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The Effect of Single Salt Solutions on the Fertilizability of the Herring Egg¹⁾

By

Ryuzo Yanagimachi

(Zoological Institute, Hokkaido University)

(With 5 Text-figures)

The effect of isotonic solutions of single salts on the fertilizability of fish eggs was first described by Newman (1905). He found that the calcium salts are the most active of the salts found in sea water in inhibiting fertilization. According to him, 95 per cent of *Fundulus* eggs are made incapable of normal fertilization even by a 5-minute treatment with N/5 CaCl₂ solution. As pointed out later by Kagan (1935), Newman seems to have made no microscopical observations beyond those necessary to determine the percentage of cleavage. It is therefore uncertain whether the locus of the effect of calcium is in the egg membrane or in the cortex of the egg. As is well known, the teleost eggs generally do have micropyles, and the site of sperm entrance into the egg is restricted to that point. Therefore, sufficient attention must be given to the micropyle, when the teleost egg loses its capacity for normal fertilization. Unfortunately, this seems not to have been done by Newman.

Yanagimachi & Kanoh (1953) have observed in herring that the spermatozoa sluggish in sea water become intensely motile when they come in contact with the surface of egg membrane in the vicinity of the micropyle. This indicates that the egg membrane in the vicinity of the micropyle of the herring egg is specific in its property. Under normal conditions it makes the sperm of the same species intensely active and facilitates the entrance of the sperm into the egg. The prime aim of the present investigation is to find out the effect of various salts on the maintenance of the fertilizability of the herring egg, with special regard to the effect of salts on the membrane around the micropyle.

Before going further the author wishes to express his sincerest gratitude to Professor Atsuhiko Ichikawa for his counsel throughout the course of this work and for his kindness in reading the original manuscript.

Material and methods

Mature eggs of the Pacific herring (*Clupea pallasii* C. et V.) were used as material

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of the present investigation. They were removed from the gonads of freshly killed fish. Every precaution was taken in order to avoid accidental sperm contamination. As much as possible, experiments were performed upon eggs from a single female.

The experiments were carried out in comparing the effects of the isotonic solutions of single salts on fresh unfertilized eggs. Chlorides of sodium and potassium were made in M/4.5 solution and those of magnesium and calcium were made in M/6.7 solution. These concentrations were assumed to be approximately isotonic with the egg of *Clupea pallasii* (Yanagimachi '53). The hydrogen ion concentration of all the solutions was adjusted to pH 7.6 by addition of sodium bicarbonate, and the factor of pH was eliminated. The volume of the test solution and the number of eggs used in each experiment were 50 ml and 50-100 respectively. After an exposure to a test solution, eggs were transferred into isotonic Ringer's solution,²⁾ and inseminated in it to examine their capacity for fertilization. The concentration of spermatozoa at the time of insemination was made constant, as nearly as possible, at about 1/5,000 ($1.8-2.2 \times 10^7$ sperm/ml). Three to five hours after the insemination the percentage of fertilization was determined by counting the eggs undergoing cleavage.

Results

(1) *The effect of NaCl and KCl solutions upon the maintenance of the fertilizability of unfertilized eggs.*

The action of NaCl solution and that of KCl solution are similar. When the unfertilized eggs were exposed to an isotonic solution of NaCl or KCl for periods up to 24 hours, no visible effect was produced either in the solution or after removal to the Ringer's solution. They were able to be fertilized when inseminated in the Ringer's solution. Large numbers of the eggs remained still capable of being fertilized, even after the maximum exposure of 100 hours (Fig. 1). For comparison, fertilizability of the eggs exposed to the Ringer's solution for the same duration is shown in the same figure.

Newman (1905) stated that M/5 KCl helps the *Fundulus* egg to retain its fertilizability longer than M/5 NaCl. Ishida (1948), on the contrary, found that M/7.5 NaCl helps the *Oryzias* egg to retain its fertilizability considerably longer than M/7.5 KCl. Thus the results obtained in *Fundulus* and that in *Oryzias* contradict each other. Since the eggs of these two forms are known to be practically immune to osmotic changes of the medium (Loeb '15, Yamamoto '41), the contrariety of the results may not be attributed to the difference in osmotic pressure of the solutions used by these investigators. In the case of *Clupea*, at any rate, no practical difference was found between NaCl and KCl, with respect to their capacity for preserving the fertilizability of the unfertilized egg.

