The Block to Polyspermy in Sturgeon and Trout with Special Reference to the Role of Cortical Granules (Alveoli)

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INTRODUCTION

When a monospermic egg is fertilized, the attachment of the fertilizing spermatozoon to the egg surface provokes a protective reaction that prevents all but one spermatozoon from entering the egg; this is the block to polyspermy. The nature of the defence mechanism against polyspermy has been the subject of many investigations, performed mainly on the eggs of sea urchins.

It was established that on fertilization of the sea-urchin egg a cortical reaction takes place consisting of morphological changes in the cortical layer, spreading in wave-like fashion from the point of the spermatozoon attachment over the whole egg surface: the light scattering and the intensity of birefringence undergo changes; extrusion of mucopolysaccharide granules takes place, accompanied by the separation of the vitelline membrane and its transformation into the fertilization membrane; the perivitelline space appears and the hyaline layer is then formed at the egg surface (see Runnström, 1952; Rothschild, 1956; Allen, 1958). Several workers have presented data suggesting that some of these changes take part in the inhibition of polyspermy.

However, the onset of the cortical reaction is preceded by a rather prolonged latent period, of the order of 10–20 seconds, while some 10–26 seconds more are necessary for the granule breakdown to sweep over the whole egg surface (Moser, 1939; Allen & Griffin, 1958). During such a time-lag many spermatozoa will collide with the egg. It was, therefore, suggested that the block to polyspermy can be ensured only by changes preceding the cortical reaction (Moser, 1939). Rothschild & Swann, 1952, Rothschild, 1956, brought forward the hypothesis that the process of block is diphasic: in the first phase invisible changes (the ‘fertilization impulse’) cover the egg surface within the course of a few seconds, leading to an incomplete block; the second phase corresponds to...
the visible cortical reaction and is completed with the formation of a sperm-impermeable layer (in *Psammechinus miliaris*, at a temperature of 16–18° C., in an average time of 63 seconds after insemination).

The purpose of this paper is to study the inhibition of polyspermy in fish and, in particular, to test on them the hypothesis of diphasic block. The mechanism of the block to polyspermy in fish has not been studied up to the present. Analysing the data found in the literature on the process of fertilization in fish, Rothschild (1958) suggested that the block to polyspermy was realized in them by means of the fertilization impulse.

The surface layer of teleost eggs is known to include cortical alveoli (cf. Rothschild, 1958), while that of Acipenseridae has cortical granules (Dettlaff, 1957, 1961); both of them contain mucopolysaccharides. As in the case of sea urchins, the contact of the fertilizing spermatozoon with the egg in fish initiates a wave of granule (or alveolus) breakdown. It seems interesting to elucidate whether this process plays some role in the block to polyspermy.

In order to find out the part played by the fertilization impulse and by the granule (alveolus) breakdown in the block to polyspermy, a series of experiments was done. (1) The course of the cortical reaction in normal development and after treatment with polyspermy-inducers was compared. (2) Sperm attachment and the onset of the cortical reaction were separated in time by activating the eggs and then inseminating them after various time-intervals; this experiment makes it possible to state the exact moment of the cortical reaction at which the penetration of the spermatozoon becomes impossible. (3) It was investigated whether the perivitelline fluid into which the content of the cortical alveoli is passed, inhibits the penetration of spermatozoa; with this aim in view (a) the perivitelline fluid was removed from activated eggs which then were inseminated, and (b) the direct action of the perivitelline fluid on spermatozoa was tested.

**MATERIAL AND METHOD**

The sturgeon *Acipenser gülstenstädti colchicus* V. Marti and the lake trout *Salmo trutta* L. morpha *lacustris* Linné were the objects of investigation. Their eggs are covered by tough membranes, and the spermatozoa can reach the surface of the cytoplasm only through micropylar canals.

Sturgeon usually possess about 10 micropylar canals forming a group in the region of animal pole. In eggs taken from different females their number varies from 1–2 to 43. Under normal conditions of insemination only one spermatozoon enters the egg as a rule; while on insemination with fairly dense sperm suspensions, polyspermy is frequently observed (Ginsburg, 1957a, 1959). Simultaneous presence of several unchanged spermatozoa in fertilized eggs of *A. ruthenus* and *A. gülstenstädti* was described by Perssov (1954, 1956, 1957). On these grounds he concluded that physiological polyspermy was natural in Acipenseridae. The penetration of supernumerary spermatozoa into the eggs of sturgeon, however, provokes far-reaching disturbances in development and
early death of the embryo (Ginsburg, 1953, 1957a) which is characteristic of physiologically monospermic eggs.

The egg of the trout has one micropyle, and specially carried out experiments showed that polyspermy is never observed, whatever the density of the sperm suspension at insemination. Thus the second object of investigation is also physiologically monospermic.

