Effects of Acidulated Ringer's Solution on the Egg of Dog Salmon, *Oncorhynchus keta*[1)]

By

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(With 2 Plates and 4 Tables)

It has been shown that salmon egg is not activated in Ringer's solution; that is, in this solution the egg does not show for a long time any sign of activation such as breakdown of cortical alveoli and formation of blastodisc containing a nucleus in an advanced stage beyond metaphase (Kanoh '50, *etc.*). On the other hand, in experiments on the removal of the chorion of salmon egg by double treatment with acidulated Ringer's solution and pancreatin dissolved in Ringer's solution, Kanoh and Yamamoto ('57) found that the egg had already been parthenogenetically activated and had formed distinct blastodisc after the dissolution of chorion had been completed. In this case, the cortical alveoli remained unchanged. These experimental results suggest the possibility that a single treatment with either acidulated Ringer's solution or pancreatin-Ringer's solution can induce an activation which is not accompanied by breakdown of cortical alveoli in the salmon egg. In order to test such a possibility, the effects of acidulated Ringer's solution on the salmon egg were studied. In this paper are described the results of such experiments.

Material and Method

The materials used in the present study were all taken from matured individuals of dog salmon, *Oncorhynchus keta*. Most experiments were performed on unfertilized eggs. As the occasion demanded, eggs inseminated in Ringer's solution were also employed (*cf.* Kusa '50). The Ringer's solution used for the experiments was made up as follows: M/6.5 NaCl 100 parts + M/6.5 KCl 2.8 parts + M/10 $CaCl_2$ 3.4 parts (pH 7.2, with $NaHCO_3$). This solution was isotonic to the salmon egg. Ca-free Ringer's solution was made by omitting $CaCl_2$ from the above constituents. The solution of M/10 $CaCl_2$ used in the experiments was isotonic to the egg, and the pH of the solution was adjusted to 7.2 by $NaHCO_3$. To acidify the solutions some amounts of conc. HCl were added. The pH was measured by a glass-electrode pH meter. In order to distinguish them from the acidulated solutions, the solutions whose pH was adjusted to 7.2 were all designated as the normal solutions in

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the present paper. All the experiments were carried out at room temperature, 12°–15°C, and the other details of the procedures used in these experiments will be given in the following description.

Results

Activation induced by acidulated Ringer's solution: A preliminary experiment is performed using the same acidulated Ringer's solution as used in the preceding work (Kanoh and Yamamoto '57). The eggs are immersed for 15 minutes in the acidulated Ringer's solution whose pH is adjusted to 1.8 with HCl, and then washed thoroughly in the normal Ringer's solution. After 17 hours' immersion in this solution, all the eggs form a blastodisc, with some of them cleaving if insemination is previously performed.

These results indicate that the acidulated Ringer's solution can induce activation of the salmon egg. Now the problems to be solved are (1) the effective range of pH and (2) the duration of immersion in the acidulated Ringer's solution sufficient to induce the egg activation.

First, the pH is varied and the duration of immersion in an acidulated Ringer's solution is kept constant for 15 minutes. After the eggs are treated in acidulated solution and then washed in several changes of the normal Ringer's solution, they are left in the latter solution. The result of this experiment is presented in Table 1. High incidence of activation occurs when the eggs are immersed in the acidulated solution whose pH is adjusted to 2.0–3.0. No appraisal of the blastodisc is made of eggs immersed in the acidulated Ringer's solution of pH 1.5 as all of them undergo cytolysis.

Table 1. Blastodisc formation of eggs subjected to the treatment with acidulated Ringer's solution of various pH for 15 minutes and transferred into the normal Ringer's solution.

pH of acid. solution	After 17 hours	
	Eggs cytolyzed (%)	Blastodisc formation
1.5	100	?
2.0	5	+
2.5	0	+
3.0	0	+
3.5	0	−
4.0	0	−

Next, the duration of immersion in an acidulated Ringer's solution is altered. The result of this experiment is presented in Table 2. Blastodisc formation is observed in the eggs immersed in the acidulated Ringer's solution of pH 2.0, 2.5 and 3.0 for over 1/4, 2 and 5 minutes respectively. There is a possibility that prolonged immersion in the acidulated Ringer's solution whose pH is higher than

3.0 can induce the blastodisc formation. In fact, the majority of the eggs immersed in the acidulated Ringer's solution of pH 3.5 for 17 hours raise blastodisc. At pH 4.0 and 4.5, however, only a small percent of the eggs are activated. Accordingly, it can be said that the limit of pH of the acidulated Ringer's solution for inducing the blastodisc formation is below 3.5 or 3.0.

