven (1966) also observed that cells of *Lymnaea* embryos swell and separate in calcium-free media.

Exchangeable sodium content of embryos with cyanide-swollen cleavage cavities

Beadle (1969*b*) observed that pulmonate embryos with enormously distended cleavage cavities could be produced by exposing them to cyanide ions. Treatment with 1 mM-CN^- immediately arrests cleavage. However, the cells appear cytologically healthy and even after several hours treatment, development is usually resumed on removal of the cyanide (although some morphogenic abnormalities may appear very much later in development). During their exposure to cyanide the normal periodic collapse of the cleavage cavity does not occur and the cavity grows to a very large size. Beadle's photographs and my own observations make it clear that the swelling is due primarily to an increase in volume of the cavity and not of the cells, the turgid embryos looking like small footballs with their cells stretched thinly over the enlarged cavity. The cavity may be burst by mechanical stimulation but it reswells again within a few hours.

It was observed in a previous section that large changes in exchangeable sodium content accompany the normal expansion and collapse of the cleavage during early cleavage. Is a similar increase in exchangeable sodium content associated with the artificially swollen cleavage cavity?

Embryos from each of five egg-masses at morula to blastula stage were decapsulated, divided into two groups of 10–20 and placed in either normal ^{22}Na-labelled isolation medium or a similar medium in which 1 mM-CN^- replaced part of the HCO_3^-. After equilibrating for periods ranging from 3–24 h, the diameters of ten of the embryos (where this number remained – a few degenerated after 24 h and in some cases the delicate very swollen embryos were burst by handling procedures) were measured to provide an estimate of mean volume of the group. They were then removed from the labelling medium, washed for 90 s (the minimum time to avoid damage to the swollen embryos) and their ^{22}Na activity measured (in groups of three where possible). Table 2 shows the volumes of normal and cyanide treated embryos and their exchangeable sodium contents (corrected for loss of ^{22}Na during washing using the regression lines in Figs. 9(*a–c*) though this is probably not accurately applicable to the swollen embryos).

An increase in volume was noted in all of the cyanide-treated groups. After 3–5 h the increase ranged from 0·7–0·9 nl. This represents a large increase in volume of the cleavage cavity whose normal volume may be roughly estimated from micrographs to be about 0·2–0·4 nl. After 23–24 h the volume increase is even more marked (1·4–5·4 nl).

The increase in volume is accompanied by a large increase in exchangeable sodium content. The mean exchangeable sodium content of embryos increases by a factor of about 3 after 3–5 h and by a factor of 4·5 after 23–24 h treatment (Table 3). The interpretation of these results will be considered in the Discussion.

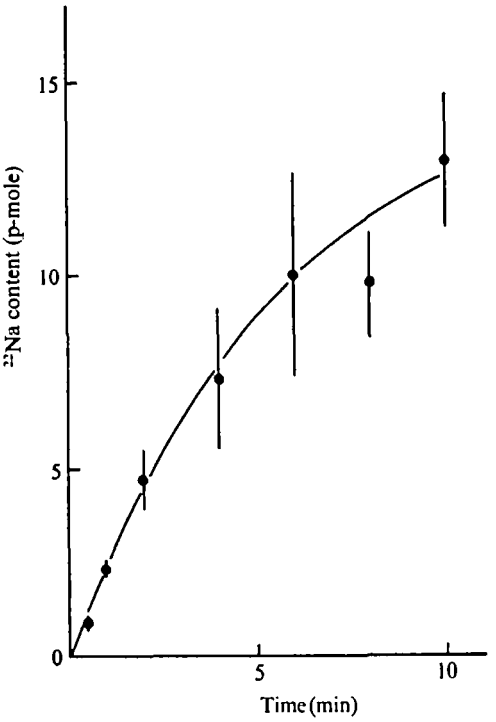


Fig. 10. Influx of ^{22}Na into decapsulated morula–gastrula embryos isolated in saline. Points and bars represent means ± S.E. Numbers of measurements (each three pooled embryos) indicated in brackets.

DISCUSSION

Beadle & Beadle (1969) demonstrated that developing embryos of the pulmonate *Biomphalaria sudanica* take up sodium from the water via the capsular fluid so that the sodium content of the encapsulated embryos increased more than seven times between blastula and hatching. It is demonstrated here (fig. 1) that the *Lymnaea stagnalis* embryos have the same ability, the sodium content of embryos plus capsules increasing from about 2 nmol to about 7 nmol. The above authors did not take into account the high sodium content of the capsular fluid itself which was thought to be negligible (see also Taylor, 1973) and when the sodium content of the individual embryos is examined (figs. 1, 2) the increase which commences after gastrulation is seen to be very much greater indeed (from about 15 pmol to 5·5 nmol, a factor of about 350). A parallel rise in calcium and potassium content occurs during the period from trochophore to veliger stage (Figs. 3, 4), accompanying the large volume increase which is seen at this time. The rise in calcium content is very dramatic and ascends through five orders of magnitude between gastrula and hatching. The rise in calcium content is of course associated with the growth of the shell. It is interesting that a high degree of calcium accumulation has taken place by trochophore stage, although visible shell secretion does not begin until elongation. However, the shell gland primordia are discernible from late gastrula stage (Raven, 1966), and it is possible that this tissue begins to accumulate calcium some time before calcium salts are deposited in the shell.

