Zoology. — *The influence of thiourea on the development of Limnaea stagnalis L.* By F. H. SOBELS. (From the Zoological Laboratory, Dept. of General Zoology and Dept. of Endocrinology, University of Utrecht.) (Communicated by Prof. J. BOEKE.)

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Introduction.

The influence of thyroxine on the development of Invertebrates has been studied by several authors. O. V. HYKES (1930, 1931) examined the influence of this compound on the development of *Physa* and *Paracentrotus*. P. WEISS (1928) stimulated metamorphosis of Ascidia larvae by means of thyroidextract; and B. CHATZILLO (1936) describes the accelerating influence of thyroid hormone on the later stages of development of *Limnaea stagnalis*. The question whether there is an accelerating or an inhibiting influence of this Vertebrate hormone on the development of eggs and larvae of Invertebrates provoked an extensive discussion.

G. BEVELANDER (1946) investigated the influence of thiourea on the eggs of *Arbacia punctulata*. Fertilized eggs, exposed to a concentration of 1 % thiourea do not cleave; at a concentration of 0.5 % a specific inhibition of gastrulation occurs. This would be due to a selective action of thiourea on the blastula stage. According to BEVELANDER a specific physiological influence of this compound, already known during several years for its influence on the Vertebrate organism, on the eggs of the sea urchin exists.

The purpose of my investigation was to determine the influence of thiourea on the cleavage and later development of eggs of the pond snail, *Limnaea stagnalis*.

Methods.

Egg-masses were obtained in the usual way by stimulation of the snails with *Hydrocharis* (RAVEN and BRETSCHNEIDER 1942); the eggs were separated from the mucus and cultured in little glass dishes. Thiourea was applied in concentrations of 0.5, 0.75, 1 and 2 % (0.07, 0.10, 0.13 and 0.26 molar, respectively) dissolved in distilled water. For the treatment of decapsulated eggs, however, the thiourea was dissolved in a 0.04 % $CaCl_2$ solution, in order to prevent abnormal cleavage due to a lack of Ca^{++}-ions (HUDIG 1946). If the eggs were to be observed for several days, they were cultured in petri-dishes without water on a bottom of 2 % agar; in some experiments, thiourea was dissolved in the agar in the concentration desired.

Decapsulation was performed by pricking the egg capsules on a dry glass plate under a binocular microscope; after this manipulation the eggs were pipetted into the thiourea solutions.

The eggs were fixed in Bouin, sectioned at 7.5 μ and stained with iron haematoxylin-saffranin or azan. To distinguish the nineteen stages until the 4th cleavage, RAVEN's normal table (1946) has been used.

Experiments.

1. *Inhibition and delay of development.*

Eggs of different egg-masses (Tu 1—5) were treated with a 1 % thiourea solution, immediately after laying. A 3 hours' treatment causes already an inhibition of development. After exposure to the thiourea solution during 4—5 hours inhibition of 1st cleavage occurs. When the eggs are transferred to distilled water, recovery is still possible after a 5 hours' treatment. A longer exposure of eggs in their capsules to a 1 % thiourea solution gives no recovery; mostly an abnormal 3rd cleavage occurs.

The eggs of different batches show great variations in susceptibility, but as a rule all eggs of one batch are arrested in the same stage of development.

By studying eggs which after a 3—4 hours' treatment were transferred to distilled water and, after thorough washing, were laid out on an agar bottom, it becomes evident that this relatively short treatment causes a marked delay of development at later stages.

When treatment is not beginning at the uncleaved stage, but somewhat later, the inhibition of development is less pronounced.

In experiment Tu-9 eggs were exposed during 15 hours to a 0.5 % thiourea solution. During the first 3 days, no difference in rate of development with the controls could be observed. After 6 days, however, the treated eggs showed a marked delay in development, which was still more pronounced after 9 days.

A 3—4 hours' treatment with a 2 % thiourea solution causes also an inhibition and delay of development.

2. *Arrest of development.*

Uncleaved eggs were placed on agar, containing 1 % of thiourea. Development was arrested at the 4-cell stage. A similar phenomenon could be observed after 10 hours' treatment with a 1 % solution. Most eggs were arrested in the 8-cell stage, some eggs, however, did not develop beyond the uncleaved stage. Exposed to a 2 % solution during 6 hours a few eggs were arrested in the 2-cell stage, most eggs did not cleave.

Decapsulated eggs in a 1 % solution (Tu 33, 34) remain uncleaved or are arrested in the 4-cell stage.

