On the Mechanism of Freezing Injury in Extracellularly Frozen Eggs of the Sea Urchin*

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Abstract

When the eggs of the sea urchin are injured by extracellular freezing, two types of cytolysis occur, namely "black" and "white" cytolysis. By exposing the eggs to hypertonic and then isotonic salt solutions similar types of cytolysis were also observed. The causal factors leading to these types of cytolysis were investigated in detail to clarify the mechanism of freezing injury.

As a result of the present experiments it was noted that the following factors may be involved in the cytolysis of eggs resulting from an extracellular freeze-thaw sequence; 1) irreversible changes of the plasma membrane caused by intense dehydration, 2) lyotropic damage of the protoplasm as a whole by concentrated salt solution, and 3) mechanical damage of surface membrane caused by a very high osmotic stress at the cell surface during rapid thawing.

After fertilization, an immediate increase of freezing tolerance in the eggs was observed. This may be attributed to the formation of a new plasma membrane and cytoplasmic change in the eggs following fertilization.

Contents

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I. Introduction

Freezing is one of the most drastic environmental phenomena encountered by animals and plants in cold and temperate climates. The effects of freezing on various overwintering organisms have been studied for many years[1,2], while preservation of animal materials at low temperatures was

increasingly performed from a medical view point especially after World War II. These investigations led to the recent developments of cryobiology. Abundant works have now been published on freezing organisms, cells and tissues[3,4]. Among these works those by LOVELOCK[5,6] may appear to be supported by many medical cryobiologists for the mechanism of freezing injury in animal cells. He postulated that red blood cells were injured by the increased salt concentration during freezing rather than by ice or low temperature itself, and that freezing injury was the result of membrane damage caused by concentrated sodium chloride. After his work was published, several hypotheses have been proposed with criticisms[7–10]. However, there is still no established theory to explain satisfactorily the freezing injuries on living cells in general.

The egg of the sea urchin have been found to be one of the best materials to observe the processes of various kinds of cellular injury during freezing and thawing. ASAHINA[11] investigated in detail the processes of freezing and thawing in the eggs of the sea urchin, and found two types of cytolysis which occurred when the eggs were injured by extracellular freezing. The purpose of the present paper is to clarify the mechanism of freezing injury in the sea urchin eggs by investigating the nature of the two types of cytolysis. Since a similar effect may be expected in the eggs in sea water between the process of freeze-thawing and that of an exposure to hypertonic and then hypotonic solutions, experiments were conducted by exposing eggs to these solutions. The results of these experiments may, the author believes, contribute to the interpretation of freezing injury.

The freezing tolerance of the sea urchin eggs remarkably changes during development to first cleavage. This may present an important clue in the study of the mechanism of freezing tolerance. Regarding this problem, therefore, some experimental results will also be referred to.

II. Materials and Methods

1) *Biological materials*

The following sea urchins were used during their breeding seasons: *Strongylocentrotus intermedius*, *Strongylocentrotus nudus* and *Hemicentrotus pulcherrimus*. *S. intermedius* was collected at Akkeshi and Oshoro (Hokkaido), *S. nudus* at Oshoro, and *H. pulcherrimus* at Asamushi (Aomori) and Misaki (Kanagawa). Gametes were obtained by the injection of 0.53 M potassium chloride into the body cavity. Eggs were washed three times with filtered sea water. After fertilization, they were washed once with filtered sea water to eliminate excess spermatozoa. Embryos were raised in Millipore-

filtered sea water with gentle stirring at the similar temperatures to that of the natural environment; namely 10°C for *H. pulcherrimus* and 15°C for both *S. intermedius* and *S. nudus*.

In the present experiments, unfertilized eggs were exclusively used unless specified.

2) *Observation of the process of freeze-thawing*

A special refrigerated microscope was used to observe the eggs during freezing and thawing. This microscope was a remote-controlled inverted type set in a cold box[12].

3) *Procedure of freeze-thawing*

Small test tubes (10×10 mm) containing a small amount (0.5–1.0 ml) of egg suspension in sea water were placed in a refrigerator at −30°C. The temperature of the egg suspension was measured by inserting the tip of a fine copper-constantan thermocouple directly into the egg suspension in the test tube. When the egg suspension was cooled to −4°C, it was seeded with a small amount of ice crystals to avoid intracellular freezing. When the frozen egg suspension was cooled to a required temperature, the test tubes were quickly transferred to another refrigerator which was kept at the same temperature. Duration of freezing was measured from the time of transfer to the latter refrigerator. With these procedures, cooling rate of the egg suspension was observed to be about 1.0°C/min between −5° and −15°C. Figure 1 shows the typical freezing curve. Test tubes containing

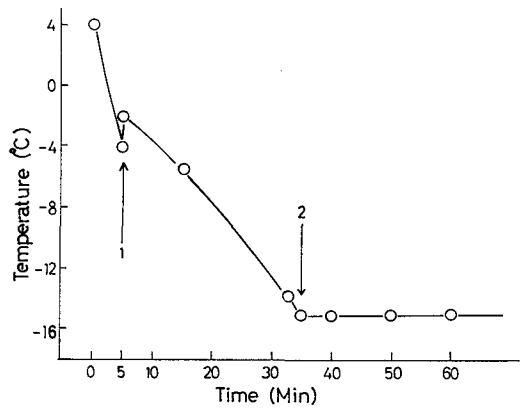

Fig. 1. A freezing curve of egg suspension (0.5 ml) in a test tube cooled in a refrigerator kept at −30°C. Arrow 1 indicates the time of ice seeding, and arrow 2 indicates the time of transferring the test tube to another refrigerator kept at −15°C

the frozen eggs uspension were usually warmed in two ways: (1) in air at 18°C (slow thawing) and (2) in a 45°C warm water bath (rapid thawing). Warming rates were about 2.5°C/min in the former and about 160°C/min in the latter between −15° and −5°C. When the temperature of the thawing egg suspension approached −2°C, the test tubes containing the egg suspension were immersed in an ice water bath for a short time until ready for use.

4) *Concentrated sea water treatment*

A large volume of sea water was frozen at −16°C. Ice formed was eliminated by filtering through gauze mesh, and the concentrated sea water was obtained. Packed eggs were suspended in the concentrated sea water for varying times and then they were transferred to normal sea water chilled at 0°C in an ice-water bath. These procedures were done in a cold room (2°–4°C).

5) *Hypertonic solution treatment*

A small amount of packed eggs was suspended in about 10 volumes of isotonic sodium chloride solution for 5 minutes. These eggs were sedimented by low speed centrifugation. The packed eggs were separately suspended for varying times in about 10 volumes of a series of hypertonic solutions of sodium chloride. After centrifugation, these eggs were transferred to isotonic sodium chloride solution. Another method was used to expose eggs in increasing concentration of sodium chloride solution. Various amounts of 4 M sodium chloride solution were added drop by drop to 50 ml of the egg suspension in isotonic sodium chloride solution with gentle stirring. By adding 4 M sodium chloride to the egg suspension at the rate of 10 ml/min, the concentration of the suspending medium increased from 0.55 M to 3.5 M within 30 minutes. The above mentioned treatments were conducted at both room temperature (18°–20°C) and 0°C. In the case of hypertonic treatment at 0°C, sodium chloride solution and egg suspension were previously chilled at 0°C in an ice-water bath and the experiments were made in a cold room. Hypertonic solution treatments were also conducted by the use of glycerol or sorbitol in place of sodium chloride.

6) *Electron microscopic observation*

a) Transmission electron microscopy

Eggs with or without previous treatments were prefixed for 1.5 hours in 2.5% solution of glutaraldehyde in sea water, washed with sea water, and postfixed in 4% solution of osmium tetroxide in sea water for 1 hour. After fixation, the eggs were dehydrated in a graded series of ethanol, and

embedded in Epon 812. Thin sections were cut with a Porter-Blum ultramicrotome, stained with uranyl acetate followed by the lead citrate stain, and observed with a JEM-100C electron microscope.

b) Cryo-scanning electron microscopy

The cryo-scanning electron microscopic observation was made according to NEI and ASADA[13] and FUJIKAWA and NEI[14]. Eggs with or without previous treatments were fixed for 4 hours in 2% solution of glutaraldehyde in sea water, and washed with sea water. The eggs were then suspended in 30% glycerol solution for 2 hours. They were frozen in cooled Freon 22 at −160°C and transferred to the chamber of a scanning microscope. They were freeze-fractured and etched in vacuum at about −100°C. The fracture faces were coated with gold and observed with a scanning electron microscope (JSM-50 A).

7) *Extraction and determination of lipids released by hypertonic sodium chloride treatment*

The lipids released from eggs which were suspended in 2 M sodium chloride solution were determined. To avoid drastic change of medium concentration, packed eggs of *S. intermedius* were first suspended in an equal volume of 2 M sodium chloride solution at 0°C and sedimented by low speed centrifugation. After the removal of the supernatant, 10 volumes of cold 2 M sodium chloride solution was added to the egg suspension. Eggs were sedimented by low speed centrifugation and the supernatant was aspirated. This procedure was repeated three times to equilibrate the eggs with 2 M sodium chloride solution. Then eggs were suspended in 10 volumes of 2 M sodium chloride solution at 0°C. After 0.5 and 2 hours, the eggs were respectively sedimented by low speed centrifugation. The supernatant was filtered through Whatman No. 1 filter paper, and centrifuged at $7{,}000 \times g$ for 20 minutes. The supernatant was mixed with 10 volumes of chloroform-methanol (2 : 1, v/v). After shaked well, the mixture was separated into two phases. The upper phase was again mixed with the solvent. The lower phases from both mixtures were combined and evaporated to dryness in vacuum at 35°C. The residue was dissolved in a small volume of benzene. The amount of phospholipids was measured by the method of SHIBUYA *et al*[15].

8) *Elimination of fertilization membrane and hyaline layer*

Immediately after insemination, eggs were rapidly suspended in about 10 volumes of artificial sea water free of calcium and magnesium. An ex-

traordinarily high membrane elevation took place on the eggs. The fertilization membranes were eliminated by passing eggs through a nylon mesh. The hyaline layer was dissolved in calcium-magnesium-free artificial sea water within 10 minutes. Then the eggs were transferred to normal sea water. Artificial sea water was prepared according to van't Hoff's prescription.

9) *Parthenogenetic activation and development*

a) Urea treatment

It was reported that fertilization membrane formation was artificially induced by immersion of eggs in urea solution[16]. In the present experiment, the following method was used. Two drops of packed eggs (0.05–0.1 ml) were suspended in 10 ml of 1 M urea solution for 2 minutes. Eggs were sedimented by low speed centrifugation and supernatant was decanted. The sedimented eggs were again suspended in 10 volumes of sea water. The percentage of membrane formation was about 90%.

b) Procaine treatment

The method to develop eggs without rupture of cortical granules by procaine treatment were reported by VACQIER[17]. In the present experiment this method was slightly modified as follows: Eggs of *S. intermedius* were exposed for 2 minutes to 0.01 M procaine (Sigma Chemical Co.) in sea water. An equal volume of diluted sperm suspension in sea water was added and eggs were allowed to sediment. After 10 minutes of insemination, the supernatant sea water was removed by aspiration and replaced with fresh sea water. Eggs subjected to this procedure did not undergo the cortical reaction. No fertilization membranes elevated and no hyaline layer formed. Eggs were raised at 18°C. The percentage of cleaved eggs was about 85%.

10) *Dissociation of the sea urchin embryo at 16-cell stage*

A sea urchin embryo at the 16-cell stage is composed of three peculiar sized cells; micromeres, mesomeres, and macromeres. The methods of the dissociation of the cells from sea urchin embryos developed by HYNES and GROSS[18], and by SPIEGEL and RUBINSTEIN[19] were slightly modified as follows: After insemination an extraordinarily high membrane elevation took place in calcium-magnesium-free artificial sea water containing 1 mM EDTA. The fertilization membranes were then removed by passing the eggs through a nylon mesh. After washing several times with normal sea water, the embryos were allowed to develop until the majority of them became 16-cell stage. Embryos at 16-cell stage collected by low speed centrifugation were suspended in about 50 volumes of calcium-magnesium-free sea water containing

1 mM EDTA. By slight agitation, the embryos were completely dissociated into individual cells. These individual cells were kept in normal sea water at 0°C until ready for use.

