Periodic sensitivity of mechanisms of cytodifferentiation in cleaving eggs of *Limnaea stagnalis*

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WITH ONE PLATE

INTRODUCTION

Investigations on cellular reproduction have led to a highly resolved and integrated picture of the cell cycle in a morphological and physiological sense. The various preparations for division, doubling of components or syntheses, follow their own time course parallel to one another. It has become evident that the various factors involved in cell division are dissociable, for example chromosome doubling and reproduction of centrioles (Bucher & Mazia, 1960), DNA replication and protein synthesis (Zeuthen, 1961).

The conditions for cell division in general are applicable to division of egg cells. However, in addition in egg cells there is a complicating system of morphogenetic factors acting, as must be postulated from the observation that in ‘mosaic’ eggs the fate of the blastomeres is fixed.

In dividing eggs differences between daughter cells may be due to local differences established during oogenesis in the mother which are parcelled out during cleavages. It is, of course, also possible that a particular blastomere becomes different in properties of morphogenetic importance, for instance by localized synthesis and/or by the accumulation of randomly distributed factors.

Such considerations lead to several alternatives which are open to verification by experimental analysis. So it is of interest to know whether factors of morphogenetic importance are present from oogenesis or from fertilization onwards, or whether they arise, for example, during early cleavage. Secondly, it is important to know whether such factors and their direct influence are dissociable and/or independent from the factors involved in cell division. Lastly, if morphogenetic factors which determine the fate and differentiation of cells are produced during early cleavage, it is of interest to study the effect of

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the mitotic cycle, the cytokinetic cycle and the energy cycle, etc. upon their creation.

For a decision between these alternatives, and as an approach to the analysis of the nature of morphogenetic factors in early development, the sensitivity to centrifugation of the developing egg of *Limnaea stagnalis* at successive stages from oviposition till third cleavage was studied. The influence of centrifugation on cell reproduction was measured as delay of the next cleavage after treatment, and the influence on morphogenesis by following the development of each treated egg.

**MATERIAL AND METHODS**

Eggs of *Limnaea stagnalis* were obtained by exposing the snails to stimulating leaves and a mild temperature rise (from 20 to 25° C.; Geilenkirchen, 1961).

In any experiment one egg mass only was used. The cleavage divisions of the eggs of one mass, however, are not synchronized. In order to obtain treatment-groups of synchronously cleaving eggs, groups of eggs which started first cleavage almost simultaneously (within 1 min.) were selected. The first group selected was used as a control. Treatment groups A, B, C, D, etc. were centrifuged (always for 10 min. at 640 g.), at 0, 15, 30, 45, etc. min. after the eggs started first cleavage. The last group was again used as a control. Before, during and after the treatment the eggs were kept in tap-water at 25° C. The time of the second and/or the third cleavage of the eggs in each treatment group was noted. In some experiments the relation of stratification to the egg axis was observed and photographed. Furthermore, serial sections for cytological observations were made of some eggs of all treatment groups fixed immediately after centrifugation.

After the third cleavage the eggs were kept in tap-water at 25° C. for 48 hr. Then the capsules, each containing a single egg, were laid out on half dry agar-agar in petri dishes and cultured at 25° C.

The development of each embryo was followed from day to day and recorded until they reached a stage at which the eyes in normal embryos were well developed. In all, five grades of developmental disturbances were distinguished (Geilenkirchen, 1961).

*First period death.* Under this heading were included all those embryos which died before, or during the gastrula stage.

*Second period death.* All embryos which developed further than the gastrula stage, but died without showing specific morphological abnormalities.

*Exogastrulation.* Exogastrulae observed in *Limnaea* experiments were vesicular embryos in which the invagination of the archenteron was suppressed. These embryos usually died within a few days.

*Unspecific malformations.* In this group were included all embryos which showed an abnormal development and which could not be described as head malformations or as exogastrulae.
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Head malformations. All microphthalmic, monophthalmic, synophthalmic, triophthalmic, cyclopic and anophthalmic embryos were classed in this group. They can all be made to develop into normally reproducing snails.

RESULTS

Delay of cleavage

As described above, groups of synchronously dividing eggs were subjected to mild centrifugation at regular intervals of time after first and second cleavage. The delay of second and/or third cleavage was measured.

Text-fig. 1 shows the results. The ordinates show the retardation in minutes of the cleavage which follows the treatment. On the abscissae a time scale is given on which the times of cleavages of the controls and the times at which treatments began are given. Each dot represents the arithmetic mean of the retardations in one group, representing between five and ten eggs. It is obvious from Text-fig. 1 that only during a limited time interval in each cycle does centrifugation cause a retardation of cleavage.

