STUDIES OF THE CLEAVAGE IN THE FROG EGG

I. ON THE TEMPORAL RELATION BETWEEN FURROW DETERMINATION AND NUCLEAR DIVISION

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INTRODUCTION

The cleavage plane in normally dividing animal cells is closely related to the position and the direction of the mitotic apparatus (cf. Gray, 1931). A displacement of the mitotic apparatus induces a corresponding shift in the position of the furrow in sea-urchin eggs (Harvey, 1935; Rappaport & Ebstein, 1965), in the grasshopper neuroblasts (Carlson, 1952; Kawamura, 1960) and in amphibian eggs (Waddington, 1952; Dan & Kojima, 1963). Recently, Zotin (1964), compressing axolotl eggs between slides to displace the mitotic apparatus, concluded that the position of the furrow is determined by the mitotic apparatus at the ana-telophase.

On the other hand, it has been shown in sea-urchin eggs that once a stage between the metaphase and mid-anaphase is reached, the cell can form the cleavage furrow even after the mitotic apparatus is destroyed (Cornman & Cornman, 1951; Swann and Mitchison, 1953; Hiramoto, 1965) or removed (Hiramoto, 1956). However, since it is still not certain whether or not the same situation exists in amphibian eggs, a test for this point is desirable.

In this paper, the eggs of *Rana nigromaculata* were employed. Two experimental procedures, (a) removal and (b) screening of the mitotic apparatus of the first cleavage, were performed during the period between the nuclear division and the appearance of the furrow, and the critical stage was studied at which the furrow formation became independent of the mitotic apparatus.

BASIC OBSERVATIONS

In eggs of *Rana nigromaculata* the furrow of the first cleavage usually appeared about 2 hr. after insemination, and that of the second cleavage about $\frac{3}{4}$ hr. after the first at 19° C. As experiments were performed on eggs from several females and under uncontrolled room temperatures, different batches of eggs showed a significant degree of variation in the time of the first cleavage, ranging from 2 hr. 38 min. (18° C.) to 1 hr. 42 min. (22° C.) after insemination. A single batch of eggs usually showed a spread of about 5 min., most of them beginning to cleave within 3 min. around the mean cleavage time.

To facilitate comparison between different experimental series the times of cleavage of individual cases were adjusted to a standard of 2 hr. This was made simply by multiplying the observed times for various stages by an over-all correction factor (2 hr./actual cleavage time).
Sequence of the nuclear division antecedent to the furrowing was observed on prepared sections because of the opaqueness of the living material. To prepare sections uncleaved eggs were fixed in cold Smith's solution at 5 min. intervals, and after orienting the egg with the presumable division plane vertical to the plane of section, the eggs were imbedded in paraffin and stained with Feulgen's fluid or bromphenol blue (Mazia, Brewer & Alfert, 1953). The division plane could be determined in the uncleaved egg by the slant and the shading of the edge of the pigment cap which foreshadowed the median plane of the future embryo, and consequently the division plane of the first cleavage in most eggs. As additional observations, fixation with Zenker's or Gilson's fluid and the triple staining of acid fuchsin, fast green, and bromphenol blue were used.

In eggs fixed 32 min. before the beginning of the first cleavage (—32), sperm and egg pronuclei were in close contact with one another. At 26 min. before the cleavage (—26), the nucleus was mostly at the prometaphase but rarely at the metaphase. At —20 min., it reached early or middle anaphase and at —16 min., late anaphase. At —12 min., dispersed minute chromosomal vesicles were formed, as telophase. At —7 to —4 min. every egg possessed a few fused chromosomal vesicles, as the reconstitution stage. The diastemata (inner walls) observed in the frog egg were very similar in origin and development to those of newt and axolotl eggs (Selman & Waddington, 1955; Zotin, 1964). For convenience for future discussion the schedule of the nuclear events is summarized in Table 1.

Table 1. 