Although the capsular cation concentrations are higher than in the external water (Taylor, 1973) it is unlikely that the capsular fluid provides a significant ion reserve for the developing embryo, except perhaps during very early stages. Approximately 13 nmol or about 60% of the original capsular sodium is left behind by the hatching embryos. Clearly, the main source of sodium for the developing embryos is, ultimately, the external water as was concluded for *Biomphalaria* (Beadle & Beadle, 1969). Similarly the total calcium content of the emerging snail is very much higher than the amount of calcium initially present in the capsule (about 10^4 pmol – from data in Taylor, 1973). By veliger stage, the potassium content of the embryos already exceeds the initial total potassium content of the capsule (approximately 250 pmol in this medium – from Taylor, 1973). Evidently ion uptake mechanisms capable of transporting ions from the dilute external medium appear early in development.

The embryos also face the problem of osmoregulation in a hypo-osmotic and hypo-ionic medium from an early stage. The colloid osmotic pressure of the capsular fluid of *L. stagnalis* is about 4–5 m-osmol (Beadle, 1969*a*) and although the cation activities in the capsular fluid are relatively higher in the capsular fluid than in the water due to Donnan effects, they are still rather low (Taylor, 1973). Raven & Klomp (1946) estimate that the uncleaved egg has an osmotic pressure of 93 m-osmol. Data for the cytoplasmic osmotic pressure and ionic activities of later cleavage stages are not available. However, it has been estimated that up to gastrula stage the exchangeable cation contents are about 15 pmol Na^+, 50 pmol K^+ and 1·3 pmol Ca^{2+} (Figs. 2–4). The volume of these embryos is about 1·6 nl (Table 2) giving an overall concentration of these cations of about 41 mM. As it is likely that an appreciable proportion of these are present as free ions it can be inferred that similar osmotic problems exist for cleavage stages as for the egg. This is also indicated by the observations of Beadle (1969*b*) and in the present work that inhibition of metabolism by anoxia, cyanide or low temperature causes a swelling of 2-cell to blastula stages.

The 'recurrent' cleavage cavity observed in many freshwater and land pulmonates has been considered to represent the precocious development of a water-excreting mechanism (Raven, 1966). A similar view is reached in this paper. Changes in size of the cavity are accompanied by changes in embryo exchangeable sodium content by 5–15 pmol. The reduction in embryo exchangeable sodium content to about 5 pmol on collapse of the cavity must be due to a net loss of sodium from the organism, presumably from the cavity. Measurements of sections and live embryos indicate that the volume of the collapsed embryos is about 1·1 nl whereas the volume of the cavity itself is only about 0·2–0·4 nl. On this basis it appears that the concentration of exchangeable sodium in the cavity is much higher (30–50 mM, assuming the larger cavities have higher sodium contents) than in the cytoplasm (about 4·5 mM, ignoring non-solvent volume).

The observed increase in exchangeable sodium content could be due to a net uptake of sodium or to mobilisation of a slowly exchanging bound sodium pool. Direct chemical analysis of the embryos is necessary to distinguish between these two alternatives. However, the latter case requires that the initial total sodium pool of the embryo be very large since at least 50–100 pmol are released by the 32 cell stages. Though bound sodium pools are commonly observed in animal tissues, e.g. freshwater protozoans (Dunham & Child, 1961; Klein, 1961, 1964), frog oocytes (Dick

