8-1-1922

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Reprinted from THE AMERICAN JOURNAL OF PHYSIOLOGY
Vol. 61, No. 3, August, 1922

THE FERTILIZATION-REACTION IN ECHINARACHNIUS PARMA

V. THE EXISTENCE IN THE INSEMINATED EGG OF A PERIOD OF SPECIAL SUSCEPTIBILITY TO HYPOTONIC SEA-WATER[1]

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Received for publication June 1, 1922

In the first paper of this series (1) on the fertilization-reaction in *Echinarachnius parma* the writer called attention to the cortical changes that manifest themselves in this egg as a response to insemination. Briefly, these are as follows:

Soon after it takes in the sperm the inseminated egg lifts off the membrane. This formation of the "fertilization membrane," as it is usually called, is due to a liquefaction throughout the cortex that brings about the formation of the perivitelline space and thus the separation of the membrane. It has often been held that membrane separation is of importance in preventing polyspermy. Now in *Echinarachnius* these observations on the separation of the membrane are of significance because they quite clearly showed that the block to polyspermy is established before the actual separation of the membrane. The change in the egg whereby polyspermy is prevented is of extreme rapidity. But during three summers sufficient observations on individual eggs were made to make clear just what takes place. With care one can observe the actual entrance of the sperm concerned in the fertilization-reaction. It was learned in this way after mixing the eggs and sperm that it takes a certain time for the sperm to reach the egg. This time will vary with several factors: the concentraton, the activity and the age of the sperm suspension, the temperature of the sea water, the vitality of the eggs and the time in the breeding season. These or at least some of these factors will likewise determine the penetration time of the sperm,

[1] The experiments were made at the Marine Biological Laboratory, Woods Hole, Mass., during the summer of 1920. They were reported at the 1920 (Chicago) meeting of American Society of Zoölogists as part of a Symposium on Fertilization. An abstract appeared in the *Anatomical Record* for January, 1921.

that is, the time that it takes the sperm to get into the egg from tip to base. Now, the actual time of penetration of the sperm within the egg—that is, the time elapsing between the moment when the tip of the sperm head touches the egg to that at which the base of the head disappears within the egg—is of extremely brief duration. This time is not easy to measure, except with slowly reacting eggs or sperm when the penetration time is lengthened. Early in the season when the temperature is low one can best follow the penetration. I did not give any figures for the penetration time in the paper cited. I did, however, relate the response of the egg to additional sperm entry to the penetration time of the fertilizing sperm for indeed this was clearly brought out in the obsrvations.

A wave of negativity moves over the egg, its rate varying with the variations in time that mark the disappearance of the sperm within the cytoplasm. And if the penetration is rapid or slow, the response of the egg to additional sperm is of the same order. It must, however, be admitted that there may not be any causal connection between these two phenomena.

In tables 1 and 2 on page 4 of the paper mentioned it is readily apparent that after the sperm is within the egg—regardless of the time the actual penetration may have consumed—there follows now a period of about 15 seconds before membrane separation begins. (Thus, in table 1 sperm are engulfed at 50, 49, 30, 45 and 30 seconds after insemination and the membrane comes off at 65, 64, 50, 67 and 50 seconds respectively after the sperm has disappeared within the egg.)

During this period of membrane separation the egg is extremely susceptible to hypotonic sea-water.

If eggs of *Echinarachnius parma* are placed under the microcsope and inseminated one may observe the rapid penetration of the sperm and with it the wave of negativity as evidenced by the behavior of supernumerary sperm around the egg. There then follows the period of about 15 seconds before membrane separation begins. The moment that the membranes begin to lift the eggs are treated with tap-water. In 15 to 20 seconds after the tap-water is added, the eggs burst. This cytolysis is violent, rapid and complete. But for the use of sea-water of graded hypotony it would have been extremely difficult adequately to interpret this behavior. With the use, however, of sea-water ranging in dilutions from 95 per cent to 5 per cent, the whole period has been clearly revealed. If the egg is treated with tap-water when the membrane is one-third or a half off instead of at the moment of membrane

separation, the result is the same—there is a complete cytolysis in some 15 seconds. If the egg is allowed to separate its membrane from its entire surface except the very last point opposite the site of sperm entry (from which the membrane lifts last) and is exposed at this instance, then cytolysis takes place as before.

