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THE CHANGES IN SEA WATER AND THEIR INFLUENCES UPON MARIEN ANIMALS

II. The Influences of Natural Salts upon the Germ Cells of Sea-Urchin

BY

TEIJIRO HAYASHI

海水の變化と其の海産動物に及ぼす影響に就て

二、「ウニ」の生殖細胞に及ぼす中性鹽類の影響

林　禎　二　郎

Introduction

In a previous paper the author has reported his studies on the fertilization and development of sea-urchin eggs in artificial sea water. Extensive investigations made on the influence of neutral salts upon living egg-cells of the sea-urchin will be described in this paper.

LOEB (1908), LILLIE (1910), HEILBRUNN (1913) and JUST (1922) noted that some neutral salts of definite concentration, caused in an unfertilized egg of the sea-urchin a membrane-separation which has long been known as the phenomenon of parthenogenesis. According to MOOR (1916) and LILLIE (1921) such eggs, in which the membrane was lifted by any parthenogenetic agents, could no longer be fertilized by insemination. However, in my case no salts were used which were noticed to cause parthenogenesis at any concentration. On the other hand, it is to be understood that the nature of the present problem has close connection with the biological character of the cell membrane.

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As an important factor which determines the rate of penetration of water through the cell membrane, LUCKE and MCCUTHEON named the osmotic pressure of the solution and MICHAELIS (1928) and GELLHORN (1929) mentioned that it depends upon the semipermeability of the membrane. According to LOEB (1913) and HEILBRUNN (1915) the permeability of the membrane is changable after fertilization.

It is known that there is an antagonistic action among the ions of the salts which affect the animal, if more than two salts come into action at the same time. This reaction is very complicated. It has long attracted the attention of invesigators.

In the following described experiments the influence of a simple ion of a salt as well as the antagonistic action of several salts upon the cell were examined. The sea-urchin, *Strongylocentrotus nudus (A. Aggasiz)*, which is abundant in Hokkaido, was used as the material. The unfertilized eggs, the spermatozoa and the fertilized eggs were employed in order to determine their specificity against the salts.

The room temperature at the time of this experiment was 18°C–20°C.

Experiments

1. The influence of neutral salts upon the unfertilized eggs.

The salts used were NaCl, KCl, $MgCl_2$, and $CaCl_2$ which are usually found in sea water. The unfertilized eggs were put into vessels of hard glass, each containing 20 ccm. of the solution. After remaining there a certain time they were removed into normal sea water. Then, after being washed twice in the fresh sea water, the eggs were inseminated by normal spermatozoa. The observations were made after two hours from the insemination. The results obtained are given in Table I.

Table I.

	1 hour			6 hours			12 hours			15 hours			24 hours			39 hours			64 hours		
	Ferti.		un-	Ferti.		un-	Ferti.		un-	Ferti.		un-	Ferti.		un-	Ferti.		un-	Ferti.		ab-
	nor.	ab-nor.	ferti.	nor.	ab-nor.	ferti.	nor.	ab-nor.	ferti.	nor.	ab-nor.	ferti.	nor.	ab-nor.	ferti.	nor.	ab-nor.	ferti.	nor.	ab-nor.	nor.
m. MaCl	2	0	10	0	0	—	0	0	—	—	—	—	—	—	—	—	—	—	—	—	—
$\frac{3}{4}$ m. NaCl	13	1	1	3	0	6	12	0	6	0	2	23	0	9	0	0	9	31	0	0	—
$\frac{1}{2}$ m. NaCl	4	0	1	6	0	1	11	0	1	28	0	2	18	3	5	0	19	10	0	0	—
$\frac{1}{4}$ m. NaCl	0	0	—	0	0	—	0	0	—	0	0	—	0	0	—	0	0	—	0	0	—
m. KCl	0	0	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
$\frac{1}{2}$ m. KCl	5	2	2	8	0	1	6	0	2	—	—	—	—	—	—	—	—	—	—	—	—
$\frac{1}{4}$ m. KCl	0	3	1	0	0	—	0	0	—	—	—	—	—	—	—	—	—	—	—	—	—
m. $CaCl_2$	0	3	12	0	0	—	0	0	—	—	—	—	—	—	—	—	—	—	—	—	—
$\frac{1}{2}$ m. $CaCl_2$	5	2	2	0	0	—	0	0	—	0	0	—	0	0	—	0	0	—	—	—	—
$\frac{1}{4}$ m. $CaCl_2$	0	5	5	0	0	—	0	0	—	—	—	—	—	—	—	—	—	—	—	—	—
$\frac{1}{2}$ m. $MgCl_2$	7	1	1	3	1	1	3	2	7	2	0	9	0	19	3	3	3	11	0	0	—
Control	6	0	1	5	1	1	3	1	1	0	3	3	0	1	8	0	0	—	0	0	—

The explanation of the table is as follows.

