

# Experiments Concerning the Cleavage Stimulus in Sand Dollar Eggs

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It is now clear that the mitotic figure of dividing echinoderm eggs plays a causal role in the determination of the furrow. Since Hiramoto ('56) showed that removal of the mitotic apparatus before appearance of the furrow did not prevent division, it is convenient to consider the apparatus the source of a stimulus which is the immediate trigger for cytokinesis. Neither the area of the cell which receives the stimulus nor the mechanism of division is known. Information concerning one aids in the understanding of the other. Detailed information concerning the particular areas of the mitotic apparatus necessary for the establishment of the furrow is highly desirable. Such information could be derived from study of healthy cells in which some part of the apparatus had been deleted.

Since direct manipulation or destruction of a portion of the mitotic apparatus may result in general cell damage, it was decided to attempt the creation of artificial, incomplete mitotic figures by bringing together regions of the two mitotic figures in a dividing, binucleate sand dollar egg. The spherical egg was distorted to a torus or doughnut shape by pressing a glass sphere through it. In the first division, the cleavage plane severed the cell in the region between the asters on one side of the torus. The cell thus became horseshoe-shaped and binucleate. In subsequent divisions, as cells divided from the free ends of the horseshoe, the asters in the binucleate cell were brought together. There ensued a cleavage furrow which cut between the polar regions of the two separate mitotic figures in an area which had never been in close proximity to a spindle or chromosomes. Furrows of this kind could

be elicited and closely observed between asters of the second, third and fourth divisions. The furrows were morphologically normal and appeared in concert with the division of other cells of the embryo. The modification of cell geometry which was employed to produce binucleate cells also yielded additional information bearing upon the source and nature of the stimulus.

In two recent hypotheses (Swann and Mitchison, '58; Wolpert, '60) concerning the mechanism of cytokinesis it has been proposed that the telophase nuclei or asters are the sources of the immediate stimulus for cytokinetic activity. These structures are considered the origin of a substance which modifies the poles of the cell but does not reach the part of the cortex that will become the walls and head of the furrow. The proper operation of this mechanism requires sharp diffusion gradients. The form of the furrow would also be altered concomitantly with the distance between the sources. In some of the experiments mentioned above, it was noted that an apparently normal furrow could be established between asters separated by a greater than normal distance. The need for further investigation of the capacity of echinoderm eggs to divide under conditions that would make difficult the establishment of sharp diffusion gradients was indicated. To this end, cells were kneaded by repeated compression or extrusion during the period when the cleavage furrow is determined. This treatment did not prevent temporally and morphologically normal division.

## MATERIALS AND METHODS

Eggs and sperm of *Echinorachnius parma* were obtained by dissection of the

gonads. In order to insure that none of the experimental cells were subjected to drastic mechanical treatment, individual eggs were gently divested of membrane and jelly by drawing them through a tapered constriction near the orifice of a braking pipette. It was necessary to insure that the temperature of the eggs did not exceed 20°C.

Since these experiments dealt primarily with the process whereby the position of cleavage plane is established, it was essential to impose experimental conditions before the time the event normally occurs. The experiments described below were begun 20 minutes after fertilization when it has been shown that the cleavage plane in this species is undetermined (Rappaport, '60).

Distortion experiments were performed with the aid of an inverted microscope (Unitron, Model BMIC). With a rack and pinion micromanipulator, a glass sphere melted on the end of a needle was pressed downward upon the egg which rested on the floor of the observation chamber. After the sphere had been centered on the egg, its downward movement was continued until it passed through the egg and rested on the floor of the chamber. Thus the egg was converted to a torus. Glass spheres of several diameters were used.

Eggs were compressed against the bottom of an observation chamber with a piece of glass or plastic coverslip moved by a manipulator. Extrusion was accomplished by forcing the eggs through a tapered constriction near the tip of a braking pipette. The constrictions employed were such as to distort the eggs to cylinders the diameter of which was one-half to one-third the length.

An air turbine centrifuge of partially original design was used when high speed centrifugation was required.

