ZOOLOGY

ON THE INFLUENCE OF DISTILLED WATER AND LITHIUM CHLORIDE UPON THE EGGS OF LIMNAEA STAGNALIS L. AT THE 2-CELL STAGE

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RAVEN (1942) exposed eggs of *Limnaea stagnalis* to weak solutions of LiCl. Characteristic malformations were induced by this treatment: part of the eggs developed to exogastrulae, whereas other eggs yielded embryos with malformations in the head region.

RAVEN, KLOEK, KUIPER and DE JONG (1947) showed that a maximum of sensibility for the production of exogastrulae exists at the time of second cleavage. Lithium treatment immediately after shedding or in the 24-cell stage (RAVEN and RIJVEN 1948) produces mostly head malformations.

DE GROOT (1948) exposed decapsulated eggs immediately after shedding to higher concentrations of LiCl, in order to study the direct effects of this treatment. Hypertonic LiCl solutions caused disturbances of the nuclear cycle of maturation and fertilization, which lead to a standstill of development at different stages, dependent on concentration, stage of treatment, temperature and susceptibility of the eggs. In isotonic solutions, the nuclear phenomena, as far as could be observed, proceeded normally, but the cytoplasmic components of the egg showed interesting abnormalities; the distribution of the subcortical plasm was quitec abnormal. The animal pole plasm did not develop either in hypertoni or isotonic solutions.

RAVEN and ROBORGH (1949) treated decapsulated eggs with isotonic and hypotonic LiCl solutions. In these eggs, the telophase chromosomes of the first maturation division show a reversible swelling into karyomeres. At the same time the sperm nucleus swells and migrates towards the animal pole. The spermaster shows an increased hydration after the second maturation division; its disappearance is delayed. The amoeboid mobility of the eggs is considerably increased after the second maturation division. The first cleavage is delayed. Various abnormalities of the first cleavage mitosis occur. The results indicate that the primary action of the lithium ions consists in a change of the state of hydration of the protoplasmic colloids, causing changes of water equilibrium between various components of the egg.

Finally, treatment of decapsulated *Limnaea* eggs at the 24-cell stage

with LiCl solutions leads to an increase in the size of the nuclei and a decrease of their distance from the cell surface (RAVEN and DUDOK DE WIT 1949). Both phenomena are most pronounced in 0.05 % LiCl and diminish in intensity with increasing concentrations of LiCl, but they are also less pronounced in distilled water.

In the present investigation the direct effects of LiCl solutions at the 2-cell stage, when the eggs show a maximum sensibility for the production of exogastrulae, have been studied.

Material and Methods.

When the eggs of an egg-mass had developed to a 2-cell stage with beginning flattening of the blastomeres after their maximum rounding off (about stage 5 according to RAVEN 1946), the egg-mass was divided into 3 equal parts. The eggs of one part were decapsulated and transferred for 1, 2 or 3 hours to a 1.0 %, 0.4 % (hypertonic), 0.2 % (isotonic), 0.1 % or 0.05 % (hypotonic) LiCl solution. Those of the second part were likewise decapsulated and put for the same time in distilled water. Both groups of eggs were fixed immediately after treatment. The eggs of the third part remained in their capsules till the end of the experiment, then they were also decapsulated and fixed simultaneously with the other eggs.

41 Egg-masses have been treated in this way. 188 Li-treated eggs have been studied cytologically, together with 156 controls and 186 distilled-water eggs.

All eggs were fixed in BOUIN's fluid. The 7.5 μ thick sections were stained with iron haematoxylin and saffranin.

Results.

1. *Controls.* In the control eggs, which develop inside their capsules, after 1 hour the second cleavage has begun. On an average, they have reached a 4-cell stage with rounded blastomeres and reconstituting karyomere nuclei (stage 12 of RAVEN 1946). After 2 hours, they have developed to a stage just preceding 3d cleavage; as a rule, the cells contain metaphase or early anaphase spindles (stage 15). Finally, 3 hours after the beginning of the experiment, the controls mostly have reached an 8-cell stage with wide cleavage cavity and interphase or early prophase nuclei (stage 19). As a matter of course, development proceeds quite normally in these eggs; they show a regular cycle of formation and extrusion of fluid in the cleavage cavity, with well-developed secretion-cones, as described by RAVEN (1946). The animal and perinuclear plasm is quite dense, whereas the rest of the cytoplasm is moderately to strongly vacuolar, the vacuoles having arisen by swelling of the γ-granules (fig. 2, 4).