The actual course of membrane separation runs over a period anywhere from 9 to 26 seconds. During this period the egg cannot withstand treatment with tap-water with anything like the resistance of the uninseminated egg. Following complete membrane lifting there ensues a period during which the membrane rounds out and becomes equidistant from the egg. The egg is now resistant again although the resistance is not so great as that at about 10 minutes after insemination. But it is clear that with the formation of the membrane and even before its rounding out the egg has recovered its resistance to tap-water. The following summary of experiments reveals these facts.

The uninseminated egg. Uninseminated eggs mounted under the low power of the microscope are treated with tap-water. With a stop watch the rate of cytolysis in 10 lots of eggs each from 10 females is determined. The time in tap-water to cytolysis is as follows:

	NUMBER									
	1	2	3	4	5	6	7	8	9	10
Time in seconds to cytolysis.....	270	243	60	113	150	148	256	247	155	240

The rate of cytolysis, in eggs from these same females, 5 to 10 seconds after insemination, is about the same.

The inseminated egg during membrane separation. Eggs from these same females inseminated in turn and exposed to tap-water during membrane separation gave the following rates of cytolysis:

	NUMBER									
	1	2	3	4	5	6	7	8	9	10
Time in seconds to cytolysis.....	14	17	19	20	9	11	18	16	7	6

The inseminated egg 2 minutes after insemination. Eggs from these same females exposed to tap-water after membrane separation cytolyzed around 120 seconds.

Once it was well established with tap-water that the susceptible period falls in exactly with the period of membrane separation, attention was directed to the susceptibility of the egg to less dilute sea-water at different stages of the process of membrane separation. This seemed important because with the 100 per cent tap-water it appeard that *when the egg cytolyzed the break always came from that part of the cortex from which the membrane was lifting at the time of exposure.* The cytolysis with tap water was, however, far too rapid to be sure of this. The less dilute sea-water proved that this interpretation was correct. *When eggs are exposed to dilute sea-water during the period of membrane separation they cytolyze by an outflow of cytoplasm at the points from which the membrane is lifting at the moment of exposure.* The data on the effects of sea-water of different dilutions are summarized in what now follows:

Eggs were tested during the period of membrane separation of sea-water diluted with tap water to give 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90 and 95 per cent sea-water.

The results with these dilutions may be briefly summarized. In 95 and 90 per cent sea-water the eggs do not show any change. In 85 and 80 per cent sea-water the eggs do not break down but merely show a slight protrusion of protoplasm during membrane elevation. Thus, in 85 per cent sea-water the eggs show 1 per cent buds. The response of the egg during membrane separation to treatment with 75 per cent sea-water is about the same as that to 80 per cent except that the percentage of buds is slightly increased and that the egg may elongate slightly. Eggs kept in these dilutions show some development; the more dilute giving no true cytoplasmic cleavage, the less dilute giving nuclear as well as cytoplasmic division.

Thirty parts tap-water plus 70 parts sea-water: When eggs are exposed to the action of this dilution during the period of membrane separation they form buds. For example, if eggs are exposed at the moment of membrane separation the membrane bulges out at the point of membrane lifting and there follows a protrusion of the cytoplasm beneath the bulging membrane. If the membrane has been lifted from half the egg then the buds form in the angles (i.e., as seen in optical section) between the lifted membrane and the part of the cortex from which the membrane is not yet lifted. If the membrane stands off from the egg at every point except the very last point of its attachment—the pole opposite that of sperm entry—then the bud forms at this point.