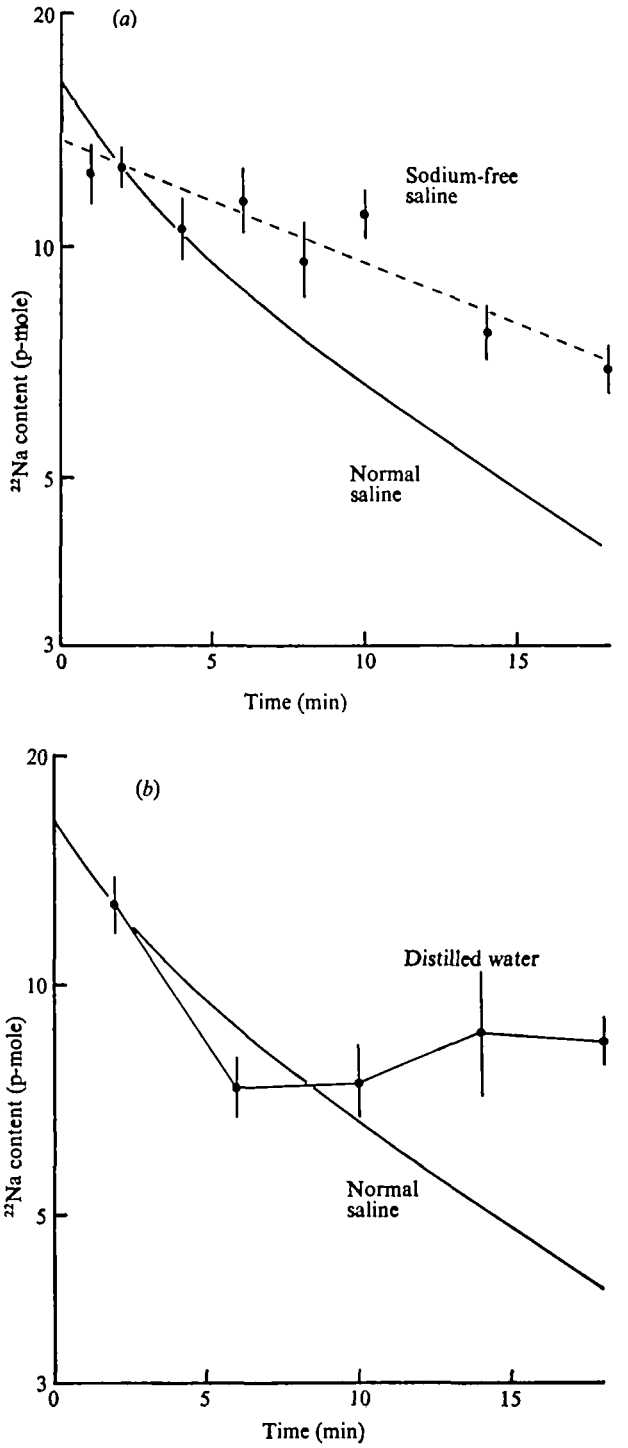


Fig. 11. Efflux of ^{22}Na in 21–22 h blastulae in sodium-free media. (*a*) Sodium-free saline, (*b*) distilled water. Points and bars represent antilog (mean log ^{22}Na content ± S.E.). Number of measurements (each three pooled embryos) indicated in brackets. Dashed line in (*a*) fitted by least squares (simple exponential regression) is the expression ^{22}Na content = $13{\cdot}76\ e^{-0{\cdot}036t}$. Continuous lines from Fig. 9(*b*) represent efflux in normal medium.

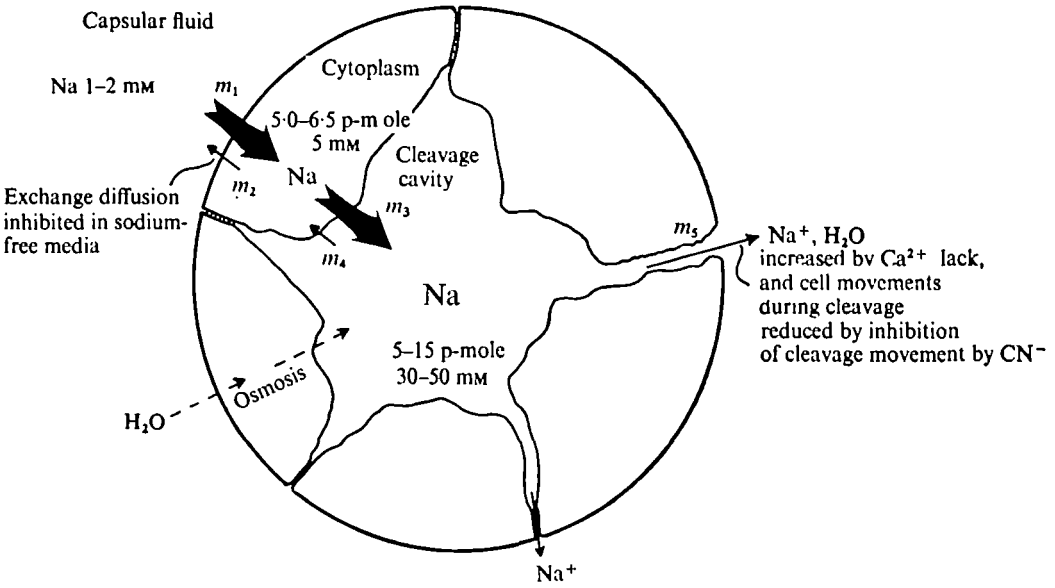


Fig. 12. A model for the main movements of sodium and water in embryos of *Lymnaea stagnalis* during cleavage to early gastrula stage. The postulated sizes and concentrations of exchangeable sodium pools are shown. m_1–m_5 are symbols used in Appendix to represent sodium fluxes. See text for further explanation.

