Calcium Efflux Following Parthenogenetic Activation of the Dog Salmon Egg

By

Tadashi S. Yamamoto

Zoological Institute, Hokkaido University

(With 3 Text-figures and 3 Tables)

Fertilization or parthenogenetic activation is accompanied by pronounced changes in the egg surface. At the same time, various substances pour forth from the egg into the surrounding medium (Ishihara, 1964; Fodor et al., 1975). For example, the fish egg discharges the contents of cortical alveoli into the interstice between the ooplasmic surface and the egg membrane. Similarly, various substances seem to be effused from other sites of the egg after fertilization or parthenogenetic activation (Ohtsuka, 1964). Among them, certain substances are diffused through the egg membrane into the surrounding medium. Kanoh (1956) demonstrated the efflux of calcium ions occurring at parthenogenetic activation of the dog salmon egg and suggested that the efflux is a manifestation of the rise of the free calcium level in the ooplasm. Recent investigations with aequorin revealed that the free calcium level of the ooplasm explosively rises at fertilization of the eggs of the sea urchin (Steinhardt et al., 1977) and the medaka (Ridgway et al., 1977). In view of the importance of calcium ions in stimulation and excitation, the calcium efflux of the dog salmon egg was analyzed in the present study.

Materials and Methods

Materials used were derived from matured individuals of the dog salmon, *Oncorhynchus keta*, captured at the Chitose Salmon Hatchery, Hokkaido. The ripe eggs were obtained by dissecting the belly of females. Those eggs which showed over 90% cleavage after fertilization were used. Before the experiments, the eggs were thoroughly washed with isotonic salt solution for removing calcium ions from the egg surface. The salt solution had the constitution of 139.0 mM NaCl, 3.9 mM KCl and 10 mM Tris-HCl (pH 7.2) (Yamamoto, 1965).

To determine the concentration of calcium in the ooplasm, the eggs were broken by cutting one by one with fine scissors in the salt solution. The sticky egg

Jour. Fac. Sci. Hokkaido Univ. Ser. VI, Zool. 22 (1), 1979.

fluid thus obtained was poured into a Visking tube and dialyzed with vigorous stirring against a definite volume of the salt solution containing an appropriate concentration of $CaCl_2$. The digestion of organic components remaining in the dialyzing bag was carried out by the method described by Bialaszewicz (1927) using concentrated HNO_3. The concentration of calcium in the samples was determined by atomic absorption spectrophotometry. The working condition of the instrument is shown in Table 1. The calcium content of the sample was

Table 1. Instrumental setting for the determination of calcium by atomic absorption spectrophotometry using Hitachi Model 208 spectrophotometer

Wavelength:	422.7 nm
Slit:	1.0 mm
H.C.Lamp current:	10 mA
Air: Supply,	1.8 Kg/cm^2
Flow rate,	12.0 l/min
Acetylene: Supply,	0.28 Kg/cm^2
Flow rate,	2.5 l/min
Sensitivity:	0.15 μg/ml Ca for 1% absorption

calculated so as to represent the calcium concentration in the original egg volume. Dividing the weight of 25 eggs by the specific gravity of the egg (1.08), the egg volume was obtained. All of the experiments were repeated from three to five times, and each value represents the mean of closely agreeing figures.

Details of the experimental procedures will be given in each description. Except for the dialysis, the experiments were carried out at 4°C.

Results

In order to verify the occurrence of calcium efflux at activation, 200 eggs each were separately treated in the following way:

Group A: Deionized water, 2 min → 60 ml of the salt solution

Group B: The salt solution acidulated, 2 min → 60 ml of the salt solution

Group C (control): The salt solution, 2 min → 60 ml of the salt solution

The acidulated salt solution was made by adding concentrated HCl to the salt solution and adjusting to pH 1.8. Two hours later, the salt solutions which had soaked the eggs were collected and their calcium contents were determined. The results are shown in Table 2.

