



HOKKAIDO UNIVERSITY

Title	Survival of Plant Tissue at Super-low Temperatures by Rapid Cooling and Rewarming
Author(s)	SAKAI, Akira; 酒井, 昭
Description	International Conference on Low Temperature Science. I. Conference on Physics of Snow and Ice, II. Conference on Cryobiology. (August, 14-19, 1966, Sapporo, Japan)
Citation	Cellular Injury and Resistance in Freezing Organisms : proceedings, 2, 119-130
Issue Date	1967
Doc URL	https://hdl.handle.net/2115/20412
Type	departmental bulletin paper
File Information	1_p119-130.pdf



Survival of Plant Tissue at Super-low Temperatures by Rapid Cooling and Rewarming*

Akira SAKAI

酒 井 昭

*The Institute of Low Temperature Science
Hokkaido University, Sapporo, Japan*

Abstract

To clarify the mechanism of cell survival at super-low temperatures some experiments were made with a combination of prefreezing and rapid cooling and rewarming, using the cortical cells from the winter twigs of a mulberry tree.

Tissue sections mounted between cover glasses with water were previously dehydrated at different degrees by extracellular freezing. Almost all of the sufficiently prefrozen cells at temperatures below -20°C could survive immersion in liquid nitrogen, irrespective of the rewarming rates. However, in the partially dehydrated cells at temperatures above -15°C , the rewarming rate seriously influenced the survival. These facts suggest that in the partially dehydrated cells, freezable water still remains to some extent in the cells after prefreezing, and the intracellular crystallization nuclei formed in the course of rapid cooling in liquid nitrogen probably grow and cause damage during subsequent slow rewarming in air, while, in rapid rewarming, there might be no time for the crystallization nuclei to grow to a damaging size.

The tissue sections immersed in liquid nitrogen after slight prefreezing were rapidly transferred to isopentane baths at temperatures ranging from -5 to -100°C and kept there for 20 minutes before rapid rewarming. In the slightly prefrozen cells, no damage was observed for 20 minutes at temperatures below -60°C , while, above -60°C , especially above -45°C , all cells were completely destroyed. It was also confirmed that when tissue sections could rapidly pass through a temperature range between the freezing point of the tissue and about -60°C , these cells suffered no damage.

The effect of cooling rate upon the survival of the cells was investigated, and the results indicated that a continued drop in survival was observed as the cooling rate rose; at higher cooling rates and with rapid rewarming, the effect was reversed and survival rose again with the increasing cooling rate.

Introduction

If various organisms are sufficiently desiccated, they can then be cooled in liquefied gases to super-low temperatures without injury. Extracellular freezing is also considered an effective method for dehydrating living cells. As has been previously reported (Sakai, 1956, 1958, 1960, 1962, 1965), if twigs of woody plants are dehydrated sufficiently by extracellular freezing, they can survive immersion in liquid nitrogen or helium.

Another method for maintaining viability at super-low temperatures using ultra rapid cooling and rewarming was presented by Luyet (1937).

This method consists of preventing the growth of intracellular crystallization nuclei which are usually formed during the course of cooling to super-low temperatures, by rapidly passing through the crystallization nuclei growth zone. Many attempts have been

* Contribution No. 802 from the Institute of Low Temperature Science.

made to check this hypothesis (Goetz and Goetz, 1938; Luyet and Gehenio, 1938, 1954; Rapatz and Luyet, 1963, 1965). The expected results were obtained, however, only with a few materials which were especially desiccated or treated with protective agents (Luyet and Gehenio 1938, 1954; Rapatz and Luyet, 1965). Luyet *et al.* (1958, 1960) have demonstrated many facts concerning phase transitions and recrystallization temperature occurring in the course of rewarming following immersion in an isopentane bath at -70°C , using water and various aqueous solutions. However, they have not obtained survival curves supporting the facts obtained with aqueous solutions.

In extremely hardy plant cells, however, this method was found to be effective in maintaining viability at super-low temperatures, especially when combined with prefreezing method (Sakai, 1956, 1958). For these reasons, to clarify the mechanism of cell survival at super-low temperatures, a systematic study was made with a combination of prefreezing and rapid cooling and warming, using extremely hardy cortical cells.

I. Materials and Methods

Cortical parenchyma cells from winter twigs of mulberry tree (*Morus bombycis* Koidz.) were used as the experimental materials. Cells from the same twig were used in each series of experiments. Thin tangential tissue sections (20 to 30 μ thick, 1 to 2 mm wide and 2 to 3 mm long) were sliced from the cortical tissue of a twig using the sharp blade of a straight edge hand razor. Ten tissue sections were used in each experiment. The osmotic concentration of the parenchyma cells was determined in a balanced salt solution by the usual plasmolytic method. This value was 0.75 M.

