Frost Resistance in Algae Cells*

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Abstract

When algae cells are subjected to freezing, they invariably undergo extracellular freezing provided they are slowly cooled. These algae cells can tolerate freezing for a long time. The frost resistant cells of green algae were found to have only a very small amount of sugars, instead, they have a considerable amount of potassium salt. They exhibit no seasonal change in frost resistance.

Algae cells were subjected to freezing in a medium consisting of various kinds of solute, such as inorganic salts, sugars, polyhydric alcohols etc. The result seems to suggest that frost injury in algae cells becomes fatal when the dehydration and contraction of protoplasm in the freezing cell reaches a critical point. Protective agents against cellular dehydration and contraction can increase frost resistance at least in fresh water green algae. Cells of marine algae, having a high resistance to plasmolysis were also highly resistant to freezing.

Introduction

Frost resistance in plant cells has long been the objective of investigations and various theories for the mechanism of resistance have been presented by many botanists (Levitt, 1956). With algae cells, however, research on frost resistance has been rather limited up to the present and the mechanism of resistance still remains uncertain.

The frost resistance in algae cells is very particular in nature. Frost hardening by temperature treatment which is well known way to produce frost hardiness in various phanerogamic plants in temperate regions cannot be seen. Algae cells are observed to exhibit none or little seasonal changes in frost resistance. Despite their remarkably high frost resistance some of the green algae are found to accumulate no cellular substaces known to be protective agents.

The present writer has been working for some years on the frost resistance mechanism mainly with various algae cells both of fresh water and marine. Some remarkable results observed on the nature of freezing algae cells is reported in the present paper, and the mechanism of frost resistance is discussed based on the behavior of these cells frozen with various kinds of environmental mediums. Considerations on the relation between frost resistance and osmotic resistance in algae cells will also be briefly referred to.

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I. Materials and Methods

As fresh water alga, lake ball, *Aegagropila sauteri* (Nees) Kütz. (Cladophoraceae) was employed. The vegetative cells of the following marine algae collected from the winter intertidal zone were also used as experimental materials.

green alga	*Ulothrix flacca*	
	Ulva pertusa	
	Enteromorpha intestinalis	
	E. linza	
	Monostroma angicava	
red alga	*Bangia fusco-purpurea*	
	Porphyra pseudolinearis	(♀)
	P. pseudolinearis	(♂)
	P. yezoensis	
	P. onoi	

Lake balls were kept in water at room temperature and marine algae were cultivated in sea water at 0°C until they were used for experiments.

Small masses of filamentous cells of a lake ball or fragments (2×2 cm) of tissue of marine algae with a small amount (2 cc) of water or sea water were placed in small dishes. These dishes were cooled in the cold room at various subzero temperatures to freeze the algae cells. Frozen algae in the dishes were removed and thawed at room temperature. The mortality of the cells was determined by means of vital staining, plasmolysis and the following deplasmolysis. A solution of balanced salts was used as the plasmolyticum and neutral red solution was employed for vital staining.

The freezing processes of cells were observed under a microscope in a special cold box, the lowest attainable temperature was −30°C. Cold fixation was conducted as follows (Terumoto, 1958 b). The frozen tissue pieces were fixed in chilled mixed solution (abs. alcohol–acetic acid mixture 19 : 1, v/v) at subzero temperatures. Fixation lasted for 4 hours and was followed by dehydration overnight in abs. alcohol at room temperature. Soluble elements such as potassium ion and sodium ion in cells were quantitatively detected by means of flame photometry. In case of lake balls, the filamentous cells were previously washed several times with deionized water and blotted on filter paper and then weighed. In case of sea-lettuce, *Ulva pertusa*, fresh thalli were washed with 1 M sucrose solution and blotted on filter paper. Both of them were desiccated rapidly at 105°C for 6 hours, and then extracted in deionized water (Scott and Haywood, 1955). Chlorine was determined by the conventional $AgNO_3$ titration.

II. Nature of the Cells of Some Green Algae

Lake ball. Filaments of lake ball are composed of a series of cylindrical cells with a diameter of 40–80 μ (Fig. 1 A). The length of the each cell is 6–9 times longer than the diameter. The cell wall is about 3–5 μ in thickness. These cells have many chloroplasts arranged in a dense net like mesh, and contain numerous nuclei and pyrenoids (Fig. 1 B). Cells immersed in 1 M balanced salt solution showed a concave type of plas-

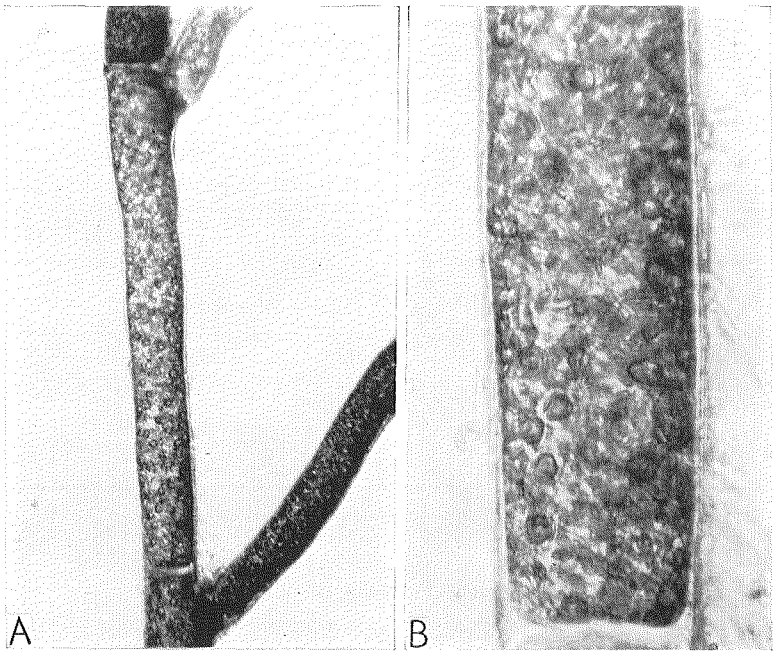

Fig. 1. Filamentous cells of a fresh water alga, lake ball, *Aegagropila sauteri*
A. ×127 B. ×500

molysis at room temperature (Fig. 2). The critical concentration of plasmolysis of cells was 0.85 M (NaCl isotonic). This value is very high as compared with other fresh water algae which have values of about 5 atm. (0.1 M NaCl isotonic) (Guillard, 1962). An accumulation of compounds in the cell by temperature treatment has been known to remarkably enhance cellular osmotic value in various phanerogamic plants. Osmotic concentration of this alga was, however, not affected by the change of environmental temperature (Terumoto, 1959). This suggests that the high osmotic value in this alga is not due to an accumulation of the nutritional materials in the cell. The soluble elements in cells of lake ball are shown in Table 1. Inorganic ions contribute to a greater part of osmotic concentration; potassium ion is very abundant, while sodium ion is rather scarce, the sugar content, likewise, is very small.