Eggs of Tu-38, decapsulated immediately after laying and transferred to a 0.75 % solution remain uncleaved, but live for several hours. We shall refer to this special case later.

Batches Tu 40—48 showed that the eggs are more susceptible to the treatment at the 2-cell stage than at later stages of development. The eggs can still be arrested at the 4-cell stage by a treatment beginning during the first cleavage (RAVEN stage 5 at the latest), and at the 8-cell stage by a treatment beginning at a late 2-cell stage (RAVEN stage 8); when the treatment is begun still later, development proceeds beyond these stages and mortality is rather low.

At a high temperature (33° C) arrest in the uncleaved or 4-cell stage is accelerated. Batches placed all night in a 1 % solution at a temperature of streaming tap water showed the same abnormalities of development as those treated at room temperature during a much shorter time. Hence the susceptibility of the eggs seems to be dependent on temperature.

An inspection of the eggs fixed after arrest at the 4-cell stage reveals that the nuclei are not always in the same phase. In some cases they have formed monasters with disorderly arranged chromosomes, in other batches development has stopped at the prophase stage or at an anaphase stage of division. Eggs arrested either in the 4- or 8-cell stage preserved the capacity to form a wide cleavage cavity; in this case the nuclei are always in the interphase- or prophase stage.

3. *Abnormalities of cleavage.*

A 1 % Tu solution applied during 5—6 hours produces interesting abnormalities of cleavage (fig. 1). These abnormalities occur especially

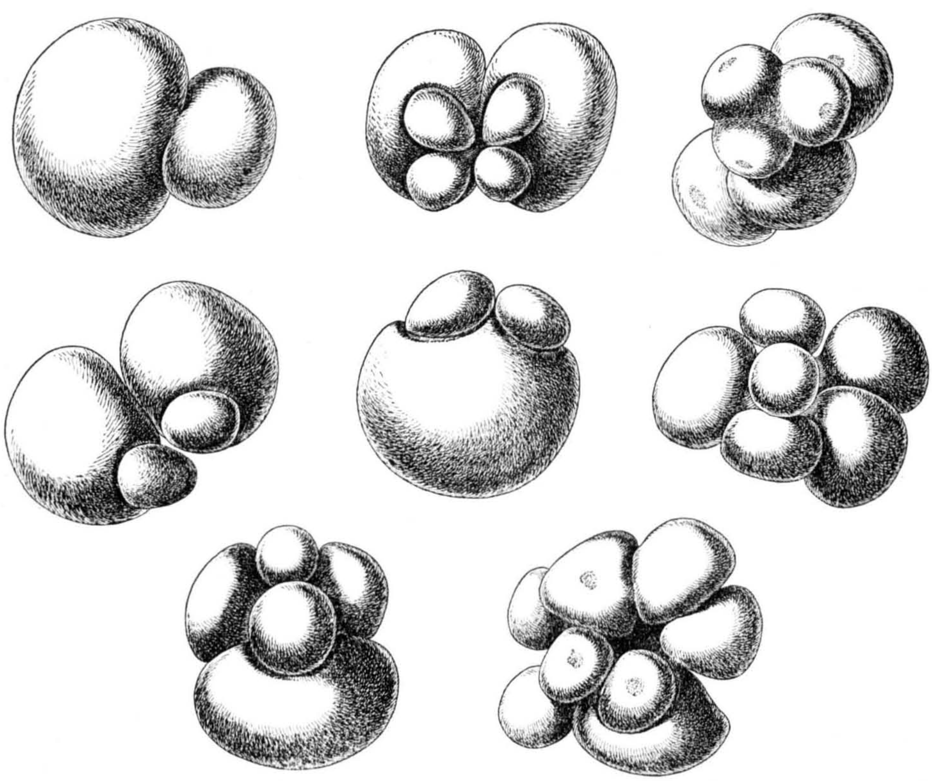

Fig. 1. Abnormal cleavage stages in *Limnaea* eggs treated with thiourea.

after the 3rd cleavage, but also at earlier stages. Abnormal 3-, 5- and 6-cell stages occur. The blastomeres show great divergences in size. A very unequal distribution of yolk among the blastomeres may occur. In many cases the synchronism of cell divisions is entirely or partially disturbed.

A treatment with a 2 % thiourea solution yields many 2-cell stages with blastomeres of different size. In some cases there is only an apparent cleavage by formation of giant polar bodies.