Tissue sections were mounted between cover glasses (18 \times 18 mm) with 0.05 ml water and cooled in one of several ways. The frozen cells were rewarmed, either rapidly in water at 30°C (rapid rewarming) or slowly in air at 0°C (slow rewarming). In order to obtain the greatest cooling and rewarming rates, an unmounted tissue section was held with a forceps and immersed directly into liquid nitrogen, and then transferred rapidly into water at 30°C .

The temperature was determined with 0.1 mm copper-constantan thermocouples and recorded by a highly sensitive recorder or oscilloscope. The cooling rate was represented as the time required for the temperature to fall from about -5°C to within 5°C of the final bath temperature. Rewarming rate was represented as the time required for the temperature to rise from the freezing bath temperature to a few degrees below the melting point of the tissue.

To dehydrate the cells at different degrees, tissue sections were frozen at -5°C and then cooled to various temperatures for 20 minutes. These prefrozen cells were then rapidly cooled in liquid nitrogen or isopentane baths at a cooling rate greater than 100°C per second. In such a case, the degree of dehydration scarcely proceeds during the subsequent cooling, as pointed by Mazur (1966).

The viability of cells was determined by the vital staining test using neutral red and the plasmolysis test in which plasmolysis and deplasmolysis were repeated twice with a twofold isotonic balanced salt solution and water. As a result of the treatment, normally stained and plasmolysed cells were regarded as normal ones.

To investigate the effect of the suspending medium on the survival of cells immersed in liquid nitrogen, the following solutes were compared; ethylene glycol, glycerol, dimethyl sulfoxide, glucose and sucrose. Tissue sections were treated in 2M solutions of these chemicals for 20 minutes at room temperature.

II. Results and Discussion

In the preliminary experiments, the following facts were confirmed: 1. Tissue sections mounted between cover glasses with 0.05 ml water could withstand slow freezing at any temperature at least above -115°C for 16 hours. 2. These frozen cells at any temperature could also survive subsequent rapid rewarming in water at 30°C , although cells from less hardy plants are well known to be sensitive to rapid thawing. 3. However, when tissue sections mounted in water were rapidly frozen by direct immersion from room temperature into isopentane baths below -20°C , all of the cells were destroyed, irrespective of the rewarming rates. These cells might be frozen intracellularly. 4. In some animal cells there exists a critical region of temperature. With the human red blood cells this region extends from -3 to -40°C ; and if more than a few seconds elapse between these temperatures, damage takes place (Lovelock, 1954). Such a critical temperature range does not exist in plant cells at least within 24 hours.

From these facts, it is reasonable to consider that the destruction of cortical cells used may be caused only by intracellular freezing. In such a sense, this material is favourable to consider the mechanism of injury on the basis of survival value. This material also simplifies the interpretation of the results obtained. Besides, tissue sections were mainly mounted with water to eliminate complicated factors resulting from the medium.

The effect of rewarming rate upon the survival of cells immersed in liquid nitrogen was investigated. For this purpose, tissue sections mounted between cover glasses with

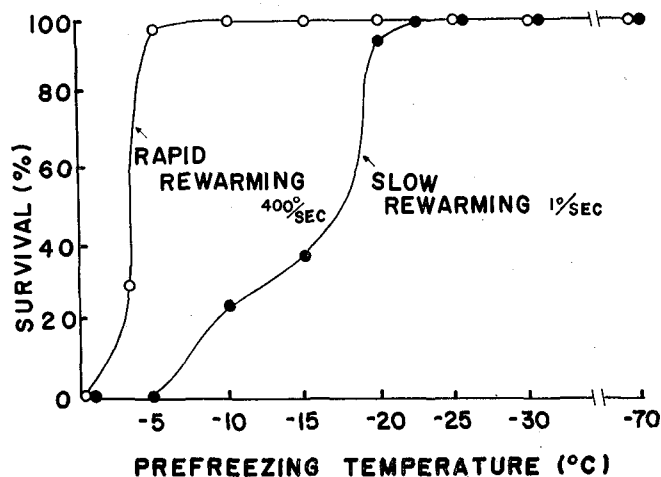


Fig. 1. Effect of rewarming rate upon the survival of cells immersed in liquid nitrogen following prefreezing at various temperatures

