Zoology. — *Direct effects of isotonic and hypotonic lithium chloride solutions on unsegmented eggs of Limnaea stagnalis.* I. By CHR. P. RAVEN and J. R. ROBORGH. (Zoological Laboratory, University of Utrecht.)

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When eggs of *Limnaea stagnalis* are treated, in their capsules, with weak (0.001 %—0.01 %) solutions of LiCl, either exogastrulation occurs or embryos with synophthalmic and cyclopean head malformations may develop in a number of cases (RAVEN 1942). In these cyclopean monsters dorsomedian parts of the head, which in normal development arise from cells surrounding the original animal pole of the egg, are suppressed (RAVEN 1947). It was concluded from these observations that the influence of lithium may be explained by a depressing action on a gradient field with high point at the animal pole.

The action of lithium chloride is not altogether specific; both exogastrulae and cyclopean malformations may also be obtained with the chlorides of sodium, potassium, magnesium and calcium. However, in a quantitative respect the action of lithium greatly surpasses that of all other kations together (RAVEN and SIMONS 1948).

The action of lithium is phase-specific. A treatment immediately after oviposition leads to the formation of cyclopean malformations; at the time of 2nd cleavage there is a maximum susceptibility for the production of exogastrulae; whereas a second maximum for the production of head malformations exists at the 24-cell stage (RAVEN, KLOEK, KUIPER and DE JONG 1947; RAVEN and RIJVEN 1948).

Lithium chloride in hypertonic and slightly hypotonic concentrations decreases the viscosity and increases the tension at the surface of *Limnaea* eggs in the egg capsules; very weak solutions (about 0.01 %), on the contrary, produce a slight increase of viscosity (DE VRIES 1947).

In order to study the direct effects of lithium chloride on the *Limnaea* egg, in further experiments the eggs were exposed to the solutions after decapsulation.

M. GRASVELD (1949) showed that LiCl is more toxic than NaCl, KCl, $MgCl_2$ or $CaCl_2$. In no concentration of LiCl development proceeds beyond the 4—8 cell stage. Hypertonic LiCl solutions cause a great increase of amoeboid activity of the eggs.

DE GROOT (1948) studied the influence of LiCl solutions varying from 4 % to 0.05 %. Development does not proceed beyond the second cleavage. It may be inhibited at different stages, dependent on concentration, stage of treatment, temperature and susceptibility of the eggs. In 0.6—0.4 %,

the eggs show a very intense amoeboid activity during and after the formation of the second polar body in the controls.

The cytological consequences of the treatment were studied by DE GROOT, using concentrations of 1 %—0.2 % (the latter is about isotonic to the eggs). In 1 % solutions, the first maturation spindle sinks into the interior of the egg and degenerates. In 0.5 %, the first polar body is formed in a normal way, but then the second maturation spindle sinks into the interior and degenerates. In 0.4 % LiCl, development may be deflected at different moments. If treatment starts at an early stage, the chromosomes may swell into karyomeres shortly after the extrusion of the first polar body. No second maturation spindle is formed, but the sperm nucleus swells into a male pronucleus and rises to the animal pole which it may have reached already 35 minutes after the formation of the first polar body. The egg karyomeres coalesce to a female pronucleus which copulates with the male pronucleus; cleavage spindles appear in which the chromosomes show an abnormal arrangement.

In other batches, treated with 0.4 % LiCl, after the extrusion of the first polar body a second maturation spindle is formed, which sinks into the egg and places itself perpendicular to the egg axis. In still other batches, both polar bodies are formed in a normal way, but either the egg karyomeres are displaced towards the centre of the egg or development stops after copulation of the pronuclei.

The cytoplasmic differentiations are greatly disturbed by treatment with lithium chloride. The distribution of the vegetative pole plasm is very abnormal, especially in 0.2 % LiCl; the animal pole plasm is not formed in any of the concentrations studied.

When 24-cell stages of *Limnaea* are treated with lithium chloride (0.05—0.4 %), a swelling of the nuclei and a decrease of their distance from the cell surface occurs. Both phenomena are most pronounced in 0.05 % LiCl, and diminish in intensity with increasing concentrations of LiCl; in distilled water they are less pronounced, too (RAVEN and DUDOK DE WIT 1949).

Summarizing, it may be said that a treatment with lithium chloride affects both the nuclei and the cytoplasm of *Limnaea* eggs. However, since most of the solutions employed by DE GROOT are hypertonic to the eggs, it is not easy to decide which of the results obtained by him are due to hypertonicity and which are specific lithium effects. Therefore, in the present investigation the effects of isotonic and hypotonic lithium chloride solutions on decapsulated undivided *Limnaea* eggs have been studied.