III. Results

1) *Cryomicroscopic observations of the process of freeze-thawing of eggs*

a) Intracellular freezing

When eggs were rapidly cooled down and frozen, intracellular freezing readily took place and the eggs became suddenly dark between ice crystals. The phenomenon, a very rapid black out of frozen cells, is the most common type of intracellular freezing in living cells and is designated as flashing[20-22]. Flashing usually took place independently in each cell when eggs were cooled somewhat rapidly with extracellular ice at temperatures below −8°C. It has been reported that sea urchin eggs exposed to rapid cooling faster than 10°C/min showed a high possibility of causing intracellular freezing, while slow cooling of about 1°C/min did not[23,24]. After thawing from flashing, eggs appeared dark and granular, showing a characteristic pattern of coagulated protoplasm. Intracellular freezing has proved fatal in various living cells except in the case of extremely rapid cooling[20,25].

b) Extracellular freezing

When eggs were cooled slowly (less than 1°C/min), ice formed around the eggs and the process of extracellular freezing began. In these eggs the ice crystal on the cell surface withdrew water from the cell interior as the cooling proceeded because of the difference in chemical potential between the ice and supercooled water within the egg. Thus a marked dehydration and contraction occurred. Even in such a contracted state, the shape of the unfertilized eggs was not remarkably changed, and as a rule was nearly spherical. But in the case of fertilized eggs the shape showed a highly irregular change. When unfertilized eggs of *S. intermedius* were extracellularly frozen at −4°C and cooled down slowly, they became dark at temperatures below −15°C within 5 minutes. Even at temperatures above −15°C, as the period of freezing time was lengthened, a darkening of the eggs began to occur. The number of darkened eggs increased with the prolonged freezing period and all eggs became dark within 4 hours at −15°C (Fig. 2 b). Such darkening of protoplasm never suggests any intracellular ice formation in these cells, but it shows a typical coagulation of destroyed protoplasm. The degree of coagulation varied according to the severity of the freezing conditions. When the condition of freezing was very severe, eggs strongly contracted and a uniform darkening of the entire egg took place. But under

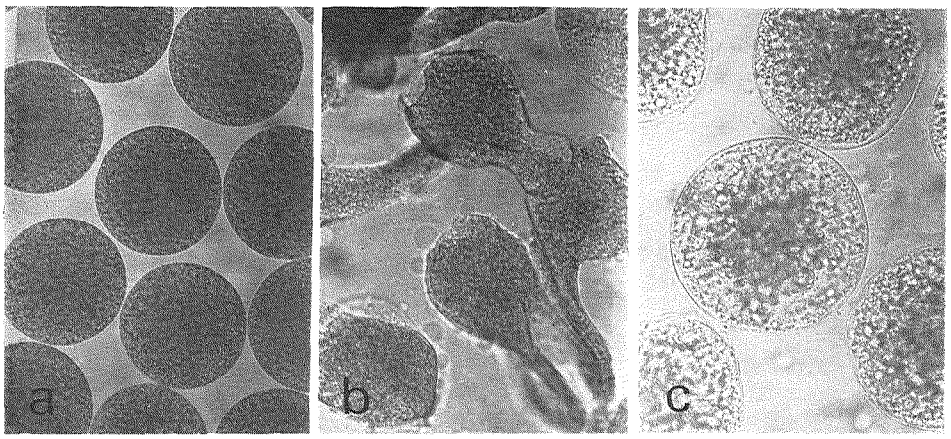

Fig. 2. Two types of cytolysis in unfertilized eggs of *S. intermedius*. × 200
a : Normal eggs
b : "Black" cytolysis
c : "White" cytolysis

a less severe conditions, a complete darkening of protoplasm occurred only at the core and the periphery of the egg remained pale with a somewhat swollen appearance. The cytolysis of dark appearance described above is designated as "black" cytolysis hereinafter.

In the process of slow thawing at a rate of about 2°C/min from extracellular freezing, some eggs showed a gradual swelling at temperature range between −10° and −5°C resulting in cytolysis. In these cytolysed eggs a considerable number of transparent vacuoles were formed in the hyaline plasma, and the eggs appeared pale in a distinctly lighter shade compared with the coloration of "black" cytolysed eggs. This type of cytolysis is designated as "white" cytolysis hereinafter (Fig. 2 c). In the thawing process from freezing at −15°C when the warming rate was high (about 10°C/min), almost all of the eggs became "white" cytolysed. Some of the eggs burst at a single site at the periphery. Their contents at times flowed out and dispersed in the medium, leaving only the crumpled membranes. When the freezing period was longer, darkening occurred also in the process of warming in freezing eggs which appeared intact prior to the commencement of warming.

In sea urchin eggs the most frequent type of cytolysis by extracellular freezing were "black" and "white" cytolysis. These two types of cytolysis were also frequently observed when the eggs were injured by various other chemical or physical factors[26-30]. "Black" cytolysis appears to be quite similar

to "dark brown cytolysis", and "white" cytolysis is comparable to "pale or greenish-yellow cytolysis" described by ÖHMAN[29]. It was easy to distinguish these two types of cytolysis from the intracellularly frozen and thawed eggs by observing their appearance under a microscope.

2) *Factors which cause "black" and "white" cytolysis in extracellularly frozen eggs*

Cryomicroscopic observations revealed that freezing injury increased in the extracellularly frozen eggs as the freezing temperature was lowered, freezing period was prolonged, and the warming rate was increased. To clarify or determine the factors which cause "black" and "white" cytolysis, cellular responses to freezing temperatures, freezing period of time, and the warming rate from various freezing temperatures were examined by estimating the percentage of these two types of cytolysis.

a) Freezing temperature

When extracellularly frozen eggs of *S. intermedius* were cooled at a rate of 1°C/min, almost all of them underwent "black" cytolysis immediately after they were cooled to temperatures below −17.5°C (Fig. 3). Such a killing temperature is designated as the critical freezing temperature hereinafter. No "white" cytolysis occurred in the eggs after cooling to a freezing temperature below the critical temperature. Almost all of eggs appeared intact after thawing from temperatures above the critical freezing temperature, provided

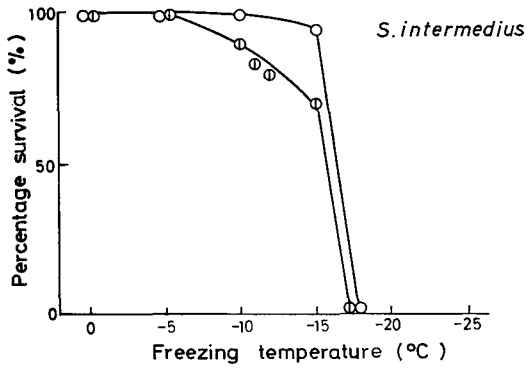

Fig. 3. Survival of frozen-thawed unfertilized eggs of *S. intermedius*. Immediately after the eggs were cooled at 1.0°C/min to various predetermined temperatures, they were thawed at 2.5°C/min. Percentage values are averages of duplicate samples. At least 500 eggs were counted in each sample

—○— intact eggs
—◑— eggs capable of 1st cleavage

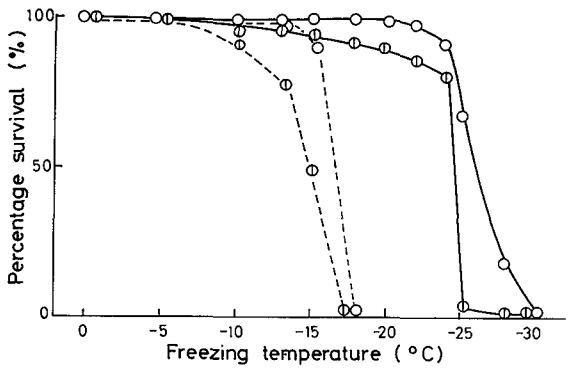

Fig. 4. Survival of frozen-thawed unfertilized eggs of *S. nudus* and *H. pulcherrimus*. Immediately after the eggs were cooled at 1.0°C/min to various predetermined temperatures, they were thawed at 2.5°C/min. Percentage values are averages of duplicate samples. At least 500 eggs were counted in each sample

—○— intact eggs (*S. nudus*)
—◑— eggs capable of 1st cleavage (*S. nudus*)
·····○····· intact eggs (*H. pulcherrimus*)
·····◑····· eggs capable of 1st cleavage (*H. pulcherrimus*)

that the freezing period was not lengthened. These intact eggs were viable and could form the fertilization membrane and cleave normally following insemination. The ability of frozen-thawed eggs to cleave decreased as the freezing temperature approached the critical temperature. The critical freezing temperature of eggs of *H. pulcherrimus* was the same as *S. intermedius* (−17.5°C), while that of *S. nudus* was about −27.5°C (Fig. 4).

b) Length of freezing period

In contrast to the fact that almost all eggs underwent "black" cytolysis after a slow freezing below the critical freezing temperature, both types of cytolysis, "black" and "white", appeared when eggs were frozen at temperatures above the critical freezing temperature and then thawed. In the case of eggs of *S. intermedius* frozen-thawed at and from −15°C, the number of "white" cytolysed eggs reached a maximum after 1 hour of freezing at −15°C, then "white" cytolysed eggs were gradually replaced by "black" cytolysed eggs as the freezing period lengthened (Fig. 5 a). After 5 hours of freezing, all eggs underwent "black" cytolysis. As the freezing temperature was lowered, the length of freezing period which gave rise to "black" cytolysis was shortened, and the curves of percentage occurrence for "black" and "white" cytolysed eggs shifted to the left (Fig. 6 ; *compare* Figs. 5 a and 7 a).

The eggs of sea urchin had a rather high tolerance to extracellular freezing, but the degree of such freezing tolerance varied according to the species. It is apparent from Figs. 5 and 6, that the eggs of *S. nudus* showed the highest tolerance to extracellular freezing and those of *H. pulcherrimus*

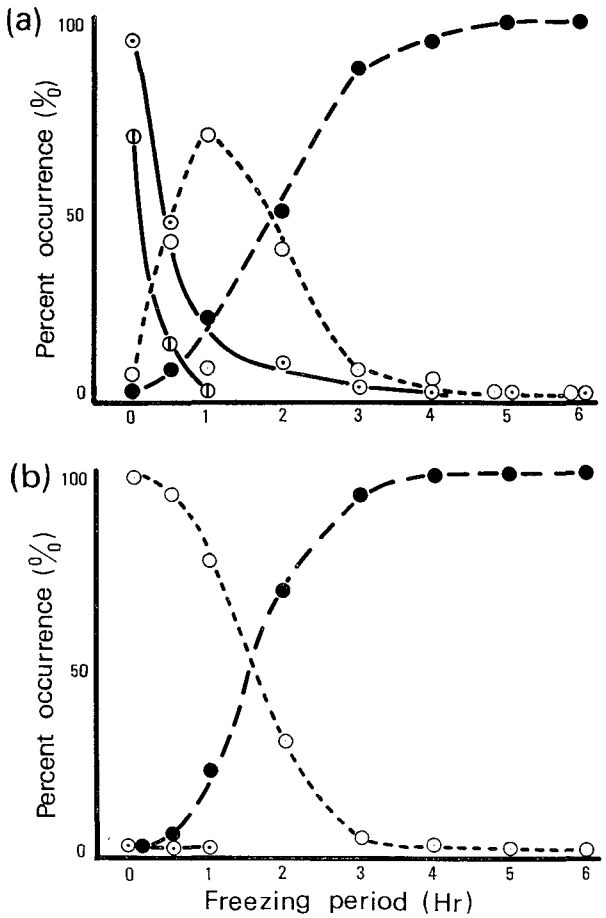

Fig. 5. Percentage occurrence of cytolysis after extracellular freezing at and thawing from −15°C. Percentage values are averages of duplicate samples. At least 500 eggs were counted in each sample. Cooling rate, 1.0°C/min. Warming rate, 2.5°C/min (a) and 160°C/min (b) Material: Unfertilized eggs of *S. intermedius*

——⊙—— intact eggs
——◑—— eggs capable of 1st cleavage
·····○····· "white" cytolysed eggs
---●--- "black" cytolysed eggs

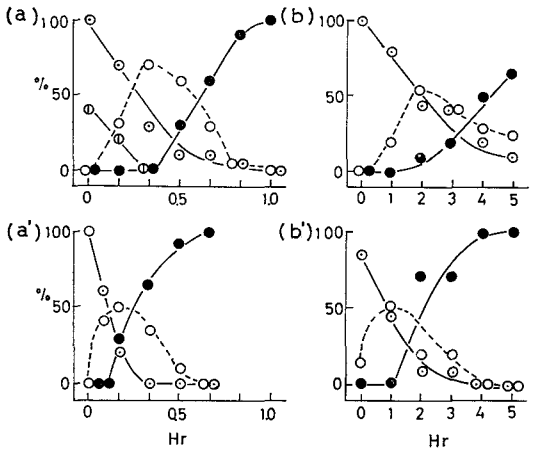

Fig. 6. Percentage occurrence of cytolysis after extracellular freezing and thawing. Percentage values are averages of duplicate samples. At least 500 eggs were counted in each sample. Cooling rate, 1.0°C/min. Warming rate, 2.5°C/min. Freezing temperature, (a) −10°C, (a′) −15°C, (b) −20°C, (b′) −22°C
Abscissa: Period of freezing time (hour)
Ordinate: Percentage occurrence of cytolysis (%)
Material: Unfertilized eggs of *H. pulcherrimus* [(a) and (a′)], and those of *S. nudus* [(b) and (b′)]
—⊙— intact eggs
—◑— eggs capable of 1st cleavage
·····○····· "white" cyto[ysed eggs
—●— "black" cytolysed eggs

were most sensitive among the three species of sea urchin. The freezing temperature at which 50% of eggs underwent "black" cytolysis within 1 hours was approximately as follows; −9°C for *H. pulcherrimus*, −16°C for *S. intermedius*, and −22°C for *S. nudus*.