2. *Distilled water.* In most eggs kept in distilled water, after 1 hour the second cleavage has begun, but a fairly large proportion of them

is still in the 2-cell stage (41 % over against 16 % in the controls). On an average, they have reached the stage of just beginning 2nd cleavage (stage 10). Hence, it seems that their development is slightly retarded. In the 2-celled eggs, the flattening of the cells against each other is often incomplete and the cleavage cavity is lacking or incompletely developed.

After 2 hours, retardation of development is still more distinct. On an average, the eggs are in stage 13 (4-cell stage with polymorphic resting nuclei); numerous eggs have reached stage 14 (prophase nuclei), but in no case the spindles of 3d cleavage have been formed in these eggs, whereas this has occurred in 81 % of the controls. Moreover, it now becomes clear that in a certain proportion of these eggs (about 10 %) development is blocked in the 2-cell stage; in other cases, 2nd cleavage has taken place in one of the blastomeres only, so that 3-cell stages are formed. The flattening of the blastomeres is mostly incomplete, and the cleavage cavity is reduced or is lacking altogether. Vacuolisation of the cytoplasm is somewhat stronger than in the controls. The dense animal pole plasm is well-developed in these eggs.

After 3 hours, it is evident that all eggs which have passed the second cleavage are blocked in stage 13 — 14 (4-cell stage with interphase or prophase nuclei); in no case the spindles of third cleavage have been formed. The flattening of the blastomeres is incomplete, the cleavage cavity is lacking or rudimentary. The nuclei are often big and have a swollen and abnormal appearance. The vacuolisation of the cytoplasm is much stronger than in the controls; often, the structure of the cells has become quite abnormal by confluence of the vacuoles to irregular cavities, while the cytoplasm has been reduced to a mesh-work of slender strings between these cavities. In this condition the cell is on the verge of cytolysis, which has occurred, as a matter of fact, in some cells. The animal pole plasm and perinuclear cytoplasm do not take part in this vacuolisation and show a rather normal structure till the end.

3. *LiCl 0.05 %*. Development in 0.05 % LiCl corresponds, generally, to that in distilled water. Again, development is retarded as compared with the controls, and comes to a standstill at stage 13 — 14. Vacuolisation of the cytoplasm is strong, but in these eggs no confluence of the vacuoles with subsequent cytolysis has been observed after 3 hours. The nuclei are big and vesicular; in some cases, their swelling even exceeds that in distilled water. Flattening of the blastomeres against each other is stronger than in distilled water, but the cleavage cavity is often reduced or does not form at all. There is a well-developed animal pole plasm and the dense perinuclear protoplasm is very distinct and shows a concentric arrangement of granules.

4. *LiCl 0.1 %*. In this concentration, after 1 hour all eggs are still at the 2-cell stage. The nuclei are markedly big and swollen, the cytoplasm is moderately vacuolar. The cells show a nearly normal flattening and the cleavage cavity has often formed in a normal way.

After 2 hours, all eggs have passed through 2nd cleavage; most of them are in stage 13 — 14 (fig. 1), one (out of 11) has formed the spindles of 3d cleavage (stage 15). The eggs are much smaller than the controls and those in distilled water, but the nuclei correspond in size to those of the distilled-water eggs. The cytoplasm has a rather dense structure and is only weakly vacuolar. Flattening of the cells is good, but the cleavage cavity is often lacking (fig. 1).

After 3 hours, all eggs are still in stage 13 — 14; evidently, development is blocked at this stage. The nuclei are still big and swollen. The cytoplasm is moderately vacuolar. The perinuclear protoplasm is very dense and exhibits a conspicuous concentric arrangement of granules. In most eggs no cleavage cavity is present.

