The primary action of lithium chloride on morphogenesis in *Lymnaea stagnalis*

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In *Lymnaea stagnalis*, as in sea urchins, lithium ions cause disturbances of development, notably exogastrulation and head malformations (Raven, 1942). As the effect of a Li-treatment presents itself only after several hours or days of development, the question was raised of the site and the nature of the primary action of the ion in the egg cells, even though it had never been proved that Li actually enters the cells.

The Li effect was described as a coarsening of cytoplasmic structure (Runnström, 1928), a condensation of the cytoplasm, caused by dehydration of certain of its colloidal components (Raven & Roborgh, 1949; Raven & van Zeist, 1950), and as an electrostatic effect on phosphatides located in the cortical layer of the egg (Raven, 1956). The controversy between these hypotheses was the original incentive to study this problem by electron microscopy. Such a study was undertaken earlier by the author (1959) but no influence of Li ions on the ultrastructure of the *Lymnaea* egg at the single cell stage could be found at that time. In later years it became clear that the type and extent of morphogenic aberrations in *Lymnaea* are very much dependent on the stage of development at which treatment with Li ions takes place (Geilenkirchen, 1964a; Verdonk, 1965).

In order to have a maximum chance of finding some Li effect, the relatively low number of eggs that can be studied in serial sections by electron microscopy has to be compensated by a high percentage, preferably 100%, of cases of morphogenic disturbance. Such a high percentage is obtained according to Geilenkirchen (1967) by a treatment of the eggs starting shortly before the second cleavage and using a solution of 0.05 M-LiCl, a nearly isotonic concentration.

The negative result obtained in the earlier study could have been due also to a preparative technique too crude to allow the detection of small changes in fine structure of the cell. Therefore the fixation and embedding technique for this embryological material have been steadily improved (Elbers, 1966). If it remains impossible to find any difference between control and experimentally treated cells, even when preservation of cell structure is improved upon as much as
possible, the search for a primary Li effect demands proof of the actual penetration of Li ions into the cell. In the earlier literature it was tacitly assumed that the Lymnaea egg is readily permeable to lithium ions. The literature on ion permeability of cells reveals however that such an assumption is not always valid. In order to support the electron microscopic observations, therefore, in this study attempts were made to measure the penetration of the noxious agent into the egg cells. By cytolizing an egg in a small quantity of distilled water and measuring the electrical conductivity of the resulting suspension (Elbers, 1966) to a certain extent uptake or exchange of salt can be detected by change in conductivity.

METHODS

Electron microscopy

According to the method described by Geilenkirchen (1967) synchronized eggs of Lymnaea stagnalis in their capsules were put into the test solutions at 6°C, 5 to 10 min before the second cleavage. The treatment lasted 2h and afterwards the eggs, which in the meantime had reached the 4-cell stage, were divided into two lots.

One group of eggs was washed with tap water at 6°C for 2h and reared in tap water at 25°C for 48h. Subsequently the capsules were laid out on moist agar in Petri dishes and cultured at 25°C. The other group of eggs was fixed by 1% osmium tetroxide in a balanced salt medium containing 25 mM-KCl, 2.5 or 25 mM-NaCl, 0.5 mM-CaCl2, 0.25 mM-MgCl2 and 0.25 mM-NaH2PO4. As a control a third group of eggs was treated with distilled water and fixed in the same way.

The decapsulated eggs were fixed at 5°C for 1h. Swelling due to fixation was tested with single egg cells, which are almost spherical. A diameter increase of only 5% was found. After dehydration the eggs were embedded in Epon. Serial sections, mounted on brass rings with a 1 mm hole, were stained with lead citrate and uranyl acetate (Venable & Coggeshall, 1965). Electron microscopy was done with the Philips E.M. 200 electron microscope at magnifications of from ×137 to ×19000.