Table 2. Blastodisc formation of eggs subjected to the treatment with the acidulated Ringer's solution for various period of time and transferred into the normal Ringer's solution.

Acid-treatment		After 17 hours	
pH	duration (min.)	eggs cytolyzed (%)	blastodisc formation
2.0	1/4	0	+
	1/2	0	+
	1	0	+
	2	0	+
	5	0	+
	10	5	+
2.5	1/2	0	±
	1	0	±
	2	0	+
	3	0	+
	5	0	+
	10	0	+
3.0	1	0	±
	3	0	±
	5	0	+
	10	0	+

Toxicity of acidulated Ringer's solution: It is interesting to note that the salmon egg does not show cytolysis after immersion in the acidulated solution, because acid solution is believed to be injurious for the animal cells. Probably the use of Ringer's solution in the treatment may be necessary for arresting the toxic action of acid on the egg. In fact, all eggs immersed in acidulated dist. water (pH 2.0) die rapidly. The mechanism of protective action of the Ringer's solution in the treatment is next analysed. In this connection, two elements in Ringer's solution are thought to be involved: The cations contained and the salt concentration.

In the first experimental series, the role of Ca ions in the acid-treatment is examined. The solutions of pH 2.0 are made with Ca-free Ringer's or $CaCl_2$ solutions. Eggs previously washed in the normal Ca-free Ringer's or $CaCl_2$ solution are immersed in the above acidulated solution for 2 minutes. Then they are thoroughly washed in several changes of the normal Ringer's solution and kept in that solution for 17 hours. The results are shown in Table 3. Fourteen percent of eggs treated with the acidulated Ca-free Ringer's solution show the cytolysis in the

normal Ringer's solution, while no eggs undergo cytolysis in the cases of the acidulated $CaCl_2$ and Ringer's solutions.

Table 3. Role of Ca ions for the survival of acid-treated egg.

Treatments		Eggs died (%)
acidulated solution	normal solution	
Ca-free Ringer	Ringer	14*
$CaCl_2$	Ringer	0
Ca-free Ringer	Ca-free Ringer	100*
$CaCl_2$	Ca-free Ringer	0
Ringer	Ca-free Ringer	30*
Ca-free Ringer	$CaCl_2$	100**
$CaCl_2$	$CaCl_2$	100**
Ringer	$CaCl_2$	45**
Ringer	Ringer	0

* Eggs undergoing cytolysis. ** Eggs showing precipitation of protoplasm.

In the second experimental series, Ca ions of the normal Ringer's solution are studied for their relationship to the acid-treated egg. After the eggs are treated for 2 minutes with the acidulated solutions used in the first experimental series, they are transferred into the normal Ca-free Ringer's or $CaCl_2$ solution. Before the transfer they are washed in several changes of these solutions. The results of this experiment are presented in Table 3. All eggs undergo cytolysis when both acidulated and normal solutions are lacking in Ca ions. Furthermore 30 percent of eggs show cytolysis in the normal Ca-free Ringer's solution, even after they are treated with the acidulated Ringer's solution. All eggs die, however, in the normal $CaCl_2$ solution, even when they are treated with the acidulated $CaCl_2$ solution. In this case the dead eggs are completely opaque. This is quite different from the case of the cytolysis in the Ca-free Ringer's solution, because in the latter case the dead eggs are transparent. These facts suggest that the death of the egg in $CaCl_2$ solution results from the precipitation of the egg protoplasm.

From the results of the above two experimental series, it may be said that Ca ions in both acidulated and normal solutions serve to protect the egg from the toxic action of acid at an appropriate concentration, though Ca ions are apparently toxic themselves in relation to the acid-treated egg.

In the third experimental series, the relation of the salt concentration to the protective action of the Ringer's solution in the acid-treatment is studied. After the eggs were immersed in the acidulated solutions (pH 2.0) whose salt concentration is varied, they are transferred into the normal Ringer's solution. The result of this experiment is presented in Table 4. Following 2 minutes' immersion in the

acidulated solution whose salt concentration is less than 2/3 of that of the Ringer's solution (hypotonic to the egg), the eggs die when transferred to the normal Ringer's solution, whereas no eggs die when the acidulated solution used is isotonic to the egg. The dead eggs are opaque suggesting the occurrence of the precipitation of the egg protoplasm.

