Title	Activation of Dog Salmon Egg by Heavy Metal Ions (With 1 Plate and 2 Tables)
Author(s)	YAMAMOTO, Tadashi S.
Citation	, 15(1), 148-156
Issue Date	1962-12
Doc URL	http://hdl.handle.net/2115/27360
Type	bulletin (article)
File Information	15(1)_P148-156.pdf

Instructions for use

Activation of Dog Salmon Egg by Heavy Metal Ions[1]

By

Tadashi S. Yamamoto

Zoological Institute, Hokkaido University
(With 1 Plate and 2 Tables)

It has been shown that salmon egg does not show any indication of activation in isotonic Ringer's solution even after fertilization (Kanoh '50, K. Yamamoto '51). This is a peculiar property of the salmon egg because eggs of other fresh water fishes, such as medaka and stickleback, can be activated in isotonic Ringer's solution when they are fertilized or parthenogenetically stimulated (T. Yamamoto '56).

Activation of salmon egg by immersion in isotonic solution has been successful in some cases. In experiments involving the treatment of the salmon egg with $CaCl_2$ and $MgCl_2$ (Kanoh '52, Kusa '53) it was found that immersion of the egg in isotonic solution of these salts brings about the activation though the breakdown of cortical alveoli is inhibited. In other experiments (Kanoh & Yamamoto '57) it was found that the activation without breakdown of cortical alveoli is also caused in the eggs subjected to double treatment with acidulated isotonic Ringer's solution (pH 1.8) and 0.2% pancreatin dissolved in isotonic Ringer's solution. These facts suggest a possibility that the eggs of salmon may be activated in isotonic Ringer's solution as in the case of the eggs of the other fresh water fishes (*e.g.* medaka), if some extra agents are added to the environmental Ringer's solution. For years the writer has been interested in analyzing the mechanism of egg activation in teleostean fishes with various agents. The present paper deals with the effects of heavy metal ions on the salmon egg.

Material and Method

The eggs of dog salmon (*Oncorhynchus keta*) were used in the present study. As heavy metals, $ZnSO_4$ was generally used in the experiment[2] but $CdCl_2$, $CoCl_2$, $MnCl_2$ and $NiCl_2$ were

---

also employed. Since the matured salmon egg can be kept in Ringer's solution without suffering any changes of activation (Kanoh '50) ,the metals were all used after having been dissolved in Ringer's solution of the following constitution: M/6.5 NaCl 100 parts + M/6.5 KCl 2.8 parts + M/10 CaCl$_2$ 3.4 parts (pH 7.2). For the observation of the blastodisc, eggs were fixed in Bouin's fluid. When necessary, sections were cut at 10μ thickness with the ordinary paraffin method and stained with Delafield's hematoxylin. Other technical details will be described in each section. All the experiments were performed at room temperature (12°–17°C).

Results

Effective concentration of Zn ions for egg activation: As reported in the previous paper (T.S. Yamamoto '57b), the unfertilized eggs of salmon were activated when immersed in Ringer's solution containing Zn-salt and raised distinct blastodiscs. Then the effective concentration of ZnSO$_4$ in Ringer's solution for inducing the formation of blastodisc was first determined. The eggs were immersed in one of Ringer's solutions containing ZnSO$_4$ of which concentration varied from 0.0001% to 0.8%.

After 17 hours' immersion in these Zn-Ringer's solutions, no eggs underwent cytolysis in any cases. Distinct blastodiscs were observed in the eggs treated with 0.01–0.4% Zn-Ringer's solution. At the concentration of 0.001% ZnSO$_4$, some eggs raised the disc but the others did not. In 0.0001% ZnSO$_4$, no eggs raised the disc. At the higher concentration of ZnSO$_4$ more than 0.4%, no distinct blastodisc was formed.

The blastodiscs formed in Zn-Ringer's solution sometimes appeared as if they had been cleaved as in the normal blastodisc at 2–4 cell stages (Fig. 1). In the sectioned material, it was revealed that furrows as a rule remained superficial. Only in rare cases however furrows comparable to those of normal cleaving eggs were observed (Fig. 2), though the nucleus remained undivided (Fig. 3). Regarding the morphological state of the nucleus in Zn-treated egg, further description will be found later.