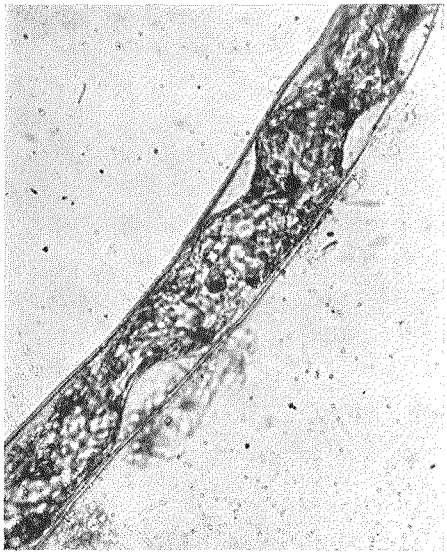

Fig. 2. A filamentous cell of lake ball plasmolysed in 1 M balanced salt solution at room temperature. ×280

Sea-lettuce. Thalli of sea-lettuce, *Ulva pertusa* consist of compact double layers of cells. The cells are long and slender, being about 10–15 μ in width and 40–50 μ in

length. They are arranged perpendicularly to the surface of the thalli and have no intercellular space. The critical concentration of plasmolysis of these cells is 0.55 M (NaCl isotonic). Inorganic soluble elements of sea-lettuce account for a greater part of osmotic concentration in the cells.

Table 1. Inorganic ions in the green algae (M)

Species	Lake ball	Sea-lettuce
Osmotic value* (NaCl isotonic)	0.85	0.55
K	0.349	0.272
Na	0.012	0.187
Cl	0.199	0.341
(Sugar	0.050	—)

* Osmotic values were estimated by means of plasmolysis

III. Freezing Process of Algae Cells

Filamentous cells in lake ball. Filamentous cells were placed with a small amount of water on a cover glass and covered with silicone oil. Materials prepared in this way were observed under a microscope generally at temperatures around −15°C. At a cooling rate of 3°C per minute, ice forms only outside the cells and fatal intracellular freezing is prevented. Under extracellular freezing conditions, the ice gradually increased in amount as the cooling proceeded withdrawing water from the cells. The appearance of the cells seemed nearly the same as that of unfrozen ones, although the cells slightly decreased in volume (Fig. 3). After thawing, cells retained their original cell structure. The cell wall (cellulose membrane) of this alga seemed to have some ability to prevent the ice seeding into cell from the outside, since the cells previously killed by intracellular freezing, in which the cellular protoplasmic membrane was entirely destroyed, could undergo extracellular freezing provided that the freezing was slow.

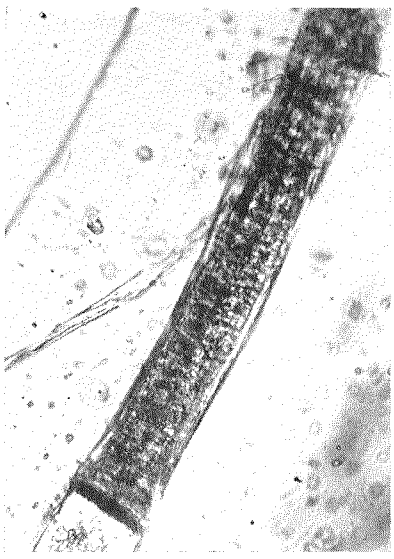

Fig. 3. A filamentous cell of lake ball, extracellularly frozen in water at −15°C for 20 minutes. Cells can survive freezing. ×290

The extracellularly frozen cells at temperatures above −20°C fixed by means of cold fixation appeared to be slightly contracted, but no change in interior structure was observed under a microscope. A prolonged freezing resulted in increased injury to the cells even at −15°C. After thawing, injured cells were observed to be remarkably contracted. They sometimes showed a pseudo-plasmolysis appearance, or a disorganized structure with remarkable vacuole formation. The highest lethal freezing

temperature in a small mass of filamentous cells seems to lie around −20°C. The same proportion of survival was obtained from the freezing experiments of these cells at −20°C for 2 hours and also for one full day. When the duration of freezing was prolonged for over 24 hours, the number of killed cells remarkably increased. Some cells, however, could survived freezing at temperatures below −25°C; 10 to 40 per cent of the total frozen cells could withstand temperatures of −25 to −30°C (Table 2).

Table 2. Percentage of cells of lake ball surviving extracellular freezing for 24 hours (Terumoto, 1959 a)

Freezing temperature (°C)	Weight* (g)	Survival (%)	Growth at 20°C one month after thawing
0	3.9	100	Normal
−10	5.2	100	Normal
−20	5.0	80–90	Surface cells fade
−25	3.9	30–40	Globular aggregations are felt somewhat elastic
−30	3.7	10–20	Globular shape maintains, but the aggregations are not elastic
−38	4.4	0	Globular shape collapses, all the cells fade

* Weight of wet globular aggregation subjected to freezing

Vegetative cells in marine algae. By means of cold fixation the freezing processes in marine algae were observed. Almost all the marine algae observed underwent extracellular freezing with a characteristic appearance of "frost-plasmolysis" in which ice formed between the protoplast and cell wall. The lethal temperatures at which marine algae were killed by extracellular freezing within 24 hours are presented in Table 3. Even

Table 3. Critical temperature in freezing marine algae
Duration of freezing was 24 hours
(Terumoto, 1960 b, 1961, 1964 a, 1965)

	Marine algae	Survived (°C)	Fatal (°C)
green alga	*Ulothrix flacca*	− 25	−35
	Ulva pertusa	− 10	−15
	Enteromorpha intestinalis	− 20	−25
	E. linza	− 20 (50%)**	−25
	Monostroma angicava	− 20 (50%)	−25
red alga	*Bangia fusco-purpurea*	− 55 (50%)	−70
	Porphyra pseudolinearis (♀)	− 55 (50%)	−70
	P. pseudolinearis (♂)	− 70 (50%)	—
	*P. yezoensis**	−196	—
	P. onoi	− 10	−15

* Collected in late April
** Percentage of survival

at higher temperatures than those presented in Table 3 prolonged freezing resulted in an increase in number of killed cells.