The action of NaCl solutions of various concentrations ranging from 1M to M/512 was tested. The same method of procedure was used throughout as in the experiments with isotonic solutions. Results of a typical series of experiments are

2) Constitution of isotonic Ringer's solution ; M/4.5 NaCl 100 ml + M/4.5 KCl 3.5 ml + M/6.7 CaCl₂ 1.5 ml + M/6.7 MgCl₂ 2.4 ml (pH was adjusted to 7.6 by addition of a small amount of sodium bicarbonate).

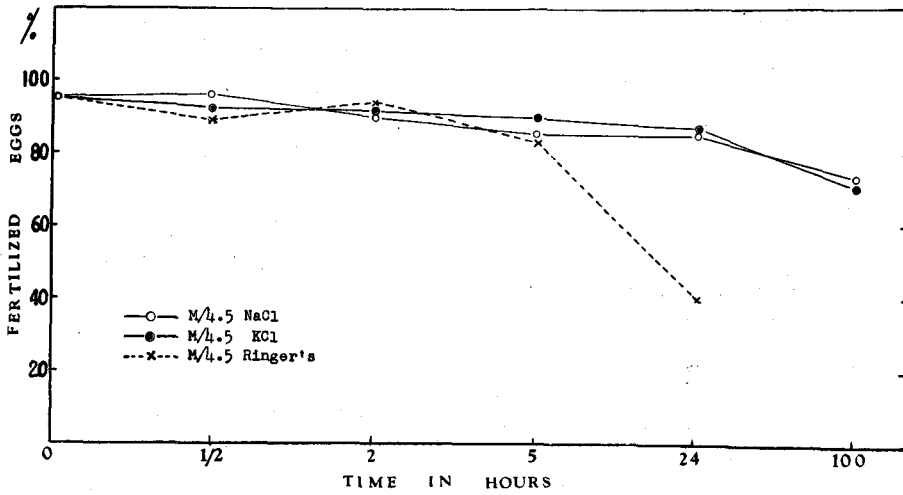


Fig. 1. Percentage of fertilization in eggs inseminated in Ringer's solution after exposure to the isotonic solutions of NaCl and KCl (Room temp. 5°-12°C.).

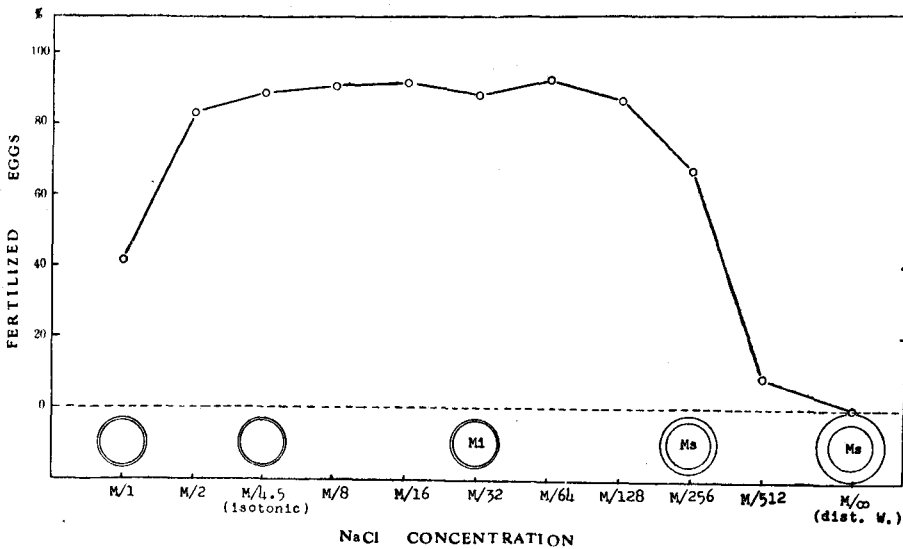


Fig. 2. Percentage of fertilization in eggs inseminated in Ringer's solution after a 2-hour-exposure to NaCl solutions of various concentrations. Ms, egg membrane swells with continuous immersion in the solution; Mi, egg membrane remains intact (Room temp. 15°C.).

shown in Fig. 2, from which it will be seen that high percentage of fertilization was obtained over a surprising range of salt concentration. However, there was a lowered percentage of fertilization in eggs exposed to the solution more dilute than M/256. This seems to be due to the fact that in this solution the egg membrane became so swollen that the micropyle was closed. Thus, low sodium concentration is not favorable to the maintenance of fertilizability of unfertilized egg. In M/ ∞ NaCl solution, i. e. distilled water, a conspicuous swelling of egg membrane took place and the eggs lost their fertilizability almost instantaneously.

