Studies of the cleavage in the frog egg

II. On determination of the position of the furrow

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In sea-urchin eggs, once karyokinesis reaches metaphase or anaphase, the cleavage furrow can be formed even if the mitotic apparatus is destroyed (Swann & Mitchison, 1953) or removed (Hiramoto, 1956). A similar result was obtained in frog eggs (Kubota, 1966). In amphibian eggs a much longer time is available for performing experiments than in sea urchins as the furrow first appears at the animal pole and slowly travels toward the vegetal pole. Taking advantage of this situation, Waddington (1952) and Dan & Kuno-Kojima (1963) performed various kinds of operations to elucidate the roles of the egg cortex and the inner cytoplasm in furrow formation, and Selman & Waddington (1955) also made cytological observations of the process.

In the present paper a shift of the inner cytoplasm relative to the cortex and its influence on the course of the furrow was analysed for eggs of the frog Rana nigromaculata.

EXPERIMENTAL PROCEDURES AND RESULTS

I. Tilting of the egg in relation to gravity

On inclining fertilized amphibian eggs, light cytoplasm located originally on the side of the animal pole moves upward to a new position in the egg and heavy cytoplasm moves downward, resulting in a relative displacement in position between the cortex and the inner cytoplasm (Pasteels, 1951). The behaviour of the furrow was investigated after such inclination.

Method

After removing most of the jelly, eggs were placed on a sheet of filter paper in a moist chamber. Immediately after the onset of the first cleavage, eggs were tilted so that the cleavage plane was approximately horizontal and kept in this position for approximately 40 min until the tip of the furrow reached the vegetal pole. If the egg began to right itself, the egg was turned to the horizontal position.

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with a hair loop. After the cleavage was completed, eggs were transferred into Smith’s fluid together with the filter paper and the position of the furrow was closely examined after fixation.

Results

After tilting the eggs could be divided into two groups: some formed furrows curving downwards in the inclined state (Fig. 1), while some formed straight furrows showing no effect due to the forced inclination. On sectioning it was found that, on the basis of the distribution of the Delafield haematoxylin stained protoplasm and uncoloured yolk grains, considerable dislocation of the inner cytoplasm had occurred in eggs with curved furrows and little dislocation in those with straight furrows. These results suggest that there is a close correlation between the curving of the furrow and the dislocation of the cytoplasm.

![Text-fig. 1. Bending of the furrow after tilting during the entire period of the first cleavage. Drawing from the photograph of a fixed egg.](image)

For further analysis, however, it is necessary to move cytoplasm more rapidly in a restricted region, and rubbing of the egg surface was adopted in place of the tilting of the whole egg.

II. Rubbing of the egg surface

Method

(1) Eggs surrounded by the vitelline membrane and a thin layer of jelly were used. Prior to the dislocation of the cytoplasm, a solution of Nile blue sulphate was injected in some eggs with a micropipette to mark by vital staining a part of cytoplasm lying below the cortex (Fig. 2, open circle). The insertion aperture of the pipette on the cortex, soon closing, became a scar, which served as a mark on the cortex itself (Fig. 2, filled circle).

(2) For rubbing, eggs were placed on a sheet of dry filter paper, and the future path of the furrow was rubbed across several or many times perpendicularly in one direction with a hair loop. After the treatment eggs were soaked in spring water and the progress of the furrow was followed under a binocular dissecting microscope, using untreated eggs as controls.
In the present paper, in order to facilitate comparison of the results of different experimental series, the standard period from insemination to the beginning of the first cleavage is taken as 2 h (Kubota, 1966) and that from the first to the second cleavage as 38 min, and various developmental stages observed in each series were expressed by times converted to these standards.

Text-fig. 2. Diagram of the operation performed prior to the furrowing at the equatorial region and the resultant alteration in the course of the furrow. (A) Vital staining by injection of Nile blue sulphate solution. The arrow is the direction of rubbing. (B) Displacement of the stained cytoplasm, caused by the rubbing of the surface with a hair loop. (C) Resultant deviation of the furrow from the normal course. Note that the furrow passes over the displaced, stained cytoplasm (open circle). See also Plate 1, fig. A.

