Further observations on the distribution of cytoplasmic substances among the cleavage cells in *Lymnaea stagnalis*

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SUMMARY

The period of development from the 4th cleavage to the 24-cell stage was studied. Both during 4th and 5th cleavage a wave of mitosis passes over the egg from the vegetative to the animal pole. At 5th cleavage it does not spread over the cells of the first quartet, however, and cell division stops for 3 h at the 24-cell stage. Nucleoli are now formed in the interphase nuclei, and many of them are extruded whole into the cytoplasm. After 5th cleavage the cleavage cavity is gradually reduced and finally disappears altogether. All cells then extend towards the centre of the egg. In this process one of the macromeres (3D) finally becomes preponderant, gets a central position and applies itself against the inner side of the animal cells. This is preceded by a period of seemingly haphazard variation in macromere positions. Lipid globules and mitochondria accumulate in the central parts of the first quartet cells. The special cytoplasm (SCA-plasm) found in the most vegetative part of the macromeres at the 8-cell stage is distributed among the cells at the next cleavages. Part of it passes into the 2nd micromeres, another part into the 3rd micromeres, while the rest remains concentrated in the vegetative part of the macromeres around the vegetative cross-furrow. At this place coarse dark composite granules, of a special kind and very rich in RNA, become visible at the 16-cell stage. At the 24-cell stage they begin to move inwards along the cell walls, and finally condense into the compact RNA-rich 'ectosomes' at the central ends of the macromeres. The significance of the SCA-plasm and of the 'ectosomes' for the determination of dorso-ventrality in *Lymnaea* is discussed.

INTRODUCTION

In an earlier paper (Raven, 1963), a description has been given of subcortical ‘patches’ of cytoplasm in the equatorial zone of the uncleaved egg of *Lymnaea stagnalis*, staining differently from the surrounding cytoplasm. There are six of these patches, arranged according to a regular pattern. They arise by ooplasmic segregation during the passage of the egg cell through the female genital duct of the parent, and point to the existence of a mosaic pattern in the egg cortex. Evidence was adduced that this pattern reflects peculiarities in the mutual positions of elements surrounding the oocyte in the gonad during oogenesis.

In a subsequent paper (Raven, 1967), a further study of these ‘patches’ (now called ‘subcortical accumulations’, SCA) has been made. They consist of a

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dense cytoplasmic matrix, containing a special kind of small granule. At certain stages lens-shaped bodies, consisting of coiled threads, can also be observed in them. At the uncleaved stage the SCA form a regular pattern, which is dorso-ventral and nearly symmetrical. Its plane of symmetry coincides with the median plane of the future embryo. Just before first cleavage, the SCA exhibit a latitudinal extension and fusion. They are distributed in a regular way among the 2, then 4, blastomeres. At 3rd cleavage they pass as a whole into the macromeres. In each quadrant they show a gradual shift toward the vegetative pole. Their positions in the 4 quadrants of the egg exhibit characteristic differences.

The distribution of the SCA-plasms among the cleavage cells was described up to the end of the 8-cell stage (stage 19). Since then it has become probable that these plasms play a part in the determination of bilateral symmetry at later stages (Raven, 1970; Raven & Bezem, 1973). Therefore it is important to follow their distribution among the cells of the embryo as accurately as possible. In the present paper their further evolution up to the 24-cell stage is described.

MATERIAL AND METHODS

This series consists of 125 eggs of *Lymnaea stagnalis*. Samples of eggs were fixed in Bouin’s fluid at intervals of 15 min, beginning at 4th cleavage. The series ends with a sample fixed 120 min after 4th cleavage, when the eggs were at the 24-cell stage. The mean number of eggs per sample is 14.

The eggs of each sample were cut partly into sections of 6 \( \mu \text{m} \), partly of 5 \( \mu \text{m} \). Special care was taken that no sections were lost. They were stained with iron haematoxylin and eosin.

The procedure employed for the investigation resembled that described in my previous paper (Raven, 1967). Graphical reconstructions were made of all eggs, in which cell boundaries and positions of SCA-plasms were marked. These positions were projected on to an idealized equatorial plane of the egg. The SCA positions found in the eggs of one group were combined into a ‘median projection’ characterizing the group as a whole.

RESULTS

1. In the eggs of the first batch, fixed during 4th cleavage, all cells are in mitosis. In most eggs, the micromeres are in metaphase, the macromeres in late anaphase or telophase.

There is a small cleavage cavity in the centre of the eggs. The inner zones of the blastomeres, bordering on the cleavage cavity, often have a hyaline appearance, corresponding to the ‘secretion-cones’ described in a previous paper (Raven, 1946). When the eggs are viewed from the vegetative pole, the macromeres are extended in a clockwise direction, in preparation for their laevotropic division (Fig. 1). The position of the beginning cleavage furrows is indicated in most eggs by a shallow depression of the cell surface.
Just as during previous divisions (cf. Raven, 1967), the SCA plasms are greatly drawn out beneath the cell membrane of the dividing cells. In all macromeres they occupy the most vegetal part of the cell, extending along the cross-furrow (in B and D) or occupying the angles at the ends of this furrow (in A and C). In some eggs, the SCA-plasms in B and D bend slightly inwards along the vegetative cross-furrow. In all macromeres, they extend from the most vegetal part of the cell in an animal direction along its right margin (as seen from the vegetative side) (Fig. 1). This extension reaches in all cases well beyond the groove indicating the position of the future cell boundary (Fig. 2), which suggest that at the ensuing cell division part of the SCA substance will pass into the 2nd micromeres. In their most animal part these cells get an appreciable quantity of the dense plasm derived from the coalescence of the animal and vegetative pole plasm. In a previous paper (Raven, 1946) it has been shown that the fusion of the two pole plasms takes place at the 4-cell stage, and that a considerable part of the common mass of dense protoplasm resulting from this fusion passes into the first micromeres at 3rd cleavage. The remainder is mainly concentrated in the animal half of the macromeres, and a great part of this passes into the 2nd micromeres at 4th cleavage.