Fig. 2 shows cases where the blastomeres at the 2nd cleavage are not

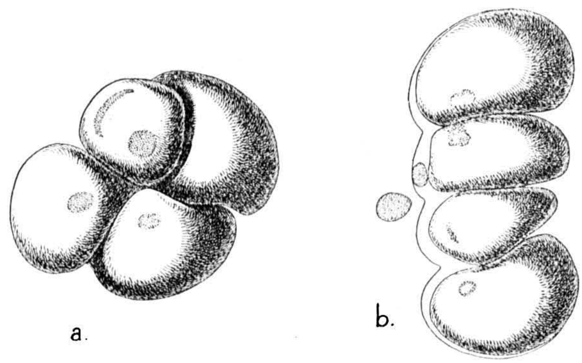

Fig. 2. Abnormal 4-cell stages.

formed in a normal way; there is a disturbance in direction of cleavages. This phenomenon can be observed especially in those cases where the 2nd cleavage is delayed in one of the blastomeres; when, after some time, cleavage begins in this blastomere too, it divides at right angles to the other one. This abnormality has been observed in several batches treated with 0.75 % Tu after decapsulation.

4. *Mechanisms of abnormal cleavage.*

In the abnormal cleavage the following points are of interest:

a. Cell division may be arrested. This may lead to uncleaved eggs with several groups of clotted chromosomes (fig. 3). At the second cleavage

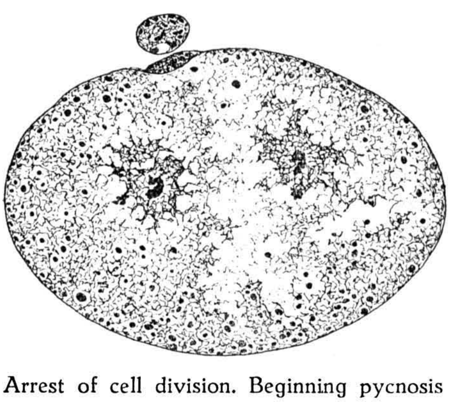

Fig. 3. Arrest of cell division. Beginning pycnosis of nuclei.

one of the cells may fail to divide, which leads to a 3-cell stage. As a rule these cells with arrested division contain 2 nuclei; apparently mitosis has taken place, but has not been followed by cell division. In most cases, no further mitosis takes place. Also at later cleavages, arrest of cell division may occur in one or more of the blastomeres.

b. The nuclei show various types of degeneration.

1⁰. *Fragmentation of chrosmosomes.* In some cases cleavage spindles with disorderly arranged and fragmented chromosomes occur; sometimes

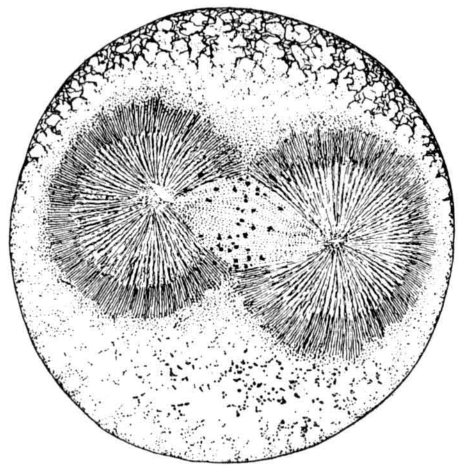

Fig. 4. First cleavage splindle with fragmented and disorderly arranged chromosomes. Spindle and asters abnormally enlarged.

the latter are widely scattered throughout the cytoplasm (fig. 4). In other cases the number of chromosomes formed in the spindles is evidently reduced.

2⁰. Often *pycnosis* occurs; this is clearly demonstrated in the case of fig. 3.

3⁰. *Monasters* are also found, especially in eggs arrested at the 4-cell stage. They contain irregular or clotted masses of chromosomes in their central part.

c. In many cases anachronisms of cleavage occur. At the 2nd cleavage one of the blastomeres may divide much later than the other, so that a temporary 3-cell stage is intercalated between the 2- and 4-cell stages. The mitosis in this half is also delayed as compared with the other one.

d. Most interesting are deviations in the position of cleavage spindles, which have been observed often in these eggs. In one batch which had been decapsulated and put into a 0.75 % Tu solution immediately after oviposition, no cleavage occurred (Tu 38); the eggs were fixed after 8 hours, when the controls were in the 8-cell stage. The maturation divisions had taken place normally; 2 polar bodies were present in all eggs. The animal pole plasm had been formed under the egg cortex of the animal side. The cleavage spindle had been formed, but at this moment the development had taken an abnormal course. The asters of the cleavage spindle had grown to enormous sizes (fig. 4); the spindle itself had also enlarged and elongated, so that in many eggs the asters were pressed against the egg cortex at opposite points of the surface (fig. 5). The