c) Rate of warming

When extracellularly frozen eggs were cooled down to −15°C and then warmed rapidly (160°C/min), almost all eggs other than "black" cytolysed ones underwent "white" cytolysis (Fig. 5 b). As the freezing period was lengthened, the number of "black" cytolysed eggs increased, while that of "white" cytolysed eggs decreased in the same manner as seen in the result in slow warming. From the fact that "white" cytolysis took place only in the process of thawing, it was expected that thawing may be an important factor to cause "white" cytolysis. ASAHINA[11] noted that rapid thawing was quite harmful to extracellularly frozen egg of *S. nudus*. The same result was obtained in the eggs of *S. intermedius* in the present experiment. To

examine the effect of thawing rates, test tubes containing frozen egg suspension were warmed by the procedures indicated in Table 1. It was clearly shown that the warming rate was one of the most important factors which caused "white" cytolysis in freezing eggs insofar as the freezing period was short. When extracellularly frozen eggs were warmed slowly (2.5°C/min) to −5°C, then rewarmed at various rates, almost all of the eggs were viable regardless of the rewarming procedures indicated in Table 1. When extracellularly frozen eggs were cooled down to −5°, −7.5°, −10°, −12.5° and −15°C respectively and then warmed rapidly at a rate of 160°C/min, the

Table 1. Effect of warming rate on the survival of unfertilized eggs after extracellular freezing

Warming procedure	Approximate warming rate (°C/min)	Intact egg (%)*	"White" cytolysed egg (%)*	Cleaved egg (%)*
a) Stand test tube in cold air (0°C)	0.5	100	0	73
b) Stand test tube in warm air (22°C)	2.5	95	5	70
c) Shake test tube in ice-water bath (0°C)	10.0	95	5	70
d) Shake test tube in running water (12°C)	60.0	35	65	18
e) Shake test tube in warm water (45°C)	160.0	0	100	0

Material: Unfertilized eggs of *S. intermedius*

Test tubes, containing 0.5 ml of frozen mass of egg suspension in sea water were cooled to −15°C (cooling rate, 1.0°C/min), then they were treated by the indicated procedures

* Percentage occurrence, the average of five measurements

Table 2. Effect of final freezing temperature on the survival of eggs after extracellular freezing and rapid thawing

Final freezing temperature (°C)	Intact egg (%)*	"White" cytolysed egg (%)*	Cleaved egg (%)*
− 5.0	100	0	98
− 7.5	95	5	90
−10.0	80	20	65
−12.5	60	40	15
−15.0	0	100	0

Material: Unfertilized eggs of *S. intermedius*

Test tubes containing extracellularly frozen eggs were cooled to each temperature (cooling rate, 1.0°C/min), and then shaked in warm water (warming rate, 160°C/min)

* Percentage occurrence, the average of five measurements

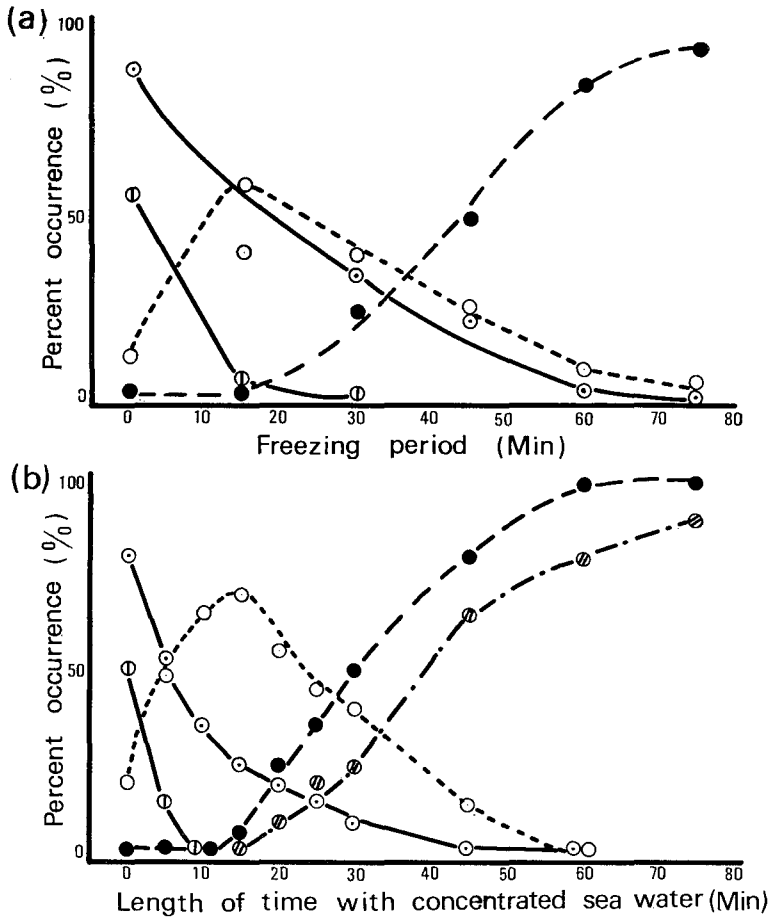

Fig. 7. Percentage occurrence of cytolysis in the eggs following an extracellular freeze-thawing at and from −16°C (a), and an exposure to concentrated sea water and then to normal sea water at 0°C (b). Percentage values are averages of duplicate samples. At least 500 eggs were counted in each sample. Cooling rate, 1.0°C/min. Warming rate, 2.5°C/min

Material: Unfertilized eggs of *S. intermedius*

——⊙—— intact eggs
——◐—— eggs capable of 1st cleavage
·····○····· "white" cytolysed eggs
— —●— — "black" cytolysed eggs
—·—▨—·— "black" cytolysed eggs before the transfer

percentage of occurrence of "white" cytolysis increased as the final freezing temperature was lowered (Table 2). From the results indicated in Table 2 the most dangerous temperature range for freeze-thawing eggs to cause "white" cytolysis by rapid warming may be between $-15°$ and $-10°C$.

3) *Occurrence of two types of cytolysis by treatment with concentrated sea water*

Upon extracellular freezing, sea water surrounding eggs is concentrated with the progressive freezing. In sea water, about 80% of water freezes out at $-10°C$, and 90% at $-12°C$[31]. To simulate the freeze-thawing process, eggs were immersed for varying lengths of time in concentrated sea water, separated from the frozen sea water mass at $-16°C$, and then transferred to normal sea water at 0°C. This caused the pattern of occurrence of both "black" and "white" cytolysis quite similar to that caused by actual freeze-thawing at $-16°C$ (Fig. 7). The occurrence of "white" cytolysis reached a maximum within 15 minutes, then it decreased with a concomitant increase in "black" cytolysis as the period of immersion was prolonged. "Black" cytolysis also occurred during the process of transferring the eggs to normal sea water, which otherwise appeared intact in the concentrated sea water. This phenomenon coincided with the fact that "black" cytolysis also occurred during the process of thawing of extracellularly frozen eggs. "Black" cytolysis occurred in a shorter period of time in eggs treated with concentrated sea water than in those which were actually frozen-thawed. When eggs were immersed in concentrated sea water at room temperature (18°–20°C), they instantly "black" cytolysed.

4) *Occurrence of two types of cytolysis by treatment with hypertonic sodium chloride solution*

It was reported that the concentration of electrolytes in the cell and the surrounding medium was the main factor to cause injury in extracellularly frozen cells[5]. The surrounding medium of the sea urchin eggs, namely sea water, is a solution of electrolytes. With the progressive freezing, the concentration of sodium chloride increases firstly and most remarkably among the electrolytes contained in sea water[32]. HOBSON[27] reported that the cytolysis of dark appearance in the sea urchin eggs occurred in a hypertonic sodium chloride solution. Based on the fact that the two types of cytolysis occurred by treatment with concentrated sea water, experiments were carried out using hypertonic sodium chloride solution instead of the concentrated sea water.

a) Minimum cell volume

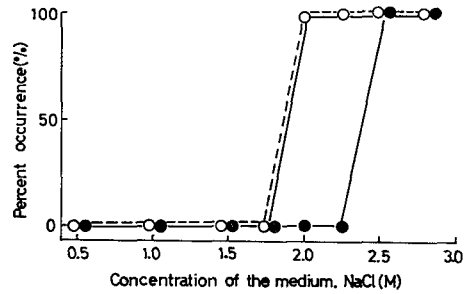

Fig. 8. Percentage occurrence of "black" cytolysis of unfertilized eggs in a hypertonic sodium chloride solution within 5 mlnutes at 18°C. Percentage values are averages of duplicate samples. At lesat 500 eggs were counted in each sample

—○— *S. intermedius*
·····○····· *H. pulcherrimus*
—●— *S. nudus*

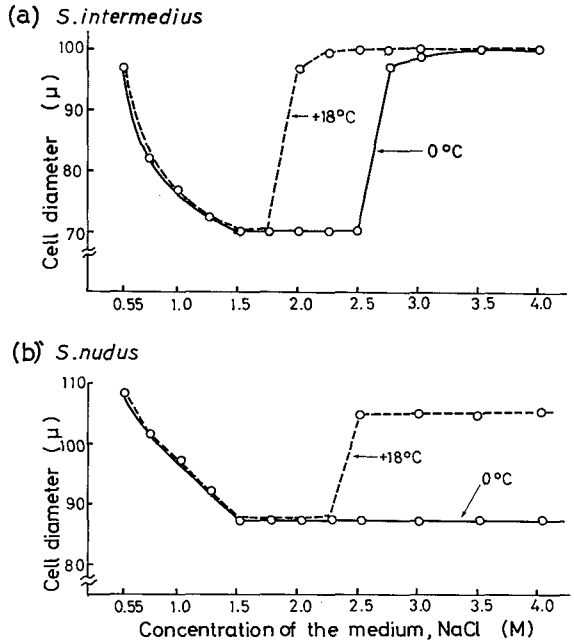

Fig. 9. Change of diameter of eggs within 5 minutes in a hypertonic sodium chloride solution at 18° and 0°C
Material: Unfertilized eggs of (a) *S. intermedius* and (b) *S. nudus*

Eggs were separately immersed for 5 minutes in a series of solutions with increasing concentrations of sodium chloride. In the case of eggs of *S. intermedius* and *H. pulcherrimus*, "black" cytolysis took place at a concentration of 2.0 M at 18°C, while "black" cytolysis occurred at 2.5 M in *S. nudus* (Fig. 8). The volume of each egg was determined by measuring the egg diameter in the media of increasing solute concentration. Each egg acted as an osmometer. The egg volume decreased gradually as the tonicity of the suspending solution increased. The egg volume then remained nearly constant to a certain value of medium concentration, after which it increased rapidly. In the case of eggs of *S. intermedius*, their volume decreased gradually to a minimum, as the concentration of the media increased to 1.5 M. It then remained constant till the medium concentration approached 1.75 M (Fig. 9 a). At concentrations above 1.75 M, egg volume increased rapidly and the eggs became dark from the periphery to the center. Thus "black" cytolysis occurred in the treated egg. When erythrosine was dissolved

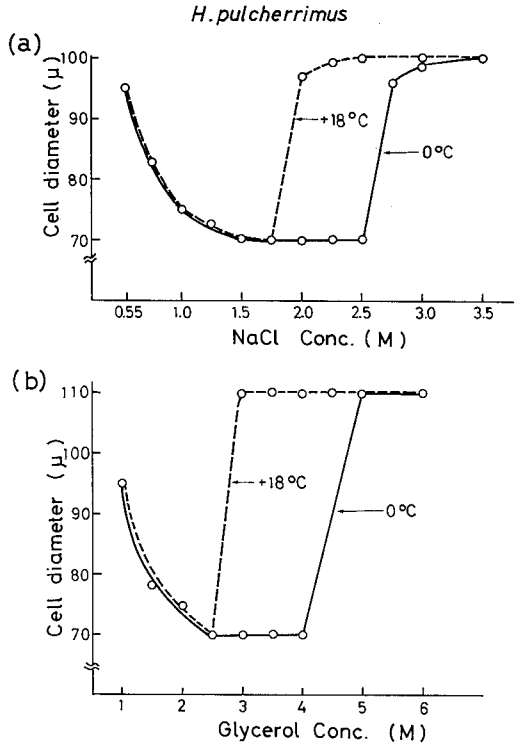

Fig. 10. Change of diameter of eggs within 5 minutes in hypertonic sodium chloride solution (a) and glycerol solution (b) at 18° and 0°C
Material: Unfertilized eggs of *H. pulcherrimus*