<table>
<thead>
<tr>
<th>Time of fixation before beginning of first cleavage (min.)</th>
<th>Nuclear stage</th>
</tr>
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<tbody>
<tr>
<td>—120</td>
<td>Insemination</td>
</tr>
<tr>
<td>—32</td>
<td>Close contact of sperm and egg pronuclei</td>
</tr>
<tr>
<td>—26</td>
<td>Mostly prometaphase, rarely metaphase</td>
</tr>
<tr>
<td>—20</td>
<td>Early or middle anaphase</td>
</tr>
<tr>
<td>—16</td>
<td>Late anaphase</td>
</tr>
<tr>
<td>—12</td>
<td>Telophase with dispersed chromosomal vesicles</td>
</tr>
<tr>
<td>—7, —4</td>
<td>Restitution stage with chromosomal vesicle fusing</td>
</tr>
<tr>
<td>0</td>
<td>Appearance of initial furrow</td>
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</tbody>
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EXPERIMENTAL PROCEDURES AND RESULTS

(a) Removal of the mitotic apparatus

In order to see whether or not the cortex of the frog egg acquires an ability to form the cleavage furrow independently of the mitotic apparatus, the removal of the latter is necessary. Its removal by suction with a micropipette was carried out at various times prior to the appearance of the first cleavage furrow, and the critical time was determined, in reference to Table 1, when the capacity for furrow formation by the cortex alone (without the aid of the mitotic apparatus) was established in the egg.
Method

(1) **Outfit.** A device used for suction was as follows. A glass micropipette with 0.15 mm. bore was attached to a hypodermic needle, which was, in turn, connected to a 1 ml. syringe filled with paraffin oil. After excluding air bubbles, junctions were air-tightly cemented with paraffin-balsam paste. The syringe piston was driven by a screw. The whole set was attached to the upright tube of a microscope.

(2) **Operation.** The object of the operation was to remove the mitotic apparatus (or nucleus) before the onset of the first cleavage. After removal of most of the jelly coat uncleaved eggs of 1.5-1.7 mm. diameter were allowed to rest on a sheet of filter paper which was placed in a Petri dish containing water to the height of the egg. Insertion of the micropipette (0.15 mm. bore) into the animal hemisphere and regulation of its depth were controlled by fine adjustment of the microscope tube on which the apparatus was fixed. Subsequent suction was made by controlling the movement of the syringe piston by the screw, and the cytoplasm with a slight tint of purplish grey was removed. When brown pigments in the peripheral zone of the egg began to be drawn out, the suction was stopped and the tip of the micropipette was shifted to a new position within the egg and more cytoplasm was removed. After the completion of the operation water was added to the dish and the eggs were left there for a period until the second cleavage would have occurred, and then they were transferred to the fixative.

Since the egg was opaque, it was impossible to ascertain, by sight, the removal of the mitotic apparatus (or nucleus) during suction. However, it would be expected that cleavage might happen once regardless of the presence or absence of the mitotic apparatus but never happen twice or more in the absence of it. From such a consideration, the removal was inferred from failure of the second cleavage in the operated egg. Thus, eggs which developed the furrow of the second cleavage or showed furrow-like figures were discarded because of a suspicion of retention of the mitotic apparatus. To avoid error, careful examination for the absence of the furrow of the second cleavage was made on the same operated egg twice, once before and once after fixation, under a binocular dissecting microscope.

Result

Sucking the cytoplasm out of the eggs was carried out at a rate of one egg per ca. 2 min. Since the first cleavage of operated eggs tended to be retarded, the cleavage time was only indirectly obtained from intact eggs placed in the same dish. An excessive suction displaced the cortical pigment and led the whole pigment cap to spread toward the vegetal pole. Such eggs cytolyzed soon after the operation.

Fig. 1 shows the result obtained from four series of experiments. The earliest cases in which only the first cleavage took place, but not followed by the second, were found among eggs sucked 23 to 19 min. before the first cleavage was due (−23 to −19), when the nuclear division should have been between metaphase and anaphase (see Table 1).