When the eggs previously fertilized in normal Ringer's solution were immersed in Zn-Ringer's solution, the eggs raised blastodiscs as in the case of the unfertilized eggs. Moreover, the blastodiscs of these eggs showed cleavings accompanying nuclear divisions. The effective concentration of ZnSO$_4$ in Ringer's solution for encouraging the cleavage of fertilized eggs was 0.01–0.4% and coincided with that for inducing the blastodisc formation.

Toxicity of Zn ions: As stated already, no eggs underwent cytolysis in 0.001–0.8% Zn-Ringer's solution. On the other hand, when the eggs were immersed in 0.2% ZnSO$_4$ dissolved in tap water, they underwent cytolysis within a short time. This seems to indicate that the toxic action of Zn ions on the egg is arrested by Ringer's solution.

What components of Ringer's solution are responsible for arresting the toxic

action of Zn ions?   To solve this problem, the eggs were immersed in one of 0.2% $ZnSO_4$ solutions made up with M/6.5 NaCl, M/6.5 KCl, M/6.5 LiCl, M/10 $CaCl_2$ or M/10 $MgCl_2$.   After 17 hours' immersion in these solutions, the eggs did not undergo cytolysis in the solutions of Zn-NaCl, -KCl and -LiCl as in the case of Zn-Ringer's solution, whereas about 60 and 70% of the eggs underwent cytolysis in the solutions of Zn-$CaCl_2$ and -$MgCl_2$ respectively.   These observations seem to indicate that the toxic action of $ZnSO_4$ is nearly arrested by the presence of monovalent cations. However the salt concentration of the Ringer's solution may have its share in the problem here concerned, because in Zn-tap water all of the eggs undergo cytolysis within a short time but in Zn-$CaCl_2$ or -$MgCl_2$ some eggs are still alive even after 17 hours' immersion.

Breakdown of cortical alveoli and nuclear change in Zn-treated egg: Breakdown of cortical alveoli, which occurred in normal development, was scarcely observed though blastodiscs were found distinctly in the eggs treated with 0.1% Zn-Ringer's solution.   Most of the alveoli remained were found in the proximal periphery of the blastodisc.   The same condition of the cortical alveoli was found in the eggs treated with 0.01–0.4% $ZnSO_4$ (Figs. 6–9).

As for the egg nucleus, maturation division proceeded in Zn-Ringer's solution and the second polocyte was extruded after about 80 minutes.   The pronucleus thus formed moved inward to the center of the blastodisc.   When the egg was immersed for about 17 hours in Zn-Ringer's solution at the concentration over 0.01 %, the pronucleus underwent some morphological changes as shown in figures 4 and 5; In figure 4, it has swelled markedly, as compared with the normal one, and was deeply stained with hematoxylin.   In figure 5, the membrane surrounding the pronucleus has gone out of sight but chromatin-like particles have become scattered in the region where the pronucleus had been situated.   In the egg immersed in Zn-Ringer's solution for more than 17 hours, the writer failed to find the egg nucleus, probably because of its disintegration.   In the egg of which the blastodisc was not clear in 0.001% Zn-Ringer's solution, the nucleus remained at the metaphase, the same stage as that of the intact egg.   In the egg previously fertilized in normal Ringer's solution, then immersed in Zn-Ringer's solution, the second polocyte was extruded as in the case of the unfertilized egg, karyogamy occurred within about 2 hours and the cleavage proceeded in normal fashion.   The cleavage pattern appeared to be normal in early stages when the eggs were kept at 10°C, but they all underwent degeneration before gastrula stage.

In order to secure development beyond the gastrula stage, the fertilized eggs were transferred into tap water after 17 hours' immersion in 0.2% Zn-Ringer's solution.   Figures 6, 7, 8 and 9 reproduce sections provided from the eggs thus treated.   As will be seen, the eggs developed to some extent after transfer into tap water.   The eggs thus treated, however, soon underwent degeneration and no eggs reached neurula stage.   It was further noticed from these figures that the breakdown of cortical alveoli did not occur even in tap water if the eggs were

treated previously in Zn-Ringer's solution.