(0.05%) in the medium of egg suspension with a solute concentration higher than 1.75 M occurrence of "black" cytolysis was always followed by simultaneous uptake of erythrosine into the eggs. However even at such a high medium concentration, the egg volume remained minimal for a few minutes immediately after the eggs were immersed in sodium chloride solutions. The critical concentration which brought about rapid increase of egg volume, resulting in "black" cytolysis, within 5 minutes at 18°C was 2.0 M in eggs of *S. intermedius* and *H. pulcherrimus*, and 2.5 M in those of *S. nudus* (Figs. 9 and 10 a). When experiments were made at 0°C, the eggs showed a tolerance to a higher hypertonic sodium chloride solution with the cell volume at a minimum. The critical concentration to cause "black" cytolysis at 0°C was 2.5 M in eggs of *S. intermedius* and *H. pulcherrimus* (Figs. 9 a and 10 a), while eggs of *S. nudus* could tolerate more than 4 M (Fig. 9 b).

b) Length of immersion period in hypertonic sodium chloride solution

When eggs were immersed for a longer period in hypertonic sodium chloride solution, "black" cytolysis also occurred even in medium with lesser concentrations than the critical concentration which causes cytolysis within 5 minutes. The number of "black" cytolysed eggs increased as the period of immersion was lengthened (Fig. 11). "Black" cytolysis occurred more

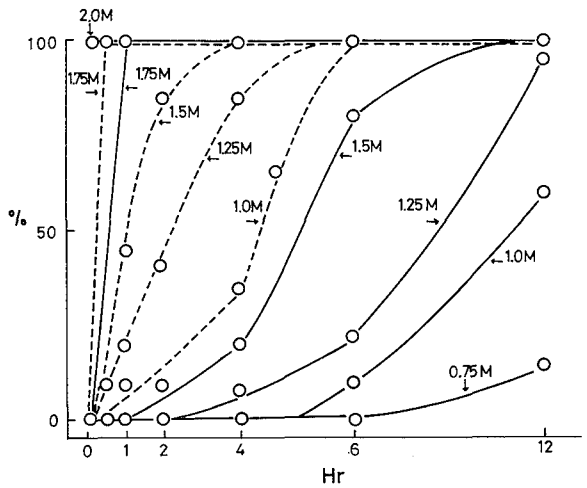

Fig. 11. Percentage occurrence of "black" cytolysis in hypertonic sodium chloride solution at 18°C. Percentage values are averages of duplicate samples. At least 500 eggs were counted in each sample
Abscissa: Period of immersion in sodium chloride solution
Ordinate: Percentage occurrence of "black" cytolysis
——○—— unfertilized eggs of *S. intermedius*
------○------ unfertilized eggs of *H. pulcherrimus*

rapidly in eggs of *H. pulcherrimus* than in those of *S. intermedius* under the same experimental conditions.

c) Transference of eggs from hypertonic to isotonic sodium chloride solution

Since one of the important effects of thawing from extracellular freezing is assumed to be the rehydration of cells once dehydrated in a hypertonic solution, the following examination was made. Eggs were immersed in a 1.5 M sodium chloride solution for varying lengths of time and then were transferred to isotonic (0.55 M) sodium chloride solution. "White" cytolysis occurred in the process of transferring eggs to the isotonic solution, and the maximum occurrence of "white" cytolysis took place in the eggs transferred at 70 minutes of the immersion period. By the same procedure the number of "black" cytolysed eggs remarkably increased in the treated eggs, as the immersion period was lengthened beyond 70 minutes (Fig. 12).

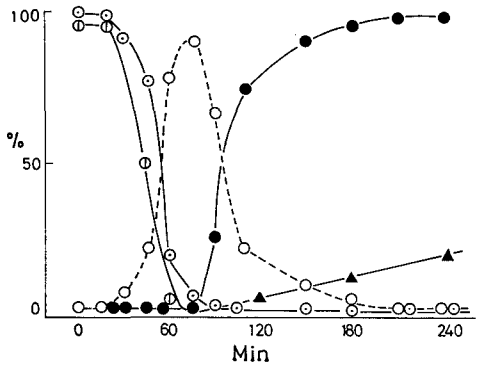

Fig. 12. Percentage occurrence of cytolysis by transferring unfertilized eggs from 1.5 M sodium chloride solution to isotonic one at 18°C. Percentage values are averages of duplicate samples. At least 500 eggs were counted in each sample

Material: Unfertilized egg of *S. intermedius*

Abscissa: The length of time for which eggs were kept in 1.5 M sodium chloride solution before the transfer

Ordinate: Percentage occurrence of cytolysis

—⊙— intact eggs
—◑— eggs capable of 1st cleavage
·····○····· "white" cytolysed eggs
—●— "black" cytolysed eggs
—▲— "black" cytolysed eggs before the transfer

5) *Cytolysis in hypertonic solutions of non-electrolytes*

Hypertonic solutions of some non-electrolytes, such as glycerol and sorbitol were observed to be quite effective to cause cytolysis in immersed eggs.

The critical tonicity of these non-electrolyte solutions to cause cytolysis was found to be nearly the same as that of sodium chloride counterparts. When immersed in a hypertonic glycerol solution, eggs shrank spherically at least for 2 minutes. With a longer period of immersion egg volume increased as glycerol increasingly enters into the eggs. This was followed by the simultaneous death of the eggs with a remarkable change in their color to dark. From the observations described above, volume change of eggs was determined after 2 minutes of immersion in solutions with graded concentration of glycerol. Egg volume decreased gradually with the increased concentration of glycerol and reached a minimum at 2.5 M at 18°C. At a concentration higher than 2.5 M egg volume increased rapidly with the simultaneous change in egg color to dark. When the eggs immersed in hypertonic glycerol solutions were maintained at 0°C, they retained their minimum cell volume even at concentrations above 2.5 M, but they cytolysed rapidly at 5 M (Fig. 10 b). In a hypertonic sorbitol solution eggs shrank and showed an irregular rugged surface. When immersed in a sorbitol solution above 2.5 M eggs lysed promptly at 18°C, while they did the same in the solutions above 3.5 M at 0°C (Table 3).

Table 3. Percentage occurrence of cytolysis in unfertilized eggs in hypertonic sorbitol solution

Temperature	Concentration of sorbitol (M)					
	1	1.5	2	2.5	3	3.5
18°C	0	0	0	100	100	100
0°C	0	0	0	0	0	100

Material: Unfertilized eggs of *S. intermedius*

Eggs were exposed to isotonic (1.0 M) and hypertonic sorbitol solutions for 5 minutes at 18° and 0°C

6) *Electron microscopic observation of cytolysed eggs*

a) Transmission electron microscopy

In a "black" cytolysed egg caused by freeze-thawing there were numerous small pores seen throughout the cytoplasm. The small size and the large number of these pores appeared sufficient to scatter the transmitting visible light so as to render the eggs dark in appearance in the bright microscopic field (Fig. 13 b). The cortical granules and the plasma membrane were completely destroyed. The same results were also observed in the cytoplasm of the "black" cytolysed eggs produced in hypertonic sodium chloride solution

(Fig. 13 c). In the latter case, the process of transferring to isotonic solution was omitted before the observation of the samples so that the egg showed a more contracted form than that caused by freeze-thawing and some cortical granules remained intact. In the egg cytolysed in hypertonic sodium chloride solution the pores in cytoplasm were observed to be smaller and more abundant throughout the cytoplasm than that in a frozen-thawed egg. The plasma membrane was completely destroyed. In the "white" cytolysed egg caused by freeze-thawing, on the other hand, there were many large pores and empty spaces (Fig. 13 d). The cortical granules and the yolk granules disappeared, and the plasma membrane was completely ruptured.

b) Cryo-scanning electron microscopy

The method of cryo-scanning electron microscopy was developed by ECHLIN[33] and NEI *et al.*[34] to observe wet biological specimens in their native state under an electron microscope. In a "black" cytolysed egg caused by freeze-thawing, one of the most distinct differences from normal was in the appearance of various granules embedded in cytoplasm (Figs. 14 a and 14 b). The granules were seen randomly distributed and a great number of small granules appeared. These granules in frozen-thawed eggs were observed to be distinctly larger and their size distribution was wider than in normal ones. In addition the granules in frozen-thawed eggs were frequently deformed. Such a deformation of the granules was more conspicuous in "black" cytolysed eggs produced in hypertonic salt solution. The cytoplasmic matrix in a "black" cytolysed egg produced by freeze-thawing and hypertonic salt solution appeared rough, while that in normal ones was smooth (Figs. 14 b and 14 c). On the other hand, "white" cytolysed egg showed a smooth surface of cytoplasmic matrix although the number of yolk granules was extremely low and the size of them was very large (Fig. 14 d). These granules might be assumed to have fused with each other during "white" cytolysis.

7) *Lipid loss from eggs in hypertonic sodium chloride solution*

LOVELOCK[6] found that phospholipids and cholesterol were dissolved out from the membranes of erythrocytes which were suspended in 0.8 M sodium

Fig. 13. Electron micrograph of cytolysed eggs

Material: Unfertilized eggs of *H. pulcherrimus*. × 5,500

a) A normal egg cell
b) A "black" cytolysed egg caused by freeze-thawing at −15°C
c) A "black" cytolysed egg caused by exposure to 2 M sodium chloride
d) A "white" cytolysed egg caused by rapid thawing