The twelve eggs used in the first series of Fig. 1 had been left in the Petri dish until the intact eggs reached blastula stage. These operated eggs remained alive with no further cleavage nor fragmentation. Further, the removal of the mitotic apparatus was checked by cytological observations made in six of the operated eggs included
in Fig. 1. In the eggs which were fixed 1½ hr. after the first cleavage, neither nucleus nor radiate structure of the aster was observed except in one blastomere.

In order to investigate the cytoplasmic figures of the eggs at the time of division, another series including eggs sucked at -20 to -15 min. and the control were fixed immediately after the appearance of the furrow, and the triple staining method was used. In control eggs rod-like elements stained with fast green (Fig. 2a, black dots) were dispersed in the entire region of the aster. But in nine eggs ascertained to have cleaved in the absence of nucleus, besides the dispersed elements, one or two aggregates were recognized, which looked to be of the same material as the elements (Fig. 2b). Such an aggregation of the normally dispersed rod-like elements may suggest an attempt on the part of the egg to re-organize the astral centre after the operation.

Fig. 1. Relation between time of removal of mitotic apparatus and occurrence of furrowing without it. Open circles represent the cases in which division took place without the apparatus, and filled circles represent failures. Position of the circles indicates the time of operation in relation to the onset of the first cleavage which is 0 min. on the abscissa.

Fig. 2. Part of section parallel to the egg axis and the spindle axis of the egg of Rana nigromaculata at the beginning of first cleavage (schematic drawing). (a), Control egg. Two polyhedral structures and rods within aster indicate respectively nuclei and dispersed elements stained with fast green. (b), Operated egg. Mitotic apparatus was previously sucked out before cleavage. Two large black spots indicate aggregates of green elements.

However, details of the cytoplasmic configuration differed considerably between the operated and the control eggs in the following points. First, in the operated eggs, a radially oriented pattern in the cytoplasm was less apparent than the pattern in the normal aster. Secondly, while there was an extensive central region of the aster (centrosphere) in the controls, which was characterized by a very coarse texture and greenish staining, the corresponding region of the operated eggs was much reduced in extent or almost absent. Finally, in some operated eggs, the green aggre-
gates of the rod-like elements were found occasionally at the periphery of the egg, or sometimes there was only one per egg. In spite of these irregularities of the aggregate, furrows of operated eggs always appeared in the centre of the animal hemisphere. Therefore, it may be reasonable to think that the furrow was induced by the original aster before its removal rather than by the reorganizing aggregates.

(b) Screening of the cortex from the influence of the mitotic apparatus

The object of this experiment is to separate the cortex from the mitotic apparatus by interposing a foreign object. Paraffin oil was adopted. Paraffin oil, injected into the egg before furrowing, rounds up in the cytoplasm under the animal pole, pushing the mitotic apparatus (or nucleus) to one side of it. Such an oil droplet would spatially prevent the effect of the mitotic apparatus from reaching the opposite side of the oil droplet. Injection was made at various times before the first cleavage, and by finding the eggs in which the furrow extended on both sides of the oil droplet, the critical time for the cortical autonomy was determined, because in such eggs the cortical autonomy must have been established on the opposite side of the oil droplet before interception.

Method

By using the same device as that in the preceding experiment, the cytoplasm was sucked out before the oil injection. In order to leave the mitotic apparatus unremoved and to suck out only the whitish cytoplasm of the vegetal hemisphere, the micropipette was inserted into the egg more deeply than in the preceding experiment. For the oil injection the same pipette was used after removing the cytoplasm, and it was inserted through the aperture formed by the previous suction.

Fig. 3. Diagram illustrating various types of furrows in eggs injected with paraffin oil. Dotted area and black circles in the figures show respectively the pigment cap and an insertion aperture of the pipette. (a-b), Eggs injected with optimal amount of oil. (c) Eggs injected with insufficient amount of oil.

