Survival of Plant Tissue at Super-Low Temperatures. IV. Cell Survival with Rapid Cooling and Rewarming

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Summary. Thin unmounted cortical tissue sections from winter twigs of the mulberry tree were held with a thin forceps and rapidly immersed in liquid nitrogen from room temperatures without prefreezing. They were rewarmed; rapidly in water at 10° to 40°, or slowly, in air at room temperatures. In those sections rapidly rewarmed, all survived. None survived in those sections rewarmed slowly in air.

Tissue sections mounted between coverglasses with water were extracellulary prefrozen at the temperatures low enough to dehydrate almost all of the freezable water in cells. These sufficiently prefrozen cells could survive immersion in liquid nitrogen, and the survival value was very little affected by the rates of cooling to and rewarming from super-low temperatures. With insufficient prefreezing at higher temperatures, however, the rewarming process seriously influenced the survival value of cells frozen at super-low temperatures. Slow rewarming in air destroyed all of the cells, while rapid rewarming in water at 30° did not affect them. An abrupt decrease in the survival value in insufficiently prefrozen cells during rewarming was also observed at temperatures above approximately −50° following immersion in liquid nitrogen. Very little decrease in the survival value was observed in any of the cells that had been sufficiently prefrozen.

These results indicate that cells which are insufficiently prefrozen may contain freezable water which nucleates during rapid cooling in liquid nitrogen and then grows during the subsequent slow rewarming into ice masses which destroy the viability of the cells. Such fatal intracellular freezing rarely occurs in sufficiently prefrozen cells, irrespective of the rate of cooling to or rewarming from super-low temperatures.

If various organisms are sufficiently desiccated, they can then be cooled in liquified gases without injury. Extracellular freezing is also considered an effective method for dehydrating living cells. As has been previously reported (10–13), if twigs of woody plants are prefrozen sufficiently by extracellular freezing, they can survive immersion in liquid nitrogen or helium.

Another method for maintaining viability at super-low temperatures using rapid cooling and rewarming, has been reported by Luyet (4). This method consists of preventing the growth of those intracellular crystalline nuclei usually formed during the course of cooling to super-low temperatures, by rapidly passing through the crystalline nuclei growth zone. Many attempts have been made to check this hypothesis for a long time. The expected results have been obtained only with a few materials, especially desiccated (5) or treated with protective agents (6). The primary difficulty encountered in using this method is to cool and warm rapidly enough to accomplish the objective. In highly hardy plant cells, however, this method is very effective in maintaining viability at super-low temperatures when used together with pre-freezing method (10–12).

Materials and Methods

Cortical parenchyma cells from winter twigs of the mulberry tree (*Morus bombycis* Koidz.) were used as the experimental material. Cells from the same twig were used in any 1 series of experiments. Thin tangential tissue sections (1 or 2 cell layers 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 edged hand razor. Ten tissue sections were used in each experiment. The osmotic concentration of the parenchyma cells was determined in a balanced salt solution (NaCl: $CaCl_2$ = 9:1, in volume) by the usual plasmolytical method. This value was approximately 0.75 M. The tissue sections were mounted between coverglasses (16 × 16 mm) with water or other aqueous solutions of 0.05 ml, and cooled in various ways. The cooling and rewarming rates were determined with 0.2 mm copper-conslantan thermocouple and recorded. The frozen cells were rewarmed, either rapidly in water at 30° (rapid rewarming) or slowly in air at 0° (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 N_2 (the cooling rate

of approximately 500° per second), then rapidly into water at 30° (the rewarming rate of approximately 400° per second).

To obtain various degrees of dehydration, tissue sections were prefrozen at various temperatures for 10 minutes before being immersed in liquid N_2 or isopentane baths at various temperatures.

The viability of the 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 twice isotonic balanced salt solution and water.

Results

In preliminary experiments, tissue sections mounted between cover glasses with water could withstand freezing at temperatures above at least −115° for 16 hours. The frozen cells at −115° could also survive even a rapid thawing by immersion into water at 30°, although less hardy plant cells are generally sensitive to rapid thawing. However, when tissue sections mounted with water were rapidly frozen by direct immersion from room temperature into isopentane baths below −20° and kept there for 5 minutes, all of the cells were destroyed, irrespective of the rewarming velocity. From these results, it is reasonable to assume that frost injuries in the cortical cells slowly rewarmed in air following immersion in liquid nitrogen, may only be caused by intracellular freezing.