In the cycle preceding the third cleavage this phenomenon is clear-cut and the sensitive stage coincides with the end of interphase and the beginning of prophase. In the cycle preceding the second cleavage the sensitive period is less evident and sharp, although undoubtedly present. In five experiments with eggs centrifuged before the second cleavage, it was investigated whether, in addition to a possible retardation of the second cleavage, the third cleavage was also retarded. In the cases studied, this was not so.

Stratification and elongation

The Plate, Figs. 1–16, shows eggs centrifuged (640 g., 10 min.) at regular intervals after first and second cleavage. Two phenomena are observed: stratification of the egg contents and elongation of the egg. With regard to

EXPLANATION OF PLATE

FIGS. 1–16. Eggs centrifuged (640 g. for 10 min.) between first and second (Nos. 1–7) cleavage; between second and third cleavage (Nos. 8–15), and after third cleavage (16). Nos. 1–7 single eggs centrifuged 0, 15, 30, 45, 60, 75 and 90 min. after first cleavage, respectively. Nos. 8–15 single eggs centrifuged 0, 10, 30, 40, 50, 60, 70 and 80 min. after second cleavage, respectively. No. 16, egg centrifuged 10 min. after third cleavage. In all cases the animal pole is at the top in the photographs. The arrows in Nos. 1 and 7 point at the first indication of the cleavage furrow. The clear zone with indention in Nos. 1 and 7 must not be taken for a furrow; this indention is due to centrifugation. In No. 2 the two blastomeres are maximally separated.

FIGS. 17 AND 18. Two consecutive sections of an egg cell centrifuged 40–50 min. after first cleavage. The spindle is broken.

FIG. 19. Section of an egg cell centrifuged 50–60 min. after first cleavage.
TEXT-FIG. 1. Retardation of second or third cleavage (ordinates) after centrifugation (640 g for 10 min.), in relation to time after first or second cleavage at which centrifugation started (abscissae).
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Stratification it is always found that the axis of centrifugation and the animal–vegetative egg axis (as indicated by the polar bodies) almost parallel each other; in our experiments the deviation never exceeded 20°. In the experiment to which the photographs refer, the yolk zone lies at the animal pole and the fat cap at the vegetative pole (see legend to Plate, Figs. 1–16); in other experiments the reverse has been found (Raven & Tates, 1961).

The stratification is not very clear immediately after cleavage (Plate, Figs. 2, 3, 9, 10, 11), then it becomes more distinct and at about 50 min. after cleavage a maximum is reached (Plate, Figs. 4, 5, 12). Until the next cleavage has passed, not much change is observed. Stratification is thus maximal from prophase until telophase of the mitotic cycle. These observations agree with the data of Heikens (1947).

With regard to the elongation of the cells it is evident that only during actual cleavage are they very susceptible in this respect (Plate, Figs. 1, 7, 8, 14, 15). A pronounced elongation and deformation is observed at the time of the first, second and third cleavage.

Cytological observations

Of the eggs treated at the various stages, some were fixed immediately after treatment in Bouin's fixative, sectioned and stained with iron-hematoxylin-eosin. At all stages stratification was observed and no attempt was made to discriminate between stages of more and less pronounced stratification. The nucleus or the mitotic figure was found in the zone between fat and yolk cap. A remarkable observation was made in eggs centrifuged at the stage which caused delay of next cleavage. In all their blastomeres it was found that the mitotic figure was disrupted and that its parts were disoriented. For instance, one aster plus remnants of the spindle was found in the yolk zone, the other aster plus remnants of the spindle plus chromosomes in the fat zone. In other cases the chromosomes or the prophase nucleus were found separated from the asters. This phenomenon can be seen in two consecutive sections in the Plate, Figs. 17, 18. It is evident that the mitotic figure at this stage is very easily broken. Centrifugation at a stage 10 min. or more later never causes disruption of the mitotic figure (Plate, Fig. 19).

Morphogenetic effects

In a first series of experiments, groups of synchronously dividing eggs from a single egg mass were centrifuged at regular intervals after first and/or second cleavage and then cultured in the normal way. The results are shown in Text-fig. 2A and B.