Green algae *Enteromorpha linza* and *Monostroma angicava* which were kept at 0°C were cultured at 20°C for a few weeks, and then they were frozen for 24 hours at graded temperatures. The frost killing temperature estimated is shown in Table 4. The resistance capacity of cells to freezing was not remarkably influenced by 12–14 days temperature treatment.

Table 4. Percentage of survival in frozen-thawed cells after cultivation at 20°C in darkness (Terumoto, 1964 a)

Enteromorpha linza					*Monostroma angicava*				
Days of cultivation	Freezing temperature (°C)				Days of cultivation	Freezing temperature (°C)			
	−10	−15	−20	−25		−10	−15	−20	−25
0	100	100	60	0	0	100	100	50	0
7	100	100	50	0	7	100	100	50	0
14	100	80	40	0	12	100	60	20	0

IV. The Effects of Mediums to Frost Resistance

1. LAKE BALL

Frost resistance was ascertained in the cells previously immersed in various kinds of mediums by which the properties of protoplasmic membrane in these cells may prob-

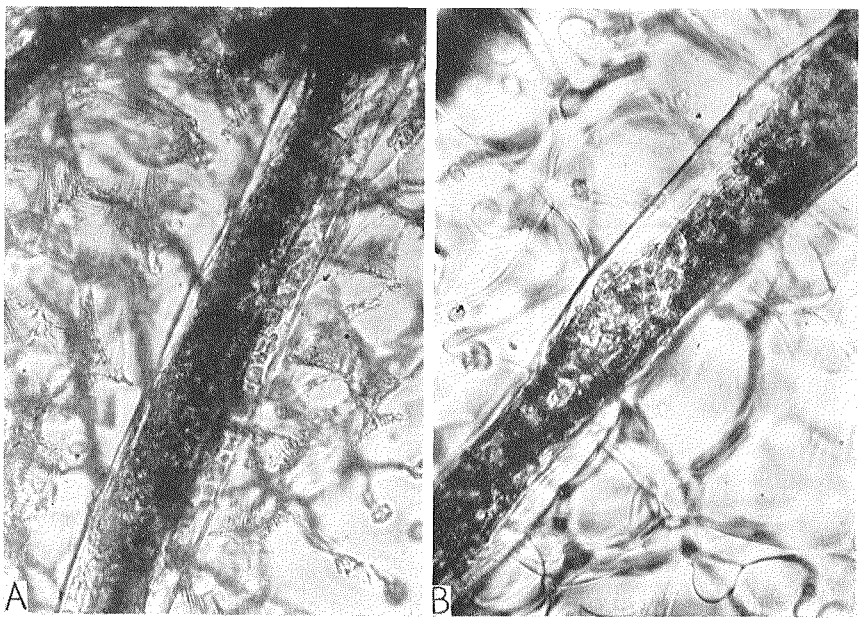

Fig. 4. Extracellular freezing of filamentous cells of lake ball in monovalent inorganic salt solution. Cells are fatally injured. ×295
A. Freezing in 0.5 M KCl at −15°C
B. Freezing in 0.5 M NaCl at −13°C

ably be affected. The frost killing rate in the cells was estimated after freezing at −15°C for 2 hours and thawing at room temperature under a microscope. The details of this experiment were described in a previous paper (Terumoto, 1962).

Effect of inorganic salts. At low concentrations, monovalent inorganic salts were generally indicated to have larger damaging effects to the cells at room temperatures than the bivalent salts. $SrCl_2$ and $CaCl_2$ in 1 M solution were shown to have no damaging effect to cells for 24 hours at room temperature. The use of monovalent inorganic salts in the solution resulted in a fatal frost injury to the cells, while bivalent salts, as a rule, produced less injury. In the inorganic salts used, calcium salts were least damaging. Frozen cells in isotonic solution of $SrCl_2$ were not injured, but in hypotonic $SrCl_2$ solution cells were severely injured. The freezing processes of the cells in some mediums were observed as follows.

When frozen in KCl or NaCl solution, cells were remarkably dehydrated and irregularly flattened; the protoplasm is contracted in the center or along one side of the cell. Frozen cells in 0.8 M solution were not capable of plasmolysis after thawing. The extracellularly frozen cells at temperatures around −15°C were frequently observed to show many plastids which were derived from the protoplast attached to the cell wall (Fig. 4 A, B). Some plasmolysed cells in hypertonic salt solution further contracted upon freez-