2. In the eggs fixed 15 min after 4th cleavage, the formation of the second micromeres has been completed. In most eggs, the 1st micromeres are in telophase, while the macromeres and 2nd micromeres have karyomeres uniting into a polymorphic nucleus. The cleavage cavity has begun to enlarge. Most
Fig. 3. Median projection of second lot; 15 min after 4th cleavage. Macromeres and 2nd micromeres of 12-cell stage.

blastomeres still exhibit 'secretion cones' or hyaline borders along the cavity (Fig. 4).

With the separation of the 2nd micromeres from the macromeres, in each quadrant of the egg the tip of the animal 'spur' of the SCA-plasm is pinched off, so that a certain amount of this substance comes to lie in the 2nd micromeres (Fig. 3). In vegetative pole view, this SCA-plasm in the 2nd micromere does not lie in the angle between the corresponding macromere and its right neighbour (as would be expected from its position at the previous stage, cf. Fig. 1), but it has shifted counterclockwise towards the middle of the new cell boundary between macromere and 2nd micromere, where these two cells are still connected by a mid-body and spindle remnant. As a matter of fact, the SCA-plasm occupies the region between this spindle remnant and the nucleus of the 2nd micromere (Fig. 4). At the sides of the nucleus it passes rather gradually into the rest of the perinuclear cytoplasm, but it is still clearly distinguishable from it. For one thing, the granules it contains are somewhat coarser than those of the perinuclear cytoplasm. Moreover, in most cases there is a cleft in the sections along the outside of the nuclear membrane in the region where this is bounded by the SCA-plasm, but not elsewhere (Fig. 4).

On the other side of the cell membrane separating the 2nd micromere from the corresponding macromere, somewhat similar relationships are found. The region bounded by this cell membrane, the cell surface, the spindle remnant and
the nucleus of the macromere is likewise occupied by SCA-plasm, which extends somewhat further in latitudinal direction than in the second micromere, however. A cleft along the nuclear membrane contiguous with the SCA-plasm may often be observed in the macromeres too.

The SCA-plasm in this region is connected by a rather thin subcortical sheet of the same substance with the SCA-plasm occupying the most vegetative part of the macromeres. This is heaped up along the vegetative cross-furrow in 2B and 2D and near the extremities of this furrow in 2A and 2C. The ingression of this plasm along the furrows, which was adumbrated in the previous stage, has become somewhat clearer; it now has occurred also in 2A and 2C (Fig. 4). Moreover, a new kind of coarse dark granule begins to appear in all macromeres in the SCA-plasm in the immediate neighbourhood of the vegetative cross-furrow. The granules are rather large, irregular, flaky bodies; probably they have arisen by clustering of smaller granules. Most of them are situated near the cell surface, but some may also be observed more inward along the cross-furrow.

3. The eggs of the next lot, fixed 30 min after 4th cleavage, have wide cleavage cavities. Spindle remnants with mid-bodies connect all pairs of sister cells of this 16-cell stage. Coalescence of karyomeres into a polymorphic nucleus is now also taking place in the cells of the 1st micromere quartet. In the macromeres and 2nd micromeres the process has further advanced, but has not yet
Table 1. Stages of the cell cycle reached by the macromeres and 2nd micromeres of eggs fixed 1 h after 4th cleavage

<table>
<thead>
<tr>
<th>Stage</th>
<th>Quartet</th>
<th>Macromeres</th>
<th>2nd micromeres</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>B and D</td>
<td>A and C</td>
</tr>
<tr>
<td>Interphase</td>
<td>—</td>
<td>7</td>
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</tr>
<tr>
<td>Prophase</td>
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<td>12</td>
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<tr>
<td>Pro-metaphase</td>
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<td>3</td>
</tr>
<tr>
<td>Metaphase</td>
<td>9</td>
<td>1</td>
<td>—</td>
</tr>
</tbody>
</table>

been completed; sometimes single karyomeres are still situated on the outer side of the membrane of an otherwise well-rounded nucleus.

No great change in the position of the SCA-plasm in comparison with the previous stage (Fig. 3) has taken place. With the flattening of the cells which accompanies the widening of the cleavage cavity, the nuclei have come closer to the cell surface. The SCA-plasm adjacent to the nuclei of the macromeres and 2nd micromeres has thereby been flattened to a rather narrow superficial layer capping the nuclei. The clefts along the nuclear membranes bordering on the SCA-plasm are still often visible. The coarse dark granules surrounding the vegetative cross-furrow in some cases clearly extend inwards along the cell membranes separating the macromeres (Fig. 5).

4. In most of the eggs fixed 45 min after 4th cleavage the cleavage cavity is greatly distended. By its growth the cells have been flattened, and the cell boundaries opened up from the cavity outwards. This holds especially for the cell boundaries in the animal hemisphere and for the vegetative cross-furrow, where the adjacent cells meet with rather sharp edges (Fig. 8).

All cells have interphase nuclei; especially those of the macromeres and 2nd micromeres are large and swollen, but still somewhat irregular in shape, with local protrusions and blebs. The spindle remnants present in the previous stage are only occasionally still visible in animal cells, but mid-bodies are still present between the other cells.

