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INITIATION OF DEVELOPMENT IN ARBACIA

IV. SOME CORTICAL REACTIONS AS CRITERIA FOR OPTIMUM FERTILIZATION CAPACITY AND THEIR SIGNIFICANCE FOR THE PHYSIOLOGY OF DEVELOPMENT

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In the egg of *Echinarachnius parma* a striking effect subsequent to insemination is the dissolution of the egg cortex. This dissolution begins at the site of sperm entry and progresses as a wave over the whole surface of the egg; thus arises the perivitelline space with the so-called "fertilization membrane" equidistant from the egg at all points. This cortical response of the egg to insemination is easily visible and readily followed (JUST, 1919). Now during this period of cortical breakdown the egg is extremely susceptible to various agents; this I have demonstrated particularly with dilute sea-water. Moreover, the egg exhibits this susceptibility to dilute sea-water by breaking down in that zone from which the vitelline membrane ("fertilization membrane") is lifting at the moment when one places the egg in dilute sea-water. Zones from which the membrane has lifted are resistant; and zones to which the membrane is still stuck to the surface of the egg are resistant. Thus, susceptibility to dilute sea-water passes as a wave over the egg surface with the wave of membrane separation; behind this wave resistance rapidly returns (JUST, 1921, 1922 a). I have also studied the susceptibility to dilute sea-water exhibited by the egg of *Arbacia* immediately following insemination (JUST, 1921). This study I have continued while working summers at the Marine Biological Laboratory, Woods

Hole, Mass., and find that the conditions in the egg of *Arbacia* differ somewhat from those in the egg of *Echinarachnius*. The present report aims to set forth these results. Still more recent studies indicate that one can correlate this susceptibility of the egg of *Arbacia* with other of its manifestations under normal and experimental conditions. The data of these studies are here, therefore, brought together because the writer feels that they are of value to other workers who use this egg. To me the data give evidence of some criteria of the optimum fertilization capacity of the egg of this Woods Hole sea-urchin.

It gives me great pleasure to acknowledge my indebtedness to Prof. Franz Schrader and to Dr. Sally Hughes-Schrader for several helpful criticisms and suggestions given during the preparation of this paper.

EXPERIMENTS AND OBSERVATIONS

The experiments and observations here reported are considered in the following order:

1. The effect of dilute sea-water on the uninseminated and the inseminated egg.
2. Observations on the rate and character of membrane separation.
3. The rôle of the egg-jelly in the fertilization-reaction.
4. Polyspermy.

THE EFFECT OF DILUTE SEA-WATER ON ARBACIA EGGS

Experiments were repeatedly made first on uninseminated eggs. Next, eggs from these same lots were inseminated in sea-water and exposed to the action of dilute sea-water. In both cases the time to cytolysis was noted with the aid of a stop watch. The method is simple. Eggs from one female were removed from sea-water and mounted under the low power of the microscope and the time to cytolysis recorded. Usually, at least three records were made on the eggs from a single female. At first, the time to cytolysis of the first, of the last, as well as of the majority of the eggs, was noted. It was found, however, that it was sufficient to record the time of cytolysis of two-thirds of the eggs. And these are the figures here given.

On the uninseminated egg

If the uninseminated egg of *Arbacia* be exposed to dilute sea-water it takes up water and swells. In dilutions of 50 parts sea-water plus

50 parts tap or distilled water and in less dilute sea-water the egg remains intact for twenty-four hours or more; with greater dilution of sea-water, the egg remains intact for a shorter time. The more dilute sea-water is, therefore, injurious. In the extreme dilutions of sea-water the egg breaks down before it reaches osmotic equilibrium.

R. S. LILLIE has made careful studies of these effects of dilute sea-water on the egg of *Arbacia*. The present writer has used a slightly different method in his study of the effect of dilute sea-water on this egg and has also used greater dilutions; he has especially employed distilled or tap water with a small drop[1] of highly concentrated eggs —in the proportions of 10 to 20 cc. of distilled or tap water plus one drop of eggs. Dilute sea-water—5, 10, and 15 parts sea-water plus 95, 90 and 85 parts distilled or tap water respectively—gives comparable results. I report here the results on tap and distilled water only. The first experiments were made during the summer of 1920. They were repeated throughout the breeding season during the summers of 1921, 1922 and 1923.

When uninseminated eggs of *Arbacia* are exposed to distilled or tap water in the proportions of 10 to 20 cc. of water plus one drop of eggs they cytolyze in 30 to 180 seconds. In 16 lots of eggs, for example, from 16 different females, the time to disintegration in tap water was as follows: 60, 120, 100, 120, 120, 180, 90, 60, 60, 60, 30, 30, 70, 125, 130, and 60 seconds. The eggs of *Arbacia* thus exhibit a considerable degree of variability with respect to their capacity to withstand exposure to tap water. Distilled water gives similar results.

The data on the rate of cytolysis in the recently inseminated egg of *Arbacia* were obtained by inseminating eggs in normal sea-water and exposing them to distilled or tap water (as well as to various dilutions of sea-water with distilled or tap water) at intervals after insemination. The following summary (Table I) gives some results from this group of experiments.

This experiment is representative of the whole group of fifty made during several seasons on this point. From the figures given in Table I, it is at once apparent that in the inseminated egg of *Arbacia* a sharply defined period of special susceptibility comparable to that existing in the egg of *Echinarachnius* is not demonstrated, if the *rate of cytolysis* be taken as the criterion.

[1] One drop is $^1/_{17}$ cc.

Table I

Rate of disintegration of inseminated eggs of Arbacia in tap water

NO.	Time in seconds after insemination when exposed to tap water	Time in seconds to cytolysis in tap water
1	5	90
2	10	90
3	15	90
4	20	60
5	25	60
6	30	30
7	35	60
8	40	60
9	45	120
10	50	120
11	55	140
12	60	100
13	65	100
14	70	120
15	75	180
17	80	120
18	85	180
19	100	180
20	120	240
21	180	300

Experiments were also made in this wise: at intervals after insemination in normal sea-water, eggs were exposed to the action of tap water (and to dilutions made up of 5, 10 and 15 parts sea-water plus 95, 90, and 85 parts tap water respectively). 5 drops of these eggs in tap water were removed to 250 cc. of sea-water after 15 and 30 seconds; later the cleavage per cent, was noted. One such experiment from a group of thirty is given in Table II.

Eggs inseminated in sea-water but not exposed to tap water gave in each case 100 per cent. of cleavage. Uninseminated eggs exposed to tap water for 15 and for 30 seconds gave no cleavage. The uninseminated control in sea-water gave no cleavage.

The experiment cited shows that following insemination *Arbacia* eggs do not reveal a sharply defined period of low resistance to tap water as measured by the per cent. of cleavage on return to sea-water. Thus, like the uninseminated egg, the inseminated egg of *Arbacia* differs from the inseminated egg of *Echinarachnius* in its response to treatment with tap water.