Results

The experimental procedure was applied in three different regions: (a) animal, (b) equatorial and (c) vegetal egg surface; and at two different stages: (1) immediately before the arrival of the furrow tip, and (2) at an earlier stage for each region. In this report, the term equatorial region indicates a girdle-like portion comprising the margin of the pigment cap and the white area below it.

(1) Treatment shortly before formation of the furrow. Equatorial (b) and vegetal (c) regions: by injecting Nile blue sulphate solution, the future path of the furrow was first marked at the equatorial (b) or vegetal (c) region. On the approach of the furrow (Text-fig. 2 A), the marked but as yet undepressed surface was rubbed perpendicularly to the path of the furrow. When rubbed, the cytoplasm stained in blue moved in the direction of rubbing (B open circle), while the insertion point remained at the initial position (B filled circle). The existing furrow halted for a while after the treatment and the newly formed part of the furrow left its straight course, invariably passing over the coloured cytoplasm (C open circle). These facts unequivocally indicate that in the equatorial and vegetal regions the subcortical cytoplasm found on the division plane possesses a capacity for inducing the furrow in the cortex, at least just before the appearance of a new furrow (see Plate 1, figs. A, B, B').

Animal region (a): if the rubbing was conducted ahead of a furrow on the animal pole region when a furrow was appearing, neither displacement of the injected dye nor alteration of the course followed, the furrow always travelling along a straight path. However, if the egg surface was broken at some distance
from the furrow with a pointed end of a razor, and then a hair loop was gently moved to traverse the future course at the marked point, the stained cytoplasm flowed with a trail toward the cut as the protoplasm was extruded from the egg. The furrows of such eggs, instead of passing through the position of the insertion scar, travelled on one side closer to the cut.

Plate 1
(Photographs of eggs fixed in Smith's fluid)

Fig. A. Curving of the furrow of the first cleavage, produced by rubbing the equatorial surface of the egg ahead of the furrow tip. The semicircular dark spot on the left side of the furrow is an artifact of fixation, not the part stained with Nile blue sulphate.

Figs. B, B'. Equatorial and vegetal views of the same egg. Curved furrow in the vegetal region, formed by rubbing at the vegetal pole when the furrow was approaching.

Fig. C. Deviation in the course of the furrow of first cleavage, induced on the animal surface by the extrusion of the cytoplasm (see Text).
The possibility that the path of the furrow of amphibian eggs can be modified by a treatment given after the onset of cleavage has been briefly pointed out by Waddington (1952, exp. vii). He scraped off some cytoplasm from dividing newt eggs and observed an alteration in the course of the furrows in only a few eggs. In the present experiment, positive results were obtained in many instances. This difference may be due to the technique employed rather than to the material used.

Text-fig. 3. Relation between time of rubbing at the equator and later bending of the furrow. Open circles represent positive cases of the occurrence of bending and filled circles represent straight furrows. The circles are placed according to the time of rubbing (on the abscissa), the onset of the first cleavage being 0 min.

(2) Treatment at earlier stages. The experimental results described above suggest that some changes directly concerned with the development of the furrow or the capacity to induce the furrow may be established in the subcortical cytoplasm before the actual appearance of the furrow. If this is the case, displacement of the cytoplasm after the establishment of such changes would be followed by a corresponding change in the position of the furrow when it appears and, inversely, displacement beforehand would cause no change. In other words, the critical stage could be found by treating eggs at regular intervals and by examining the position of the furrow formed. For this reason the experiments were repeated at earlier stages.

In the following experiments the times of the initiation of cleavage in operated eggs were determined indirectly from those of eggs simultaneously inseminated and not subjected to operations.

Equatorial region (b): the first cleavage plane of Rana nigromaculata passes close to the point of sperm entry. After finding a black spot indicating this point on the egg surface, the presumptive course of the furrow in the equator was
rubbed at various stages before and after the onset of the first cleavage, and the course of the furrow was followed.