## RESULTS

### 1. Cleavage of distorted eggs

Immediately after the glass spheres contacted the bottom of the chamber, the eggs were flattened. In the following 30 minutes some appeared to round up about the shaft supporting the sphere and regain nearly normal diameter. Whether the egg

does so or not has no bearing upon the outcome of the experiment unless the size of the sphere is great enough to create generalized compression that completely inhibits cytokinesis (Danielli, '52). *Echinorachnius* eggs are excellent material for this experiment as the absence of cytoplasmic pigment granules permits easy observation of the mitotic apparatus (Chambers and Chambers, '61).

The amphiaster appeared in the restricted region afforded by the torus synchronously with the controls (figs. 1a, 2a). The furrow was initiated either on the peripheral surface or on that immediately adjacent to the glass sphere. The intrusion of the furrow was accomplished smoothly and at the same time as the controls. There were no indications of distension in the vicinity of the asters or attenuation in other parts of the cell. Although division at one side of the torus was complete, the daughter nuclei were still contained within the same cell membrane at the tips of the horseshoe shaped cell (figs. 1b, 2b). It follows that if division were restricted to regions in close proximity to a spindle, there would always be one binucleate cell in the progeny of this distorted cell. It was found however that subsequent divisions consistently resulted in the production of a group of uninucleate cells.

Shortly after completion of the first division the apposed surfaces produced by cleavage frequently flattened against each other and then rounded up and moved apart before the appearance of the furrows of the second division. This activity is comparable to the flattening and rounding of normal echinoderm blastomeres which

Fig. 1 Cleavage of torus-shaped cell. Condition of the mitotic apparatus shown in line drawings. The position of the spindle is indicated by a double line. Note synchrony with controls. Initial temperature 19.5°. Timing begins at fertilization.

- a. Immediately before furrowing. 69 min.
- b. First cleavage completed, resulting in binucleate cell. 79 min.
- c. Second cleavage. Two cells have divided from the free ends of the horseshoe and the binucleate cell is dividing between the polar regions of the asters of the second division. 142 min.
- d. Division completed. Each cell contains one nucleus. 144 min.

have been interpreted by Chambers ('19) as due to the waxing and waning of the asters. The mitotic apparatus of the second divisions usually appeared parallel to the long axis of the cell. The cleavages which followed cut cells from each of the two free ends of the horseshoe (fig. 2c, d). As in normal echinoderm eggs, these two

divisions are not always precisely synchronous. The two free cells thus established were uninucleate but the binucleate cell, representing the bend of the horseshoe, contained an aster and nucleus from each of the mitotic figures. The asters in the binucleate cell moved closer together as the two cleavages were completed. An-