Conductivity measurements

Eggs in the 2-cell stage, shortly before second cleavage, were treated with different salt solutions as specified in the experimental part. They were then decapsulated, washed with distilled water and transferred individually to the conductivity cell filled with distilled water. Intact eggs, showing no sign of salt leakage, were subsequently cytolized by an electrical shock. The salt release was registered as an increase in conductance, the end value of which was obtained as a scale number on the registering instrument. This number refers to the salt content of the egg but depends of course also on the size of the cells and the specific ionic composition of the egg cytoplasm. The sensitivity of this method is such that a change in concentration of 2·10^{-5} M-NaCl in the conductivity cell
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can be detected. This means a concentration change in the egg of about $5 \times 10^{-4}$ M. Uptake or loss of salts from the eggs during experimental treatment is assessed with more difficulty because of the biological variability of size and/or salt content of different eggs. Only the mean salt content of control and treated eggs can be compared.

**EXPERIMENTS AND RESULTS**

A full description of all the constituents of a normal 4-cell *Lymnaea* will not be given here. The cell organelles, such as mitochondria, Golgi bodies, yolk granules and vacuoles, lipid droplets, the nucleus and the cell membrane, look the same as those from other stages described earlier (Elbers, 1957, 1959; Bluemink, 1967), (Fig. 1).

Of importance, however, is the description of the cytoplasmic matrix which contains vesicular and granular components and occasionally microtubules (Fig. 2). The vesicular elements presumably represent the endoplasmic reticulum. It is difficult to state how far these vesicles are interconnected, but anyhow this endoplasmic reticulum, like that of undifferentiated cells in general, is only weakly organized, indicating a low level of protein synthesis in the egg cells (Haguenau, 1958). The granules are found partly in connexion with the outside of the vesicles, partly free in the cytoplasm. They have a diameter of about 200 Å and represent the ribosomes. They occur also in clusters as polysomes (Fig. 3). A layer of the cytoplasmic matrix, about 1 μ in thickness, immediately beneath the cell membrane generally does not contain cell organelles, although occasionally mitochondria and fat droplets are found as near as about 0.1 μ to the cell membrane (Figs. 1, 2). The distribution of vesicles and granules in the cytoplasmic matrix is essentially the same throughout the whole cell volume.

Often, however, on the micrographs, a cortical layer can be distinguished in which the cytoplasmic matrix looks a little darker than in the more central part of the cell (Fig. 2). This layer is thicker than the layer which is relatively free of organelles, but its inner border is very diffuse. Generally it is 2 to 3 μ thick, but in some places it cannot readily be distinguished. At the border between two cells it is often much thicker in one cell than in its neighbour. It contains all the same organelles as are found in the rest of the cytoplasm. The darker appearance of the matrix is caused either by a higher number of ribosomes per unit volume in this region, or by a difference in chemical properties of the ribosomes by which they become more heavily stained than those in the central part of the cell. As the ribosomal particles are much less distinct than those found in most types of cell, it is very difficult to decide between these two possibilities.

The above description is based on 1,385 photographs, from a group of 36 normal eggs in the 4-cell stage. Control groups of normal eggs were cultured along with each experiment. At least 95% of the control eggs developed into normal snails.
Ninety to a hundred per cent of the eggs treated with 0.05 M-LiCl on the other hand developed into exogastrulae. From this experimental group 44 eggs were serially sectioned and 1,174 photographs were taken. No difference in cell structure could be found between treated and control eggs.

![Fig. 1. Nearly equatorial section of a 4-cell Lymnaea egg (control).](image)

It was thought that the primary Li effect could perhaps be enhanced by using more concentrated (and therefore hypertonic) LiCl solutions. In order to single out possible effects of hypertonicity as such, or some general salt effect, experiments were done with 0.75 M-, 0.1 M- and 0.2 M-LiCl, 0.1 and 0.2 M-NaCl and 0.2 and 0.4 M-sucrose.

Eggs treated with these solutions showed considerable shrinkage, increasing with concentration (Fig. 4). With the higher salt concentration the cytoplasm becomes so condensed that its matrix components are only rarely discernible on
Fig. 2. Vesicular and granular components of the cytoplasmic matrix are easily discerned especially in the cortical region of the egg (control). Here also spindle remnants are found (SR). $M =$ mitochondrion; $y =$ yolk granule; $L =$ lipid droplet; $VM =$ vitelline membrane.
Fig. 3(A). Free and membrane bound ribosomes are found, the free ribosomes very often in clusters (control).

3(B). A tangential view of membrane bound ribosomes occasionally shows them ordered into a spiral structure (50 mM-LiCl).
Fig. 4(A). Two-cell stage egg shrunken by 100 mM-LiCl. Note the cortical region of higher electron optical density.