Table II

The effect of exposing inseminated eggs of Arbacia to tap water as revealed by the per cent. of cleavage on return to normal sea-water

No.	Time in seconds after insemination when exposed to tap water	Per cent. of cleavage	
		Following 15 seconds exposure	Following 30 seconds exposure
1	5	80	17
2	10	59	23
3	15	42	30
4	20	56	26
5	25	75	20
6	30	96	24
7	35	95	28
8	40	73	33
9	45	83	50
10	50	86	56
11	55	94	61
12	60	84	70
13	75	96	70

I have pointed out above that the inseminated egg of *Echinarachnius* exposed to tap water during the process of membrane separation breaks down in the zone from which the membrane is lifting at the instant of exposure; this breakdown thus indicates a wave of susceptibility to extreme hypotonicity which runs parallel with the wave of membrane separation. Comparison with the uninseminated egg, with the inseminated egg before the beginning of the separation of the membrane, or with the egg after complete separation of the membrane reveals the egg of *Echinarachnius* during its process of membrane separation is in a stage of maximum susceptibility to hypotony as measured by the rate of disintegration (Just, 1922a). No such disintegration either as to *locus* or as to *rate* is evident in the egg of *Arbacia* exposed to tap or distilled water at intervals after insemination (and, therefore, certainly in some cases during the process of membrane separation). I attribute this behaviour of the egg while in tap or distilled water to the nature of the normal cortical changes leading to the separation of the membrane[1]. These changes are briefly described in the following section.

[1] My observations on the cortical changes in the eggs of *Arbacia* induced by insemination were made on (a) living eggs, (b) fixed whole eggs, and (c) sectioned eggs.

OBSERVATIONS ON THE RATE AND CHARACTER OF MEMBRANE SEPARATION

When eggs of *Arbacia* in sea-water are mounted under the microscope and inseminated with thin sperm suspension, the sperm reach the eggs in two to three seconds. The first effect of the sperm is to indent the membrane—actually to push it in, for one can observe the membrane give beneath the sperm. Twenty seconds after insemination the cortex is turbid. Now like a flash, beginning at the point of sperm attachment a wave sweeps over the surface of the egg, lightening the cortex as it passes—the cortex becomes a lattice beneath the membrane. Below the sperm head a minute cone (described by MATHEWS) forms twenty-five seconds after insemination; one might almost say that the cone forms around the sperm head. The sperm head is suddenly pulled into the cone; the cortical strands release the membrane at the site of sperm attachment and beginning here the membrane separates in a wave from the surface of the egg, leaving in its wake collapsing cortical strands. Thirty seconds after insemination the membrane is separated from the egg by a narrow perivitelline space. During the ensuing twenty-five seconds the perivitelline space increases in with; the cortical strands are more sharply defined giving the cortex the appearance of a striated membrane. The vitelline membrane is equidistant from the egg at all

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All observations were made with the best optical equipment procurable—Zeiss apochromatic objectives and compensating oculars and Zeiss aplanatic, plankton, and change-over darkfield condensers. With the change-over condenser I used the Zeiss apochromatic objective "X". I believe that many workers make the mistake of using inferior optical equipment in their studies of fertilization and similar processes in the living marine egg.

In all observations on the living eggs the few eggs mounted under the microscope were well flooded with sea-water. I never use large numbers of eggs in a small drop; nor do I use eggs in a hanging drop because of the injury that results when such eggs get caught in the angle made by the surface of the glass and the water film. Only when using immersion lenses were the eggs ever under a cover slip and then never for more than a few seconds. Care was also exercised to avoid heat from the lamp used.

After trying out about a dozen fixing fluids, I found one that faithfully reproduces the structural changes observed in the living egg. Without the study of fixed eggs, both *in toto* and in sections, I should hesitate to speak with surety concerning some of the details here mentioned. Well fixed cells are still of some value though some histologists profess nowadays a contempt for any but studies on the iving cell.

points and the perivitelline space is at its greatest width one hundred and twenty seconds after insemination. *The striated cortex is the hyaline-plasma layer*[1].

Thus, in the egg of *Arbacia*, the time for the separation of the membrane from the egg, spreading in a wave over the whole surface of the egg from the site of sperm attachment, is five seconds. This is a far more rapid process than that described for the egg of *Echinarachnius*. Moreover, in the larger egg of *Echinarachnius*, one may readily observe the cortex going into solution as the membrane separates droplets passing from the cortex across the perivitelline space. These differences may be sufficient to account for the failure of the egg of *Arbacia* to disintegrate in tap or distilled water with the rapidity characteristic of the egg of *Echinarachnius* during the period of membrane separation; it would be difficult indeed to expose the egg to the action of the dilute sea-water during the five second interval of membrane separation. Moreover, the restitution of the cortex following in the wake of its breakdown must be similarly brief and thus, the susceptibility in the zone from which the membrane is separating at the instant of exposure must be of short duration. It is therefore easy to appreciate the practical difficulty of observing the eggs in sea-water, getting them into dilute sea-water at the instant the membrane begins to lift and under focus of the microscope in time. The difference between these two eggs are undoubtedly correlated with differenees in size, physical make-up, etc. In the larger *Echinarachnius* egg the cortical response to insemination is spread over a period of time great enough to resolve the process into stages; in the smaller *Arbacia* eggs the response is more rapid and the stages compressed, so to speak.

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[1] These observations must be made on eggs inseminated in an open drop. Once inseminated they may be covered by a slip. Apparently, eggs cannot withstand pressure at the time of insemination—HEILBRUNN has noted this. I have made an extensive study of eggs enmeshed in fibres of lens paper (previously thoroughly washed in distilled then in sea-water) by placing a mat of fibres above and below them and find that the eggs will not respond to insemination. Eggs thus placed immediately after insemination develop normally. This failure of insemination might mean that such pressure diminishes the capillary spaces in the cortex. Any one such space with which the spermatozoon normally reacts to set up the explosive fertilization-reaction thus diminished is now too small for this explosion since highly explosive materials fail to react in capillary spaces (Sir HUMPHRY DAVY). I have tried to study the effect of varying the temperature and pressure at the time of insemination with no clear cut results, but such a study might be illuminating. In nature *Arbacia* eggs must be frequently fertilized at fairly great depths; in the laboratory even greater pressure is possible.

The difference in the cortical response to insemination of the eggs of *Echinarachnius* and of *Arbacia* is brought out in another way. In the eggs of *Echinarachnius* the separation of the membrane in tap water is rapidly followed by disintegration; eggs of *Arbacia* in tap or distilled water form membranes in a few seconds and remain intact thereafter for some time. If the reader will refer to the figures on the rate of cytolysis given above he will note that in no case does the uninseminated eggs of *Arbacia* cytolyse under thirty seconds, and this is most exceptional for in the majority of cases cytolysis does not take place at such a rapid rate.

Membrane separation by dilute sea-water

That dilute sea-water will initiate membrane separation in echinid ova has been demonstrated by several workers (cf. McClendon, Glaser) since Schücking's original observation. I find that brief exposures to tap or distilled water will readily bring about membrane separation in the egg of *Arbacia*, the results comparing favorably with those obtained with butyric acid; such eggs go as far as the monaster streak stage showing on one side of the periphery an accumulation of pigment, in the equator of the axis of the streak, which may be in the form of a bud or an excavated area in the cortex. My results, though in no wise novel, are of interest in aiding us to interpret the cortical changes underlying membrane separation. The experiments now cited are concerned with the *rate* of membrane separation in dilute sea-water.

I found that uninseminated eggs of *Arbacia* exposed to tap or distilled water (5 drops of eggs plus 5·5 cc. of tap or distilled water) on return to sea-water frequently showed 95 per cent. beautifully separated membranes. I therefore made observations on the egg while in the dilute sea-water, with the following results:

> 10 lots of uninseminated eggs from 10 different females exposed to tap water — 5 drops of eggs plus 5·5 cc. of tap water in each case in a watch glass mounted under the low power of the microscope. The time to membrane separation in the majority of the eggs noted with a stop watch:

No.	1	2	3	4	5	6	7	8	9	10
Time in seconds to membrane separation while eggs in tap water	7	5	5	6	5	8	5	8	5	5

10 lots of eggs from 10 different females exposed to distilled water — 5 drops of eggs plus 5·5 cc. of distilled water — gave time to membrane separation around five seconds.

