Zoology. — *The osmotic properties of the egg of Limnaea stagnalis L.* By CHR. P. RAVEN and H. KLOMP. (From the Zoological Laboratory, University of Utrecht.) (Communicated by Prof. G. KREDIET.)

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It has been observed by one of us (RAVEN 1945) that the eggs of *Limnaea stagnalis* in the 4—5 hours between oviposition and 1st cleavage show a considerable increase in volume. Increases of 36—56 % (average: ± 45 %) have been observed in different egg-masses. When volume is plotted against time, one gets no quite regular curve (l.c., fig. 6). A rapid rise during the formation of the 1st polar body is followed by a nearly horizontal part, in some cases even a slight drop, shortly afterwards; similar irregularities are found during the formation of the 2nd polar body and (to a less extent) about one hour before 1st cleavage. Though a similar course has been found in many experiments, these fluctuations have, perhaps, no real significance, and must be attributed to systematic errors in the volume determinations. The eggs show, shortly after the extrusion of either of the polar bodies, some amoeboid activity, by which the outline of the egg becomes rather irregular; therefore, the calculation of volume from the measured longest and shortest diameter (using the equation $V = {}^1/_6 \pi ab^2$) will yield, at this moment, too high results.

The swelling of the egg is due, apparently, to the absorption of substances, especially water, from the surrounding egg capsule fluid. It seemed important, therefore, to study the osmotic properties of the *Limnaea* egg.

1. *The swelling in distilled water.*

When the eggs are prepared out of their capsules shortly after oviposition and transferred to distilled water, a considerable swelling, much exceeding the normal increase in volume in the egg capsule fluid, takes place. Fig. 1 gives an example.

Experiment PD. Oviposition at 9 h. 25. 10 Minutes later 6 control eggs (Co), in the egg capsule, were measured; average volume = 653.000 μ^3. Then, 12 eggs were prepared out of the capsules and transferred to distilled water at 9.52 (A). The first measurement of this sample took place at 10.01, then at regular intervals of 6½—7½ minutes measurements were taken till 10.22; at 10.26 the controls were measured again. The further course of the experiment may be taken from the figure. The controls reached after 145 minutes a volume of 1.009.000 μ^3 (swelling: 55 %); the eggs in distilled water after 190 minutes a volume of 1.336.000 μ^3 (swelling: 105 %); in the controls at this moment cleavage had begun.

The further development of the eggs in distilled water is as follows: The first cleavage takes place nearly always, be it often somewhat delayed as compared with the controls. It shows a number of peculiarities

which will be treated below. In some cases development comes to a standstill at the 2-cell stage; mostly, however, the 2d cleavage takes

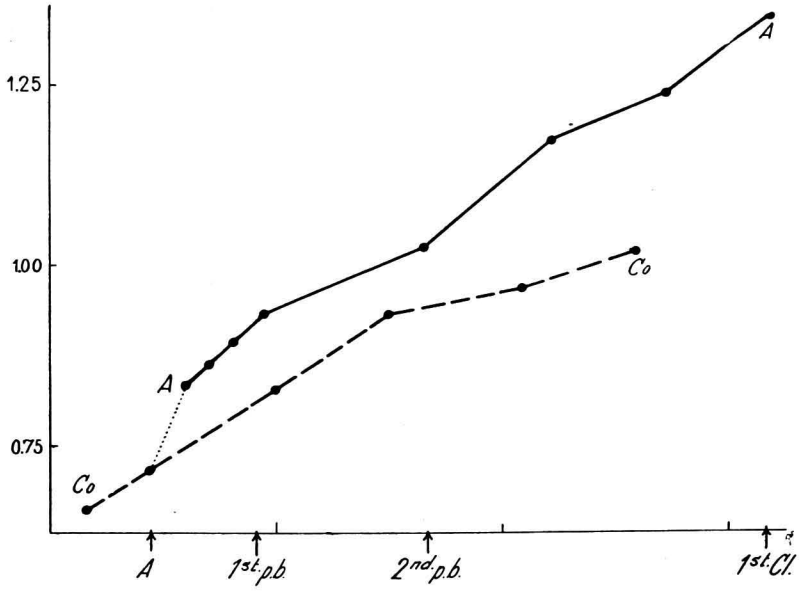

Fig 1.