5. *LiCl 0.2 %*. In this LiCl solution, which is isotonic with the freshly-laid *Limnaea* egg, but, in consequence of the swelling of the eggs which has meanwhile taken place, presumably must be regarded as hypertonic to 2-celled eggs, after 1 hour all eggs are still at the 2-cell stage. Flattening of the cells has occurred and a lenticular cleavage cavity is present in most eggs. The cytoplasm is rather dense and only weakly vacuolar.

After 2 hours, in most eggs development has made no progress. The cytoplasm has become still more dense, but the nuclei are big and vesicular (fig. 3). In some eggs, big round sharply-delimited cavities have appeared in the cytoplasm. A few eggs show a beginning of 2nd cleavage, which is, however, quite abnormal, the blastomeres being very unequal in size.

After 3 hours, part of the eggs are still in the 2-cell stage, but in most eggs an abnormal second cleavage is taking place. All kinds of cleavage anomalies have been observed: unequal cleavage of both cells, in extreme cases leading to the splitting off of very small cells by both blastomeres; formation of non-nucleated cytoplasmic lobulations at the animal end of the cells, which may likewise be pinched off; cleavage of one blastomere only, so that 3-celled eggs are formed. All eggs possess a very dense deeply-staining cytoplasm, in which only few minute vacuoles have formed around γ-granules.

6. *LiCl 0.4 %*. In this strongly-hypertonic solution development is still more inhibited. No more cleavage takes place, even after 3 hours. The eggs are much reduced in size. The cytoplasm is very dense and contains only a few very small vacuoles around γ-granules. The nuclei, as a rule, have an abnormal appearance, and contain some heavily-staining chromatin masses in a clear karyolymph. The cleavage cavity between the cells is often very wide, while the cells are reduced to narrow crescents bordering this cavity. Apparently, a strong dehydration of the cytoplasm has taken place.

7. *LiCl 1 %*. The same phenomena, but still more pronounced, appear in 1 % LiCl solutions. Apart from flattening of the blastomeres

against each other and formation of a cleavage cavity between them, no further development has taken place. The cytoplasm is extremely dense and stains deeply with saffranin; no vacuoles are present. The nuclei in most cases are very abnormal, with condensed and clotted chromatin often forming a coarse network. They have a markedly shrivelled appearance. Dehydration of the cells is extreme; the cleavage cavity is often very wide and the cells are narrow crescents. In other cases, however, no cleavage cavity has been formed and the cells are small and more or less spherical.

Discussion.

1. *The inhibition of development by distilled water.*

RAVEN and KLOMP (1946) showed that in eggs transferred to distilled water shortly after shedding the first cleavage nearly always takes place, although often somewhat delayed as compared with the controls. In some cases development comes to a standstill at the 2-cell stage; mostly, however, the 2nd cleavage also takes place. Development does not proceed beyond the 4-cell stage, even when the eggs are transferred to distilled water after the 1st or even the 2nd cleavage.

RAVEN (1949*b*) and RAVEN and ROBORGH (1949) observed that eggs put in distilled water immediately after oviposition develop normally till 1st cleavage. Both the maturation divisions and the formation of a cleavage spindle take place in an entirely normal way; no differences with normal eggs developing in their capsules can be observed.

The first signs of a detrimental effect of distilled water on the *Limnaea* egg occur at the time of 2nd cleavage. A small part of the eggs are blocked at the prophase of this cleavage; in other eggs only one of the blastomeres divides. Most eggs cleave, however, without external signs of injury, but with some delay as compared with the controls. Reconstitution of the nuclei takes place, and in most cases the prophase stage of the next division is reached. None of the cells, however, is able to form the spindles of 3d cleavage. Soon, the first signs of impending cytolysis appear by the confluence of cytoplasmic vacuoles.

It may be concluded from these observations that the eggs are not very susceptible to distilled water at the uncleaved stage. Susceptibility increases, however, towards the end of the 2-cell stage, and reaches a maximum at the 4-cell stage. The changes provoked in the egg by this medium affect especially the mechanism of division, preventing the dissolution of the prophase nuclei and the formation of metaphase spindles. Whether the observed swelling of the nuclei is directly responsible for this effect, or the primary point of attack is on the cytoplasm, cannot be decided from these observations.