In order to be sure of these results I would take the time to membrane separation of eggs of each female in the dilute sea-water at least three times. Since 1923, I have made it part of my routine work to determine the rate of membrane separation in distilled water. In this way determinations have been made on eggs from a large number of females throughout each breeding season. *Eggs in optimum condition give membranes in tap or distilled water in five seconds and in some cases even earlier.*

The experiments cited show that highly diluted sea- water initiates membrane separation at a more rapid rate than spermatozoa. What is more, if eggs are inseminated and exposed to tap or distilled water before membrane separation, the membranes come off in the dilute sea-water at the same rate as from uninseminated eggs. This observation first made in 1920 was repeated at great length during 1922 and subsequently. There is thus no criterion here for the difference between an uninseminated and inseminated egg. I present now a brief summary of the experiments made on this point during the week, July 21—27, 1922:

Effect of tap water following insemination in sea-water. Eggs inseminated in sea-water and 5, 10, 15 and 20 seconds later exposed to tap water — 5.5 cc. of tap water plus 5 drops of eggs. Results on three lots of eggs from each of twenty females show that the membranes come off from the eggs while in the tap water. The time for membrane separation is five seconds. On return to sea-water these eggs develop. Uninseminated eggs in tap water give the same rate of membrane separation.

In 1923 these experiments were repeated with distilled water with the same results. In addition I found that eggs inseminated with a more dense sperm suspension than that usually employed and placed in distilled water three seconds after insemination form membranes at the same rate as uninseminated eggs in tap or distilled water; such eggs returned to sea-water fifteen to thirty seconds later develop. This appears to me to be a highly interesting observation. Practically, there is no difference between the response of the uninseminated egg and the inseminated egg, that has not yet separated a membrane, to treatment with dilute sea-water. That sperm are attached before the dilute sea-water calls forth membrane separation cannot be doubted since

the eggs develop after thirty seconds exposure on return to sea-water. Observations show that sperm lose their fertilizing power if exposed to tap or distilled water since eggs inseminated with sperm suspension exposed to the action of tap or distilled water for thirty seconds respond as eggs exposed to tap or distilled water alone; that is, the sperm do not fertilize the eggs.

These experiments concerning the effect of dilute sea-water on inseminated eggs give some hint as to the rapidity of the fertilization-reaction. The reaction must be practically instantaneous —the time elapsing between insemination and the reaction being largely a function of the density of the sperm suspension employed: the more dense the suspension the more rapidly the fertilizing spermatozoa reach the eggs. Thus, if eggs are heavily inseminated, and three to five seconds later are exposed to distilled water, on return to sea-water the per cent. of development is increased. This might be objected to on the ground that polyspermy is induced and so vitiates the experiment. It will be shown beyond, however, that this is not a valid objection.

The experiments with dilute sea-water are also suggestive for an explanation of the mechanism of membrane separation[1]. They would seem to lend support to the surface tension theory of membrane separation (TRAUBE, HEILBRUNN, 1915). However, earlier observations of mine, JUST, 1922 (questioned by HEILBRUNN, 1924) showed that eggs of *Arbacia* (and

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[1] When the uninseminated egg is exposed to tap or distilled water the intake of water by the cortex brings about distention of the membrane so rapidly that it stands off from the egg. When, on the other hand, the uninseminated egg is exposed to strong hypertonic sea-water the egg contracts from the membrane showing at first cortical strands connecting vitellus and membrane; these strands collapsing leave a clear perivitelline space. Inseminated eggs before membrane separation give one the impression of a temporary increased rigidity of the cortex. This condition is exaggerated in eggs with delayed membrane separation showing thickened cortices (cf. my observations on eggs of *Echinarachnius*); this rigidity is quickly followed by disintegration of the cortex except at first for strands extending to the membrane. The membrane due to pressure now set up by the solution of the cortical colloids below it distends and stands off from the egg. If this interpretation be correct, one may conclude that insemination has two effects: first, increassed rigidity of the cortex and second, cortical breakdown which increases the pressure in the perivitelline space. But in addition, the cortex builds up the hyaline plasma layer—delicate radial strands later covered by an extremely thin membrane—which suggests contraction. Thus, neither hypo- nor hyper- tonic sea-water initiates membrane separation in exactly the same manner as the spermatozoon; the former brings about rapid distention of the membrane; the later, rapid contraction of the egg initiated in the cortex.

of *Echinarachnius*) separate membranes *while in hypertonic sea-water* — a finding which BATAILLON and later BATAILLON and TCHOU have obtained with the eggs of several urchins. HEILBRUNN, 1915, makes a strong point of the fact that "hypertonic membranes" are swollen membranes, and, therefore, quite different from those obtained by insemination: "hypertonic membranes" do not collapse in sea-water containing egg albumen but sperm induced membranes do, as LOEB years ago showed. BATAILLON, on the other hand, finds that "hypertonic membranes" do collapse in such sea-water.

Effect of exposure to dilute sea-water during the period of membrane separation

I refer again to Table II wherein the reader will note that inseminated eggs cleave on return to sea-water following an exposure of fifteen or thirty seconds to tap water. Previously I had noted when carrying these eggs through to the pluteus stage a peculiar form of blastulae — spheres larger than the normal made up of cuboidal cells except at one pole where the cells are squamous. The cilia are much longer than those of the control. In 1923 during July and August I made a thorough-going study of the origin of these forms.

If inseminated eggs of *Arbacia* be exposed to tap or distilled water — 10 cc. of water plus 5 drops of eggs — and then transferred to 250 cc. of sea-water a certain per cent. develop into swimming forms. This *percentage* depends upon the length of the exposure; the *normality* of the swimming forms depends upon the time after insemination that the exposure is made. The highest per cent. of large blastulae with the long cilia results from exposure to the dilute sea-water around 45 to 60 seconds after insemination. In some cases every single blastula resulting from exposure at this time is of this type. A few citations follow:

July 6. Eggs from each of four females in turn at 10 second intervals up to three minutes after insemination exposed to tap water for *one* minute then placed in 250 cc. of sea-water.

July 7. Eggs similarly treated with tap water for *three* minutes.

July 9. Eggs from one female divided into three lots. Lot A; exposed to tap water for fifteen seconds, 15, 30, 45, 60, 120, 180 and 240 seconds after insemination and returned to sea-water. Lot B: as Lot A except exposure is for *thirty* seconds. Lot C: fifteen seconds exposure 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 120, 180 and 240 seconds after insemination.

In all these experiments large blastulae were found as the result of exposures 45 to 100 seconds after insemination. The highest per cent. of abnormal blastulae was found in the exposure made 45 to 60 seconds after insemination.

July 16. Eggs exposed to tap water for fifteen seconds, 15, 30, 45, 60, 75, 90, 120, 135, 150, 165 and 180 seconds after insemination. In each case eggs returned to 250 cc. of sea-water.

A few abnormal blastulae found among the eggs exposed 45, 75 and 90 seconds after insemination. 100 per cent. large blastulae result from eggs exposed 60 seconds after insemination.

July 17. Eggs exposed for thirty seconds 30, 45, 60, 75, 90 and 100 seconds after insemination gave 17, 73, 76, 64, 51 and 30 per cent. respectively of abnormal blastulae with cuboidal cells and long cilia.