As reported already (Yamamoto, 1967), the eggs of Group A formed a narrow perivitelline space in the salt solution and clearly showed the evidence of activation (breakdown of cortical alveoli, extrusion of the second polar body, *etc.*). In the eggs of Group B, no appreciable space was found between the egg membrane and the ooplasmic surface. In these eggs, however, an activation

without breakdown of cortical alveoli occurred (Yamamoto, 1964). It was verified by the observation of the extrusion of the second polar body in sectioned materials. Although all eggs previously treated with the acidulated salt solution underwent cytolysis within 17 hours after immersion in the salt solution (Yamamoto, 1964), no pathological change of the egg was detected in this experiment (immersion

Table 2. Concentration of calcium effused from dog salmon egg during the first 2 hours after activation (mM)

Group	A	B	C (control)
Stimulator	Deionized water	Acid salt sol.	Salt sol.
Ca concentration	0.191±0.004	0.015±0.001	0.003±0.001

in the salt solution for 2 hours). No morphological change was observed in the eggs of Group C. From the results shown in Table 2, it is clear that the dog salmon egg effuses a statistically significant amount of calcium following activation.

In the above experiment, the salt solution which had soaked the eggs appeared transparent. When the solution of Group A was stored for one or two days, however, it formed a small quantity of precipitate. The precipitate did not disappear after addition of concentrated HCl. No precipitate was formed in the solutions of Group B and C.

As reported already, the membrane of the dog salmon egg undergoes some morphological and chemical changes following egg activation (Aoki, 1941; Kanoh and Yamamoto, 1957). Furthermore it seems to possess a calcium-binding capacity (Hamano, 1949; Yamamoto, 1957). These facts might suggest that calcium effused following egg activation derived from the egg membrane. To check this possibility, the calcium content in the egg membranes of Group A, B and C was determined. The membranes were isolated from 100 eggs of each group and were repeatedly washed with the salt solution. After over-night immersion in a large amount of the salt solution with vigorous stirring, the clean egg membranes were digested with HNO_3. It was revealed that the calcium content in the egg membrane of three groups was nearly the same. Assuming the amount of calcium determined in the egg membrane is homogeneously distributed not only in the egg membrane but also in the egg protoplasm, the concentration was 0.25 μM, which is much lower than that effused from the egg. Therefore the amount of calcium effused following egg activation is not explained by that contained in the egg membrane.

Then, the time course of the calcium efflux following egg activation was examined. One hundred eggs were continuously immersed in 30 ml of deionized water and the calcium concentration in the surrounding medium of the eggs was determined at intervals. The results are shown in Figure 1. It is clear that the efflux following immersion of eggs in deionized water is divided into two phases:

In the first phase, about 70% of the total efflux occurred. This phase was terminated at about 30 minutes after immersion of eggs in deionized water. The second phase of the efflux was initiated at about 90 minutes after immersion. Furthermore the total amount of calcium effused within 2 hours was apparently less than that in the previous experiment, where the egg stimulated with deionized water was transferred into the salt solution (Group A); the amount of calcium effused from the eggs continuously immersed in deionized water was about one fifth of that from the eggs kept in the salt solution after stimulation with deionized water. Since the breakdown of cortical alveoli has equally occurred in these eggs and the contents of the cortical alveoli have been discharged into the interstice between the egg membrane and the ooplasmic surface, it is possible to deduce that the effused calcium derives from intracellular stock other than the contents of the

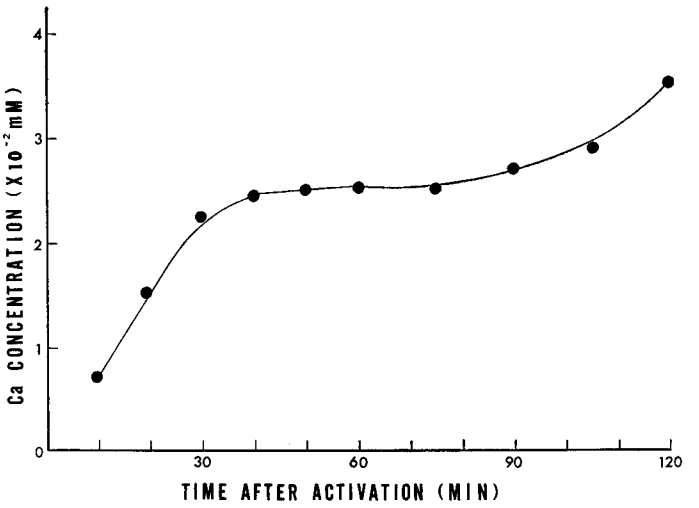

Fig. 1. Time course of calcium efflux in the dog salmon egg. The eggs were continuously immersed in deionized water and the amount of calcium effused into the surrounding medium was determined.

cortical alveoli. This may also be supported by the occurrence of calcium efflux in the eggs which showed an activation without breakdown of the cortical alveoli (Group B).