Experiment PD. Swelling of *Limnaea* eggs in distilled water. Abscissae: time. Ordinates: average volume of eggs in $10^6\ \mu^3$. A: eggs in distilled water. Co: controls in egg capsule. 1st p.b., 2nd p.b., 1st cl.: time of 1st polar body, 2nd polar body, 1st cleavage.

place also. Development does not proceed beyond the 4-cell stage, even when the eggs are transferred to distilled water after the 1st or even the 2d cleavage. Apparently, the susceptibility of the egg to hypotonicity is great at the 4-cell stage.

The blastomeres swell further until cytolysis begins. The moment of cytolysis is very unequal even in the blastomeres of one egg; it varied from 8—28 hours after oviposition. When cytolysis occurs late, vacuoles become visible in the egg, which can protrude far from the egg surface. Immediately before cytolysis sets in, they sink somewhat back into the egg, probably because one of the vacuoles has broken through to the exterior and expelled its fluid contents; soon afterwards, the yolk granules begin to pour out. This vacuolization process reminds that described by LEITCH (1936) in eggs of *Echinometra lucunter* swelling in dilute seawater, which occurred also shortly before the eggs underwent cytolysis.

In eggs, which have been transferred to distilled water with a slight addition of $CaCl_2$, and, therefore, show a normal cleavage with formation of a cleavage cavity (cf. below), these vacuoles are not formed. This may be due to the active fluid output by the periodic contraction of the egg, with expulsion of the fluid filling the cleavage cavity (cf. COMANDON and DE FONBRUNE 1935).

2. *The osmotic pressure in the egg immediately after oviposition.*

In order to determine the osmotic pressure in the recently laid eggs they were transferred to solutions of sucrose and urea of different concentrations. The equilibrium volumes, reached after some hours, were found to depend only upon the molar concentration of the solution; in isotonic solutions of urea and sucrose, equilibrium volumes were equal. This shows that the swelling of the egg is an osmotic process.

In solutions above 0.10 M the eggs shrink; in solutions below 0.09 M they swell; at the concentrations of 0.09 M and 0.10 M the volume remains nearly constant. It may be concluded that the recently laid eggs are isotonic with 0.09—0.10 M solutions of non-electrolytes. (The average internal osmotic pressure of the egg, as calculated from our experiments, equals that of a 0.093 M solution; cf. fig. 3).

In solutions of 0.03 M, the rapidity of swelling equals that of the controls in the egg capsule fluid. This observation does not prove, of course, that this fluid is isotonic with a 0.03 M solution. We have to reckon, in fact, with an influence of the composition of the external medium upon the permeability of the eggs; therefore, the rapidity of swelling can be no measure of the osmotic pressure of the medium. The equilibrium volume was not reached in these solutions, as the swelling continues till the entrance of 1st cleavage, after which an accurate volume determination is no longer possible.

3. *The non-solvent volume.*

Fig. 2 gives an example of an experiment, in which eggs of one egg-mass have been transferred to various concentrations of urea.