No great changes have taken place in the position of the SCA-plasms (Fig. 6). In most macromeres the connexion between the two parts of this plasm, located near the vegetative cross-furrow and above the nucleus, respectively, has greatly diminished or is no longer visible. The last-mentioned portion of this plasm has moved, together with the nucleus, in counterclockwise direction, when viewed from the vegetative pole. A similar displacement of the nuclei and SCA-plasms in the 2nd micromeres can be observed.

Both the SCA-plasm surrounding the vegetative cross-furrow and the coarse dark granules lying in this plasm have further extended into the depth along the cell membranes (Fig. 7). In those cases where the cell boundaries in this region are opened up by the extension of the cleavage cavity, the macromeres meeting
in this furrow are 'folded down', so to speak, the former surfaces of contact now adjoining the cleavage cavity. The SCA-plasm and the coarse granules are taken along in this movement, so that they now extend for a certain distance along the inner surfaces of the flattened macromeres (Fig. 8).

5. In the eggs fixed 1 h after 4th cleavage, mitosis has begun in the macromeres and 2nd micromeres. On an average, it has advanced to prometaphase in the macromeres, while most 2nd micromeres are in prophase. There are, however, differences in the stage of mitosis both between different eggs and among the cells of a single quartet within a same egg. Particularly, it is evident that the cells in the B- and D-quadrants are slightly ahead of those in the A- and C-quadrants. This holds especially for the macromeres; though the effect is also indicated in the 2nd micromeres, here it is, probably, hardly significant (Table 1).

No asynchronism with respect to the dorsoventral axis, such as becomes apparent at a later stage in the animal hemisphere (van den Biggelaar, 1971a), can be detected at this stage. To be sure, such asynchronism might be expected to be most pronounced with regard to the cells in the B- and D-quadrants, respectively. However, only in 2 out of 11 eggs are the macromeres 2B and 2D in a different stage of mitosis, whereas a difference in mitotic stage between 2A and 2C is found much more often (7 out of 11 eggs).

The cleavage cavity at this stage decreases in size. Apparently, this diminution of the cleavage cavity takes place rather gradually, progressing simultaneously with the advance of mitosis. When the macromeres are in prophase or early prometaphase, the cleavage cavity is still rather wide, as a rule, but according as mitosis progresses the cavity becomes smaller. Presumably, the fact that mitotic cells contract and round off to a more nearly spherical shape facilitates the extrusion of the blastocoelic fluid (Raven, 1946).

When the cleavage cavity decreases in size, the diameter of the embryo as a whole diminishes and the walls of the cavity thicken. This means that even the animal cells, which do not prepare for division, become less flattened, that the unfolded cell boundaries close up once more, and that adjacent cells get large surfaces of contact again. At the same time 'secretion cones' are once more visible.

As far as can be made out, the position of the SCA-plasms has not changed appreciably with respect to the previous stage, but the delimitation of those parts lying near the nuclei of the macromeres and 2nd micromeres from the perinuclear cytoplasm is rather difficult at this stage. The position of the SCA-plasms surrounding the vegetative cross-furrow varies with the configuration of the macromeres. In some eggs with rather wide cleavage cavity and 'open' vegetative cross-furrow, part of the SCA-plasm still extends along the inner side of the cells along the cavity, as in Fig. 8. When, however, the contents of the cleavage cavity are extruded, and the macromeres once more apply themselves against each other, thus closing the cross-furrow, these parts of the SCA plasms are folded back again to a more vertical position on either side of the
Table 2. Stages of the cell cycle reached by the macromeres and 2nd micromeres at 1 h 15 min after 4th cleavage

<table>
<thead>
<tr>
<th>Stage</th>
<th>Macromeres</th>
<th>2nd micromeres</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Quartet</td>
<td>B and D</td>
</tr>
<tr>
<td>Prometaphase</td>
<td>—</td>
<td>3</td>
</tr>
<tr>
<td>Metaphase</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>Early anaphase</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Middle anaphase</td>
<td>4</td>
<td>11</td>
</tr>
<tr>
<td>Late anaphase</td>
<td>14</td>
<td>4</td>
</tr>
</tbody>
</table>

Cleavage cells of Lymnaea

common cell membrane in 2B and 2D and in the inner angles of 2A and 2C.

Curiously, we find at this stage no coarse dark granules at these places. These granules are located at this stage at the vegetative pole and along the cross-furrow immediately beneath the surface, but they do not extend inwards along the cell membranes. Either a renewed concentration of coarse granules in superficial parts of the macromeres has taken place, or the granules found more inwards at earlier stages have been dissolved or become indistinguishable. The observation that the general visibility of the granules has diminished at this stage argues for the latter explanation.

6. At 1 h 15 min after 4th cleavage, mitosis of the macromeres and 2nd micromeres has further progressed. As Table 2 shows, the majority of the macromeres are in middle to late anaphase, whereas most 2nd micromeres are in metaphase or early anaphase. The lead of the B- and D-quadrants over the A- and C-quadrants is still evident at this stage, especially with respect to the macromeres. When the macromeres have reached the middle anaphase stage, they begin to elongate in preparation for the next dexiotropic division, extending in a counterclockwise direction, when viewed from the vegetative pole (Fig. 9).

Figures 2, 4, 5, 7, 8

Fig. 2. 4th cleavage. Division of macromere 1B. SCA-plasm in subcortical position (between arrows), extends into the future micromere 2b. × 875.


Fig. 5. Thirty minutes after 4th cleavage. Coarse granules (C.G.) along vegetative cross-furrow. × 875.

Fig. 7. Forty-five minutes after 4th cleavage. Coarse granules (C.G.) along vegetative cross-furrow. × 875.