Then, the concentrations of dialyzable and non-dialyzable calcium in the egg were determined. Twenty eggs each were separately treated with deionized water, the acidulated salt solution or the salt solution for 5 minutes and were immersed in 20 ml of the salt solution for 15 minutes. The eggs were cut with fine scissors in every dish of the solution in which the eggs had been soaked, and the egg fluids were dialyzed against 200 ml of the salt solution for 24 hours at 1°C. After dialyzation, the calcium concentration of the outer salt solution of the dialyzing

bag was determined. This calcium is tentatively named "free" calcium of the egg fluid in this paper. The contents of the dialyzing bag were measured in volume with a graduated cylinder. Then they were digested with HNO_3 and the calcium concentration was determined. This calcium is called "fixed" calcium. The direct determination of the concentration of total (free + fixed) calcium in the egg was carried out without dialysis. The results are shown in Table 3. The

Table 3. Concentration of calcium in dog salmon egg (mM)

Stimulator	Deionized water	Acid salt sol.	Salt sol.	—
Free Ca	11.8±0.2	12.7±0.5	12.0±0.3	—
Non-dialyzable Ca	1.6±0.3	1.2±0.3	1.7±0.5	—
Total Ca[1)]	13.3±1.1	13.9±0.8	13.6±0.3	13.5±0.7[2)]

1) Theoretical calculation. 2) Direct determination.

calcium concentrations in the broken eggs which had been treated differently agreed well each other and suggested that no remarkable increase in the concentration of intracellular free calcium at activation is detected by the method used in the present study.

In this experiment, the outer salt solutions of the dialyzing bag always formed a small quantity of precipitate within 24 hours. The precipitate did not disappear with the addition of concentrated HCl. Furthermore, the calcium concentration in the solution did not change after removal of the precipitate by straining through filter paper.

In order to calculate the concentration of radicals, which are supposed to bind with calcium in the egg, and the dissociation constant between calcium and the radicals (Nakamura and Yasumasu, 1974), the relationship between free and bound calcium concentrations was studied by dialyzing the egg fluid against salt solutions containing various amounts of $CaCl_2$. In this experiment, bound calcium represents the difference between the concentrations of fixed calcium and of calcium remaining in the dialyzing bag, after dialysis against the salt solution containing specific amounts of calcium ions. The eggs were first treated with deionized water or with the salt solution (control) for 5 minutes and were immersed in the isotonic calcium-bearing salt solution for 15 minutes. Then, they were cut in the solution which had soaked the eggs and the resulting egg fluid was dialyzed against a solution having the same constitution as the soaking solution. Regardless of the first treatment, the relationship between the free and bound calcium concentrations were the same (Fig. 2). The dissociation constant between calcium and radicals and the concentration of calcium radicals in the egg fluid were calculated by the formula, $(R-B)/B=K/F$. In this formula, the following abbreviations were used: B, bound calcium concentration; F, free calcium concentration; K, dissociation constant; R, total radical concentration (Nakamura

and Yasumasu, 1974). The relationship between reciprocals of the free and bound calcium concentrations is shown in Figure 3. From this figure, it appears that the egg fluid contains two kinds of calcium radicals: the dissociation constants were 4.08×10^{-4} M and 5×10^{-3} M, respectively. The concentrations of these radicals in the egg fluid were 5.1 mM and 15.4 mM.