Then an experiment was undertaken in order to ascertain what length of time of immersion in Zn-Ringer's solution was enough to inhibit the breakdown of cortical alveoli before transfer into tap water. Eggs were immersed in 0.1% Zn-Ringer's solution for a certain length of time, then transferred into tap water. Sixty minutes later, they were fixed and sectioned. The results showed that the breakdown of cortical alveoli was inhibited by 2–4 hours' immersion. It has been ascertained in the preliminary control experiments that the breakdown of cortical alveoli in tap water did not begin within one minute. On the other hand, when the eggs were placed in Zn-Ringer's solution after treatment with tap water for one minute, cortical alveoli broke down. Therefore, it can be said that the breakdown of cortical alveoli is not inhibited in Zn-Ringer's solution if the stimulation for the breakdown of the alveoli is established in tap water.

Comparison of Zn ions and tap water in the activation of eggs: Results obtained in the present experiments suggest that the effect of Zn ions on the salmon egg is quite similar to that of tap water, though the former does not cause the breakdown of cortical alveoli in the activated eggs. Thus the effect of Zn ions was compared with that of tap water.

It has been shown that the activation of salmon egg can be induced in Ringer's solution if it is previously treated with tap water for a few minutes (Kanoh '56). In order to compare the effect of tap water with that of Zn-Ringer's solution, the following experiments were performed. Eggs fertilized in normal Ringer's solution were treated with 0.1% Zn–Ringer's solution for a certain length of time, then kept in normal Ringer's solution. About 12 hours later, the cleaved eggs were counted. As a control, tap water was used instead of Zn-Ringer's solution. A typical result of this experiment is presented in table 1. From this table, it may be supposed that the effect of tap water is manifested more rapidly than that of Zn-Ringer's solution.

It was revealed in a preliminary experiment that the formation of the blastodisc and the cleavage of the fertilized egg in tap water were prevented if

Table 1. Comparison of the effect of 0.1 % Zn-Ringer's solution and of tap water on the fertilized salmon egg. Eggs were treated with Zn-Ringer's solution or tap water for a certain number of minutes, then kept in normal Ringer's solution.

Duration of immersion in the solution	Eggs cleaved (after 12 hours)	
	Zn-Ringer's solution	Tap water
30 sec.	0%	0%
1 min.	0	0
3	0	30
5	50	90
10	90	100

1×10^{-4}M KCN was contained in tap water but that they were not prevented at the concentration of 1×10^{-5}M. That is to say, a critical concentration of KCN to prevent the development is found between 1×10^{-4}M and 1×10^{-5}M. Considering this fact, the effect of KCN on the development in Zn-Ringer's solution was studied, with the result that 1×10^{-4}M KCN was found to prevent also the development in Zn-Ringer's solution but 1×10^{-5}M KCN has no such effect as in the case of tap water.

It has been reported that the chorion of the unactivated salmon egg is dissolved by the double treatment with acidulated Ringer's solution and pancreatin but the chorion of the activated egg in tap water is not dissolved by the same double treatment. It is supposed, therefore, that some chemical changes have taken place in the chorion following egg activation in tap water (Kanoh & Yamamoto '57, T.S. Yamamoto '57a). In the eggs activated with Zn-Ringer's solution, the chorion was dissolved by the double treatment mentioned above. It is clear, therefore, that the susceptibility of the chorion of Zn-treated egg to the enzymatic double treatment remained unchanged even after the egg activation. This is a marked difference between the Zn-activated egg and the tap water-activated egg. In this connection, it must be noted that the chorion becomes opaque after activation in tap water but no recognizable change of the translucency of the chorion was observed after the activation in Zn-Ringer's solution.