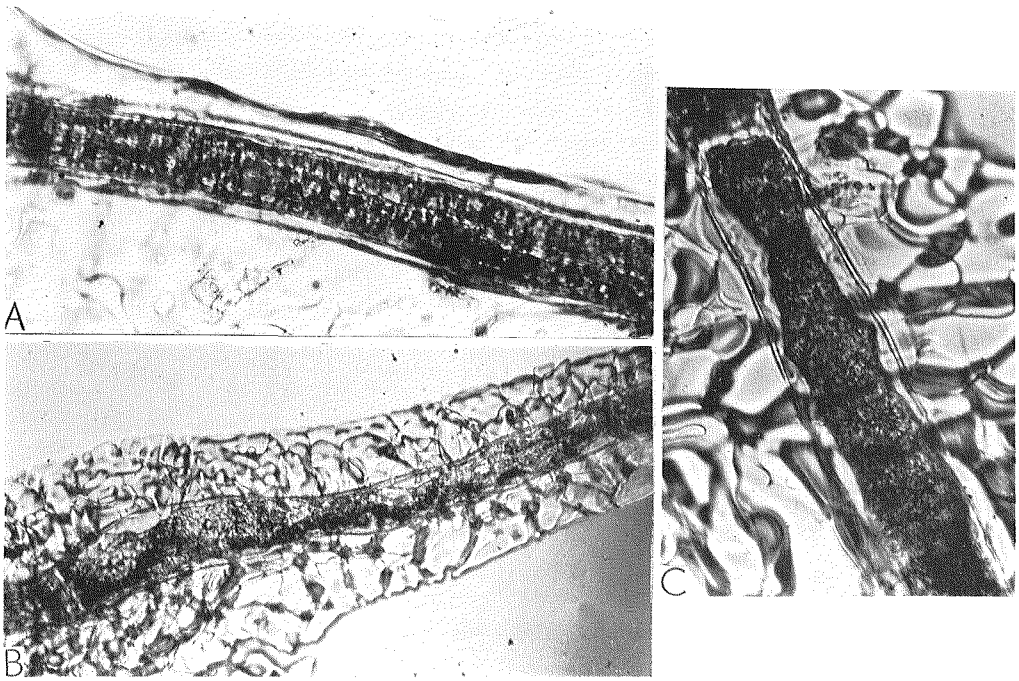

Fig. 5. Extracellular freezing of filamentous cells of lake ball in bivalent inorganic salt solution at −15°C

A. Freezing in 0.1 M $CaCl_2$. Freezing is innocuous to the cells. ×295

B. Freezing in 0.5 M $CaCl_2$, showing concave plasmolysis in the cell. Freezing is innocuous to the cells. ×147

C. Freezing in 0.5 M $MgCl_2$, showing irregular plasmolysis in the cell. Freezing is fatal to the cells. ×295

ing, but most of them were killed and protoplasts in these cells were coagulated in the form as they were. The interior of the cell appeared to be roughly granular. After thawing concave plasmolysis disappeared in the cell.

The damaging effect of Ca-salts was found to be rather slight when compared with that of Na-salts. Among Ca-salts CaI_2 was rather harmful. Frost resistance of cells frozen with various Ca-salt solutions were higher than those frozen with other salt solutions (Table 5). The cells frozen with $CaCl_2$ solution underwent frost-plasmolysis; the form of plasmolysed cells was concave which was much the same as the cell form in hypertonic balanced salt solution at room temperature (Fig. 5 B). The thawed cells were found to be alive, and their contracted protoplasts soon deplasmolysed and recovered their normal figure. The cells frozen in hypotonic solution of 0.1 M $CaCl_2$ showed no plasmolysis and the cells survived freezing (Fig. 5 A).

Tartrate, sulfate and iodide were remarkably injurious to cells of lake ball even in

Table 5. Lethal critical concentrations of various salt solutions (at 20°C in darkness for 24 hours) and the effect of these solutions to frost resistance of lake ball (at −15°C for 2 hours)

Solutions*	Lethal critical concentration (M)	Effect to frost resistance	
		Survived (M)	Fatal (M)
KCl	0.4–0.5	0.1–0.3 (30%)***	0.4 –1.0
NaCl	0.5–0.6	—	0.1 –1.0
LiCl	0.6–0.7	0.1–0.2 (50%)	0.3 –1.0
$BaCl_2$	1.0 **	0.1 (30%)	0.2 –1.0
$SrCl_2$	1.0 **	0.4–1.0	0.2 –0.3
$MgCl_2$	0.5–0.6**	0.6–0.7	0.1 –0.5, 0.8 –1.0
$CaCl_2$	1.0 **	0.1–1.0	—
NaCl	0.5 –0.6	0.01–0.05	0.06–0.1
NaI	0.2 –0.3	0.01 (50%)	0.02–0.1
NaBr	0.4 –0.5	0.01 (80%)	0.02–0.1
NaSCN	0.3 –0.4	0.01 (30%)	0.02–0.1
Na_2SO_4	0.1 –0.2	0.01 (50%)	0.02–0.1
$NaNO_3$	0.3 –0.4	0.01 (30%)	0.02–0.1
Na-Tart.	0.05–0.06	0.01 (50%)	0.02–0.1
Na-Acet.	0.3 –0.4	0.01 (50%)	0.02–0.1
$CaCl_2$	1.0	0.1–1.0	—
CaI_2	0.7 –0.8	—	0.1 –1.0
$CaBr_2$	0.8 –0.9	0.1–1.0	—
$Ca(NO_3)_2$	0.9 –1.0	0.1–1.0	—

* In these salt solutions the range of concentrations tested were from 0.1 to 1.0 M, except for the sodium salts with various anions which were used with a concentration detween 0.01 and 0.1 M

** The cells plasmolyse in solutions of a concentration higher than 0.6 M

*** Percentage of survival

very low concentrations. Chlorine ion was rather innocuous than the other anions (Table 5). Cells frozen with NaCl solution of a concentration of over 0.06 M were killed, while in other solutions, the critical concentration to cause frost injury to cells was about 0.01 M.

Extracellularly frozen cells in monovalent salt solutions never underwent plasmolysis during freezing and were remarkably dehydrated resulting in an irreversible coagulation of plotoplasm. On the other hand, freezing cells in bivalent salt solutions, as a rule, underwent plasmolysis. In such cases, plasmolysed cells with a rather smooth surface of protoplast, of both concave and convex types, suffered none or little injury, while those with irregular surfaces of protoplast suffered severe injury (Fig. 5 C). The form of frozen protoplast in diluted salt solutions, regardless of the type of salts, was very similar to the frozen figure of the cells in pure water and these cells suffered little injury.

Effect of sugar. It is well known that sugar is very useful to protect plant cells from the injury of freeze-thawing (Levitt, 1956; Terumoto, 1957). However, neither sucrose nor glucose exerted any protective effect against frost injury in the cells of lake ball. About 50 per cent of frozen cells in 1 M sucrose solution were killed at −5°C. All the frozen cells were entirely destroyed at temperatures below −5°C. The figure of frozen cells in 1 M sucrose solution observed by cold fixation at −5°C indicated that frozen cells with contracted protoplast were alive. In a concentrated sucrose solution higher than 1 M, the frost resistance of cells was almost lost.

Glucose was less harmful to the cells than sucrose, especially at low concentrations. Upon freezing the cells in 1 M glucose solution were extremely dehydrated and flattened, the center part of filamentous cells became slender, but no plasmolysis occurred at all, in these cells. When thawed, the protoplast shrank and coagulated, and pseudoplasmolysis was observed in a part of the cells. Frozen cells in 1 M sucrose solution showed the same freezing process as in 1 M glucose. When the cells was frozen in a nearly isotonic sugar solution, they underwent plasmolysis, but even under plasmolysed conditions, they were invariably killed by freezing.