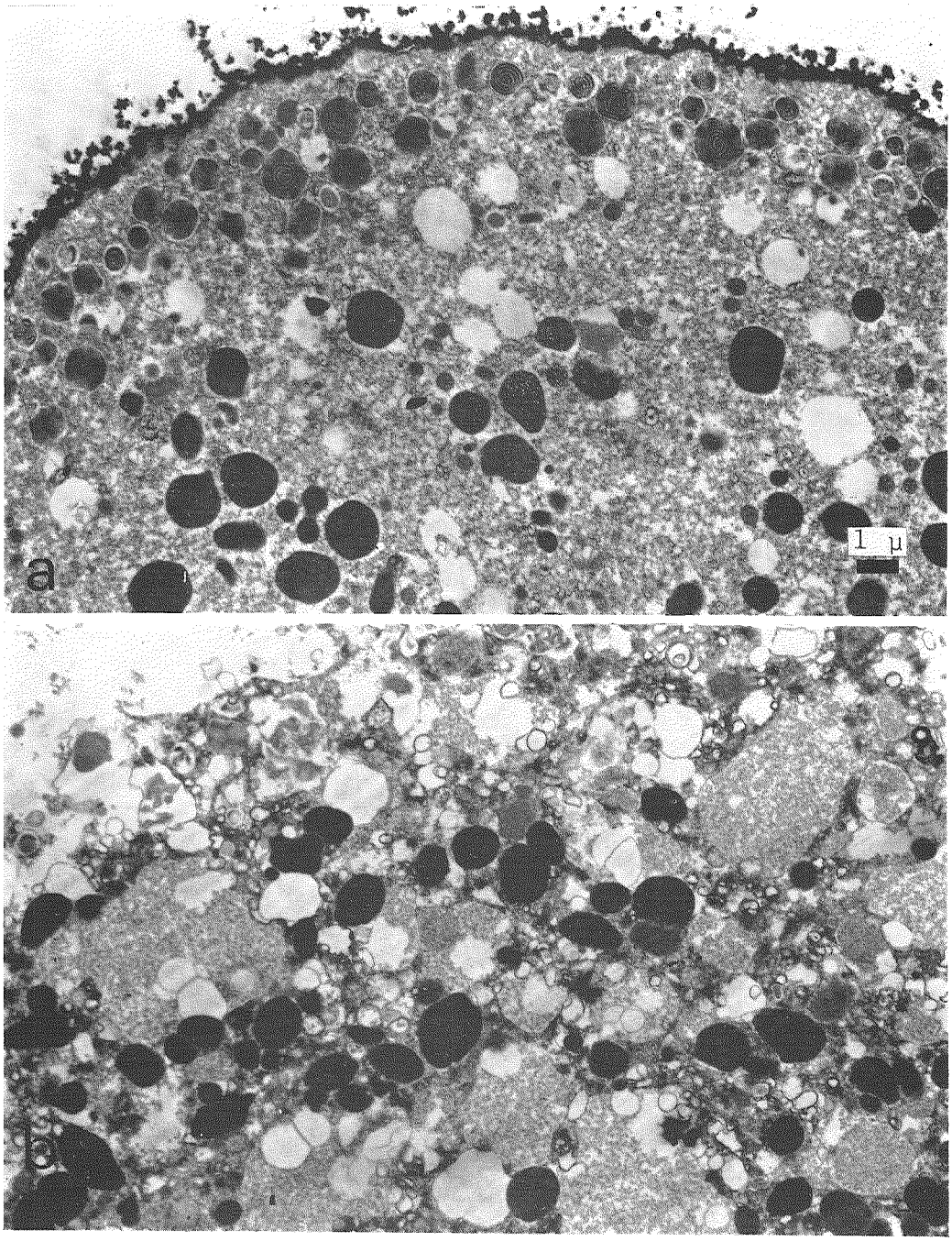

Fig. 13. a–b

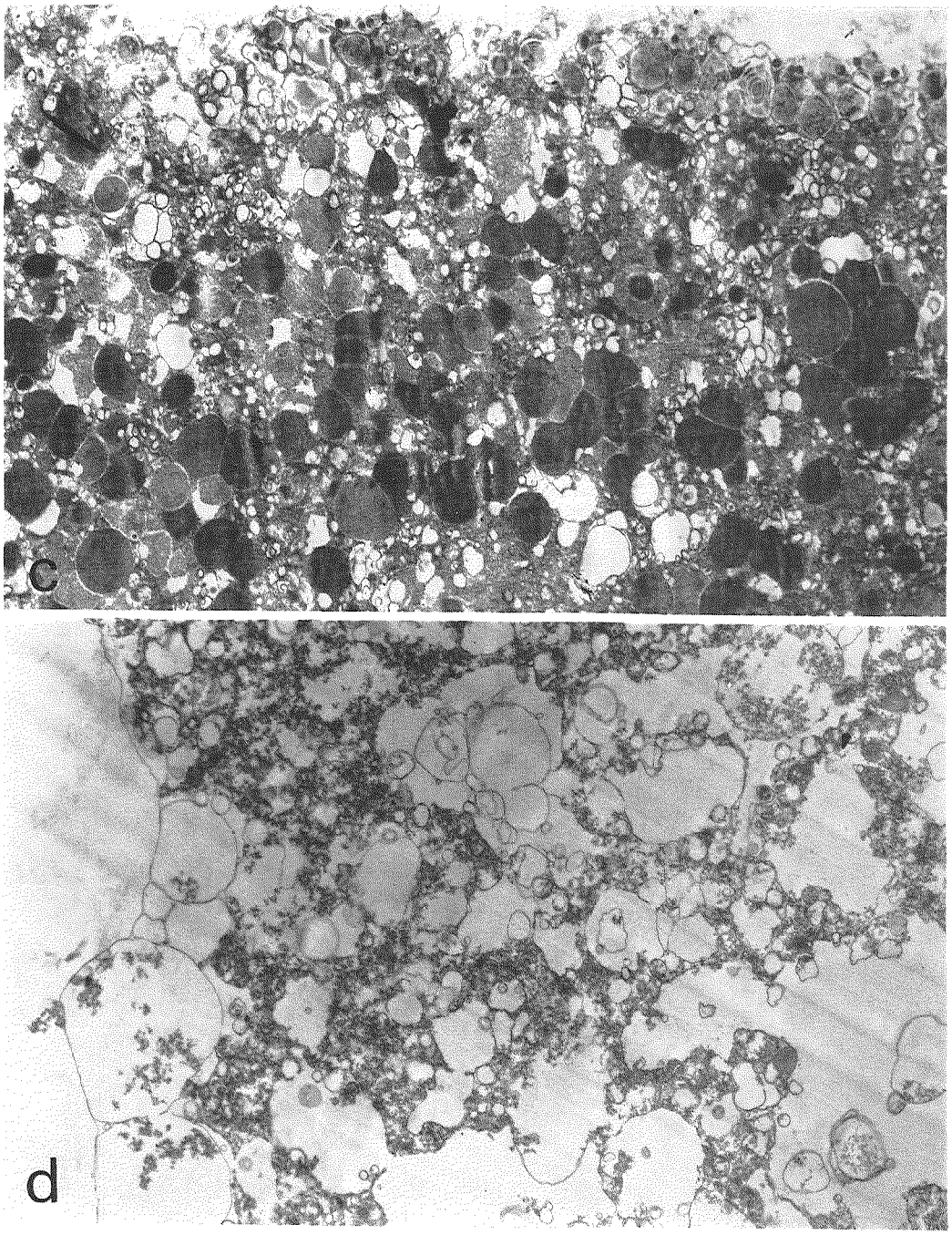

Fig. 13. c–d

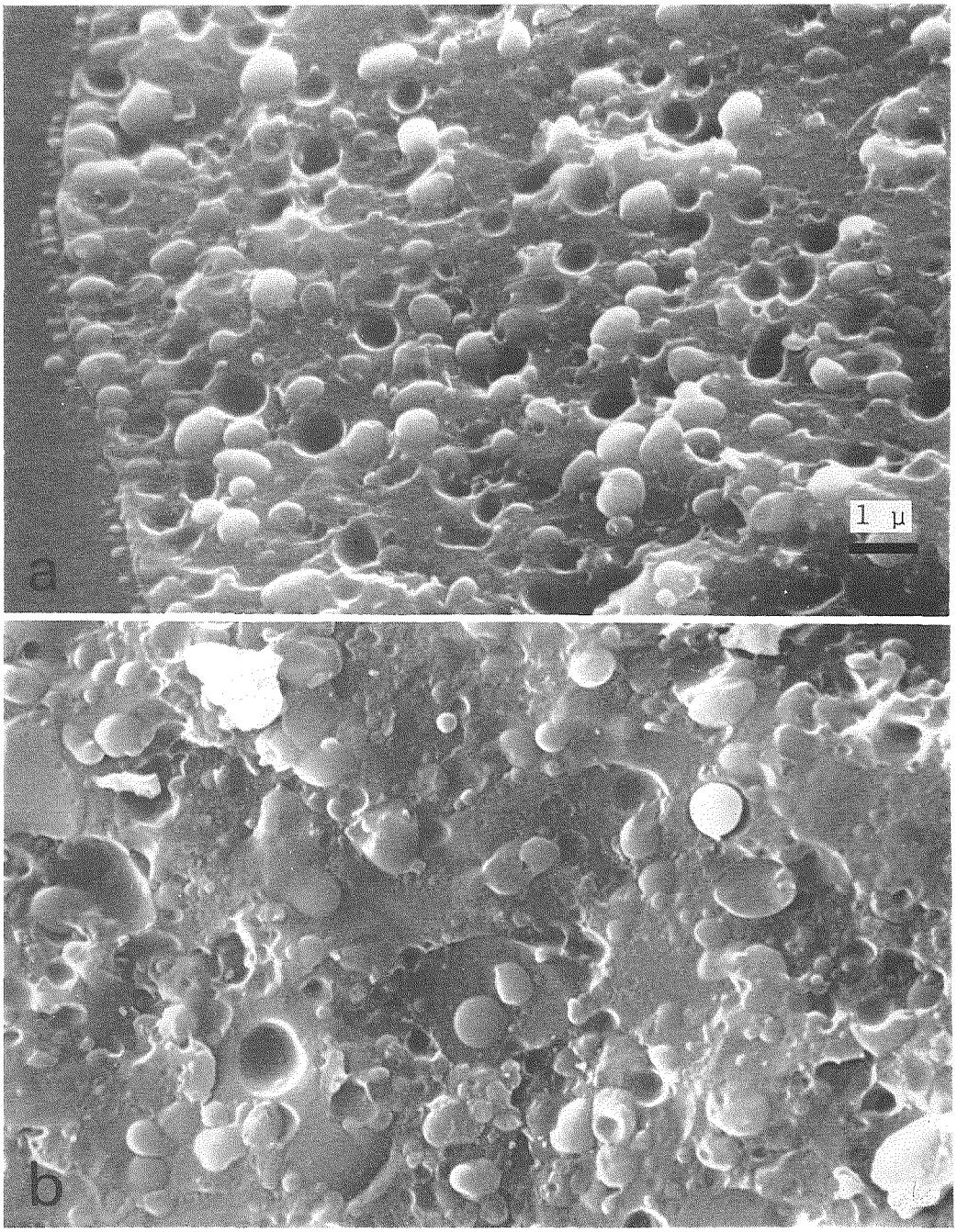

Fig. 14. a-b

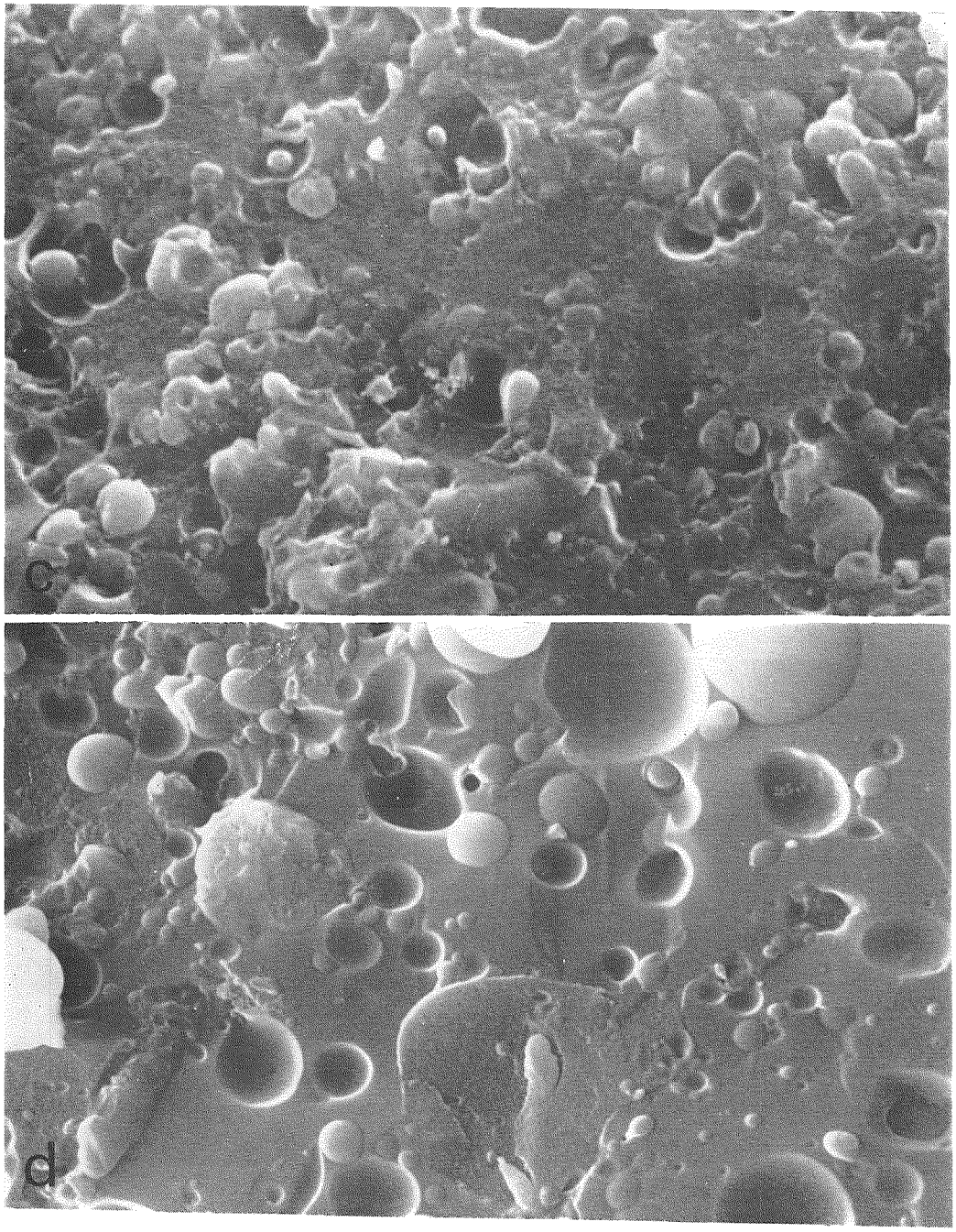

Fig. 14. c–d

chloride solution. In the case of the eggs it was expected that during the exposure to hypertonic salt solutions the loss of lipids from the surface membrane would take place in the eggs which might cause cytolysis at the time of the following rehydration. Therefore, the phospholipids in the surrounding medium were determined after suspending eggs in a medium of 2 M sodium chloride at 0°C for 0.5 and 2 hours, respectively. Eggs remained shrunk to the minimum cell volume in the media. When eggs were transferred to isotonic solution, no cytolysis occurred after exposure for 30 minutes to 2 M sodium chloride solution. But "white" cytolysis occurred in 85% of eggs transferred after exposure for 2 hours to the medium and no "black" cytolysis was seen, while eggs were intact before the transfer. No phospholipid was detected in the surrounding media after suspending eggs for 30 minutes, while 5 ng/egg of phospholipid was detected after suspending eggs for 2 hours.

8) *Increase in tolerance to extracellular freezing in eggs following fertilization*

It was reported by ASAHINA and TANNO[35] that tolerance increased rapidly following fertilization within 5 minutes after insemination in eggs of *S. nudus*. In the present work some experiments were also made concerning the mechanism of the increase in freezing tolerance in fertilized eggs.

