**Zoology.**. — *The influence of higher concentrations of lithium chloride on maturation and first cleavages of the egg of Limnaea stagnalis.* I. By A. P. DE GROOT. (Zoological Laboratory, University of Utrecht.) (Communicated by Prof. CHR. P. RAVEN.)

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The effect of lithium chloride on early development has first been described by HERBST (1892) in the eggs of sea urchins. After LiCl treatment the invagination of vegetative material during gastrulation may be prevented by an extension of the presumptive entoderm at the expense of the presumptive ectoderm, resulting in the occurrence of so-called "exogastrulae".

With the same agent LEPLAT (1920) obtained an abnormal development of amphibian eggs. Gastrulation was normal, but during further development abnormalities occurred, resulting in cyclopic or anophthalmic larvae. As shown by LEHMANN (1937) with the aid of vital staining, in the trunk the differentiation of notochord tissue may be suppressed, whilst the presumptive notochord cells differentiate in an abnormal direction under the influence of LiCl.

In 1942, RAVEN discovered the disturbing effect of LiCl on the egg of a Gastropod. Both exogastrulae and embryos with head malformations were obtained by treating the eggs of *Limnaea stagnalis*. In a series of investigations, RAVEN and his co-workers (1947) found that the appearance of either exogastrulae or larvae with head malformations was dependent on the stage of treatment. For the production of exogastrulae there is a distinct maximum of sensibility just before and during the second cleavage, whereas two periods of high sensibility exist with regard to the production of head malformations, viz. one immediately after oviposition and a second, some hours after the 24-cell stage.

It is likely that the nature of the damaging influence of LiCl is the same in each of the three kinds of eggs. However, it is not yet clear in which way LiCl affects the egg. Possibly this problem might be elucidated by studying the primary effects of the treatment. However, when the eggs of *Limnaea* are treated with the diluted LiCl solutions used for the production of the above-mentioned abnormalities, no visible effects on the structure of the eggs immediately after treatment can be observed. Therefore, in the present investigation the initial effects of LiCl were studied after treatment with higher concentrations than those used in previous experiments. Whereas the experiments of RAVEN c.s. (1942 and 1947) were carried out with concentrations varying from 0.001 % up to 0.01 %, in the present investigation solutions of 0.05 % up to 4.0 % have been used.

*Material and methods.*

Snails were stimulated to deposit egg-masses by means of *Hydrocharis* leaves, cf. RAVEN and BRETSCHNEIDER (1942). Considering the differences in stage of development in different parts of an egg-mass, the latter was divided lengthwise into halves; one half was treated and the other one used as a control. The treated eggs were decapsulated and washed in distilled water, in order to remove the capsule fluid, and then transferred to the solution. The control eggs developed inside the egg capsules. Twelve different concentrations of LiCl have been used, varying from 4.0 to 0.05 %. The solutions were prepared by diluting a stock solution of 4.0 % with distilled water. In addition a number of batches were treated with 3.0 % and 1.5 % $CaCl_2$, and some with 4.2 % and 3.2 % urea. Several batches were treated with each solution and the course of development was studied in vivo. In this way 74 egg-masses, with a total number of more than 1300 eggs, were studied.

7 Batches, treated with 4 different concentrations of LiCl, were studied cytologically. Parts of these batches were fixed at different moments, embedded in paraffin and sectioned in the usual way (5 $\mu$). The sections were stained partly with azan, partly with iron haematoxilin and saffranin, in such a way that both staining methods were applied to each group of eggs.

*Observations in vivo.*

In 4.0, 2.0, 1.5 and 1.0 % LiCl the eggs shrink considerably, showing a decrease of volume of more than 50 %. Their greenish colour changes into brownish-yellow. They become more or less flattened perpendicular to the egg axis, and show a bright area in the centre in transmitted light. The latter phenomenon is connected with the orientation of the eggs in these strongly hypertonic solutions. Some minutes after being transferred into LiCl, the eggs turn the animal pole downwards and remain in this position. This movement is to be taken into account, in order to avoid unexact observations on polar body formation. As a result of this remarkable orientation, the egg averts its animal pole from the observer, and polar bodies are not visible without turning the eggs. If the microscope is focussed deeply a little ring may be seen in the centre of the bright area, i.e. the outline of a polar body. It is likely that this orientation is caused by an unequal withdrawal of water from different parts of the egg. According to DALCQ (1923) the permeability of the *Asterias* egg is highest at the animal pole. If the *Limnaea* egg has the same property, then the orientation phenomenon is understandable. It would imply that the withdrawal of water is highest at the animal pole region, which causes an increase in density, resulting in a turning downwards of this part of the egg.