Thirty-five parts tap-water plus 65 parts sea-water and 40 parts tap-water plus 60 parts sea-water: Eggs exposed to these dilutions show

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changes comparable to those shown in 30 parts tap-water plus 70 parts sea-water, but the changes come on more quickly. Thus, in 70 per cent sea-water the eggs exposed during membrane lifting form buds in from 1 to 1½ minutes; but in 60 per cent sea-water these buds form in about 40 seconds. Moreover, while the percentage of eggs with membranes that respond to the treatment of 70 per cent sea-water with bud formation beneath the lifted membrane is from 70 to 80 per cent, the percentage of eggs with membranes that thus respond to the 60 per cent sea-water is always close to a hundred. Such eggs however tend to disintegrate rather than to form buds. The formation of these buds is always beneath the membrane at the point at which it is lifting. They never arise from the region from which the membrane has lifted or from the region in which the membrane is still stuck to the egg.

Hypotonic sea-water of 55 parts sea-water plus 45 parts tap-water and sea-water of greater hypotony: These dilutions are cytolytic and in them the eggs respond much as do eggs in 100 per cent tap-water except that the onset of cytolysis is not so rapid. They are indispensable for working out the wave-like nature of the cytolysis in the membrane eggs. If eggs are placed in the dilution 40 parts sea-water plus 60 parts tap-water at the moment of membrane lifting they disintegrate instead of budding in the region of membrane separation. At this point the egg protrudes rapidly. This is followed by an outflow of cytoplasm through the region of liquefying cortex. The cytoplasmic outflow results in the formation of a dumb-bell shape mass of egg material within the partially lifted membrane. If eggs are exposed to the action of these dilutions of sea-water after the membranes are one-third or one-half off, then the disintegration takes place in the zones from which the membrane is just lifting. Such eggs exhibit a girdle of protruding cytoplasm within the membrane. In optical section this girdle appears as buds in the angles between the lifted membrane and the egg. The outflow of cytoplasm that leads to disintegration is in this angle around the egg. If eggs are exposed just before the membrane is lifted from the last point of the egg—the pole opposite sperm entry—the cortical break is in the zone immediately around this point. If the eggs are exposed at the very instant that the membrane is lifting from its last point of attachment, the break comes at this point: there is a bud of cytoplasm which precedes the cortical outflow leading to complete cytolysis. Such eggs, therefore, respond like those that receive an exposure at the very beginning of membrane lifting. The two are, however, readily distinguished by the great difference in the extent of completely lifted membrane.

It is thus seen first that the suceptibility of the egg of *Echinarachnius* falls in with the period of membrane separation. But what is more important is that like the process of membrane separation the susceptibility travels at the same rate as the membrane lifting wave. As the cortex goes into solution, thus lifting the membrane,—a process easily followed under the microscope—droplets of ectoplasm move across the perivitelline space before they completely disappear. Any point on the egg surface where this dissolution is taking place becomes the point of susceptibility at the instant of the dissolution.

In the next place, one must note the most remarkable characteristic of this period of susceptibility during membrane lifting. The susceptibility of the cortex is rapidly reversible. I have made observations on thousands of eggs and have yet to see an egg exposed during membrane separation break in those regions from which the membrane has not lifted. This is especially well brought out by exposing eggs during the early stages of membrane separation. Any part of the egg from which the membrane is not lifted is resistant to the dilute sea-water. And it is equally true that any part of the egg from which the membrane has lifted is resistant. This is strikingly shown by exposing eggs just at the moment that the membrane is being lifted from the last point on the egg surface. In this case the breakdown is only at this point; the zones from which the membrane is already off are resistant. When the membrane is fully off, the egg leaves the period of susceptibility. We may say, therefore, that a wave of resistance to dilute sea-water follows in the wake of the wave susceptibility. There is an exceedingly rapid restitution process in the cortex following a momentary loss of resistance through the normal cortical breakdown that pushes off the vitelline membrane.