At the time of the observation the eggs in the normal sea water had attained the 4-cell stage of development.

NaCl.

1 mol sol. One hour influence had little to do with the development of the eggs. The development was normal but a little retarded as compared with the control. More than six hours influence caused a change in the eggs, so that no eggs were fertilized. Most of them disintegrated in the solution.

¾ mol sol. No effect was noticed after subjection for 1 hour, 6 hours and 12 hours, respectively. After remaining more than 15 hours in the solution the cleavage of the egg became incomplete and so it was destined to destruction.

½ mol sol. This had an influence almost the same as ¾ mol solution, at any duration of exposure.

¼ mol sol. The eggs were so injured in this solution in only one hour that none of them were fertilized by normal spermatozoa. All eggs were swollen up and then broken into pieces.

KCl.

1 mol sol. The solution was injurious. Therefor, no eggs were observed to be fertilized under the influence of this concentration.

½ mol. sol. The eggs were normal as far as 12 hours influence of this solution.

¼ mol. sol. After remaining one hour in the solution the eggs were already affected. The fertilization membrane was not sufficiently formed and the segmentation of the eggs was also abnormal.

$CaCl_2$.

1 mol sol. A few eggs were fertilized after one hour exposure. So far as the development of the eggs was concerned they were all abnormal. Many eggs had the normal fertilization membranes with undivided cytoplasm which suffered from severe cytolysis (Fig. 1). More than 6 hours influence caused the disintegration of the eggs.

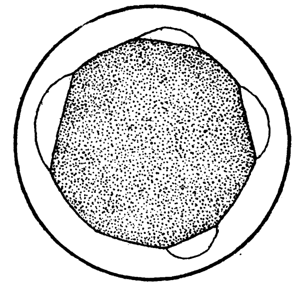

Fig. 1.

½ mol sol. The fertilization and development were normal in the eggs of one hours exposure. No eggs were fertilized after exposure of more than 6 hours.

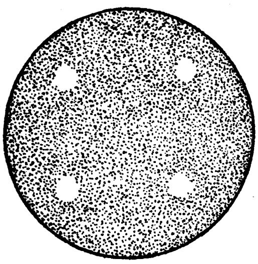

Fig. 2.

¼ mol sol. One hour in the solution was enough to injure the eggs. The fertilization membrane was formed irregularly and the segmentation of the eggs was never set though the nucleus divided into 4 (Fig. 2). Thus the polynucleated cell was obtained. The other unfertilized eggs in this solution were enormously swollen up. All the other series had injured the eggs.

$MgCl_2$.

½ mol sol. The solution acted normally as the sea water. It is to be noticed that not only the salt solution but also the natural sea water affect the eggs injuriously when they bear their influence for more than 24 hours on unfertilized sea-urchin eggs. We see from the data that a one mol solution of any salts was harmful to the unfertilized eggs while ½ mol or ¾ mol solutions which have almost equal osmotic pressure to that of the natural sea water, had no disadvantagous effect. Therefrom $CaCl_2$ is an only exception which affects the egg injuriously even in the ½ or ¾ mol solution.

2. The influence of neutral salts upon the fertilized eggs.

In this case normally fertilized eggs were employed as the material. Three hours after fertilization they were removed into hard-glass vessels each containing 20 ccm. of the following solution; ½ m. NaCl, ½ m. KCl, ½ m. $CaCl_2$ and ½ m. $MgCl_2$. At that time the majority of the eggs in the sea water have developed into the thirty-two cell stage. Observations were made once after ninety minutes and twice after six hours of the experiments. The results were as follows:

After ninety minutes.

In the ½ mol NaCl solution all eggs were at the early morula stage. Each blastomere was slightly swollen. It had become somewhat transparent, losing the normal appearance.

In the ½ mol KCl and in the ½ mol $CaCl_2$ solution all eggs attained the normal morula stage. The development of the eggs was retarded in the ½ mol $MgCl_2$ solution while the blastomeres in it remained normal.