Cleavage in distilled water is abnormal; the flattening of the cells against each other and the formation of a cleavage cavity are prevented

or reduced (RAVEN and KLOMP 1946). One might think that this latter circumstance be responsible for the inhibition of development, since the extrusion of fluid from the egg through the cleavage cavity, as takes place in normal eggs (RAVEN 1946), is not possible. However, the addition of a small quantity of $CaCl_2$ to the medium leads to normal cleavage, but still development stops at the 4-cell stage; however, cytolysis is somewhat delayed as compared with eggs in pure distilled water (RAVEN and KLOMP 1946).

On the other hand, the block of development at the prophase of 3d cleavage may be broken by the addition of egg capsule fluid or of crushed eggs to the distilled water (HUDIG 1946).

The same holds true of 0.0004 M $CaCl_2$, 0.0016 M $MgCl_2$ and 0.0125 M NaCl and KCl solutions (GRASVELD 1949). These observations, and especially the quantitative difference between the effects of bivalent and monovalent kations, point to some specific action of the ions; this may be due to their influence on the water permeability of the egg cortex or the state of hydration of the cytoplasmic colloids.

2. *The effects of lithium.*

Contrary to NaCl and KCl, LiCl in no concentration with certainty breaks the block of development at the prophase of 3d cleavage. As a matter of fact, GRASVELD (1949) observed a few cases, in which the eggs in 0.2 % (0.05 M) LiCl developed to an 8-cell stage. We found among our material one egg that had formed the spindles of 3d cleavage in 0.1 % LiCl. These cases remain exceptions, however; in general, development in hypotonic LiCl solutions agrees with that in distilled water.

In 0.05 % LiCl, the state of the cytoplasm does not differ very much from that in distilled water. Vacuolisation is strong, as it is both in the controls and in distilled-water eggs. On the contrary, in 0.1 % LiCl the cytoplasm has a rather dense structure and is only weakly vacuolar; moreover, the cells are much smaller than those of the controls (fig. 1 and 2). This is still more pronounced in 0.2 % solutions, where the eggs possess a very dense deeply-staining cytoplasm with only a few minute vacuoles (fig. 3 and 4). Finally, in 0.4 % and 1.0 % LiCl the dehydration of the cytoplasm becomes extreme.

On the contrary, the nuclei have a swollen appearance not only in 0.05 % and 0.1 %, but also in 0.2 % LiCl solutions; in the former, in some cases they even exceed the nuclei of distilled-water eggs in size. Even in 0.4 % LiCl solutions, they appear not to be markedly smaller than in the controls; in 1 % solutions, however, they are small and shrivelled down. We may conclude, therefore, that lithium affects differently the cytoplasm and the nuclei. It provokes a swelling of the nuclei, which is only prevented by strongly hypertonic solutions. This corresponds to the observations of RAVEN and DUDOK DE WIT (1949) and RAVEN and ROBORGH (1949). On the other hand, lithium has a

dehydrating effect on the cytoplasm. The observation that this is obvious already in 0.1 % solutions, which are hypotonic and have an osmotic pressure which is, presumably, only slightly higher than that of the egg capsule fluid, indicates that it is no mere osmotic phenomenon. Moreover, we have compared the structure of eggs in 0.2 % (0.05 M) LiCl with that of eggs in isosmotic (0.1 M) urea and glucose solutions. Since these eggs did not come from the same egg-mass, and had not been fixed and stained simultaneously with the other ones, no absolute value may be attached to this comparison. The experiment has to be repeated with greater precautions. Still, the preliminary results indicate that the density of the cytoplasm in LiCl is markedly greater than that in isosmotic non-electrolyte solutions.

Hence, it appears that the conclusion drawn by RAVEN and ROBORGH (1949) from their experiments viz. that the primary action of the lithium ions consists in a change of the state of hydration of the protoplasmic colloids, causing changes of water equilibrium between various components of the egg, is correct. Notably the nuclei and the cytoplasm react in a different manner to these ions.