In Text-fig. 2A the percentage of exogastrulation and first-period death plus exogastrulation are plotted against the time after first or second cleavage at
TEXT-FIG. 2. The percentages of normal and abnormal development against stage of treatment. A. Ordinates: Percentages of exogastrulae (○) and first period death plus exogastrulae (×). Abscissae: Time of start of treatment with regard to first or second cleavage. B. Ordinates: Additive percentages of normal (○), aspecific (●), head malformations (©) and second period death (×). Abscissae: Time scale as in A.
which centrifugation started. In Text-fig. 2B, normal development, unspecific malformations, head malformations and second-period death are plotted in the same way. Up to 40-50 min. after first and second division the eggs are rather insensitive to centrifugation. Normal development lies between 70 and 90 per cent. At this time a gradual decrease of normal development sets in and a corresponding increase of first-period death plus exogastrulation is observed. A minimum in normal and a maximum in abnormal development occurs at the

**Text-fig. 3A and B:** The percentages of normal and abnormal development against the time of treatment (centrifugation 640 g. for 10 min.) after first and second cleavage. Legend as in Text-fig. 2.
time of cleavage. The other malformations are found at a more or less constant and small rate. A distinct maximum in morphogenetic effect by centrifugation is observed at the time of first, second and third cleavage.

In a second series of experiments the morphogenetic effect of centrifugation at stages between oviposition and first cleavage has been studied. The results are also shown in Text-fig. 2. It is evident that the effects are small and that with the centrifugal force used, no sensitive periods before first cleavage are found. The whole period is rather insensitive compared to the cleavage periods later on.

In a third set of experiments the sensitivity in the period immediately after first and second cleavage has been studied in detail with small time intervals. Groups of eggs were centrifuged as soon as first or second cleavage started and 5, 10, 15, 20 and 25 min. afterwards. Text-fig. 3A and B shows the results. It can be seen that sensitivity decreases sharply within 10–15 min. after the onset of cleavage.

In all, 2640 eggs have been used in these experiments.

**DISCUSSION**

*Delay of cleavage*

It might be thought that centrifugation causes a delay of cleavage on account of an abnormal arrangement of the cell contents, by which, for example, syntheses are delayed by a temporary displacement of reaction partners. It seems more likely, however, that the cleavage delay found is the result of a disruption of the mitotic apparatus at about prophase. Obviously the mitotic apparatus gains in cohesive strength as the mitotic cycle proceeds, because later on in the cycle disruption is never observed. In some blastomeres it was found that asters and prophase nucleus were lying in different regions of the cell. One of the asters was found in the yolk zone, the other one and the nucleus in the fat zone. At this stage the two asters seem to have a different density. A detailed study of the disruption and recovery of the mitotic apparatus will be published separately.

*Morphogenetic effects*

Morphogenetic effects caused by mild centrifugation are undoubtedly linked to first, second and third cleavage. From oviposition till first cleavage, the period of maturation divisions and amphimixis, the egg cell is fairly insensitive to the treatment. This leads to the hypothesis that the morphogenetic factors involved arise at the time of first division. An argument in favour of such a view can be derived from the observation that giant polar bodies can be formed after centrifugation at the time of polar body formation. The polar body is then almost as big as a blastomere at first cleavage. In the cases studied development was normal, but, the embryos were very small. Thus loss of almost the whole
animal half of the egg, surface and cytoplasm, does not seem to hamper normal development.

It is also possible that the morphogenetic factors, already present before first cleavage, can be influenced only from the time of first cleavage onward, either because they are previously insensitive, or because the cleavage causes definite damage which is not observed if the time between centrifugation and first cleavage is long enough. The easiest explanation would be a disarrangement of the distribution of cytoplasmic factors which is made definite by the cleavage furrow. This would be an extension to the first and second cleavage of the hypothesis proposed for morphogenetic effects of centrifugation around third cleavage by Raven & Tates (1961). But, as said above, the direction of the axis of centrifugation and the egg axis are parallel and it is difficult to assume a different distribution of cytoplasmic factors over the blastomeres at first and second cleavage. Furthermore, one would rather expect in that case a weak effect at first and second, and a strong effect at third cleavage, since the equatorial third cleavage will always ‘fix’ a disarrangement of the cytoplasm in micro- and macromeres. But the reverse is found: the morphogenetic effect seems smaller at third cleavage (Text-fig. 2). Another argument against this hypothesis is derived from Text-fig. 3. If centrifugation is started up to 10 min. after the onset of the second cleavage, morphogenetic effects still occur. During those 10 min. the cleavage furrow certainly has been completed.