Activation by other heavy metal ions: The other heavy metals used activated the egg without breakdown of cortical alveoli as in the case of $ZnSO_4$. The effective concentrations for the activation in Ringer's solution are presented in table 2. As will be seen, Cd ions were effective at very low concentrations similarly to Zn ions. It must be noticed that the former ions had a marked toxic action on the egg and at high concentration more than the effective one for the formation of blastodisc, the egg rapidly underwent cytolysis.

Table 2.   Effective concentration of heavy metal ions for inducing
the formation of the blastodisc in salmon egg.   Heavy metals
were all dissolved in isotonic Ringer's solution.

Heavy metal	Effective range
$CdCl_2$	0.025–0.2 %
$ZnSO_4$	0.01 –0.4
$NiCl_2$	0.1 –1.0
$CoCl_2$	0.2 –1.0
$MnCl_2$	0.5 –1.0

The maturation division of the egg proceeded in Ringer's solution containing these heavy metal ions and the second polocyte was extruded from the egg. The pronucleus thus formed underwent some morphological changes as stated in the case of $ZnSO_4$ but did not show any nuclear division in the case of unfertilized egg. On the other hand, the eggs fertilized previously in normal Ringer's solution

developed to some extent in Ringer's solution containing these heavy metal ions similarly to the case of $ZnSO_4$.

Discussion

As stated already, salmon egg does not show any indication of activation in isotonic Ringer's solution even after fertilization. To induce egg activation, immersion of the egg in hypotonic Ringer's solution less than $M/10$ or in nonelectrolyte solution has been reported to be necessary (Kanoh '50, K. Yamamoto '51). On the other hand, activation without breakdown of cortical alveoli has been successfully evoked by the immersion of the egg in isotonic solutions of $CaCl_2$ and $MgCl_2$ (Kanoh '52, Kusa '53).

In the present study it was revealed that heavy metal ions contained in isotonic Ringer's solution activate the egg without breakdown of cortical alveoli. It does not seem likely that the effect of heavy metals in the concentration used were due to osmotic factors. Although measurements of the osmotic pressure of the solution were not made, no shrinakge of the test egg was noticed. In previous experiments by Aoki ('39), Kanoh ('56) and others, Ringer's solutions in concentration of $M/6.5$, $M/7$ and $M/8$ have been used for the isotonic solution of the salmon egg and no distinct changes of the egg owing to these osmotic variations have been found in these Ringer's solutions. In fact the writer found no significant difference of effect between the heavy metal solution made up with $M/6.5$ Ringer's solution and the solution made up with $M/8$ Ringer's solution. Probably minor changes (of the order of the concentrations used in the present study) in salt content of Ringer's solution produce little or no effects. In this case, no eggs underwent cytolysis within 48 hours in isotonic Ringer's solution containing heavy metal ions. Furthermore, that solution did not inhibit the cleavage of fertilized egg. The development proceeded to the morula or blastula stage but beyond the gastrula stage. It is supposed therefore that heavy metal ions used may produce an effect not antagonized by Ringer's solution, and consequently the egg undergoes degeneration. Probably the heavy metal ions exert their toxic action on the egg in the later stages of development.

By deduction from the results concerning suitable concentrations of heavy metal ions for the initiation of development in the salmon egg, the following order can be arranged; $Mn < Co < Ni < Cd \leqq Zn$. In the study of the animalizing effect of heavy metal ions in sea urchin eggs, Lallier ('56) has stated: "Les ions de métaux lourds forment avec les protéines des complexes de stabilité élevée dans les conditions physiologiques normales. En général l'ordre de stabilité de ces complexes est: $Mn < Fe < Co < Ni < Cu < Zn$." This order seems to coincide well with that of the heavy metal ions mentioned above in the present study, which is a matter of interest worth being studied further.