Material and methods.

The snails were stimulated to oviposit by means of *Hydrocharis* in the usual way (RAVEN and BRETSCHNEIDER 1942). Immediately after oviposition, the eggs were decapsulated and washed three times in distilled water. Part of the eggs were kept in this medium as controls, the other

ones were transferred immediately to the LiCl-solutions. Solutions of 0.2 %, 0.15 %, 0.1 % and 0.05 % LiCl have been used. The former is about isotonic to the eggs, the latter three are hypotonic. After 1, 2, 3 and 4 hours part of the eggs, both of experimental and control series, were fixed in BOUIN's fluid. They were embedded in paraffin, sectioned at 5 μ, and the sections stained either with iron haematoxylin and saffranin or with azan.

In total, 495 treated eggs have been studied cytologically. Table I gives their distribution according to concentration and duration of treatment. They have been compared with 341 control eggs developed in distilled water.

TABLE I.

Duration of treatment \ Concentration LiCl	0.20 %	0.15 %	0.10 %	0.05 %	Total
1 h.	47	20	26	23	116
2 h.	49	55	43	30	177
3 h.	3	35	38	29	105
4 h.	20	29	25	23	97
Total	119	139	132	105	495

In our previous investigations, azan-stained sections of BOUIN-fixed material proved to be very valuable for the study of cytoplasmic differentiations in the egg; especially the vegetative pole plasm showed a very clear elective staining in this way. However, in this year's preparations the azan staining did not give any satisfactory results, though various modifications of the technique have been tried. Therefore, the cytoplasmic differentiations have not been considered in this paper; this point needs further study with improved staining techniques.

Results.

The following effects of the treatment with isotonic and hypotonic LiCl solutions have been observed:

1. a swelling of the telophase chromosomes of the first maturation division into karyomeres, accompanied with an accelerated migration of the sperm nucleus towards the animal pole;
2. an increased hydration of the spermaster after the second maturation division, with a delay in its disappearance;
3. a considerable increase of amoeboid mobility of the eggs after the second maturation division;
4. a delay of the beginning of cleavage;
5. various abnormalities of the first cleavage mitosis.

1. *The swelling of chromosomes between the first and second maturation division.*

We will begin with a short description of the period between both maturation divisions in normal eggs (RAVEN 1945, 1949). The first maturation spindle possesses a well-developed aster at both ends. At the end of anaphase, the inner aster is big and has a large clear "central body". When the central group of dyads reaches the margin of the "central body", their movement stops; the 18 dyads arrange themselves along the outer surface of the "central body" into a more or less irregular ring. Simultaneously, the "central body" increases in size and transforms into the second maturation spindle. The dyads, which have retained their individuality and their compact structure, now arrange themselves into the equatorial plate of this spindle. At the outer end of the spindle, astral radiations are formed in the cytoplasm. The inner aster of the second maturation spindle, however, is provided by the spermaster. This has appeared during the telophase stage of the first maturation division, has grown rapidly in size during the formation of the second maturation spindle, and now fuses with the deep end of the latter. Then the second maturation division begins. Immediately after the second polar body has been extruded, the telophase chromosomes remaining in the egg begin to swell into karyomeres, which assemble immediately beneath the egg cortex at the animal pole. The remaining aster (the former spermaster) shifts to a deeper position; it has a big, clear, vacuolated "central body" and a ring of short astral rays; soon, however, it becomes inconspicuous and vanishes altogether. The sperm nucleus, which had retained a subcortical position and compact structure till the end of the second maturation division, begins to move towards the animal pole at the moment the egg chromosomes begin to swell into karyomeres. During its migration, it swells and develops into a male pronucleus, which meets the female karyomeres at the animal pole.

In the controls of the present investigation, which have developed in distilled water, these processes take place in an entirely normal way. No deviations in the course of the maturation divisions as compared with that in normal eggs can be observed.

Two points in this cycle of events must be especially stressed: first, the fact that the chromosomes (dyads) between both maturations divisions remain compact and are arranged as such into the second maturation spindle; secondly, that the sperm nucleus does not begin its migration and its transformation into the male pronucleus before the end of the second maturation division. In both respects, the eggs treated with lithium solutions differ from the controls.