Our results, therefore, support the views upheld by RANZI (e.g. 1947*a* and *b*) according to which the influence of lithium upon embryonic determination in various animals depends on its tightening action upon filamentous proteins.

Eggs in 0.05 % and 0.1 % LiCl solutions agree with those in distilled water in possessing greatly swollen nuclei. The state of the cytoplasm, on the other hand, especially in 0.1 % LiCl, differs markedly from that in distilled water. It seems probable, therefore, that the block of development at the prophase of 3d cleavage, which occurs in all these eggs, may rather be connected with the excessive swelling of the nuclei than with the state of the cytoplasm. On the other hand, the delayed and atypical 2nd cleavage observed in 0.2 % LiCl, and the immediate inhibition of development in stronger LiCl solutions, may be due to the dehydration of the cytoplasm in these media.

According to DE GROOT (1948) and RAVEN and ROBORGH (1949), eggs transferred to 0.2 % LiCl immediately after shedding show a rather normal development up to first cleavage. This cleavage occurs in most of the eggs, though with some delay; part of the eggs exhibit disturbances in the formation of the cleavage spindles, however. Also a second cleavage may take place, but, according to DE GROOT, nearly always a 3-cell stage is formed at this cleavage. No further development has been noted by this author. Hence, development comes to a final stop at the same stage, whether the eggs are transferred to the solution immediately after oviposition or not before the 2-cell stage. Evidently, the susceptibility to the direct effects of lithium chloride is low during the uncleaved stage, and increases markedly towards the end of the 2-cell stage. The same has been found above to be the case with regard to the action of distilled

water. Previous experiments have shown that the eggs at the time of 2nd cleavage are also highly susceptible against the action of urea (RAVEN and KLOMP 1946), thiourea (SOBELS 1948) and various chlorides (RAVEN and SIMONS 1948).

According to DE GROOT (1948), in eggs transferred to 0.2 % or stronger LiCl solutions immediately after shedding, the distribution of cytoplasmic substances is abnormal and the animal pole plasm does not form at all. In the eggs put in 0.05 % and 0.1 % LiCl at the 2-cell stage, the animal pole plasm and perinuclear plasm are well-developed and quite distinct even after 3 hours. In the eggs kept in 0.2 % or higher concentrations, these plasms are not clearly distinguishable on account of the great density of the whole egg; in many cases, however, even in these eggs the animal pole plasm is indicated by its somewhat darker staining. It seems, therefore, that lithium does not suppress these plasms once they are formed. The difference in density between these plasms and the rest of the cell, which is striking in normal eggs, is diminished by the general dehydration of the cytoplasm, however. Perhaps this attenuation of the density contrast between the animal and the vegetative side of the egg may bear a relation to the depression of the polar gradient field which is apparent from the morphogenetic results of lithium treatment (RAVEN 1949*a*).

Summary.

1. The direct effects of distilled water and lithium chloride solutions upon 2-celled eggs of *Limnaea stagnalis* have been studied.

2. Development in distilled water is blocked at the prophase of 3d cleavage.

3. In hypotonic (0.05 % and 0.1 %) LiCl solutions development is blocked at the same stage. In 0.2 % LiCl 2nd cleavage is abnormal. In hypertonic (0.4 %, 1.0 %) LiCl solutions development stops nearly immediately.

4. Lithium provokes a swelling of the nuclei, which is only prevented by strongly hypertonic solutions.

5. Lithium has a dehydrating effect on the cytoplasm.

6. The primary action of the lithium ion consists in a change of the state of hydration of the cytoplasmic colloids.

7. At the time of 2nd cleavage the eggs are very susceptible against various injurious agents.

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CHR. P. RAVEN and W. VAN ZEIST: *On the influence of distilled water and lithium chloride upon the eggs of Limnaea stagnalis L. at the 2-cell stage.*