Lea, 1967), barnacle muscle fibres (Allen & Hinke, 1970; Caille & Hinke, 1972) they normally represent only 30–50% of the total sodium. Extended immersion in ^{22}Na for up to 50 h (Figs. 2, 6) provides no evidence for a slowly exchanging fraction. Thus the simplest explanation of Fig. 6 is that during cleavage there is a net uptake of sodium from the medium (normally capsular fluid), this is secreted into the cleavage cavity by the blastomeres and is periodically expelled by collapse of the cleavage cavity.

Sodium influx into uncleaved eggs is very low and has not been measured accurately. Elbers (1966) demonstrated that electrolytes are only very slowly lost from uncleaved *Lymnaea* eggs. The sodium influx increases about an hour before cleavage and has been estimated as 0·03–0·04 pmoles min^{-1} for encapsulated and saline-isolated eggs. Another sharp increase in influx is observed which exactly coincides with the first signs of cleavage (0·23 pmol min^{-1}).

From the data in Fig. 6 it is possible to estimate the average rate of change of exchangeable sodium content over the period since the previous cleavage for each of the measurements of ^{22}Na content of embryos with swollen cavities. This has a mean value of $0{\cdot}28 \pm 0{\cdot}003$ (S.E., $n = 12$) pmol min^{-1} and for some embryos was as high as 0·4 pmol min^{-1}. Since this represents a minimal value for the sodium influx (whether or not there is a net sodium uptake), another increase in sodium influx probably occurs at the time of the first appearance of the cleavage cavity (Fig. 6*e*). According to the suggested hypothesis, this also represents a minimal estimate of the rate of sodium secretion into the cleavage cavity.

The estimates of the exchangeable content of both encapsulated and decapsulated morula to gastrula stages exhibit a high degree of variability and it is possible th

This is also partly explained by changes in the sodium content of the cleavage cavity though this has not been directly examined in this study. Morulae and blastulae have occasionally been observed to emit fluid from the cavity into the capsular fluid. At least from early morula stage onwards the rate of sodium turnover is very high. The minimal estimates of sodium efflux and influx obtained by simple exponential regression for morula to gastrula stages are in the range 1·4–3·4 pmol min^{-1}. The measurements of efflux in sodium-free saline (Fig. 11*a*) suggest that a large proportion (about 65% of the estimated efflux for blastulae) of these fluxes may represent an exchange component. The efflux into distilled water (Fig. 11*b*) does not seem to confirm this. However, there is evidence that this medium affects the integrity of cell junctions. If the cleavage cavity of these stages also has a high sodium concentration then the high loss rate initially in this medium may represent an increased rate of leakage of the cavity contents.

The non-linearity of the semi-logarithmic plots of the efflux data for morulae, blastulae and gastrulae in normal sodium medium indicates that the sodium is not present in these stages as a single pool from the point of view of exchange with the medium.

It is possible that the location of sodium pools and the sodium fluxes are essentially similar in all stages from first cleavage until gastrulation and Fig. 12 shows a simple model which is consistent with all of the observations and which provides a mechanism for osmotic regulation in these embryos. The main features of the model are that there are two main sodium pools in the embryo which are located in the cytoplasm and in the cleavage cavity. There is a net transport of sodium plus an anion from the external medium (normally capsular fluid) across the blastomeres into the cleavage cavity. Water which enters the cells due to their osmotic imbalance with the medium follows the sodium into the cavity by local osmosis. The sodium salt and water leave the cavity by diffusion across the cell junctions and by bulk movements when temporary separations of the cells occur either by build up of hydostatic pressure or by movements of the cells relative to each other during cleavage. In early cleavage stages these bulk movements are observed as the periodic extrusions of fluid from the 'recurrent' cleavage cavity.

It is not clear whether such emissions should be considered as the result of a definite active explusion process. It is possible that they simply represent the return of the blastomeres to a less strained shape when the cell junction bursts at a particular point. The cell junctions are of the septate desmosome type (Bluemink, 1967, and personal observations). Presumably they are frequently broken down and reformed in order to accommodate cell movements occurring during cell division. Leakage of cavity contents to the exterior may occur through discontinuities created in this way. During early cleavage the blastomeres are large and cell division occurs in all of them more or less simultaneously. Thus periodic release of the entire cavity contents may be an unavoidable consequence.

The computation of the total exchangeable sodium content and total sodium efflux using a simple exponential regression will slightly underestimate the sodium content and perhaps considerably underestimate the efflux if sodium is exchanging as a two or more compartment system. In the Appendix, equations are derived which describe the expected time course of sodium efflux in the model proposed in Fig. 12, under

steady-state conditions (possibly a reasonable approximation to the conditions in the precisely age-standardized blastulae, but probably not accurately applicable to the other stages). It is seen that in order to specify the time course it is necessary to know a minimum of five variables (three fluxes and the sizes of the two sodium pools). None of these are known with great precision. However, by a process of trial and error it is possible to select reasonable values of these variables from which one can compute curves (solid lines in Figs 9*a–c*) which provide a good fit (by eye) to the data. This gives some additional confidence in the plausibility of the model.