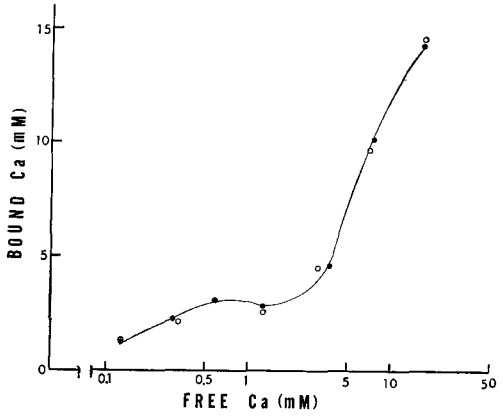

Fig. 2. Relationship between free and bound calcium concentrations in the egg fluid of the dog salmon. Open circle, ripe egg; solid circle, activated egg.

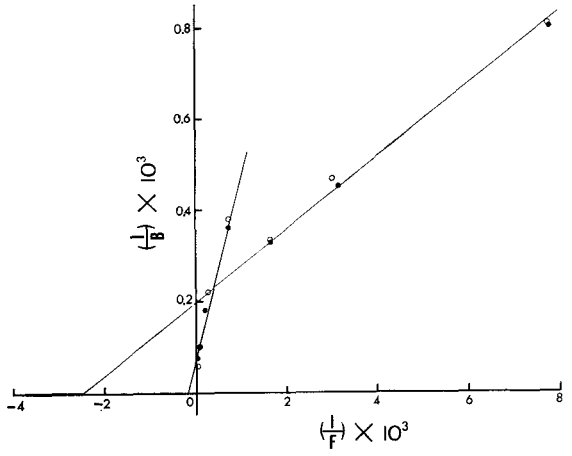

Fig. 3. Relation of reciprocals of the concentrations in bound and free calcium. 1/B and 1/F indicate reciprocals of bound and free calcium concentrations and are calculated from the values shown in Fig. 2. Open circle, ripe egg; solid circle, activated egg.

Discussion

It was revealed in the present study that the concentration of calcium in the ripe unfertilized egg of the dog salmon is about 13.5 mM. Bialaszewicz (1927) and Hayes et al. (1946) determined the inorganic constituents of the egg in salmon, *Salmo salar*, and reported that the coelomic (probably ripe) egg contains 0.122 mg or 0.084 mg calcium per 118 mg egg weight. If the specific gravity of the egg of this fish is the same as that of the dog salmon, the concentrations of

calcium in the *Salmo* egg are 22.33 mM or 15.38 mM, which do not markedly vary from the present data.

When the unfertilized egg of the dog salmon is immersed in deionized water, it is activated parthenogenetically (K. Yamamoto, 1951). Namely, it shows breakdown of cortical alveoli, extrusion of the second polar body and formation of a distinct blastodisc. Simultaneously with these morphological changes, the egg effuses calcium. Using the sodium alizarin sulfonate method, Kanoh (1956) demonstrated that the eggs of the dog salmon effuse 2.45 μg calcium/egg during the first 2 hours after activation. Since he did not describe the egg volume, the exact comparison of his data with the present result is not possible. However his value is the same order of magnitude with that obtained in the present study, where the egg effused about 1.4% of its total calcium during the first 2 hours after immersion in deionized water. In the study of the time course of calcium efflux, it was shown that the efflux is divided into two phases: the first phase corresponds to the time of the breakdown of cortical alveoli and the second to the period of the extrusion of the second polar body or the formation of a distinct blastodisc (K. Yamamoto, 1951). The egg may repeat the efflux during embryonic development and lose much more calcium. In fact, Hayes et al. (1946) showed the loss of calcium from the *Salmo* egg to be about 8% of the total calcium during the first 2 days after initiation of embryonic development.

The present result clearly demonstrates that calcium effused from the egg does not derive from the membrane of the ripe egg; it is a part of the intracellular stock other than the contents of cortical alveoli. In the egg of medaka, *Oryzias latipes*, an explosive rise in the free calcium level of ooplasm at the time of activation was already reported by T. Yamamoto (1954), Ridgway et al. (1977) and Gilkey et al (1978). A similar rise in the free calcium level of ooplasm at fertilization was also reported in sea urchin and echiuroid eggs (Mazia, 1937; Nakamura and Yasumasu, 1974; Steinhardt et al., 1977; Johnston and Paul, 1977). Calcium effused from the dog salmon egg and detected in the surrounding salt solution of the egg may therefore be free calcium released from intracellular calcium stock in the ripe egg. It may be concluded that the explosive rise of free calcium in ooplasm seems to be a general feature of egg activation.

