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STUDIES OF CELL DIVISION

I. The Effect of Dilute Sea-Water on the Fertilized Egg of Echinarachnius parma during the Cleavage Cycle

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Several workers (1), (2), (3), (4), (5), (6) have demonstrated with various methods that in the egg of *Arbacia* there is a rhythm of susceptibility to toxic agents that accompanies the first cleavage cycle. Chief among these are Mathews (2) and R. S. Lillie (5) who have likewise formulated theories of cell division based on their results. During the summer of 1920 at the Marine Biological Laboratory, Woods Hole, Mass., the writer (7), while investigating the very short period following insemination during which the egg of *Echinarachnius parma* is markedly susceptible to dilute sea-water, found it advisable for comparison to study the effects of dilute sea-water on the egg through the cleavage cycle. The present paper embodies the results of this study.

I. When inseminated eggs of *Echinarachnius parma* are exposed to the action of dilute sea-water they take up water and swell; in sea-water of slight dilution they reach a point of equilibrium without disintegrating. They remain thus for a longer or shorter time which depends upon the degree of the dilution of the sea-water. If the dilution be great (30 per cent of sea-water and less) the eggs swell and cytolyze. The rate of this cytolysis in a given dilution of sea-water is most rapid in eggs that are exposed to the dilute sea-water just before cleavage. The period of susceptibility of the *Echinarachnius* egg to dilute sea-water is thus similar to that found by R. S. Lillie (5) in the egg of *Arbacia*.

The rate of cytolysis of eggs while in dilute sea-water constitutes an excellent criterion for determining this period of susceptibility in the developing egg. The results are sharp, well-defined, and admit of little dispute. This is particularly true if the cytolysis comes on rapidly; for this reason I used 100 per cent tap-water in most of the observations. Again, study of the rate of cytolysis involves observations on individual eggs during the process of disintegration—a method which has been fruitful of interesting results. Thus, with it the writer has established the fact that during the period of membrane lifting the egg passes through a very critical period as shown by its high degree of susceptibility to dilute sea-water. The value of this method is also seen at once if among eggs of a given lot there are individual variations with respect to the cleavage rhythm. For example, after the first cleavage the difference in cleavage tempo of a lot of eggs increases making it difficult to obtain clear-cut results on eggs *en masse.* But it is very simple to mount such eggs under the microscope and watch them individually.

To the use of tap-water for determining the susceptibility periods in the cleaving eggs, there is, of course, a certain objection; it is a brutal method, not capable of nice results. The situation, therefore, calls for a method that sharply marks off the periods of susceptibility—a dilution that has no very serious effect on the egg except during these periods. An approximation of this is to be found in the dilutions of sea-water around 50 per cent. They form a check on the results obtained by tap-water.

I summarize now experiments on the rate of cytolysis of the developing egg while in tap water which show the periods of susceptibility.

Eggs inseminated in sea-water. At 2 minute intervals drops of these eggs are exposed to tap-water and the rate at which they cytolyze while in the tap water recorded. In ten experiments eggs reach the height of resistance 26 to 32 minutes after insemination. At this time they withstand tap-water treatment for 300 to 330 seconds. At 40 to 43 minutes after insemination resistance drops—time to cystolysis is 30 seconds. Seventy-three to 84 minutes after insemination the egg is extremely susceptible—time to cytolysis is about 20 seconds. Ninety-five to 98 minutes after insemination (first cleavage) the egg is resistant—time to cytolysis is 120 to 130 seconds.

Experiments in dilute sea-water (60 parts tap-water plus 40 parts sea-water) give comparable results. Eggs in which one or more blastomeres cleaved before others—especially eggs in the eight cell stage at the time of micromere formation—likewise show a susceptibility before cleavage. One can, therefore, obtain very pretty demonstrations of the separate rhythms of cell division in the blastomeres of the cleavage stages. The method is thus differential and may be capable of precise use in the study of cleavage problems.

A variation of the method so far considered is to obtain the per cent of cytolysis of the eggs after a given number of minutes in the dilute sea-water. Thus, eggs are inseminated in sea-water and at 2- or 5-minute intervals they are transferred to dilute sea-water up to and through the first cleavage. Then after 20 or 40 minutes the per cent of intact eggs in the various dishes is taken. A number of experiments was made in this way both with tap-water and with different dilutions particularly that made up of 60 parts tap-water plus 40 parts sea-water. In eleven experiments of this group the eggs were found susceptible at 40 to 44 minutes after insemination and again about 10 minutes before cleavage.