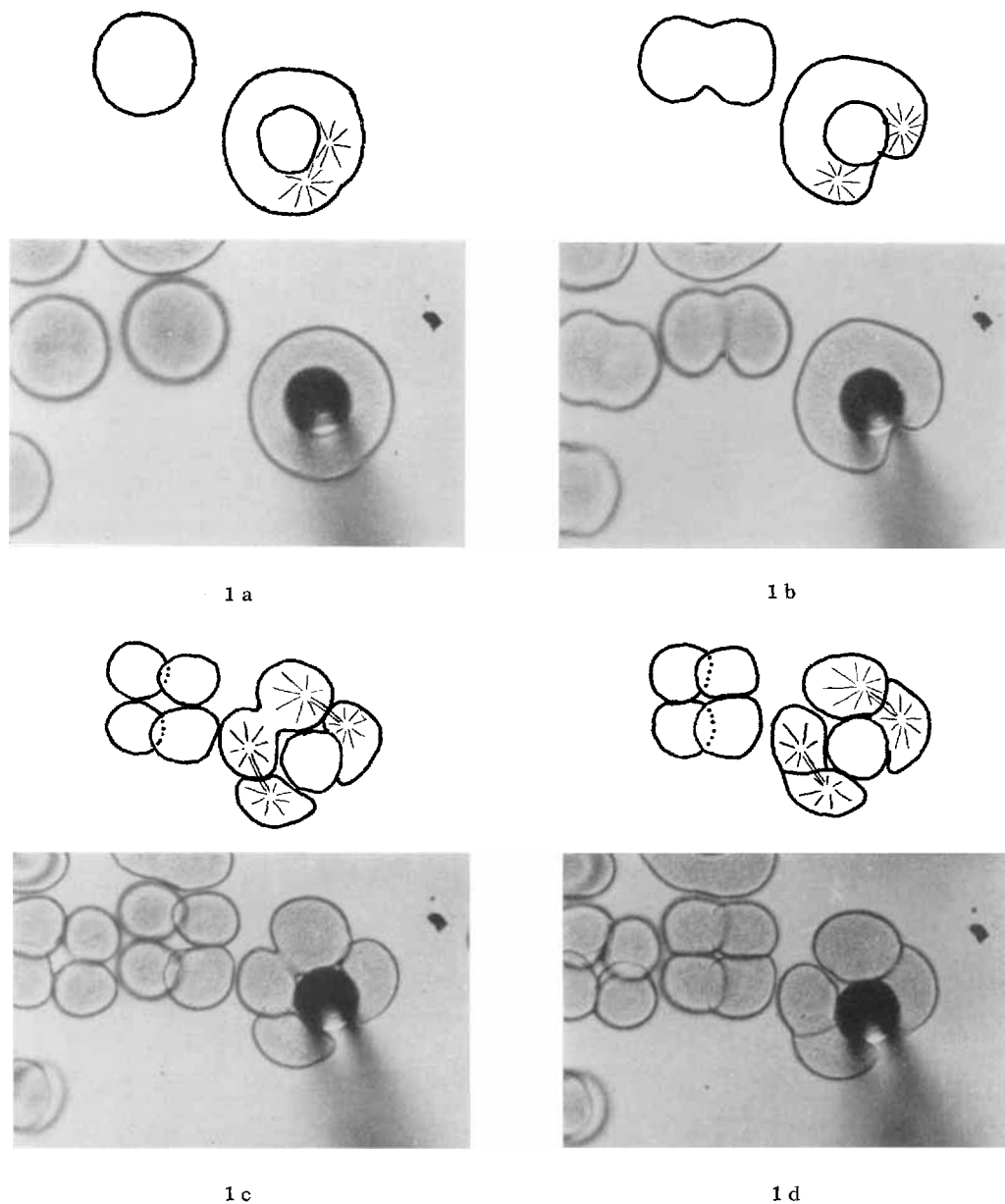


Figure 1 a - 1 d

other furrow appeared between the asters in the binucleate cell and was completed in normal time (figs. 1c, 1d, 2d). The embryo was thus converted to a 4 cell stage. Except in cases where it was deferred to a subsequent cleavage period, the division of the binucleate cell always began within 5 minutes of the divisions which established the two free cells at the tips of the horseshoe.

The completion of the furrow between asters that were not joined by a spindle (or "non-spindle furrow") could be deferred by vibrating the glass sphere several times while the furrow deepened. It also occurred when the zone forming the bend of the horseshoe was greatly attenuated by the use of a relatively large distorting sphere. When the furrow was vibrated, the indentation receded (fig. 2e), as does the normal furrow (Chambers, '19). The blastomeres flattened against each other in the interkinetic period. Coincident with indications of furrowing activity of the third cleavage in the free cells of the embryo, a non-spindle furrow appeared between two asters. In some cases this furrow developed normal to and contemporaneously with a furrow that separated a normal mitotic apparatus, (fig. 2g, h).

By this method, non-spindle division could be deferred until the 4th cleavage. Identification and observation of the multinucleate cell became exceedingly difficult in later cleavages. Following numerous repetitions of this experiment it became clear that the furrow could appear between any two astral regions regardless of the orientation of the mitotic figures involved. Thus furrows appeared between asters whose apposed surfaces were polar to polar, polar to lateral and lateral to lateral. Further, non-spindle division was always initiated and completed in phase with the division of the other cells of the embryo.

## 2. Cleavage of compressed and extruded cells

Beginning 20 minutes after fertilization, eggs were compressed until their diameters were about half again as great as the controls. After release, the discoidal cells returned to spherical form. As soon as the diameters of compressed eggs equaled

those of controls, they were recompressed. The interval between compression was between two and three minutes. By alternate compression and release, the form of the egg was constantly changing until the appearance of the cleavage furrows. It would seem that this manipulation must have affected a kneading of the cell contents. Cleavage furrows appeared simultaneously in compressed and uncompressed eggs and were completed in normal fashion.