In the control sea water, however, all eggs were at the early blastula stage.

After 6 hours.

In the ½ mol NaCl solution development did not advance and each blastomeres was greatly swollen. In the meanwhile the pigment granules became obscure. Next, in the ½ mol KCl solution all blastomeres of the eggs were swollen up abnormally. Most eggs in the ½ mol $CaCl_2$ solution showed abnormal morula stage. In the ½ mol $MgCl_2$ solution the blastomeres were broken into several pieces. By this time the control had the eggs at the late blastula stage.

From the above data it is evident that the fertilized eggs were more or less affected by the simple salt solution in spite of the isotonic pressure of the solution.

3. The influence of neutral salts upon the spermatozoon.

In this series of experiment, at first two drops of the testicular fluid of the sea-urchin were dropped into vessels containing 10 ccm. of sea water, and next one drop from this vessel was transferred into the other vessels, each containing 20 ccm. of ½ mol NaCl, KCl, $CaCl_2$ and $MgCl_2$ respectively. Thus the spermatozoa were treated in each solution, for 10 minutes in the first series and for 30 minutes in the second series. Then in order to test the activity of these spermatozoa fresh eggs were inseminated by them. The result are given in Table II.

Table II.

	10 Minutes		30 Minutes	
	Fertlized Eggs	Unfertilized Eggs	Fertilized Eggs	Unfertilized Eggs
1/2 m. NaCl	13	31	4	81
1/2 m. KCl	3	80	0	—
1/2 m. $CaCl_2$	0	0	0	—
1/2 m. $MgCl_2$	0	0	0	—
Control	18	3	12	4

Ten minutes treatment.

In the ½ mol NaCl and ½ mol KCl solutions spermatozoa were moving very actively and some eggs were normally fertilized. On the contrary, the ½ mol $CaCl_2$ and ½ mol $MgCl_2$ solution were injurious to the spermatozoa and no eggs were fertilized by them. In the control the spermatozoa fertilized the eggs normally.

Thirty minutes treatment.

In the ½ mol NaCl solution the spermatozoa were moving in lively manner so the eggs were fertilized normally. In the ½ mol KCl, ½ mol $CaCl_2$ and ½ mol $MgCl_2$ solutions the spermatozoa stood still and no eggs were fertilzed by them. On the other hand, most eggs were normally fertilized in the control.

As a whole the spermatozoa were very susceptible to the ions of the solution.

4. On the antagonistic action between NaCl and some other neutral salts.

In this experiment fertilized eggs were used as they were more susceptible to the salts than the unfertilized eggs as is shown in the former experiment. Seven different combinations were prepared, four of which were combination of two salts while three were of three salts.

A	½ m. NaCl 20 ccm.	½ m. KCl 0.44 ccm.	
B	”	½ m. $CaCl_2$ 0.30 ccm.	
C	”	½ m. $MgCl_2$ 1.56 ccm.	
D	”	½ m. $MgSO_4$ 0.76 ccm.	
E	”	½ m. KCl 0.44 ccm.	½ m. $CaCl_2$ 0.30 ccm.
F	”	”	½ m. $MgCl_2$ 1.56 ccm.
G	”	”	½ m. $MgSO_4$ 0.76 ccm.

The proportion of each component of the solution was taken after VAN'T HOFF's formula which was employed in the author's previous work (1929). Table III gives the results of the observation.

Table III.

	1 hour		5 hours					12 hours					
	1c.	2c.	1c.	4–8c.	16–32c.	moru.	ab. s.	1c.	2–32c.	moru.	blast.	ab. s.	dis.
A	10	9	3	0	0	14	1	1	2	11	0	3	0
B	7	0	3	0	0	0	27	0	0	0	0	9	0
C	10	2	6	2	0	0	27	1	0	0	0	16	4
D	20	1	14	3	0	0	21	13	2	0	0	15	2
E	10	1	0	1	10	2	4	2	1	9	0	6	1
F	10	8	0	0	12	1	6	0	0	0	(1)	11	0
G	4	4	3	0	4	13	2	0	3	13	0	4	0
Cont.	3	11	0	0	0	(30)	4	0	0	0	(7)	4	0

Explanation

Combination A. One hour after the treatment nine eggs started to develop and attained the normal two cell stage. After five hours fourteen eggs were at the stage of early morula. However, these morulae could not develop beyond that stage even though they kept normal appearance still several hours later.