What is true of the first cleavage is also true for the second. The susceptibility is therefore a rhythmical phenomenon bound up with the cleavage process.

We may correlate the results obtained by the study of cytolysis in dilute sea-water with another method which briefly is as follows:

Eggs are inseminated in normal sea-water and at intervals transferred to hypotonic sea-water from which after 1 minute they are removed to normal sea-water and their development noted. One minute should be sufficient for the exposure since during the critical period the eggs (as shown above) while in the dilute sea-water cytolyze in less than 1 minute. The object is to give a short exposure that will just cover the critical period. By counting about three hundred eggs in each exposure one gets a good percentage index for the critical period. This method eliminates the personal factors as far as possible and the results are fairly reliable.

11:10 a.m. Two lots of eggs from two females, each lot in 10 cc. of sea-water. Eggs in good condition without a trace of accidental insemination, not a single membrane formed.

11:20 a.m. The two lots of eggs mixed and inseminated with light sperm suspension in 300 cc. of sea-water. One drop of these eggs pipetted off at 2-minute intervals to 10 cc. of tap water where they remained for 1 minute, then collected in a pipette and transferred to 10 cc. of sea-water. Controls: uninseminated in sea-water; inseminated in sea-water.

2:00 to 4:00 p.m. Counts of cleaving eggs made. Eggs exposed 38 minutes after insemination gave 30 per cent cleavage. Eggs exposed during the period 80 to 90 minutes after insemination gave no cleavage.

Results with dilute sea-water (60 parts tap-water plus 40 parts sea-water) give comparable results. Eggs are also shown susceptible just before the second cleavage.

This method can be checked by the estimation not only of the number of eggs that cytolyze but also of the eggs that cleave. The cytolyzed eggs and the cleaving eggs may be estimated in each dish or separate observations may be made to cover each in turn. Both methods were followed.

A word concerning these observations may not be out of place. The eggs were from fresh animals used as soon as procured, when this was possible. Control eggs were kept inseminated in sea-water to determine the per cent of eggs that would divide under normal conditions; and an uninseminated control to make sure that the results would not be obscured by the mixed conditions in a given lot of eggs due to accidental insemination were always run. *No experiment was ever considered if the control showed less than 95 per cent of development or if the uninseminated control showed a single membrane.* It is possible with extreme care never to have a single membrane formed in the uninseminated control. Nothing so vitiates results as the heterogeneity of the egg population due to accidental fertilization; for then the cleavage which it is desired to have as nearly the same throughout the lot as possible is arhythmic and the observation is really worthless.

The methods of these observations differ somewhat from those used by R. S. Lillie (5) in his study of the resistance of *Arbacia* eggs to dilute sea-water, but the results obtained are of the same kind. My methods emphasize the complete disintegration. In addition, I have merely carried out the simple corollary of estimating the cleavage. He allowed the eggs to remain in the hypotonic sea-water and I transferred mine to sea-water after a minute.

The uninseminated Echinarachnius egg will withstand treatment with tap-water for 2 and in some cases 5 minutes before cytolyzing. Some 10 to 20 minutes after insemination the eggs are even more resistant. A slight drop in resistance comes on midway between insemination and cleavage, followed by a rise in resistance. This in turn is followed by a sharp drop in resistance. Recovery then follows and the egg divides. Any single experiment made would establish this, and the observations together leave no doubt of the fact.

Dilute sea-water is not the only agent that betrays the susceptible period before cleavage. Recently, Baldwin (6) has shown that *Arbacia* egg is susceptible to alcohols. Previously, Moore (4) has shown a diminution in the resistance of this egg to hypertonic sea-water. Finally, the pioneer work in this field by Lyon (1) should be mentioned. Despite the fact, as subsequently shown by Mathews (2), that Lyon missed the exact stage at which the egg of *Arbacia* is least resistant to the action of KCN, this work of Lyon's is as important as any of the remarkable investigations with which he has enriched the field of experimental embryology. Spaulding (3) likewise demonstrated susceptibility in *Arbacia* by various methods.