Since the distortion resulting from compression occurred in only one axis, it was decided to repeat the experiment using an extrusion method which would provide a better chance of affecting distortion in different axes in successive manipulations. Eggs were aspirated into and blown out of silicone treated braking pipettes with constrictions as described above. After extrusion they were lined up serially so that the time interval between handlings would be relatively uniform. In several experiments, cells were permitted to round up between extrusions in order that they would not always pass through the constriction in the same orientation. This required allowing each egg about 5 minutes between extrusions and permitted about 15 extrusions per egg before the appearance of furrows. By cooling with ice, it was possible to extend the period before cleavage and accomplish 37 extrusions at 3 minute intervals before the appearance of furrows. Extruded cells cleaved at the same time as controls. As furrows ap-

Fig. 2 Cleavage of torus-shaped cell, condition of the mitotic apparatus shown in line drawings. Position of the spindle indicated as in fig. 2. Note synchrony with controls. Initial temperature 17°. Timing begins at fertilization.

- a. Immediately before furrowing. 92 min.
- b. First cleavage completed. 99 min.
- c. Second cleavage, beginning separation of cells from the free ends of the horseshoe. 145 min.
- d. Second cleavage. Division from ends of horseshoe completed, non-spindle furrow appears in the binucleate cell. 155 min.
- e. Appearance of cell following vibration which caused the disappearance of the non-spindle furrow. 190 min.
- f. Beginning of third cleavage, divisions are normal to and parallel with the plane of the page. 199 min.
- g. Beginning of non-spindle division. The position of the non-spindle furrow is shown by the arrow in the line drawing. 204 min.
- h. Completion of non-spindle division. 206 min.