Combination B. No eggs began to develop within one hour, but five hours later nine eggs were at the four-cell stage. After twelve hours, these eggs developed into the morula stage, of which the blastomere was, on the contrary, the abnormal form.

Combination C. In this series two eggs developed within one hour into the normal two-cell stage. After five hours most eggs showed the abnormal 32–cell stage, beyond which the development advanced no further.

Combination D. In this case only one egg developed into the four-cell stage within one hour. After five hours three eggs were at the normal four-cell stage and the other fourteen eggs possessed poly-nuclei in each blastomere. No eggs developed further than the four-cell stage but some eggs disintegrated after twelve hours.

Combination E. Within one hour one egg started to develop. After five hours two eggs were found at the normal morula and ten eggs at the sixteen-cell stage while four eggs showed an abnormal sixteen-cell stage.

Combination F. After one hour eight eggs were at the normal two-cell stage and five hours later thirty one eggs were at the morula stage while twelve eggs showed abnormal forms. After twelve hours there were one blastula and eleven morulae which were all abnormal in form.

Combination G. In this case four eggs developed into the two-cell stage in one hour. After five hours thirteen eggs reached the normal morula stage and four eggs were at the sixteen-cell stage. Further development did not occur in this solution and some of the eggs showed abnormality.

Control. The eggs which were kept in the normal sea water developed into two-cells within one hour and after five hours most eggs were at the late morula stage. The observation after twelve hours got seven normal and four abnormal blastulae.

We see from the above that the antagonistic action of three salt ions is unfavorable than that of two salt ions.

Conclusion

When the unfertilized eggs of sea-urchin were treated with the hypertonic or the hypotonic salts solution they lost the power of being fertiled. However, ¾ mol or ½ mol solutions of any single salt, which is approximately isotonic with the sea water, was not so toxic for the unfertilized eggs. As is shown by HEILBRUNN (1913, 1915, 1926) the unfertilized sea-urchin egg in this case is perhaps surrounded by a semipermeable plasmmembrane which controls the osmotic pressure of the medium within a certain limit. Though it has been reported that even the isotonic solution causes in unfertilized eggs the separation of the parthenogenetic membrane, in my case, the formation of such membrane at any concentration of the salt did not occur. On the other hand, in the one mol solution of NaCl or KCl a few eggs formed a membrane which was quite different from the fertilization membrane. The eggs which lifted these membranes always showed abnormal appearance and of course these eggs were destined to disintegrate.

The reaction of the unfertilized eggs to the isotonic solution, however, are variable according to the ionic valency of the salts. Na acts to dissolve or disintegrate the plasmmembrane in a long exposure. However, this ion is not toxic for the egg plasm, as in case of a broken piece of plasm after a long exposure to the influence of the solution. The pigment granules keep a normal appearance. On the contrary, the bivalent Ca is very toxic for the egg-plasm although it does not injure the egg membrane. As the result of the treatment in Ca solution, the inner hyaloplasm urges out on the egg membrane forming small small gelatinous globules.

It is very interesting to note that the duration of life of the unfertilized eggs of sea-urchin differs according to the medium.

LILLIE (1911) observed that the *Asteria* eggs after three hours exposure to the isotonic NaCl produced more larvae than the control eggs in sea water.

In my case the unfertilized sea-urchin eggs kept their susceptibility to fertilization more than 39 hours in isotonic solutions of neutral salts except $CaCl_2$, though in the control no eggs were fertilized at this time. The order of toxic action of salts for the unfertilized eggs is as follows; $Ca \gg Mg > K \geqq Na$. It is noticed that this order is the same as that of the ionic valency. LILLIE (1910) stated on the *Arbacia* eggs that the influence of K is weaker than Na and GELLHORN (1927) also gave the following order for *Strongylocentrotus*; $Ca > Na > K$. Recently CHAMBER and PAUL (1928) studied the effect of various chlorides upon *Amoeba proteus* and found the toxic order to be $KCl > NaCl > CaCl_2 > MgCl_2$ against the *exterior* of *Amoeba*. The oder was reversed, however, against the interior structure of the animal.

It is well known that spermatozoa are very susceptible to the changes of medium and as is shwon in Table II the spermatozoa lost their power of fertilization even after a short exposure to an isotonic salt solution except NaCl. The toxic order is as follows; $Ca > Mg > K > Na$.