The furrow was bent even after treatment given before cleavage. However, a difference from the case induced by rubbing just ahead of the furrow (Text-fig. 2) lies in the fact that the curving of the furrow was less and occurred only in the white part of an equatorial strip. As shown in Text-fig. 3, occurrence of the bending varied considerably in the experimental series. In the third series, the curved furrow was found at the earliest in eggs rubbed 22 min before the first cleavage (—22 min).

Text-fig. 4. Relation between time of pre-cleavage rubbing of the vegetal region and occurrence (open circles) or failure (filled circles) of bending of the furrow in the succeeding first cleavage.

Text-fig. 5. The critical time of rubbing after the first cleavage for the bending of the furrow of the second cleavage. Compare with Text-fig. 4.
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Vegetal region (c): a similar experiment was performed in the vegetal region and the results illustrated in Text-fig. 4 were obtained. As can be seen in the figure, the critical time for the furrow bending in the vegetal region is approximately $-33$ min, which sections showed to be the time when the centrosomes start to migrate towards opposite sides of the nucleus. The figure of $-33$ min obtained for the first cleavage practically coincides with that for the second cleavage, $-32$ min (Text-fig. 5). Thus, the critical time in the vegetal region occurred earlier than that in the equator and, furthermore, in many cases the degree of furrow bending was greater than in the equatorial region.

Table 1. Influence of the time of puncturing of uncleaved eggs in the animal region (arrows) on the position of the furrow of the first cleavage, expressed as the ratio of unequally divided eggs/total

<table>
<thead>
<tr>
<th>Time before cleavage (min.)</th>
<th>$-70$</th>
<th>$-60$</th>
<th>$-50$</th>
<th>$-40$</th>
<th>$-30$</th>
<th>$-20$</th>
<th>$-10$</th>
<th>$0$</th>
</tr>
</thead>
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<td>Series I</td>
<td>$\uparrow$ (8)</td>
<td>$\uparrow$ (30)</td>
<td>$\uparrow$ (82)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Series II</td>
<td>$\uparrow$ (83)</td>
<td>$\uparrow$ (60)</td>
<td>$\uparrow$ (80)</td>
<td>$\uparrow$ (43)</td>
<td>$\uparrow$ (20)</td>
<td>$\uparrow$ (70)</td>
<td>$\uparrow$ (30)</td>
<td>$\uparrow$ (20)</td>
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<td>$\uparrow$ (27)</td>
<td>$\uparrow$ (58)</td>
<td>$\uparrow$ (73)</td>
<td>$\uparrow$ (71)</td>
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Animal region (a): as described above, no displacement of the furrow occurred by simply rubbing with a hair loop. As a result, in order to achieve a dislocation of the furrow, cutting the animal surface had to be combined with rubbing. It is therefore impossible to compare this result, in a strict sense, with those obtained in the equatorial (b) and the vegetal region (c) by rubbing alone.

Following puncturing and rubbing, the eggs returned in a few minutes to the round form and ultimately many of them entered cleavage. After completion of the cleavage, the eggs were fixed with Smith's fluid and the relationship between the time of operation and the frequency of the furrow being displaced to the side of the cut was studied. On surveying Table 1, one finds that the frequency of displaced furrows is high in groups of eggs operated less than $45$ min before cleavage ($-45$ min) and low in groups of eggs operated earlier. From sections, it is seen that the time of $-45$ min corresponds to the stage subsequent to completion of syngamy. An interpretation of this phenomenon is extremely difficult at present. However, a possible explanation would be that when a disturbance occurs early enough, the pronuclei migrate and meet at the egg centre or a newly formed synkaryon has a greater freedom to move back to
the centre, both resulting in equal cleavage, while as the time passes it becomes harder for a nucleus to move, causing unequal divisions in various degrees.