Table 4. Relation of salt concentration of Ringer's solution for the survival of acid-treated egg. The salt concentration was changed by diluting the Ringer's solution with dist. water. For example, 2/3 Ringer means the solution of 2 parts of the Ringer's solution plus 1 part of dist. water.

Treatments: Salt concentration of		Eggs died (%)
acidulated solution	normal solution	
2/3 Ringer	1/1 Ringer	100
1/2 Ringer	1/1 Ringer	100
1/3 Ringer	1/1 Ringer	100
1/1 Ringer	2/3 Ringer	100
1/1 Ringer	1/2 Ringer	100
1/1 Ringer	1/3 Ringer	100
1/1 Ringer	1/1 Ringer	0

The last experimental series is designed to show whether the salt concentration of the normal solution serves to protect the egg from the death. After the eggs are treated with the acidulated Ringer's solution for 2 minutes, they are transferred into the normal solution of various salt concentrations. The result of this experiment is shown in Table 4. When the acid-treated eggs are transferred into the normal solution whose salt concentration is less than 2/3 of that of the Ringer's solution (hypotonic to the egg), they become opaque and die in that solution. On the other hand, if the eggs are transferred into the normal solution isotonic to the egg, they survive for at least 17 hours in that solution.

These experimental results indicate that the salt concentration of both acidulated and normal solutions is one of the factors responsible for the survival of the acid-treated eggs.

Cytological study of acid-treated egg: Sections of the egg reveal that the activation induced by the treatment with the acidulated Ringer's solution is not accompanied by breakdown of cortical alveoli. Most of the alveoli remaining are found at the margin of blastodisc (Fig. 1). If insemination is performed prior to the acid-treatment, some eggs cleave normally but others undergo abortive cleavage (Fig. 1). In the latter case, incipient furrows appear but do not cut through the blastodisc. The nuclear divisions proceed normally in some eggs and are found particularly in the eggs subjected to the prolonged acid-treatment (*e.g.*, 10–20 minutes at pH 2.0).

For the karyological study of fertilization in the acid-treated egg, several batches of eggs from different females are used. The inseminated eggs, treated with the acidulated Ringer's solution (pH 2.0) for 30 seconds and then immersed in the normal Ringer's solution, are fixed in Bouin's fluid at certain intervals up to 6 hours after the treatment. Unfortunately the results vary from batch to bacth, though they are uniform within a batch. The behavior of the egg nucleus is roughly divided into two groups.

In the first group, the metaphase spindle of the second meiotic division is, as found in the intact egg, located in the animal pole of the egg after the acid-treatment (Fig. 2). Its outer pole is intimately associated with the egg surface. Up to 20 minutes after the treatment the chromosomes are arranged as a typical metaphase plate. Highly condensed dyads, arranged in a circle, are seen in the polar sections. Anaphase movements are initiated within 30 minutes after the treatment. Although some eggs are still at metaphase, the other exhibits complete separation of dyads. Anaphase movements are rapid and are completed in about 10 minutes. At 40 minutes after the treatment, all eggs are at anaphase (Fig. 3). The daughter chromosomes are at either ends of the pole of the spindle. The surface of the egg at this stage is smooth, *i.e.*, no bulge is evident. At 50–60 minutes, a distinct bulge occurs on the surface of the egg. By 60–90 minutes, the bulge is squeezed from the egg surface and the polar body is completely formed (Fig. 4). The haploid egg pronucleus moves toward the central part of the blastodisc and unites with the sperm nucleus at 2 hours after the treatment. The above mentioned sequence of the karyogamy is quite identical with that observed in the normal fertilization of salmon egg by K.Yamamoto ('52), though the time required for the karyogamy is shorter in the acid-treated egg.

In the second group, the anaphase movements of the second meiotic division are initiated after the treatment and at 40 minutes all eggs are at late anaphase as in the first group. The separation of the two haploid sets of chromosomes at anaphase appears to be normal. However, the extrusion of the polar body does not occur in this group. The surface of the egg remains smooth throughout and no bulge is formed. The late anaphase spindle is submerged below the smooth surface of the egg and rotates into an oblique position (Fig. 5). The daughter chromosomes are at either ends of the pole of the submerged spindle. Thereafter the spindle show morphological changes and often it changes into a tri- or tetra-polar one (Fig. 6). Each end of the pole receives a share of the daughter chromosomes of the anaphase spindle. At 2 hours after the treatment, the submerged nucleus forms a single pronucleus (Fig. 7). The pronucleus thus formed may contain two sets of chromosomes and, at 4 hours after the treatment, it unites with the sperm nucleus (Fig. 8). At 6 hours after the treatment, the egg is at prophase of the first cleavage.