Experiments on sturgeon were carried out at the Rogozhkin sturgeon hatchery (the Lower Don) in the spring of 1958 and 1959. Eggs and sperm were procured from fish matured as a result of an injection with a homogenate of acetonized sturgeon pituitaries. Experiments on trout were performed at the Svir hatchery (Leningrad region) in October–November 1959. The fish were kept in river stews up to maturity.

The procedure of individual experiments will be dealt with in the corresponding sections. In the experiments that required an estimation of polyspermy incidence, the eggs with the characteristic cleavage pattern were counted. Hagström & Allen (1956) pointed out, in experiments involving nicotine treatment of sea-urchin eggs, that abnormal cleavage could not be used as a criterion of polyspermy. In the experiments on Acipenseridae, however, polyspermic eggs were always readily distinguishable from monospermic ones (even from those cleaving atypically) by the presence of superfluous blastomeres. The incidence of polyspermic eggs is completely revealed only at the stage of the second cleavage division, when counting was carried out. Special experiments showed that the percentage of eggs with superfluous blastomeres at this stage practically coincides with that of polyspermic eggs found in the same aliquot of eggs by means of cytological investigation (Ginsburg, 1957a and recent unpublished data). It is permissible therefore to regard the criterion chosen as a reliable one.

For cytological investigation the eggs were fixed with Sanfelice fluid and embedded in paraffin. In sturgeon only the animal part of the egg was as a rule embedded; the vegetative part was taken only for special purposes, since, because of its large amount of yolk, it is difficult to obtain satisfactory sections. In trout the cortical cytoplasmic layer or, later, the blastodisc was removed from the surface of the yolk. Serial sections of 7 μ, less often of 10 μ, were prepared. They were stained with Heidenhain’s azan stain (cortical granules and the colloidal content of cortical alveoli are electively stained with the aniline blue) and with Heidenhain’s iron haematoxylin. Drops and smears of trout-sperm were fixed with the vapour from 1 per cent. osmium tetroxide solution and stained with iron haematoxylin.

EXPERIMENTAL RESULTS

1. Changes in the duration of the latent period and of the spread of the cortical reaction on pretreatment with polyspermy inducers

The first attempt to approach the analysis of the block to polyspermy in fish consisted in a comparison of changes occurring in the sturgeon egg at
normal monospermic fertilization with those at experimental polyspermic fertilization induced by the action of chemical agents. It was shown on sea-urchin eggs (Rothschild & Swann, 1950; Rothschild, 1953; Hagström & Allen, 1956) that one such agent, namely nicotine, acts through the egg, prolonging the conduction time of the block to polyspermy (and not through increase of sperm motility). According to some results (Rothschild & Swann, 1950) nicotine does not affect the visible changes in the cortical layer, but according to others (Hagström & Allen, 1956), it inhibits the breakdown of the cortical granules and the formation of the hyaline layer. A retardation of the cortical reaction in sea-urchin eggs was also found on treatment with urethane, another polyspermy-inducing agent (Sugiyama, 1956).

In preliminary experiments on polyspermy induction in Acipenseridae under the action of urethane we were also able to confirm that its action was realized not through the spermatozoa but through the eggs. When the sperm was diluted in urethane solution, the percentage of polyspermic eggs either did not differ from that in the control or was less, but when eggs were treated prior to fertilization with the same solution, the polyspermy percentage constantly increased (Table 1).

| Table 1 |

<table>
<thead>
<tr>
<th>Incidence of polyspermy after treatment of sturgeon eggs and spermatozoa with 2 per cent. urethane</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Sperm density, 5.55 × 10⁷/ml.; temperature, 19.7° C.)</td>
</tr>
<tr>
<td>Eggs treated</td>
</tr>
<tr>
<td>Spermatozoa treated</td>
</tr>
<tr>
<td>Control (without treatment)</td>
</tr>
</tbody>
</table>

With the purpose of studying the mechanism of the action of polyspermy inducers, urethane, ethyl ether, and acetone were used. Ripe sturgeon eggs were pretreated for 5 minutes with a 2 per cent. urethane solution in river water. In two other variants of the experiments they were exposed to the action of ethyl ether or acetone vapours. To do this the eggs were distributed in one layer on gauze stretched over a glass ring; the ring was then immersed for 1 minute in a dish containing ether or acetone in such a manner that the layer of eggs was 10–12 mm. from the surface of the fluid. Immediately after the treatment the eggs were inseminated and aliquots of them were fixed at various time-intervals (from 10 seconds to 2 minutes) for cytological investigation (203 eggs were studied in sections). The remaining eggs developed up to the stage of the second cleavage division; the count of polyspermic eggs showed all the treatments applied to be effective (Table 2).
TABLE 2

Incidence of polyspermy in sturgeon eggs pretreated with urethane, acetone, and ethyl ether

(Sperm density, 7.04 × 10⁷/ml.; temperature, 21.2° C.)