Result

Paraffin oil (sp. gr. c. 0·8) was located at the highest position in the cytoplasm, usually under the animal pole. Eggs injected with an optimal amount of paraffin oil formed two types of furrows. In one type the furrow appeared only on one side of the insertion aperture (Fig. 3a), and in the other type the furrow appeared diametrically across the aperture (Fig. 3b).

Eggs injected with a smaller amount of oil formed either a curved furrow passing beside the aperture (Fig. 3c) or a straight furrow across the aperture. The straight furrow was accompanied with an upheaval of the egg surface on one side, suggesting
that the oil droplet was pushed aside by the furrow. These eggs were excluded, because there is room to think that the furrowing occurred under incomplete interception of the mitotic apparatus. On the other hand, eggs injected with an excessive amount of oil formed a swelling by local expansion of the egg surface and did not cleave.

The oil injection was carried out at intervals of 2–3 min. to analyse a relation between the occurrence of the two types of furrows and the time of injection. The results are illustrated in Fig. 4. The furrows which appeared diametrically on both sides of the aperture (Fig. 3b) were found at the earliest in eggs operated about 20 min. before the beginning of the first cleavage (−20). The critical time again corresponds to the anaphase of the nuclear division (see Table 1).

Eggs which received an optimal amount of oil were fixed at the beginning of the first cleavage and sectioned. The furrow was seen on a thin cytoplasmic layer which overlay the oil droplet generally exceeding two-fifths of the egg diameter. The mitotic apparatus, pushed aside by the oil droplet, was found only on one side of the furrow at some distance from it. These observations suggest that, under a furrow extending on both sides of the aperture, the other side of furrow was formed on the cortical region spatially separated from the mitotic apparatus, which in turn further supports the conclusion that the critical stage of the cortical autonomy for furrowing is the anaphase of the nuclear division.

Fig. 4. Relation between time of oil injection and occurrence of furrowing in the region which was screened off from mitotic apparatus by the paraffin oil. Open circles, furrow occurring on both sides of the oil droplet, implying that the determination of the furrow had occurred earlier than the injection. Filled circles, furrow forming only on a side nearer to mitotic apparatus, suggesting no determination yet at the time of the injection.
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DISCUSSION

The critical periods for the cortical autonomy for furrowing found by the two different approaches were in good agreement and fell during the metaphase and the anaphase of karyokinesis.

Similar conclusions have been reported in other materials, such as sea-urchin eggs (Cornman & Cornman, 1951; Swann & Mitchison, 1953; Hiramoto, 1956, 1965), newt neuroblasts (Zirkle, 1957), or cultured chick fibroblasts (Davis & Smith, 1957) by impairing the nuclear function. Thus, the same mechanism must be at work in exceptionally large cells, such as frog eggs, as in smaller cells.

In amphibian eggs a few other workers have attacked the question of whether or not the endoplasm is involved in the furrow formation. In newt eggs Waddington (1952) and Dan & Kojima (1963) concluded that the factor immediately responsible for the deepening and advancing of the furrow was located in the cortex itself. However, they admitted that an influence from the mitotic apparatus may be concerned in the initiation of the furrow. Further, by displacing the mitotic apparatus of axolotl eggs, Zotin (1964) reported that the determination of the furrow position was established at late anaphase–early telophase when the diastema approached the egg surface. This critical time is a little later than that observed in the present experiment. The reason of the disagreement, however, is not obvious, since there are differences in both material and method used between Zotin’s experiments and mine.

SUMMARY

1. The time scale of nuclear division with reference to cleavage time was prepared for the normal egg of Rana nigromaculata.

2. Two experimental procedures, (a) removal and (b) screening of the mitotic apparatus, were performed in the eggs at various times before the beginning of the first cleavage, in order to determine when the furrowing activity of the cortex became independent of the mitotic apparatus.

3. The two experiments concordantly indicated that an autonomous capacity for furrowing was established in the cortex between the metaphase and the late anaphase.

4. Notwithstanding its extremely large size, this critical stage for the frog egg is practically the same as those which have been reported in other smaller cells.

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REFERENCES


