Effects of Ethylenediaminetetraacetate on the Egg of the Dog Salmon, *Oncorhynchus keta*[1,2)]

By

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(*With 1 Text-figure*)

In the physiological study of fertilization of the medaka egg, T. Yamamoto ('54, '61) found that Ca ions are released from the cortical protoplasm when the egg is stimulated to show an activation. The release of Ca ions in the process of activation was also reported by Kanoh ('56) in the salmon egg. These facts seem to indicate that there occur some molecular changes involving Ca ions in the protoplasm during egg activation. Furthermore it was reported in the previous paper (T.S. Yamamoto, '64) that short exposure of the salmon egg to acidulated Ringer's solution induces an activation not accompanied by breakdown of the cortical alveoli in normal Ringer's solution. Since acid is known, in general, to readily dissolve calcium compounds, it may be supposed in this case that acid had forcibly removed Ca ions from the salmon egg. If this is true, it is possible to anticipate that the removal of Ca ions from the protoplasm induces an activation of the egg. It is therefore interesting to test the effects on the protoplasm of the salmon egg of ethylenediaminetetraacetate as a metal-chelating agent. In the present paper are described the results of such experiments.

Material and method

The materials used were all taken from matured individuals of dog salmon, *Oncorhynchus keta*. As has been shown, the salmon egg does not show any indication of activation in Ringer's solution (Kanoh, '50, '52a). Therefore ethylenediaminetetraacetate (EDTA) was used after having been dissolved in Ca-free Ringer's solution[3)]. Eggs to be immersed in the solution of EDTA were previously washed thoroughly in Ca-free Ringer's solution.

1) Contribution No. 726 from the Zoological Institute, Faculty of Science, Hokkaido University, Sapporo.

2) This paper is dedicated to Professor Sajiro Makino, Zoological Institute, Hokkaido University, Sapporo, in honor of his sixtieth birthday. June 21, 1966.

3) Composition of the Ringer's solution: M/6.5 NaCl 100 parts + M/6.5 KCl 2.8 parts + M/10 $CaCl_2$ 3.4 parts (pH 7.2). Ca-free Ringer's solution used in the present study was made by omitting $CaCl_2$ from the above constituents.

When fertilized eggs were demanded in the experiments, the eggs were inseminated in Ringer's solution, then washed in Ca-free Ringer's solution[3,4]. For cytological study, the eggs were fixed in Bouin's fluid and embedded in paraffin by the ordinary method. Sections were prepared 10μ thick and stained with Delafield's hematoxylin. All the experiments were carried out at room temperature, 12°–15°C: other details of the experimental procedure used will be given in the following description.

Results

It was found that the eggs immersed in M/100 EDTA (pH 7.0) for 24 hours raised a distinct blastodisc without formation of perivitelline space. In sections, it was certified that the cortical alveoli remained unbroken in these eggs and occupied the marginal region of the blastodisc. The degenerating nucleus could be found in this blastodisc. When eggs previously fertilized in Ringer's solution were immersed in the same solution, the egg and sperm nuclei degenerated in most cases, and no indications of cleavage were appreciable in the blastodisc. In rare cases however the blastodisc contained several resting nuclei and had a bumpy surface suggesting that it underwent incomplete cleavings during the immersion. Since the salmon egg does not form the blastodisc after mere immersion in Ca-free Ringer's solution, it is reasonable to conclude that the above mentioned blastodisc formation is due to the action of EDTA.

The same blastodisc formation was observed at pH ranging from 6.0 to 10.0, suggesting that it has no relation to the pH of the solution. By changing the concentration of EDTA, it was revealed that the blastodisc is distinctly formed at M/100–M/500 but is less distinct at M/1000–M/10000. At M/50000, no apparent effect of EDTA was observed in the egg.

In order to ascertain whether the above mentioned blastodisc formation is elicited as a result of real activation of the egg or not, the behavior of the egg and sperm nuclei in the EDTA-treated egg was studied. Eggs previously inseminated in Ringer's solution were immersed in M/100 EDTA and fixed in Bouin's fluid at 30 minute intervals up to 6 hours after immersion.

Up to 4 hours in EDTA the metaphase spindle of the second meiotic division was, as in the intact egg, still found in the animal pole of the egg. Anaphase movements were initiated within 5 hours after immersion. Although some eggs were still at metaphase, the others exhibited complete separation of dyads in the spindle. By 6 hours after immersion, the daughter chromosomes of the meiotic division were, in most eggs, at either ends of the pole of the spindle. The outer pole of the spindle was found in the bulge occurring on the surface of the egg, exhibiting the complete formation of the second polar body. At this time, however, some eggs were at anaphase and a few others were at metaphase of the second meiotic

4) The fertilizing spermatozoon obviously penetrates, by this procedure, into the egg but cannot evoke any changes in the egg following fertilization, so long as the eggs are kept in the Ringer's solution or the Ca-free Ringer's solution (Kusa, '50a, '64).

division. This suggests that a portion of the eggs does not respond to the action of EDTA.