(2) *The effect of CaCl₂ and MgCl₂ solutions upon the maintenance of the fertilizability of unfertilized eggs*

The action of CaCl₂ solution and that of MgCl₂ are worthy of notice. When the eggs were exposed to the isotonic solutions of these salts, rapid loss of fertilizability took place and practically all the eggs became incapable of fertilization after two hours (Fig. 3). However, no visible sign of deteriorative effect was produced in any part of the egg either in the solution or after removal to the Ringer's solution. When they were pricked with a glass needle, all of them initiated the cortical changes, i. e. breakdown of cortical alveoli³⁾, and extruded the polar body. Even after a 24-hour-exposure to CaCl₂ solution, eggs were still able to respond to pricking. It was also found that when the membrane of the egg exposed to CaCl₂ solution was removed by means of iridectomy-scissors (with some difficulty) the egg becomes fertilizable. It is likely, therefore, that the site affected by CaCl₂

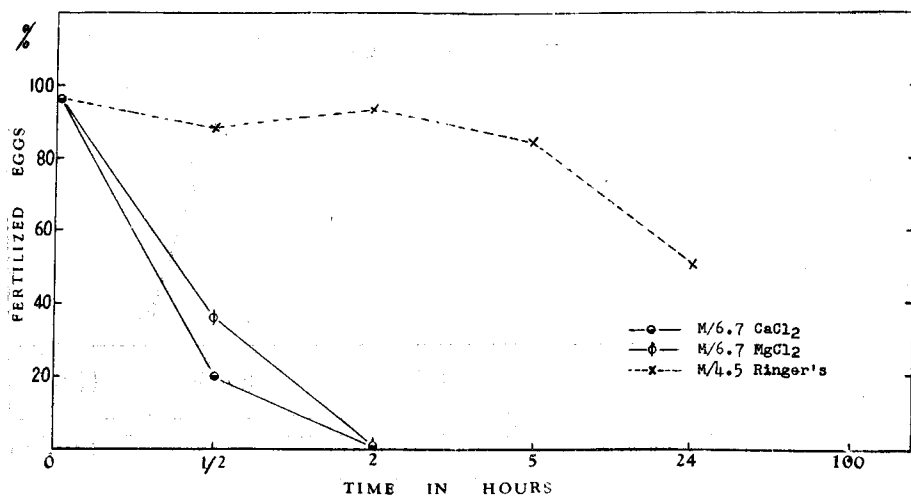


Fig. 3. Percentage of fertilization in eggs inseminated in Ringer's solution after exposure to the isotonic solutions of CaCl₂ and MgCl₂ (Room temp. 5°–12°C.).

3) For the breakdown of the cortical alveoli of herring egg, see Kanoh ('53).

is not the cortex of the egg, but the egg membrane. Further discussion on this problem will be offered in the next section.

The action of isotonic solution of $MgCl_2$ was tested and was found to be similar to that of $CaCl_2$, though $MgCl_2$ was less effective than $CaCl_2$ in inhibiting fertilization. Thus, $CaCl_2$ is the most active of four salts in inhibiting fertilization. The action of $MgCl_2$ is intermediate between those of $CaCl_2$ and $NaCl$ or KCl . These four salts may be arranged, therefore, in the following order with respect to the power to prevent fertilization: $CaCl_2 > MgCl_2 > NaCl, KCl$.

The action of $CaCl_2$ solutions of various concentrations is summarized in Fig. 4. The eggs exposed to M/6.7-M/16 solution of $CaCl_2$ for two hours were not fertilized at all, whereas the eggs exposed to dilute solutions of $CaCl_2$ (such as M/512 solution) were maintained fertilizable. Thus, low calcium concentration is very favorable to the maintenance of fertilizability of unfertilized egg.