Without further citations I think that we may conclude that here is a striking case of *differential recovery* (cf. CHILD's important work). Following insemination eggs at given intervals receive the same treatment but they do not respond in like manner—they show a maximum susceptibility that lies around 45 to 60 seconds after insemination. The explanation is equally clear: at the time of the cortical changes incident to the separation of the vitelline membrane the eggs are susceptible to hypotonicity though this susceptibility does not sharply reveal itself. A permissable assumption is that the membrane separates from the egg explosively and a wave of restitution follows in the wake of the wave of the cortical breakdown underlying membrane separation. This wave moves at a rate sufficiently rapid so that cytolysis does not take place in the dilute sea-water as rapidly as in the egg of *Echinarachnius*. The eggs are nevertheless hard hit as we realize in the blastula stage where recovery is not complete.

Production of extra-ovates

After the eggs have passed stages favorable for the production of abnormal blastulae, i.e., when the membrane is equidistant from the egg at all points and the perivitelline space of the same width throughout, they enter the stage of maximum resistance to hypotonicity (cf. R. S. LILLIE). The membrane now an inert shell no longer plays any part in the metabolism of the egg; the mobile hyaline plasma layer is the plasma membrane of the egg regulating exchange with the environment. Exposure of the egg to distilled or tap water now gives a high percentage of

extra-ovates. This effect of hypotonicity first noted by LOEB has been studied also by R. S. LILLIE. Some workers, (cf. RAWITZ, YATSU), however, have experienced difficulty in obtaining extra-ovates that form double embryos.

I find that it is easy to obtain 80 to 90 per cent. extra-ovates by placing the eggs in tap or distilled water three minutes after insemination. Such a dilution is more effective than the less dilute solutions used by LILLIE. I simply mount the eggs in the tap or distilled water under the microscope and watch until the cortex ruptures and the cell contents begin to flow out leaving a part behind within the ruptured membrane. Undoubtedly the formation of extra-ovates is due to the state of the egg cortex at this time. The eggs, however, must be of optimum condition and the fertilization-reaction must have been optimum. *Extra-ovates do not form readily in eggs in poor condition.* A single experiment may be cited:

August 4, 1923. Eggs from 6 females inseminated in turn and exposed to tap water at 30 seconds intervals up to ten minutes after insemination. Eggs from each female were successively inseminated and exposed to tap water until the most favorable stage for the production of extra-ovates was learned. The time for the formation of the extra-ovates in the dilute sea-water was noted. The results may be summarized:

No. Female	Time of insemination	Time of formation of highest percent of extra-ovates	Time in seconds for formation of extra-ovates
1	1:48 P. M.	1:51 P. M.	25
2	1:56 „	1:59 „	40
3	2:08 „	2:11 „	30
4	2:19 „	2:21·5 „	32
5	2:35 „	2:38 „	35
6	3:01·5 „	3:04 „	23

Brief reference may now be made to some other experiments made during several seasons:

1. Uninseminated eggs treated with tap water and as quickly as possible after separation of the membrane removed to sea-water were at intervals again exposed to tap water. No extra-ovates formed.
2. Inseminated eggs exposed to tap water five to twenty seconds after insemination for ten seconds gave extra-ovates when again returned to tap water three minutes after insemination.

3. Eggs induced to form membranes by treatment with xylene and toluene gave no extra-ovates when placed in tap water.
4. Eggs with "butyric acid" membranes gave about three per cent. extra-ovates.
5. Eggs treated with "butyric acid" for thirty-five seconds, five to twenty seconds after insemination gave extra-ovates when exposed three minutes after insemination.
6. Shaking, centrifuging, putting the eggs through bolting silk under pressure gave extra-ovates three minutes after insemination.
7. Uninseminated eggs treated with hypertonic sea-water frequently form buds on return to sea-water. (See JUST, 1922).

Nos. 1, 3, and 4 may be of importance as a means of analyzing the mechanism of membrane separation by experimental procedures. Nos. 2 and 5 would seem to indicate that in the combination of sperm and experimental agent the effect of the sperm predominates. No. 6 emphasizes that one must exercise care in treating eggs after full membranes are formed. To this point I shall return. The buds formed by eggs in sea-water after exposure to hypertonic sea-water (No. 7) are not extra-ovates in the sense here used since these buds no matter how large never develop (see JUST, 1922). On the other hand, extra-ovates from fertilized eggs always develop, remaining attached to the part of egg within the membrane and thus producing twin swimming forms if the connecting bridge of cytoplasm does not break.

Study of both the living and the sectioned extra-ovate eggs shows that the first cleavage spindle may lie in any plane with reference to the axis of "extra-ovation". It may lie wholly in that part of the egg enclosed by the membrane parallel or at right angles to the axis of "extra-ovation". At the first cleavage, therefore, the extra-ovate may not in the latter case contain a daughter nucleus. If one pole of the first cleavage spindle lies in the extra-ovate, the cleavage plane passes through or parallel to the cytoplasmic bridge connecting the two portions of the egg. The original hyaline-plasma layer is around that part of the egg within the membrane and not around the extra-ovate[1]. Mitosis is induced by a zygote nucleus not by the sperm and the egg nuclei independently.

[1] The vitelline membrane of the egg of *Arbacia* present before insemination is built by the egg; so too after insemination is the structurally well defined hyaline plasma layer. Neither is to be compared with the films (precipitation membranes?) around endoplasmic spheres, that is, egg fragments without cortex, or with films around ruptured portions of cytolyzed eggs. There is undoubtedly regeneration of the hyaline plasma layer around extra-ovates; I regret, however, that I have not studied this in sectioned eggs.

A word in passing concerning the optimum period for the production of extra-ovates may not be out of place here. Workers (cf. MORGAN, YATSU) have noted that soon after fertilization the plasma of echinid ova increases in viscosity as revealed by the greater ease of cutting up the eggs into fragments. The predominating factor in this increased viscosity must be cortical—the greater ease in cutting the egg falls in with the optimum period for the production of extra-ovates. This production is itself an expression of increased viscosity; and „extra-ovation" itself is due to the altered physical state of the hyaline plasma layer at this time. Moreover, although the method of measuring changes in viscosity of fertilized egg cells by centrifugal force so beautifully refined by HEILBRUNN fails to reveal any marked changes in the endoplasm, as indicated by the width of the hyaline zone, until ten or fifteen minutes after insemination (HEILBRUNN, 1915), yet we know that at the periphery of the cell there is increased viscosity soon after insemination as shown by the behaviour of the pigment granules[1]. In the uninseminated egg (*Arbacia* and eggs of other echinids) the pigment granules lie near the surface. These are easily massed as a disc by centrifugalization. After insemination the granules are held at the cortex and are not readily displaced by centrifugal force. Moreover, in eggs inseminated after centrifuging the disc of pigment remains unaltered throughout the cleavage stages.

Because these results are all cortical effects they become of value for the interpretation of the fertilization-reaction. For the most part I have presented the data without comment. These are no casual observations but the accumulation of several seasons' work. As far as I could determine every experiment was made under uniform conditions;

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[1] I have other evidence of this increased viscosity. Uninseminated eggs may be drawn out into fine filaments by putting them among fibres of lens paper; they may be put through bolting silk the mesh of which is less than $1/4$ the diameter of the egg. This also is an indication of the elasticity of the vitelline membrane. This is true of other egg cells—cf. BECKWITH's work on eggs of hydroids deformed by centrifugal force. Paramoecia likewise can squeeze themselves through fine tubes and assume most bizarre shapes returning again to normal when the pressure is released.