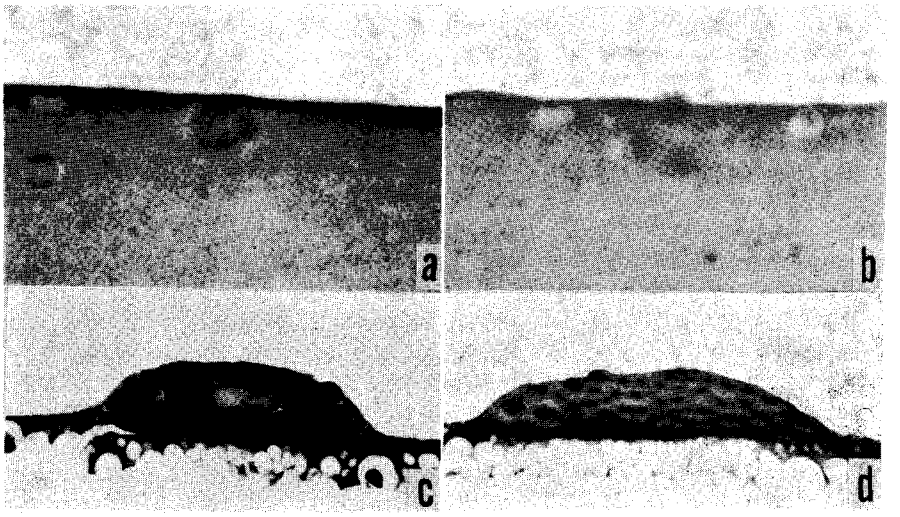

Fig. 1. Sections through the blastodisc of the fertilized egg immersed in M/100 EDTA. a, Bipolar structure originating from the penetrated spermatozoon. The egg was immersed in EDTA for 4 hours. ca. ×530. b, Tripolar structure found in the egg immersed for 6 hours. Note a small body resembling the polar body on the egg surface. ca. ×530. c and d, The blastodisc of the egg kept in tap water for 24 hours after 4 hours' immersion in EDTA. The breakdown of cortical alveoli was incomplete and some of the alveoli remaining were found in the margin of the blastodisc. ca. ×100.

On the other hand, the penetrated spermatozoon did not form the pronucleus in up to 6 hours' immersion and its development during immersion in EDTA showed a considerable divergence from that in normal fertilization. To describe this in detail, the sperm head swelled slightly within 2 hours of immersion and the aster was formed near the base of the head. With the lapse of time, the sperm aster was enlarged in the protoplasm near the egg surface and, by 4 hours after immersion, it developed into a bipolar structure comparable to the mitotic spindle, though there was no typical arrangement of chromosomes in this structure (Fig. 1a). The structure lay close to the egg surface, with its long axis perpendicular, or somewhat oblique to the latter. Five hours after the treatment, the structure persisted in the same position near the egg surface and both its lateral sides were slightly disrupted. By 6 hours' immersion, a new pole was formed on a lateral side of the spindle-shaped structure, probably owing to the lateral disruption of the bipolar structure. One of these poles protruded outward from the egg surface, where formed a small body resembling the polar body (Fig. 1b). As mentioned already, there were no eggs with sperm pronucleus.

The above observations indicate that most eggs slowly react to the action of EDTA and form the second polar body. After penetration the spermatozoon, however, does not develop into the pronucleus and probably undergoes degeneration without causing conjugation with the egg nucleus.

The intact salmon egg does not show any indication of activation in Ringer's solution but is activated and shows the breakdown of cortical alveoli when immersed in hypotonic solution, such as tap water. Thus the final experiment was designed to show whether the cortical alveoli remaining in the egg after the action of EDTA break down when the egg is transferred into hypotonic solution. Eggs previously inseminated in Ringer's solution were immersed in M/100 EDTA for a certain length of time, then transferred into tap or dist. water. After 24 hours' immersion, the eggs were fixed and sectioned to examine the state of the blastodisc.