Effect of polyhydric alcohols. Polyhydric alcohols such as glycerol and ethylene glycol are known to be one of the most effective agents to protect cells against frost injury. The effects of 6 kinds of polyhydric alcohols on the frost resistance of cells of lake ball were investigated.

Glycerol, like sucrose, can not penetrate into the cells of lake ball. Frozen cells in 1 M glycerol solution were not injured at all, at temperatures down to −15°C, but at lower temperatures frozen cells were clearly injured.

Table 6. Glycerol and frost resistance of cells of lake ball. Duration of freezing at −20°C was 2 hours (Terumoto, 1962)

Concentration (M)	Pure water	0.2	0.5	1.0	1.2	1.4	1.6	1.8	2.0
Survival (%)	80–90	50–60	40–50	20–30	0	0	30	50	50

In the medium with the glycerol concentration between 0.2 and 1.4 M, the frost resistance of cells decreased. An observation of the freezing process of cell revealed

that the coagulation of protoplasm which actually occurred by excessive dehydration from these cells had a flat appearance. All of the frozen cells in the 1.2 M glcerol solution at −19°C for 30 minutes were dead.

Ethylene glycol rapidly penetrate into the cells of lake ball. Cells immersed in a hypertonic ethylene glycol solution underwent plasmolysis at first, and thereafter deplasmolysed to the normal state. In general, ethylene glycol increases frost resistance in plant cells (Terumoto, 1957, 1960 a). The most effective concentration of ethylene glycol for the increasing of frost resistance in cells of lake ball were 2 to 3 M. In 2 M ethylene glycol, cells were observed to plasmolyse simultaneously with the beginning of freezing (Fig. 6). The plasmolysed cell form was of a concave type, such as seen in a balanced salt solution at room temperature. Frozen cells in polyhydric alcohols such as ethylene glycol, propylene glycol, diethylene glycol, triethylene glycol and polyethylene glycol at −33°C for 2 hours showed a good survival rate after thawing. Ethylene glycol and propylene glycol exerted the best effect. Diethylene glycol showed a 50–60 per cent survival. Triethylene glycol and polyethylene glycol could not readily permeate into the cells, and the protective effect of these large molecular polyhydric alcohols were small (Table 7).

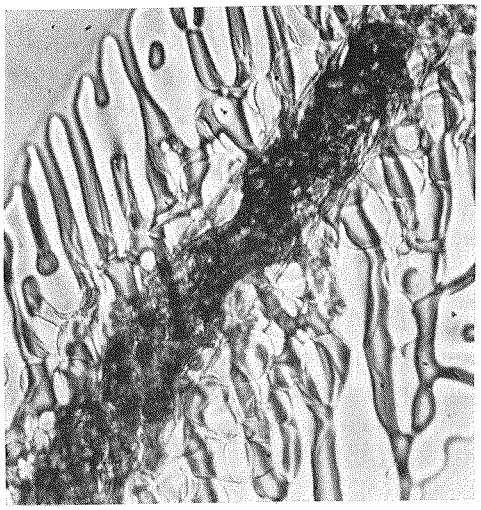

Fig. 6. A filamentous cell of lake ball, extracellularly frozen in 2 M ethylene glycol at −19°C, showing concave plasmolysis. Cells can survive freezing. ×295

Table 7. Protective effect of various polyhydric alcohols related to their ability to permeate into the cells of lake ball (Terumoto, 1960 a)

Solutes	Molecular weight	Concentration (M)	Permeability to solute	Survival (%) after freezing at −33°C for 2 hours
Ethylene glycol	62	1	Complete	100
Propylene glycol	76	1	Complete	80–90
Diethylene glycol	106	1	Complete	50–60
Triethylene glycol	150	1	Impermeable	40–50
Polyethylene glycol	400	0.5	Impermeable	20–30
Control (deionized water)				0–10

Effect of fat solvent. Fat solvent may reasonably alter the nature of the protoplasmic membrane. A previous application of fat solvent on cells may, therefore, change the degree of frost resistance in these cells. The following three experimental procedures were applied.

a) Cells soaked in various solutions of fat solvents for 24 hours at room temperature were frozen in the same medium in which they were immersed.

b) Cells soaked in various solutions for 24 hours at room temperature were washed with water, they were then frozen in pure water.

c) Cells were frozen with various solutions, without any pre-treatments.

In procedure a) all of the frozen cells except in the case of methanol, ethanol and acetone, were fatally injured. Cells soaked in methanol exhibited a higher frost resistance than the control: They invariably survived at −30°C for 12 hours. In procedure b), even if the cells were washed well with water after the soaking in *n*-, *iso*-propanol, *n*-, *sec*-, *tert*-butanol and ethyl "cellosolve", about 50 per cent of these cells could not survive freezing. That is to say, these fat solvents presumably caused some irreversible changes in the protoplasm of the cells. In procedure c), cells frozen in 7 kinds of solutions suffered fatal injury. Previous soaking of cells in acetone, ether and chloroform gave no influence on the frost resistance of these cells after they were washed with pure water. The examined fat solvents can be classified into three groups according to the degree of injury in the cells of lake ball.

1) methanol and ethanol.
2) acetone, ether and chloroform.
3) *n*-, *iso*-propanol, *n*-, *iso*-, *sec*- and *tert*-butanol.

While methanol and ethanol did not decrease the original frost resistance, methanol showed a specially high protective effect. Methanol easily permeated into the cell and showed no toxicity to cells. The survival rate of the cells and the figure of cells observed by means of cold fixation after freezing at −15°C for 2 hours in various solutions of the solvents are shown in Table 8.