Until now we have left out of the discussion the observations made with regard to elongation and stratification of the cells. From the Plate it is obvious that from a stage 10 min. after first or second cleavage the elongation and stratification are minimal, whereas at the time of cleavage the elongation and stratification are maximal. It can also be observed that at 15, 30, 45, 60 and 75 min. after the first cleavage and at 10, 30, 40, 50, 60 and 70 min. after the second cleavage the elongation first increases slowly and then increases rapidly at the time of the next cleavage (Plate, Figs. 1, 7, 14, 15). The time relations of the cycle of cell elongation and the cycle for morphogenetic effects resemble each other very closely. This is an argument in favour of a hypothesis which states: the fixation of the fate of the blastomeres in mosaic eggs is due to a mosaic of morphogenetic factors contained in the cell membrane and these factors are parcelled out during cleavages (Geilenkirchen, 1961). The cell membrane is a useful substrate for such a pattern of localized structural differences as has been argued for Limnaea eggs by Elbers (1959). It is also clear that even a minor change may hamper or alter the ‘local’ physiological function of the cell membrane and that morphogenesis may be changed as a result (Geilenkirchen, 1961).

The amount of new surface to be produced at each cleavage is considerable, 28 to 50 per cent. according to Wolpert (1960). The insertion of new surface is a rather fast process and it takes place in the region of the cleavage furrow (Selman & Waddington, 1955; Swann & Mitchison, 1958). For sea urchin eggs
it has been shown that with proceeding partitioning of the egg cell the original cell membrane of the one-cell stage remains situated at the surface of the embryos (Wolpert & Mercer, 1963).

In our experiments we found that the elongation of the egg cells is most pronounced at the time of cleavage. This may be due to a weakening of the surface layer of the egg at that time, or to a change in density of cytoplasmic inclusions. However, elastic stretch of the cell membrane seems to be excluded since it has been observed by electron microscope that the membrane (100 Å units thick) remains of the same thickness on centrifugation (Elbers, 1959). Elbers centrifuged eggs at 2000 g. for 10 min. shortly after the formation of the second polar body. At this stage the egg cells are easily elongated by centrifugation (Raven, 1945). As we have found in separate experiments, an elongation amounting to a doubling of the cell surface was obtained in Elbers’ experiments. If this elongation was merely due to elastic stretch, the thickness of the membrane should have been reduced from 100 to 50 Å. Such an effect would have been easily visible. Nothing of the kind was observed, however, the cell membrane showing its normal thickness in centrifuged eggs (Elbers, personal communication).

It must be concluded, therefore, that the stretching of the membrane which takes place when the egg is elongated by centrifugal force reflects insertion of membrane material, and that after centrifugation a corresponding amount of membrane material is resorbed.

It is found that stretching and insertion is particularly easy around division, i.e. at the time when the cell is prepared to respond to a mitotic signal by a membrane expansion. The present hypothesis suggests that when the impulse is not localized to the side of the presumptive furrow but is general and represents forces of stretch, the new membrane material is not localized in the furrow but randomly distributed around the cell. So is the resorption when the cell rounds up after centrifugation.

When this idea is connected with the hypothesis of determinants fixed on or in the peripheral membrane, then insertion of new membrane or resorption of superfluous membrane can alter the position of areas of morphogenetic importance contained in the cell membrane and also their distribution and ‘concentration’. Thus it is suggested that a fixed distribution of morphogenetic ‘substance’ in or at the cell surface can be disturbed as a result of the two random processes insertion and resorption referred to above.

Since the cell membrane only around cleavage is prepared to answer a stretch stimulus, it would then be only natural that around this stage the morphogenetic effect of centrifugation is most pronounced. In line with this hypothesis is the observation that after treatment development initially proceeds normally, and that only later, e.g. at gastrulation, are disturbances observed in development. It is conceivable that abnormalities must arise as soon as the surface properties of the cell membrane become active as determin-
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In mosaic development the first readily observable determining event in differentiation is seen in the cell lineage, e.g. *Physa* (Wierzejski, 1905) and *Planorbus* (Holmes, 1900). (*Limnaea* shows only minor differences with these species, N. H. Verdonk, personal information.)