Rapid rewarming: In water at 30°C . Slow rewarming: In air at 0°C . The tissue sections were mounted between cover glasses with 0.05 ml water

water were sufficiently dehydrated by extracellular freezing at various temperatures, and then immersed in liquid nitrogen for 20 minutes (the cooling rate was approximately 100°C per second). These cells were rapidly rewarmed by immersion in water at 30°C (approximately 400°C per second), or slowly in air at 0°C (approximately 1°C per second). Almost all of the sufficiently prefrozen cells below -20°C could survive immersion in liquid nitrogen, irrespective of the rewarming methods used. However, if the cells were partially dehydrated at temperatures above -15°C , the rewarming rate seriously influenced the survival at super-low temperatures. Especially in prefrozen cells at -5°C , all were completely destroyed by slow rewarming in air, but all survived rapid rewarming (Fig. 1).

These facts suggest that in the sufficiently prefrozen cells below -20°C , almost all of the freezable water in a cell may be withdrawn by extracellular freezing. In such a case, these cells are not damaged by rapid immersion in liquid nitrogen and even the subsequent slow rewarming in air at 0°C . On the other hand, in the partially dehydrated cells, freezable water still remains to some degree in the cells after prefreezing, and the intracellular crystallization nuclei formed in the course of rapid cooling in liquid nitrogen probably grow and cause damage during the subsequent slow rewarming in air, while in rapid rewarming, there might be no time for the crystallization nuclei to grow to a damaging size.

The next problem is that in partially dehydrated cells the damage occurs at any temperature during rewarming following removal from liquid nitrogen. To investigate this problem, tissue sections immersed in liquid nitrogen following prefreezing at -5 , -10 and -20°C were rapidly transferred to isopentane baths at temperatures ranging from -5 to -100°C at five degree temperature intervals, and were kept there for 20 minutes before rapid rewarming. The results obtained are represented in Fig. 2.

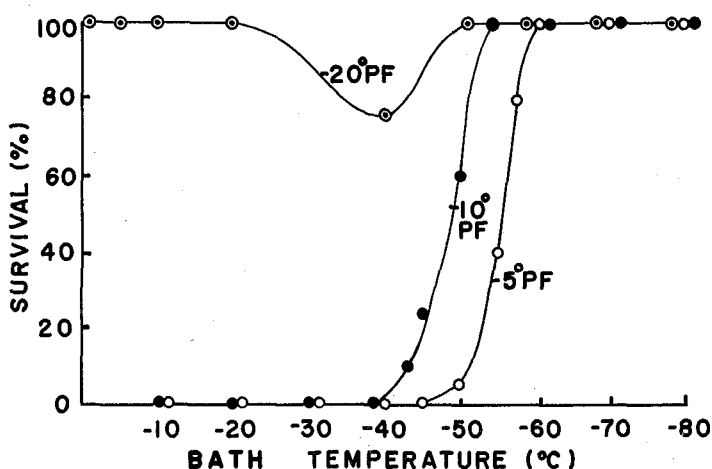


Fig. 2. Survival of prefrozen cells transferred to isopentane baths cooled at various temperatures following removal from liquid nitrogen

-5°PF , -10°PF and -20°PF indicate that tissue sections have been prefrozen at -5 , -10 and -20°C , respectively. The prefrozen cells at various temperatures were kept for 20 minutes in isopentane baths maintained at various temperatures following removal from liquid nitrogen, before being rapidly rewarmed in water at 30°C

In the prefrozen cells at -20°C , a slight decrease in survival was noted in the temperature range from -30 to -40°C . However, in the prefrozen cells at -5 and -10°C , at the temperatures below -60°C , no damage was observed for 20 minutes, while above -60°C , especially above -40°C all cells were completely destroyed. The main characteristic in the survival rate is that in a limited temperature range the survival abruptly decreases with raising the temperature from -55 to -40°C and from -60 to -45°C in the prefrozen cells at -10 and -5°C , respectively. These facts seem to suggest that the growth rate of crystallization nuclei varies with the degree of dehydration of cells, in other words, concentration of cells.

The survival rate also gradually decreased with the length of time kept at -60°C , especially in the prefrozen cells at -5°C (Table 1). Also, the length of time kept at temperatures below -70°C at least for 24 hours had no influence on the survival rate in the cells prefrozen at temperatures below -10°C .