Fig. 8. Forty-five minutes after 4th cleavage. Expansion of cleavage cavity, flattening of cells, opening up of vegetative cross-furrow. SCA-plasm and coarse granules (C.G.) partly extending along cleavage cavity. × 875.
The cells of the first quartet are in interphase. They possess spherical nuclei, in which distinct nucleoli have been formed. The cleavage cavity generally has further decreased in size, in comparison with the previous stage, though there are great differences between individual eggs of this lot. They range from a cleavage cavity of moderate size to no central cavity at all. In this latter condition, which is found in one or two eggs of the lot, the macromeres and micromeres meet in the centre of the egg with attenuated, more or less hyaline protrusions (Fig. 10, p. 50). These correspond to the 'secretion cones' described earlier. One gets the impression that this assembly of the cells in the centre is an active process, in which the macromeres take the lead. This is indicated by the fact that in such eggs small pits in the surface appear at the extremities of the vegetative cross-furrow (Fig. 10). It must be emphasized that at this stage all macromeres take part in this movement to the same extent; no differences between the quadrants of the egg are visible as yet.

More or less independent of the gradual decrease in size of the central cleavage cavity seems to be another process, through which lenticular fluid-filled cavities are formed between the cells of the animal hemisphere. In those eggs where the central cavity is either small or absent, they remain more or less isolated, but in eggs with wider cleavage cavity they may open into this cavity by means of narrow clefts or communicate with each other.

There is no clear correlation between the size of the cleavage cavity and the
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stages of mitosis. Rather wide cavities are found both in the beginning and towards the end of the range of stages covered by this lot.

The SCA-plasm lying in the neighbourhood of the nuclei has become much more distinct than at previous stages. This is caused by the fact that the perinuclear cytoplasm becomes greatly drawn out with the elongation of the spindles at anaphase. The SCA-plasm still forms a subcortical layer of rather dense cytoplasm. An intermediate layer of vacuoles, each of which contains a γ-granule of the proteid yolk, now separates the perinuclear cytoplasm from the SCA-plasm. Since these vacuoles indent and often nearly interrupt the SCA-plasm, the latter has a more or less variegated appearance.

The coarse dark granules near the vegetative cross-furrow have further diminished. In some eggs some rather small granules are still seen at the vegetative pole, but in most eggs they are invisible at this stage.

7. One and a half hours after 4th cleavage, division of the macromeres and 2nd micromeres has taken place, so that the egg has reached the 24-cell stage. In the cells of the second quartet, the nuclei are in a late telophase or early reconstitution stage; in the macromeres and 3rd micromeres reconstitution nuclei or polymorphic interphase nuclei are found. Sister cells are still connected by spindle remnants. The cells of the first quartet have big vesicular interphase nuclei with several nucleoli. Most nucleoli are situated against the inner side of the nuclear membrane; often, the membrane bulges out at this place, forming a bleb containing the nucleolus at its tip (Fig. 11). In several cases a nucleolar body seems to lie against the outer side of the nucleus, without being surrounded by a membrane (Fig. 12).

The size of the central cleavage cavity varies from moderate to small; in one egg a cavity is lacking. There is no very distinct correlation between the size of the cavity and the stage of the eggs, though most of the older eggs of the lot have small cavities. ‘Secretion cones’ generally have disappeared at this stage. The cells of the first quartet have begun to extend more or less conical protrusions with rounded ends towards the cleavage cavity. These cell parts are poor in yolk granules, but contain a dense agglomeration of small, more or less grey-blue globules. The lens-shaped clefts between these cells are still present and show the same relationships as at the previous stage.

In the cleavage cavity of the eggs of these stages, often one or more (up to 4) dark bodies are found. They lie either freely in the cavity or are attached to the surface of the cells bordering it, showing a certain preference for the inner tips of the macromeres. Presumably they are mid-bodies of early cleavage divisions, which have become isolated by the ingrowing cell walls and extruded into the cleavage cavity (Berendsen, 1971).

With the division of the 2nd micromeres, the SCA-plasm in these cells has been distributed more or less equally among the two daughter cells. The SCA substance that was located at previous stages near the nuclei of the macromeres has passed for the greater part into the 3rd micromeres, while a smaller portion
of it has remained in the macromeres along the furrow with the 3rd micromeres (Fig. 13). The SCA-plasms in these cells are clearly delimited at this stage; they contain a dense population of eosinophil granules, which are much more conspicuous than at previous stages. On the other hand, the SCA plasm surrounding the vegetative cross-furrow in the most vegetal part of the macromeres is not very distinct. Coarse dark granules are situated along the cell boundaries in this region, but they are not very conspicuous.

8. One hour 45 min after 4th cleavage, the nuclei of all cells are in interphase. Those of the first quartet cells have big nucleoli; in the other blastomeres small nucleolar bodies have appeared. In the former cells, in several cases nucleoli are found in the cytoplasm at some distance from the nuclear membrane, so that the extrusion of whole nucleoli from the nucleus in these cells can hardly be doubted (Fig. 14). Spindle remnants connecting neighbouring cells are still visible.

The central cleavage cavity has, on an average, further diminished in size; in 5 cases it is lacking altogether. The cavities between the animal cells are still very conspicuous in several of the eggs. The cells of the first quartet in those cases are mushroom-shaped in section, the broadened outer part of the cell representing the cap, while the protruding inner part extending toward the egg centre forms the stalk of the mushroom (Fig. 15). Grey-blue globules are
accumulated in the tips of these protrusions. In 4 eggs the cavities in the animal part are lacking, and the animal cells are tightly apposed to one another. The same holds for the cells of the vegetative half in most eggs, which are conical and fit closely together.