The selected values of the variables and the mathematical expression which describes these efflux curves are given in Table 4. These should not be taken to represent best estimates of these parameters. A number of combinations of these values could produce curves which give an equally good fit to the data. In particular the value of m_4 (the sodium flux from cavity to cytoplasm) was assumed to be zero (which is reasonable if there is a sodium-secreting mechanism on the inner plasma membrane) in order to simplify the computations. It is unlikely that m_4 is high relative to m_2 (the flux cytoplasm to medium) since in this case the non-linearity of the efflux curves virtually disappears but small finite values of m_4 can be incorporated into the calculations to produce curves which fit the data equally well. m_2 could partly arise from an exchange component across the outer plasma membrane. It is necessary for this flux to be high relative to m_1 (the influx) in order to achieve curves of the observed shape. When m_2 is zero the model behaves as a single compartment system with efflux equal to the net flux through the system, m_5 ($= m_3$, if $m_4 = 0$). This is consistent with the observation that in sodium-free saline the efflux from blastulae does appear to be a simple exponential (Fig. 11*a*). It is encouraging that the value for this net flux found to provide a good fit to the 21–22 h blastulae efflux curves (0·6 pmol min^{-1}, Table 4) is very similar to the efflux from blastulae of the same age in sodium-free saline (0·5 pmol min^{-1}, Fig. 11*a*). Verification or refutation of this model must await more refined means of estimating the sizes of the sodium fluxes and pools concerned.

Even if part of the fluxes represents an exchange component, the rates of sodium turnover by the morula to gastrula stages are very high for freshwater organisms of this size (volume about 1·5 nl, total rate constant at least 0·1–0·15 min^{-1}, $T_{\frac{1}{2}}$ 4–8 min). For example the freshwater protozoan *Chaos* (volume 23 nl) has a sodium efflux rate constant of only 0·0033 min^{-1} (Chapman-Anderson & Dick, 1962) and *Tetrahymena* with a volume of only 0·01 nl has sodium turnover only a little higher than *Lymnaea* embryos ($T_{\frac{1}{2}}$, 1–3 min). In terms of unit body weight the sodium fluxes are approximately 80 mmol kg^{-1} h^{-1}. Most larger freshwater animals have steady state sodium fluxes several orders of magnitude lower than this (see Potts & Parry, 1964; Shaw, 1963 for comparative tables). For example, the value for the embryo may be compared with the value for the sodium efflux from adult *L. stagnalis* in balance with 0·35 mM-Na of only 0·132 mmol kg^{-1} h^{-1} (Greenaway, 1970). About 40% of this is thought to be an exchange component. If the model developed above for movements of sodium and water through the embryo is correct, then the cycling of sodium through the embryo from a low concentration in the medium to a relatively high concentration in the cavity and then back to the medium again must involve an active transport mechanism (though not necessarily for sodium ions). This implies that the relative metabolic burden of ion transport is very much greater for the embryos of *Lymnaea* than for adult

Table 3. *Volumes and calculated exchangeable sodium concentrations in normal and cyanide-swollen morula to blastula stages (based on data in Table 2)*

	Mean volume (nl)	Mean exchangeable sodium content (pmol)	Mean exchangeable sodium concentration (mM)	Mean Δ_{Na}/Δ_{vol} (mM)
Normal	1·6	17·0	11·09	—
3–5 h CN	2·3	51·8	23·3	43·7
23–24 h CN	6·3	72·5	15·0	17·7

Individual values of concentrations were calculated using estimates of volume and sodium content obtained from the same egg-mass and the means calculated from these.

The expansion of the cleavage cavity, which occurs on treating morulae and blastulae with 1 mM cyanide and which has also been observed in earlier stages including the 2-cell stage (Beadle, 1962*b*), can also be interpreted in terms of the model shown in Fig. 12. The treatment immediately arrests cell division and the relatively more stable cell junctions which result could eliminate the normal route for fluids leakage.