From these facts it may reasonably be considered that if tissue sections can rapidly pass through a temperature range between freezing point of the tissue and about -60°C ,

Table 1. Survival of cells held for different lengths of time at -60°C following removal from liquid nitrogen, before being rewarmed in water at 30°C
Tissue sections were mounted in water between cover glasses

Prefreezing temp. ($^{\circ}\text{C}$)	At -60°C before rapid rewarming				
	1 min	20 min	1 hr	6 hrs	16 hrs
- 5	100*	100	90	25	0
-10	100	100	100	100	30
-20	100	100	100	100	100

* Percentage of survival

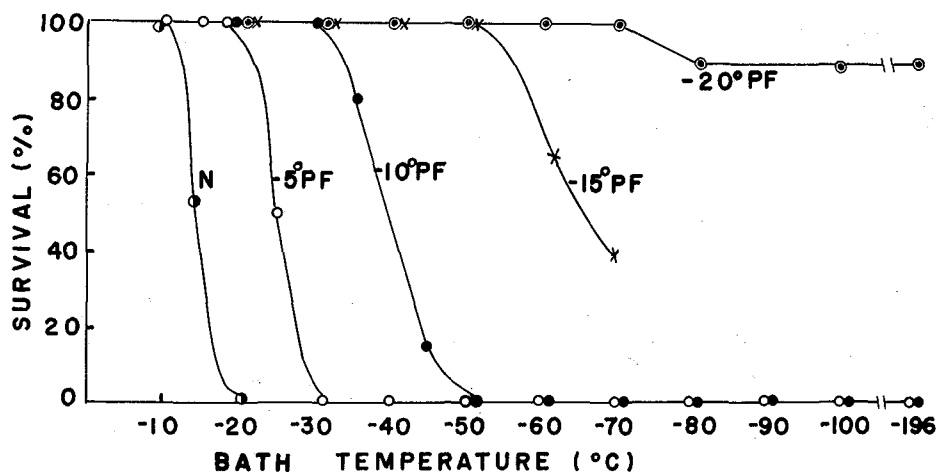


Fig. 3. Survival of cells immersed in isopentane baths at various temperatures and subsequently rewarmed slowly in air at 0°C

N indicates tissue section not prefrozen, PF indicates prefreezing. Immersion time in isopentane bath: 10 minutes. Tissue sections were mounted between cover glasses with water

these cells suffered no damage.

To check this problem and to investigate further the effect of rewarming and cooling rates upon the survival, tissue sections mounted between cover glasses with water were pre-frozen at various temperatures and then immersed in isopentane baths for 10 minutes before being rewarmed slowly in air at 0°C or rapidly rewarmed in water at 30°C. The results obtained in slow rewarming are presented in Fig. 3. The survival curves may be indicative of the degree of intracellular freezing which pre-frozen cells at various temperatures can withstand, because tissue sections are rapidly cooled and then slowly rewarmed. The survival rates varied remarkably with the degree of dehydration of cells. Almost all of the cells pre-frozen at temperatures below -20°C could withstand even direct immersion in isopentane baths below -70°C and subsequent slow rewarming in air at 0°C.

With rapid rewarming, at temperatures below -40°C, quite different survival curves, U-shape and V-shape were obtained in the cells pre-frozen at -5 and -10°C respectively (Fig. 4). For comparison, the curves obtained at slow rewarming rate are represented

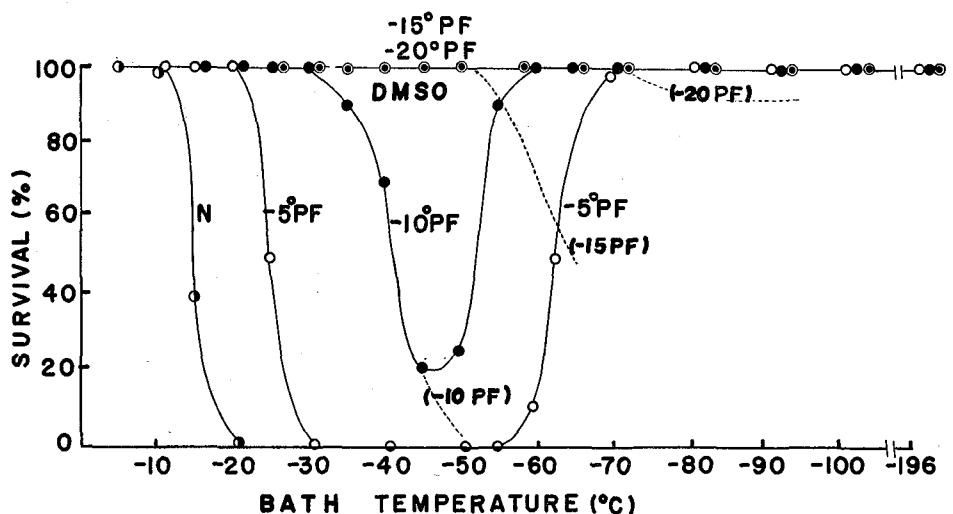


Fig. 4. Survival of cells immersed in isopentane baths at various temperatures and subsequently rewarmed rapidly in water at 30°C. N indicates tissue sections not pre-frozen, PF indicates pre-freezing. DMSO indicates tissue sections treated with 2M dimethyl sulfoxide. Dotted lines indicate the survival curves of the cells rewarmed slowly