It was also confirmed that prefreezing temperatures below −20° were required to allow the cortical parenchyma cells to withstand immersion in liquid nitrogen and subsequent slow rewarming in air at 0°.

Tissue sections mounted between coverglasses with water were sufficiently prefrozen at temperatures below −20°, and then immersed in liquid N_2 for 5 minutes (the cooling rate of approximately 150° per second) and thawed rapidly by immersion in water at 30° (approximately 80° per second), or slowly, in air at 0° (approximately 1° per second). These prefrozen cells could survive immersion in liquid nitrogen, irrespective of the rewarming method used. However, if the cells were insufficiently prefrozen at −5° or −10°, the rewarming rate seriously influenced the survival value at super-low temperatures. These cells were completely destroyed by slow rewarming in air, but unaffected by rapid rewarming in water at 30° (fig 1).

In order to determine whether prefrozen cells at various temperatures are rapidly destroyed within a limited temperature range in the course of rewarming, tissue sections were immersed in liquid nitrogen following prefreezing at −5°, −10° and −20°, were rapidly transferred to isopentane baths at temperatures ranging from −5° to −100°, and kept there for 20 minutes, before being rapidly rewarmed in water at 30°.

The results of this experiment were as follows:

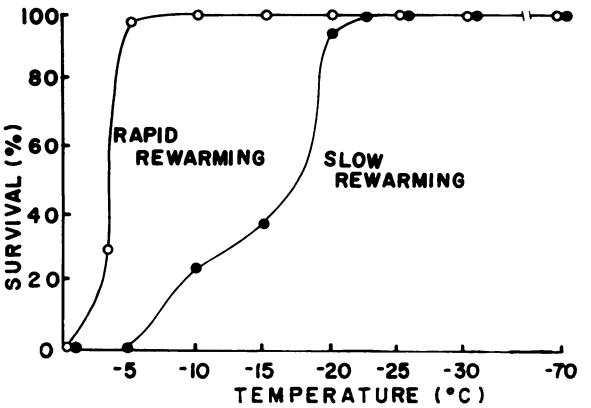

FIG. 1. Effect of rewarming rate upon the survival of cells immersed in liquid nitrogen following prefreezing at various temperatures. Rapid rewarming: in water at 30°, slow rewarming: in air at 0°. The tissue sections were mounted between coverglasses with water of 0 05 ml.

Table I. *Survival Value of the Cells Remaining for Different Lengths of Time in Isopentane Bath at −60° Following Removal from Liquid N_2, Before Being Rewarmed in Water at 30°*

Tissue sections were mounted between coverglasses with water.

Prefreezing Temp	Time in isopentane bath at −60° 1 min	20 min	1 hr	6 hr	16 hr
− 5°	100*	100	90	25	0
−10°	100	100	100	100	30
−20°	100	100	100	100	100

* Percentage of survival.

during rewarming, the percent survival of cells prefrozen at −5° to −10° and rewarmed following immersion in liquid nitrogen, decreased abruptly in the temperature ranges from −50° to −60° and from −45° to −55°. In general, the percent survival of cells prefrozen at −20° was unaffected by rewarming, even when the cells were maintained at −5° to −25°, although a slight effect was noted in the temperature range from −30° to −40° (fig 2). It was also noted that at −60°, the percent survival decreased slowly with time (table I).

All of the cells were destroyed in mounted tissue sections that were not prefrozen prior to immersion in liquid nitrogen, even when the sections were rapidly rewarmed in water at 30°. However, cells in tissue sections treated with 2 M dimethyl sulfoxide survived such immersion and subsequent rapid rewarming in water at 30°.

Unmounted tissue sections held with forceps were rapidly immersed into liquid nitrogen from room temperature without prefreezing at the cooling rate of approximately 500° per second, and then rewarmed rapidly in water at 10° to 40° (approximately 400° per second), or slowly in air at

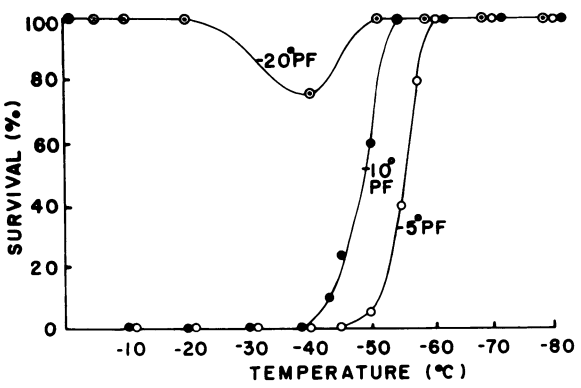

FIG. 2.