After insemination *Arbacia* eggs deprived of membranes do not flow in fine filaments among fibres of lens paper, nor will they readily pass through bolting silk the diameter of whose mesh is half that of the egg. Indeed, I find that inseminated eggs before membrane separation will not pass through this bolting silk which a few seconds before as uninseminated eggs they passed through easily. But this may not be so much evidence for increased viscosity as for increased pressure in the cortex below the membrane about to separate.

each was repeated several times during four seasons. I realized after the work got well under way that these data served to establish some criteria for the optimum fertilization capacity of this egg. This therefore meant that I had to test every lot of eggs for 1) the time to membrane separation, 2) the quality of membranes (whether narrow, eccentric, or full with perivitelline spaces of equal width throughout), 3) capacity to separate membranes in tap or distilled water, and 4) capacity to form extra-ovates and the speed at which these form. Such tests revealed that the results depend upon the physiological condition of the eggs.

Slowly reacting eggs

There is evidence that a given lot of eggs uncontaminated with perivisceral fluid showing high fertilizin content as revealed by the power of the sea-water above them to agglutinate species sperm, form at almost uniform rate full clearly defined membranes equidistant from the egg at all points. The uniform rate of membrane separation, granted proper insemination with freshly prepared and clean suspensions of sperm in optimum condition, is remarkable. The merest tyro can discern that the membranes stand off from such eggs at sixty seconds after insemination; with careful observation he can determine that membrane separation begins twenty-five seconds earlier. One hundred twenty seconds after insemination the membrane is separated from the spherical egg by a wide perivitelline space of equal width throughout.

There are lots of slowly reacting eggs, however, whose rate of membrane separation is greater than this. Such eggs, moreover, fail to exhibit uniformity of *rate* in separating membranes. Similarly, such eggs may separate membranes so closely stuck to the surface of the egg that some workers might say that membrane separation does not take place. These are "tight" membranes closely stuck to the thickened cortex; failure of normal cortical break-down reduces the width of the perivitelline space to a minimum[1]. All gradations from these conditions to the normal are encountered. Thus, the perivitelline space may not

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[1] Here, doubtless, lies the explanation of HEILBRUNN's "swollen membranes". In eggs of both *Arbacia* and *Echinarachnius* I have observed under various experimental conditions that the cortex thickens beneath a thin membrane closely stuck to the egg surface. In some cases this membrane undoubtedly disappears (see my paper on the fertilization of *Arbacia* eggs in KCN-sea-water, Anat. Rec. **37**, 1927.) Cf. also my paper on *Echinarachnius,* Biol. Bull. **36**, 1919, page 44; this description applies to eggs with thick cortices to which the membranes are closely applied.

be of equal width throughout and so the membrane is eccentric; in some cases the eggs are not spherical but flattened at one pole.

Finally, the membrane may be stuck to the egg surface in one zone and elsewhere separated. In all these conditions one can rule out the sperm as responsible for the effects by making inseminations with sperm from each of several males in turn on eggs of the same female in which case the eggs respond alike. The sperm from each male in turn may be tested on eggs known to be in the optimum condition; if the sperm induce normal membranes this proves that the sperm are not responsible for the effects observed.

Uninseminated eggs from lots known by previous inseminations to be in the best physiological condition as measured by the rate and character of membrane separation when exposed to tap or distilled water show membranes in five seconds. About three minutes after insemination in sea-water such eggs form extra-ovates in twenty-five to forty seconds. Eggs in poor condition, as shown by their membranes, give poor or no response to treatment with tap or distilled water. Thus:

a) Five lots of eggs each from a different female showed after insemination membranes separating at a varied and slow rate—two to four minutes. Uninseminated eggs from these same females gave an average time for membrane separation in distilled water of sixteen seconds. Other lots of eggs from these females were inseminated and at intervals placed in distilled water. They gave an average of thirteen per cent. extra-ovates in an average time of seven and one half minutes after insemination. Average time to extra-ovate formation, sixty seconds.

b) Four lots of eggs which were known by the poor quality and rate of membrane separation to be below standard, when exposed uninseminated to tap water gave membranes as follows: 15, 17, 14 and 15 seconds respectively. These eggs formed extra-ovates in 5, 5·5, 6, and 7 minutes after insemination. They remained in the tap water for *85 seconds* before extra-ovates were noted.

c) Five lots of eggs, known in the morning by trial inseminations to be in optimum condition, when inseminated after seven hours in sea-water gave very poor membranes. Uninseminated eggs from these same females gave *no* membranes when placed in tap water. They formed a few extra-ovates in the average time of five minutes after insemination but only after *three minutes* exposure.

I could cite other experiments which would give the same story. Frequently I failed to procure either membranes or extra-ovates in tap

or distilled water. Before I realized what the significance was I worked to obtain membranes and extra-ovates on eggs not previously tested for the membrane reaction following insemination. After I realized that the physical behaviour of the eggs in hypotony is an index of their physiological condition I appreciated the cause of my failures.

On the basis of these findings I should say that the worker who wishes to use the eggs of *Arbacia* in their best possible condition should ascertain, in addition to the facts that the eggs are uncontaminated with perivisceral fluid and have a high fertilizin content, they form full membranes at the minimum and uniform rate, that they separate membranes in distilled or tap water rapidly and that after insemination they give extra-ovates in a high per centage of cases after the membranes have separated to their greatest distance from the eggs.

There remain now two other criteria for the optimum fertilization capacity: the presence of the egg jelly and the effect of heavy insemination. These we shall discuss in turn.

RÔLE OF THE EGG JELLY IN THE FERTILIZATION-REACTION

Periodically the statement recurs that the jelly enclosing echinid ova is necessary for the separation of the vitelline membrane. Thus GRAY[1], following MC CLENDON and ELDER, claims that eggs of *Echinus*

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[1] In a footnote GRAY says that after he had reached the conclusion that the jelly is necessary for the separation of the vitelline membrane he found that MCCLENDON in a paper published eight years previously likewise had concluded that membrane "formation" is due to precipitation which only takes place in the presence of the egg-jelly. According to HOBSON, however: "GRAY admits (personal communication) and the present writer has also observed, that prolonged washing in normal sea-water restores to a certain extent the power of membrane formation .... The acid sea-water described by GRAY has a pH of about 2·4. This is more acid than is necessary for the rapid removal of the zona pellucida. Sea-water pH 3·2, or higher, removes the zona pellucida quite satisfactorily, and does not prevent membrane formation".

Egg-jellies are worthy of more study. Those surrounding ova of Asteroids and of Holuthurians show striations which thus make them appear canaliculated or give the ova the appearance of bristling with filaments. The structure of the jelly around the egg of *Asterias* which is enclosed by squamous epithelium was first described by FOL. During maturation and fertilization of this egg, I find that the epithelium gathers in a disc at one pole; according to FOL, however, it drops off in shreds when the egg comes into sea-water.

When the inseminated egg of *Echinarachnius* is exposed to the action of tap or distilled water during the process of membrane separation, the pigment in its jelly hull is decolorized, the wave of decolorization beginning in the zone of cortical disintegration brought on by the exposure (JUST, 27b).

freed of jelly do not separate membranes after insemination. Certainly this is not true of the eggs of *Arbacia,* according to Harvey and to Lillie, 1914. I too have found that this egg without jelly, like that of *Echinarachnius,* will separate the membrane.