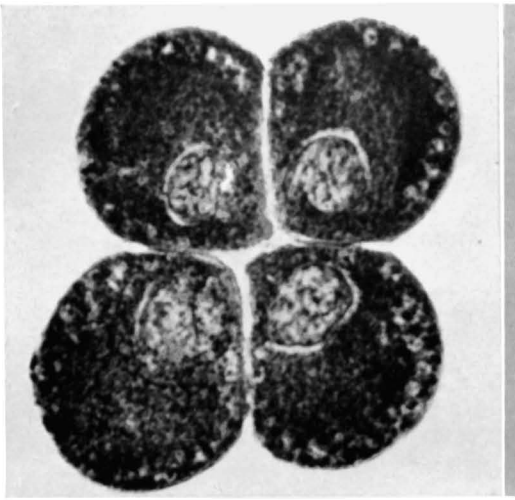

Fig. 1

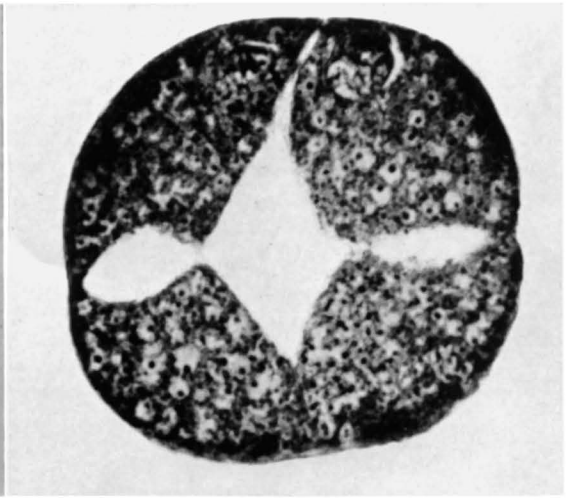

Fig. 2

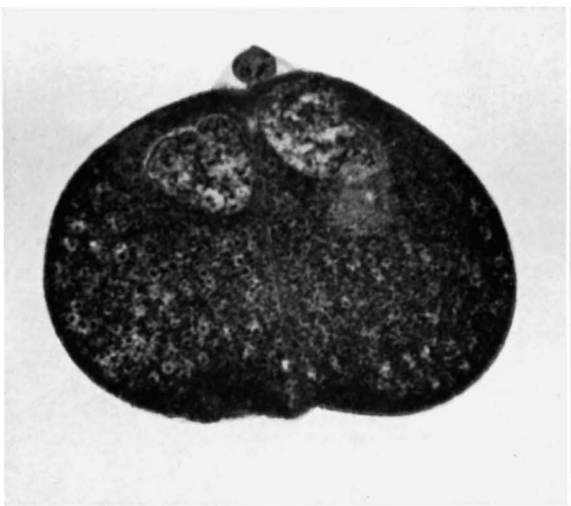

Fig. 3

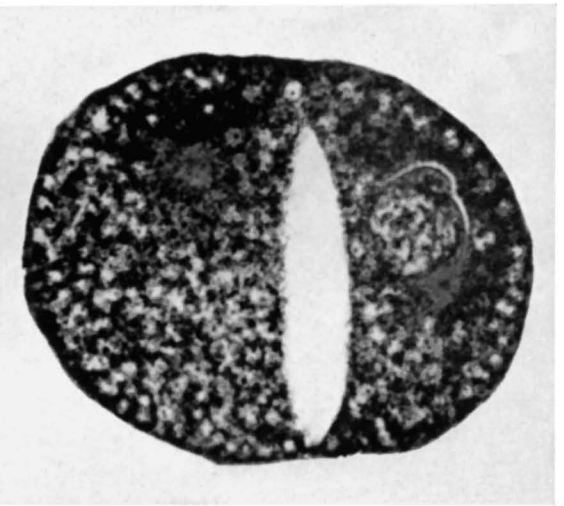

Fig. 4

Fig. 1. Egg of *Limnaea*, 2 hours in 0.1 % LiCl. Stage 14.

Fig. 2. Normal egg at the same stage.

Fig. 3. Egg of *Limnaea*, 2 hours in 0.2 % Li Cl. Stage 8.

Fig. 4. Normal egg at the same stage.

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