The chorion of salmon egg immersed and activated in tap water is not digested by the double treatment with acidulated Ringer's solution and pancreatin, whereas that of unactivated egg is digested by the same treatment (Kanoh &

Yamamoto '57, T.S. Yamamoto '57a). Such change in the susceptibility of the chorion to the enzymatic solution has been reported to be apparently related to the activation of the egg proper, because the chorion isolated from the unactivated egg is digested by the double treatment even after immersion in tap water, whereas the chorion isolated from the egg once activated is not digested (T.S. Yamamoto '57a). Deducing from the similarity of behavior of the cortical alveoli of the teleost egg and that of the cortical granules of the sea urchin egg, the writer surmised in a previous study that the content of cortical alveoli extruded from egg cortex following activation might be related to this change of the chorion (T.S. Yamamoto '57a). In the present study, the chorion of the egg activated by immersion in Zn-Ringer's solution was digested by the double treatment. Since the cortical alveoli remained unbroken in the blastodiscs of eggs thus treated, the results of the present experiment seem to support the surmise mentioned above. However, the writer has no reason to neglect the following posibilities; 1) Besides the content of cortical alveoli, there may be some substances in the egg which are extruded from the egg proper following activation in tap water but are not extruded in Zn-Ringer's solution. These substances may probably be directly related to the change of the chorion mentioned above (Zotin '58, Ohtsuka '60). 2) Zn ions may act on the chorion to keep it in digestible state even after activation. In the eggs treated with calcium, in which the cortical alveoli remain unbroken, the chorion becomes insoluble on account of the combination of calcium (Hamano '49, T.S. Yamamoto '57a). Certainly the subject deserves further study.

An explanation of the mechanism of the activation by heavy metals is not easy. Ca and Mg ions activate the salmon egg without breakdown of the cortical alveoli as in the case of heavy metal ions. Therefore, it is assumed that the action mechanism of the heavy metal ions may be the same of both Zn ions and Ca and Mg ions. In fact, these cations are all bivalent. Nevertheless the following facts suggest that there may be a different mechanism of the action of heavy metal ions from that of Ca and Mg ions: 1) The effective concentration of Ca ions for the formation of the blastodisc is more than 50% in Ringer's solution (Kusa '53), whereas that of the heavy metal ions is less than 1% in Ringer's solution. 2) In the unfertilized eggs, the pronucleus undergoes disintegration without any change of nuclear division when treated with calcium or magnesium, but it swells and comes to be deeply stained when treated with heavy metals.

The writer has come to a tentative conclusion as to the action of heavy metals. According to biochemical knowledge, heavy metal ions are closely related to the enzymatic activity in living organisms and, *in vitro*, various sorts of heavy metal ions have been reported to activate various peptidases, phosphatases and lecithinases (Vallee '59). On the other hand, it is here worthwhile to recall Runnström's hypotheses in sea urchin eggs (Runnström '49). According to him, the incitement of development may be traced to activation of the enzymatic system in the egg. He has proposed that an enzyme-inhibitor complex might be

present in the cortex of the unfertilized sea urchin egg and that penetration of the spermatozoon might remove the inhibitor. Furthermore, Kanoh ('54) has measured NH_4 ions released from the salmon egg during activation and suggested the possibility that the release might be a result of proteolysis occurring in the egg. Since heavy metal ions activate peptidases, *in vitro*, and in the salmon egg they can induce the formation of the blastodisc in isotonic Ringer's solution, activation of the egg might proceed in comany with the activation of the enzymatic system by heavy metal ions permeated into the egg.

Probably, morphological change of pronucleus in the eggs activated by heavy metal ions may be due to the effect of the ions which have permeated. In this connection, it is of interest that in the cleavage of echinoderm egg, chromosomes have been found to become laden with zinc on the initiation of mitosis, particularly on entering the metaphase (Fujii '55).

Summary

1. By immersion in isotonic Ringer's solution containing heavy metal ions (Zn, Cd, Ni, Co and Mn), eggs of dog salmon (*Oncorhynchus keta*) were parthenogenetically activated without breakdown of cortical alveoli.

2. Eggs previously fertilized in normal Ringer's solution developed to some extent in the Ringer's solution containing heavy metal ions.

3. The action of heavy metal ions in the Ringer's solution on salmon egg seems to be a peculiar one that is different from the action of hypotonic Ringer's solution and isotonic solutions of $CaCl_2$ and $MgCl_2$.