The increase in volume of cyanide-treated embryos is accompanied by a large increase in their exchangeable sodium content (Tables 2, 3). In addition the overall sodium concentration in the embryos increases on swelling (Table 3). One interpretation of these events is that the blastomeres, like most other cells, possess a sodium extrusion mechanism which removes intracellular sodium and thereby controls the cells' volume by preventing the attainment of Donnan equilibrium for all ions. In this case cyanide, by inhibition of cellular metabolism, might be expected to increase intracellular [Na^+] and hence cell volume. However, although there is an increased degree of vacuolization and small volume changes of the cells cannot be discounted, it is clear from the microscopical observations which were made in the course of the present study and those of Beadle (1969*b*, plate I) that the main volume changes are in the cleavage cavity and hence it seems likely that this is the site of sodium accumulation also. Table 3 also shows values of the increase in sodium content divided by the increase in volume (i.e. the concentration of fluid added to the cleavage cavity and assuming that there is no change in volume or sodium content of the blastomeres). The mean value for this concentration is 43·7 mM after 3–5 h, which is in the range of sodium concentration postulated in the normal cleavage cavity. If the sodium is mainly in the cleavage cavity this result suggests that although cell division immediately ceases on treatment with cyanide, sodium transport into the cavity is not completely arrested, at least in the 3–5 h treatment. Perhaps this is not unreasonable as it is known that many adult pulmonates have the capacity for anaerobic metabolism (von Brand, Baernstein & Mehlman, 1950). Although volume and sodium content are further increased after 23–24 h the mean sodium concentration of the added fluid declines to 17·7 mM.

According to Raven (1966) fluid secretion into the cleavage cavity up to the 16-cell stage occurs through conical prominences of vacuolated cytoplasm projecting from the inner wall of the blastomeres, the 'secretion cones'. The precise significance of these structures needs to be further investigated. It is possible that they are the sites of intense exocytotic activity, perhaps releasing vesicles of high osmotic concentration to the cavity, or other structural devices associated with water/solute coupling.

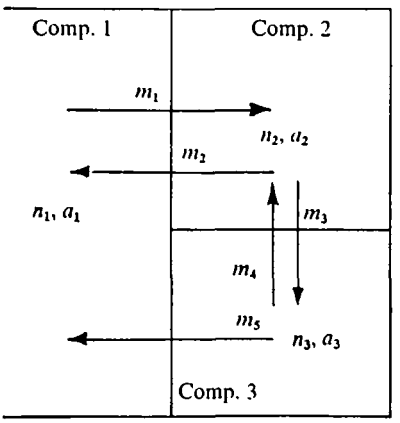
Possibly the main significance of fluid transport into the cleavage cavity is the excretion of excess water entering the blastomeres from the hypo-osmotic medium. Other excretory products may be entrained in the fluid flow but this is probably not an important aspect of its function in such small animals. Such an excretory system could be compared with the contractile vacuole of freshwater protozoans, although it is interesting to note that cyanide prevents the filling of the latter structure (Kitching, 1952) whereas it prevents the collapse of the cleavage cavity. A somewhat analogous system of fluid excretion is seen in the freshwater coelenterate *Hydra*. Fluid is secreted into the enteron and periodically expelled from the hypostome (Marshall, 1969). In both of these examples, as in *Lymnaea* embryos, fluid secretion is apparently coupled to sodium transport (Schmidt-Nielsen & Schrauger, 1963; Marshall, 1969).

It is concluded that the developing embryos of *L. stagnalis* do not avoid the problems of osmotic and ionic regulation in a dilute medium and that ion uptake and water excretion systems are developed in the earliest cleavage stages. There is at present no evidence to suggest that uncleaved eggs have any special osmoregulatory mechanism though they are hypertonic to the capsular fluid. It appears that the exchange of ions between the medium and uncleaved eggs is very slow and it is possible that they have a sufficiently low permeability to water and ions to allow compensatory devices to be delayed until cells with a polar morphology appear. In fact they swell continuously during this stage (Raven & Klomp, 1946).

The appearance of these regulatory mechanisms is associated with a 50- to 100-fold increase in sodium influx during early cleavage. Raven, Bezem & Isings, (1952) estimate that a more than 5-fold increase in water transport also occurs at first cleavage. These changes in flux rate occur quite quickly (over a few minutes) and it would be interesting to investigate the mechanism whereby these sudden changes are switched on.

APPENDIX

Consider the following three-compartment system representing the model shown in Fig. 12.



Compartments 1, 2 and 3 represent the medium, cytoplasm and cleavage cavity respectively.

Let n_1, n_2, n_3 be the numbers of ^{22}Na ions and a_1, a_2, a_3 be the total numbers of sodium ions in compartments 1, 2, 3 respectively. m_1 to m_5 represent the sodium fluxes as indicated.

Let $\bar{n}_0$ and $\bar{n}_t$ be total number of ^{22}Na ions in embryos, i.e. (n_2+n_3) at time zero and time t respectively.