Summarizing, on the basis of differences in the ease of a shift of the median cytoplasm by rubbing, three levels can clearly be distinguished along the egg axis. In the animal region (a), shifting is impossible so that a cut is needed to cause a deviation of the furrow. The equatorial region (b) is intermediate and may include a steep gradient and the vegetal region (c) is the most liable to shifting of the internal cytoplasm. This may indicate three levels of rigidity in the subcortical protoplasm of the egg.

**DISCUSSION**

In a previous paper (Kubota, 1966) the author reported that amphibian eggs from which the mitotic apparatus had been removed could still form cleavage furrows. In the present experiments, further analysis has been made possible.

Treatment during the cleavage stage. As far as the equatorial (b) and the vegetal (c) regions are concerned, the fact that movement of the median subcortical cytoplasm bends a furrow appearing subsequently may mean that the internal cytoplasm just ahead of the advancing furrow tip has already acquired the capacity for inducing a furrow in the overlying cortex before it is pushed to a new position, while the cortex itself remains passive.

The close dependence of the appearance of the furrow on the mitotic apparatus has been shown for various animal cells of relatively small size (Harvey, 1935; Carlson, 1952; Swann & Mitchison, 1953; Hiramoto, 1956, 1965; Yamamoto, 1964; Rappaport & Ebstein, 1965; cf. also Gray, 1931), as well as in amphibian eggs (Dan & Kuno-Kojima, 1963; Kubota, 1966). In the division of amphibian eggs, however, it is known that a diastema is formed in addition to the mitotic apparatus. Selman & Waddington (1955), working with newt eggs, observed that extension of the furrow was preceded by the diastema. Zotin (1964) reported in Axolotl eggs that diastema dispersed with heavy water (D₂O) induced a fragmentation on the egg surface. Since the diastema is found in the animal hemisphere of the present material, the possibility remains that the cytoplasmic factor directly involved in altering the course of the furrow may be the diastema, as long as this structure was displaced by the operation. However, the same interpretation is not applicable to the vegetal hemisphere where no figure like the diastema has ever been found.

Treatment of the precleavage stage. A bending of the course of the furrow results even when the cell surface is rubbed some time before the appearance of the furrow. The earliest time of operation causing a deviation is, in the vegetal region (c), at the stage of centrosome separation (−33 min) and in the equatorial region (b) at a later stage (−22 min). Parenthetically, in the animal region (a), it turned out to be a stage shortly after syngamy (−45 min), although this datum was derived from a combination of cutting and rubbing.

In the equatorial region (b) the degree of bending is less than for the case of
rubbing just ahead of an advancing furrow. In other words, if an egg marked with Nile blue sulphate is rubbed earlier, the furrow which appears does not quite reach the mark but falls between it and the scar on the median plane. From this, it looks as if there is a factor which tends to pull back or rectify the distortion if ample time is allowed before the formation of a furrow. The observed critical time (—22 min) coincides well with a figure reported in the previous paper (—23–20 min) as the time of the establishment of the autonomy for cleaving by the cortex of the animal half of the egg (Kubota, 1966).

On the other hand, the critical time for the vegetal region (c) is considered to be determined more reliably, partly because the effect of the rubbing was more conspicuous than in the equator (b) and partly because a good agreement was obtained between the critical times for the first and the second cleavages. This critical time is —33 min, corresponding to the stage of separation of the centrosomes.

As for the animal region (a), the critical time (—45 min) is derived from considerably variable data and furthermore may belong to an entirely different category because of the difference in technique employed. At any rate, since the procedure is so drastic as to cause a dislocation of the nucleus (or mitotic apparatus), the above result might be ascribed merely to a loss of the capacity of the synkaryon to return to the centre of the egg. However, the possibility cannot be excluded that the cleavage plane could somehow be determined as early as the stage of syngamy. The possibility of so early a determination of the division plane finds some support from the early critical time of determination characteristic of the vegetal region and from the fact that the direction of the furrow of the first cleavage can be anticipated from the point of sperm entry in *Rana nigromaculata*. A definite pattern of the propagation of gelation in the animal cortex reported by the present author (Kubota, 1967) would be a good mechanism correlating the sperm entrance point and the direction of the first cleavage furrow. The idea of a very early determination of the future cleavage plane is also compatible with the opinion of Roux (1887) that the division plane of the first cleavage in the frog egg coincides with the copulation path of the sperm pronucleus.