with the dotted lines. These survival curves abruptly rose again with the decreasing temperature, from -55 to -70°C and from -45 to -60°C in the cells pre-frozen at -5 and -10°C respectively, which was characteristic in rapid rewarming.

Tissue sections pre-frozen at -15 and -20°C and treated with 2M dimethyl sulfoxide survived rapid cooling to any temperature tested including that of liquid nitrogen, if they were rapidly rewarmed in water at 30°C.

The effect of the length of time upon the survival of the cells cooled rapidly in isopentane baths below -60°C was investigated. Tissue sections pre-frozen at -5, -10 and -20°C and treated with 2M dimethyl sulfoxide without pre-freezing were directly

Table 2. Effect of the length of time upon the survival of the cells cooled rapidly in isopentane baths below -60°C

Tissue sections prefrozen at -5 , -10 and -20°C and treated with 2M dimethyl sulfoxide without prefreezing were directly immersed into isopentane baths at temperatures below -60°C , and then kept there for 24 hours, before being rewarmed rapidly in water at 30°C

Prefreezing temp. ($^{\circ}\text{C}$)	Temperature ($^{\circ}\text{C}$)				Liquid nitrogen
	-60^*	-60	-70	-80	
-5	100**	0	0	0	100
-10	100	50	100	100	100
-20	100	100	100	100	100
Dimethyl sulfoxide (2M)	100	50	100	100	100

* Tissue sections kept at -60°C for 10 minutes only

** Percentage of survival

immersed into isopentane baths at temperatures below -60°C , and kept there for 24 hours, before rewarming rapidly in water at 30°C . As shown in Table 2 the survival varied with the degree of prefreezing and the length of time kept at temperatures below -60°C . However, the length of time for which the tissue sections were kept below -70°C had no influence on the survival of the cells prefrozen at the temperatures below -10°C and treated with dimethyl sulfoxide.

Different bath temperature influences the cooling and rewarming rates and growth rate of crystallization nuclei. However, it was noted that the effect of bath temperature varies considerably with the dehydration of cells, cooling and rewarming conditions and the types of suspending medium. From the results obtained, (Figs. 3, 4) four temperature ranges can generally be distinguished concerning cell survival. 1) At the temperatures above -15°C , most of the cells immersed in isopentane baths above -15°C survived, irrespective of the rewarming rate. In this temperature range the cooling rate is rather small. From these reasons, most of cells may have frozen extracellularly. 2) At the temperatures between -20 and -50°C , almost all of the cells were killed, irrespective of rewarming rates used. This fact suggests that death was caused by fatal intracellular freezing during cooling and standing before being rapidly rewarmed. Presumably, this means that the crystals that form in the cells during cooling are large enough to be immediately damaging. This suggests that the growth rate of crystallization nuclei is very great in this temperature range. 3) At temperatures between -50 and -70°C , the survival rate increases with the decreasing temperature and the rewarming rate has a marked influence on the survival. These facts seem to suggest that hardly any harmful effect occurred during cooling and standing. Besides, the rate of survival is remarkably affected by the length of time. These facts imply that the growth rate of crystallization nuclei has become much smaller than that in higher temperatures. 4) At temperatures below -70°C , the rate of survival is affected remarkably with the rewarming rate. However, the length of time has hardly any influence on the survival within 24 hours.

To investigate the effect of additives on the survival at super-low temperatures, one experiment was performed using 2M solutions of dimethyl sulfoxide, ethylene glycol,

Table 3. Effect of suspending medium on the survival of cells immersed in liquid nitrogen without prefreezing

Tissue sections mounted between cover glasses with each solution were immersed in liquid nitrogen and then rewarmed in water at 30°C. 2M solution of each solute was used

Suspending medium	Percentage of survival
Dimethyl sulfoxide	100
Ethylene glycol	100
Glycerol	0
Glucose	70
Sucrose	40
Water	0