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Polyspermic eggs (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 per cent. urethane solution, 5 minutes</td>
<td>46.2</td>
</tr>
<tr>
<td>Acetone vapour, 1 minute</td>
<td>30.3</td>
</tr>
<tr>
<td>Ethyl ether vapour, 1 minute</td>
<td>26.9</td>
</tr>
<tr>
<td>Control (without pretreatment)</td>
<td>12.4</td>
</tr>
</tbody>
</table>

In an unfertilized egg the surface cytoplasmic layer that contains the cortical granules closely adheres to the egg membrane (Plate 1, fig. A). Ten seconds after insemination it can be seen that the cortical layer in almost all the animal region of the egg is strongly vacuolated, as a result of which the granules acquire a columnar form; the extrusion of the granules starts at some points, with the concomitant separation of the membrane from the egg surface (Plate 1, fig. B). In some eggs this process, as in the case illustrated, occurs only in small surface areas near the micropyles, while in other eggs it involves the whole top of the animal region. In the pretreated eggs at this time there have not yet appeared any indications of cortical reaction (Plate 1, fig. C). In acetone-pretreated eggs single large vacuoles (Plate 1, fig. D, SV), the result of the treatment applied, occur here and there at the egg surface.

Twenty seconds after insemination of the control eggs the granules are extruded and the membrane is separated over a large area (Plate 1, fig. E). In urethane- and ether-pretreated eggs the expulsion of the granules has also started, but in the first case it has spread only over a small distance (Plate 1, fig. F), while in the second case this process is just beginning (Plate 1, fig. G). After acetone pretreatment, the cortical layer is strongly vacuolated in the micropylar region, but the secretion of granules has not yet started (Plate 1, fig. H).

After 30 seconds the membranes in the control eggs are elevated in the whole animal region, but after urethane and ether pretreatment only in the centre of this region, while after acetone pretreatment granule breakdown has just started. After 1 minute the elevation of the membranes in most of the control eggs is completed (in 6 out of 9, or 67 per cent.), while in the remainder the membranes have failed to separate from the surface of the egg only in a small region at the vegetative pole. In urethane-pretreated eggs the membrane elevation is at this time finished in a smaller percentage of cases (3 eggs out of 8, or 38 per cent.); after ether and acetone treatment the membranes of all eggs are not elevated at a considerable distance around the vegetative pole. After 2 minutes the
elevation of membranes is completed in all the experimental variants (with rare exceptions in ether- and acetone-pretreated eggs).

Thus all the treatments tested in this work that induced an increase in the incidence of polyspermy prolonged the latent period of the cortical reaction and apparently somewhat retarded the cortical granule secretion itself. The differences from the control are most clearly manifested early (10–30 seconds) after insemination, while later they become obscured. Most labile is the inhibiting action of urethane which easily penetrates into cells and can be rapidly released from them (Cornman, 1954). Since in sturgeon the establishment of contact between the spermatozoa and the eggs occurs in the first 10–15 seconds after insemination (Ginsburg, 1957b), it is just at this time that the inhibition of the block to polyspermy is of importance for the penetration of supernumerary spermatozoa.

The data of this set of experiments favour the existence of a direct correlation between the spread of the cortical reaction and the block to polyspermy. However, they do not provide indisputable evidence, since the chemical agents applied could have affected not only the cortical reaction but also the rapidly proceeding changes in the first phase of the block to polyspermy that have been suggested in the hypothesis of Rothschild & Swann.

2. Loss of fertilizability after artificial activation of the egg

(a) Activation of sturgeon eggs by pricking

In order to obtain more definite data on the relationship between the spread of the cortical reaction and the block to polyspermy, experiments were carried out in which the penetration of the spermatozoon and the onset of the cortical reaction were artificially separated in space and time. The results obtained have been published elsewhere (Ginsburg, 1960).

In these experiments the method applied was that used by Dettlaff (1961) when studying the role of fertilization impulse and cortical reaction in egg activation in Acipenseridae. Dettlaff pricked eggs at various points on their surface and thus varied the difference between the time taken by the fertilization impulse to reach the site of the nucleus and the time taken by the slower wave of cortical granule breakdown to reach the same place. Her experiments showed that the cortical reaction induced by pricking spreads at the same velocity as in fertilization.