When the unfertilized egg is treated with the acidulated Ringer's solution, the egg nucleus behaves in exactly the same manner as in the inseminated egg and changes into the tri- or tetra-polar spindle (Fig. 9). It forms the pronucleus at 2

hours after the treatment, but the pronucleus thus formed does not show any sign of division until 6 hours after the treatment. In the unfertilized eggs fixed at 24 hours after the treatment, the pronucleus shows some morphological changes; the membrane surrounding the pronucleus disappears and Feulgen-positive particles scatter in the region where the pronucleus had been situated (Fig. 10).

When the duration of the treatment of the inseminated eggs with the acidulated Ringer's solution (pH 2.0) is prolonged to 5–10 minutes, the behavior of the egg and sperm nuclei of the second group becomes somewhat modified. In this case the anaphase movements of the second meiotic division are not initiated or, even if initiated, stop at early phases of the separation of dyads. At 30 minutes after the treatment, the metaphase or early anaphase spindle is submerged below the smooth surface of the egg and rotates into an oblique position (Fig. 11). With time lapse, the distance of the spindle from the egg surface increases gradually (Fig. 12). In most eggs the anaphase movements of the meiotic division do not proceed further, and the submerged metaphase spindle is found even at 17 hours after the treatment. Peculiar facts are also observed in the behavior of the sperm nucleus. At 30 minutes after the treatment, the sperm aster develops near the base of the sperm head as in the case of normal fertilization (Fig. 13, *cf.* K. Yamamoto '52). After 50 minutes the sperm head shows a little swell being slightly larger in size than in the preceding stage and is not stained homogeneously with hematoxylin. It appears as a mass of indistinct basophilic particles and is enclosed in the well developed sperm aster (Fig. 14). In the eggs fixed at 70 minutes after the treatment, there is a peculiar fibrous structure resembling the mitotic spindle in the protoplasm near the smooth egg surface (Fig. 12). However, its fibers are feeble in appearance, and indistinct basophilic particles are irregularly spaced among them. It is none other than the sperm nucleus, because the distinct submerged spindle is found in the same egg. In most eggs such conditions of the sperm and egg nuclei are maintained for at least 17 hours after the treatment. The above mentioned sperm nucleus is always found in the protoplasm near the surface of the egg. This fact suggests that, differing from the case of the normal fertilization, the sperm nucleus does not move from the penetrating point toward the central part of the blastodisc.

Discussion

The results of the present study indicate that the activation not accompanied by breakdown of cortical alveoli observed after double treatment (Kanoh and Yamamoto '57) is mainly induced by treatment with the acidulated Ringer's solution.

The method of causing artificial parthenogenesis by the use of inorganic acids was first elaborated by Loeb and Neilson (*cf.* Loeb *et al.* '01), who employed a solution of 3–5 ml of N/10 inorganic acid plus 10 ml of sea water, and immersed for 3–20 minutes. In the case of *Asterias*, they succeeded by this means in bringing about 20 percent of eggs to gastrula stage. Since these experiments, inorganic

acids have been used with some success as parthenogenetic agents by several authors on the eggs of marine forms. In fish eggs, the use of inorganic acids in artificial parthenogenesis is due to T. Yamamoto ('61), who found the method to be remarkably successful with the eggs of medaka, *Oryzias latipes*. The eggs were placed in 10^{-3}N HCl-, H_2SO_4- or HNO_3-Ringer's solution, and after immersion in these solutions practically every egg showed activation, *viz.*, the breakdown of cortical alveoli and the elevation of the chorion from the egg surface. The cleavage did not occur, however, in these parthenogenetically activated eggs, and the development ceased at the stage of bipolar differentiation.

In the present study it was found that HCl was an efficient parthenogenetic agent for the salmon egg. The eggs raise the blastodisc in the normal Ringer's solution, if they were previously treated with the acidulated Ringer's solution (pH 2.0) for more than 15 seconds. In contrast to the results of T. Yamamoto's experiment ('61), the activation of the salmon egg induced by the acidulated Ringer's solution was not accompanied by breakdown of cortical alveoli. In this connection it is worthy to note that the salmon egg does not show any sign of activation in the Ringer's solution, even if it is fertilized. Immersion in nonelectrolyte solution or in hypotonic solution is known to be indispensable for inducing the breakdown of cortical alveoli and the blastodisc formation in the salmon egg (Kanoh '50, '51). On the other hand, *Oryzias* eggs activate in the Ringer's solution after fertilization and the cortical alveoli break down (T. Yamamoto '61). These facts suggest that the inorganic acids may act on the salmon egg as they do on the *Oryzias* egg. Furthermore, Ito ('55, '56) reported that the activation of the eggs of *Plecoglossus* is facilitated by the acidulated Ringer's solution of pH 3.0–5.2, though it is also observed after mere immersion in the normal Ringer's solution. Considering these facts, it may be supposed that the inorganic acid stimulates the eggs of freshwater fish to induce the blastodisc formation.