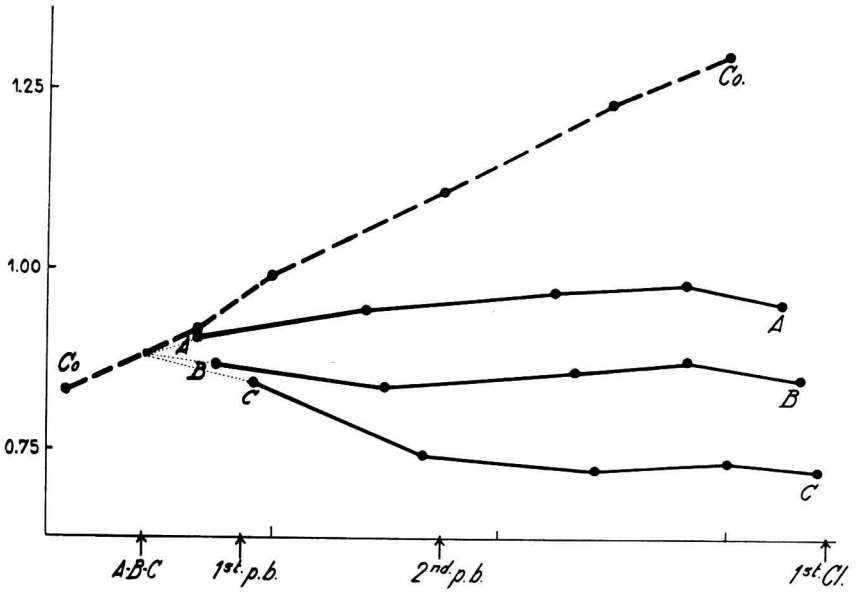

Fig. 2.

Experiment OR. Swelling of *Limnaea* eggs in 0.08 M (A), 0.10 M (B) and 0.15 M (C) urea solutions. Explanation cf. fig. 1.

Experiment OR. Oviposition at 14 h. 10. After 5 minutes volume determination of 6 controls in the egg capsule (Co): 833.000 μ^3. The controls show normal swelling: 56 % in 3 hours. A number of eggs was prepared out of the capsules, and transferred at 14.35 to 0.08 M. (*A*: 4 eggs), 0.10 M. (*B*: 6 eggs) and 0.15 M. (*C*: 11 eggs) solutions of urea. The equilibrium volumes after about 3 hours were: $A = 967.000\ \mu^3$, $B = 862.000\ \mu^3$, $C = 728.000\ \mu^3$.

These figures show that the product of volume and external osmotic pressure is by no means constant; the swelling and shrinking are less than inversely proportional to the osmotic pressure. This must be attributed to the presence in the egg of substances which are osmotically inert. By means of the equation $P(V-b) = P_0(V_0-b)$, in whicht P is the osmotic pressure, V the total egg volume and b the volume occupied by these osmotically inactive substances ("non-solvent volume"), the latter can be calculated (cf. BROOKS & BROOKS 1941). In the above-mentioned experiment the non-solvent volume, as determined in this way, is 453.000 μ^3, constituting 54 % of the original egg volume.

In other experiments, similar values have been obtained. In fig. 3, the observed equilibrium volumes (reduced to percentage of original volume) have been plotted against the reciprocal of the relative pressures; a straight line is fitted to the points and produced to the ordinate at $1/M = 0$,

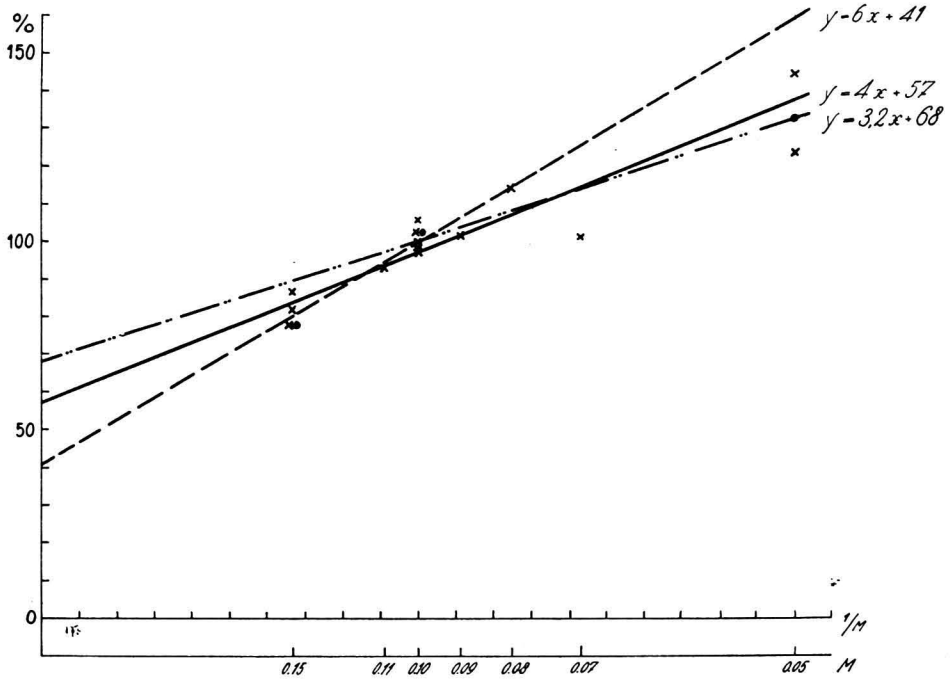

Fig. 3.