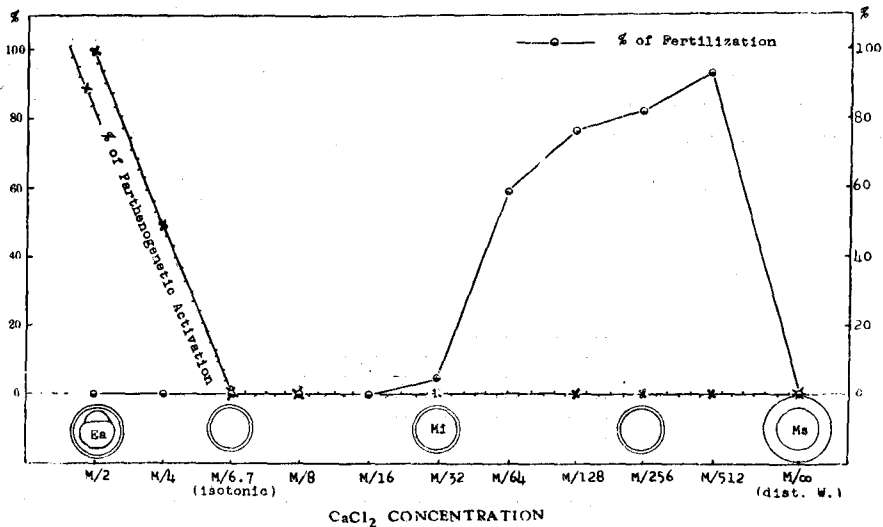


Fig. 4. Percentage of fertilization in eggs inseminated in Ringer's solution after a 2-hour-exposure to $CaCl_2$ solution of various concentrations. Ms. egg membrane swells with continuous immersion in the solution, Mi, egg membrane remains intact, Ea, egg activates parthenogenetically by continuous immersion in the solution (Room temp. 15°C.).

(3) *The nature of the fertilization preventing action of $CaCl_2$*

(A): As stated already the site affected by $CaCl_2$ solution seems not be the

4) Hypertonic solution of $CaCl_2$ (M/2-M/4) is specific in its action. By continuous immersion in this solution large numbers of eggs were activated partheongenetically. Submitting the eggs for two hours in M/2 solution, for example, 100 per cent of eggs was activated and formed blastodisc (cf. Fig. 4).

cortex of the egg, but the egg membrane. Careful observation, however, revealed that neither deteriorative deformation of the egg membrane nor closing of micropyle was produced by the action of CaCl_2 . It seems justifiable to consider, therefore, that an invisible change was brought about in the egg membrane.

As pointed out by Yanagimachi & Kanoh (1953), the membrane around the micropyle of *Clupea* egg has some specific properties. Under normal conditions it makes the sperm of the same species active⁵⁾ and facilitates the entrance of the sperm into the egg. Inhibition of fertilization by CaCl_2 may be due to the loss of the specific properties of the membrane around the micropyle. The procedure of the experiment to elucidate this point was as follows. Fresh eggs, exposed the isotonic CaCl_2 solution for varying periods of time, were transferred into Ringer's solution and washed thoroughly in it. Insemination was performed in Ringer's solution by adding fresh sperm suspension. The final concentration of spermatozoa at the time of insemination was made constant at about 1/5,000. Within 5-20 minutes after the insemination, the microscope was focused upon the micropyle, and spermatozoa swarming at the micropyle were observed. According to the total number of spermatozoa which came in contact with the mouth part of micropyle for a period of one minute (average of several determinations), the degree of sperm activation was classified into six grades (Table 1).

Table 1. Representation of the degree of sperm activation (for explanation see text).

Number of spermatozoa counted	Degree of sperm activation
100 <	5
50-100	4
20-50	3
10-20	2
1-10	1
0	0

With the same procedure the effect of the isotonic solutions of NaCl , KCl , and MgCl_2 was investigated. The results of a typical series of experiments are shown in Fig. 5, from which it will be seen that an intimate relation is found between the degree of sperm activation around the micropyle and the rate of fertilization. On the surface of the membrane of the egg exposed to CaCl_2 or MgCl_2 solution for two hours or longer, sperm activation did not take place at all, and no spermatozoon could enter into the micropyle. On the contrary, intense sperm activation took place around the micropyle of the egg exposed to NaCl or KCl solution, and large numbers of eggs were fertilized upon insemination.