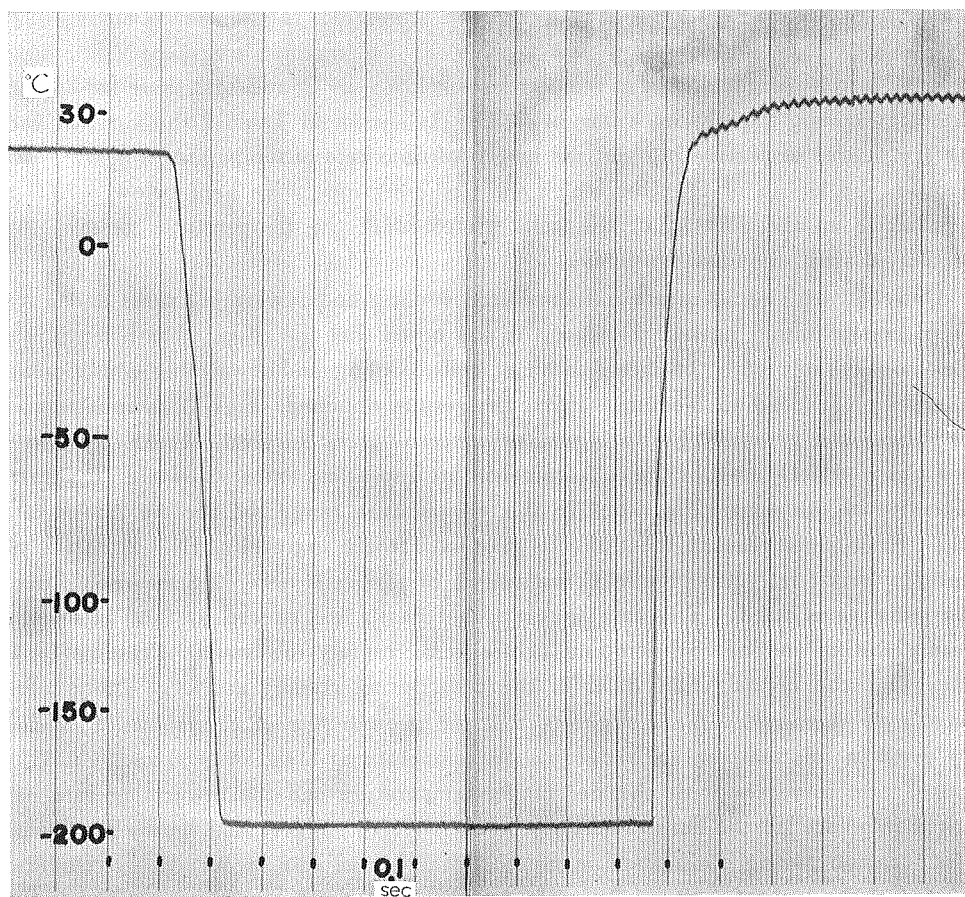


Fig. 5. Oscillogram of a tissue section immersed into liquid nitrogen and subsequently rewarmed in water at 30°C

An unmounted tissue section (20 to 30 μ thick, 1 to 2 mm wide and 2 to 3 mm long) held with forceps was rapidly immersed in liquid nitrogen from room temperature and then rapidly rewarmed in water at 30°C

glucose, sucrose and glycerol. With the exception of sucrose and glucose, all of these solutes can easily penetrate into cells. Tissue sections treated with each solution were immersed in liquid nitrogen without prefreezing, and then rapidly rewarmed in water at 30°C. As shown in Table 3, the protective effect differed considerably with the solutes used.

In all experiments undertaken so far, tissue sections mounted between cover glasses with 0.05 ml water were prefrozen at various temperatures, before being immersed into liquid nitrogen or isopentane baths. To obtain the greatest cooling and rewarming rates, an unmounted tissue section at room temperature was held with a thin forceps and rapidly immersed into liquid nitrogen. The cooling rate was approximately 2,500°C per second (Fig. 5). An unmounted tissue section was then rewarmed rapidly in water at 30°C or slowly in air at room temperatures. All of the cells survived rapid rewarming and all were destroyed by slow rewarming in air.

The effect of the degree of frost hardiness on the survival of cells rapidly cooled to and rewarmed from super-low temperatures was investigated using tissue sections with different frost hardiness. As shown in Table 4, in the case of extremely hardy

Table 4. Effect of the degree of frost hardiness on the survival of cells cooled and rewarmed rapidly without prefreezing

An unmounted tissue section held with forceps was rapidly immersed into liquid nitrogen and then rewarmed in water at 30°C

Degree of frost hardiness	Rewarming conditions (°C)		
	10 (air)	10 (water)	30 (water)
Hardy cells*	0***	0	50
Extremely hardy cells**	0	100	100

* Cortical cells from a twig collected in December

** Cortical cells from a twig collected in late January

*** Percentage of survival

cells from a winter twig all survived rapid rewarming in water at 10 and 30°C, while only 50 per cent of the cells survived in water at 30°C in the cells from a twig taken in early December.