Eggs transferred to a 4.0 % solution immediately stop their development and cytolysis occurs after a certain lapse of time. If the eggs are transferred

to a 2.0 % solution immediately before the formation of the first polar body in the controls, a partly extruded polar body is formed, ranging in shape from a slight prominence of the egg surface to a nearly totally extruded body, with a narrow connection with the egg surface. If the eggs are transferred to the solution somewhat earlier, then only a slight prominence occurs. With still earlier treatment even this bulging out is suppressed.

In 1.5 % and 1.0 % a first polar body may or may not be formed. Its formation is prevented only, when treatment begins more than 30—35 minutes before. If it begins at a later moment, the body is extruded in nearly all eggs. It is, however, deformed, more or less oblong, with its largest axis parallel to the egg axis. Development does not proceed further and cytolysis occurs.

In 0.6 and 0.5 % a first polar body was always extruded; mostly it had an abnormal shape, often with a conical protuberance at the top. A second polar body was never observed. During its formation in the controls, the treated eggs showed abnormally strong amoeboid movements.

In 0.4 % the behaviour of the eggs varies. A first polar body is always extruded. Often a second polar body may also be formed, especially when treatment begins less than 20 min. before the formation of the first polar body. It was noted that the percentage of eggs extruding a second polar body is not only higher, when treatment begins later, but also when the temperature is higher. At the time of second polar body formation in the controls, treated eggs may show a highly irregular outline, caused by intensive amoeboid movements. The shape is normal again after a certain time. Cleavage activity was never observed in this solution.

In 0.35, 0.3, 0.2, 0.1 and 0.05 %, both polar bodies were nearly always formed. As well as in higher concentrations the moment of polar body formation is normal; a retardation did never occur. Giant polar bodies were observed in 0.4, 0.35 and 0.2 %, but only in a very small number of eggs.

A 0.35 % solution is the highest concentration in which a cleavage furrow was observed, but it was only a temporary one. Although the eggs may be divided totally into two blastomeres, no two-cell stages could be found after some hours; so the cleavage furrows had disappeared again.

In 0.3 %, the cleavage furrow is permanent, but mostly abnormal. In some cases a cleavage furrow appears between the animal and the vegetative poles, dividing the egg perpendicularly to the egg axis. As well as in 0.35 % the cleavage furrow appears with considerable delay and the blastomeres never flatten, but remain spherical. The percentage of eggs showing a first cleavage increases and the delay in cleavage decreases with decreasing concentrations; in 0.05 % there is no delay at all. The same may be said with regard to the second cleavage, which occurs first in 0.3 % solutions. However, in 0.05 % the second cleavage is less frequent than in 0.1 %. In 0.2 % nearly always a three-cell stage arises at the second

cleavage. The blastomeres formed at cleavage in the higher concentrations remain spherical; in 0.1 % and 0.05 % they flatten themselves against each other and a cleavage cavity is formed. Especially in the latter concentration a quite normal two-cell stage is obtained.

*Cytological observations.*

7 Egg masses, treated with four different concentrations, have been studied cytologically. The choice of the concentrations was determined by the results of the observations in vivo. In order to investigate the internal processes, attended with the suppression of polar bodies, 1.0 % and 0.4 % LiCl were used, by which the extrusion of the first and second polar body, respectively, might be prevented. In contrast with previous observations in vivo, the eggs in 0.4 % for a large part extruded both polar bodies; this discrepancy was ascribed to abnormal high temperatures, rising sometimes to more than 30° C. Suppression of the second polar body was obtained, therefore, by treating two other egg-masses with 0.5 % LiCl. Finally, one egg-mass was treated with a 0.2 % solution, which is about isotonic to the eggs. Lots of eggs were fixed at different moments after the formation of first and second polar body, after the first cleavage and some after being replaced to distilled water. The behaviour of different egg-masses treated with the same concentration was not identical. However, a distinct increase of the damaging influence with increasing concentrations was observed.