In the experiments described below the sturgeon eggs were activated by pricking (a) near the animal pole (therefore near the micropylar canals), and (b) at the vegetative pole (at the maximum distance from the canals), and then inseminated. If changes blocking the entrance of spermatozoa spread over the egg surface in the first seconds after the activating treatment, the fertilizability of eggs in both experimental variants will be lost (or, at any rate, sharply decreased) almost simultaneously; if the inhibition is connected only with the visible cortical reaction, then in variant (b) the capacity to be fertilized will be
lost much later than in variant (a), the difference corresponding to the time required for the spread of cortical reaction over the egg (Dettlaff, 1957, 1961).

Sturgeon eggs were put into water one by one, pricked with a glass needle (diameter 20–40 μ), and after various time-intervals, from 1–2 seconds to 5 minutes, inseminated with rather dilute sperm (dilution 1:500 to 1:1000; sperm density, 1·18 × 10⁶ to 1·21 × 10⁷/ml.). In some lots the eggs stood such a treatment well, in others cytolysis occurred in many eggs. After pricking, all the eggs showed signs of activation. The difference between fertilized and activated but unfertilized eggs was manifested during cleavage (in activated eggs cleavage furrows do not appear at all or show an irregular pattern and appear with a considerable delay, Dettlaff & Ginsburg, 1954) and later, when fertilized eggs undergo gastrulation while activated ones gradually degenerate.

**Table 3**

*Experiment (No. 3) on pricking animal and vegetative regions of sturgeon eggs with subsequent insemination*  
*(Temperature, 13·8° C.)*

<table>
<thead>
<tr>
<th>Time from pricking to insemination</th>
<th>Pricking the animal region</th>
<th>Pricking the vegetative region</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total number of eggs</td>
<td>Fertilized eggs (%)</td>
</tr>
<tr>
<td>2 seconds</td>
<td>60</td>
<td>2 (3·3)</td>
</tr>
<tr>
<td>5 seconds</td>
<td>40</td>
<td>4 (10·0)</td>
</tr>
<tr>
<td>10 seconds</td>
<td>20</td>
<td>0 (0)</td>
</tr>
<tr>
<td>1 minute</td>
<td>..</td>
<td>..</td>
</tr>
<tr>
<td>2 minutes</td>
<td>..</td>
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<tr>
<td>3 minutes</td>
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<td>..</td>
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<tr>
<td>4 minutes</td>
<td>..</td>
<td>..</td>
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<tr>
<td>5 minutes</td>
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<td>..</td>
</tr>
</tbody>
</table>

In all, 6 sets of experiments were performed and more than 1,000 eggs pricked, but only in 576 of them was development followed for a sufficient period of time. For cytological investigation eggs were fixed after the same time-intervals as when insemination was carried out. Seventy-four eggs were studied in sections.

All the experiments gave similar results. One of them will be taken as an example (Table 3). On pricking the animal region, the eggs were, as a rule, unfertilizable with the shortest intervals between pricking and insemination. Single eggs, however (an average of 6 per cent. in all experiments) became fertilized.

One can see in sections of the eggs that 5 seconds after pricking the wave of cortical reaction, spreading asymmetrically, reaches the border of the vegetative region at the side of the prick, while in the opposite direction it involves the whole top of the animal region (Text-fig. 1 A). At the position of the micropylar canals the escape of cortical granules is finished and the membrane is separated.
from the egg surface (Plate 2, fig. A). It is evident that no spermatozoon entry into such eggs is possible.

Why occasional eggs become fertilized becomes clear as a result of living observations made after pricking. In many lots of eggs, in particular in darker pigmented ones, the wave of granule breakdown can be clearly seen: pricking is followed by a short latent period (usually lasting 1–2 seconds), a bright spot then appears at the site of pricking and spreads in all directions, towards the animal pole much faster than towards the border with the vegetative region. This wave reached the animal pole 3–5 seconds after pricking (at a temperature of 21–22°C). It should be noted that some seconds must usually pass after insemination until spermatozoa cover the distance to the aperture of the micropyle and along the micropylar canal to the egg cortex (Ginsburg, 1957b). Even when insemination is done as soon as possible after pricking, the spermatozoon reaches the surface of the cytoplasm only when the cortical reaction has already spread over the micropylar region.

In occasional eggs, however, the cortical reaction proceeded more slowly, mainly at the expense of a prolongation of the latent period (similar differences in the duration of the latent period of cortical reaction were found in sea-urchin eggs by Allen & Griffin, 1958). In these eggs the wave of granule discharge reached the animal pole in 7–11 seconds. In such cases the spermatozoon can penetrate the egg when insemination occurred 2–5 seconds after the pricking.

On pricking the vegetative region (Text-fig. 1 b–d) fertilization occurred not only when insemination was a short time after pricking (5–30 seconds), but also after intervals of 1–3 minutes (Table 3). With intervals of 1 and 2 minutes, the fertilization percentage was high (87.5 per cent. on average) and practically corresponded to the fertilization frequency in the same lot of eggs inseminated.
without pricking (84.5 per cent.). After an interval of 3 minutes the fertilization percentage halved and no fertilization occurred at longer intervals.