4(B). Two-cell stage egg shrunken by 200 mM-NaCl.
the electron micrographs and fat globules are seen to be forced out of the cell surface. The difference in electron density between the cortical plasma layer and the more central cell regions is much enhanced in the shrunken cell (Fig. 4). This picture is the same regardless of whether the medium was made hypertonic by LiCl, NaCl or sucrose. Macroscopically the eggs get the same glassy appearance as well. They do not re-swell even after a stay of many hours in the hypertonic solutions. The combined evidence indicates that the effect on cell structure is purely osmotic and that these three substances at least do not readily permeate the cell membrane.

In order to see whether there could be some salt penetration, not expressed by re-swelling of the cells, conductometric measurements of salt content were carried out after treatment of the cells with hypertonic LiCl, NaCl and KCl solutions. The salt content of normal *Lymnaea* eggs at the 2- to 4-cell stage amounts to 79 scale divisions on the conductometer ($n = 60, \text{S.D.} = 7.4$). After treatment with 0-075 M salt solutions for 2 h a value of 83 ($n = 12, \text{S.D.} = 4.7$) was found. Likewise with 0-1 M solutions a value of 82 ($n = 34, \text{S.D.} = 8.2$) was obtained. After a 24 h stay in 0-075 M-LiCl and KCl solutions the conductivity value was 84 ($n = 10, \text{S.D.} = 11$). Previously it was found that salt does not leak out of the eggs in distilled water. If LiCl entry had occurred within these 24 h, to equalize the concentration inside the egg with that of the medium, then a conductometric salt value of about 150 would have been found.

The variability in the salt content and the size of *Lymnaea* eggs makes the limit of detection of salt accumulation rather high. For LiCl it is estimated that a permeation of about 10% of the treatment concentration of 0-075 M would have been detected. But even in the experiments with salt concentrations of up to 0-1 M, no significantly higher conductivity is found than in normal eggs. It may be concluded that the eggs do not take up salt from the medium or give it off either, unless there is a process of salt exchange such that differences in equivalent conductivity of the ions are just cancelled: this would involve a deviation from a one-for-one exchange.

If a small amount of salt is normally taken up by active transport into the egg from the capsule fluid in the period of second cleavage, Li ions perhaps could use this system as well. Since g-strophantin is known to inhibit active cation transport in a number of biological systems and dinitrophenol (DNP) as an uncoupler of oxidative phosphorylation acts in the same direction, these substances were tested for their possible influence on the lithium effect in *Lymnaea*. 0-5 mM strophantin and 1 mM-DNP in no way diminished the effect of a 0-05 M-LiCl solution so that in both cases about 100% exogastrulae were obtained. An active transport system could prevent the gradual release of salt from the cell along the gradient of chemical potential if the cell membrane were normally permeable to salts. If such active transport could be inhibited by strophantin or DNP, this would result in an enhanced loss of salt from the egg. Therefore eggs at the 2-cell stage were treated with 1 mM strophantin and 1 mM-
DNP, for 2 h. Their mean salt content afterwards proved, however, to be equal to that of the control eggs.

If some carrier-mediated, metabolic-energy-dependent transport of Li ions across the cell membrane plays a rôle in their effect, it can be predicted that lowering the temperature would diminish this effect. Therefore parallel experiments were done at 6 °C and at 28 °C with the LiCl concentrations 50 mm, 25 mm, 12-5 mm and 6 mm (Table 1). It is seen that the Li effect is practically the same at the two temperatures. Such a low temperature coefficient is more typical of simple diffusion, if it exists, or surface exchange than of a metabolically linked process. These tests show that active transport of ions is unlikely to take place in the Lymnaea egg at the 2-cell stage.

Table 1. Percentage of normal development (N), exogastrulation (E) and cytolysis (C) 3 days after treatment with LiCl at 6° or 28°C

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Concentration of Lithium Chloride</th>
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<tbody>
<tr>
<td></td>
<td>50 mM</td>
</tr>
<tr>
<td></td>
<td>N  E  C</td>
</tr>
<tr>
<td>6</td>
<td>5  95  0</td>
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<tr>
<td>28</td>
<td>0  74  26</td>
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DISCUSSION

No primary effect of LiCl on cell ultrastructure could be demonstrated and no indication was found of actual uptake of salt by the egg cells in our experiments.