I have frequently used shed eggs that were devoid of jelly as shown by examining them in a suspension of Chinese ink in sea-water. Such eggs fertilize and show separated membranes. It is however true that eggs either shed or taken from the ovaries which show no jelly are not in optimum condition; similarly, eggs that have stood in sea-water for some time lose their jelly. Goldfarb has shown that with increasing age outside the body the eggs of *Toxopneustes, Hipponoë,* and *Arbacia* reveal "progressive changes in size, in loss of jelly, in retarded membrane formation, in decreased total cleavage and decreased rate of cleavage". But clearly this does not mean that loss of jelly *per se* makes for the poor condition of the eggs, for if the jelly be gently but rapidly removed by successive washings in sea-water, the eggs are by no means impaired.

The following experiments were frequently made: Eggs from one female were divided into two equal lots. One lot was allowed to remain in sea-water with jelly hulls intact; from the other, the jelly was removed by successive washings during an hour. Approximately equal numbers from each lot were placed in 2 cc. of sea-water giving dense suspensions. Each suspension was inseminated. The eggs of that lot with jelly hulls showed well separated membranes; those of the other lot without jelly hulls showed poorly separated membranes. If, however, inseminations were made in large volumes of sea-water, eggs from both lots separated membranes equally well.

Again, if a thick drop of eggs from the lot with jelly was spread on a slide and inseminated membranes were separated. But an equal drop of eggs from the lot without jelly treated in the same way showed few if any well separated membranes. If, however, these latter were inseminated while freely suspended in sea-water and then placed on the slide they separated membranes.

This type of experiment indicates that eggs without jelly if neither crowded nor flattened at the time of insemination will separate membranes. The jelly hull would thus seem to be a buffer for the egg. It would also seem to be a protection for the egg in other ways. For example, my experiments show that the blood inhibitor is more difficult to remove from jelly-free eggs than from those with jelly.

That the egg-jelly may be removed without impairment of normal membrane separation is further evident from the following. Eggs of high fertilization capacity were put through bolting silk the mesh of which was about equal to the diameter of the egg. Examined in a suspension of Chinese ink in sea-water the eggs were found devoid of jelly. On insemination they separated beautiful full membranes; the rate and character of membrane separation were in no wise different from those of the control eggs within jelly hulls. The use of bolting silk to remove jelly is superior to any method that I know of.

Eggs put through bolting silk at the time of membrane separation or very shortly thereafter lose their membranes; they develop in perfectly normal fashion. This shows that the use of bolting silk is innocuous whereas removal of the membranes by shaking or by sucking the eggs up into fine bore pipettes is distinctly injurious[1]. BOVERI long ago, and PAINTER subsequently, showed that eggs shaken just after membrane separation tend to halt in the monaster stage. Application of centrifugal force at this time is also capable of producing abnormal development.

Hydrochloric acid and shaking are methods commonly employed for the removal of jelly. Both are apt to injure the eggs. Where such eggs fail properly to respond to insemination this failure is due to injury rather than to loss of jelly. Recently VLÈS, REISS and VELLINGER have pointed out that KCN in sea-water will remove the jelly from sea urchin eggs. In this connection I cite now some experiments of mine made in 1921.

Eggs of *Arbacia* were placed in 250 cc. of M/2000 and M/1000 KCN in sea-water. One hour later each lot was inseminated in the solutions. Four hours later the eggs showed no cleavage, only a broad streak about the nucleus. On return to sea-water 40 per cent. of the eggs cleaved an hour later.

Subsequently stronger solutions of KCN in sea-water were employed, the eggs being kept in these for varying periods of time up to two

[1] Cf. PLOUGH's recent results on the development of isolated blastomeres. This work is open to serious objection because of the method used for removing the membranes at a stage when the eggs are highly susceptible to centrifuging, to shaking, and to the pressure put upon them by drawing them up into fine capillary tubes. PLOUGH's results may be correct but it would have been far better had he used a method for removing the membranes that is less deleterious or had he removed the membranes at the time that he separated the blastomeres. Eggs of *Arbacia* and even more those of *Echinarachnius* are unable to withstand the drastic treatment PLOUGH gave them. And these eggs are not unique in this respect.

hours and inseminated in the solutions. These eggs never cleaved in the KCN sea-water, going only as far as the monaster stage — a large clear area extending over two thirds the diameter of the egg.

Observations were next made on the cortical response to insemination of eggs while in KCN sea-water after exposures varying from fifteen to one hundred twenty minutes. Such eggs separate very thin membranes over the entrance cones two minutes after insemination beneath which the cortices thicken and push up to the thin membranes. As the hyaline-plasma layers grow in width the membranes lose visibility. Three experiments are cited:

a) Eggs that have been in M/200 KCN in sea-water for 74 min. inseminated in the solution. Two minutes later membranes off over the cones. Well defined hyaline-plasma layers distinctly visible 6 minutes after insemination; membranes not visible.

b) Eggs that have been in M/200 KCN in sea-water for 63·5 min. inseminated in the solution. 2·5 minutes later membranes off, widest over the cones. 6 minutes after insemination cones are gone, hyaline-plasma layers well defined, membranes faintly visible. 11 minutes after insemination membranes no longer visible; wide hyaline-plasma layers.

c) Eggs that have been in M/200 KCN in sea-water for 80 min. inseminated in the solution. 1·5 minutes later membranes off beginning at entrance point of sperm. 10·5 minutes after insemination membranes barely visible over wide hyaline-plasma layers.

Experiments with M/100 KCN in sea-water gave similar results.

Had I not followed the process of membrane separation in these "KCN eggs" but had made my observations ten minutes after insemination I should doubtless have concluded that they did not separate membranes. Membrane separation is abnormal in KCN solutions, not because of loss of jelly but because of the injury to the cortex as revealed by its exaggerated activity, the increased size of the entrance cone, and the increased width of the hyaline plasma layer. These observations of the fertilization of eggs in KCN sea-water have led to a fruitful line of inquiry which I shall later report. But may I digress for a moment?

Many workers are agreed that KCN depresses oxygen consumption (cf. BUCHANAN's important note). How far then is oxygen necessary for the fertilization-reaction? And if, as I believe that I can show, the reaction is resolvable into stages, at what stage is oxygen necessary? With respect to oxygen, the fertilization-reaction may resemble muscle contraction. It would be interesting to know if fertilization be possible in *oxygen-free* sea-water.

One other point. These eggs in "KCN sea-water" develop as far as an abnormally large monaster stage — sperm penetration and formation of the zygote nucleus are normal — and the eggs remain thus for hours. On return to normal sea-water the eggs resume mitosis[1]. A high per cent. develop, though the plutei show abnormalities. The fertilization-reaction is thus complete despite failure of the eggs to divide while in the KCN solution. This is in striking contrast to inhibition of fertilization by blood or by copper (LILLIE, 1921).

We may conclude that the egg-jelly is not necessary for the separation of the membrane. The jelly acts as a buffer, a protecting coat. If it be removed by agents otherwise not harmful the process of membrane separation is normal. Harmful agents interfere with normal membrane separation because of their effect on the eggs themselves and especially on the cortex. However, eggs in optimum condition for fertilization should possess jelly.

It is evident from the foregoing that the egg of *Arbacia* in its response to insemination is a rapidly reacting system. A measure of its reaction rate might be approximated through study of heavily inseminated eggs. This appears to be true.

POLYSPERMY

Biological processes like nerve conduction and muscle contraction are alike capable of repetition; the fertilization-reaction on the contrary is non-repetitive. Once fertilized the egg or fragments thereof (but cf. MORGAN[2], 1895) are incapable of additional response to insemination (JUST, 1923). The inseminated egg remains indefinitely refractory to the action of the spermatozoon. How soon after insemination does this refractory stage set in? The evidence indicates that in the egg of *Arbacia* the fertilization-reaction is complete the instant that the spermatozoon touches its surface.