In 1.0 % LiCl, development stops soon. The first maturation spindle looses its contact with the animal pole and sinks into the interior of the egg. It shows a degenerated appearance and in its centre a nucleus-like body is situated. In the degenerating spindle and especially at its periphery a great number of vacuoles appear, each containing a heavily stained rod-shaped body, which, sometimes, seems to be paired. In eggs fixed half an hour later, these bodies prove to be scattered over a wider area, and their number, shape and dimensions may vary greatly (fig. 1). We cannot yet

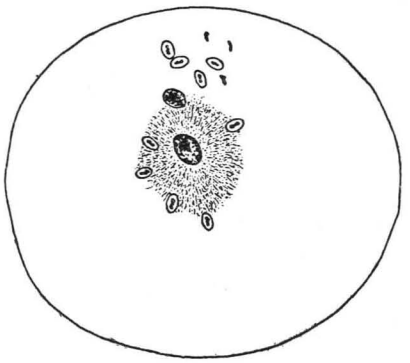

Fig. 1. Egg treated with 1.0 % LiCl, showing a degenerated first maturation spindle with a nucleus-like structure and scattered vacuoles containing a rod-shaped body.

decide whether these bodies originate from chromosomes, or are to be considered as coagulation products of the degenerating spindle.

In 0.5 % LiCl, the first polar body is formed without any visible abnormality. However, the second maturation spindle sinks into the interior of the egg and degenerates. In its centre a nucleus-like structure may appear. Like the degeneration of the first maturation spindle in 1.0 % LiCl, that of the second maturation spindle is accompanied with the appearance of vacuoles scattered in and around the spindle (fig. 2). The heavily-

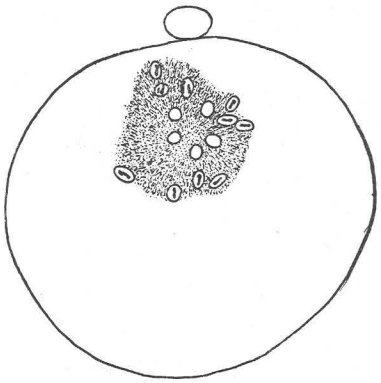

Fig. 2. Degenerated second maturation spindle in 0.5 % LiCl. In some of the vacuoles, the chromosome-like bodies are lying in pairs.

staining bodies in these vacuoles are often lying in pairs; sometimes they exceed many times the dimensions of chromosomes. The subcortical plasm extends beneath the egg cortex, leaving free an area at the animal pole. This gap does not close as it happens in normal eggs after completion of the second maturation division. Even in eggs fixed after the first cleavage in the controls, the subcortical plasm has not yet spread round the whole egg. So, the normal movement of this cytoplasmic component is either delayed or suppressed altogether. Formation of an animal pole plasm has never been observed in these eggs. After transfer of the treated eggs to distilled water, large multipolar spindles appeared, whereas nuclei or chromosomes could no longer be observed. The formation of these spindles may, possibly, be attributed to an evolution of the spermaster.

In 0.4 % LiCl the development proceeds somewhat further than in higher concentrations and the effect of the treatment is less destructive. Very interesting abnormalities occur, varying greatly in different egg-masses. Several cases may be distinguished:

*a.* If treatment starts at an early stage, the chromosomes may swell into karyomeres shortly after the extrusion of the first polar body, taking a position immediately beneath the egg cortex at the animal pole in the disappearing rests of an aster (fig. 3). So a situation is reached, which in normal eggs occurs only after the second maturation division. The second maturation spindle is not formed. The sperm nucleus migrates towards the animal pole and swells into a male pronucleus much earlier than normally

(fig. 3). In some cases, the animal pole had been reached already 35 minutes after first polar body formation, i.e. 1—1½ hour too early. The karyomeres coalesce to a female pronucleus which copulates with the male pronucleus. A cleavage furrow does not appear, but a cleavage spindle is