In the process of slow cooling at 1°C/min, the critical freezing temperature to cause freezing injury in fertilized eggs of *S. intermedius* was observed to be about −22.5°C, while that of unfertilized ones was −17.5°C (Fig. 15). Viability in unfertilized eggs were suddenly lost between −15° and −17.5°C, while in fertilized ones it gradually decreased in a temperature range around −20°C. The lower critical freezing temperature to cause fatal injury in fertilized eggs as compared with unfertilized ones appears to be a general character in eggs of various kinds of the sea urchin (TAKAHASHI, unpublished).

As already noted by ASAHINA[11] with materials of *S. nudus*, a clear fluctuation in freezing tolerance of eggs following fertilization was observed also with *S. intermedius*. Upon fertilization, a high freezing tolerance was

Fig. 14. Scanning electron micrograph of cytolysed eggs
Material: Unfertilized eggs of *S. intermedius*. × 10,000
a) A normal egg cell
b) A "black" cytolysed egg caused by freeze-thawing at −15°C
c) A "black" cytolysed egg caused by exposure to 2 M sodium chloride
d) A "white" cytolysed egg caused by rapid thawing

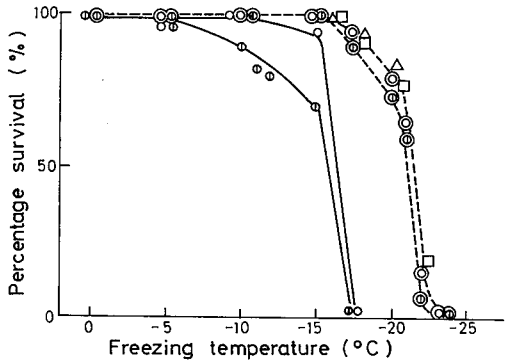

Fig. 15. Survival of frozen-thawed eggs of *S. intermedius*. Immediately after the eggs were cooled at 1°C/min to various predetermined temperatures, they were thawed at 2.5°C/min. Percentage values are averages of duplicate samples. At least 500 eggs were counted in each sample

Unfertilized eggs: ——○—— intact eggs, ——◐—— eggs capable of 1st cleavage
Fertilized eggs: ·····◎····· intact eggs, ·····◍····· eggs capable of 1st cleavage
Fertilized eggs without fertilization membranes and hyaline layers: ·····△····· intact eggs
Urea treated eggs: ·····□····· intact eggs

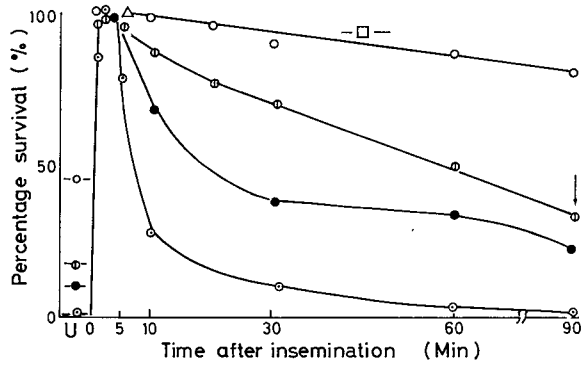

Fig. 16. Change in tolerance to freeze-thawing in eggs of *S. intermedius* during the 1st cleavage. Coolinge rate, 1.0°C/min. Warming rate, 2.5°C/min. Percentage values are averages of duplicate samples. At least 500 eggs were counted in each smaple. An arrow indicates time of cleavage. U in abscissa indicates unfertilized eggs

——○—— Intact eggs after freezing at −15°C for 30 minutes
——◐—— Eggs capable of 1st cleavage after freezing at −15°C for 30 minutes
——●—— Intact eggs after freezing at −15°C for 1 hour
——⊙—— Intact eggs after freezing at −20°C for 1 hour
——□—— Intact eggs after freezing at −15°C for 30 minutes (material, urea treated eggs)
——△—— Intact eggs after freezing at −15°C for 30 minutes (material, fertilized eggs eliminated fertilization membranes and hyaline layers)

instantly produced, reached a maximum within about 5 minutes after insemination and then gradually decreased as the development of the fertilized egg proceeded. The fertilized eggs still retained a higher tolerance than unfertilized ones during the first cleavage. It became increasingly apparent that the highest tolerance was exhibited within 5 minutes after insemination as the freezing period was lengthened and the freezing temperature was lowered (Fig. 16).

One of the remarkable morphological changes in the eggs of the sea urchin immediately following fertilization is the formation of the fertilization membrane and hyaline layer. To verify the possibility that fertilization membrane and/or hyaline layer might participate in the very rapid acquisition of high freezing tolerance of fertilized eggs, these were eliminated from the fertilized eggs, then the denuded fertilized eggs were frozen and thawed. Figures 15 and 16 show that these newly formed components of fertilized eggs did not play any role in exhibition of the high freezing tolerance.

As already shown in Table 1, unfertilized eggs were very susceptible to rapid thawing after extracellular freezing. By using the fertilized eggs the effect of the warming rate on the thawing cells from extracellular freezing was examined by the procedure indicated in Table 4. As a result of this experiment, a remarkable improvement of cellular tolerance to rapid thawing following fertilization was demonstrated.

Table 4. Effect of warming rate on the survival of fertilized eggs after extracellular freezing

Warming procedure	Approximate warming rate (°C/min)	Intact egg (%)*	Cleaved egg (%)*
a) Stand test tube in warm air (22°C)	2.5	100	90
b) Shake test tube in ice-water bath (0°C)	10.0	100	51
c) Shake test tube in running water (12°C)	60.0	100	27
d) Shake test tube in warm water (45°C)	160.0	100	18

Material: Fertilized eggs (5 minutes after insemination) of *S. intermedius*

Test tubes, containing 0.5 ml of frozen mass of egg suspension in sea water were cooled to −15°C (cooling rate, 1.0°C/min), then they were treated by the indicated procedures

* Percentage occurrence, the average of five measurements

9) *Change in cell shape of fertilized eggs in weak hypertonic sodium chloride solutions*

It was reported by ASAHINA[23] that fertilized eggs showed a remarkable

shrinking during extracellular freezing. This was also the case in the present work (*see* Result 1). From an assumption that the mechanism of freezing injury may be involved in cellular dehydration and contraction resulting from extracellular ice formation, changes in appearance of eggs immersed in a weak hypertonic solution were observed after 0, 2, 10, 30, 60 and 90 minutes respectively from the time of insemination. In a weak hypertonic solution (about 1.25 M), prepared by adding a 2 M sodium chloride solution to the same volume of normal sea water, eggs underwent various modes of cell contraction, but did not cytolyse at least for 10 minutes. Unfertilized eggs shrank uniformly with a wrinkled surface retaining their spherical shape. Eggs at 2 minutes after insemination contracted in a spherical shape with a smooth surface. In a period from 10 to 40 minutes after insemination eggs remarkably shrank showing an irregular rugged surface, while in the period from the diaster stage to the first cleavage, they shrank showing a smooth surface. Immediately after the first cleavage, divided cells again underwent a remarkable contraction showing a rugged surface (Fig. 17).

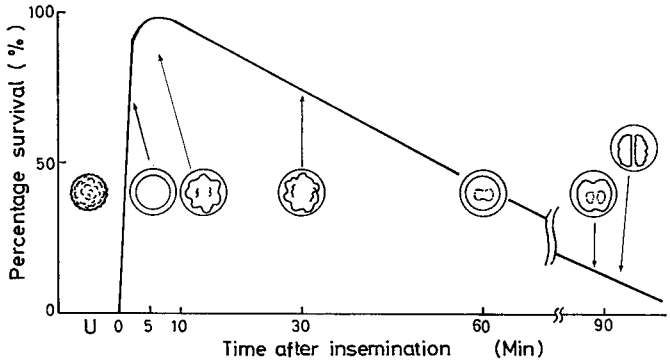

Fig. 17. A schematic presentation of change in appearance of eggs when immersed in hypertonic salt solution (about 1.25 M) during the 1st cleavage, with the freezing tolerance curve indicated in Fig. 16
U: Appearance of an unfertilized egg

10) *Freezing tolerance of eggs activated artificially*

When eggs were suspended in a 1 M urea solution for a few minutes and then transferred to normal sea water, fertilization membranes were formed without insemination. The urea treated eggs showed a freezing tolerance as high as fertilized eggs. The urea treated eggs showed a critical freezing temperature of about −22.5°C, and could tolerate long freezing periods (Figs. 15, 16 and Table 5).

A very rapid and complete breakdown of the cortical granules in the cortex of eggs is one of the remarkable changes at the time of fertilization. Prior to the insemination a treatment of eggs with a procaine suspension in sea water was very effective in preventing the breakdown of the cortical granules. To examine the relation of the breakdown of the cortical granules to the rapid increase of freezing tolerance in fertilized eggs, procaine treated eggs with or without subsequent insemination were frozen and thawed. It was noted that the procaine treated and thereafter fertilized eggs somewhat increased their freezing tolerance. The critical freezing temperature was about −21°C, but they could not tolerate freezing longer than 30 minutes at −17.5°C (Table 5).

Table 5. Freezing tolerance of procaine treated eggs

Final freezing temperature / Freezing period	Intact egg (%)*						
	−15°C	−17.5°C	−20°C	−22°C	−17.5°C		
		0.5 min			30 min	60 min	90 min
a) Unfertilized egg cell	100	9	0	0	0	0	0
b) Fertilized egg cell	100	100	56	25	85	72	29
c) Procaine treatment+sperm (89% cleavage)**	100	82	24	0	0	0	0
d) Procaine treatment*** only	100	9	0	0	0	0	0
e) Urea treatment	100	85	61	20	74	72	20

Material: Eggs of *S. intermedius*

Eggs were extracellularly frozen at 1.0°C/min and thawed from various temperatures indicated

* Percentage occurrence, the average of five measurements

** Eggs treated by procaine and then inseminated cleaved after 90 min of sperm addition. Percentage of cleaved eggs was 89%

*** Eggs were treated by procaine, but not inseminated

11) *Tolerance of fertilized eggs to hypertonic solutions of sodium chloride and glycerol*

It was difficult to observe the volume change of fertilized egg in hypertonic sodium chloride solution because of their irregularly contracted form in this solution. The percentage occurrence of "black" cytolysis in fertilized eggs with an increasing medium concentration of sodium chloride was determined (Fig. 18). At 18°C, fertilized eggs cytolysed in less concentrated sodium chloride solutions than in those to cause cytolysis of unfertilized ones. The mean value of medium concentration at which 50% cytolysis took place in fertilized eggs was 1.5 M, while that in unfertilized eggs was 2.0 M. But

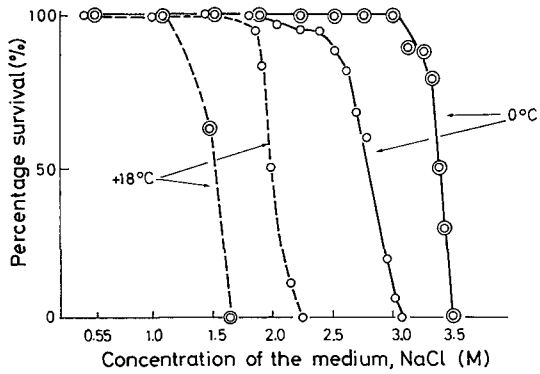

Fig. 18. Survival of fertilized and unfertilized eggs in increasing concentration of sodium chloride solution. Percentage values are averages of duplicate samples. At least 500 eggs were counted in each sample

Material: Fertilized (5 minutes after insemination) and unfertilized eggs of *H. pulcherrimus*

○ unfertilized eggs
◎ fertilized eggs

Table 6. Percentage occurrence of cytolysis in eggs in hypertonic glycerol solution

Temperature	Egg	Concentration of glycerol (M)										
		1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0	6.0	7.0
18°C	Unfertilized	0	0	0	0	100	100	100	—	100	—	—
	Fertilized	0	0	0	100	100	—	100	—	100	—	—
0°C	Unfertilized	0	0	0	0	0	0	0	0	100	100	100
	Fertilized	0	0	0	0	0	0	0	0	0	0	0

Material: Fertilized (5 minutes after insemination) and unfertilized eggs of *S. intermedius*

Eggs were exposed to isotonic (1.0 M) and hypertonic glycerol solution for 5 minutes at 18° and 0°C

at 0°C, fertilized eggs did not cytolyse even at 3.0 M, namely they were more tolerant to hypertonic sodium chloride solution than unfertilized ones. A similar result was obtained in hypertonic glycerol solutions (Table 6).