Table 8. Survival of frozen cells in various fat solvents. Duration of freezing at −15°C was 2 hours (Terumoto, 1964 b)

Fat solvents	Concentration (M)	Survival (%)	Figure of freezing cells
Methanol	1	100	Normal figure. Degree of contraction small
Ethanol	1	100	
Acetone	1	50	Extremely dehydrated figure
iso-Propanol	1	0	Extremely dehydrated figure
tert-Butanol	1	0	
Ethyl "cellosolve"	1	0	Normal figure
Deionized water		100	

At freezing temperature above −20°C the degree of contraction of the cells in methanol was clearly smaller than that of the cells frozen in water; plasmolysis did not occur after thawing in these cells. No change in internal structure of cells was observed except for the plastids which were slightly aggregated (Fig. 7). After thawing all of the cells survived. Frozen cells in 2 M acetone were remarkably dehydrated and contracted without any occurrence of plasmolysis. Almost all cells frozen in 2 M acetone at −15°C for 30 minutes died. Frozen cells in the 0.1 M *n*-butanol were strongly de-

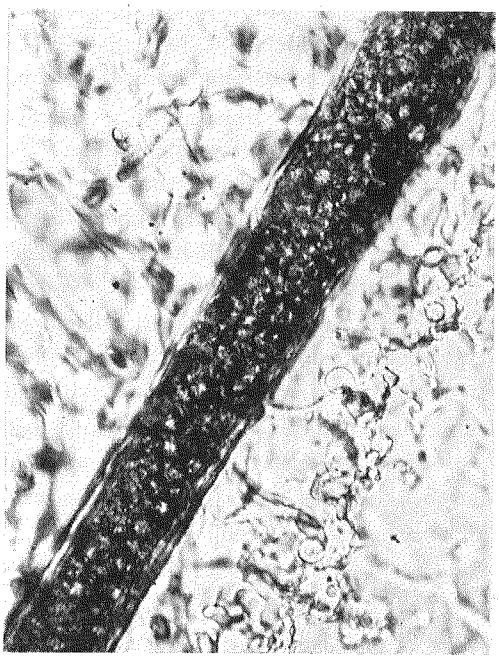

Fig. 7. A filamentous cell of lake ball, extracellularly frozen in 2 M methanol at −18°C. Note the unchanged appearance of the frozen cell. Freezing is entirely innocuous to the cell. ×350

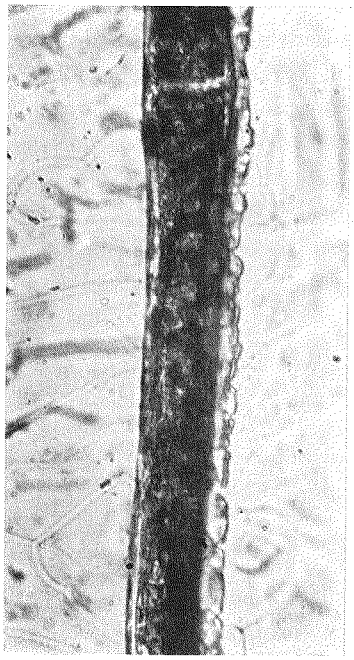

Fig. 8. Extracellular freezing of filamentous cells of lake ball at −15°C which was previously treated with 0.1 M Na-oxalate and washed with water before freezing. Freezing was fatal to the cells. ×350

hydrated and became remarkably slender except for the nodes. After thawing protoplasts of these contracted cells clearly collapsed. Frozen cells in methanol, ethanol and ethyl "cellosolve" were also dehydrated by extracellular freezing, but the state of protoplast was as normal as that of intact cells. Protoplast of cells frozen in *iso*-propanol and *tert*-butanol appeared to collapse. Treated cells in toluene and xylene were fatally injured by freezing.

Effect of surfactant. Surfactants can not permeate into cells and only affect the surface of cells. The surfactants used were as follows.

Anionic group (Monogen LH, Emal O, Aerosol OT)
Cationic group (Amer Dex, Levenol A, Osvan)
Nonionic group (Span 20, Tween 20, Tween 80)

A detailed report on the effect of surfactants on freezing cells were given in a previous paper (Terumoto, 1964 b). It was shown that among these three groups of surfactants anionic surfactants were most injurious to the freezing cells, cationic ones were less injurious, while nonionic ones were least injurious.

Effect of Ca-precipitant. The results obtained from the freezing experiment of cells in salt solutions suggest that calcium ion in the medium is effective to protect cells against frost injury. The effect of previous treatment with Ca-precipitants on cells was, there-

fore, examined. The cells were treated previously with Na-oxalate and Na-citrate solutions at various concentrations from 10^{-1} to 10^{-2} M for 15 minutes and washed with pure water. Freezing in these treated cells at −15°C for 2 hours was remarkably injurious (Fig. 8), although the freezing at −5°C for 2 hours was not harmful for these cells. The freezing process of these treated cells was almost the same as that of the control cell frozen in water.

Table 9. Effect of pre-treatment with Ca-precipitants on frost resistance of cells. Duration of pre-treatment with 0.1 M Na-oxalate was 15 minutes. Cells were washed for 15 minutes with pure water and then frozen in water for 2 hours (Terumoto, 1964 b)

Freezing temperature (°C)	−5	−10	−15
Survival (%)	90–100	60	10

2. A RED MARINE ALGA, *PORPHYRA YEZOENSIS*

Cells soaked in various solution, isotonic to sea water, of inorganic salts, sugars,

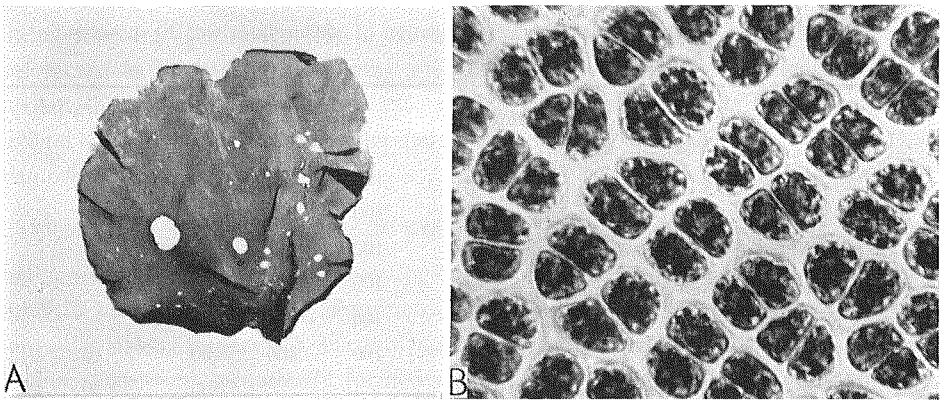

Fig. 9. A marine red alga, *Porphyra yezoensis*
A: A thallus. ×1 B: Cells in a thallus. ×625

Table 10. Freezing of cells of *Prophyra yezoensis* soaked in various solutions. Duration of freezing at −100°C was about 24 hours (Terumoto, 1965)

Solutions	Concentration (M)	Survival (%)
Sea water		100
NaCl	0.5	80–100
Sucrose	1	0
Glycerol	1	0
Ethylene glycol	1	100
Methanol	1	0
Deionized water		0

polyhydric alcohols, fat solvents, and deionized water were frozen slowly to −100°C to examine the effects of these agents on cellular frost resistance.