4. A tentative explanation was proposed as to the mechanism of the action of heavy metal ions on salmon egg.

## Literature cited

Aoki, K. 1939. Ueber die Wasseraufnahme der Lachseier, I. J. Fac. Sci. Hokkaido Imp. Univ. (Zool.), 7: 27–38.

Fujii, T. 1955. Presence of zinc in nucleoli and chromosomes and its possible role in mitosis. J. Fac. Sci. Univ. Tokyo (Zool.), 7: 313–325.

Hamano, S. 1949. On the swelling layer of egg membrane of salmon, *Oncorhynchus keta* (in Japanese with English Résumé). Physiol. and Ecol., 3: 43–46.

Kanoh, Y. 1950. Ueber Wasseraufnahme und Aktivierung der Lachseier. I. Annot. Zool. Japon., 24: 13–21.

———— 1952. Ueber die Beziehung zwischen dem Zerfallen der Kortikalalveoli und der Entwicklung bei Fischeiern (in Japanese with German Résumé). Jap. J. Ichthyol., 2: 99–103.

———— 1954. On the metabolism of the salmon egg at the time of activation (in Japa-

nese). Zool. Mag., 63: 160.

————— 1956. Ueber Wasseraufnahme und Aktivierung der Lachseier. IV. Vorläufiges Experiment in Betreff des Calcium-problems. J. Fac. Sci. Hokkaido Univ. (Zool), 12: 259–263.

Kanoh, Y. & T.S. Yamamoto 1957. Removal of the membrane of the dog salmon egg by means of proteolytic enzyme. Bull. Jap. Soc. Sci. Fish., 23: 166–172.

Kusa, M. 1953. Significance of cortical change in the initiation of development of the salmon egg (Physiological analysis of fertilization in the egg of the salmon, *Oncorhynchus keta* II). Annot. Zool. Japon., 26: 73–77.

Lallier, R. 1956. Les ions de métaux lourds et le probléme de la determination embryonnaire chez les échinodermes. J. Embryol. Exp. Morph., 4: 265–278.

Ohtsuka, E. 1960. On the hardening of the chorion of the fish egg after fertilization. III. The mechanism of chorion hardening in *Oryzias latipes*. Biol. Bull., 118: 120–128.

Runnström, J. 1949. The mechanism of fertilization in metazoa. Adv. Enzymol., 9: 241–327.

Vallee, B.L. 1959. Biochemistry, physiology and pathology of zinc. Physiol. Rev., 39: 443–490.

Yamamoto, K. 1951. Activation of the egg of the dog-salmon by water and the associated phenomena. J. Fac. Sci. Hokkaido Univ. (Zool), 10: 303–318.

Yamamoto, T. 1956. The physiology of fertilization in the medaka (*Oryzias latipes*). Exp. Cell Res., 10: 387–393.

Yamamoto, T.S. 1957a. Some experiments on the chemical changes in the membrane of salmon eggs occurring at the time of activation. Jap. J. Ichthyol., 6: 54–58.

————— 1957b. Some morphological and physiological aspects of the eggs of teleostean fishes. J. Fac. Sci. Hokkaido Univ. (Zool.), 13: 484–488.

Zotin, A.I. 1958. The mechanism of hardening of the salmonid egg membrane after fertilization or spontaneous activation. J. Embryol. Exp. Morph., 6: 546–568.

Explanation of Plate V

Fig. 1. Blastodisc of unfertilized salmon egg formed by immersion in 0.2% Zn-Ringer's solution for 17 hours. In this case, the blastodisc appears as if it was cleaving. ca. ×17.

Fig. 2. Section through the blastodisc of unfertilized salmon egg immersed in 0.1% Zn-Ringer's solution for 17 hours. A cleavage furrow can be seen. ca. ×60.

Fig. 3. The nucleus (n) of the egg presented in figure 2. Nuclear division has not occurred. ca. ×50.

Figs. 4–5. Nuclei of unfertilized salmon egg immersed in 0.1% Zn-Ringer's solution for 17 hours. ca. ×430.