Nor yet have observations and experiments been limited to the eggs of echinoderms. Other ova pass through a period of low resistance prior to cleavage. Thus, F. R. Lillie (8) says of the egg of *Nereis* that centrifuging during the anaphase and telephase tends to inhibit cleavage. Conklin (9) using various agents found that the egg of *Crepidula* is not susceptible just before cleavage. It may well be, therefore, that ova generally exhibit rhythmical resistance to environmental changes. If this be true the study of this phenomenon becomes important for any analysis of the physiology of cell division. And indeed, the susceptibility of the *Arbacia* egg at least plays a large part in the formulation by R. S. Lillie (5) of a theory of cell division.

It seems to me, however, that any speculation concerning the significance of the susceptibility period appearing before cleavage is of value only if the period be rather sharply defined. Merely to say that the egg suffers a drop in resistance before the cleavage begins leaves too wide a gap in a process so well ordered as that of mitotic cell division in animal ova. When one considers the whole mechanism of this type of cell division—not merely the mitotic spindle or the cytoplasm alone—and the synchrony of events in division, one must offer a postulate that considers every part of the mechanism. Mathews made, therefore, an important contribution when through the study of sectional material he fixed the susceptibility period of *Arbacia* egg to KCN as the time when the asters are at the height of their development—a finding highly consonant with his theory of cell division.

Thus, from this point of view F. R. Lillie's (8) statement that in the egg of *Nereis* centrifuging during anaphase and telephase tends to inhibit cleavage becomes vastly important. The susceptible period is here clearly defined. In what follows I shall present observations that attempt similarly to fix the susceptibility period in the living egg of *Echinarachnius*. The report on the study of sectioned eggs I must leave to a future date.

II. If on the evidence submitted we concede that the egg of *Echinarachnius* exhibits before cleavage a marked drop in resistance to dilute sea-water we must address ourselves to the query, at just what stage in the cleavage process does this drop in resistance manifest itself. According to Mathews (2) and F. R. Lillie (8) the susceptibility period in *Arbacia* and *Nereis* is well defined. In the egg of *Echinarachnius* the susceptible period is likewise well defined.

Wilson (10), (11), (12) in several places has given descriptions of the normal fertilization and mitosis as seen in the egg of *Toxopneustes;* the events leading to first cleavage in the living egg of *Echinarachnius* are closely similar. In addition I have noted certain changes in the hyaline plasma layer. In *Echinarachnius* the events leading to first cleavage are briefly as follows:

The sperm and its aster move toward the female nucleus. With conjugation of the nuclei the astral rays stream outward reaching the cortex. The hyaline plasma layer now exhibits delicate irregularities in its contour. This "amoeboid" phase becomes more pronounced. The rays die down. There then appears in the nuclear region a clear streak which is the intact zygote nucleus with hyaloplasm substance at the nuclear poles. *At this time the egg is susceptible.* The "streak" grows and widens—so that it is dumb-bell shape. This means that the nucleus breaks down, the nuclear content escapes and the hyoplasm spheres at the spindle poles increase in size. The "dumb-bell" gives rise to a well-defined mitotic complex with hyaloplasm spheres that increase in size and astral rays that are more extensive. The asters are meantime moving apart to their definite positions. The egg is still spherical. Meantime the hyaline plasma layer is again active. With the stage of greatest growth of the hyaloplasm sphere, when the astral rays are very extensive, the hyaline plasma layer exhibits an amoeboid activity from over the poles toward the equatorial zone of the spindle. This results in an accumulation of hyaline plasma layer material in the zone of the future cleavage plane. The egg is most susceptible at the time when the hyaloplasm sphere is largest, the rays extended, and the hyaline plasma layer is about to move toward the equator of the spindle from the area over the spindle poles. When the hyaline plasma layer heaps up at the equator the egg is again resistant. The egg now rapidly elongates in the spindle axis. Beneath the equatorial girdle of hyaline plasma layer a cleft appears, triangular in optical section, which is the beginning of the first cleavage plane. The hyaline plasma layer rapidly moves in and the two blastomeres arise. The period of susceptibility thus falls in the late anaphase and the telephase.

Mathews gives a table which compares his results with those of Lyon on the effect of KCN on the egg of *Arbacia.* There are here also two periods of susceptibility. These coincide with the development of the asters; the period of immunity coincides with the regression of the asters and the development of the nucleus.

Lillie finds the egg of *Nereis* most susceptible to centrifuging during the anaphase and the telephase. In *Crepidula,* according to Conklin, "the greatest changes are produced when the eggs are in some phase of kinesis at the beginning of the experiments, while stages of interkinesis are affected relatively little."