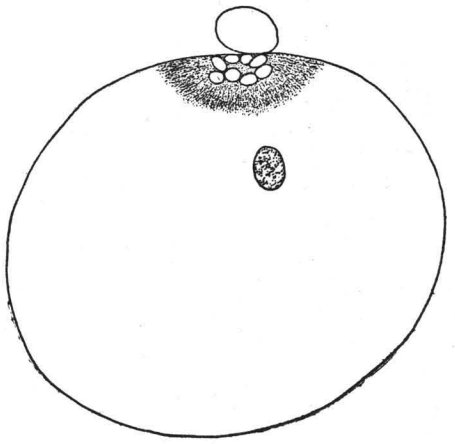

Fig. 3. Egg in 0.4% LiCl, fixed 20 min. before second polar body formation in the controls. Accelerated migration of the sperm nucleus and early formation of karyomeres.

formed in which the chromosomes show an abnormal arrangement. Karyomeres arise again, if treated eggs are transferred, at this stage, to distilled water. They do not take a position immediately beneath the egg cortex, but sink into the interior. The subcortical plasm has spread around the egg in a normal way. In none of the eggs, however, an animal pole plasm could be observed. Its formation seems to be suppressed.

*b.* In other eggs, treated with the same concentration, the disturbance seems to be less serious. The premature formation of karyomeres did not take place and a second maturation spindle was formed. In about half of these eggs the latter did not take a normal position, but sank into the interior and placed itself perpendicular to the egg axis. This is the phenomenon which has been observed by RAVEN and MIGHORST (1946) after treatment of the eggs with a slightly hypertonic $CaCl_2$ solution.

*c.* The second maturation spindle is formed and remains in a normal position; a second polar body is extruded. The chromosomes swell into karyomeres, then the latter sink into the interior of the egg (fig. 4). In these eggs the migration of the sperm nucleus is delayed. A first cleavage spindle is not formed. Karyomeres and sperm nucleus remain in the interior of the egg. The animal pole plasm is not formed. The subcortical plasm shows some irregularities in its distribution.

*d.* A fourth group of eggs extruded both polar bodies. The male and female pronuclei, situated at the animal pole, copulate. Any further development is inhibited. The animal pole plasm is not formed. The subcortical plasm is distributed rather abnormally.

In 0.2 % LiCl, as far as could be observed, the nuclear phenomena of maturation and fertilization are normal. Development proceeds further and a first segmentation occurs in most of the eggs. As far as the cytoplasmic components are concerned, however, all the eggs show very interesting

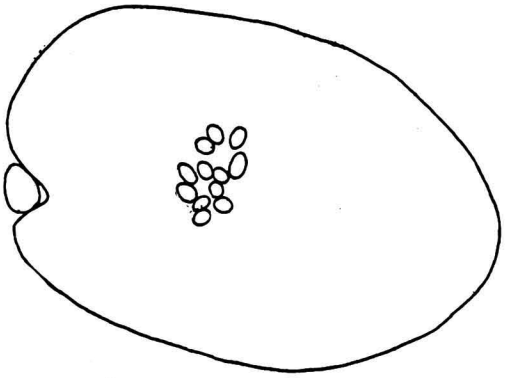

Fig. 4. Egg with second polar body extruded in 0.4 % LiCl. Karyomeres sunk into the interior. (Globe at left is no polar body.)

abnormalities. An animal pole plasm does not develop. The subcortical plasm shows a conspicuously abnormal distribution. At first, it does not spread evenly beneath the whole egg cortex. It is heaped up locally in considerable amounts whereby no preference for a certain region seems te exist (fig. 5). An accumulation may occur at the vegetative or at the animal pole, or at both, or somewhere between both poles. At other places it may lack altogether. This abnormal situation is not maintained; for at the time of first cleavage and after, the disturbance is less pronounced.