In these eggs, the first maturation division takes place in a normal way (fig. 1 *a*—*b*). However, as soon as the dyads have reached the "central body" of the inner aster of the spindle and even before the first polar

body has entirely been pinched off, the chromosomes begin to swell. At first, they appear each surrounded by a clear space in the sections (fig. 1 *c*). Shortly after, they have formed small vesicles with a distinct wall, while the chromatin begins to disperse in the interior of the vesicle (fig. 1 *d*).

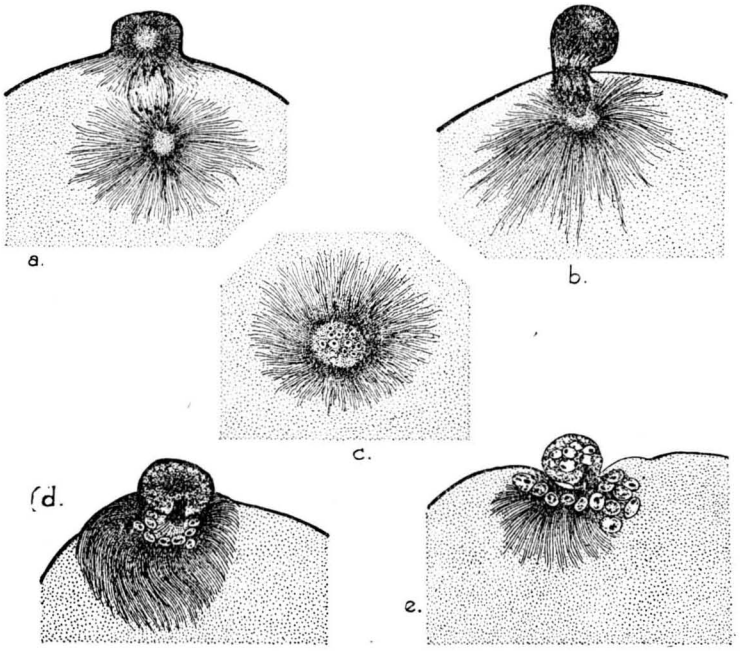

Fig. 1. *Limnaea stagnalis*, 0.05 % LiCl, 1 hour. *a*. Late anaphase, *b*. Telophase of first maturation division. *c*. Swelling of chromosomes. *d, e*. Karyomeres surrounding stalk of first polar body.

These karyomeres, which swell still further until the largest ones have reached a size of about $7\frac{1}{2} \times 6\frac{1}{2}\ \mu$ (fig. 1 e), surround the connecting stalk of the first polar body, which has been condensed into a heavily-staining "mid-body", forming an irregular ring around its base. They are surrounded, in their turn, by the astral radiations of the inner maturation aster, which can be followed quite a distance into the cytoplasm. The centre of this aster, between the ring of karyomeres, is occupied by an area of rather dense protoplasm, representing the "central body" of the aster; this is hardly visible, however, because it is flattened against the surface, contrary to its position in normal eggs of this stage, where it is situated at some distance beneath the surface and forms a round or ellipsoid body.

The chromosomes of the first polar body, like those of the egg, swell into karyomeres, be it somewhat delayed as compared with the egg chromosomes (fig. 1 e).

This abnormal development is not restricted to a certain concentration of LiCl only; we have observed it both in isotonic (0.20 %) and in hypotonic (0.10 %, 0.05 %) solutions. Moreover, DE GROOT (1948), as

stated above, found a similar phenomenon in eggs treated with a hypertonic (0.40 %) LiCl solution. On the contrary, in the control eggs in distilled water no swelling of chromosomes occurs at this stage. Hence, this swelling is not a purely osmotic phenomenon, but must be due to a specific action of the LiCl.

The swelling of the maturation chromosomes into karyomeres after the extrusion of the first polar body resembles the process normally occurring after the second maturation division. It is, therefore, interesting to observe that it is accompanied with another phenomenon taking place in normal development simultaneously with the formation of egg karyomeres: the migration of the sperm nucleus towards the animal pole and its transformation into a male pronucleus. Though the sperm nucleus is not always easily detectable in the eggs, in all cases in which it is found it is situated at this stage as a compact dark body beneath the egg cortex in the controls. On the contrary, in those Li-treated eggs in which the first maturation chromosomes have formed karyomeres, the sperm nucleus without exception has begun its migration towards the animal pole, which it has reached already in a number of cases, lying immediately beside or even in the peripheral part of the maturation aster surrounding the karyomeres. At the same time, it has swollen into a big vesicular pronucleus, in which the individual chromosomes have, evidently, retained some individuality so that it looks as if it were composed of a number of fused karyomeres.