Figs. 6–9. Sections through the blastodiscs of fertilized salmon eggs. The eggs fertilized in Ringer's solution were immersed in 0.2% Zn-Ringer's solution for 17 hours, then transferred into tap water. Figure 6 is the egg without treatment with tap water, figure 7 is that immersed for 4 hours and figures 8 and 9 for 24 hours in tap water. These figures indicate that the eggs develop to some extent after transfer into tap water. See nuclei in the blastodisc. al, cortical alveoli remained. ca. ×50.
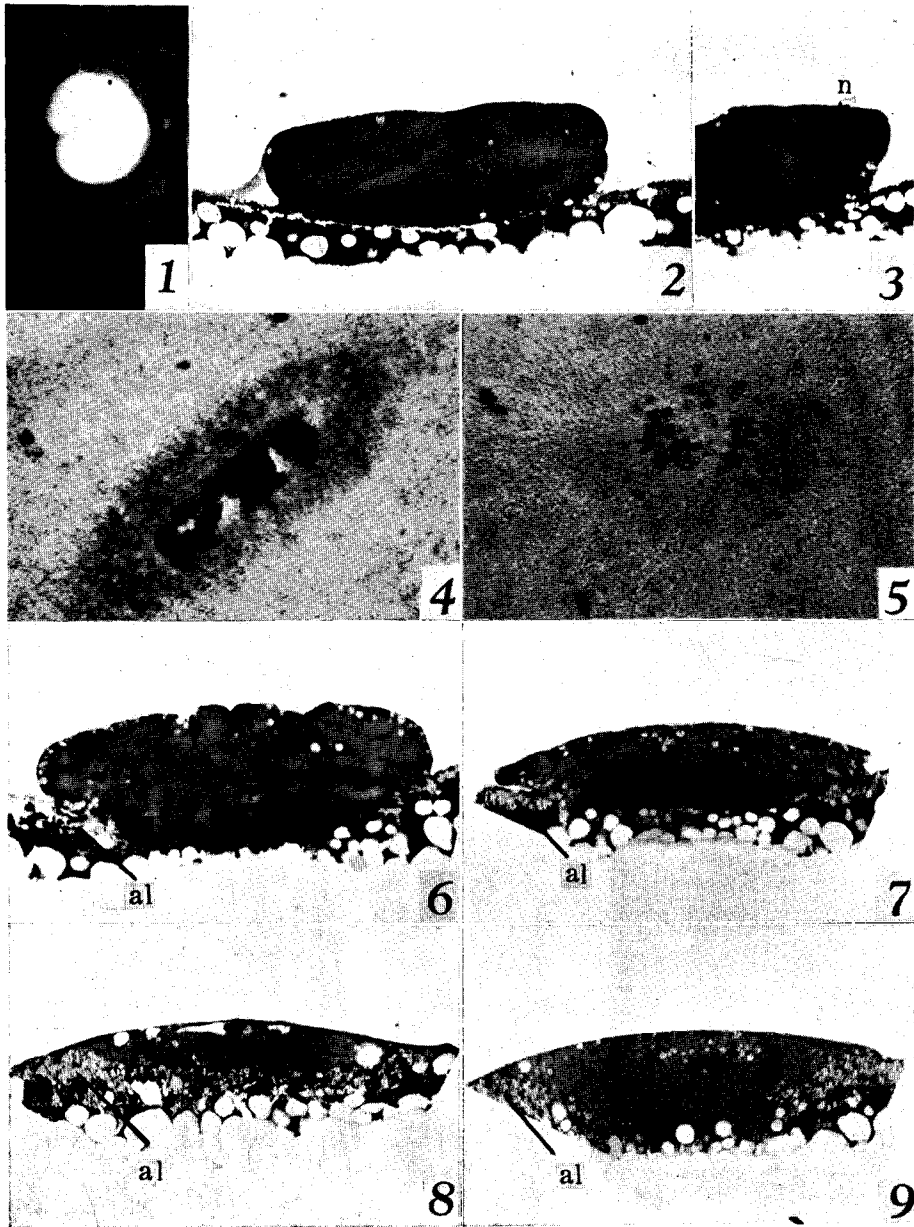
T. S. Yamamoto: *Activation of Dog Salmon Egg*