5) As is well known, the spermatozoa of most species of marine animals display rapid motion when they are discharged into sea water. In marked contrast to such spermatozoa, those of *Clupea pallasii* are almost motionless in sea water or in Ringer's solution.

Thus, the chief cause of the loss of fertilizability of the egg by CaCl_2 or MgCl_2 seems to be the loss of the sperm activating property of the membrane around the micropyle.

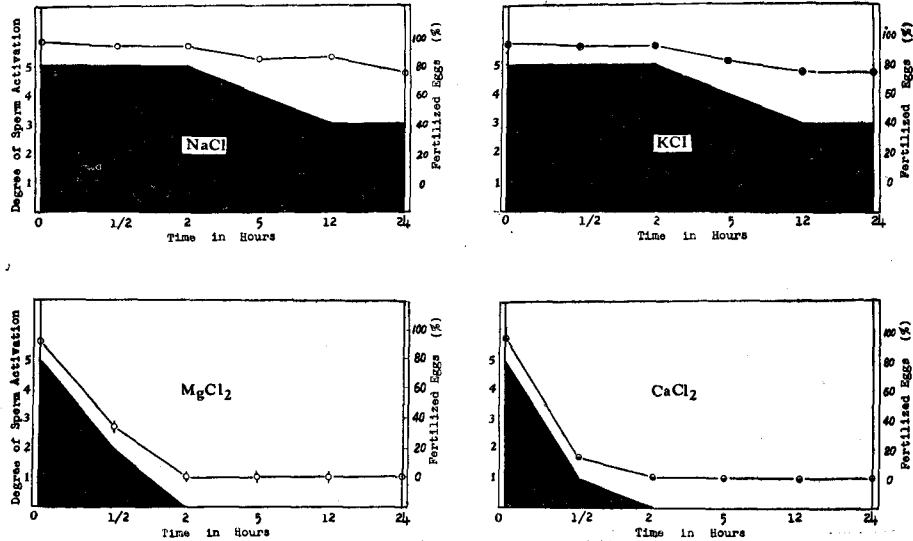


Fig. 5. Relation between the degree of the sperm activation and the rate of fertilization. Explanation in text. (Room temp. 10°-15°C.).

(B): According to Newman (1905), the prevention of fertilization of *Fundulus* egg by calcium salts seems to be of the nature of a precipitation of the colloids of the egg membrane. He stated "Supposing the egg to be a colloidal system with a membrane negatively charged it should be possible to precipitate or to coagulate this membrane by salts of high positive tension coefficient, such as CaCl_2 , and to redissolve this precipitate or coagulum by use of salts of high negative tension coefficient, such as Na_2SO_4 . This was done successfully, fertilization being inhibited by CaCl_2 and restored by Na_2SO_4 ."

So the author has attempted a series of experiments, according to Newman's method, to ascertain whether the fertilizability of *Clupea* egg exposed to CaCl_2 can be restored by use of Na_2SO_4 . Eggs 100 per cent fertilizable in fresh condition were exposed for two hours to the isotonic solution of CaCl_2 and divided into two dishes, one containing 30 ml of the isotonic (M/6.7) Na_2SO_4 solution and the other containing the isotonic Ringer's solution as control. The control eggs were immediately inseminated, and were allowed to remain in the dish, but not a single egg cleaved. The eggs transferred into Na_2SO_4 were allowed to stand there for 1/6~10 hours, and were then put into the isotonic Ringer's solution and inseminated. No eggs fertilized at all through the whole course of experiments. The sperm

activating property of the membrane around the micropyle also was not restored. The action of M/6.7 K_2SO_4 , M/4.5 NaCl or KCl, isotonic Ringer's solution (pH 7.6-11.0), and distilled water was tested by the same method. The results were, however, negative in all the cases. Thus, the author could not confirm the result obtained by Newman.

Summary

(1) The eggs of *Clupea pallasii* exposed to the isotonic solution of NaCl or KCl for 100 hours are still able to be fertilized when inseminated in Ringer's solution. Eggs exposed to the isotonic solution of $CaCl_2$ or $MgCl_2$, on the other hand, lose their fertilizability within a short time. When the eggs are exposed to these solutions for two hours or longer none of them is fertilized even after return to Ringer's solution.

(2) The four salts NaCl, KCl, $CaCl_2$, and $MgCl_2$ inhibit fertilization of *Clupea* egg in the following order: $CaCl_2 > MgCl_2 > NaCl, KCl$.