When the eggs of medaka are stimulated by pricking with a glass needle, they show a series of changes of activation. If eggs previously anesthetized are stimulated by pricking, the rise in the free calcium level of the ooplasm is slight and the breakdown of cortical alveoli is not initiated (T. Yamamoto, 1954). A similar correlation between the breakdown of cortical alveoli and the free calcium level was also suggested in dog salmon eggs which are stimulated in deionized water or in acidulated salt solution. As reported already, the dog salmon egg immersed in the acidulated salt solution extrudes the second polar body and formed the distinct blastodisc without breakdown of cortical alveoli (Yamamoto, 1964). In this case, the amount of calcium effused is about one tenth of that from the egg which is stimulated in deionized water and showed the breakdown of cortical

alveoli. It is known that the intracellular concentration of free calcium is closely correlated with the exocytosis (Kanno et al., 1973). Since the breakdown of cortical alveoli seems to be an example of massive exocytosis (Hart et al., 1977), it is possible to suppose that a certain level of free calcium in ooplasm is required for the breakdown of cortical alveoli. The breakdown may not be initiated at concentrations of free calcium lower than the threshold. In support of this supposition, Yamaguchi (1958) succeeded in inducing the breakdown of cortical alveoli in herring eggs by intracellular application of calcium ions. Therefore the intracellular free calcium level of the dog salmon egg may arrive at the threshold following stimulation by deionized water but will not attain the threshold with the acidulated salt solution.

The occurrence of slight efflux of calcium from the eggs treated with the acidulated salt solution suggests that the extrusion of the polar body and the formation of a distinct blastodisc are also correlated with the rise of intracellular free calcium level. According to several authors, the inseminated eggs of the dog salmon initiate the embryonic development without breakdown of cortical alveoli after immersion in isotonic solution of $CaCl_2$ (Kanoh, 1952; Kusa, 1953; Yamamoto, 1967). It is simply deduced from the present experiment that the free calcium level of ooplasm in these eggs rises slightly as a result of an ionic exchange which might occur between intracellular cations and extracellular calcium ions in the isotonic solution (Kanoh, 1951). In this case the level may not arrive at the threshold to induce the breakdown of cortical alveoli.

In the eggs of medaka, Gilkey et al. (1978) suggested that the release of free calcium from the intracellular stock is stimulated by calcium ions carried into the egg through the previous calcium influx. The calcium efflux in sea urchin and echiuroid eggs is also preceded by a transient calcium influx (Nakazawa et al., 1970; Paul and Johnston, 1978). If the rise of the ooplasmic free calcium level is indispensable for the breakdown of cortical alveoli in the dog salmon egg, however, it is hard to suppose that the rise in level is induced by extracellular calcium ions, because the egg causes the breakdown of alveoli in deionized water even after previous removal of calcium ions from the environment (Yamamoto, 1965). In this sense, the underlying mechanism of the breakdown of cortical alveoli in the dog salmon egg might resemble that observed after treatment of the medaka egg with ionophore, A23187 (Gilkey et al., 1978). It should be mentioned here that, differing from the case of the medaka egg, the breakdown of cortical alveoli in the dog salmon egg is induced only after immersion of the egg in hypotonic solution or in nonelectrolyte solution of various concentrations and does not require external calcium; without immersion in these solutions, the eggs do not show any morphological changes even after insemination (Yamamoto, 1966).

The amount of calcium effused from eggs kept in salt solution after stimulation in deionized water is evidently larger than that from eggs continuously immersed in deionized water. This fact indicates that the plasma surface of the egg shows some physico-chemical changes under the influence of the extracellular medium and

that the egg may easily lose intracellular calcium in the salt solution. In the observation of embryonic development of fertilized dog salmon eggs previously stimulated with deionized water and kept in various salt solutions, the author reported that each blastomere shows a tendency to separate in isotonic Ringer's solution which contains 3.2 mM calcium ions (Yamamoto, 1967). The blastomeres were however firmly touching each other in the eggs kept in tap water, which is hypotonic to the egg and contains a very small (less than 0.02 mM) amount of calcium ions. The separation of blastomeres was also observed in the cleavage of herring eggs which were allowed to develop in a calcium-free salt solution (Yanagimachi, 1957). Therefore it is probable that the tendency to blastomere separation in the dog salmon egg is induced by the insufficiency of calcium in the surface of the blastomeres. If this is true, the insufficiency of calcium may be a result of the above mentioned properties of the plasma surface in the salt solution.