If the latter possibility is accepted, it will follow that in the egg of *R. nigromaculata* the cleavage plane is determined roughly during the period from the union of the pronuclei to the separation of the centrosomes.

**SUMMARY**

1. When the internal cytoplasm of the eggs of *Rana nigromaculata* is displaced during the first cleavage by keeping the cleavage plane horizontal, the subsequent course of the furrow deviates in the direction of gravitation.

2. When a furrow tip is approaching the equator or the vegetal pole, if the subcortical cytoplasm lying ahead of the tip of the advancing furrow is dis-
placed by rubbing the egg surface, the furrow deviates from a straight line and passes through the new position of the displaced cytoplasmic mass.

3. In the animal region, the double treatment of cutting and rubbing the cell surface produces a flow of the internal cytoplasm, followed by alteration of the path of the furrow. These facts indicate that the subcortical cytoplasm of the median zone has the ability to induce a furrow on the overlying cortex. There is no autonomous capacity of the superficial cortical layer for forming a furrow.

4. When the presumptive course of the furrow is rubbed much earlier to displace the underlying cytoplasm, a local bending of the furrow occurs. The earliest time of successful operation causing the bending of a later furrow is a stage of initiation of karyokinesis (33 min before cleavage) in the vegetal region and somewhat later in the equatorial region. In the animal region no dislocation can be brought about by rubbing alone.

5. In the animal region, the critical time as determined by the extrusion of cytoplasm by the combination of cutting and rubbing the surface turns out to be immediately after the apposition of the two pronuclei (45 min before cleavage). It is possible to infer that the determination of the division plane of the first cleavage occurs in the cytoplasm during the period between syngamy and centrosome migration.

RÉSUMÉ

Etudes sur la segmentation de l’œuf de grenouille.

II. Sur le déterminisme de la position du sillon

1. Lorsque le cytoplasme profond des œufs de Rana nigromaculata est déplacé pendant la première segmentation en maintenant le plan de segmentation horizontal, la suite du sillon dévie dans la direction de la gravitation.

2. Lorsque l’extrémité d’un sillon s’approche de l’équateur ou du pôle végétatif, dans un œuf dont le cytoplasme subcortical se trouvant en deçà de cette pointe de sillon a été déplacé en frottant la surface de l’œuf, le sillon devie de sa ligne droite et contourne la masse cytoplasmique déplacée.

3. Dans la partie animale de l’œuf, un traitement double, consistant à couper et à frotter la surface de l’œuf, produit un flux de cytoplasme interne suivi d’une altération du cheminement du sillon. Ces faits montrent que le cytoplasme subcortical de la zone médiane a la possibilité d’induire un sillon dans le cortex sus-jacent. Il n’y a pas de capacité autonome de former un sillon dans la couche corticale superficielle.

4. Lorsque le trajet présomptif du sillon est frotté beaucoup trop tôt pour pouvoir déplacer le cytoplasme sous-jacent, on observe une incurvation locale du sillon. Le moment le plus précoce où il soit possible de provoquer l’incurvation d’un futur sillon correspond au stade du début de la caryocinèse (33 minutes avant le clivage); dans les parties végétatives et dans la région équatoriale, ce moment est un peu plus tardif. Dans la région animale, aucune dislocation ne peut être obtenue par simple frottement.
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5. Dans la région animale, le temps critique qui a pu être déterminé par l'extrusion du cytoplasme à la suite d'une combinaison de section et de frottement de la surface a pu être démontré correspondre au moment suivant immédiatement la copulation des pronœcules (45 minutes avant le clivage). En considérant la méthode employée et les résultats obtenus, il est possible d'inférer que la détermination du plan de division du premier clivage apparaît dans le cytoplasme pendant la période s'étendant entre la syngamie et la migration des chromosomes.

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REFERENCES


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