Graph representing relation between egg volume and osmotic pressure. Abscissae: reciprocal of molar concentration. Ordinates: equilibrium volumes in percentage of original volume. Crosses: urea experiments. Dots: sucrose experiments.

— . . — . . — . . — swelling. — — — — — — shrinking. ———— both combined.

where the average non-solvent volume can be read off (MC.CUTCHEON. LUCKÉ and HARTLINE 1931). It is found to equal 57 % of the original egg volume.

This non-solvent volume is exceptionally high. In marine eggs, the osmotically determined non-solvent volumes range between 0 % an 48 % (BROOKS & BROOKS, p. 68); the higher of these values are already unduly high, as compared with the analytically determined dry weights, which range from 13 % to 30 % with a distinct crowding between 18 % and 23 %. Although the latter determinations concern only echinoderms and worms, it seems very improbable that the volume of dry matter in the egg of *Limnaea* will much exceed these values. Therefore, the unduly small osmotic volume changes of this egg must be due to other factors. In this connection, it is of interest to note that in our experiments the calculated non-solvent volume for swelling alone (68 %) exceeds considerably that for shrinking (41 %; cf. fig. 3); this means that the eggs swell less than would be expected on the basis of shrinking experiments. This may be due to a resistance of the egg surface or vitelline membrane to stretching when swelling occurs. Possibly, the vitelline membrane offers, in a less degree, also some resistance to shrinking; that it is rather inelastic in these solutions is shown by the fact that it is thrown into folds in shrunken eggs.

4. *The permeability constant.*

The permeability constant for swelling in distilled water has been calculated using the formula

$$P = \frac{dV}{dt} \cdot \frac{1}{S_0 (a_i - a_e)_0}$$

in which the quantities have been expressed in the standard units of GM.cm^{-2}.sec^{-1}. (GM.l^{-1})$^{-1}$ (cf. BROOKS & BROOKS 1941).

In the case of fig. 1, for the period between the first two measurements (9—15½ min. after the eggs have been transferred to distilled water) the increase of volume dV = 31.200 μ^3 = 31.2 × 10^{-9} cm^3 = 1.73 × 10^{-9} GM; the time dt = 6½ minutes = 390 sec. The surface S_0, as calculated from the diameter of the eggs, is 428 × 10^{-6} cm^2, while the activity gradient $(a_i - a_e)_0$ equals the internal osmotic pressure of the egg = 0.093 GM/l. This yields for the permeability constant P the value 1.13 × 10^{-7}. In another experiment, a permeability constant for swelling in distilled water P = 2.14 × 10^{-7} was determined. This suffices to show that the permeability constant in *Limnaea* is of the same order of magnitude as in various marine eggs (mostly echinoderms) ,where most of the values lie close to 2 × 10^{-7} (BROOKS & BROOKS 1941, p. 86). In view of the difference in systematical position and natural external medium the agreement is rather remarkable.

5. *Development of the eggs in urea and sucrose.*

In a 0.15 M solution of urea, the eggs extrude one or both polar bodies, but do not cleave. In a 0.10 M solution, however, the eggs undergo the 1st and, as a rule, also the 2d cleavage; the 3d cleavage can be indicated in some of the blastomeres, but is not finished. Therefore, in most eggs development stops at the 4-cell stage; soon, cytolysis sets in. In all weaker solutions of urea employed (0.03—0.10 M), the same behavior of the eggs has been observed: cytolysis at the 4-cell stage. Apparently, the susceptibility of the egg to the action of urea is great at the 4-cell stage.

It has been shown above that at this stage there is also an increased susceptibility to hypotonicity. The influence of weak urea solutions cannot be attributed, however, to their hypotonicity. In the first place, the eggs behave in the same way in a 0.10 M urea solution, which is nearly isotonic to the egg or even slightly hypertonic. Secondly, the natural external medium of the eggs, the egg capsule fluid, is, evidently, also strongly hypotonic. Finally, the behavior of the eggs in sucrose solutions is quite different.