With up to 2 hours' immersion in EDTA, the eggs seemed to remain in the normal physiological state, showing the complete breakdown of cortical alveoli and the formation of wide perivitelline space in water. The cleavage proceeded in the normal fashion in these eggs. With 4 hours' immersion in EDTA, the eggs also formed wide perivitelline space in water. When observed in sections, however, the breakdown of cortical alveoli was incomplete and some of the alveoli remained unbroken in these eggs. They occupied the marginal region of the blastodisc (Fig. 1c). In most eggs the periblast was of considerable size and the blastomeres were few compared with the normal control. In some eggs which contained a larger number of remaining alveoli in the blastodisc, cleavage did not occur though a number of large asters were found in the blastodisc (Fig. 1d). In these eggs, no distinct nuclei were found in the blastodisc but several small masses of chromatin were detected among some asters. When the period of immersion in EDTA was prolonged to 6 hours, the effect of EDTA was very evident. About 20% of the eggs thus treated died in water within 24 hours. Although wide perivitelline space was also observed in the living eggs, a number of cortical alveoli remained unbroken in the blastodisc. No eggs were cleft in tap water but there were a number of small asters in the disc. No nuclear structure was detected in these eggs. After 24 hours' immersion in EDTA, about 25% of the eggs died in water. The survivors formed wide perivitelline space but the incomplete breakdown of cortical alveoli was more evident than in eggs immersed in EDTA for 4–6 hours. No trace of protoplasmic division or nuclear structure was found in the blastodisc though a few abortive asters were observed.

Discussion

It was found in the present study that the salmon egg forms a distinct blastodisc without breakdown of cortical alveoli after immersion in EDTA. There are several reports indicating that the breakdown of cortical alveoli itself is dispensable in the initiation of the development in fish eggs (Kanoh, '52b; Kanoh and Yanagimachi, '56; Devillers *et al.*, '53; Kusa, '53; T.S. Yamamoto, '62, '64; Sakai, '64). The perivitelline space was

hardly observed in eggs with a distinct blastodisc after immersion in EDTA. Since the perivitelline space should be formed in Ringer's solution when some of the cortical alveoli are eliminated from the egg cortex (Kanoh, '50, '51; T. Yamamoto, '61), it may be concluded that hardly any of the cortical alveoli have been broken down in these EDTA-treated eggs.

In the salmon eggs which formed a distinct blastodisc without breakdown of cortical alveoli and extruded the second polar body by other techniques (Kanoh, '52b; T.S. Yamamoto, '62, '64), the male and female pronuclei conjugated in the center of the blastodisc and cleavage followed. In the eggs immersed in EDTA, the spermatozoon after penetration remained for at least 6 hours in the protoplasm near the egg surface, where it developed a sperm aster and formed a bipolar structure comparable to the mitotic spindle. The process of meiotic division in EDTA was considerably retarded compared with that in normal fertilization but the spermatozoon formed the aster at nearly the normal rate (K. Yamamoto, '52). This fact indicates that there is a chronological disharmony (Beetschen, '59) in the development of the male and female pronuclei in the egg immersed in EDTA. Probably the failure of migration of the spermatozoon toward the center of the blastodisc and the chronological disharmony in the development of the pronuclei cause the failure in conjugation of male and female nuclei which reflects on the failure in cleavage of the fertilized egg in EDTA. Agrell ('57) observed in the partially activated sea urchin egg that the male pronucleus remains in the egg periphery without migration, while the female pronucleus migrates toward the center of the egg. These facts suggest that the effect of incomplete activation of egg protoplasm appears more evidently on the migration of the male nucleus than of the female nucleus. Osanai ('64) suggested from his experimental results on the partial fertilization of sea urchin eggs that an ectoplasmic activation attending the cortical change induces an endoplasmic activation correlating with the developmental change of the egg components, such as the nucleus and the aster. These statements suggest that, in the salmon egg, the blastodisc is formed as a result of the incomplete activation of egg protoplasm by EDTA.

An explanation of the underlying mechanism of activation by EDTA is not easy. As mentioned already, the egg completed the second meiotic division in EDTA and extruded the second polar body, though the process was much slower than in normal fertilization (K. Yamamoto, '52). It is evident from this observation that the blastodisc formation by EDTA is not to be ascribed to the hypertonicity of the solution, because the salmon egg immersed in hypertonic Ringer's solution (M/4) does not proceed to the second meiotic division, though it forms a distinct blastodisc (Kanoh, '51). It can be supposed that EDTA slowly stimulates the salmon egg by removing metallic ions from the surface protoplasm of the egg, then the egg extrudes the second polar body: as the protoplasmic surface seems to be almost impermeable to EDTA (Kanoh, '52a), the removal of the metallic ions from the egg is restricted to only the superficial area; for this reason or owing to the absence of free Ca ions in the egg, the protoplasmic activation is incomplete, thus the

spermatozoon cannot migrate toward the center of the blastodisc. Recently Ohtsuka ('64) reported that, without causing any visible cortical changes of the medaka egg, EDTA induces the hardening of the chorion which normally appears only after egg activation. Probably this may also be regarded as incomplete activation of the egg by EDTA. As the spermatozoon of the sea urchin can induce the development of the egg by itself without the co-operation of the egg nucleus (*cf.* Hiramoto, '62), it is not surprising that the spermatozoon of salmon showed an indication of mitotic activity and formed a bipolar structure comparable to the mitotic spindle in the protoplasm near the egg surface.