FIG. 2. Temperature range rapidly destroyed in the course of rewarming following removal from liquid nitrogen. 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°. The tissue sections were mounted between coverglasses with water.

room temperatures (approximately 30° per second). All of the cells survived rapid rewarming and all were destroyed by slow rewarming in air. Unmounted tissue sections held with forceps that were not prefrozen were rapidly immersed in isopentane baths cooled at various temperatures ranging from −10° to −100° and kept there for 20 seconds before being rapidly rewarmed in water at 30°. The results revealed that all of the cells were destroyed in the tissue sections immersed in isopentane baths at temperatures ranging from −30° to −40°, and that the percentage of survival was unaffected by immersion at temperatures below −60° (fig 3).

The percentage of survival at various rates of cooling was determined by other experiments. Tissue sections prefrozen at −5°, −10°, −15° and −20° were cooled rapidly by immersion in isopentane baths maintained at temperatures ranging from

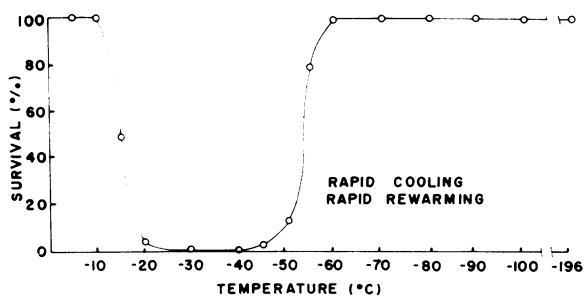

FIG. 3.

FIG. 3. Survival of cells cooled and rewarmed rapidly without prefreezing. An unmounted tissue section held with forceps was rapidly immersed in isopentane baths at various temperatures from room temperature without prefreezing, and then kept there for 20 seconds, before being rewarmed rapidly in water at 30°.

−5° to −100°. The sections remained in the baths for 10 minutes before being rewarmed slowly in air at 0° (fig 4), or rapidly in water at 30° (fig 5). Cells prefrozen at −20° could withstand even direct immersion in isopentane baths below −70° and subsequent slow rewarming in air at 0° (fig 4).

The curves shown in figure 4 may be considered as indicating the degree of intracellular freezing that cells prefrozen at various temperatures can withstand, since tissue sections were rapidly immersed in isopentane baths at various temperatures and then slowly rewarmed in air at 0°.

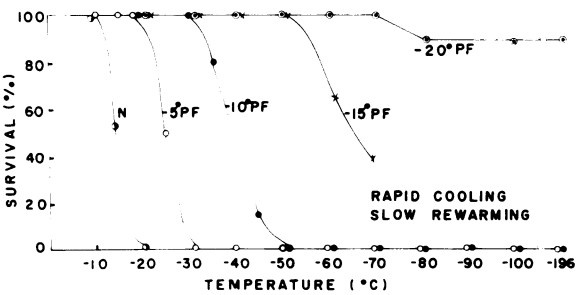

FIG. 4.

FIG. 4. Survival of cells in tissue sections immersed rapidly in isopentane baths at various temperatures for 10 minutes and subsequently rewarmed slowly in air at 0°. N indicates tissue sections not prefrozen, PF indicates prefreezing. Tissue sections were mounted between coverglasses with water.

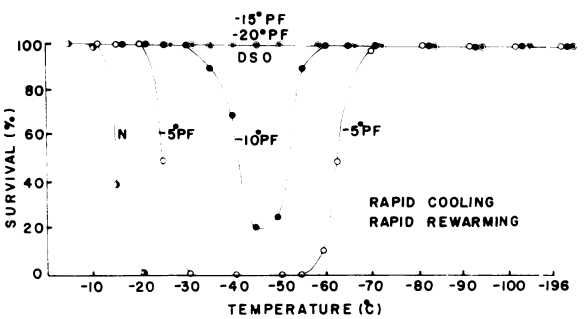

FIG. 5.

FIG. 5. Survival of cells in tissue sections immersed rapidly in isopentane baths at various temperatures and subsequently rewarmed rapidly in water at 30°. DSO indicates tissue sections treated with dimethyl sulfoxide. N indicates tissue sections not prefrozen.