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[1] Three of my students, Messrs. ANDREWS, CHASE and DULANEY, have worked out completely the history of these eggs (sectioned material) through first cleavage.

[2] Says MORGAN: "The non-nucleated fragments may be entered by one or more spermatozoa, and this takes place indifferently whether fragments have been obtained before or after the process of fertilization has taken place. ...... nucleated egg-fragments which have been obtained after the spermatozoön has entered the egg may be fertilized, and the spermatozoa will also enter the non-nucleated pieces and there undergo their transformation into a segmentation spindle."

Using fragments of inseminated eggs of *Echinarachnius*, I was unable to confirm this point made by MORGAN (JUST, 1923).

If a 3 cc. suspension of *Arbacia* eggs uncontaminated with perivisceral fluid and in optimum condition as shown by tests of their fertilizin content, of the rate and quality of membrane separation, and of their response to tap or distilled water, be inseminated with the sperm suspension ordinarily employed for normal fertilization and later be reinseminated one second later with 1 cc. of dry sperm, polyspermy does not result. That sperm are attached one second after insemination may be demonstrated by squirting sperm into the egg suspension and flooding at once with fixing fluid, with adequate microscopical preparation.

Repetitions of this experiment with initially more dense sperm suspensions gave the same results. For example, to a 3 cc. suspension of eggs as quickly as possible after insemination were added 5 cc. of dry sperm. At 10 second intervals up to 2 minutes a drop of these eggs was carried over to 250 cc. of sea-water. The eggs developed into normal plutei without any signs of polyspermy.

Seven experiments were likewise made thus: Seven 3 cc. suspensions of eggs known to be in optimum condition from one female were inseminated with thin sperm suspension and with 1, 2, 3, 4, 5 and 6 drops of dry sperm respectively. Sixty seconds after insemination each lot of eggs was carried over to 250 cc. of sea-water. No polyspermy was noted.

In six such experiments eggs that had had a heavy insemination developed into top swimming blastulae before the controls (the average time for the former was 330 minutes, for the latter, 360). In a seventh experiment there was no difference in time to swimming at the surface. In all these experiments the blastulae began to rotate on the bottom of the dish at the same time.

Polyspermy in the eggs of *Arbacia*, in my experience, means eggs below normal. Eggs in optimum condition respond too rapidly to insemination to permit polyspermy. But any agent that injures the cortex cuts down the reaction time and thus renders polyspermy possible.

The classic investigation of the Hertwigs on polyspermy in echinid ova may be cited. Eggs were rendered susceptible to polyspermy by treatment with nicotine, strychnine, quinine, etc. With nicotine, for example, the cortex gives sharp evidences of injury. One such that I have often noted is the formation of large "entrance" cones. Susceptibility to polyspermy is comparable to capacity for cross fertilization since in both cases the cortex must be weakened to insure fertilization. This fact might also suggest, in those cases in which the possibility of cross fertilization is enhanced by heavy insemination, that it is not the *number*

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of sperm that initiates cross fertilization but the *activity* of the dense sperm suspension around the egg; this activity, by the high production of CO_2, for example, might so weaken the cortex to permit entrance of foreign sperm; this too in heavy insemination with species sperm especially if the eggs and sperm be densely suspended in a small volume of sea-water. Eggs inseminated with dense sperm suspensions should be quickly brought into large volumes of sea-water to obviate this.

That the block to polyspermy is in the egg cortex rather than in the separated membrane, as suggested by FOL, is I think, now generally admitted. The separation of the membrane is far too slow to act as a block. Moreover, we must keep in mind, as LILLIE (1919) has emphasized, that "the primary change in activation is not something visible in the morphological sense". The visible cortical changes, which differ in different ova, are themselves merely the sequelae of the fertilization-reaction which we assume to be the same in all types of ova though the speed of the reaction varies with ova of different species. It must be more than a coincidence that normal polyspermy occurs generally in eggs with large amounts of yolk and so with large surfaces. With large surface goes slow reaction time, hence polyspermy. Insect ova though often possessing micropyles, which one might consider a mechanism to block polyspermy are no exception, hence are frequently polyspermic.

It is only fair to point out, however, that the egg of *Cumingia* appears to be easily polyspermic. This egg has long been a puzzle to me. Its fertilization should be carefully studied. Such study should be made on eggs from animals known to be in the best possible condition; the animals should be used as soon after collected as possible. Further, the optimum time for fertilization after egg-laying should be rigorously determined; eggs of uncertain age after laying should not be used. If, for example, one were to pipette off eggs from a female during egg-laying which has been going on for some time, one would have a mixed population of eggs with respect to their age in sea-water. Such a mixed lot of eggs would mitigate against the observation if it prove that on coming into sea-water eggs of *Cumingia* gradually reach an optimum condition for fertilization then fall from this. Obviously, the age of the sperm suspensions employed likewise must be known.

In terms of LILLIE's fertilizin theory of fertilization, the fertilization-reaction is a chemical union between sperm and a cortically located ovogenous substance (LILLIE, 1919; LILLIE and JUST, 1924). Experimental polyspermy in normally monospermic eggs thus becomes an important criterion for the speed of this reaction. That this reaction takes

place at great speed is no argument in favor of a physical (electrical) theory as GRAY[1] seems to think though once the reaction is complete

[1] GRAY's assumptions merit more than passing notice. Says GRAY: "Each unfertilized egg is surrounded by a wide gelatinous zona pellucida; this substance appears to be of a proteid nature—it is readily soluble in dilute acids, and so we may infer that it is electro-positive. If the zona pellucida is not removed before fertilization then the electro-negative colloid set free when the lipoid layer of the vitelline membrane is emulsified will come into contact with a colloid of opposite electrical charge. Mutual precipitation must occur—and this, it is here suggested, is the origin of the fertilization membrane. If however, the zona pellucida be removed prior to fertilization, then no fertilization membrane is formed. Nevertheless the egg develops normally.

"The complete mechanism of 'membrane formation' may therefore be as follows:

"The unfertilized egg is surrounded by two membranes—the hyaline vitelline membrane, and the gelatinous zona pellucida. The vitelline membrane consists of a continuous lipoid structure, in which an electro-negative protein exists in solution as a dispersed phase (or below the lipoid structure is a layer of electro-negative protein). The zona pellucida consists of an electro-positive protein. When the continuous lipoid phase of the vitelline membrane is destroyed by emulsification, the enclosed protein comes into contact with the zona pellucida, i. e. with a colloid of opposite sign. Mutual precipitation must occur, giving rise to fertilization membrane. This membrane is impermeable to the remainder of the protein, which draws in water through the fertilization membrane by osmosis. In this way the fertilization membrane is extruded from the surface of the egg".

This would be beautiful if true. But unless the egg of *Echinus* be unlike that of *Arbacia* and of *Echinarachnius* the jelly is not necessary for the separation of the membrane. Nor is it certain that the vitelline membrane is made up as GRAY postulates. Moreover, I find it difficult to reconcile the quotation just given with GRAY's earlier statement (pages 423—424) that the membrane is pushed off the echinoderm egg as off that of *Nereis* egg, namely, by disintegration and hydration of the egg surface immediately under the vitelline membrane. Besides, the egg-jelly is present around the egg of *Nereis* after but not before fertilization.