In several of the eggs it was observed that one of the macromeres extends further toward the animal side than the other ones. When the central cavity is lacking, the tip of this macromere extends beyond the egg centre and reaches the inner side of the first quartet cells of the animal hemisphere, whereas the other three macromeres are separated from the animal cells by the inner ends of the 3rd and 2nd micromeres. As a rule it is one of the macromeres 3B and 3D that occupies this central position, but in a few cases it seems to be replaced by one of the cells 3A or 3C. Apparently, the disposition of the macromeres is still variable at this stage.

No great changes have taken place in the SCA-plasms in comparison with the previous stage. Coarse dark granules along the vegetative cross-furrow are clearly visible, in many cases extending deeply into the macromeres.

9. The last lot of eggs studied was fixed 2 h after 4th cleavage, 30 min after the beginning of the 24-cell stage. All nuclei are in interphase, and have several rather large nucleoli. Extrusion of whole nucleoli into the cytoplasm by way of blebs of the nuclear membrane is taking place in all cells, hence also in those of the second and third quartet and in the macromeres.

The central cleavage cavity is either very small or has disappeared altogether. The clefts between the deeper parts of the animal cells are still present in most eggs, however, and sometimes have united to rather wide cavities. The cells of the vegetative hemisphere extend in a radial direction toward the egg centre and are tightly apposed to each other. In several cases one of the macromeres 3B or 3D protrudes beyond the egg centre and reaches the lower side of the animal cells, but in other eggs the cells 3B and 3D extend inwards to the same degree (Fig. 16), and several macromeres are in contact with the inner side of the first quartet cells.

The accumulation of grey-blue spherules in the inner parts of the cells of the first quartet is very conspicuous. The location of the SCA-plasm has little changed in comparison to Fig. 13. This plasm is characterized by the presence of rather coarse eosinophil granules. The coarse dark granules in the macromeres have further progressed along the vegetative cross-furrow toward the inner end of these cells; in several eggs a nearly continuous file of these granules along the cell boundaries can be observed, and they have begun to accumulate at the central extremities of the macromeres (Fig. 16).

**DISCUSSION**

In recent years, various investigations of our group have pointed to the importance of the 24-cell stage for further development in *Lymnaea* (Raven,
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1970; Raven & Bezem, 1973). At this stage, a break in the progress of cleavage occurs. While the preceding divisions followed each other at intervals of about 1 h, on an average, cleavage is now interrupted for 3 h (Verdonk, 1965). During this period, the cleavage cavity disappears. The macromere 3D partly withdraws from the surface, and applies itself against the inner side of the animal cells. Coarse dark granules, which have been formed in the SCA-plasm at the vegetative pole, accumulate at the central ends of the macromeres, where they coalesce into irregular complexes, which are very rich in RNA. Certain cytological and cytochemical particulars point to an exchange of substances between the cells in the area of contact. When cleavage is resumed, macromere 3D divides into the small superficially located macromere 4D and the primary mesoblast 4d. By this division, the dorsoventrality of the embryo first becomes visible in the cell pattern. Soon thereafter, it is indicated also at the animal side, at first by asynchronous divisions of corresponding cells in different quadrants (van den Biggelaar, 1971a). Later the dorsoventrality also becomes expressed in differences in the direction of divisions among the quadrants, leading to a bilaterally symmetric cell pattern (Verdonk, 1965).

From the study of both normal development and the abnormal development resulting from lithium treatment of the egg at early stages, it has been concluded that bilateral symmetry in the animal hemisphere is induced by vegetative blastomeres during and after the 24-cell stage (Verdonk, 1965, 1968a; Raven, 1970; van den Biggelaar, 1971a, b; Raven & Bezem, 1973). Therefore, a study of the developmental processes leading up to the 24-cell stage was indicated.

The first lot of eggs used for this investigation was fixed during 4th cleavage. On an average, the micromeres were in metaphase, the macromeres in late anaphase or telophase. This difference does not agree with Verdonk’s (1965) report, according to which the division of the 1st micromeres takes place about 1 h after that of the macromeres. However, it corresponds to the interval of 16 min between the two divisions observed by Van den Biggelaar (1971a). As a matter of fact, van den Biggelaar gives a diagram of this stage in his figure 2B.

A similar ‘wave of mitosis’, starting at the vegetative pole and progressing in

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**Figures 10-16**

Fig. 10. Seventy-five minutes after 4th cleavage. Diminution of cleavage cavity. ‘Contraction pit’ at vegetative cross-furrow. × 560.

Fig. 11. Early 24-cell stage. Extrusion of nucleolus in bladder-like projection of nucleus. × 1400.

Fig. 12. Early 24-cell stage. Nucleolus on external side of nuclear membrane. × 1400.

Fig. 14. Early 24-cell stage. Nucleolus outside nuclear membrane. × 1400.

Fig. 15. Twenty-four-cell stage. Small central cleavage cavity, accessory cavities between animal cells. × 560.

Fig. 16. Twenty-four-cell stage. Central cleavage cavity reduced. Coarse granules (C.G.) moving along vegetative cross-furrow to central end of macromeres. × 875.
animal direction through the egg, can be observed at the 5th cleavage. Not only are the macromeres distinctly ahead of the 2nd micromeres, corresponding to the difference of about 10 min observed by van den Biggelaar (1971a), but more subtle differences exist within each of these quartets, the cells in the B- and D-quadrants being slightly ahead of the A- and C-cells. Since the B- and D-quadrants are situated somewhat nearer the vegetative pole than the other two quadrants, this asynchronism of cleavage is apparently likewise related to the position of the cells with respect to the main axis of the egg. Table 1 (and, less clearly, Table 2) demonstrate the progression of this ‘wave of mitosis’ through the vegetative part of the egg.