In a 0.15 M sucrose solution, both polar bodies are formed, but, as a rule, the eggs do not cleave; only in some cases a 1st cleavage groove appears. With falling concentration development improves; in 0.07—0.08 M solutions, on an average, a stage of about 30 blastomeres is reached; then, with still weaker solutions development gets worse again (end stage in 0.03 M solutions: ± 8 blastomeres). Hence, the injurious action of sucrose on the eggs differs in its effects from that of urea; probably, the chemical properties of both substances play a part.

6. *The influence of $Ca^{\cdot\cdot}$-ions on cleavage.*

Both in distilled water and in sucrose and urea solutions, the eggs show a remarkable deviation of the normal course of cleavage.

In normal cleavage, the cleavage groove, beginning at the animal pole, extends around the egg to the vegetative side; then, it deepens from all sides, the blastomeres pull apart, until they are entirely separated, remaining in contact in one point only. After that, the blastomeres begin to flatten against each other, with formation of a cell boundary between them; finally, the cleavage groove disappears altogether, the outline of the egg becoming nearly spherical again (fig. 4). Soon thereafter, a lens-shaped cleavage cavity becomes visible between the two blastomeres; its contents is extruded periodically to the exterior, as described by COMANDON & DE FONBRUNE (1935). At the next cleavages, this cycle of events is repeated: deepening of the cleavage grooves, pulling-apart and rounding off of the blastomeres; then, flattening of the blastomeres and disappearance of the cleavage grooves, followed by formation of a wide cleavage cavity.

In eggs cleaving in distilled water or in solutions of sucrose or urea,

the first phases of the division process proceed normally: deepening of the cleavage groove, pulling-apart of the blastomeres. After the daughter

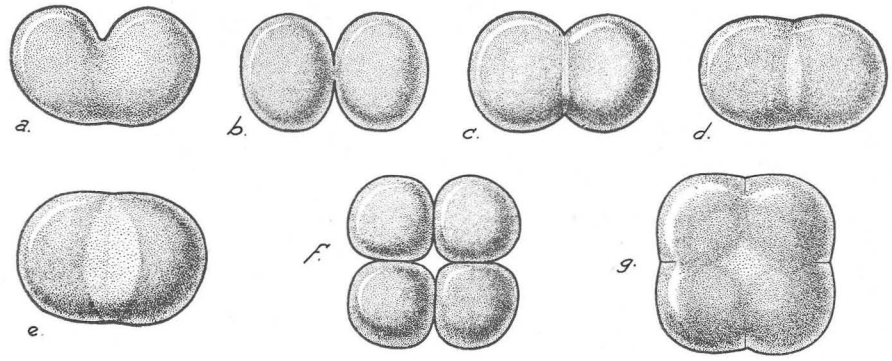

Fig. 4.
Normal cleavage in *Limnaea stagnalis*. *e.*—*c.* First cleavage. *d.*—*e.* Formation of cleavage cavity. *f.* Second cleavage. *g.* Reappearance of cleavage cavity.

cells have maximally rounded off, they beginn to flatten against each other; however, this process stops soon, and the blastomeres remain connected by a rather narrow stalk, the whole egg being dumb-bell-shaped (fig. 5). No cleavage cavity is formed. This state of things lasts

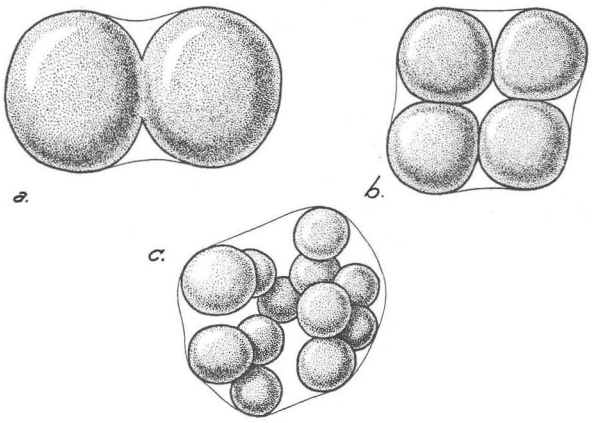

Fig. 5.
Cleavage in $Ca^{\bullet\bullet}$-free media. *a.* 2-cell stage. *b.* 4-cell stage. *c.* Later cleavage stage.

till the next division, which leads to the formation of four contiguous spherical cells, soon losing every connection. When cleavage advances further (in sucrose solutions), each of these cells divides independently in the same way; finally, a loose aggregate of spherical cells is formed.