(3) The chief cause of the loss of fertilizability of the eggs by $CaCl_2$ or $MgCl_2$ was found to be due to the change of the property of the membrane around the micropyle.

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Material and methods

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of the present investigation. They were removed from the gonads of freshly killed fish. Every precaution was taken in order to avoid accidental sperm contamination. As much as possible, experiments were performed upon eggs from a single female.

The experiments were carried out in comparing the effects of the isotonic solutions of single salts on fresh unfertilized eggs. Chlorides of sodium and potassium were made in M/4.5 solution and those of magnesium and calcium were made in M/6.7 solution. These concentrations were assumed to be approximately isotonic with the egg of *Clupea pallasii* (Yanagimachi '53). The hydrogen ion concentration of all the solutions was adjusted to pH 7.6 by addition of sodium bicarbonate, and the factor of pH was eliminated. The volume of the test solution and the number of eggs used in each experiment were 50 ml and 50-100 respectively. After an exposure to a test solution, eggs were transferred into isotonic Ringer's solution,²⁾ and inseminated in it to examine their capacity for fertilization. The concentration of spermatozoa at the time of insemination was made constant, as nearly as possible, at about 1/5,000 ($1.8-2.2 \times 10^7$ sperm/ml). Three to five hours after the insemination the percentage of fertilization was determined by counting the eggs undergoing cleavage.

Results

(1) *The effect of NaCl and KCl solutions upon the maintenance of the fertilizability of unfertilized eggs.*

The action of NaCl solution and that of KCl solution are similar. When the unfertilized eggs were exposed to an isotonic solution of NaCl or KCl for periods up to 24 hours, no visible effect was produced either in the solution or after removal to the Ringer's solution. They were able to be fertilized when inseminated in the Ringer's solution. Large numbers of the eggs remained still capable of being fertilized, even after the maximum exposure of 100 hours (Fig. 1). For comparison, fertilizability of the eggs exposed to the Ringer's solution for the same duration is shown in the same figure.

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The action of NaCl solutions of various concentrations ranging from 1M to M/512 was tested. The same method of procedure was used throughout as in the experiments with isotonic solutions. Results of a typical series of experiments are

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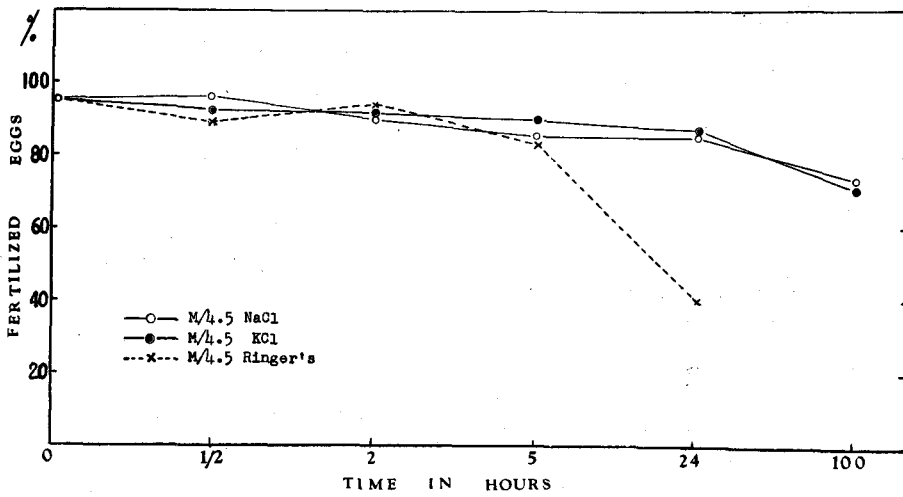