It appears, on the other hand, that this ‘wave’ does not spread over the descendants of the first quartet of micromeres. Taking into account the time-lag this quartet has incurred at the 4th division, one might expect its mitosis to begin about half an hour after that of the second quartet. Actually, however, its next division is postponed for another 4-5 hours. One might imagine that further progression of the ‘wave of mitosis’ is overtaken by the general paralysis of cell division that characterizes the 24-cell stage, but this does not explain why the next division of the first quartet cells is amply preceded by that of the second quartet cells. Apparently other, still unknown factors regulating the succession of cell divisions have come into play here.

After each division, sister-cells remain for some time connected by spindle remnants with mid-bodies. They are useful cues for recognizing matching cell pairs (Fig. 4). The spindle remnants disappear in about half an hour, but the mid-bodies remain visible for a longer time as oblong black bodies in the interspaces between adjacent cells. Moreover, similar black bodies may be found freely in the cleavage cavity. It has been observed by Berendsen (1971) that mid-bodies of early cleavage divisions become isolated by ingrowing of the cell walls on either side of them and become extruded either to the exterior or toward the cleavage cavity. The mid-bodies in the cleavage cavity often get attached secondarily to the inner surfaces of the cells, especially to the inner tips of the macromeres. They must not be confounded with the RNA-rich SCA-granules, which at a later stage accumulate in the macromeres at this place.

No distinct nucleoli are formed in the nuclei of the blastomeres during early stages of cleavage. They first become apparent in the cells of the first quartet at the end of the 16-cell stage, while the macromeres and 2nd micromeres are in mitosis for their next division. The nucleoli in the first quartet cells gradually increase in size. Meanwhile, the formation of nucleolar bodies also begins in the other cells of the embryo, as soon as they have completed their division and reached an interphase stage. Hence, half an hour after the beginning of the 24-cell stage all nuclei have several moderately large nucleoli. van den Biggelaar (1971c) has shown that they become engaged in RNA synthesis shortly after their formation.
Extrusion of whole nucleoli from the nucleus into the cytoplasm is very conspicuous at these stages (Figs. 11, 12, 14). The process, which first becomes recognizable in the cells of the first quartet at the very beginning of the 24-cell stage, but has appeared in the nuclei of all cells half an hour later, takes place in the following manner (Fig. 17): several nucleoli formed in a nucleus apply themselves tightly against the inner side of the nuclear membrane. At such places bladder-like protrusions of the membrane may be formed, with a nucleolus occupying the tip of each bladder. These blebs are constricted off from the nucleus. They lie for some time as closed vesicles against the outside of the nuclear membrane, but soon their wall is resorbed and the nucleolus is set free in the cytoplasm.

Extrusion of whole nucleoli at later cleavage stages of *Lymnaea* has been reported by me in an earlier paper (Raven, 1946). At that time the particulars of the process could not be elucidated. It now appears that it even occurs much earlier, and begins immediately after nucleoli are first formed in the cleavage nuclei. Extrusion of nucleoli by way of protrusions of the nuclear membrane during oogenesis has been described, e.g. in fish, insects and holothurians (Eggert, 1929; Ries, 1932; Kessel & Beams, 1963).

Nothing definite can be said on the significance of the process for further development. The extruded nucleoli soon become unrecognizable in the cytoplasm. Presumably, however, the extrusion process forms a convenient way for the rapid building of a new population of ribosomes in the cell. More generally, it may promote the flow of information from the nucleus to the cytoplasm during this decisive phase of development.

In the eggs of *Lymnaea* a cleavage cavity is already formed at the 2-cell stage. This so-called ‘recurrent cleavage cavity’ exhibits a regular sequence of expansion and contraction concurrent with the rhythm of cleavage divisions (Raven, 1946, 1966). It is small at the time of cell division, shows a slow swelling after the blastomeres have flattened against each other, followed by a rapid contraction when its contents are expelled to the exterior; as a rule, this occurs when some blastomeres round off prior to the next division.

The same holds for the 16-cell stage. The centrally situated cleavage cavity
is small at the time of 4th cleavage, and enlarges gradually in the next $\frac{3}{4}$ h. By the expansion of the cleavage cavity the surrounding blastomeres are greatly stretched and flattened, and their cell boundaries are opened up from within. In this process the original surfaces of contact of neighbouring cells are ‘folded down’, and become part of the lining of the cleavage cavity (Figs. 4, 5, 8).

The reverse occurs when the cleavage cavity diminishes in size once more at the end of the 16-cell stage. However, in contradistinction to what takes place at preceding divisions, this reduction of the cleavage cavity at 5th cleavage is no rapid contraction, but a very gradual process, which moreover occurs with greatly variable rate in different eggs. Fifteen minutes after the beginning of its diminution, in some of the eggs the central cleavage cavity has disappeared already, but it takes one hour before this has occurred in all eggs. The time during which this gradual disappearance of the cleavage cavity takes place (the first hour following upon the division) corresponds to the period in which the cavity enlarges after other cleavage divisions. Again it becomes evident that with the beginning of the 24-cell stage a new regimen prevails. The regular succession both of cell divisions and of fluctuations in size of the cleavage cavity is interrupted for 3 h, then recommences again.

In an earlier paper, hyaline zones along the inner borders of the blastomeres, bounding the cleavage cavity, have been described in *Lymnaea* (Raven, 1946). They were called ‘secretion cones’, since it was assumed that they played a part in the secretion of the fluid filling the cavity. Similar ‘secretion cones’ have also been described in *Succinea* (Jura, 1960). In the present investigation they were again observed during 4th cleavage (Fig. 4), and for some time immediately after 5th cleavage. With the diminution of the cleavage cavity at the 24-cell stage they disappear, though the inner tips of the macromeres stretching towards the centre may still be somewhat hyaline (Figs. 10, 16). Lately some doubt has arisen regarding the reality of the ‘secretion cones’ in the living embryo, since it has appeared that their presence in the slides depends on fixation. In eggs fixed in osmic acid no hyaline cell borders along the cleavage cavity are visible (W. Berendsen, personal communication). Notwithstanding their regular occurrence in eggs treated with other fixatives it is possible, therefore, that they are fixation artifacts.