Again, GRAY believes that it is the degree of activity of the sperm which determines one condition of fertilization, not its structure or chemical constitution. Thus in one statement he rules out all fertilization-reactions initiated by spermatozoa that normally show little or no movement—those of nematodes and decapod crustacea, for example; and what is more serious, specificity in fertilization which undoubtedly is due to chemical not physical structure. GRAY also strongly suspects that the developmental phase of fertilization is associated with two asters, one from the egg nucleus and one from the sperm. Were he sufficiently familiar with fertilization processes in various types of ova—or with fertilization in enucleated eggs—he could not long entertain this suspicion since ova differ with respect to the origin of the cleavage centres.

Finally, though I might cite other errors I make one more adverse criticism: GRAY does not understand the difference between agglutination by species egg-water—iso-agglutination—and that by toxic agents. Nor does he appreciate the difference between aggregation and agglutination of sperm. Since these are true, his criticism of LILLIE's fertilizin theory of fertilization falls to the ground.

physical epiphenomena may evidence themselves. Indeed, the *effect* of the fertilization-reaction may travel in a wave around the egg at a faster rate than the 0·00001 seconds that GRAY assumes. But this does not mean that the reaction itself is not chemical. There are cases of practically instantaneous chemical reactions.

Finally, eggs cannot be inseminated and exposed to tap or distilled water quickly enough to arrest the fertilization-reaction. Each egg that is inseminated separates a membrane in the dilute sea-water and on return to sea-water thirty seconds later develops. It was the possibility of such an arrest that led me to expose inseminated eggs to the action of dilute sea-water. As soon as I learned that dilute sea-water calls forth membrane separation, I made a number of these experiments. If sperm have reached the egg at the instant of exposure to hypotony though this lasts for thirty seconds the eggs develop on return to sea-water. The failure of polyspermic fertilization is both an index of the optimum fertilization capacity of the egg and a measure of the practical instantaneity of the reaction.

In conclusion, therefore, these observations and experiments strongly suggest some criteria for the optimum fertilization capacity of the egg of *Arbacia* in terms of its cortical response. They serve thus to emphasize anew the leading rôle played by the cortex in the fertilization-reaction. Every phase herein dealt with involves the cortex. This raises some interesting questions concerning not only the physiology of fertilization but also the physiology of development. It is of the latter that I now wish to speak.

RÔLE OF THE EGG CORTEX IN DEVELOPMENT

A notable structural characteristic of egg cells is their differentiation into endo- and ecto-plasm; in this respect they resemble other kinds of cells. Protozoa exhibit highly diverse cortical structure: trichocysts, "polar capsules", pseudopodia, contractile vacuoles" with their canals when present, myonemes, neuromotor apparatus. The nerve fibre may be regarded as cortical protoplasm drawn out to great length. As striated muscle cells develop, granules at first scattered throughout migrate toward the periphery forming rudimentary fibrillae. The flagellate spermatozoon is largely nucleus and ectoplasm. Rapidly reacting cells show cortical architecture or are relatively rich in cortex. So with the egg cell.

No one who has studied egg cells can fail to appreciate their cortical structure. The ova of *Nereis*, *Platynereis*, *Phascolosoma*, *Chaet-*

opterus, *Asterias*, *Arbacia*, *Echinarachnius*, etc., clearly exhibit a differentiation between endo- and ecto-plasm. Cortical architecture is thus an expression of a fundamental organization of the cell. But it is something more: the cortex is of significance for vital phenomena—the medium of exchange and of balance between the cell and its environment; and, in echinid ova at least, the seat of oxygen consumption and of heat production. As a response to insemination the ova mentioned above show definite cortical responses to insemination never again shown and with these consume oxygen (SHEARER and others) and produce heat (ROGERS and COLE)—echinid ova—in quantities never again equalled. The non-repetitive nature of the fertilization-reaction is due to these changes. Even though the egg be in stages of mitosis artificially induced (e. g., *Arbacia*, JUST, 1922) the cortical response to insemination may take place.

Moreover, from fertilization on and especially at each cleavage these ova exhibit changes in the cortex. The biologist with a leaning toward physical interpretation sees in the activity of the external protoplasmic layer manifestations of changes in surface tension, adsorption, precipitation, differences in electrical potential and the like. He interprets this layer of measurable thickness as a mere film more or less inert if not actually dead; he compares it to a collodion membrane. In some instances he sees in it no more than the surface of an oil drop. No one, however, who has studied the structure and activities of the cortex, observing its changes in width, its capacity for growth, its prolongations now here, now there can content himself with these naïve physical "explanations". I am wholly sympathetic with a physico-chemical approach to the problems of the cell. The elucidation of vital phenomena in terms of physico-chemical laws is surely desirable; but to ignore the complexity of structural changes and to simplify processes that are not simple can in the end lead only to confusion. I can only emphasize that the surfaces of egg cells may do all that these physically minded folk would have us believe—but they are not thereby mere surface films.

The egg of *Echinarachnius* during the process of membrane separation reveals a cortical gradient of susceptibility to dilute sea-water. There is doubtless at this time also a *cortical gradient of metabolism*, if in this egg, as in those of other echinids, there are increased consumption of oxygen and increased production of heat due to break-up of the cortex. Such a gradient one could doubtless demonstrate in the egg of *Arbacia* were not the process underlying membrane separation so rapid. However,

one can otherwise demonstrate a susceptibility to hypotony during this period. I refer to the effect of hypotony in producing the abnormal blastulae mentioned above. *This is a modification of development depending upon the state of the egg cortex at the time of exposure.*

Again, *the effect of hypotony on the inseminated eggs in producing extra-ovates is likewise a modification of development depending upon the cortical state.* An egg with one portion still within the membrane and attched to the extraovate develops twins. If the portions drop apart, two blastulae result.

Experiments still underway show that by this simple method of exposing eggs of *Arbacia* at various stages to dilute sea-water modifications of development result. *These depend upon the state of the cortex at the time of exposure.* It may well be, therefore, that not only the non-repetitive nature of the fertilization-reaction but also the *non-repetitive nature of each step in the progressive differentiation from egg to embryo depends upon progressive structural and physiological changes in cortical protoplasm.*

Cell lineage gives us little clue to the problem of differentiation. I have studied the cell lineage of the egg of *Nereis,* both living and in sections, in terms of the history and fate of the oil, yolk, and other cytoplasmic inclusions without coming to any clearer understanding of this problem. A theory of differentiation based on the distribution of pigment granules, in those eggs that possess such, is now of historic interest only. The gene theory of heredity, geneticists themselves admit—either tacitly, or by what the lawyers call undesigned testimony—to-day offers no answer. I do not for a moment suggest even that the experiments and observations here reported "prove" that the egg cortex is responsible for progressive differentiation. I do however firmly believe on the basis of the observations here reported and those to be reported that *the cortex plays a definite rôle in the physiology of development.* Future work alone will reveal to what extent this is more than a suggestion.

SUMMARY

1. Eggs of optimum fertilization capacity exhibit high fertilizin content and separate membranes uniformly and rapidly after insemination. Uninseminated, such eggs separate membranes in five seconds in tap or distilled water; they form extra-ovates in thirty seconds when

exposed to tap or distilled water three minutes after insemination. All of these reactions are associated with the best physiological condition of the egg.

2. Under proper conditions, eggs without jelly fertilize, separating normal membranes. They develop at well as eggs with jelly.

3. The speed of the fertilization-reaction is so great that polyspermy cannot be induced in eggs in optimum condition by any excess of sperm. Nevertheless, the fertilization-reaction may be chemical in nature.

4. These results serve to emphasize anew the leading rôle played by the cortex in the fertilization-reaction.

5. The cortex of the egg is significant in other ways for the physiology of development.

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