This accelerated swelling and migration of the sperm nucleus, which has also been observed already by DE GROOT in similar eggs, appears to be strictly correlated with the swelling of the egg chromosomes into karyomeres. The fact that both phenomena show the same synchronicity in normal development, be it at a later stage, strongly speaks in favour of the supposition that they are causally related. Presumably, the egg chromosomes and the sperm nucleus react similarly to a certain condition of the cytoplasm by swelling, and the sperm nucleus only begins to respond to the attractive forces directing it towards the egg karyomeres or towards the animal pole after it has undergone some swelling. The effect of the Li-treatment could be explained, in this case, by the supposition that it induces in the cytoplasm the condition provoking the swelling of the karyomeres and sperm nucleus.

In DE GROOT's eggs in 0.4 % LiCl, in which karyomeres were formed after the extrusion of the first polar body no second maturation spindle developed. The karyomeres coalesced to a female pronucleus which copulated with the male pronucleus. A cleavage spindle with abnormal arrangement of the chromosomes formed afterwards.

One might expect a similar development to occur in our eggs in 0.2—0.05 % LiCl with premature karyomere formation. However, it appears that this is not the case. Of course, there is no direct evidence as to what might have become of eggs like those of fig. 1 *d* and *e*, had they be allowed to develop further. However, the following indirect evidence is available:

1°. according to DE GROOT (1948) and M. GRASVELD (1949), in isotonic and hypotonic solutions of LiCl both polar bodies are formed;
2°. in no batches fixed 2—4 hours after the beginning of treatment we ever observed any indication that the second maturation division had been suppressed.

It seems, therefore, that the premature swelling of the chromosomes into karyomeres is a reversible process, which may be followed by a normal second maturation division. Some of our batches seem to show in which way this takes place. In batch *C 4—1* (0.10 % LiCl, 1 hour), some eggs still show a cluster of karyomeres surrounding the stalk of the first polar body in the centre of a large maturation aster (fig. 2*a*). In other eggs, the astral radiations have been reduced, the "central body" is situated somewhat deeper and has rounded off; the chromosomes are arranged in a circle around it (fig. 2*b*). Though they still have a swollen

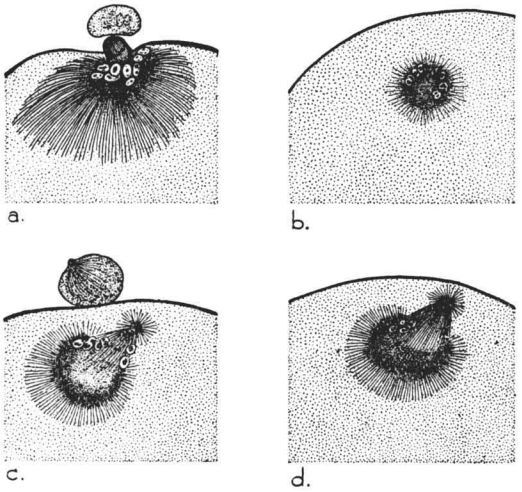

Fig. 2. *Limnaea stagnalis,* 0.10 % LiCl, 1 hour. *a.* Karyomeres of first maturation division. *b.* Swollen chromosomes surrounding central body. *c, d.* Formation of second maturation spindle. Chromosomes still somewhat swollen.

appearance, the chromatin in each of them has condensed again into a compact body. Fig. 2*c* and *d* show further transitional stages in the formation of the second maturation spindle ("acorn-stage" of the latter); the chromosomes still are somewhat swollen. In batch *P—1* (0.20 % LiCl, 1 hour), besides eggs with karyomeres (fig. 3*a*) also normal-looking anaphase and telophase stages of the second maturation division (fig. 3 *b—d*) have been found, in which the chromosomes have the appearance of compact dark bodies. Apparently, therefore, the swelling of the chromosomes immediately after the completion of the first maturation division is followed by a deswelling when the second maturation spindle forms.

Some points in this cycle of events are not yet entirely clear and make

a further study desirable. This is especially the case as regards the fate of the male pronucleus after its premature formation and migration. Though we have paid special attention to this point, we have not yet been able to find out what becomes of the male pronucleus when the