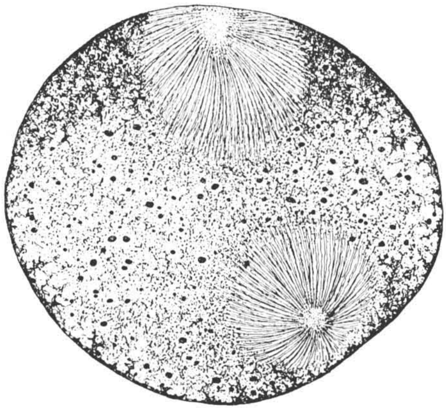

Fig. 5. Enlarged and rotated cleavage spindle in egg with inhibition of first cleavage.

chromosomes had lost their regular arrangement, they were lying scattered over the whole cytoplasm. Most interesting is, however, the fact that in many of these eggs the spindle had rotated into the direction of the egg axis; one of the asters is situated at the animal pole, pushing aside the

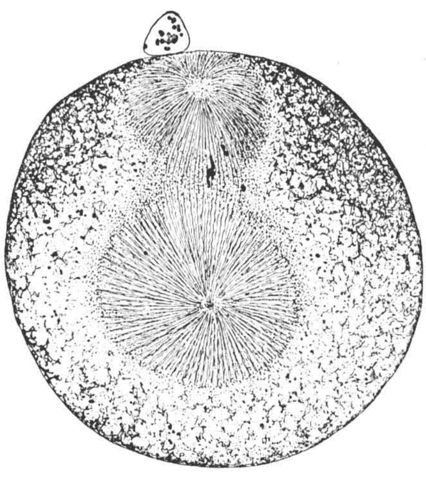

Fig. 6. Enlarged cleavage spindle, rotated into the direction of the egg axis, in egg with inhibition of first cleavage.

animal pole plasm, the other is lying at or near the vegetative pole (fig. 5, 6). Among 17 eggs, in 4 cases this had occurred; in 4 other eggs the spindles had an oblique position intermediate between that described above and its normal position perpendicular to the egg axis.

In the embryos in which cleavage is delayed in one of the blastomeres of the 2-cell stage, the spindle of this cell rotates in such a way that it comes to lie parallel to the egg axis. In this way, 4-cell stages with a tetraëdic position of the cells are formed (fig. 2*a*).

In other cases at the second cleavage one of the spindles may place itself perpendicularly to the plane of first cleavage which leads to a *T* shaped 4-cell stage (fig. 7). Sometimes both cleavage spindles take this position which gives rise to a cleavage stage with 4 cells in a line (fig. 2*b*).

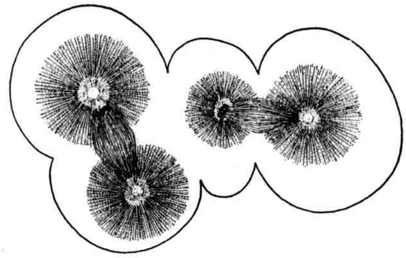

Fig. 7. Deviation of cleavage spindle in 2nd cleavage of *Limnaea* egg treated with thiourea.

Discussion.

Eggs in their capsules subjected to a thiourea solution of 1 % show an inhibition of development at the first cleavage stages. Until 5 hours of treatment this inhibition is reversible; but during further development the influence of thiourea shows itself by a delay of development. A treatment begun in the uncleaved stage causes the most pronounced inhibition and delay, possibly the influence on the nuclear apparatus is much stronger in this case.

A solution of 0.5 % applied for a long time shows itself to be poisonous too. Probably a higher temperature increases the susceptibility of the eggs for the influence of thiourea.

The inhibition of development seems to be in accordance with the facts found by BEVELANDER (1946) in *Arbacia*. In my experiments, however, there was no specific arrest of development in the gastrula stage.

My experiments show that an arrest of development at the 4-cell stage can still be brought about by a treatment beginning during the first cleavage, whereas development can be arrested at the 8-cell stage, when treatment begins at the utmost at a late 2-cell stage. When treatment begins still later even a relatively longer thiourea treatment causes less injury.

In WOKER's experiments (1943, 1944), *Tubifex* eggs subjected to colchicine show an irreversible arrest of cleavage in the 2-cell stage, as colchicine is acting directly on the cell nucleus in the interphase stage immediately after first cleavage. WOKER speaks, therefore, of a "critical phase" of greatest susceptibility. In the thiourea treatment in *Limnaea* the arrest of development does not occur immediately, but only after a certain lapse of time. This may indicate that the "critical concentration" of thiourea within the eggs is only gradually reached by the endosmosis of the agent into the cells.