So far as the present determinations are concerned, the concentrations of dialyzable calcium in the eggs treated in various ways well agreed each other. Since the dog salmon egg undoubtedly effused detectable amounts of calcium during activation and this calcium is probably in free state, it seems peculiar that the concentration of free (dialzyable) calcium in the egg does not change after immersion of the egg in deionized water. To explain these results, it may be assumed that calcium ions released at the early phase of activation are again bound with calcium radicals in the egg within 20 minutes after stimulation in deionized water. This assumption might be supported by the observations on the time course of the luminescent reaction between aequorin and free calcium released at fertilization in sea urchin and medaka eggs (Steinhardt et al., 1977; Gilkey et al., 1978). Furthermore two kinds of calcium radicals, differing from each other in their dissociation constants, were demonstrated in the dog salmon egg. On the other hand, Hamaguchi and Mabuchi (1978) reported that in starfish oocytes homogenization alters the calcium-binding ability of calcium radicals and releases a large amount of calcium ions from intracellular calcium stock. Although a conclusive interpretation can not be drawn from the experiment, there is a possibility that the dialyzable calcium determined in the present study includes calcium ions released from calcium radicals during preparation and dialysis of the egg fluid.

Summary

Through quantitative estimation of calcium in a salt solution, in which were soaked the ripe eggs of the dog salmon (*Oncorhynchus keta*) previously stimulated by deionized water or an acidulated salt solution, the calcium efflux of the eggs at activation was studied. The egg stimulated by deionized water effused about 1.4 % of the calcium originally contained in the ripe egg (total calcium). When an acidulated salt solution was used as the stimulator, the egg showed an activation without breakdown of cortical alveoli and effused only about 0.1% of its total calcium. The amount of calcium effused was apparently small in the eggs

continuously immersed in deionized water, suggesting that the egg is apt easily to lose intracellular calcium in the salt solution. The effused calcium was not derived from the egg membrane. Probably a sudden rise of free calcium level in the ooplasm was induced as a result of the release of calcium from the intracellular stock at egg activation. The effused calcium seems to be a part of this ooplasmic free calcium. The breakdown of cortical alveoli appeared to be initiated after the intracellular free calcium level arrived at the threshold.

The author is very grateful to Professor Yasuhiko Kanoh, Akkeshi Marine Biological Station, for many stimulating discussions on the problem. Thanks are also expressed to Professors Hitoshi Yoshida and Mitsuhiko Taga, Department of Chemistry, for their technical advice in the determination of calcium, and to the members of the staff of the Hokkaido Salmon Hatchery who help me the collection of materials used in the present study.

References

Aoki, K. 1941. On the swelling of the membrane of salmon egg. Kagaku, Tokyo **11**: 517. (In Japanese)

Bialaszewicz, K. 1927. Contributions à l'étude de la composition minérale des cellules-oeufs. Pubbl. Staz. Zool. Napoli **8**: 355–369.

Fodor, E. J. B., H. Ako and K. A. Walsh 1975. Isolation of a protease from sea urchin eggs before and after fertilization. Biochemistry **14**: 4923–4927.

Gilkey, J. C., L. F. Jaffe, E.B. Ridgway and G. T. Reynolds 1978. A free calcium wave traverses the activating egg of the medaka, *Oryzias latipes*. J. Cell Biol. **76**: 448–466.

Hamaguchi, Y. and I. Mabuchi 1978. Measurement of intracellular free calcium concentration of the starfish egg by means of the microinjection of aequorin. Cell Struct. and Function **3**: 259–263.

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

Hart, N.H., S. F. Yu and V. A. Greenhut 1977. Observations on the cortical reaction in eggs of *Brachydanio rerio* as seen with the scanning electron microscope. J. Exp. Zool. **201**: 325–331.