To investigate the effect of bath temperature upon the survival of unrefrozen cells, unmounted tissue sections held with forceps at room temperature were rapidly immersed into isopentane baths cooled at various temperatures and kept there for 20 seconds before being rapidly rewarmed. Nearly the same survival curves as Fig. 4 were obtained (Fig. 6). All of the cells were alive in the tissue sections immersed in isopentane baths below -60°C and above -10°C. While, almost all of the cells were destroyed in an intermediate temperature range from -20 to -50°C. The differences in cooling and rewarming rates of the tissue sections immersed into isopentane baths at -50 and -60°C were small. Nevertheless, their rates of survival were quite different. As already mentioned, this fact may be explained from the difference of the growth rate of intracellular crystallization nuclei formed during the rapid cooling.

To clarify the effect of cooling rate upon the survival of unrefrozen cells, the

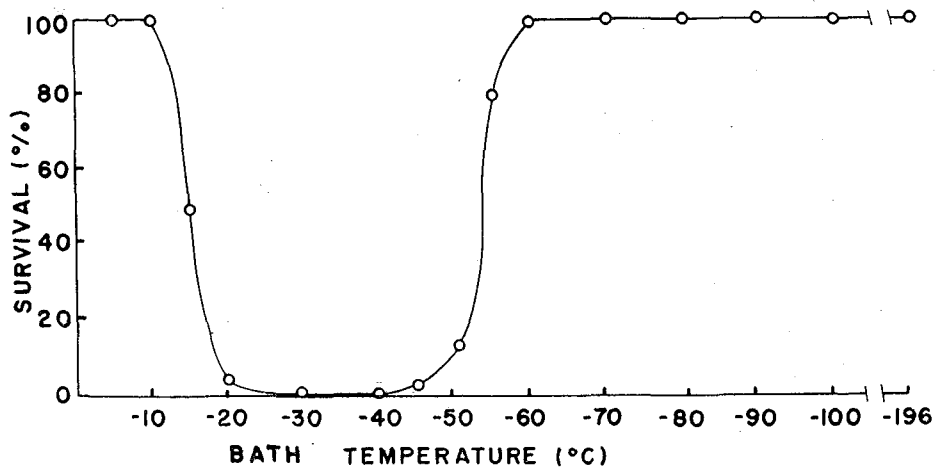


Fig. 6. Survival of cells cooled and rewarmed rapidly without prefreezing

An unmounted tissue section at room temperature held with forceps was rapidly immersed in isopentane baths at various temperatures without prefreezing and kept there for 20 seconds before being rewarmed rapidly

survival rate was plotted against the cooling rate, using the data presented in Fig. 6. (Fig. 7). Cooling and rewarming rates were calculated from oscilloscopic traces. The survival rate dropped continuously as the cooling rate rose towards about 3,000°C per minute. However, at cooling rates above 20,000°C per minute, survival rate rose again

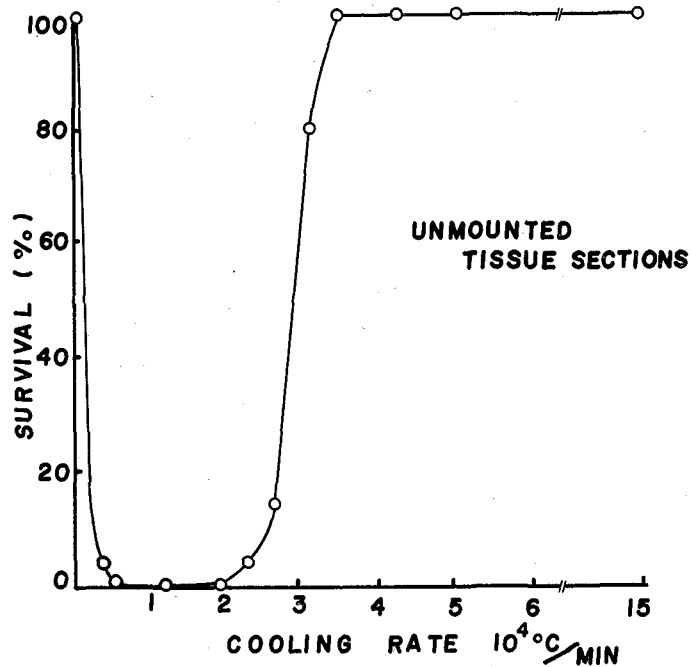


Fig. 7. Effect of cooling rate upon the survival of unrefrozen cells
The survival rate was plotted against the cooling rate, using the data presented in Fig. 6

remarkably with the increasing cooling rate.