Considering the eggs with abnormal cleavage, it is remarkable that many of these eggs show the tendency to continue the normal sequence of spiral cleavage, but by the influence of thiourea certain divisions are skipped. As soon, however, as a deviation of the direction of cleavage spindles takes place a total aberration of the normal type of cleavage occurs.

These spindle rotations may be explained by weakening of the factors, governing the position of the spindles in normal development, by the action of thiourea. However, many of these cases call forth another explanation. In most cases, the spindle rotations occur in cells in which cell division has been delayed for a considerable time. In the eggs described above (p. 905) where the first cleavage spindle had rotated into a vertical position, meanwhile the 3rd cleavage had taken place in the controls. It might be supposed that the conditions governing the succession of spindle positions in normal development change relatively independent of the other developmental processes, so that in these eggs, which have skipped two cleavages, the spindles are forced into a position corresponding to that of 3rd cleavage.

This explanation can also be applied to those cases described above, where a delay of 2nd cleavage in one of the blastomeres causes the spindle of this cell to rotate into the position of a 3rd cleavage spindle.

HÖRSTADIUS (1928) reports on comparable phenomena in eggs of *Paracentrotus lividus* caused by shaking or exposure to diluted seawater. In these cases the formation of mitotic spindles can be delayed, but the spindles are turning synchronously with those of the control eggs. In this sea urchin a cytoplasmic factor, determining the formation of micromeres and located at the vegetative side of the egg, plays a part. We do not know, however, which is the determining factor for spindle rotation preceding the 3rd cleavage in the *Limnaea* egg. Similar observations have been made by CONKLIN (1938) in *Crepidula* eggs treated with low temperatures; after returning the eggs to 20°, some divisions may be skipped, but the subsequent cleavages (e.g. formation of micromeres) nevertheless take place in the normal direction.

RAVEN and MIGHORST (1946) report on a deviation of the second maturation division in eggs of *Limnaea* exposed to a 0.5 % $CaCl_2$ solution. In some egg masses a depolarization occurs. The second maturation spindle looses its contact with the animal pole, becomes centrally located and assumes a position at right angles to the polar axis.

Our hypothesis seems to give a possible explanation of these facts as there is a tendency of the second maturation spindle to assume the position of the first cleavage spindle in normal eggs.

PASTEELS (1931) displaces the 1st cleavage spindle in eggs of *Barnea candida* by ultraviolet radiation. There is no alteration, however, of the position of the spindle to the egg axis or of the spiral cleavage type.

PASTEELS (1930) reports on some cases of orientation of the first cleavage spindle in the direction of the egg axis. According to his interpretation, the spermatozoon has penetrated in these cases at the lateral side of the egg and the male pronucleus has been formed in an abnormal place. As soon as the spindle figure moves to the egg cortex, as occurs in all eggs of Lamellibranchiates, regulation takes place and the axis of mitosis places itself at right angles to the egg axis. PASTEELS explains these phenomena by the action of a cytoplasmic factor, which determines the position of the spindle.

Possibly, treatment with thiourea solution influences cytoplasmic factors responsible for cell division. It seems probable that these factors are located in the cortical or subcortical plasm.

On the other hand, the phenomena of nuclear degeneration seem to point to a direct action of the thiourea solution on the nuclear apparatus.

Summary.

1. Eggs of *Limnaea stagnalis* were treated at several stages with solutions of thiourea varying between 0.5 and 2 %.
2. Treatment of eggs within the capsules with a 1 % thiourea solution immediately after oviposition causes an inhibition of development. When the duration of the treatment is 5 hours or less, recovery occurs, but at later stages development is delayed as compared with the controls.
3. A longer treatment with thiourea solutions varying between 0.75 and 2 % causes an arrest of development either in the uncleaved or in the 4- or 8-cell stages. With respect to this arrest, the stage in which treatment begins is of great importance.
4. A longer treatment also causes many abnormalities of cleavage. The synchronism of cleavage divisions may be entirely or partially disturbed.
5. Cell divisions may be arrested; the nuclei show various types of degeneration, e.g. fragmentation of chromosomes, pycnosis and monasters; anachronisms of cleavage also occur.
6. In many cases, deviations from the normal direction of spindles occur, especially in delayed cleavages. They point to a relative independence of the factors governing the direction of the spindles in spiral cleavage.

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