With the gradual disappearance of the cleavage cavity during the hour following upon 5th cleavage all cells surrounding the cavity extend in a centripetal direction. However, this process takes a somewhat different course in the vegetative and the animal hemisphere. In the vegetative part, while the cleavage cavity diminishes in size the opened-up cell boundaries become folded together again, the cells apply themselves once more against one another and enlarge their common surfaces (Figs. 10, 15, 16). The impression that one has to do with an active process of junction by increased adhesivity of the cells is strengthened by the observation that a contraction of the outer cell surfaces along the vegetative cross-furrow, leading to the appearance of small pits at its extremities,
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seems to take place (Fig. 10). With the further diminution of the central cavity the macromeres and 3rd micromeres extend more and more towards the centre as somewhat truncated pyramids (Fig. 16).

The cells of the animal hemisphere, on the other hand, especially those of the first quartet, behave differently. They are joined together only with their broadened outer parts, from which conical or more or less club-shaped protrusions are extended towards the egg centre (Fig. 15). Between these inner cell parts cavities remain, which mostly appear more or less lens-shaped in section. They are interconnected, and may open into the central cleavage cavity by means of narrow clefts; sometimes they even join to a larger cavity beneath the animal pole. Half an hour after the beginning of the 24-cell stage they are still present. They disappear at a later stage, and the animal cells then become tightly joined together like the cells of the vegetative hemisphere.

Originally, all macromeres take part to the same degree in the extension toward the egg centre. Soon after the beginning of the 24-cell stage, however, one or other of the macromeres may have taken the lead, extending further toward or even beyond the centre of the egg. In such a case as a rule it is only this macromere that makes contact with the inner border of the first quartet cells, the other macromeres remaining separated from these cells by the inner extensions of the 2nd and 3rd micromeres. As a rule, the leading macromere is one of the cells 3B or 3D, broadly meeting at the vegetative cross-furrow, but this does not hold in all cases. Moreover, even half an hour after the beginning of the 24-cell stage in some eggs no inequality between the macromeres has yet become visible (Fig. 16). Apparently, the characteristic configuration of the 24-cell stage—in which 3D has a nearly axial position, extends markedly beyond the egg centre and applies itself against the inner side of the first quartet cells, while the other macromeres 3A, 3B and 3C remain broader and lower, and have no contact with the cells of the first quartet—is not reached in a direct and straightforward way but, so to speak, only waveringly.

When the cells of the first quartet, at the very beginning of the 24-cell stage, form conical protrusions extended toward the egg centre, a dense accumulation of small more or less grey-blue spherules is first seen in the inner ends of these protrusions. These regions of the cell are at the same time poor in yolk granules. It is these cell parts that later get a very close contact with the macromere 3D. Cytochemical staining seems to show that they contain glycogen (Raven, 1970). Electron microscope observations have shown that this region of the cells is filled with lipid globules, while dense masses of mitochondria occupy the inter-spaces between these globules and are heaped up beneath the cell membrane (M. R. Dohmen, personal communication). Since most of the lipid will have been removed in paraffin sections, it seems likely that the pale grey-blue colour must be ascribed to the mitochondria.

At fourth cleavage, when the macromeres elongate in a sinistral direction in preparation for the formation of the 2nd micromeres, the SCA substance is
drawn out beneath the cell surface in the same direction (Fig. 1). All 2nd micromeres acquire a small part of this substance when they are split off from the macromeres (Fig. 3). It remains visible for some time in these cells, filling the space between the cell surface, the nucleus and the spindle remnant. When they next divide, it passes in about equal amounts to their daughter cells (Fig. 13). The main mass of the SCA-plasm remained at 4th cleavage in the macromeres. One part of this substance is mainly concentrated above the nuclei, while a second portion remains concentrated in the most vegetative region of the cells, occupying the space on either side of the vegetative cross-furrow and the angles between adjacent furrows at its extremities. The two portions in each macromere are connected by a thin subcortical layer of SCA-plasm, which becomes disrupted before long. The SCA substance near the macromere nucleus follows the nucleus in its migration from a ‘sinistral’ to a ‘dextral’ position, and passes at the next division for the greater part into the 3rd micromere, a smaller portion of it remaining in the macromere along the furrow with the 3rd micromere (Fig. 13). The SCA-plasm surrounding the vegetative cross-furrow tends to extend into the depth along the interblastomeric planes. When the vegetative cross-furrow is opened up from within by the expansion of the cleavage cavity, part of this material temporarily comes to lie along the border of this cavity, but with the subsequent diminution of the cleavage cavity and the closure of the furrow they are folded back into their original position.

The SCA-plasm in the neighbourhood of the nuclei at certain stages merges rather gradually into the perinuclear plasm lying at a deeper level in the cells. It remains recognizable, however, by its superficial position just beneath the egg membrane and by a somewhat larger content of basophil granules. Moreover, a clef along the nuclear membrane of macromeres and 2nd micromeres, though apparently an artifact due to shrinking, is highly characteristic, since it is only present on the side where the nucleus is bounded by the SCA-plasm (Fig. 4), and therefore seems to point to special properties of this substance. When the mitotic spindles of 5th cleavage appear, the perinuclear plasm and SCA-plasm become separated again, the former being drawn out along the spindles, whereas the latter retains its subcortical position. A layer of γ-vacuoles now separates the two. With the beginning of the 24-cell stage, rather coarse eosinophil granules, which are probably identical with the β-granules of the proteid yolk, begin to accumulate in the SCA-plasm.