The cells around the animal pole derive from the cells 1a–1d. In *Physa* the cells 1a\(^2\) and 1b\(^2\) divide only once and form prototroch cells. The cells 1c\(^2\) and 1d\(^2\) divide only once and form head vesicle cells. After three successive cleavages of 1a\(^1\)–1d\(^1\), their descendants, together with the trochoblasts, constitute the animal half of the embryo. It is a remarkable fact that at this stage most of these cells stop dividing and remain as larval cells, viz. apical plate (1a\(^{111}\)–1d\(^{111}\), 1a\(^{121}\), 1b\(^{121}\), 1b\(^{1211}\)), prototroch (1a\(^{21}\), 1b\(^{21}\), 1a\(^{22}\), 1b\(^{22}\)), and head vesicle cells (1a\(^{122}\), 1c\(^{122}\), 1d\(^{1211}\), 1d\(^{1212}\), 1d\(^{1221}\), 1d\(^{1222}\), 1c\(^{21}\), 1c\(^{22}\), 1d\(^{21}\), 1d\(^{22}\)). Only eight cells at this stage, the cephalic plate cells, continue to divide. Ultimately they form the head and head organs.

Central to the problem of morphogenesis of the head region, with its determinate cleavage pattern, is the mechanism by which cell division stops in most cells but continues in a limited number of cells. Many ways are conceivable, but in view of the data on centrioles and cell division (for ref. see Mazia, 1961) it would be interesting to study the reproduction and distribution of those bodies during early development. It is evident that one blastomere gives rise to qualitatively different sister cells, because the mechanism which controls the decision to divide or not to divide is distributed according to a fixed plan whose disturbance leads to abnormal development.

It can be concluded that a cleaving blastomere in mosaic development has two functions: (a) to divide and produce two viable cells; (b) to parcel out in the proper way the factors which determine the cleavage pattern and thus morphogenesis. Obviously the first function is only slightly and reversibly disturbed by mild centrifugation around prophase. The second function is strongly disturbed by centrifugation at actual cleavage. It is known from experiments by N. H. Verdonk (unpublished), that treatment of early developmental stages with LiCl causes deviations in the cleavage pattern only at the time when the decision is made between cells which remain further undivided and cells which will continue to divide. After centrifugation primary effects of the same nature are found.

These results agree very well with the observations made about development after centrifugation. Furthermore they fit the hypothesis given above. The mosaic egg seems very well suited for a study of the unknown mechanism which decides the choice for a cell either of differentiation and no further division or of continued division without differentiation.

The following conclusions can be drawn:

(1) In early development morphogenetic factors are present which show periodicity in sensitivity to centrifugation, synchronized with the cleavage cycle. The period of greatest sensitivity lies at the time of actual cleavage.
(2) Cell division is delayed by the same treatment; mitotic prophase is the most sensitive stage.

(3) Morphogenetic factors in Limnaea eggs are dissociable and independent from cell division factors.

(4) The morphogenetic factors demonstrable at first, second and third cleavage are not demonstrable before first cleavage.

(5) The morphogenetic factors present at early cleavages determine the differentiation of animal pole cells into larval non-dividing cells, and cells which continue the process of division.

SUMMARY

1. Eggs of Limnaea stagnalis, of ages differing by 15-min. intervals between oviposition and the third cleavage division, have been centrifuged at 640 g. for 10 min.

2. Centrifugation before first cleavage did not have any effect on development.

3. Centrifugation at first, second and third cleavage caused a strong morphogenetic effect.

4. Centrifugation during prophase of second and third division caused delay of cleavage. The mitotic figure is disrupted.

5. It is suggested that the morphogenetic effect of centrifugation can be ascribed to a disturbance of the normal distribution of morphogenetic 'substance' at or in the cell surface, by random insertion or resorption of membrane after stretching the cell.

RÉSUMÉ

Sensibilité périodique des mécanismes de cytodifférenciation dans les œufs de Limnaea stagnalis en voie de segmentation

1. Des œufs de Limnaea stagnalis, d’âges échelonnés de 15 en 15 minutes entre l’oviposition et la troisième mitose de segmentation, ont été centrifugés à 640 g. pendant 10 minutes.

2. La centrifugation avant la première segmentation n’a eu aucun effet sur le développement.

3. La centrifugation à la première, deuxième et troisième division de segmentation a un effet morphogénétique marqué.

4. La centrifugation pendant la prophase de la deuxième et troisième division produit un retard de la segmentation. L’édifice mitotique est disloqué.

5. Il est suggéré que l’effet morphogénétique de la centrifugation peut être attribué à une perturbation dans la distribution normale d’une ‘substance’ morphogénétique à la surface de la cellule ou dans cette surface, en raison du fait qu’après l’élongation de la cellule la membrane interblastomérique s’insère au hasard ou se résorbe.
REFERENCES