In sections of eggs fixed 2 minutes after pricking it can be seen that the wave of granule discharge has not yet reached the equator (Text-fig. 1B); the granules in the region of the animal pole are still unchanged (Plate 2, fig. B). After 3 minutes this wave has already spread over the animal region (Text-fig. 1C), in some of the eggs reaching its top, while at the opening of the terminal canal of the micropyles the contact between cytoplasm and membrane is still preserved (Plate 2, fig. C); in other eggs the cortical reaction is finished and the membrane separated (Plate 2, fig. D). It seems that in the first case the spermatozoon is still able to penetrate the egg, while in the second it is already impossible. Four minutes later the separation of membranes is completed in all cases (Text-fig. 1D; Plate 2, fig. E).

**Table 4**

*Experiments on pricking the vegetative region of sturgeon eggs at various temperatures*

<table>
<thead>
<tr>
<th>Time from pricking to insemination (minutes)</th>
<th>Temperature (°C.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>13.8</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
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</table>

The remaining experiments gave similar results. On pricking the vegetative region at intervals of 5, 10, 20, and 60 seconds, 100 per cent. of eggs were fertilized in some aliquots. The loss of fertilizability in different experiments took place at a different time after pricking: the higher the temperature the earlier the loss (Table 4). We can see convincingly in sections that the moment of the loss of fertilizability coincides in all cases with the secretion of cortical granules by the cytoplasm adjacent to the micropyles.

Thus in all experiments no decrease of fertilizability was observed for a long time after pricking in the region of the vegetative pole (up to 1–2 minutes); on the other hand, the loss of this ability coincided precisely with the completion of the cortical reaction and the separation of membranes in the micropylar region.

(b) *Activation of trout eggs by water treatment*

In the experiments with trout eggs water was used as an activating agent instead of pricking. Trout eggs were inseminated at different times after putting
them into water; simultaneously, other egg aliquots were transferred from water into Ringer and 0.1 M versene solutions to reveal the onset of early activation changes. It is known that salmonid eggs are not activated in Ringer solution (or in 0.1 N sodium chloride) but that activation once started continues to develop (Kusa, 1950; Devillers, Thomopoulos, Colas, 1954; Zotin, 1954). Activation also fails in 0.1 M versene solution (Dettlaflf, 1959).

The experiment was carried out twice. It turned out (Text-fig. 2) that 10 seconds after putting into water the eggs were not yet activated, 20 seconds later half of them were activated, while all were activated after 30 seconds: if these eggs are put into Ringer and versene solution, a perivitelline space of normal size, the zone of fat droplets, and the blastodisc are formed. At the same time on insemination 30 seconds after putting the eggs into water all of them can still be fertilized. Fertilizability rapidly decreases after 60 seconds and is completely lost after 120 seconds.

![Text-fig. 2. The loss of fertilizability in trout egg with time after activation by immersion into water (sperm density on insemination $1.63 \times 10^7$/m). Continuous line, percentage of fertilized eggs; dotted line, percentage of eggs which proved to be activated when put into Ringer solution.](image)

A study of sections of 53 eggs fixed at different time intervals after they were put into water (temperature 4-0°C) showed that the cortical egg layer preserved for 40 seconds the same appearance that it had prior to immersion (Plate 3, fig. 1A). After 60 seconds the layer of cortical alveoli remains still unchanged in
some of the eggs (Plate 3, fig. 1b), while in others the breakdown of alveoli has begun. After 80 seconds an intense secretion process was observed in the micropylar region in all cases: the alveolar wall adjacent to the surface burst and the colloidal content was released under the egg membrane, where it could be readily distinguished on staining with Heidenhain’s azan method in the form of light blue clots usually adhering to the inner surface of the membrane (Plate 3, fig. 1c). 120 seconds after the immersion of eggs in water the process of breakdown of cortical alveoli had spread further, while a smoothing of the cytoplasm surface and a solution of the released content of the alveoli had started in the micropylar region (Plate 3, fig. 1d).

The data obtained are in good agreement with the observation made by Kusa (1950) that eggs of the dog salmon (Oncorhynchus keta) when put into water (temperature 10° C.) lost their fertilizability in 120 seconds; but these data are at variance with those of K. Yamamoto (1951), also obtained on the dog salmon, according to whom the eggs in water ceased to undergo fertilization only after 30 minutes (temperature 5–7° C.).

Thus no decrease of fertilizability is observed in trout eggs during a considerable time after the application of the activating agent. Thirty seconds after immersion in water some initial changes (that seem to correspond to the fertilization impulse) have already occurred in eggs, since they behave in Ringer and versene solutions like activated ones. However, the alveoli are unchanged at this time and the spermatozoon enters the egg despite the initiated change. The fertilizability is lost only at the moment of breakdown of cortical alveoli in the micropylar region.