This mode of cleavage is reminiscent of the abnormal cleavage of sea urchin eggs in $Ca^{\cdot\cdot}$-free sea water, as first described by HERBST (1900). It seemed probable, therefore, that the lack of $Ca^{\cdot\cdot}$-ions in the external medium was the cause of this deviation of cleavage in *Limnaea*, too.

This was proved by the fact that the addition of a small quantity of $CaCl_2$ to the medium leads to normal cleavage. In distilled water with $CaCl_2$, the swelling of the eggs is like that in pure water. Cleavage proceeds normally, the blastomeres flatten against each other, and a cleavage cavity is formed, which is wider than in control eggs in egg capsule fluid. Development stops at the 4-cell stage, but cytolysis is somewhat delayed as compared with eggs in pure distilled water.

In sucrose solutions with addition of $CaCl_2$, the eggs die at the same stage as in the corresponding solutions without $CaCl_2$; but cleavage, as far as it occurs, is normal; the blastomeres remain in connection and cleavage cavities are formed. The minimum concentration of $CaCl_2$, which had an appreciable effect, was 0.005 %; in 0.01—0.08 % $CaCl_2$ the effect is clearly observable.

Apparently, the lack of $Ca^{\cdot\cdot}$-ions brings about a change of the egg surface, by which the coherence of the blastomeres is seriously disturbed. Our observations give some indications as to the point of attack of this influence.

The uncleaved egg is surrounded by a fine plasmatic lamella, the vitelline membrane. In normal cleavage, this membrane remains in contact with the egg surface throughout; it folds inwards with the cleavage grooves.

In eggs cleaving in $Ca^{\cdot\cdot}$-free media, already at the 1st cleavage the vitelline membrane loses its contact with the egg surface in the region of the cleavage groove; it does not fold inwards, but bridges the cleavage groove from one blastomere to the other (fig. 5). At the 4-cell stage, the membrane surrounds tightly the spherical blastomeres. At later stages, it detaches itself still more from the blastomeres, at last forming a loose envelope surrounding them.

It is probable, therefore, that a change in the properties of the vitelline membrane is the primary cause of the abnormal cleavage in $Ca^{\cdot\cdot}$-free media.

Summary.

1. The eggs of *Limnaea stagnalis*, in their egg capsules, show a swelling of 36—56 % between oviposition and 1st cleavage.

2. In distilled water, swelling exceeds considerably that in egg capsule fluid. The eggs reach the 4-cell stage, then they cytolyse.

3. In urea and sucrose solutions, the equilibrium volumes depend upon the molar concentration of the solution. The recently laid eggs are, on an average, isotonic with 0.093 M solutions of non-electrolytes.

4. The average non-solvent volume, as calculated from the osmotic volume changes, is 57 % of the original egg volume. This high value is, probably, due to a resistance to swelling and shrinking by the vitelline membrane.

5. The permeability constant for swelling in distilled water lies close to 2×10^{-7}.

6. In 0.03—0.10 M urea solutions, development stops at the 4-cell stage. With sucrose, in 0.07—0.08 M solutions a stage of about 30 blastomeres is reached; in stronger and weaker solutions, development comes to a standstill at an earlier stage.

7. In $Ca^{\cdot\cdot}$-free media, cleavage has an abnormal course; the blastomeres remain spherical in shape and lose connection at an early stage. Addition of a small quantity of $CaCl_2$ leads to normal cleavage. The primary action of the lack of $Ca^{\cdot\cdot}$-ions seems to be a change in properties of the vitelline membrane, which loses its contact with the egg surface in $Ca^{\cdot\cdot}$-free solutions.

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