When the eggs were put into tap water, the breakdown of cortical alveoli normally occurred if the duration of immersion in EDTA was shorter than 2 hours. In proportion to the prolongation of immersion in EDTA, however, some of the alveoli remained unbroken in tap water and were found in the margin of the blastodisc after the formation of the distinct disc. The number of the remaining cortical alveoli increased as the duration of immersion in EDTA was prolonged. Probably the egg protoplasm may undergo changes with the action of EDTA as evidenced by the incomplete breakdown of the cortical alveoli after transfer into tap water. It is of interest to note that the duration of immersion in EDTA for the initiation of the anaphase movement of the second meiotic division is enough to induce the above mentioned protoplasmic changes in the egg. Furthermore it was noted that, in proportion to the increase in the number of the remaining cortical alveoli, the protoplasmic division of the blastodisc in tap water is affected. Probably this indicates that the presence of cortical alveoli disturbs in some way the protoplasmic division.

The importance of Ca ions in the process of activation in fish eggs has been pointed out by T. Yamamoto ('54, '61). In the salmon egg, however, Kusa ('50b) found that eggs previously treated with an isotonic solution of sodium oxalate show activation in dist. water. He supposed that, unlike the case of medaka, Ca ions in the environmental solution are unnecessary for the activation of salmon egg. On the other hand, Dettlaf ('59) reported that the activation of the salmon egg previously immersed in M/10 EDTA for 4–5 minutes is retarded or suppressed in water. From this result, he concluded that Ca ions are indispensable for the activation of the salmon egg. In the present study, the normal activation of the egg was observed in dist. water after 2 hours' immersion in M/100 EDTA dissolved in Ca-free Ringer's solution. Accordingly it may be said that, as reported by Kusa ('50b), the activation of the salmon egg does not require the presence of Ca ions in the environmental solution. This conclusion seems to be contradictory to the case of the medaka egg. In this connection, it is here worthwhile to recall T. Yamamoto's statement on the medaka egg (T. Yamamoto, '54, '61). He separated the reception of stimulation in the activation process from the evocation of excitation. According to him, Ca ions are not only unnecessary in the primary phase of the process (reception of stimulation) but they are released from the cortical

protoplasm at this phase. He considered that Ca ions are necessary, or at least have a favorable effect, in the secondary phase (evocation of fertilization wave or breakdown of the cortical alveoli). Since the release of Ca ions in the process of activation has been actually reported by Kanoh ('56), there is a possibility that sufficient Ca ions are released at the primary phase to cause the secondary phase of the process of activation in the salmon egg. The fact that Ca ions are not needed in the environmental solution for the activation of salmon egg will be accounted for in this way.

Summary

1. Effects of a metal-chelating agent, ethylenediaminetetraacetate (EDTA), on the egg of the dog salmon, *Oncorhynchus keta*, were studied.

2. On the immersion of unfertilized and fertilized eggs in M/100 EDTA, it was observed that the egg protoplasm moved toward the animal pole without breakdown of cortical alveoli, and formed a distinct blastodisc. The eggs completed the second meiotic division during immersion and extruded the polar body. The spermatozoon could not migrate to the center of the blastodisc and remained in the protoplasm near the egg surface where it formed a bipolar structure comparable to the mitotic spindle. The conjugation of egg and sperm nuclei seemed not to occur in these eggs, thus the cleaving of the blastodisc did not take place. It was suggested that EDTA slowly stimulates the salmon egg; but the induced activation of the egg protoplasm is incomplete for the continuation of development.

3. The eggs previously immersed in M/100 EDTA for 2 hours showed the complete breakdown of cortical alveoli and blastodisc formation after transfer into water. With longer immersion in EDTA, inhibition of the breakdown of cortical alveoli occurred in the egg and some of the alveoli remained unbroken in the blastodisc after transfer into water. It was concluded that the activation of salmon egg by hypotonic solution does not require the presence of Ca ions in the environmental solution.

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