## *Discussion.*

1. *Stage of beginning of treatment.*

During the observations in vivo it was noted that the effect of a certain solution on maturation was not only dependent on the stage of development at which the eggs are exposed to the solution. It may be concluded that, within certain limits, the longer the time between the beginning of treatment and the formation of the first polar body in the control eggs, the lower the concentration, sufficient for the suppression of a polar body. For instance, in 1.0 % the first polar body was only suppressed if the treatment began more than 32 minutes before its formation in the controls. In 1.5 % it was already suppressed, if the treatment began about 25 min. before. As far as the second polar body is concerned, the same relation was noted. Moreover, it was observed that the connection between polar body formation and the beginning of treatment was influenced by temperature. The higher the temperature, the earlier the treatment with a certain solution had to be started in order to prevent the formation of a polar body.

2. *Hypertonicity.*

According to the determinations of RAVEN and KLOMP (1946) the internal osmotic pressure of the *Limnaea* egg equals that of a 0.093 Mol. solution of a non-electrolyte. The osmotic pressure of such a solution corresponds to 2.1 atm. Most of the solutions, employed in the present

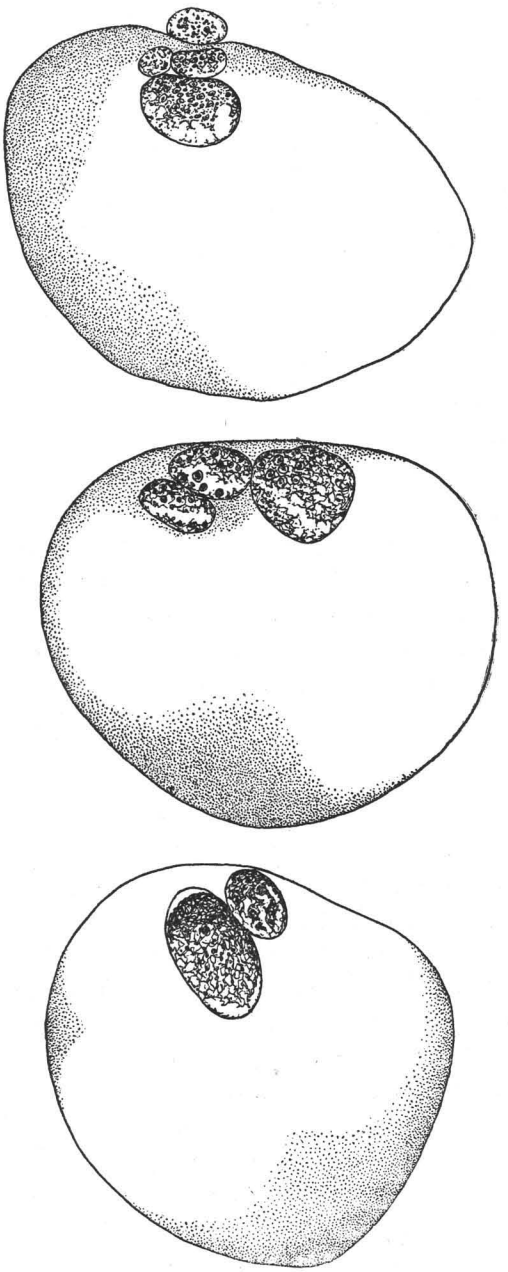

Fig. 5. Three eggs treated with 0.2 % LiCl, fixed 30 min. before first cleavage, showing a highly abnormal distribution of the subcortical plasm.

investigation, have an osmotic pressure considerably higher. For the calculations of the osmotic pressures of the solutions employed it was assumed that LiCl is completely dissociated.

TABEL I.

| % LiCl. . . . | 4.0 | 2.0 | 1.5 | 1.0 | 0.6 | 0.5 | 0.4 | 0.35 | 0.3 | 0.2 | 0.1 | 0.05 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| osm. press. in atmosf. | 42.2 | 21.1 | 15.8 | 10.5 | 6.3 | 5.3 | 4.2 | 3.7 | 3.2 | 2.1 | 1.1 | 0.5 |

Hence, a 0.2 % LiCl solution is isotonic to the *Limnaea* egg; all higher concentrations are hypertonic. So one has to take into consideration that the observed effects need not be due to a specific influence of LiCl, but may be caused primarily by hypertonicity of the medium. Mostly, it is impossible to distinguish between the results of these different factors. One is confronted here with an important disadvantage of fresh water eggs for experimenting with higher concentrations. Only in the investigation of marine eggs it is possible to vary the concentration of a certain ion between sufficiently wide limits, without necessarily changing the osmotic pressure of the medium. Therefore, the interpretation of our results meets with considerable difficulties. A comparison with the results of experiments with marine eggs is important to decide whether an effect is due to non-isotonicity or to a specific property of LiCl.