The problem is to derive an expression to describe the fall in radioactivity of the whole embryo ($\bar{n}_t$) during washout in unlabelled medium.

$$\frac{dn_2}{dt} = m_1\frac{n_1}{a_1} - m_2\frac{n_2}{a_2} - m_3\frac{n_2}{a_2} + m_4\frac{n_3}{a_3}, \tag{1}$$

$$\frac{dn_3}{dt} = m_3\frac{n_2}{a_2} - m_4\frac{n_3}{a_3} - m_5\frac{n_3}{a_3}. \tag{2}$$

At steady state:

$$m_1 - m_2 = m_3 - m_4 = m_5. \tag{3}$$

Assuming comp. 1 is an infinite reservoir, $n_1 = 0$. Hence equation (1) becomes:

$$\frac{dn_2}{dt} = -\frac{m_2+m_3}{a_2}n_2 + \frac{m_4}{a_3}n_3 \tag{4}$$

and equation (2) becomes:

$$\frac{dn_3}{dt} = -\frac{m_3}{a_3}n_3 + \frac{m_3}{a_2}n_2. \tag{5}$$

Differentiating equation (4), and substituting from (5)

$$\frac{d^2n_2}{dt^2} = -\frac{m_2+m_3}{a_2}\cdot\frac{dn_2}{dt} + \frac{m_4}{a_3}\left(-\frac{m_3}{a_3}\cdot n_3 + \frac{m_3}{a_3}\cdot n_2\right).$$

From equation (4),

$$n_3 = \left(\frac{dn_2}{dt} + \frac{m_2+m_3}{a_2}n_2\right)\frac{a_3}{m_4},$$

therefore

$$\frac{d^2n_2}{dt^2} = -\frac{m_2+m_3}{a_2}\cdot\frac{dn_2}{dt} + \frac{m_4}{a_3}\left[-\frac{m_3}{a_3}\left(\frac{dn_2}{dt} + \frac{m_2+m_3}{a_2}n_2\right)\frac{a_3}{m_4} + \frac{m_3}{a_2}\cdot n_2\right]$$

$$= -\left(\frac{m_2+m_3}{a_2} + \frac{m_3}{a_3}\right)\frac{dn_2}{dt} - \frac{m_1m_3}{a_2a_3}\cdot n_2. \tag{6}$$

Differentiating equation (5), and substituting from (4)

$$\frac{d^2n_3}{dt^2} = -\frac{m_3}{a_3}\cdot\frac{dn_3}{dt} + \frac{m_3}{a_2}\left(-\frac{m_2+m_3}{a_2}n_2 + \frac{m_4}{a_3}n_3\right).$$

From equation (5),

$$n_2 = \left(\frac{dn_3}{dt} + \frac{m_3}{a_3}n_3\right)\frac{a_2}{m_3}$$

therefore

$$\frac{d^2n_3}{dt^2} = -\frac{m_3}{a_3}\cdot\frac{dn_3}{dt} + \frac{m_3}{a_2}\left[-\frac{m_2+m_3}{a_2}\left(\frac{dn_3}{dt} + \frac{m_3}{a_3}n_3\right)\frac{a_2}{m_3} + \frac{m_4}{a_3}n_3\right]$$

$$= -\left(\frac{m_3}{a_3} + \frac{m_2+m_3}{a_2}\right)\frac{dn_3}{dt} - \frac{m_1m_3}{a_2a_3}\cdot n_3. \tag{7}$$

Table 4. *Values of parameters used to calculate efflux curves in Figs 9(a–c) (see text for details)*

	Fig. 9(*a*) morula	Fig. 9(*b*) blastula	Fig. 9(*c*) Early gastrula	Fig. 9(*c*) Mid gastrula
$\bar{n}_0$	16·0	16·5	11·5	22·0
a_2	6·0	6·5	5·0	6·0
a_3	10·0	10·0	6·5	14·0
m_1	4·0	2·5	2·0	5·0
m_2	2·8	1·9	1·7	3·0
$m_3 = m_5$	1·2	0·6	0·3	2·0
m_4	0·0	0·0	0·0	0·0
A	12·20	11·84	7·35	18·82
B	3·80	4·65	4·15	31·8
λ_1	−0·120	−0·060	−0·046	−0·125
λ_2	−0·667	−0·385	−0·400	−0·833

$\bar{n}_t = Ae^{\lambda_1 t} + Be^{\lambda_2 t}$,
$\bar{n}_0 = A + B = a_2 + a_3$ (in units used).

Units: sodium pools, pmol; ^{22}Na activities, pmol at medium S.A.; fluxes, pmol min^{-1}; rate constants, min^{-1}.