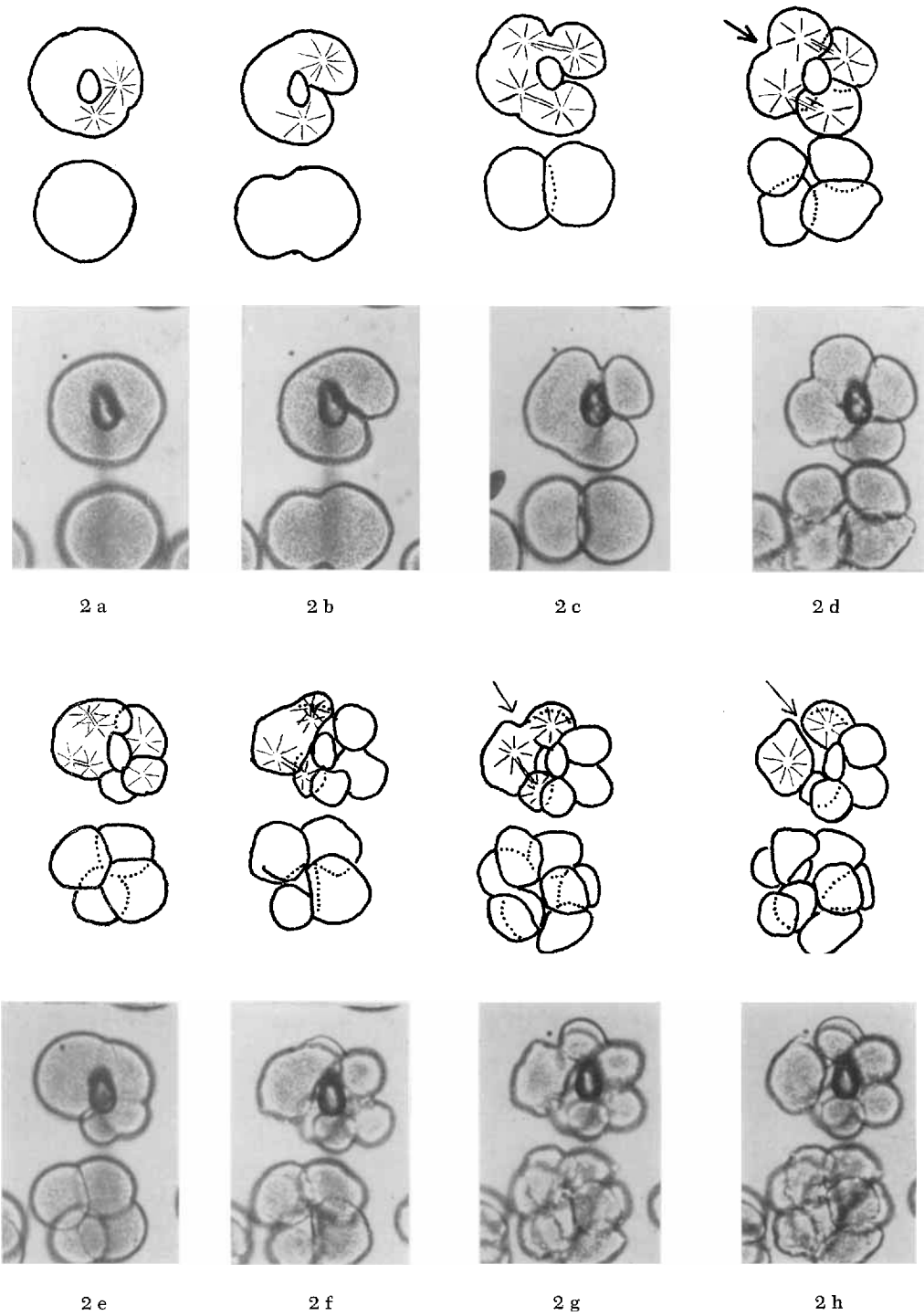


Figure 2 a – h

peared, cells were set aside to permit study of the form of the furrow and the relation of the cleavage plane to the axis of the cell. The great majority of furrows were normal. The plane of cleavage bore no fixed relation to the long axis imposed upon the cell by the last extrusion. In some cases the plane of cleavage coincided with the long axis (fig. 3).

In order to estimate how much mixing the extrusions accomplished, cells were stratified by centrifugation for 3 minutes at  $24,000 \times g$ , beginning 5 minutes after fertilization. Centrifuged eggs were elongate and the contents were distributed according to Harvey's ('56, pg. 127) description. Because of its roughly cylindrical shape the centrifuged egg tended to pass through the constriction in the same orientation in successive extrusions. The estimate of the degree of mixture was thus conservative. Extruded centrifuged eggs divided with centrifuged controls. The only clear remaining indication of stratification that persisted after extrusion was a small concentration of yolk at the heavy end. In controls the oil cap and lighter zone beneath as well as the yolk layer could be seen. Of the hundreds of control eggs, all but a few divided at right angles to the plane of stratification. The plane of division of extruded eggs bore no fixed relation to the remnant of the yolk layer. Thus the extrusions have been demonstrated to affect a redistribution of the cytoplasm and negate the relationship between the plane of cleavage and the plane of stratification which has been demonstrated in centrifuged echinoderm eggs (Morgan and Spooner, '09).

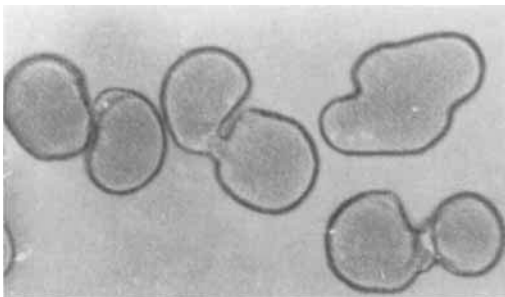


Fig. 3 First cleavage following 17 extrusions.

## DISCUSSION

Despite the severe distortion which produced the torus-shaped cells, non-spindle division was accomplished by a morphologically normal furrow in the normal time period in concert with the division of the other cells of the embryo. Numerous other observations of cell division with varying degrees of chromosomal and spindle deficiency have been made. In his review of this work, Swann ('52) emphasized the retardation which accompanied cleavage in the presence of incomplete mitotic figures and interpreted it as indicating that some essential part, the chromosome, was missing. Chambers and Chambers ('61) expressed a similar opinion. Retardation of division is most difficult to interpret unless the underlying mechanism is thoroughly known. Most of the cases previously reported involved cells that had been parthogenetically activated, centrifuged or subjected to a variety of other treatments. In these cases, it would be interesting to know whether the delay preceded or followed the formation of the asters or cytoasters. If, however, we follow the line of reasoning of Swann ('52) and Chambers and Chambers ('61), we must conclude that the temporal normality of the non-spindle furrow in these experiments indicates that no influence necessary for normal cytokinesis is absent. These results indicate that the presence of the spindle and its contents are unnecessary for the initiation and completion of a cytokinetic process which is, according to all criteria that may now be applied, normal. Thus the essential role proposed for the spindle in division of grasshopper neuroblasts proposed by Kawamura ('60) cannot be applied in *Echinorachnius*.