The activation not accompanied by breakdown of cortical alveoli is elicited by several methods. Immersion in the normal isotonic solutions of $CaCl_2$ or $MgCl_2$ or in the Ringer's solution containing heavy metal ions brings about such activation in the salmon egg (Kanoh '52, Kusa '53, T.S. Yamamoto '62). The author cannot find any factor common to these methods including the immersion in the acidulated Ringer's solution. To clarify the underlying mechanism in the acid-induced activation, further study is desirable.

As for the toxic effect of acids, Loeb ('15) obtained the results that the embryos of developing *Fundulus* eggs immersed in M/500 acetic acid are soon killed, and that the addition of certain amounts of any one of several salts serves to protect the embryos from the toxic action of the acid. These results coincide with those obtained in the present study. Some eggs underwent cytolysis after treatment with the acidulated Ca-free Ringer's solution. This may indicate that, among the cations contained in the Ringer's solution, Ca ions are the most effective for antagonizing the acid. Furthermore, after transfer into the normal Ca-free Ringer's

solution, the acid-treated eggs underwent cytolysis. It appears from this result that Ca ions in the normal solution also serve to protect the acid-treated egg from injurious effects. Loeb ('15) considered that the antagonism of the acid by the salt took place at the chorion and the presence of the salt prevented or retarded the penetration of the acid through the chorion. In the embryos of *Fundulus* freed from the casing chorion, however, Armstrong ('28) recognized the same acid-salt antagonism. It is, therefore, not necessary to assume any specific role for the chorion. According to Armstrong ('28), the primary toxic effect of acid is to kill the surface of the embryo; the penetration of acid into the embryo occurs after the surface has been damaged. In the present study the acid-treated egg died in either the normal solution of $CaCl_2$ or the normal solutions hypotonic to the egg. Referring to Armstrong's statement, this may be explained in the following way: The surface of the salmon egg is altered or damaged to some extent by the treatment with the acidulated Ringer's solution. The injurious action of the acid is weakened by the presence of Ca ions. After transfer into the normal solution, the surrounding solution may penetrate into the egg through the affected surface. When the normal solution is the Ca-free Ringer's solution, the damage to the egg surface is not repaired but extends inward, thus causing the egg to undergo cytolysis. On the other hand, the damage is repaired to some extent in the solution containing Ca ions. The acid-treated eggs survive, therefore, in the normal Ringer's solution. If the normal solution is $CaCl_2$ which contains negligible amounts of Na ions, however, the protoplasm is precipitated by the Ca ions which have penetrated into the egg through the incompletely repaired surface. The same holds true in the case of the hypotonic solution. The precipitation causes the death of the egg and can be recognized externally by the opaque appearance of the egg. In favor of this explanation, Yamaguchi ('58) found in his microinjection experiment that the egg protoplasm of the salmon is coagulated by a solution of $CaCl_2$ and dist. water. Furthermore, it may be deduced from his data that the injected Ringer's solution is diffused through the protoplasm, since the latter shows no indication of coagulation.

The diploid egg pronucleus is formed in some batches after the treatment with the acidulated Ringer's solution. In these cases, the anaphase spindle of the second meiotic division is submerged below the surface of the egg. As a result the meiotic division is prevented from going to completion and chromosome duplication is caused by the retention of the haploid set of chromosomes normally going into the second polar body. In such way the diploid nucleus is produced. Makino and Ozima ('43) reported concerning the carp that refrigeration of the egg shortly after insemination prevents the second meiotic division and causes chromosome duplication in the egg leading to the production of the diploid egg nucleus. According to them, however, such cold shock did not cause the dislodgement of the meiotic spindle and the diploid nucleus is formed within the egg cortex. The submergence of the second meiotic spindle below the egg surface has also been reported as occurring in the experimental induction of triploidy in amphibians. Fankhauser and

Godwin ('48) exposed freshly fertilized newt eggs to 36°C for 10 minutes and submerged the meiotic spindle below the egg surface to form the diploid egg nucleus. Dasgupta ('62) succeeded in obtaining triploid frog embryos causing the submergence of the meiotic spindle. He treated the eggs shortly after insemination with hydrostatic pressure of 5000 lb/in^2 for 6 minutes. From his experimental results, Dasgupta ('62) concluded that the submergence of the spindle is due to a change in sol-gel equilibrium of the egg cortex.