3. Blocking action of perivitelline fluid

(a) Insemination of activated trout eggs after the removal of perivitelline fluid

After the completion of the cortical reaction the content of the alveoli passes into the perivitelline fluid. If it is just this secretion that creates a barrier preventing the entering of supernumerary spermatozoa, then polyspermic fertilization should occur in the absence of perivitelline fluid. In order to examine this suggestion, trout eggs were activated by immersion in water, an aperture (diameter c. 2 mm.) was then cut in the vitelline membrane (chorion) through which the end of a pipette was introduced into the space between the egg surface and the membrane, and perivitelline fluid was washed out with a stream of water. The sperm then was introduced under the chorion by means of a pipette. Insemination was carried out from 25 to 120 minutes after immersion of the eggs into water. Untreated eggs that were simultaneously inseminated served as control. The results are presented in Table 5 (experiment 1). It can be seen that fertilization of activated eggs is possible even 120 minutes after the application of the activating agent.

The cleavage of those eggs that became fertilized shows features characteristic of polyspermy. In some cases on the periphery of the blastodisc some small
blastomeres separate, while the bulk of the cytoplasm remains undivided. In other cases the whole blastodisc divides into many blastomeres (Plate 3, fig. 2b), while control eggs are at the same time at the stage of the second division (Plate 3, fig. 2A). The cleavage pattern of such eggs is very similar to that observed after experimental polyspermy in frogs (Brachet, 1910) and sturgeon (Ginsburg, 1953; Dettlafl & Ginsburg, 1954). In sections of trout eggs fixed 6 hours after insemination (temperature 8·3–8·7° C.) up to 9 pronuclei were found.

### Table 5

| No. of experiment | Mode of insemination | Interval between egg immersion into water and insemination | Total number of eggs | Fertilized eggs (%)
<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>(1)</td>
<td>Washing out perivitelline fluid and introduction of sperm under the membrane</td>
<td>(minutes) 25–45 60 120</td>
<td>33 59 37</td>
<td>23 32 21</td>
</tr>
<tr>
<td>(2)</td>
<td>Introduction of sperm into the water</td>
<td>20–45 60, 120</td>
<td>16 18</td>
<td>0 0</td>
</tr>
</tbody>
</table>

In the second experiment an orifice in the chorion was also cut but the perivitelline fluid was not washed out, while insemination was carried out by introduction of a large amount of sperm into the water. No fertilization took place (Table 5).

Thus the surface layer of the activated trout egg becomes accessible to spermatozoa after removal of the perivitelline fluid.

(b) The action of perivitelline fluid on spermatozoa

Fertilized or activated trout eggs were superficially dried on filter paper, their chorion was pricked and perivitelline fluid removed by means of a fine glass pipette. If a drop of this fluid was mixed with a drop of trout sperm, an energetic agglutination of spermatozoa was observed (Plate 3, fig. 3A).1

Perivitelline fluid caused agglutination from the earliest time it was procured, i.e. 20 minutes after egg activation. After 24 hours this effect had not decreased and had even increased (clearly marked agglutination was observed on 1:4 dilution of this perivitelline fluid in water, while no agglutination took place after such dilution of earlier fluid). It can be seen in the smears of agglutinated sperm that the sperm tail becomes sticky and forms characteristic loops (cf. Plate 3, figs. 3 B, c).

**DISCUSSION**

The penetration of the spermatozoon into activated sturgeon and trout eggs becomes impossible at the moment when the cortical granules (the contents of 1 In autumn 1960 the sperm-agglutinating agent in the perivitelline fluid of trout was shown to be inactivated by trypsin (by 0·1 per cent. trypsin solution in 1 per cent. NaHCO₃ in 15–20 seconds and by 0·001 per cent. solution in 6 minutes at 4·0° C.) and by boiling (in 1 hour–1 hour 40 minutes). These data suggest that the agglutinating agent is protein in nature.
the alveoli) are secreted in the micropylar region. On the other hand, retardation of the discharge of cortical granules in sturgeon eggs pretreated with urethane, ethyl ether, or acetone is accompanied by an increased incidence of polyspermy. These facts indicate the existence of a causal connexion between the discharge of substances contained in the cortical structures and the block to polyspermy. The results obtained are in good agreement with several observations made on sea-urchin eggs; these latter show that when the discharge of cortical granules is inhibited, or even retarded, then fertilization is polyspermic (Moore, 1916; Okazaki, 1956; Hagström & Allen, 1956; Hagström, 1957; Perlmann & Hagström, 1957). The same was observed in experiments involving the inhibition of cortical alveolar breakdown in the dog salmon (Kanoh & Yamamoto, 1957).