Wolpert ('60) has proposed that the aster of the dividing echinoderm egg induces relaxation of the adjacent polar cortex. The presumptive furrow receives the stimulus to a lesser degree and division is accomplished as a result of the contractile tension inherent in the cortex of the unstimulated regions of the cell. If this mechanism existed, the first division of the torus shaped cell should be accompanied by marked distention in the astral region (the area which presumably has been stimulated) and attenuation of the side of the

torus opposite the furrow (another area in addition to the furrow which would not receive the inducing substance). The attenuation does not occur. A small increase in size of the astral region may occur but may be attributed to astral growth. Chambers ('19) also observed that an artificially created cell bridge failed to divide when the remainder of the egg divided. The cell bridge could be construed as an area not under astral influence. The appearance of a single furrow in the first cleavage of the torus shaped cell cannot be reconciled with any hypothesis in which the position of the furrow is established by the absence of stimulus.

The results of the compression and extrusion experiments indicate that the mechanism whereby the stimulus is distributed to the appropriate region of the egg is either very resilient or can function in less than 3 minutes. Two current extensive hypotheses (Swann and Mitchison, '58; Wolpert, '60) concerning the source and mode of distribution of the stimulus for cytokinesis propose two sources (either telophase nuclei or asters) which alter the cortex in the polar and lateral regions but, due to the form of the cell, fail to reach the region destined to become the furrow. Swann and Mitchison ('58) considered diffusion the method of stimulus distribution. Wolpert ('60) accepts the idea of a space between the aster and the cortex at the time the furrow is determined but does not speculate further on stimulus distribution. If the stimulus were conveyed from two sources to the cortex by diffusion and the position of the furrow determined as described above, then the mixture of cell contents which occurs during the compression and extrusion experiments would be expected to prevent division as the establishment of an unstimulated zone under those conditions would be highly improbable. That cells so manipulated can divide suggests that simple diffusion cannot, of itself, determine the position of the furrow.

Further, Hiramoto ('56) demonstrated that the conditions necessary for division are achieved while the echinoderm egg is spherical, probably during metaphase. Thus, according to Swann and Mitchison ('58) and Wolpert ('60), the distribution

of the stimulus to the polar regions has occurred; the change or differentiation of the membrane has been initiated and requires no further stimulus for the completion of cytokinesis. If the mitotic apparatus were then moved so that the zone between the asters lay near a portion of the membrane that had previously been differentiated, the relocation should be without effect since the cleavage plane could presumably occur only where the membrane has failed to receive the stimulus. Further, relocation of the mitotic apparatus would expose the undifferentiated furrow zone to the asters; thus the entire egg surface would receive the stimulus and cleavage, according to the mechanisms proposed, would be impossible. Harvey ('35) has shown, however, that if sea urchin eggs are centrifuged immediately before division and before rupture of the nuclear membrane the cell will have two furrows, one presumably corresponding to the original position of the apparatus and the other, usually in the polar region of a cell resulting from the first division, corresponding to the new position of the apparatus. The furrows may be simultaneously active. We have repeated and confirmed this experiment using *Echinorachnius* and find frequent persistence of both furrows. Marsland *et al.* ('60) differ with Harvey as to which furrow is primary but the point is not here germane. For this discussion, the importance of these results is the demonstration that the presumably differentiated polar region of a cell can respond to the presence of a mitotic apparatus by formation of a furrow. Kawamura ('60) has also shown that relocation of the mitotic apparatus of the grasshopper neuroblast after appearance of a furrow can result in the establishment of a second furrow in normal relation to the spindle. These results are most difficult to reconcile with any hypothesis which would have the presumptive furrow region determined by the absence of stimulus or differentiation. Nonetheless some scheme such as that of Wolpert is a necessary consequence of the hypothetical location of the necessary forces or areas of cell response outside the region of the furrow. Since the publication of Wolpert's ('60) excellent review, several reports

have appeared which raise serious question whether the necessary forces or areas of cell response can be considered to reside outside the furrow region.