We may conclude: The period of susceptibility to dilute sea-water exhibited by the inseminated egg falls in with the period of membrane elevation. In eggs exposed during this period the cortex breaks and allows an outflow of cytoplasm, a bud-like protrusion, in the less dilute sea-water; or a more pronounced outflow with complete disintegration in the more dilute sea-water. This break in the cortex takes place only in the zone of membrane separation that is, where the cortex through secretion is lifting the membrane at the instant of exposure The cortex is therefore weakest in the zone of membrane separation and this weakness travels over the egg with the wave of membrane separation. Zones of the egg from which the membrane has lifted are resistant; zones of the cortex to which the membrane is still stuck are resistant. *The suscepti-*

bility of the egg is thus due entirely to the progressive cortical secretion which brings about membrane lifting. It is a manifestation of local (cortical) changes.

These observations are likewise of fundamental significance in establishing that the period of susceptibility is an expression of a period of profound physical changes. A study of these might well furnish us with a means of a closer approach to the fertilization problem. These manifestations are, however, of importance for their own sake.

. THE NATURE OF THE PERIOD OF SUSCEPTIBILITY. 1. We may turn our attention first to the rôle of the so-called "fertilization membrane," during the susceptible period. The question as to whether the membrane is preformed or arises *de novo* as the result of fertilization need not seriously concern us here, although it seems to me that the bulk of the evidence indicates in *Echinarachnius* and in *Arbacia* that the membrane is preformed. We should nevertheless remember that in our experiments made to determine the presence of the membrane on the uninseminated egg the very procedure of the experiment itself might be sufficient to call forth membrane separation. The very fact that such diverse agents may cause membrane separation proves or at least suggests that the membrane separation process is highly reactive. Therefore, experiments to show the presence of the membrane of the uninseminated egg may themselves produce the membrane by the action of the agents employed. But this is apart from the question now under consideration; namely, what part does the separating membrane play in the period of cortical breakdown when the egg is so highly susceptible to the effect of hypotonic sea-water?

In the first place, as shown above, the egg may cytolyze within the membrane which remains intact. In the second place, in the uninseminated egg the membrane does not burst but remains intact while the egg substance goes into solution. The effect of the hypotonic sea-water is to harden the membrane so that it stands off from the egg but it does not usually rupture. Since, however, the membrane is evidently undergoing a change in its physical properties just about the time that it begins to lift we should naturally expect the most pronounced change in its behavior at this time. In other words, the membrane may actually play a rôle in the behavior of the egg when subjected to the hypotonic sea-water but this rôle may be so small that it is obscure; the best time to observe any change in the behavior would be at the time when this change is at its height. This would be at the very beginning of the membrane separation process. When the egg, at the moment mem-

brane separation is beginning, is put in the hypotonic sea-water, the membrane bulges out to quite a perceptible degree at this point. This effect as elsewhere noted in this paper could not have been adequately studied had not the hypotonic sea-water been used in closely graded series. With hypotonic sea-water of varying strength this process can be worked out in great detail and is about like this: When the membrane begins to separate at the point of sperm entry and the egg put in hypotonic sea-water, the membrane lifts off from the egg at a greater distance than otherwise. If this hypotony is very great, tap-water for example, the egg bursts. If the degree of hypotony is less, the egg forms a bud at this point. To quote from my notes:

> July 8, 9:20 a. m. Inseminated eggs in sea-water 35 seconds later eggs in 30 tap-water plus 70 sea-water.
>
> First the membrane bulges out at the point at which it starts lifting; this is immediately followed by the budding of the cytoplasm just beneath. This behavior is not so clearly shown in eggs put into the tap-water or hypotonic sea-water when the membrane is off the greater part of the egg. It is best observed in those eggs put into hypotonic sea-water at the very instant that the membrane is beginning to elevate.