A characteristic element of the Lymnaea egg is formed by the coarse dark granules, arising in the SCA-plasm in the immediate neighbourhood of the vegetative cross-furrow. When they first become visible in iron haematoxylin–eosin-stained slides, towards the end of 4th cleavage, they have a rather irregular, flaky appearance, suggesting that they have arisen by the aggregation of smaller granules. This is confirmed by the observation that in galloycyanin-stained preparations already during 3rd cleavage small deeply stained spherical granules are found in the SCA-plasm on the vegetative side of the macromeres
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near the vegetative cross-furrow. They are lacking after RNase treatment. The coarse dark composite granules soon after 4th cleavage begin to move inwards along the vegetative cross furrow. In 2B and 2D, they move in file on either side of the cell membrane separating these cells (Figs. 5, 7); in 2A and 2C they follow the medially directed angle between adjacent cell membranes. When the cross-furrow is opened up by the expansion of the cleavage cavity, they partly come to lie beneath the cell membrane bounding this cavity (Fig. 8).

With the beginning of 5th cleavage and the diminution of the cleavage cavity, resulting in a restitution of the previous situation of the cross-furrow, curiously no coarse dark granules are found in the expected positions in the deeper parts of the macromeres. In general, their visibility greatly decreases during this cleavage. It is possible that a deconcentration or partial dissolution of the granules occurs. When the 24-cell stage has been reached, they reappear and once more begin their migration inwards along the cell membranes of the macromeres (Fig. 16). At the last stage studied, 30 min after the beginning of the 24-cell stage, in several eggs the coarse granules have begun to accumulate at the central extremities of the macromeres. Here, they will finally condense into the compact RNA-rich ‘ectosomes’ (Raven, 1946, 1970), which possibly play a part in the determination of bilateral symmetry in the head region.

It has been shown in a previous paper (Raven, 1967) that the SCA in the uncleaved eggs of *Lymnaea stagnalis* are arranged according to a dorsoventral and nearly symmetrical pattern. The plane of symmetry of this pattern coincides with the median plane of the future embryo. It was impossible at that stage to decide which was the dorsal and which the ventral side of the egg. At cleavage the SCA are distributed according to a definite programme along the blastomeres. Their positions in the four quadrants exhibit characteristic differences up to the 8-cell stage. Since early cleavage follows a radial pattern, it was not yet possible at this stage to identify the quadrants. For convenience sake, preliminary denotations were given to the quadrants. It was expected that by tracing development up to the 24-cell stage, when the dorsoventrality of the embryo becomes evident, it would be possible to check these provisional attributions. This expectation has not been fulfilled, however.

As a matter of fact, the average positions of the SCA-plasms and their distribution over the cells exhibit slight differences in the four quadrants of the egg also in the period between the 8-cell and 24-cell stage, as can be seen from the figures (Figs. 1, 3, 6, 9, 13). However, these differences are too small and not sufficiently consistent to permit of distinguishing the B- from the D-quadrant, or the A- from the C-quadrant.

It appears, therefore, that there is at these stages no essential difference between the four quadrants in the quantity and the distribution of the SCA-substance. This makes it unlikely that the dorsoventrality of the embryo is based upon an unequal distribution of the SCA-plasm between the two sides. In so far as the arrangement of the SCA at the uncleaved stage foreshadows the
dorsoventrality of the future embryo, this arrangement is not the cause, but a consequence of the dorsoventral structure of the egg, which itself is presumably bound to the cortex (Raven, 1970).

The fact that the SCA-plasm gives rise to the RNA-rich granules migrating to the inner pole of the macromeres at the 24-cell stage argues for its morphogenetic significance. No clear difference between the macromeres in the number of these granules prior to or during their migration has been observed, however. This suggests that a roughly equal distribution of the SCA-substance among the four quadrants suffices for normal development. A similar conclusion has been drawn recently from other observations (Raven, 1972). This conclusion does not disprove the significance of the SCA-granules for further development, but accentuates the importance of subsequent processes of topogenesis, by which the 'ectosomes' of the D-quadrant are carried into the immediate proximity of the first quartet cells, in contradistinction to those in the other quadrants.

When the dorsoventrality of the embryo, which becomes first visible at the 24-cell stage by the different topogenetical behaviour of macromeres B and D, is not due to differences in the amount of SCA-substance passing into these cells, it is obvious to conclude that it is derived in some other way from the invisible dorsoventral structure of the egg cortex.

One other possibility has to be considered, however. Our observations have shown that the preponderance of one of the blastomeres at the 24-cell stage does not come about in a direct and straightforward way, but after a period of seemingly haphazard variations. One might think that there is some kind of tug-of-war between the macromeres, in which one or the other of the two cells meeting at the vegetative pole finally overcomes and becomes the D-blastomere. In this case only the position of the median plane would be preformed at least since the time of oviposition, but the direction of the dorsoventral axis would be determined in an epigenetic way at the 24-cell stage.

Though this possibility cannot be excluded on the basis of the known facts, it must be recalled that a similar view of the epigenetic determination of dorsoventrality has been put forward with respect to the eggs possessing a polar lobe. According to Schleip (1925) the first polar lobe in Dentalium fuses in an arbitrary way with one of the two blastomeres, which thereby becomes the CD-cell. However, Verdonk's (1968b) experiments have made it very likely that also in Dentalium dorsoventrality is predetermined in the uncleaved egg. Therefore, for the moment it seems most probable that the same holds for Lymnaea.
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REFERENCES


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