It is shown in Table 10 that there was no frost damage of the cells in solutions of NaCl and ethylene glycol, but in the solutions of sucrose, glycerol, methanol and deionized water no cell survive freezing.

V. Resistance to Plasmolysis of Cells in Marine Algae

By the use of graded high concentrations of plasmolyticum the tolerance of protoplast to dehydration and contraction was observed in a number of marine algae. Critical concentrations of plasmolysis and survival rate in the cells after plasmolysis in various high concentrations of balanced salt solutions are indicated in Table 11. In general, the resistance to plasmolysis in red algae was higher than that in green algae. Especially, *Porpyra yezoensis* could tolerate plasmolysis in the highest concentration of the solution. Tolerance of *Porphyra onoi* was lowest in red algae tested. After the treatment with 3 M solution, the survival rate of this alga was only 10 per cent. It was observed that cells of marine algae having a high tolerance to plasmolysis were also highly resistant of freezing (Tables 3 and 11).

Table 11. Tolerance to plasmolysis. Duration of plasmolysis was 10 minutes (Terumoto, 1961, 1964 a, 1965)

Species	Critical conc. of plasmolysis (NaCl isotonic) (M)	Conc. of plasmolyticum (M) 2	2.5	3	3.5	4	5
Ulothrix flacca	0.85	100*	100	100	0		
Ulva pertusa	0.55	100	90	10			
Enteromorpha intestinalis	0.79	100	70	0			
E. linza	0.65	100	60–70	0			
Monostroma angicava	(?)	100	60–100	0			
Bangia fusco-purpurea	0.83	100	100	50		10	0
Porphyra pseudolinearis (♀)	0.83			100		30	0
P. pseudolinearis (♂)	0.83			100		20–30	0
P. yezoensis	(?)	100		100		100	100
P. onoi	(?)	100		10	0		

(?) Obscure
* Percentage of survival

VI. Discussion

1. FROST RESISTANCE IN ALGAE CELLS

Lake ball. Cells of lake ball frozen in water could tolerate temperatures as low as −20°C. Some of the cells survived freezing even at −30°C. Such high frost resistance of this alga is unique for fresh water algae. Upon freezing they invariably underwent extracellular freezing provided they were slowly cooled. Neither frost resistance nor the osmotic value in cells were changed by temperature treatment (Terumoto, 1959 a). This may be understood, at least to some extent, from the fact that osmotic value in

algae cells is maintained mainly by inorganic salts but not by sugars produced within the cells. For example, *Valonia* contains a large amount of potassium estimated at about 0.5 M (Blinks, 1951), and the potassium content in lake ball is also assumed to contribute to their high osmotic value (Terumoto, 1962).

Many hardy plant cells are known to survive extracellular freezing with very contracted or flattened forms (Asahina, 1956). However, the figure of frozen cells of lake ball was almost the same as that of normal ones. Frozen cells in harmful medium, on the other hand, showed a flat figure of cells and they were invariably killed. Even when the cells were frozen extracellularly at −20°C, at least for a short time, the degree of contraction of the freezing cells was not so high as that of dead cells frozen at the same temperature. In addition, the number of killed cells increased during a few days of freezing (Terumoto, unpublished). All of these observations seems to suggest that the high frost resistance in the cell of lake ball is assumably based on the characteristic structure of the protoplasm of this alga, and that the high frost resistance is a character of cells associated with the integrity of protoplasmic structure which is maintained by a continuous metabolism.

Marine algae. Under extracellular freezing conditions, cells of marine algae were dehydrated and contracted, resulting in convex frost-plasmolysis, nevertheless some cells tolerated freezing (Terumoto, 1965). It is well known that the frost resistance of hardy plants increases by cold treatment as a result of anatonosis, and decreases by a warm treatment (Levitt, 1956; Terumoto, 1958 a). However, frost resistance of *Enteromorpha linza* and *Monostroma angicava* were not influenced by cultivation at 20°C for 2 weeks (Terumoto, 1964 a).

2. EFFECTS OF VARIOUS SOLUTES IN THE MEDIUM ON FROST RESISTANCE IN LAKE BALL

Inorganic salt. Except for Ca-salts, inorganic salts generally decreased the frost resistance in various plant cells (Terumoto, 1959 b). In the solutions of monovalent salts frozen cells never underwent plasmolysis. As freezing proceeded, cells were flattened and an ice cap was secondarily formed around the cells. It has been known that previously plasmolysed cells can escape injury resulting from severe stress which may reasonably arise at the time of extracellular freezing. Almost all of the monovalent salt solutions employed seems to have an effect on the cells of lake ball making their protoplasm brittle. Accordingly, even in the cells previously plasmolysed in these salt solutions, the increase of frost resistance cannot be expected. Indeed, the higher the concentration of these salt solutions, the greater the frost injury becomes in the soaked algae cells. On the other hand, frozen cells in bivalent solutions, as a rule, underwent plasmolysis. They can escape frost injury under a plasmolysed condition. In $CaCl_2$ solution the frost injury was least and, the cells underwent a typical concave plasmolysis simultaneously with freezing. Accordingly, it appears that the surface of frozen cells in $CaCl_2$ solution is in a nearly normal state as regards the fluidity and elasticity of the protoplasm. Cells frozen in $MgCl_2$ or CaI_2 solution showed an irregular concave or convex plasmolysis, and their frost resistance was usually less. In case of animal cells especially in erythrocytes, one of the main causes of frost injury has been attributed to the concentration of salt solutions inside and outside the cells with freezing (Lovelock,

1953). However cells of lake ball contain 0.3 M of potassium within them, and frozen cells in water were not injured at −15°C, but the cells of the same alga when frozen with 0.3 M KCl solution suffered a remarkable injury. Therefore, the frost injury in algae cells may not be assumed to be the so-called salt injury at least within the cells.