I interpret this as follows: The membrane before insemination is a very plastic substance. After it begins to lift or rather at the moment it begins to lift it becomes more hardened; it sets. Before it begins to separate from the egg surface it has the general properties of the cell. But once it separates from the cell surface it is no longer a part of the metabolic machinery. Now during the time that it bulges out from the egg it is in its most elastic condition. As fast as it lifts from the egg it rapidly hardens and loses the properties of the cell protoplasm. The bulging at this time is due to the elasticity which soon passes off.

That something like the above is true seems to be supported from what we know of exovates. The best time to secure exovates is soon after the membrane is formed; after this they are more difficult to obtain. In other words, it is easiest to rupture the membrane soon after it has arisen. Moreover, the best time to shake off the membrane from either inseminated eggs or butyric-acid-treated eggs is immediately after the membrane has separated. This would seem to lend support to the view given above, namely, that the membrane changes its properties after it lifts from the surface of the egg.

The conclusion of this whole matter is therefore that the egg membrane is not a living part of the egg once it is separated from the egg surface and that it plays no rôle in the susceptibility of the egg. The

phenomena osmotic or otherwise go on in virtue of the cell substance and not through or because of the vitelline membrane.

2. If indeed it be true that the membrane once off the egg plays none but a passive rôle in the metabolic activity of the egg, we must conclude that there is some alteration in the egg itself. And this is indeed the case as shown by a comparison of the physical character of the uninseminated and the inseminated egg with a membrane after treatment with hypotonic sea-water.

The uninseminated egg when subjected to the action of tap water becomes granular and disintegrates. At one point in the cortex just under the membrane the cytoplasm forms bubbles. These gradually form throughout the egg. The membrane now stands out as clearly as the so-called "fertilization membrane." After the cortex goes into solution in this way the interior of the egg is destroyed. The cytoplasm resembles crystals of salt through which water is seeping, so granular is its appearance. The effect of the tap water in lifting the membrane is often lost sight of because the swelling of the cytoplasm is so closely connected with the breakdown of the cortex that the water-logged cytoplasm fills out the distended membrane. Disintegration now takes place *within* the membrane. This is the usual procedure. Occasionally, however, the egg ruptures at one point and cytoplasm pours out at this point from the egg. The two striking characteristics of the behavior of the uninseminated egg in response to the treatment with tap-water are the granular appearance of the cytoplasm and the general uniform disintegration of the egg. It is as though the cytoplasm is washed away.

With the inseminated egg during the susceptible period the case is quite different. In the inseminated egg treated with tap-water the egg ruptures at one point and flows out. It may form a ball after it flows out and so maintain itself in sea-water for some time. The cytolyzed plasma tends to cohere. This outflow usually does not pass beyond the membrane which remains intact. Or, if the outflow forces itself through the membrane the rest of the membrane remains intact and spherical. In other words, the physical properties of the inseminated and the uninseminated egg are quite different as shown by the difference in reaction to hypotonic sea-water. This change is apparent the *moment the membrane begins to lift* and can be very clearly revealed by the study of the behavior of the egg in hypotonic sea-water of graded strength.

3. The susceptible period here described is not, however, the only one in the egg after insemination. As is well known particularly from the very careful experiments of R. S. Lillie (2) the egg of *Arbacia* is quite susceptible to hypotonic sea-water just before cleavage. In my study of *Echinarachnius*, in order to get all the data I could I have studied the susceptibility of this egg before cleavage. It is practically like that of *Arbacia*. Just before cleavage a period of great susceptibility to hypotonic sea-water sets in. It was deemed advantageous to study this in detail in order to determine what the two periods have in common. A brief comparison may here be made. In the case of the recently inseminated egg that is susceptible to hypotonic sea-water, the susceptibility is due to the breaking up of the cortex in that part of the egg where the membrane is lifting. In the case of the egg that is susceptible to the hypotonic sea-water just before cleavage the hyaline plasma layer is intact. The conditions therefore are quite different for in the one case we have the cortex breaking up later to build the hyaline plasma layer, in the other we have the hyaline plasma layer already formed. But the susceptibility of the egg at the time just before cleavage is due in part to the changes in the hyaline plasma layer as I have elsewhere shown for at this time the susceptibility is clearly localized over the spindle poles.