For an evaluation of this negative result one must take a general view of the action of salts on living cells. As Reznikoff (1927) stated, 'any agent with which the cell is treated may (a) affect the plasma membrane only, (b) affect both the plasma membrane and the internal protoplasm, (c) injure the internal protoplasm only or (d) may not enter the cell but affect it by abstracting a necessary constituent'. For a decision between these possibilities the first problem is that of the mode of transport of salts through biological membranes. One has to consider free diffusion, diffusion through pores, pinocytosis and carrier mediated transport, the last subdivided into facilitated diffusion, exchange diffusion and active transport, which utilizes some source of energy inside the cell (Csáky, 1965).

Studies of the transport problem in a large number of cell types reveal that no generalization applicable to all biological cells is possible. No pertinent data on salt transport in embryonic cells and in Lymnaea egg cells in particular are available however, and, as Ussing (1960) states with respect to our problem, it is a surprising fact that in no case has the Li effect been correlated with the Li
content of the organism in question. The next best thing to do is to look for data on permeability in cells which have some physiological property in common with the *Lymnaea* egg cells, for instance in that they also live in an environment of which the salt composition is not regulated by other cells of the same organism, or in that they are single cells without special permeability adaptations associated with their mutual interconnexion in a tissue organization. With the exception of red blood cells, this type of cell is seldom used for permeability studies. Some relevant data however can be gathered from the literature about the salt permeability of amoebae and large plant cells.

Chambers & Reznikoff (1926), from immersion and injection experiments on *Amoeba dubia*, conclude that NaCl and KCl readily penetrate the cell from the exterior but that CaCl₂ and MgCl₂ do not. The lethal action of NaCl and the antagonizing effect of CaCl₂, however, occur at the surface of the cell. Reznikoff & Chambers (1927) studied the effect of Na, Ca and K salts of weak acids like lactic, boric, acetic and carbonic acid on *Amoeba dubia*. These salts can be injected in high concentrations without toxic effects but amoebae can only be immersed in very dilute solutions of them without injury. These experiments indicate that in the amoeba the important factor in the production of lethal effects by salt is not their penetration into the interior but rather their action on the plasmalemma from the outside. The same conclusion is reached by Reznikoff (1927) in his study on the action of the chlorides of lead, mercury, copper, iron and aluminium on *Amoeba dubia*. None of the salts gave visible internal evidence of penetrating into the amoeba, with the exception of aluminium chloride, for which the similarity in appearance of the internal protoplasm in immersion and injection experiments would indicate a high degree of permeability.

The mechanism whereby *Acanthamoeba sp.* is capable of maintaining freely exchangeable K in the cell against a concentration gradient by means of active transport was studied by Klein (1961). Sodium permeability seems to be entirely passive, the amoeba lacks a pump to maintain a low intracellular Na⁺ concentration but it may achieve a similar end by binding most of this cation. The exchangeable cations could be removed from the cells by washing them with isotonic 0.3 M sucrose for 2–4 h. Klein (1964) found that when the suspending medium is made hypertonic by the addition of sucrose there occurs a net loss of about ⅓ of the cell K but not of cell Na, possibly by inhibition of the active K-transport mechanism. Dinitrophenol had the same effect, but strophanthin had no effect on this type of K-transport. From further analysis of subcellular fractions Klein & Breland (1966) conclude that *Acanthamoeba* does not possess a typical Na–K dependent Mg-ATP hydrolysing enzyme, or a Na–K coupled active cation transport like that demonstrated in many cells of higher organisms. In the amoeba a Mg-apyrase of the microsomal fraction very probably does provide the energy for the active K-transport. The stimulation by K salts is not specific, but is also obtained by the addition of Li salts.
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While the possible penetration of LiCl was not tested with the amoeba, some data are available on the penetration of Li into the cell sap of the fresh water alga *Nitella*. Brooks (1921) found by spectroscopic analysis that the presence of Li in the sap can be demonstrated after treatment of the cells with 0.05 M-LiCl for 24 h. At this concentration the salt was not toxic. With lower LiCl concentrations the demonstration of Li required an accordingly longer time of treatment. With regard to the character of cation penetration into *Nitella* cells, suggestion was obtained by Kitasato (1968) that $K^+$ is distributed passively across the cell membrane as a result of an $H^+$ pump working to accumulate $K^+$ in the cell by keeping the membrane potential more negative than the $H^+$ equilibrium potential. Barr (1965), however, thinks that $K^+$ is actively pumped inward, possibly by a Na–$K$ coupled pump. The effect of dinitrophenol on membrane potential was considered by Kitasato as the most conclusive evidence of a metabolic energy dependent ionic pump in *Nitella*.