But the data on *Echinarachnius* certainly show that the eggs are susceptible before cleavage at the time when the hyaloplasm spheres are largest, the astral rays widely extended, and the hyaline plasma layer is about to move from the area over the spindle poles to the equator. The amoeboid movement of the hyaline plasma layer certainly shows that it is no mere inert sheath. This behavior of the hyaline plasma layer is not peculiar to the egg of *Echinarachnius.* Thus, Lillie (13) has described amoeboid waves in *Chaetopterus* eggs. The early descriptions of von Erlanger are well known. Observers have described the equatorial girdle of hyaline plasma layer in echinid eggs; McClendon (14), for example, as well as a number of others. Andrews (15) has given a particularly good description of the hyaline plasma layer.

During membrane lifting (16) the egg of *Echinarachnius* cytolyzes by breaking only in that zone from which the membrane is lifting at the time of exposure. At what point does this egg break when exposed to dilute sea-water during the susceptible period before cleavage? The observations give a categorical answer to this question. *In the period before cleavage when the egg of Echinarachnius is susceptible to sea-water it breaks down by bursting over the poles of the spindle.*

If fertilized eggs of *Echinarachnius* in a Syracuse watch of normal sea-water are mounted under the microscope during the susceptible period before cleavage they may be readily observed when dilute sea-water is added to them. If tap-water be added they burst within 15 to 30 seconds. With dilute sea-water (10 to 30 per cent sea-water) the results are about as rapid. With 40 per cent sea-water (60 parts tap-water plus 40 parts sea-water) the eggs do not burst so quickly. In a dilution of sea-water made up of equal parts of tap-water and sea-water, the rate of bursting is slightly lower. With a dilution of 60 parts sea-water plus 40 parts tap-water the eggs tend to form buds rather than to burst.

I employed these dilutions mentioned in order to determine the exact point at which the eggs burst when put in dilute sea-water during the susceptible period before cleavage. In general, less dilute sea-water causes the eggs to form buds; a greater dilution causes them to burst; and the more dilute, the more rapid the rate at which they burst.

If one depends solely on the use of tap-water for these observations and is slow in adding the tap-water, allowing the eggs to settle, and bringing them under focus, etc., one may miss the process entirely. In all cases observed the eggs cytolyze during the susceptible period just prior to cleaving by bursting over the poles of the cleavage spindle. This is true not only of the first cleavage but of every cleavage studied as far as the blastula stage. The egg never breaks in any other way. In the less dilute sea-water, the cytoplasm bulges over the poles of the spindle. In the more dilute sea-water the break and outflow come on violently; and indeed in many cases it is hard to catch the process so rapidly do the eggs cytolyze.

An egg, then, is susceptible to dilute sea-water prior to cleavage because of a drop in resistance of the egg cortex (hyaline plasma layer) at this time. Such an egg rapidly takes up water and bursts quickly because of the low tensile strength of the hyaline plasma layer in the area over the poles of the spindle.

An egg that has passed the period of susceptibility on exposure to dilute sea-water does not cytolyze by bursting over the poles of the spindle. If such an egg has elongated, it merely takes up water, becomes spherical, and disintegrates without giving evidence of a region of highest susceptibility. If the cleavage plane is forming, this disappears. The hyaline plasma layer heaps up at the cleavage furrow, swells and becomes very conspicuous. The endoplasm rushes forcibly toward the poles—indeed, it gives one the impression of a violent contraction. If the blastomeres are actually apart, each disintegrates separately. Quite frequently in the two-cell stage, one blastomere remains intact and the other disintegrates rapidly; the number of eggs in which this happens is correlated with the number of eggs in which one blastomere is slightly in advance of the other in the cleavage rhythm. When the egg in the two-cell stage reaches the susceptible period just before the second cleavage, the blastomeres rupture over the spindle poles, that is, in the spindle axis parallel to the first cleavage plane.

A point worthy of emphasis brought out by these observations on the egg of *Echinarachnius* (*cf.* too Conklin's (9) observations) is that the egg is susceptible *before* it begins to elongate in the spindle axis and before the hyaline plasma layer has fully heaped up at the site of the future cleavage plane. This would seem to indicate first that the drop in resistance over the poles of the spindle is not due to the thrust of the spindle. Secondly, the egg does not elongate because of the moving of the spindle poles toward the egg periphery. Rather, the evidence would seem to show that the egg elongates through the force exerted at the equator by the accumulated hyaline plasma layer in this zone.