4. If we attempt to come to some conclusion as to the meaning of this susceptibility we must take into account the following facts. First, the very rapid recovery of the cortex after membrane lifting. Second, the resistance of the cortex from which the membrane is not yet lifted. Third, the apparent failure of the cortex at the site of sperm to dominate the rest of the cortex in any way, either in greater resistance or susceptibility, or in persistence of the effects of the hypotonic sea-water. In brief, we must keep in mind that this susceptibility is clearly localized; or, what is more correct, that the break in the egg which is the expression of decreased resistance to the hypotonic sea-water is clearly at the site of membrane lifting. Now this does not mean that this is the point at which the water enters the egg. Rather, it means that the point at which the membrane is lifting is the point at which the cortex is weakest. We are not dealing with an imperforate membrane but rather *with the cortex* that is not continuous; it shows a break in the zone of membrane lifting. The normal process of the cortical secretion is doubtless bound up with water movement. The cortex is washed away. The material goes into solution. Now, this process is one of rapid recovery not only in the cortex itself but toward the interior of the egg for the interior

of the egg never becomes involved. In the normal process just enough water is present to carry out the normal process, but when the egg is placed in dilute sea-water, the picture is different. The recovery in the zone of membrane formation cannot take place, and the cortex breaks. Moreover, with the cortex gone, the endoplasm is without the cortical protection and it too becomes involved so that complete cytolysis is the result. We are here dealing, then, with a sensitivity due to actual progressive dissolution of colloids in the cortex of the egg.

5. To the reader it is at once apparent that the process of membrane separation exhibited by the *Echinarachnius* egg closely resembles that of transmission in various tissues. The way in which the cortex responds to hypotonic sea-water may be additional evidence of this similarity. Certainly, the response of the cortex during membrane formation to the hypotonic sea-water reminds us of the action current in a stimulated nerve. The high susceptibility of the cortex in the zone of membrane elevation and the relatively resistant zones from which the membrane has not separated suggest the electronegative condition of a nerve fiber set up wherever the excitation may be at a given instant as it sweeps along the fiber. It may well be that this is more than a superficial resemblance and that the study of this cortical response would warrant further investigation from this point of view.

6. It seems that this wave of lowered resistance in the cortex of the egg during membrane separation is worthy of study as a secretion. Our knowledge of the actual secretory processes in cells as such is not so abundant that we may ignore any process that even merely seems a secretion. The striking cortical behavior of the egg of *Echinarachnius* might, therefore, yield valuable information for the problem of secretion.

These then are some of the points which the study of the susceptibility in the inseminated egg of *Echinarachnius* has raised. For its own sake it will repay further study. Far more significant for us, however, at this time is this question: what is the relation of this susceptibility period to the so-called fertilization-reaction? If the susceptibility period be interpreted in terms of the permeability theory or if it should be found that there are changes in oxidation and in electrical potential at this time, would these findings be interpreted as meaning that the one or the other or all these changes are the "cause" of fertilization? The writer has consistently held the point of view that the essential phase in the whole fertilization process is a reaction which is practically instantaneous between sperm and a substances of the egg, namely, the fertilization-reaction. If therefore, the findings on the

susceptible period be interpreted as the leading event in the whole fertilization process, then the reaction between egg substance and sperm is far from instantaneous but is indeed spread over an appreciable length of time. In a forthcoming paper evidence is presented which attempts to establish that this susceptible period is not the fertilization-reaction, but rather is a local change which is a sequela of the reaction between egg substance and sperm.

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