The role in the block to polyspermy assigned to the substances contained in the cortical granules (alveoli) which, after the latter break down, pass into the perivitelline fluid, finds support also in the experiments where the removal of these substances renders an egg already fertilized or activated accessible to spermatozoa. This was shown in the experiments described above on the washing out of perivitelline fluid in activated trout eggs. It seems that the results obtained on the Pacific herring (*Clupea pallasii*) should be interpreted in the same sense. Yanagimachi (1957) removed the egg membrane in Pacific herring mechanically, partially cutting it with iridectomy scissors and forcing the egg out through the aperture formed. On inseminating such denuded eggs, both non-fertilized and previously fertilized or activated by pricking, the penetration of numerous spermatozoa was observed, accompanied by an atypical cleavage characteristic of polyspermy. T. S. Yamamoto (1958) dissolved the membrane of herring eggs by a double treatment with acidified Ringer's solution and 0.2 per cent. trypsin in Ringer's solution. On insemination of these naked eggs polyspermic fertilization was observed, probably because perivitelline fluid was not retained at the egg surface in the absence of the chorion.

Similar results were obtained on sea-urchin eggs. Nakano (1954) mechanically removed the membrane of *Hemicentrotus pulcherrimus* eggs activated with saponin solution; thereafter these eggs could undergo fertilization (which was, as a rule, polyspermic).

Tyler, Monroy, & Metz (1956) mechanically removed the membrane of fertilized eggs of *Lytechinus pictus* and *L. variegatus*; on re-insemination polyspermic fertilization was observed. However, in experiments on other sea-urchin species mechanical de-membranation was insufficient for re-fertilization. It was obtained only after the application of treatments that dissolved the hyaline layer: treatment with Ca- and Mg-free media or urea solution (Sugiyama, 1951; Hagström & Hagström, 1954; Nakano, 1956). After treatment of activated *M. pulcherrimus* eggs with urea solution their fertilizability appreciably increased (Nakano, 1954).

Since the hyaline layer dissolved in these experiments is an extracellular
structure (Harvey, 1934) and seems to be formed at the expense of substances previously contained in cortical granules and then passed to the perivitelline fluid (Parpart & Cagle, 1957; see also Allen, 1958), the results obtained in the experiments on fish and sea-urchin eggs appear to be in principle similar: in both cases the block to polyspermy is eliminated after the removal of polysaccharide-containing derivatives of cortical granules (alveoli).

The detection of the agglutinating action of perivitelline fluid on spermatozoa, which is in good agreement with the analogous data of Motomura for sea urchin (see Metz, 1957), points to the probable mechanism of the prevention of polyspermy. It should be borne in mind, however, that, along with the substances previously contained in cortical structures, other secretions of the egg can enter the perivitelline fluid (as shown, for example, for Acipenseridae by Dettlaff & Ginsburg, 1954; Dettlaff, 1957). Therefore the effect obtained cannot be unreservedly ascribed to the action of the content of the alveoli, though the role played by the discharge of these substances in the block to polyspermy, which was found in other experiments, makes this interpretation seem the most likely.

During the period from the moment of the application of the activating agent until the completion of the cortical reaction in the micropylar region, in both sturgeon and trout, no decrease of the ability of the egg cytoplasm to accept the spermatozoon was observed. This tells against the existence in fish of any rapidly propagated block to polyspermy connected with invisible changes (fertilization impulse). Here the block to polyspermy seems to be realized only by means of the discharge of cortical granules (content of alveoli).

The conception of the diphasic block to polyspermy in sea urchins remains so far a hypothesis. The experiments with ‘partially fertilized’ sea-urchin eggs (Allen & Hagström, 1955 a, b; Hagström & Runnström, 1959) tell against the existence of any stable block preceding the cortical reaction. In these experiments the cortical reaction was interrupted several seconds after the fast block should have been established, but on re-insemination spermatozoa easily penetrated such eggs through that part of the surface which preserved cortical granules.

If the hypothesis of a diphasic block is nevertheless proved by further thorough study of the early developmental stages of sea-urchin eggs, then the difference in the polyspermy-preventing mechanism in echinoderms and fish should be interpreted in relation to the different modes of sperm−egg collision in these animals. In fish spermatozoa penetrate into the egg only in strictly localized sites, through micropyles. Consequently the blocking action of the discharged content of cortical structures is sufficient to prevent polyspermy. Salmonid eggs possess but one micropyle. The width of its terminal canal is such that the fertilizing spermatozoon fills the whole aperture and, until it is drawn into the cytoplasm, the penetration of other spermatozoa is physically impossible; but by then the breakdown of cortical alveoli is already accomplished.