12) *Freezing tolerance in blastomeres at the 16-cell stage*

Since the nature of protoplasm is different among blastomeres even in a whole embryo, various freezing tolerance levels among them would be expected. This led to a freezing experiment of dissociated blastomeres at

Table 7. Percentage survival in blastomeres at 16-cell stage after extracellular freezing at −15°C for 9 hours

	Experiment No.				Average
	1	2	3	4	
Unfertilized egg	0	0	0	0	0
Macromere	0	0	0	0	0
Mesomere	35.1	47.1	30.4	25.9	34.6
Micromere	100.0	100.0	100.0	100.0	100.0

Material: Isolated blastomeres from embryos at 16-cell stage of *S. intermedius*
Cooling rate: 1.0°C/min
Warming rate: 2.5°C/min
Percentage survival is based on the number of cells not stained in a 0.025% erythrosine solution within 5 minutes and given in average of ten measurements in each lot

the 16-cell stage of the embryo. Tolerance to extracellular freezing of blastomeres at −15°C was indicated in Table 7. There were apparent differences in the freezing tolerance among the three classes of blastomeres. The highest tolerance to extracellular freezing was exhibited in micromeres, while the lowest was observed in macromeres.

IV. Discussion

When sea urchin eggs were injured by extracellular freezing, two types of cytolysis, "black" and "white", were observed to occur following or during thawing[11]. The factors to cause these two types of cytolysis were investigated by various approaches. As a result of the present study, it may be safely said that freezing injury increases in extracellularly frozen eggs as the freezing temperature is lowered, the freezing period is prolonged, and the thawing rate is increased. As already pointed out by LOVELOCK[5], the cause of freezing injury may be due to the concentration of the surrounding media during freezing process and the dilution of that during thawing process. With progressive freezing, the concentration of sodium chloride increases most remarkably among the electrolytes in sea water[32]. The change in concentration of salts, mainly of sodium chloride, in the surrounding medium may be one of the most important factors to cause freezing injury of the sea urchin eggs. The results in Fig. 8 clearly show that the pattern of occurrence of cytolysis of eggs in sodium chloride solutions is quite similar to that with freeze-thawing (Figs. 3 and 4). Eggs which could tolerate a low freezing temperature could also tolerate a high concentration of sodium chlo-

ride. For example, eggs of *S. intermedius* and *H. pulcherrimus* had the same critical freezing temperature and cytolysed at the same concentration of sodium chloride within a short period. The most freezing tolerant eggs among the three species of sea urchin employed in the present study were those of *S. nudus*, which were most tolerant to hypertonic sodium chloride solution. Egg volume decreased gradually to a minimum as the medium concentration of sodium chloride increased. The percentages of the minimal egg volume to the original ones in the three species of eggs were as follows: 36.4%, *S. intermedius*; 38.7%, *H. pulcherrimus*; 54.0%, *S. nudus* (calculated from the data indicated in Figs. 9 and 10). Through a further increase in concentration of the medium, the volume in these eggs still remained nearly constant for a while, but increased suddenly with a simultaneous occurrence of "black" cytolysis when the medium concentration exceeded a critical value (Figs. 9 and 10). A possible interpretation of these results may be that a further dehydration beyond the minimal cell volume in eggs causes a destruction of cell membrane followed by a flow of the outside medium into the contracted eggs. Since the osmolarity to reduce their cell volume minimum were the same (1.5 M sodium chloride) among the three species of eggs, an important factor to tolerate a low freezing temperature as well as a high medium concentration might be the resistance to further reduction in cell volume. During freezing the concentration of the surrounding salt solution may increase to above 3.0 M at -15°C, however, eggs were not injured. This may suggest that a higher tolerance to a high medium concentration can be achieved a lower temperature. In fact, cellular tolerance to a high medium concentration increased at 0°C in all of the species employed (Figs. 9 and 10). Reduction in egg volume to a minimum one and rapid cytolysis in certain concentrations of hypertonic solution were also observed in non-electrolyte solutions of glycerol and sorbitol (Fig. 10; Tables 3 and 6). It seems, therefore, likely that the cytolysis in contracted eggs at least within a short period of immersion in hypertonic solutions may be caused by osmotic dehydration rather than the loss or denaturation of cell components by the salt solution. In case of cytolysis in non-electrolyte solutions, however, a possible injury caused by intracellular electrolytes would remain to be solved, even though the effect of extracellular salts may be ruled out.

MERYMAN[36] showed that red blood cells have a minimum critical cell volume, and osmotic dehydration due to the extracellular ice formation is considered as a major cause of freezing injury in erythrocytes. This appears to be quite interesting in relation to the above observations. He presented

the "minimum cell volume" theory for the mechanism of injury by extracellular freezing of red blood cells[10,36]. The development of extracellular ice leads to a concentration of the extracellular solutes which do not normally penetrate the cells. Water leaves the cells and osmotic equilibrium is maintained across the membranes. With continuing cell volume reduction and the compression of cell contents, if the cells cannot shrink freely in response to the concentration gradient, then an osmotic pressure difference must develop across the membranes. When this pressure gradient exceeds the tolerance of the membranes, irreversible changes in membrane permeability results. Additional evidences for a relationship between the degree of dehydration and cell damage were obtained from the study of marine mollusk[37], and the grana from spinach leaf chloroplast[38].

As shown in Fig. 11, however, "black" cytolysis can be caused in eggs even at medium concentrations below 1.5 M sodium chloride at which concentration the minimum cell volume of the eggs was not attained as yet. Similar results were obtained in both cases of freezing and exposure to hypertonic solutions (Figs. 5, 6, 7 and 12). These results indicate that the degree of injury depends on the length of freezing or period of exposure to hypertonic solutions. Under these experimental conditions, therefore, it appears that injury to eggs may be caused by the loss or denaturation of cell components.

The freezing injury hypothesis presented by LOVELOCK[5,7] appears clear and most interesting. With particular reference to red blood cells, he proposed that freezing injury was due to the concentration of the electrolytes. His conclusion was based on the observation that haemolysis in red cells began to be evident whenever the extracellular sodium chloride concentration reached 0.8 M. The concentrated electrolytes dissolve the lipid or denature the lipoprotein in the red cell membranes. In the present experiment, when eggs were exposed to 2 M sodium chloride solution for 30 minutes at 0°C, the egg volume decreased to a minimum, but no phospholipid was released from the eggs. After 2 hours of exposure, however, phospholipids were significantly released from eggs, and the eggs underwent "white" cytolysis following rehydration (Result 7). The amount of the phospholipids released within 2 hours at 0°C, 5 ng/egg, is so large that it is equal to 10% of the total phospholipids in a single egg (calculated from the data of HOSHI and NAGAI[39]). This suggests that the released phospholipid might not have come from the plasma membranes alone. At present it is difficult to determine from where the phospholipid is released, namely the plasma membrane and/or cell interior. The isolation of plasma membranes from eggs have been tried

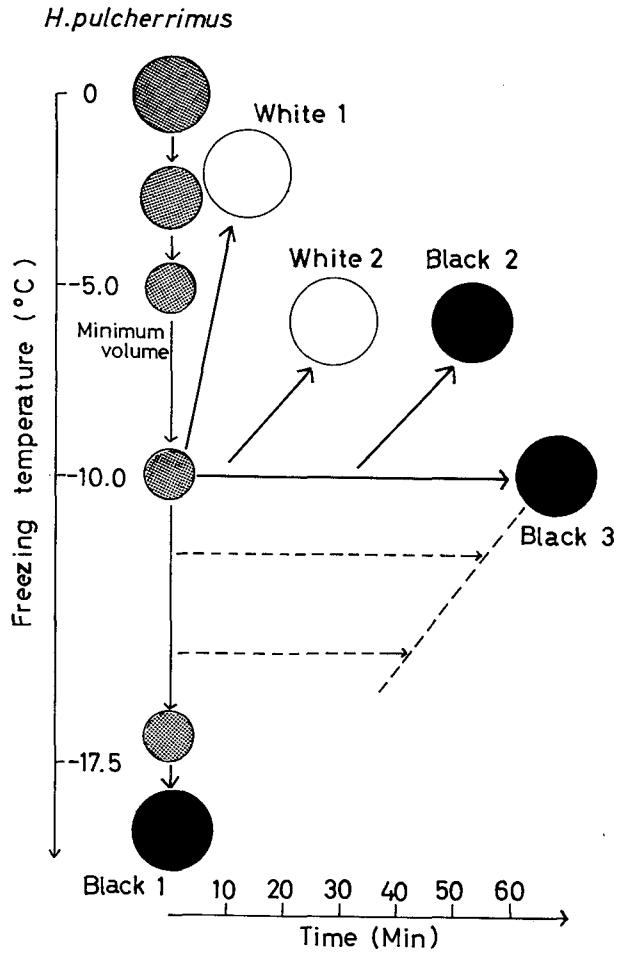

Fig. 19. Schematic presentation of the occurrence of "black" and "white" cytolysis in extracellularly frozen eggs

Arrows downward indicate frrezing treatments. Arrows upwards indicate thawing treatments. The larger the degree of angle of an upward arrow, the larger the warming rate. Arrows sideward indicate the length of freezing period. Broken lines indicate the length of freezing period in which cytolysis (Black 3) occurs at lower freezing temperatures. Freezing temperature and freezing period indicated in this figure are exclusively applicable to the eggs of *H. pulcherrimus*.

An extracellularly frozen egg gradually shrinks with decreasing temperature. During further cooling egg cell volume reaches to a minimum, and then remains constant for a while. Below the critical freezing temperature of −17.5°C the egg is mechanically destroyed since it cannot resist to a further contraction, extracellular solution enters the egg with simultaneous occurrence of "black" cytolysis (Black 1). When an egg is frozen above the critical freezing temperature for a short period and then thawed

by many investigators[40-42], however, the method for complete isolation has not yet been successful. Quantitative analysis of the released phospholipids and their fatty acids is now in progress. From the results mentioned above it seems likely that not only dehydration beyond the minimum cell volume, but also the release of phospholipids from the eggs may be the main cause of freezing injury to the eggs. As shown in Fig. 11, eggs of *H. pulcherrimus* were more susceptible to hypertonic salt solution than those of *S. intermedius*. In the case of actual freeze-thawing above the critical freezing temperature, eggs of the former cytolysed more easily than those of the latter (Figs. 5 and 6). Thus, eggs of *S. intermedius* were found to be more tolerant to a long period of freezing than those of *H. pulcherrimus*. These differences in freezing tolerance between two species of eggs might be due to the difference in resistance to denaturation of egg protoplasm by hypertonic salt solutions. It seems reasonable to assume that highly freezing tolerant eggs are very resistant to denaturation of protoplasm caused by the concentrated surrounding medium.

It has long been known that a rapid thawing from extracellular freezing is highly injurious to some plant cells[2,43], while the same procedure is usually better than slow thawing to obtain good survival in various kinds of animal cells[3]. However, LEIBO *et al.*[44] reported that the survival of extracellularly frozen-thawed mouse embryos was enhanced by slow rather than rapid warming. As shown in Fig. 5 and Table 1, eggs of the sea urchin, too, suffer serious injury by a rapid thawing. The same result was also reported by ASAHINA[11] in eggs of *S. nudus*. It has been demonstrated that fertilized eggs, which are more permeable than unfertilized ones[45], well tolerated a fairly rapid thawing (Table 4). This suggests that the injury by rapid thawing may possibly be a mechanical damage to the structure of surface cell membrane caused by a great osmotic stress at the egg surface. Such a

rapidly, "white" cytolysis occurs (White 1). When an egg is frozen for a little longer period above the critical freezing temperature, it may be injured by the concentrated salt solution. Cell membrane becomes so susceptible that the egg lyses easily even with slow thawing (White 2). During a longer freezing period, protoplasm in the contracted egg is increasingly injured by hypertonic salt solutions, both extra- and intracellular, although the egg appears still intact during freezing. Such a potential injury becomes evident when the egg is exposed to an isotonic or hypotonic solution at the time of thawing and apparent coagulation of a whole egg results (Black 2). After a still longer period of extracellular freezing, salt injury in protoplasm of the egg may proceed to a critical extent so that the surface membrane fails to prevent an inflow of concentrated solution outside, a remarkable coagulation of cytoplasm results without thawing (Black 3). The lower the freezing temperature, the shorter the period of time required to produce each type of cytolysis.

destruction of surface membrane may cause a very rapid flow of water into the egg at the time of rapid thawing, resulting in "white" cytolysis. A similar appearance of cytolysis was known to occur by various mechanical stress and was designated as an internal surface precipitation reaction of protoplasm[30].