If the extrusion of polar bodies has to be understood as a process of osmotic activity (DALCQ 1923), it is clear that the osmotic pressure of the medium is of much importance. The osmotic attraction of water by the egg is counteracted by the osmotic value of the medium. With increasing concentrations of the medium, a stage will be attained, at which the osmotic power of the egg is unable to bring about the extrusion of a polar body. An equilibrium between both may become visible by a persistent protrusion at the animal pole, i.e. a partly extruded polar body. Since the permeability of the egg cortex may be changed by ions, a specific influence of the $Li^+$-ion may exist, but the asmotic pressures of the egg and the medium are the main factors in this phenomenon. This is shown by the action of hypertonic solutions of non-electrolytes, e.g. sucrose and urea, of about equal osmotic pressures as employed in the present investigation. With such solutions polar bodies may be suppressed as well (RAVEN and KLOMP 1946; own observations of the author).

In 1.5 and 1.0 % LiCl, the first polar body was only suppressed if the treatment began more than 32 min. beforehand. The osmotic pressure of these solutions is 15.8 and 10.5 atm., respectively. According to RAVEN and MIGHORST (1946) no polar body is formed in 3.0 % and 1.5 % $CaCl_2$. These solutions have an osmotic pressure of 18.1 and 9.1 atm. respectively, presuming that $CaCl_2$ is completely dissociated. As compared with LiCl, a lower osmotic pressure of a $CaCl_2$ solution would be sufficient to suppress the first polar body. This seemed to be a remarkable difference, since it was supposed that the osmotic pressure of the medium was of

primary importance. Therefore, we treated 7 batches with 3.0 % and 1.5 % $CaCl_2$. The results showed that the first polar body was only suppressed, if the treatment started more than 25 min. beforehand. In $CaCl_2$ too, the egg places itself with the animal pole downwards and polar bodies were only visible after turning the eggs. It is possible that the above-mentioned data of RAVEN and MIGHORST (1946) are based on experiments in which the treatment began sufficiently early. At any rate the statement that in 3.0 % and 1.5 % $CaCl_2$ no polar bodies are formed, proved to be only partly correct.

From DALCQ's investigations on the influence of hypertonic seawater on the egg of *Asterias glacialis* (1923) it was concluded that in solutions of 35 atm. the first polar body was always suppressed. The treatment began at the end of metaphase I, i.e. about 10 min. before the extrusion of the first polar body. As seawater has an osmitic pressure of 25 atm., an excess pressure of 10 atm. proved to be sufficient to suppress the first polar body formation. This is considerably less than the excess pressure, required for *Limnaea* eggs. In 1.5 % LiCl the first polar body was only suppressed if treatment began more than 25 min. before; even in 2.0 % it was not always suppressed. The *Limnaea* egg is isotonic with a solution of 2.1 atm. the osmotic pressure of 1.5% LiCl equals 15.8 atm. So at least an excess pressure of 13.7 atm. is necessary to prevent the first polar body formation, i.e. 37 % more than for the *Asterias* egg. The difference is still more pronounced if one considers the fact that the treatment of the *Limnaea* eggs had to begin considerably earlier in order to be efficient. However, the results with the *Asterias* egg were obtained with solutions, made hypertonic by adding sucrose to seawater, i.e. with the aid of a non-electrolyte. By treating *Limnaea* eggs with hypertonic solutions of urea, we found an excess pressure of 9.9 atm. to be sufficient to suppress the first polar body; this is in agreement with the findings on the *Asterias* egg. Hence the properties of the osmotically active agent result in a quantitatively different behaviour of the eggs, possibly due to changes, caused by ions, in the permeability of the egg cortex.