Sturgeon eggs have several micropyles and hence the possibility of the penetra-
tion of several spermatozoa exists. Since micropyles are located in a group on a small area of the egg surface, and as the cortical reaction extends over this area in a very short time, a considerable incidence of polyspermy can occur only on insemination with very dense sperm suspensions, as has been shown experimentally. In nature such density can occur only as a rare event, since sturgeon spawn in rapid current where spermatozoa are scattered by the water stream.

Unlike fish, the whole egg surface in Echinoderms is accessible to spermatozoa, which may therefore require a more complicated defence mechanism against polyspermy.

**SUMMARY**

1. Treatment of sturgeon eggs with the polyspermy-inducers urethane, ethyl ether, and acetone prolongs the latent period of the cortical reaction and apparently retards the actual secretion of the cortical granules.

2. On activation of sturgeon eggs by pricking and of trout eggs by immersion in water, they lose their fertilizability at the moment of the discharge of cortical granules (the content of the alveoli) in the micropylar region. Before this no decrease in ability of the egg cytoplasm to accept a spermatozoon is observed.

3. After removal of the perivitelline fluid (containing substances discharged from the cortical alveoli) penetration of numerous spermatozoa into activated trout eggs becomes possible.

4. Perivitelline fluid of trout causes an agglutination of spermatozoa.

5. The data obtained provide evidence that the block to polyspermy in fishes is a one-step process realized by means of the discharge of cortical granules (the content of the alveoli).

Блокирование полиспермии у осетра и форели и роль кортикальных гранул (альвеол) в этом процессе.

А. С. Гinzбург

**Выводы**

1. При воздействии на яйца осетра раствором уретана, парами этилэфира и ацетона (агентами, стимулирующими полиспермию) наблюдается удлинение латентного периода кортикальной реакции и, по-видимому, некоторое замедление самого процесса выделения кортикальных гранул.

2. При активации яиц осетра уколом и яиц форели водой они утрачивают способность к оплодотворению в момент выделения кортикальных гранул (содержимого альвеол) в области микропиле. До этого не наблюдается какого-либо снижения способности цитоплазмы яйца к восприятию спермии.

3. После удаления перивителлиновой жидкости (содержащей вещества, выделенные из кортикальных альвеол) возможно множественное проникновение спермиев в активированные яйца форели.
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Explanacion of Plates

Key to abbreviations

CA, Cortical alveoli; CCA, colloidal content of cortical alveoli discharged from the egg; CG, cortical granules; FA, funnel-shaped aperture of micropyle; J, jelly coat; M, metaphase of the second maturation division; PG, pigment granules; SV, large-sized single vacuoles; TC, terminal canal of micropyle; V, vacuoles in the cortical layer; VM, vitelline membrane (in sturgeon external and internal vitelline membranes; the border between them is indistinguishable on photomicrographs; in trout the vitelline membrane is also called chorion). YG, yolk granules.
Plate 1
Changes in cortical reaction in sturgeon eggs pretreated with polyspermy inducers. Egg sections in micropylar region. Fixation with Sanfelice fluid, staining with Heidenhain’s Azan.

Fig. A. Non-fertilized ripe egg.
Fig. B. Control (untreated egg), 10 seconds after insemination.
Fig. C. Urethane pretreatment, 10 seconds after insemination.
Fig. D. Pretreatment with acetone vapour, 10 seconds after insemination.
Fig. E. Control, 20 seconds after insemination.
Fig. F. Urethane pretreatment, 20 seconds after insemination.
Fig. G. Pretreatment with ethyl ether vapour, 20 seconds after insemination.
Fig. H. Pretreatment with acetone vapour, 20 seconds after insemination.

Plate 2
Cortical reaction in micropylar region of sturgeon egg following activation by pricking. Fixation with Sanfelice fluid, staining with Heidenhain’s Azan.

Fig. A, 5 seconds after pricking into animal region; Figs. B, 2 minutes; C and D, 3 minutes; E, 4 minutes after pricking into vegetative region.

Plate 3
Fig. 1. Cortical reaction in micropylar region of the trout egg activated by contact with water. Fixation with Sanfelice fluid, staining with Azan. A, non-treated egg; B, 60 seconds, C, 80 seconds; D, 120 seconds after immersion into water.

Fig. 2. Blastodisc of the trout eggs. A, control egg at the stage of the second cleavage division; B, an egg inseminated through an aperture in its membrane 25 minutes after immersion in water.

Fig. 3. Trout spermatozoa. Fixation with the vapour of osmium tetroxide, staining with Heidenhain’s iron haematoxylin. A, a drop of sperm after the addition of perivitelline fluid taken 40 minutes after egg activation; B, a smear of agglutinated spermatozoa; C, control, a smear of sperm diluted with Ringer solution.

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Plate 3