In his study on the permeability of human erythrocytes to Na and K Solomon (1953) found that the addition of LiCl to the medium affected Na transport into the red cell. Li itself penetrated into the cells from an approx. 0.1 M solution to a concentration of about 20 m-equiv/l cells. The cells swell appreciably in the presence of Li, presumably due to its entry into the red cell. While Li thus appears to compete with Na for inward transport it is found that K and Rb do not. Since it is observed that the cells swell more in high Li solution than in any other alkali ion solution of equal concentration, it follows either that more Li enters than can be carried by the Na-mechanism or that the Na-mechanism operates to take Li in, but not to transport it out. Post (1957) also found an active, strophanthidin inhibited, transport of Li into the human red cell. Maizels & Remington (1959) demonstrated that the erythrocyte has associated with it 2–3 m-equiv Na/l cells which exchanges very rapidly with, and in proportion to, Na, K or Li in an external medium. It is thought that this fraction is closely associated with the cell surface. In favour of this view is the speed with which the exchanges occur and the absence of a temperature effect between 7 and 37 °C. Lepke & Passow (1968) used a 40 mM-LiCl solution for the determination of trapped extracellular fluid in an erythrocyte ghost sediment, assuming that red cells are impermeable to Li.

Immediate physiological effects of LiCl may give a clue to a possible site of action in the cell. According to Gallego & Lorente de Nó (1951) the inexcitability of nerve fibres after long treatment with LiCl must be the result of specific changes in the properties of the nerve membrane. Schou (1957) reveals as the most striking feature of the biology of the Li ion its partial similarity to the Na ion in cells in which a Na-pump mechanism has been demonstrated or is supposed to be at work. For instance, a high Na concentration protects rat kidney against toxic injury by Li, presumably due to competition between Li and Na for entrance into and re-absorption through the tubule cells (Schou, 1958).

Turkington (1968), with mouse mammary epithelial cells *in vitro*, found that
Li and NH₄ ions in a 30 mM concentration delay the onset of DNA synthesis in cells preparing for proliferation.

Suzuka & Kaji (1968) studied the effect of LiCl on isolated *E. coli* ribosomes. The ribosomes were completely dissociated into 50 S and 30 S subunits in the presence of 0.2 M-LiCl. Moreover the binding of RNA to ribosomes was inhibited. Some inhibitory effect of LiCl on the interactions of t-RNA with nucleoprotein in general seems to occur. This effect is partially reversed by the presence of NH₄⁺ or K⁺ in concentrations between 1 and 100 mM.

In *Lymnaea* an antagonistic action on the Li effect can be demonstrated for CaCl₂ (Elbers, 1952; de Vries, 1953) while KCl shows a slight synergism with LiCl. Ammonium chloride and ammonium acetate in concentrations of up to 0.05 M have no effect on development in the *Lymnaea* egg and these salts do not antagonize the Li effect (P. F. Elbers, unpublished). The antagonistic effects of CaCl₂ and alkali chlorides in *Lymnaea* were analysed more closely by Geilenkirchen (1964b). There appears to exist a reciprocal antagonism between these salts which can be explained by their electrostatic interaction with phosphate colloids.

Li⁺ imitates the action of insulin in promoting glucose uptake and glycogen deposition in rat hemidiaphragm (Bhattacharya, 1959; Clausen, 1968). Clausen considers the possibility that Li ions, like insulin, cause a certain structural modification of the cell membrane and so induce a change in the permeability of the cell membrane to glucose.