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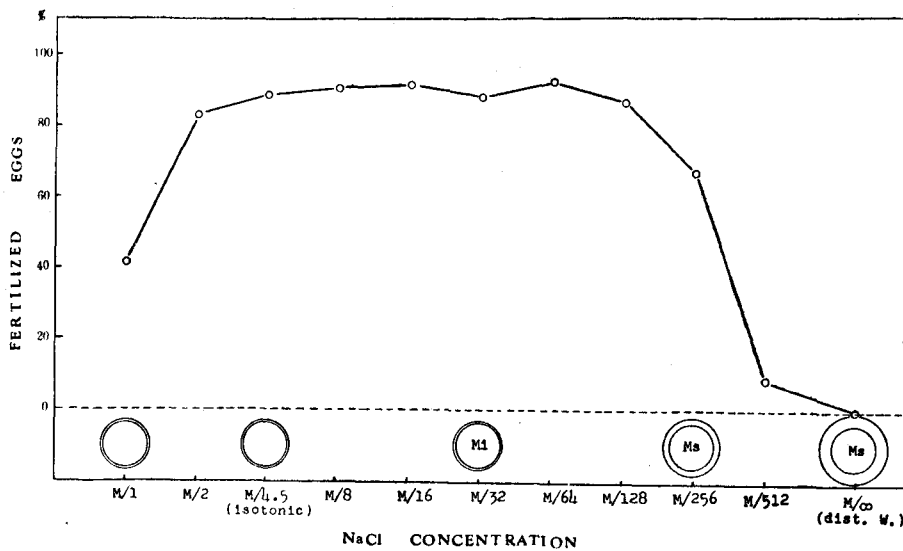


Fig. 2. Percentage of fertilization in eggs inseminated in Ringer's solution after a 2-hour-exposure to NaCl solutions of various concentrations. Ms, egg membrane swells with continuous immersion in the solution; Mi, egg membrane remains intact (Room temp. 15°C.).

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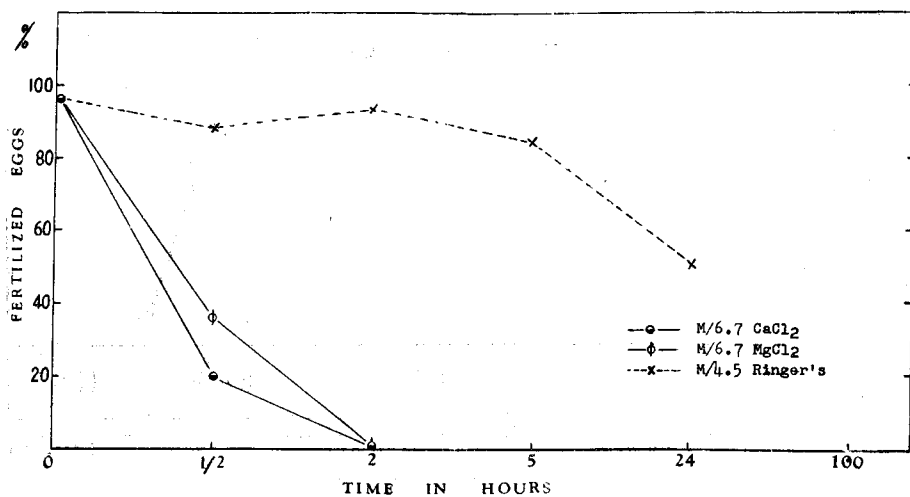


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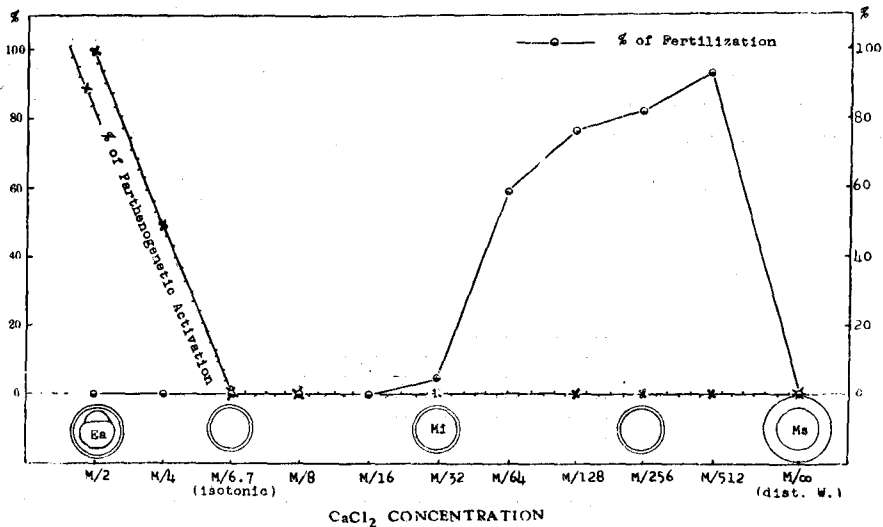