There are two possibilities to account for the underlying mechanism in the submergence of the spindle occurring in the acid-treated egg; one is that it is caused by the protoplasmic movement and the other that it is due to a change in the properties of the egg surface.

The movements of the egg protoplasm into the blastodisc may occur differently from the case in which the polar body is extruded. Roosen-Runge ('38) and Huver ('64) observed complicated movements of the egg protoplasm during the course of the blastodisc formation. If the direction of the streaming of the protoplasm into the blastodisc is altered in the acid-treated egg, it is probable that the streaming of protoplasm near the egg surface of the acid-treated egg dislodges the meiotic spindle from its original site. The submergence of the spindle may be accounted for in this way. When this possibility is fully conceded, one would expect the sperm nucleus, shortly after fertilization, to be found in the region far from the surface of the egg, because the penetrated sperm is first found in the protoplasm near the egg nucleus (Ginsburg '63) and should be subjected to the effect of the protoplasmic stream. However, this is not the case, but instead the sperm nucleus is always found in the protoplasm near the surface of the egg after the prolonged treatment with the acidulated Ringer's solution. Furthermore, in the study of the artificial parthenogenesis of an echuroid, *Thalassema mellita*, Lefevre ('07) observed that the meiotic spindle of some eggs treated with dilute solution of acids is submerged below the egg surface. But it is to be noticed that the eggs of this species show holoblastic cleavage, thus the protoplasmic stream in the egg may occur in a different way from the meroblastic fish egg.

On the other hand, the acidulated Ringer's solution alters the properties of the protoplasm at the egg surface and may possibly cause the loss of anchorage of the outer pole of the meiotic spindle to the egg cortex. It is still uncertain whether the change in the properties of the surface protoplasm of the egg, as pointed out by Dasgupta ('62), is related to the sol-gel equilibrium of the egg protoplasm or not. Anyhow, the matter is a subject for further study.

Summary

Exposure of the egg of the dog salmon, *Oncorhynchus keta*, to acidulated Ringer's solution (pH 2.0–3.0) for 1/4 –5 minutes induces an activation not accompanied by breakdown of cortical alveoli in the normal Ringer's solution (pH 7.2). Calcium ions, as well as salt concentration of both acidulated and normal solutions,

serve to protect the egg from damage. If the eggs are transferred into the normal $CaCl_2$ solution after immersion in the acidulated Ringer's solution, the toxic effect of the acid is arrested but at the same time Ca ions penetrate into the egg and cause a precipitation of egg protoplasm, leading to the death of the egg. Cytological study of acid-treated eggs fixed during the first 6 hours after treatment reveals that in some batches the second polar body is extruded in normal fashion. In others, however, suppression of the polar body formation occurs, and the acid-treated eggs give evidence of a submergence of the anaphase spindle of the second meiotic division below the egg surface. In this case the failure of the spindle mechanism occurs during telophase of the submerged nucleus, resulting in the formation of probably diploid egg pronucleus. Later on, the egg nucleus unites with the sperm nucleus if the eggs are previously inseminated.

The principal effect of the acid-treatment appears to alter the protoplasmic surface of the egg.