From equations (6) and (7),

$$\frac{d^2n_2}{dt^2} + \left(\frac{m_2+m_3}{a_2} + \frac{m_3}{a_3}\right)\frac{dn_2}{dt} + \frac{m_1m_3}{a_2a_3}.n_2 = 0,$$

$$\frac{d^2n_3}{dt^2} + \left(\frac{m_2+m_3}{a_2} + \frac{m_3}{a_3}\right)\frac{dn_3}{dt} + \frac{m_1m_2}{a_2a_3}.n_3 = 0,$$

therefore

$$\frac{d^2(n_2+n_3)}{dt^2} + \left(\frac{m_2m_3}{a_2} + \frac{m_3}{a_3}\right)\frac{d(n_2+n_3)}{dt} + \frac{m_1m_3}{a_2a_3}(n_2+n_3) = 0.$$

The general solution of such an equation is therefore

$$n_2+n_3 = \bar{n}_t = Ae^{\lambda_1 t} + Be^{\lambda_2 t}, \tag{8}$$

where

$$\lambda^2 + \left(\frac{m_2+m_3}{a_2} + \frac{m_3}{a_3}\right)\lambda + \frac{m_1m_3}{a_2a_3} = 0.$$

Therefore

$$\lambda_{1,2} = \frac{1}{2}\left\{-\left(\frac{m_2+m_3}{a_2} + \frac{m_3}{a_3}\right) \pm \sqrt{\left[\left(\frac{m_2+m_3}{a_2} + \frac{m_3}{a_3}\right)^2 - 4\frac{m_1m_3}{a_2a_3}\right]}\right\}.$$

At $t = 0$,

$$\bar{n}_0 = A + B,$$

therefore

$$\bar{n}_0\lambda_1 = A\lambda_1 + B\lambda_1, \tag{9}$$

and

$$\bar{n}_0\lambda_2 = A\lambda_2 + B\lambda_2 \tag{10}$$

and total efflux, at $t = 0$ by differentiation of (8)

$$-\frac{m_1\bar{n}_0}{a_2+a_3} = A\lambda_1 + B\lambda_2. \tag{11}$$

Subtracting (11) from (9),

$$B(\lambda_1-\lambda_2) = \bar{n}_0\lambda_1+\frac{m\bar{n}_0}{a_2+a_3}$$

therefore

$$B = \frac{\bar{n}_0}{\lambda_1-\lambda_2}\left(\lambda_1+\frac{m_1}{a_2+a_3}\right).$$

Subtracting (10) from (11),

$$A(\lambda_1-\lambda_2) = -\frac{m_1\bar{n}_0}{a_2+a_3}-\bar{n}_0\lambda_2$$

therefore

$$A = -\frac{\bar{n}_0}{\lambda_1-\lambda_2}\left(\frac{m_1}{a_2+a_3}+\lambda_2\right).$$

Therefore

$$\bar{n}_t = -\frac{\bar{n}_0\left(\frac{m_1}{a_2+a_3}+\lambda_2\right)}{\lambda_1-\lambda_2}.e^{\lambda_1 t}+\frac{\bar{n}_0\left(\lambda_1+\frac{m_1}{a_2+a_3}\right)}{\lambda_1-\lambda_2}.e^{\lambda_2 t}$$

where

$$\lambda_{1,2} = \frac{1}{2}\left\{-\frac{m_2+m_3}{a_2}+\frac{m_3}{a_3}\pm\sqrt{\left[\left(\frac{m_2+m_3}{a_3}+\frac{m_3}{a}\right)^2-4\frac{m_1m_2}{a_2a_3}\right]}\right\}.$$

Thus the time course of ^{22}Na efflux from this system is always resolvable into the sum of two exponentials. However, it is important to realize that the defining constants; A, B, λ_1, λ_2 generally do not even approximately describe any of the individual sodium pools or fluxes in the system.

The efflux curves fitted to the points in Figs 9(*a–c*) were computed from the above expression by a trial and error procedure estimating values of the variables $\bar{n}_0$, a_2, a_3, m_1, m_2, m_3 until a good fit to the data was obtained by eye. Plausible values for these parameters were chosen as follows:

$\bar{n}_0$ (pmol at specific activity of labelling medium) and m_1 (pmol min^{-1}). Minimal estimates of these are determined by simple exponential regression (Table 1).

$a_2+a_3 = \bar{n}_0$ (pmoles). Figs 6, 8 suggest a_2, the cytoplasmic sodium content, is about 5·0–6·5 pmol for early cleavage stages at least.

$m_2 = m_1-m_3+m_4$ (pmoles min^{-1}).

m_3 (pmol min^{-1}). A starting value of $m_3 = 0{\cdot}5$ was chosen from estimates of efflux in sodium-free saline in blastulae (Fig. 11*a*).

$m_4 = 0$ (pmol min^{-1}). The simplest possible case and a reasonable value for a sodium secreting mechanism (in fact calculations based on $0 < m_4 < 0{\cdot}2$ produced very similarly shaped curves).

Table 4 shows the final values of these parameters which were found to give a good fit to the data in Fig. 9(*a–c*) and the calculated values of the constants in the efflux expression. More than 50 trial efflux curves were computed before selection of these.

I am grateful to Professor L. C. Beadle for helpful and enthusiastic discussion during the course of this work. Professor J. Shaw derived the expression used in the Appendix. Financial support was provided by the Medical Research Council of Great Britain.

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