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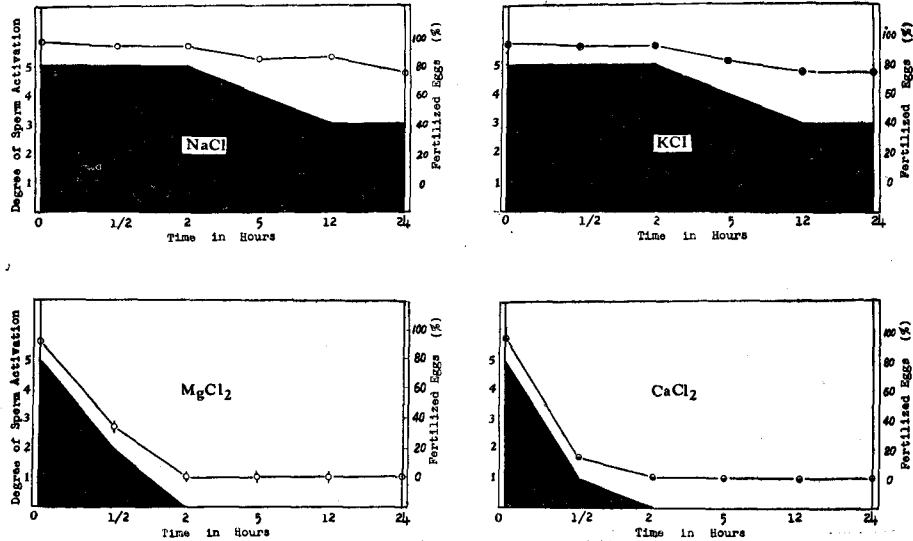


Fig. 5. Relation between the degree of the sperm activation and the rate of fertilization. Explanation in text. (Room temp. 10° - 15°C .)

(B): According to Newman (1905), the prevention of fertilization of *Fundulus* egg by calcium salts seems to be of the nature of a precipitation of the colloids of the egg membrane. He stated "Supposing the egg to be a colloidal system with a membrane negatively charged it should be possible to precipitate or to coagulate this membrane by salts of high positive tension coefficient, such as CaCl_2 , and to redissolve this precipitate or coagulum by use of salts of high negative tension coefficient, such as Na_2SO_4 . This was done successfully, fertilization being inhibited by CaCl_2 and restored by Na_2SO_4 ."

So the author has attempted a series of experiments, according to Newman's method, to ascertain whether the fertilizability of *Clupea* egg exposed to CaCl_2 can be restored by use of Na_2SO_4 . Eggs 100 per cent fertilizable in fresh condition were exposed for two hours to the isotonic solution of CaCl_2 and divided into two dishes, one containing 30 ml of the isotonic (M/6.7) Na_2SO_4 solution and the other containing the isotonic Ringer's solution as control. The control eggs were immediately inseminated, and were allowed to remain in the dish, but not a single egg cleaved. The eggs transferred into Na_2SO_4 were allowed to stand there for $1/6\sim 10$ hours, and were then put into the isotonic Ringer's solution and inseminated. No eggs fertilized at all through the whole course of experiments. The sperm

activating property of the membrane around the micropyle also was not restored. The action of M/6.7 K₂SO₄, M/4.5 NaCl or KCl, isotonic Ringer's solution (pH 7.6-11.0), and distilled water was tested by the same method. The results were, however, negative in all the cases. Thus, the author could not confirm the result obtained by Newman.

Summary

(1) The eggs of *Clupea pallasii* exposed to the isotonic solution of NaCl or KCl for 100 hours are still able to be fertilized when inseminated in Ringer's solution. Eggs exposed to the isotonic solution of CaCl₂ or MgCl₂, on the other hand, lose their fertilizability within a short time. When the eggs are exposed to these solutions for two hours or longer none of them is fertilized even after return to Ringer's solution.

(2) The four salts NaCl, KCl, CaCl₂, and MgCl₂ inhibit fertilization of *Clupea* egg in the following order: CaCl₂ > MgCl₂ > NaCl, KCl.

(3) The chief cause of the loss of fertilizability of the eggs by CaCl₂ or MgCl₂ was found to be due to the change of the property of the membrane around the micropyle.

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