As already mentioned, the basic factor in this problem is not whether ice is present within the cells, but rather the size of the ice crystals. The results obtained may be explicable in terms of crystal size. At high cooling rates, the ice crystals that form in the cells may be small enough to be innocuous; hence, when rewarming is carried out rapidly, they melt before they have time to grow, and all cells remain viable. At intermediate cooling rates, the rate of survival remains very low even with rapid rewarming. This means that ice crystals that form in the cells during cooling are large enough to be immediately damaging. At low cooling rates, the crystals should be still larger, but they form outside the cells. However, no direct evidence supporting this consideration is presented in this paper. To obtain some direct evidences, especially to determine the damaging size of intracellular ice crystals, the writer intends to investigate this problem further with an electron microscope.

Ultra rapid cooling and rewarming method was successfully applied to the cortical cells of woody plants. The main reasons for the success might be attributed to the following characters of the cells used: high frost resistance, small water content (50 per cent per fresh weight) and high sugar content. Besides, tissue sections used were very thin and small and they were frozen without immersing in any suspending medium. Therefore, unmounted tissue sections could be easily cooled ultra rapidly. The tissue sections mounted between cover glasses with water were previously frozen extracellularly before being cooled rapidly in liquid nitrogen or isopentane bath, and therefore, the mounted tissue sections also could be cooled and rewarmed rapidly. These reasons may be considered to be the main factors contributing to this success. It was also confirmed that extremely hardy cells maintained their viability more readily with this method than in less hardy cells. However, the reason is still unknown.

References

- GEHENIO, P. M., RAPATZ, G. L. and LUYET, B. J. 1963 Effects of freezing velocities in causing or preventing hemolysis. *Biodynamica*, **9**, 77-82.
- GOETZ, A. and GOETZ, S. S. 1938 Death by devitrification in yeast cells. *Biodynamica*, **2**, No. **43**, 1-8.
- LOVELOCK, J. E. 1954 The protective action of neutral solutes against hemolysis by freezing and thawing. *Biochem. J.*, **56**, 265-270.
- LUYET, B. J. 1937 The vitrification of organic colloids and of protoplasm. *Biodynamica*, **1**, No. **29**, 1-14.
- LUYET, B. J. and GEHENIO, P. M. 1938 The survival of moss vitrified in liquid air and its relation to water content. *Biodynamica*, **2**, No. **42**, 1-7.
- LUYET, B. J. and GEHENIO, P. M. 1954 Effect of the rewarming velocity on the survival of embryonic tissues frozen after treatment in ethylene glycol. *Biodynamica*, **7**, 213-223.
- LUYET, B. and RAPATZ, G. 1958 Patterns of ice formation in some aqueous solution. *Biodynamica*, **8**, 1-68.
- LUYET, B. 1960 On various phase transitions occurring in aqueous solutions at low temperatures. *Ann. N. Y. Acad. Sci.*, **85**, 549-69.
- MAZUR, P. 1966 Theoretical and experimental effects of cooling and warming velocity on the survival of frozen and thawed cells. *Cryobiology*, **2**, 181-192.

- RAPATZ, G. and LUYET, B. 1963 Effects of cooling rates on the preservation of erythrocytes in frozen glycerolated blood. *Biodynamica*, **9**, 125-136.
- RAPATZ, G. and LUYET, B. 1965 Effects of cooling rates on the preservation of erythrocytes in frozen blood containing various protective agents. *Biodynamica*, **9**, 333-50.
- SAKAI, A. 1956 Survival of plant tissue at super-low temperatures. *Low Temp. Sci.*, **B 14**, 17-23.*
- SAKAI, A. 1958 Survival of plant tissue at super-low temperatures. II. *Low Temp. Sci.*, **B 16**, 41-53.*
- SAKAI, A. 1960 Survival of the twig of woody plants at -196°C . *Nature*, **185**, 392-94.
- SAKAI, A. 1962 Survival of woody plants in liquid helium. *Low Temp. Sci.*, **B 20**, 121-122.*
- SAKAI, A. 1965 Survival of plant tissue at super-low temperatures. III. Relation between effective prefreezing temperatures and degree of frost hardiness. *Plant Physiol.*, **40**, 882-87.

* In Japanese with English Summary.