Sugar. Frozen cells in sugar solutions (sucrose and glucose) suffered increased frost injury. Sugar solutions of concentrations above 1.6 M were severely toxic to cells at room temperature. Frozen cells in isotonic (near 1 M) concentrations of sugar solution never underwent plasmolysis during freezing. Previously plasmolysed cells in hypertonic sugar solution at room temperature coagulated upon freezing. These results showed a strong resemblance to the case of cells frozen in monovalent salt solution. It may be that concentrated sugar solutions may change the fluidity and elasticity of protoplasm.

Fat solvent. As regard to the effect on frost resistance of cells of lake ball, fat solvents used were divided into three broad classes.

1) Methanol, ethanol: They did not decrease the original frost resistance.

2) Acetone, ether, chloroform: Soaked cells in these solutions for 24 hours were not affected when they were frozen in water after washing. Frozen cells in the medium of these fat solvents however showed increased frost injury.

3) *n*-, *iso*-propanol, *iso*-, *sec*-, *tert*-butanol, ethyl "cellosolve": Frozen cells in the medium were fatal. Treatment with these solutions for 24 hours caused considerable frost injury to the cells, even when they were well washed and frozen in pure water.

From the results obtained it seems that most of these solutes, even at very low concentrations, increased frost injury. Such an injurious effect can not be removed by a washing previous to freezing. Methanol and ethanol can penetrate into the cell in large amounts and therefore keep the cellular osmotic value high. Methanol was the least toxic among all of the additives used and penetrates easily into cells (Terumoto, 1964 b). Frozen cells in 2 M methanol solution did not plasmolyse and failed to contract appreciably. Accordingly, the remarkable protective effect of methanol to frost injury can be explained as a result of the prevention of excessive contraction of the protoplast brought about by an extracellular freezing in the cell.

Surfactant. Generally, members of the cationic group are markedly toxic, nonionics, as a class, are relatively non-toxic, while the anionics are of intermediate toxicity (Cruuier, 1956). Without exception treated cells with these surfactants for 24 hours were injured to some extent. Especially, anionics are injurious. Nonionics gave the smallest injury to the freezing cells. Surfactants of cationic group gave intermediate injury to freezing cells. These surfactants may be accumulated gradually on the cell surface, and cause some change on the protoplasmic surface which is favorable to the mechanical damage of protoplasm by contraction. Such as injurious effect on a protoplasmic surface may not be nullified by washing with water.

Ca-precipitant. It is well known that calcium ion has an important influence upon the maintenance of structure of protoplasm (Heilbrunn, 1952; Terumoto, 1959 b). As previously noted, Ca-salt in the medium never decreases the original frost resistance of lake ball. A pre-treatment of cells with Ca-precipitant, however, remarkably decreased the frost resistance in these cells. For example, cells treated with 0.1 M solution of Na-oxalate or Na-citrate for 15 minutes did not survive freezing at −15°C for 2 hours.

But at −10°C some of them, and at −5°C all of them could survive freezing. Therefore, even the cells in which some changes in protoplasmic structure has occurred by the treatment with Ca-precipitant can tolerated freezing provided the freezing temperature is not too low.

Polyhydric alcohol. Glycerol solution increased the frost injury. Glycerol could not penetrate into cells, and, therefore, did not reduce the degree of contraction of the cell at the time of freezing. Frost injury in the cells frozen with 1.6–2 M solution of glycerol was less than that frozen with low concentrations of glycerol. As previously stated, in plasmolysed cells the frost injury is less than in not plasmolysed cells. Since 1.6 M glycerol was over the critical concentration of plasmolysis of the cells, the increase of frost resistance in the cells soaked in 1.6 M glycerol solution can, therefore, be explained to be the result of the previous plasmolysis in the freezing cells. In spite of the injurious effect of glycerol, the other 5 kinds of polyhydric alcohol used protected cells against frost injury (Terumoto, 1960 a). The protective effect of polyhydric alcohols to frost injury in a cell was hitherto attributed to the limited dehydration of the cell at the time of freezing. These solutes well penetrate into the cell. High concentrations of these solutes can be tolerated and may cause high hydration within cells. The penetrated hydrophilic solute may, therefore, prevent excessive dehydration and contraction of cells during freezing and frost injury may be reduced.

VII. Conclusion

Frost injury and frost resistance in the cells of lake ball

All the observations described above indicated that a primary cause of the frost injury in the cells of lake ball might be an excessive dehydration and contraction of protoplasm as a result of extracellular freezing. The degree of contraction of the protoplast in a harmless state of freezing cell was usually less than that in a dangerous state of freezing cell. A harmless small molecule hydrophilic substance, which can readily permeate into the cell protects frost injury and increases the frost resistance in the cell. The degree of contraction of frozen cells in the solution of these protective substances was actually smaller than that in the cells frozen in water. Even harmless solutes cannot increase frost resistance, provided they had not previously penetrated into the cell. The protoplasm of cells of the lake ball seems to have a specific structure which can tolerate dehydration and contraction to some extent. Calcium ions bound in the protoplasm may play an important role to maintain such a specific protoplasmic structure, since a previous treatment with Ca-precipitant can cause a clear decrease in cellular frost resistance. Perhaps the main cause of frost injury in this alga may be a mechanical stress in protoplasm which becomes fatal when the contraction of the protoplast in freezing cell has reached a certain degree.

Frost injury and frost resistance in the cells of marine algae

A red alga, *Porphyra yezoensis* is one of the most resistant seaweeds to freezing; it can survive freezing in sea water even at −100°C for 24 hours. In this alga, NaCl and ethylene glycol as additives were found to be harmless when the cells were frozen at −100°C for 24 hours, while sucrose, glycerol and methanol were very harmful to the

cells under the same freezing conditions. Some intertidal marine algae have been observed to survive a severe desiccation under natural conditions (Biebl, 1962). Also in the present experiment many of the marine examined showed a high resistance to the injury of plasmolysis. The degree of resistance to plasmolysis in marine algae also showed a good correlation with that in their frost resistance. It is therefore reasonable to assume that the main factor of frost resistance in marine algae may be the high capacity of cells to withstand the severe contraction of the protoplast.

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