The fertilized egg is also susceptible to the salt dependent of course upon the specificity of the fertilization membrane. According to LOEB (1913) and HEIBRUNN (1615) the membrane is readily permeable to electrolytes which effect the egg harmfully. On this point my experiments support their view. In fact the fertilized eggs could not continue their development as long as they remained in the simple salt solution. The order of susceptibility of germ cells to the salt solution may be stated as follows; spermatozoon $>$ fertilized egg $>$ unfertilied egg.

The mixture of two salts, each of which was otherwise poisonous to the eggs, did little hurt, as there was among the salts some mutually antagonistic action which weakened the influence of the ion. In my experiment this antagonistic action prevails most in the combination of Na and Ca, next in Na and Mg and least in Na and K.

As for the antagonism of three salts it generally acts more favorably than that of the two salt csmbination and there are no remakable differences among the various combinations.

The antagonistic of the injurious salts has also been observed in other animals. According to CHAMBER and REZINIKOFF (1928) the antagonistic action of the chloride of Na, K and Ca on the protoplasm of *Amoeba proteus* was also noticed and they concluded that "the toxic effects of NaCl and KCl on the exterior of the cell can be antagonised by $CaCl_2$, Na still penetrate but at a slower rate than of the *Amoeba* immersed in a solution of this salt alone." BODINE (1928) also ascertained the same fact for the Fundulus egg: "Na + Ca mixtures were not so toxic for egg".

This means that the osmotic pressure of the surrounding medium is not the only factor which affects the sea animals, but there are still other factors, for example the nature of the ions of the salts and the antagonistic action of these ions.

Summary

1. In the present work the influence of chlorides of Na, K, Ca and Mg upon germ cells of *Strongylocentrotus nudus* was examined.

2. The unfertilized sea-urchin eggs did not form the parthenogenetic membrane in the isotonic solutions of these neutral salts. In the isotonic solutions of chlorides of Na, K and Mg the unfertilized eggs kept their susceptibility to fertilization more than twelve hours.

2. The toxic order of these salts for the unfertilized sea-urchin eggs is as follows; $Ca \geqslant Mg > K \geqq Na$.

4. The fertilization membrane is permeable to the cations and accordingly

the fertitized eggs are more susceptible to these cations than the unfertilized eggs.

5. There is an antagonism among neutral salts against the toxic actions upon the eggs.

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摘　　要

余は玆に、北海道沿岸に普通なるエゾムラサキウニ *Storongylocentrotus nudus* (*A. Agassiz*) に就き、其の生殖細胞に及ぼす NaCl, KCl, $CaCl_2$ 及び $MgCl_2$ の影響を見たるも、之等鹽類の作用に就ひては其の溶液の濃度如何が最も關係を有するものにして、一モル又は、四分の一モル溶液に於ては該動物の卵は、其の受精能力の上に著るしき障害を受くるものなり。而して海水さ略等壓の溶液内に於ては、所謂、單性生殖膜 (Parthenogenetic membrane) の生成も認められず、$CaCl_2$ 以外の溶液内に於て長時間に渡り良く受精能力を保有し得る事を示せり。然りど雖も、之等鹽類の單獨溶液内に於ては時間の經過と共に、各鹽類獨時の有害作用を受くるものにして、一般に原子價の大なるものは小なるものに比し其の作用甚だし。

受精後の卵に就き行ひたる實驗によれば、卵質より隔離されたる受精膜は未受精卵の卵膜とは其の透過性を異にし、イオンの通過を容易ならしむ事に依つて受精後の卵に於ては、之等鹽類の影響を蒙る事大なり。

活動中の精虫は又，之等鹽類に對する抵抗力微弱にして NaCl 以外の等壓溶液内に於て極めて短時間内に其の活動を停止し、其の受精能力を失ふに至る。

更らに、余は、イオンの透過容易なる受精膜にて圍まれたる受精後の卵に就き、イオンの拮抗作用をも檢べたるも、且て、多くの學者に依つて說へられたるが如く．イオン間には明らかに該用存し、各イオンの有毒作用は互に他のイオンニ依つて或程度迄阻止さるるものなり。

附記　尙前報告に於てムラサキウニを *Strongylocentrotus intermedius* させしは、エゾバフンウニの誤にして、エゾムラサキハ *St. nudus* (*A. Agassiz*) なるを以つて玆に訂正す。