We may sum up the results of the study of the period of susceptibility which the egg of *Echinarachnius* exhibits just before cleavage as follows: It undoubtedly comes on *a*, during the anaphase and telephase, *b*, while the egg is still spherical and *c*, before the hyaline plasma layer fully heaps up at the equator (site of the future cleavage plane) due to its movement from the area over the spindle poles. This movement brings about a thinning of the hyaline plasma layer in the polar areas. The susceptibility is due to a development of weakness in the cortex over the poles so that when the egg takes up water it rapidly bursts at these points. An earlier period of susceptibility is found just before the zygote nucleus breaks down.

These observations on the egg of *Echinarachnius* showing that in the susceptible period just before cleavage the egg breaks in the area over the spindle poles are by no means new. They are really but a confirmation of some earlier work by Conklin (9) on the egg of *Crepidula*. With various agents he frequently obtained a protrusion of the cytoplasm always from the areas over the spindle poles. This protrusion was most pronounced if the spindle were formed. Conklin, therefore, concluded that in cell division there is a decrease in surface tension over the spindle poles. Now Conklin has also made the interesting observation that the egg plasma may protrude in the area over the future spindle pole before the spindle forms. He therefore reached the important conclusion that the decrease in surface tension in the polar regions is not brought about by "mitotic pressure."

Conklin (17) has likewise described the production of large polar bodies in the egg of *Crepidula* by centrifuging at the time of polar body formation. Now, I think that we may easily explain this. The polar area becomes more fluid due perhaps to activity of cortical substance at the animal poles. The spindle meets little resistance as it moves into this area. If now we centrifuge the egg, it tends to rupture at the animal poles, hence the protrusion of cytoplasm to form a bud larger than the normal polar body. Chambers (18) was able to induce what we might call accessory polar bodies in the polar region of the egg of *Cerebratulus* simply by pricking the egg in this region; this seems to indicate a greater fluidity here than elsewhere on the egg surface at this time.

As I see it, my observations on the egg of *Echinarachnius*, which show that during the period of susceptibility before cleavage the egg in dilute sea-water bursts over the spindle poles, indicate a drop in the tensile strength by the hyaline plasma layer. At this time the hyaline plasma substance is about to move away from the polar areas to the equator. We may perhaps say that the egg is thus being denuded of hyaline plasma material in the polar areas. It is as though the plasma layer which protects the egg through its presence by thickening down at this time exposes the endoplasm at the poles. Eggs exposed during this period to dilute sea-water take up water—perhaps no more rapidly than at any other period after fertilization. But there is this important difference: the eggs that take up the water during the susceptible period are eggs with local weaknesses. Such eggs burst rapidly in these weakened zones. The egg gives way at the poles because of the activity of the hyaline plasma prior to moving from the poles.

Now Conklin (19) has made an observation that lends credence to this view. According to this worker, new cell walls at division are formed chiefly at the poles. In *Echinarachnius*, certainly, that part of the cell wall (hyaline plasma layer) over the spindle poles moves down to the site of the future cleavage plane and then moves inward to form the new cell walls between the two blastomeres. In the meantime, in the exposed areas over the poles with little or no hyaline plasma layer, new hyaline plasma material is rapidly constituted by the underlying cytoplasm. And it is this reconstitution which makes for a return in resistance.

Finally, I may call attention to the movement of peripheral granules in the egg of *Echinarachnius*. Granules at the equatorial zone early move inward toward the spindle. This is clearly shown in the dumb-bell stage. Wilson particularly has called attention to this point. Meves' (20) beautiful figures show the relation of the granules to the spindle area very clearly. In addition, as is well known from the descriptions of Mrs. Andrews, Gardiner, Ziegler, Fischel, McClendon and others, at the time of cleavage granules come to lie inside the cell walls. The classic work on this point is of course that of von Erlanger.

In the egg of *Echinarachnius parma*, then, by means of dilute sea-water we find a period of susceptibility just before cleavage. In this period we are able to correlate the behavior of the hyaloplasm spheres, the astral rays, the nucleus, the movement of ectoplasmic granules and the amoeboid activity of the cortex. And the behavior of these parts of the cell are but expressions of the rhythmical exchange between nucleus and plasma. In any attempt at the solution of the problem of cell division we must consider this streaming behavior of the protoplasm.

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