From the results of the present experiments, the following hypothesis for the mechanism of freezing injury of the sea urchin eggs may be proposed (Fig. 19). As water is frozen out of surrounding medium, increasing concentration of extracellular solutes leads to a egg contraction up to a concentration of about 3 × isotonic of egg cell fluid, and a minimum cell volume results. As the egg cannot behave as an osmometer beyond this value in concentration, possibly because of the cellular character of each material, a gradual increase in osmotic stress at the surface membrane results. As freezing progresses further, increasing osmotic stress may mechanically destroy the fine structure of the surface membrane. Thereafter, the extracellular concentrated salt solution flows into the egg to achieve physical equilibrium of the salt concentration. This may cause a coagulation of protoplasm resulting in "black" cytolysis (Black 1). Upon rapid thawing from an extracellular freezing at temperatures slightly above the critical one, eggs would be exposed to a very dilute solution. This may cause a very high osmotic stress at the cell surface leading to a mechanical destruction of the membrane structure. As a result of such destruction of cell membrane an extraordinarily rapid flow of water may occur into the egg. Under such conditions eggs may undergo "white" cytolysis (White 1). Even in a salt solution below the critical concentration to cause immediate "black" cytolysis, electrolytes may also injure the eggs if eggs are kept longer in a concentrated solution produced by freezing. The lipid and/or lipoprotein in protoplasm of the freezing eggs may be attacked by the salt solution, both extra- and intracellular. Such salt injury would progress with prolonged freezing period, and finally destruction in both surface membrane and inside protoplasm of the eggs occurs resulting in "black" cytolysis (Black 3). During the early process of extracellular freezing, eggs may be injured to some extent without any change in appearance before thawing. In these eggs the potential damage in the membrane may become apparent in the process of flowing of water into the eggs during thawing. This may produce swollen destroyed eggs of the type of "white" cytolysis (White 2). In eggs which have been frozen for a longer period, the maintenance of fine structure of both surface membrane and inside protoplasm may become increasingly difficult by the lyotropic action of concentrated salt solution, although the eggs still retain a normal

appearance during freezing. When they are thawed, however, such potential injuries become apparent at the time of rehydration resulting in "black" cytolysis (Black 2). In some eggs frozen for a longer period, cytoplasm may coagulated during freezing to a certain extent. When thawed, this results in an incomplete swelling of the eggs with an appearance of partial "black" cytolysis. In potentially injured eggs in a concentrated salt solution, whether their cytolysis at the time of thawing results in "black" or "white" appears to depend on the degree of coagulation which increasingly occurs in inside protoplasm of the eggs. The facts that the longer the period of immersion of the eggs in a hypertonic solution, the higher the ratio in occurrence of "black" cytolysis to "white", and that after a certain period of immersion eggs invariably undergo "black" cytolysis with or without rehydration may support the present assumption. It also appears that an inflow of outside medium to the injured eggs is prerequisite to produce a typical "black" cytolysis which means an increase of cell volume to some extent and remarkable darkening of coagulated protoplasm in appearance.

Among the numerous hypotheses for the mechanism of freezing injury, the SH-SS theory proposed by LEVITT[7] appears unique, since it may present an explanation on molecular basis. He proposed that the removal of cell water at the time of extracellular freezing could reduce the distance between proteins or portions of a protein in the protoplasmic structure, and that, as these proteins approach in distance to each other, abnormal disulfide are formed during extracellular freezing which would produce new stresses in protoplasm at the time of swelling of contracted cells. The newly formed SS bonds would now pull the protein folds, leading to unfolding and fatal denaturation. It was also reported that soybean protein was denatured by freezing, and that the denaturation of the protein was due to SS bond formation[46]. In favor of LEVITT's hypothesis. ASAHINA and TANNO[35] assumed that the increase of SH groups at least in some components of cytoplasm may be a possible factor to increase the cellular freezing tolerance. To examine the SH-SS hypothesis. TAKAHASHI and ASAHINA[49] determined the amount of protein SH groups in both "black" and "white" cytolysed eggs and unfrozen ones, but no significant change was observed among them. The determination of the amount of SH groups in the isolated cortical layer was also conducted from the idea that the most susceptible portion of an egg to freezing injury might be the cortical layer with plasma membrane. However, in such a plasma membrane rich fraction, no significant change was observed. Quantitative studies on SH groups in sea urchin eggs also revealed that immediately after fertilization the fluctuation in protein SH was very

small[47,48]. The SH-SS theory, therefore, could not be applied to the freezing injury of the eggs of the sea urchin. More detailed discussion on this problem was made elsewhere[49].

In the eggs of the sea urchin, a remarkable increase in freezing tolerance occurs immediately after fertilization[35]. Fertilized eggs showed a lower critical freezing temperature to cause "black" cytolysis than unfertilized ones (Fig. 15). However, newly formed fertilization membrane and hyaline layer at the time of fertilization could not participate in the exhibition of a high freezing tolerance in fertilized eggs (Figs. 15 and 16). What then is the main factor to increase freezing tolerance in fertilized eggs?

Upon fertilization, a new plasma membrane is formed at the cell surface which is produced by fusing the surface plasma membrane of an unfertilized egg and membranes of cortical granules together[50]. Many chemical and physical changes begin to occur promptly in cytoplasm simultaneously with fertilization[51-53]. Therefore, various properties in fertilized eggs may well differ from those in unfertilized ones. Urea treated eggs, in which cortical granules completely ruptured and new plasma membranes were produced, showed a critical freezing temperature as low as that of fertilized eggs (Fig. 15 and Table 5). In addition they, too, are tolerant to a long freezing period (Fig. 16 and Table 5). On the other hand, when eggs were treated with procaine and then inseminated, they could begin development with intact cortical granules. Such eggs showed nearly the same critical freezing temperature as that of fertilized eggs, but completely could not tolerate long freezing period (Table 5). From these observations, it appears that the important factors to increase freezing tolerance in eggs after fertilization may firstly be the formation of the new plasma membrane, and then the subsequent changes occurred in cytoplasm.

RUNNSTRÖM[54] observed a remarkable deformation of fertilized eggs of the sea urchin when they were immersed in a weak hypertonic solution. He considered that the periodical change of cell form in a hypertonic solution might show the change of the viscosity of the cortices. Many investigators[54-57] also reported that protoplasmic viscosity and viscoelasticity fluctuate from the time of fertilization to the onset of cleavage. Cryomicroscopic observations in the present study revealed that the shape of unfertilized eggs was not remarkably changed, but was nearly spherical during freezing, while that of fertilized eggs changed into a very irregular form. The difference in the degree of cell deformation between fertilized and unfertilized eggs was also observed in hypertonic sodium chloride solutions. As shown in Fig. 17, fertilized eggs in a weak hypertonic solution showed a series of

remarkable deformation according to the stage of development to the first cleavage. This may suggest that the state of cortices may be involved in the mechanism of increasing freezing tolerance.

In connection with the above facts, the report by ÖHMAN[29] is very interesting. He demonstrated that heat tolerance of eggs increased rapidly within 5 minutes after fertilization, and then decreased gradually to the level of unfertilized eggs at the time of cleavage. A passing increase of the tolerance again occurred before the first cleavage. These phenomena are very similar to the pattern of appearance of freezing tolerance, except for the later increase in heat tolerance (Fig. 16). ÖHMAN[58] also reported that the tolerance of eggs to crude lysolecithin and bee venom (lecithinase) increased rapidly within 5 minutes after insemination. The present author also found that fertilized eggs were quite tolerant to pure lysolecithin (TAKAHASHI, unpublished data). BARBER and MEAD[59] reported that lipid composition of sea urchin egg ghosts changed before and after fertilization. BARBER[60] also demonstrated that fertilized eggs were less sensitive to a treatment with mycostatin, a sterol binding drug. These results suggest that there may be a great difference in the chemical components of plasma membranes between fertilized and unfertilized eggs.

As fully demonstrated in the present study eggs which tolerated low freezing temperatures also tolerated strong dehydration in hypertonic solutions. Fertilized eggs, however, could not tolerate an immersion in hypertonic solutions at an ordinary temperature of 18°C which was tolerated by unfertilized ones, in spite of the distinctly higher freezing tolerance in the former than in the latter (Figs. 15 and 18). The fact that fertilized eggs are more susceptible to a hypertonic salt solution than unfertilized ones was already reported by HOBSON[27]. The above mentioned complicated tolerance in the eggs to hypertonic solutions at an ordinary temperature and to freezing may be understood by an observation obtained in the present work in which the grade of tolerance to dehydration in unfertilized and fertilized eggs was reversed at a low temperature (Fig. 18).

ASAHINA[61] reported that half cells produced from unfertilized eggs by centrifugation showed different freezing tolerance between heavy and light halves. The light halves contain oil drops, nuclei, clear cytoplasm including mitochondria and the fine pigment granules, and a little yolk, while the heavy halves contain most of the yolk and clear cytoplasm. Freezing tolerance in the light halves was nearly the same as that in unbroken whole eggs and was always distinctly higher than that in the heavy halves. From these results he proposed that susceptibility to extracellular freezing may

vary in the different cellular components and that the surface cytoplasmic layer may not be the most susceptible part in a single cell. However, an alternative interpretation may be possible that the difference in freezing tolerance in both halves might be due to the difference in the tolerance to cell shrinkage during freezing. Such tolerance to cell shrinkage may also mean an easy innocuous shrinkage of surface membrane. During extracellular freezing, if the packed components in any cell half prevent the membrane to shrink smoothly, a dangerous stress would occur at the membrane as dehydration proceeds resulting in a fatal injury. A possible interpretation of ASAHINA's results may be that the smooth contraction of the membrane during freezing in light halves is easier than in heavy halves, since the latter contains a much larger amount of yolk granules than the former.

Each class of blastomere isolated at the 16-cell stage, showed an apparent difference in freezing tolerance (Table 7). Similar results were obtained when the entire embryos at the 16-cell stage were frozen at $-15°C$ for 30 minutes and thawed. The macromeres and mesomeres were cytolysed completely, but the micromeres could survive and even cleave further (TAKAHASHI unpublished data). The results suggest that the response to freezing and thawing of the whole embryo, at least in a very early stage, also reflect that of the component cells. Experiments designed to observe the effects of hypertonic solutions on each classes of blastomeres is now in progress. The variation in cellular tolerance according to the nature of cell protoplasm in these blastomeres, may present useful clue to solve the freezing tolerance mechanism in sea urchin eggs.

V. Summary

When eggs of the sea urchin were injured by extracellular freezing and thawing, two types of cytolysis, "white" and "black" cytolysis, were observed. "Black" cytolysis took place in the process of both freezing and thawing, while "white" cytolysis occurred only during thawing. Eggs of each species of sea urchin had a respective critical freezing temperature at which temperature almost all eggs "black" cytolysed. Below the critical temperature freezing eggs invariably underwent "black" cytolysis without thawing. Above the critical temperature, if freezing eggs were thawed rapidly, they underwent "white" cytolysis. In a freeze-thaw procedure, if the rate of warming was increased, the number of "white" cytolysed eggs increased. Above the critical temperature, the number of "black" cytolysed eggs increased during freezing with a prolonged freezing period. In the early period of extracellular freezing above the critical temperature "white" cytolysis was apt to occur even

if eggs were thawed slowly. As the freezing period was lengthened, "white" cytolysis was gradually replaced by "black" cytolysis following thawing.

When eggs were immersed in a series of hypertonic media cell volume decreased to a minimum as the medium concentration increased, and then remained constant for some ranges of increasing concentrations. When the medium concentration exceeded a critical value, cell volume suddenly increased with simultaneous occurrence of "black" cytolysis. At low temperatures, cellular tolerance to further increase in medium concentration increased. Eggs of each species of sea urchin showed a respective critical concentration of salt solution to cause cytolysis. Those which were tolerant to lower freezing temperatures could also tolerate more concentrated hypertonic sodium chloride solutions. Below the critical concentration to cause cytolysis, the number of "black" cytolysed eggs increased as the period of exposure to salt solutions was lengthened. "Black" cytolysis also occurred in the process of transferring the eggs to isotonic solution from hypertonic ones. When the period of exposure to hypertonic salt solutions was short, "white" cytolysis only appeared in the process of transferring the eggs to isotonic solution. During the 2 hours of immersion in sodium chloride solution at 0°C, phospholipid was significantly released from the eggs, in spite of the intact appearance of the eggs in the same medium.

From these results, it may be said that at least the following factors may be involved in the cytolysis of eggs resulting from an extracellular freeze-thawing; namely 1) irreversible change of the plasma membrane caused by intense dehydration, 2) lyotropic damage on protoplasm as a whole by concentrated salt solution, and 3) mechanical damage of surface membrane caused by a very high osmotic stress at the cell surface during rapid thawing.

After fertilization, an immediate increase of freezing tolerance in eggs was observed. Fertilized eggs showed a lower critical freezing temperature, and they also tolerated longer freezing periods than unfertilized ones. Parthenogenetically activated eggs, too, showed a high freezing tolerance. These results suggest that both a formation of new plasma membrane and cytoplasmic change in eggs following fertilization might participate in the remarkable increase in freezing tolerance.

Each cell class of blastomeres at 16-cell stage of sea urchin embryo showed different freezing tolerance to extracellular freezing. Micromeres were most tolerant and macromeres were most susceptible.

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