One gets the impression from the above data that salt uptake is realized either directly by means of metabolic-energy-dependent active transport of the ions, or indirectly as a passive distribution of the ions resulting from active transport of other ions. In the Li effect on *Lymnaea*, however, no sign of active transport was found. In the first place the Li effect is not altered by the presence of strophanthin. In other cells where active transport is not inhibited by strophanthin, because it is not Na dependent, the transport is still inhibited by low temperature and metabolic inhibitors (Csák, 1965; Klein, 1964). In *Lymnaea*, however, neither dinitrophenol nor low temperature diminishes the Li effect. The antagonism or synergism of Li⁺ towards other ions, observed by some authors, is not reflected in the Li effect on *Lymnaea* egg development.

The passive permeability of the egg towards alkali cations also must be very low or completely absent. In hypertonic NaCl, KCl and LiCl solutions the cells shrink and do not re-swell. They do not take up a measurable amount of salt, and in hypertonic sucrose solution they do not lose salt, which indicates that the permeability barrier remains intact. The passive permeability of K, Na and Li through the cell membrane may decrease in that order due to the increasing hydration energies of the respective cations, according to Shanes (1958). On the basis of the difference in hydration energy Stein (1967) expects Na⁺ and Li⁺ to penetrate more slowly than K⁺ into cells when permeability is passive only. Such a difference was found by Monnier (1968) to exist with
regard to penetration through an artificial lipid membrane. Since there is appar-
ently no permeability to NaCl or KCl in the Lymnaea egg, it is less likely to
occur with LiCl. Because neither active nor passive transport of Li\(^+\) into the
Lymnaea egg, nor any effect on cell ultrastructure is demonstrable, it must be
concluded (within the limits mentioned in the Introduction) that Li\(^+\) exerts its
primary action in morphogenesis at the surface of the embryo in the 2-cell stage,
that is at the level of the molecular structure of the cell membrane. Therefore
the phosphate colloid apparently involved in this action must be located in the
cell membrane. A logical extension of this conclusion is that certain properties
of the cell membrane are normally directive in embryonic development. Such
an idea, only in other terms, was put forward by Dalcq in 1941.

**SUMMARY**

1. Almost 100% of eggs of *Lymnaea stagnalis* develop into exogastrulae when
treated for 2 h with a solution of 0.05 M-LiCl, starting shortly before second
cleavage.
2. Electron microscopic analysis of both treated and untreated eggs revealed
no differences in ultrastructure.
3. Conductometric estimation of the salt content of both treated and untreated
eggs showed no difference, even when the eggs were treated with highly hyper-
tonic salt solutions.
4. Eggs treated with hypertonic salt or sucrose solution shrink and do not
re-swell.
5. Strophantin, dinitrophenol and a 22 °C difference of treatment tempera-
ture have no influence on the Li effect.
6. No active or passive transport of Li ions into the Lymnaea egg being
demonstrated, it is concluded that LiCl exerts its primary action at the surface
of the egg; that is, at the cell membrane. The cell membrane therefore plays a
directive rôle in development of the egg.

**RÉSUMÉ**

*L' action primaire du chlorure de lithium sur la morphogénèse chez Lymnaea
stagnalis.*

1. Les œufs de *Lymnaea stagnalis* se développent à peu près cent pour cent
comme des exogastrulae quand ils ont été traités pendant deux heures à une
solution de LiCl 0.05 M juste avant le deuxième clivage.
2. Une analyse au microscope électronique d'œufs traités et non-traités ne
révèle aucune différence dans leur ultrastructure.
3. Une estimation conductométrique du contenu de sels dans les deux
groupes d'œufs ne montre aucune différence, même quand ils ont été traités
aux solutions fortement hypertoniques.
4. Des oeufs traités aux solutions hypertoniques de sels ou de saccharose se contractent et ensuite ne se gonflent plus.
5. L’ouabain, le dinitrophénol et une différence de 22 °C dans la température du traitement n’ont aucune influence sur l’effet du lithium.
6. Puisqu’aucun transport, active ou passive, du chlorure de lithium dans l’œuf de Limnée a été démontré, la conclusion la plus rationnelle qu’on peut tirer est que les ions lithium exercent leur action primaire à la surface de l’œuf, c’est à dire au niveau de la membrane cellulaire. Par conséquent cette membrane doit posséder des éléments, qui déterminent certains traits du développement de l’œuf.

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