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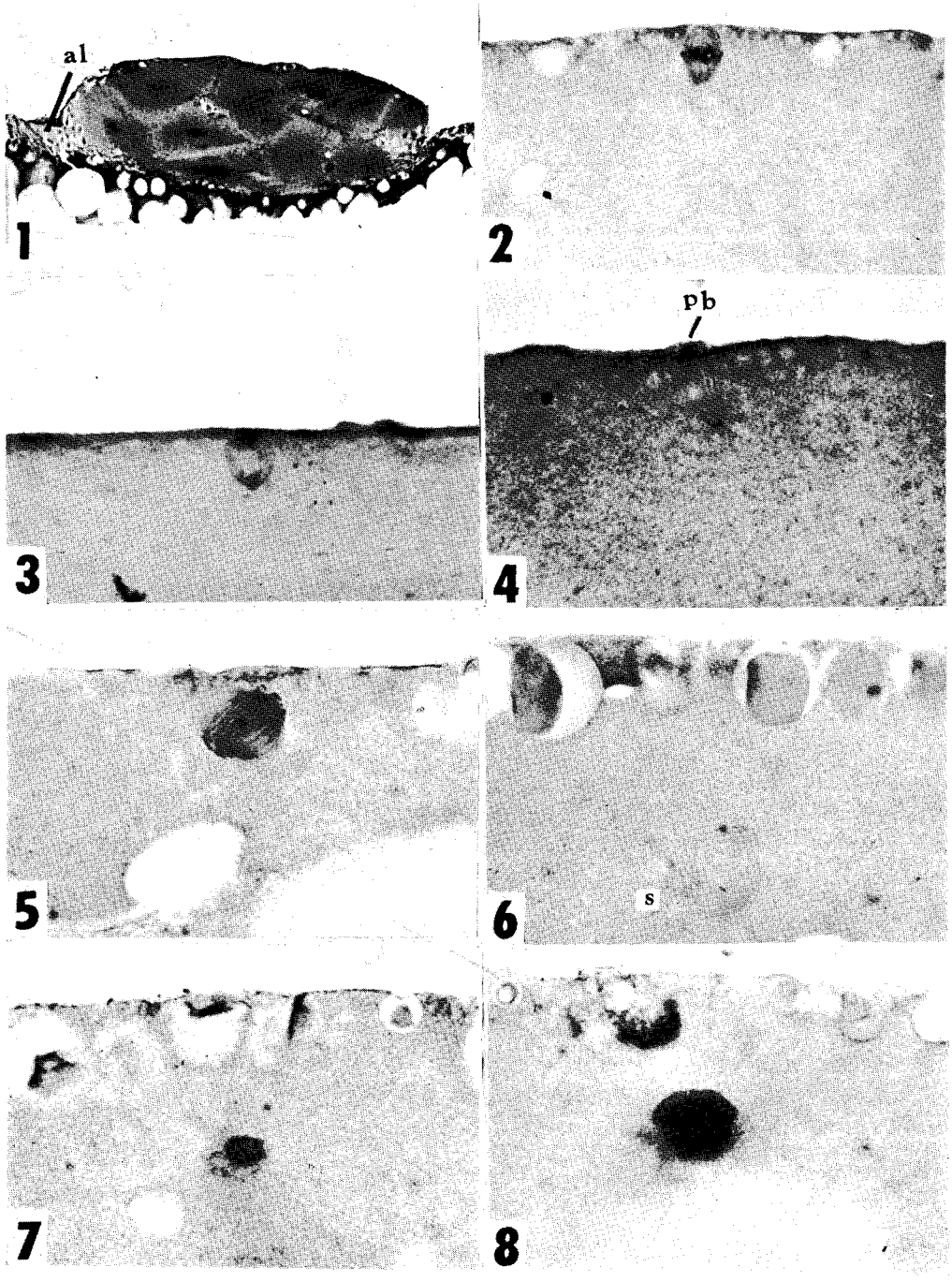
Explanation of Plates XIII-XIV

Fig. 1. Section through the blastodisc of fertilized egg. The eggs inseminated in the Ringer's solution were treated with the acidulated Ringer's solution (pH 2.5) for 3 minutes, then immersed in the normal Ringer's solution (pH 7.2) for 17 hours. The cortical alveoli remaining (al) are found at the margin of blastodisc. The cleavage pattern appears to be normal. ca. × 60.

Figs. 2–4. Second meiotic division of the eggs classified as the first group in the present paper. The eggs from a female were immersed in the acidulated Ringer's solution (pH 2.0) for 1/2 minute. ca. × 620. Fig. 2. Metaphase spindle from side view. Outer pole of the spindle is located at the surface of the egg. At 15 minutes after the treatment. Fig. 3. Anaphase spindle showing complete separation of dyads. At 40 minutes. Fig. 4. Extrusion of polar body (pb). At 70 minutes.

Figs. 5–10. Second meiotic division of the egg classified as the second group in the present paper. The eggs from a female were immersed in the acidulated Ringer's solution (pH 2.0) for 1/2 minute. ca. × 620. Fig. 5. Submerged anaphase spindle. Chromosomes are found at either poles of the spindle. The spindle appears to be slightly disrupted at both of its lateral sides. At 40 minutes after the treatment. Fig. 6. Tri-polar spindle (s) found in the fertilized egg. Spindle fibers are very indistinct. At 80 minutes. Fig. 7. Egg pronucleus in the interior of the egg. At 2 hours. Fig. 8. Conjugation pronuclei. At 4 hours. Fig. 9. Tetra-polar spindle (s) found in the unfertilized egg. Chromosomes are found at each pole of the spindle. At 2 hours. Fig. 10. A mass of Feulgen-positive particles found in the unfertilized egg. This is derived from the egg nucleus. At 24 hours.