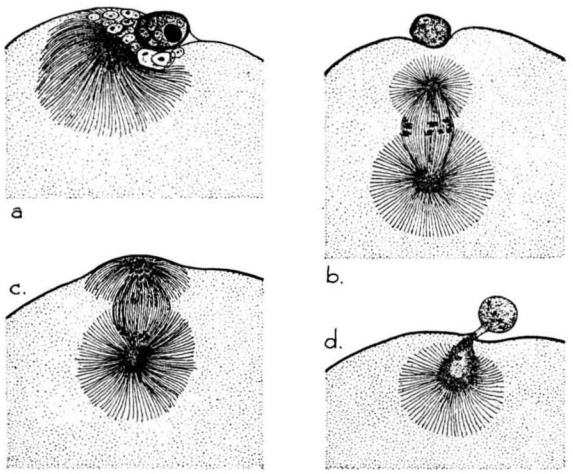

Fig. 3. *Limnaea stagnalis,* 0.20% LiCl, 1 hour. *a.* Karyomeres of first maturation division. *b.* Anaphase, *c.* Telophase of second maturation division. *d.* Extrusion of second polar body.

deswelling of the karyomeres occurs and they arrange themselves again into the maturation spindle. If it is true that egg chromosomes and sperm nucleus react in the same way to the condition of the cytoplasm, it might be supposed that the deswelling of the karyomeres will be accompanied with a similar process in the male pronucleus; in this case the latter, reverted to the state of a compact strongly basophil body, will be extremely difficult to detect among the basophil granulations of the egg cytoplasm.

Summarizing, it may be concluded that a first effect of the Li-treatment is a temporary change in the state of the cytoplasm, bringing about the swelling of both egg chromosomes and sperm nucleus, which in its turn causes their mutual attraction and the migration of the male pronucleus towards the animal pole. In isotonic and hypotonic solutions it is soon followed by a phase, in which again the deswelling influences prevail and the egg chromosomes return to their normal condition.

2. *Increased hydration of the spermaster.*

As has been stated above, in normal development the spermaster, after its temporary fusion with the deep end of the second maturation spindle, becomes independent again after the formation of the second polar body and shifts to a deeper position. Its "central body" meanwhile has greatly enlarged; it forms a big, clear, somewhat vacuolated space, surrounded by a ring of denser granular cytoplasm, in which a few short astral rays are visible for some time (fig. 4*a*). The latter soon become blurred, the

difference between the clear central area and the darker ring fades away and after some time only a somewhat clearer area in the cytoplasm indicates the position of the former spermaster; then this disappears too. The sperm nucleus, during its migration towards the animal pole, may temporarily be located within the spermaster or in its margin (fig. 4*a*).

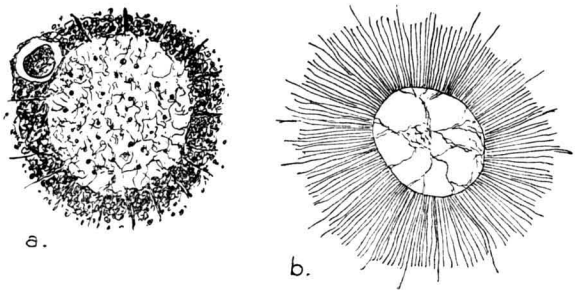

Fig. 4. Spermaster shortly after extrusion of second polar body *a*. Control egg. *b*. Egg treated with 0.20 % LiCl, 1 hour.

In the controls of the present experiments, the appearance and evolution of the spermaster is entirely the same as that just described. In the eggs, which had been treated with 0.20—0.15 % LiCl, however, the spermaster showed a different picture (the fixation and staining techniques being the same). The central area ("central body") is much more highly vacuolated in this case; the big vacuoles, which appear empty in the sections, are separated by fine protoplasmic meshes. Externally, this central area appears to be bounded by a definite fine protoplasmic lamella against the dense rim surrounding it. In eggs fixed shortly after the extrusion of the second polar body, this rim consists entirely of tightly packed astral rays, fine but very distinct and of moderate length (fig. 4*b*). Later, the astral radiations gradually fade away, but the highly vacuolated central area remains visible for a considerable time as a definite structure. As a matter of fact, the disappearance of the spermaster is greatly delayed, at least in part of the eggs, as the following batches show:

B 6—2 (0.15 % LiCl, 2 hours): controls 24 eggs, spermaster disappeared in all. 30 Li-eggs, 14 with distinct highly vacuolated spermaster, 16 spermaster disappeared.

B 4—3 (0.15 % LiCl, 3 hours): 23 controls, no spermaster left. 23 Li-eggs, 12 with highly vacuolated spermaster, 11 without spermaster.

We may conclude, therefore, that treatment with 0.20—0.15 % LiCl solutions leads to an increased hydration of the central area of the spermaster and a delay in its disappearance. This effect only becomes visible after the extrusion of the second polar body; as a matter of fact, in earlier stages neither the spermaster nor the other asters of the maturation spindles exhibit any difference as compared with those of the controls.

(To be continued.)