Scott ('60) has shown that the furrow region and parts thereof can continue to function following isolation from the lateral and polar cell regions, suggesting that activity in the furrow may be mechanically independent of other areas of the cell. I, (Rappaport, '60) have shown that the echinoderm egg will divide while subjected to considerable tensile stress. The implications of this observation concerning the membrane expansion hypothesis have been discussed in the reference just cited. Concerning the astral relaxation theory of Wolpert ('60) it would be expected that relaxation in the vicinity of the asters would result in consistent elongation of the cell during division under tensile stress and indications of yielding in the astral region. Elongation was not a constant feature of division under this circumstance and carbon particles did not in any case reveal stretching or relaxation in the vicinity of the aster. Further, in cells where the line of action of the load was parallel to the plane of cleavage the furrow formed and deepened despite firm local attachment to a sheet of glass. Membrane expansion and polar relaxation appear to exhaust possible mechanisms whereby the source of the necessary force or areas of cell response may be hypothetically located in areas outside the furrow. No evidence directly supporting either scheme has been adduced and both are inconsistent with a significant body of information as regards both physical mechanism and mode of distribution of stimulus they require.

Information thus far accumulated is insufficient for formulation of a comprehensive hypothesis concerning the mechanism of cytokinesis of echinoderm eggs. It is, however, possible to render a consistent interpretation of data cited in this discussion and the observations recorded above under Results. Wolpert ('60, Section VI, C, 2) has emphasized the importance of the asters in cleavage. Elsewhere in this report it has been shown that furrows will appear between polar and lateral regions of the aster. It may be that all

parts of the astral surface are capable of delivering the stimulus. When the distance between asters is too great, no furrow appears. The first cleavage of the torus-shaped cells takes place only in the zone where the distance between asters is small, although there are two zones between asters in these cells. Thus the distance between asters may be of considerable importance in determining whether division occurs even though the necessary apparatus may be present. In the intact mitotic figure, the spindle surfaces of the asters appear closely joined at metaphase. Any stimulus originating from the asters would therefore have twice the concentration or activity in the spindle area. All parts of the cell would thus be subjected to astral influence but only in the zone of confluence of the asters would stimulus activity be sufficient to elicit a furrow. The existence of a general stimulus insufficient to cause a furrow except in the zone of confluence might explain some of the physical changes outside the furrow which accompany division. The stimulus is thus interpreted as positive and, in normal cells, localized in the same region as the spindle. If the presumptive furrow region is considered the site where the stimulus takes effect, it should also be the locus of the mechanical activity which accomplishes division. A constriction ring hypothesis (Lewis, '42; Marsland and Landau, '54) is the simplest such mechanism that has been proposed. No evidence, either inconsistent with or directly substantiating a constriction mechanism has yet been produced.

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#### SUMMARY AND CONCLUSIONS

When the form of a sand dollar egg is modified to that of a torus before the cleavage furrow is determined, it divides only in the region of the spindle. The torus is thus converted into a binucleate horseshoe-shaped cell. The furrow is tem-



porally and morphologically normal. In this experiment, a large portion of the cell was at a considerable distance from the asters but did not exhibit any signs of furrowing. The inconsistency of this observation with hypotheses in which the position of the furrow is established by the absence of stimulus is discussed.

The second division of the torus-shaped cell occurred in concert with the controls and resulted in isolation of two uninucleate cells from the free ends of the horseshoe. The bend of the horseshoe was binucleate but within 5 minutes of the completion of the two normal divisions, a furrow appeared between the polar regions of the two asters in the binucleate cell. A furrow was thus completed in a region that was never in close proximity to a spindle or chromosomes. The non-spindle division could be delayed until the third or fourth cleavage. It appeared normal; it was completed in normal time and in concert with the division of the other cells of the embryo. Since normal division has here been demonstrated to occur in the absence of the spindle and its contents, it is proposed that neither of these entities play any necessary role in cytokinesis of sand dollar eggs.

Following frequent kneading, accomplished by compression and release and by extrusion, temporally and morphologically normal division occurs. The mechanism whereby the stimulus is delivered is therefore either very resilient or very rapid.

It is suggested that the position of the cleavage furrow may, in the normal cell, be determined by the zone of confluence of the asters.

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