Hayes, F. R., D. A. Darcy and M. Sullivan 1946. Changes in the inorganic constituents of developing salmon eggs. J. Biol. Chem. **163**: 621–631.

Ishihara, K. 1964. Release of acid polysaccharides following fertilization of sea urchin eggs. Exp. Cell Res. **36**: 354–367.

Johnston, R. N. and M. Paul 1977. Calcium influx following fertilization of *Urechis caupo* eggs. Develop. Biol. **57**: 364–374.

Kanno, T., D. E. Cochrane and W. W. Douglas 1973. Exocytosis (secretory granule extrusion) induced by injection of calcium into mast cells. Canad. J. Physiol. Pharmacol. **51**: 1001–1004.

Kanoh, Y. 1951. Ueber Wasseraufnahme und Aktivierung der Lachseier. II. Die Wirkung der hypertonischen Salzlösung. J. Fac. Sci. Hokkaido Univ. Ser. VI, Zool. **10**: 260–270.

———— 1952. Ueber die Beziehung zwischen dem Zerfallen der Kortikalalveoli und der Entwicklung bei Fischeiern. Japan. J. Ichthyol. **2**: 99–103. (In Japanese with German Summary)

——— 1956. Ueber Wasseraufnahme und Aktivierung der Lachseier. IV. Vorläufiges Experiment in Betreff des Calciumproblems. J. Fac. Sci. Hokkaido Univ. Ser. VI, Zool. **12**: 259–263.

——— and T. S. Yamamoto 1957. Removal of the membrane of the dog salmon egg by means of proteolytic enzymes. Bull. Japan. 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.

Mazia, D. 1937. The release of calcium in *Arbacia* eggs on fertilization. J. Cell. Comp. Physiol. **10**: 291–304.

Nakamura, M. and I. Yasumasu 1974. Mechanism for increase in intracellular concentration of free calcium in fertilized sea urchin egg. A method for estimating intracellular concentration of free calcium. J. Gen. Physiol. **63**: 374–388.

Nakazawa, T., K. Asami, R. Shoger, A. Fujiwara and I. Yasumasu 1970. Ca^{2+} uptake, H^+ ejection and respiration in sea urchin eggs on fertilization. Exp. Cell Res. **63**: 143–146.

Ohtsuka, E. 1964. Studies on the invisible cortical change of the fish egg. Embryologia **8**: 101–114.

Ridgway, E. B., J. C. Gilkey and L. F. Jaffe 1977. Free calcium increases explosively in activating medaka eggs. Proc. Natl. Acad. Sci. **74**: 623–627.

Steinhardt, R., R. Zucker and G. Schatten 1977. Intracellular calcium release at fertilization in the sea urchin egg. Develop. Biol. **58**: 185–196.

Yamaguchi, T. 1958. Microinjection into animal single cells. Symp. Cell. Chem. **8**: 93–99. (In Japanese with English Summary)

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

Yamamoto, T. 1954. Physiological studies on fertilization and activation of fish eggs. V. The role of calcium ions in activation of *Oryzias* eggs. Exp. Cell Res. **6**: 56–68.

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

——— 1964. Effects of acidulated Ringer's solution on the egg of dog salmon, *Oncorhynchus keta*. J. Fac. Sci. Hokkaido Univ. Ser. VI, Zool. **15**: 426–438.

——— 1965. Effects of ethylenediaminetetraacetate on the egg of the dog salmon, *Oncorhynchus keta*. *Ibid.* **15**: 644–651.

——— 1966. Independence between sperm-entry and activation in dog salmon egg. Zool. Mag., Tokyo **75**: 297–302. (In Japanese with English Summary)

——— 1967. Development of the dog salmon egg without breakdown of cortical alveoli. J. Fac. Sci. Hokkaido Univ. Ser. VI, Zool. **16**: 186–196.

Yanagimachi, R. 1957. Studies of fertilization in *Clupea pallasii*–V. The role of calcium ions in fertilization and development. Bull. Japan. Soc. Sci. Fish. **23**: 290–294. (In Japanese with English Summary)