Figs. 11–14. Second meiotic division and behavior of sperm nucleus in the eggs suppressed the polar body formation. The eggs from a female were immersed in the acidulated Ringer's solution (pH 2.0) for 10 minutes. ca. × 620. Fig. 11. Submerged metaphase spindle. Outer pole of the spindle is leaving the egg surface. At 50 minutes after the treatment. Fig. 12. Submerged metaphase spindle (en) and sperm nucleus (sn). The distance of the metaphase spindle from the egg surface is considerably increased. The sperm nucleus takes a fibrous appearance resembling the mitotic spindle and is located in the protoplasm near the egg surface. At 70 minutes. Fig. 13. Sperm aster developed near the base of the sperm head. At 30 minutes. Fig. 14. Well developed sperm aster enclosing the sperm head. At 50 minutes.



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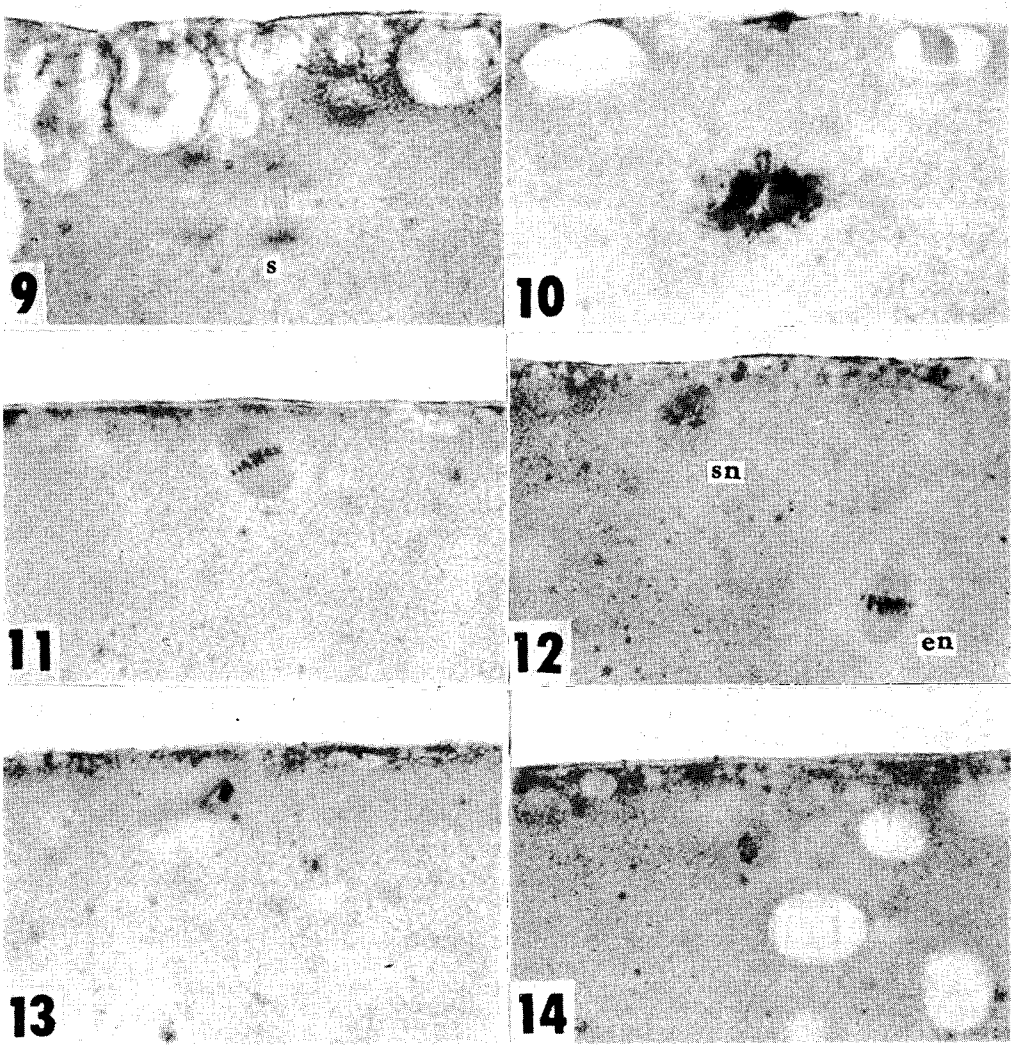

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