With rapid rewarming, the survival value of cells immersed in isopentane baths at temperatures ranging from −5° to −70°, increased with decreasing temperatures (fig 5).

Tissue sections prefrozen at −5°, −10° and −20° and treated with 2 M dimethyl sulfoxide could survive rapid cooling to any temperature, if they were rapidly rewarmed in water at 30°. However, the percentage of survival varied with the length

Table II. *Survival Value of the Cells Remaining Various Temperatures for 24 Hours Following Rapid Cooling in Isopentane Baths Below −60°.*

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

Prefreezing Temp	Temp −60°*	−60°	−70°	−80°	Liquid N_2
− 5°	100**	0	0	0	100
−10°	100	50	100	100	100
−20°	100	100	100	100	100
Dimethyl sulfoxide	100	50	100	100	100

* Tissue sections kept at −60° for 10 minutes only.
** Percentage of survival.

of time kept even at temperatures below −60° (table II).

To investigate the effect of additives on the percentage of survival at super-low temperatures using this method, an experiment was performed using 2 M solutions of dimethyl sulfoxide, ethylene glycol, 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°. The percentage of survival obtained was as follows: dimethyl sulfoxide −100, ethylene glycol −100, glucose −70, sucrose −40, and glycerol −0.

Discussion

Luyet (4) proposed the hypothesis that formation of ice in protoplasm induces death; but vitrification, which causes less structural alternation is assumed to be less injurious and perhaps entirely innocuous. Many attempts have been made to check this hypothesis. It has been found, however, very difficult to maintain vibility by this method, especially in less hardy cells suspended in aqueous solutions at super-low temperatures (9). The expected results have been obtained only with a few materials, especially those desiccated (5) or treated with protective additives (6). Mosses with different water content were rapidly immersed in liquid N_2 and rewarmed; either slowly in air or rapidly in water at 20° (5). The results showed that mosses containing more than 65 % H_2O survived rapid rewarming but were destroyed by slow rewarming. Mosses containing less than 30 % water survived immersion no matter what rewarming procedure followed. Mosses containing from 65 % to 30 % H_2O survived immersion in liquid N_2 and rapid rewarming, but when they were rewarmed slowly, the percentage of survival increased gradually with decreasing water content. Goet et al. (2) immersed films of yeast cultures supported on metal loops in isopentane at −160° and then exposed them to crystallization temperatures from −150° to −5° for 30 minutes. The survival value showed that the number of cells destroyed gradually increases with increasing recrystallization temperatures from −150° to −5°. Sakai (10, 11) also succeeded in maintaining viability in undehydrated cortical cells of winter twigs, using the rapid cooling and rewarming method.

Recently, Luyet et al. (7-8) have increased our knowledge of recrystallization, using water and various aqueous solutions. However, almost all of their attempts to maintain the viability of blood cells without any protective agent using the rapid cooling and rewarming method, have resulted in failure (1, 9).

Highly hardy cortical parenchyma cells from winter twig of woody plants, even when mounted between coverglasses with water, can withstand freezing at any low temperature when cooled slowly in air, and even with subsequent rapid rewarming in water at 30°. There exists a critical region of temperature in which some animal cells are rapidly damaged during freezing and thawing (3). With the human red blood cell this region extends −3° to −40°; and if more than a few seconds are spent between these temperatures damage takes place. Such a critical temperature range does not exist in plant cells. It is reasonable to consider from these facts that the destruction of cortical cells may only be caused by intracellular freezing.

Cells dehydrated by extracellular freezing at temperatures below −20° can survive immersion in liquid N_2 irrespective of the rewarming methods used, in contrast to cells prefrozen at temperatures above approximately −15°. Also, the percentage of survival of insufficiently prefrozen cells decreased abruptly during rewarming following removal from liquid N_2. In the prefrozen cells at −20°, however, a slight cell destruction was only observed in the temperature range from —30° to −40°. These sufficiently prefrozen cells can also withstand rapid cooling by direct immersion in isopentane at temperatures ranging from −10° to −100° and subsequent slow or rapid rewarming.

It is reasonable to assume from these results that almost all of the freezable water in a cell may be withdrawn by extracellular freezing at temperatures below −20°. If any freezable water remains in the cells following prefreezing, the intracellular crystallization nuclei that are formed during the very rapid cooling in liquid N_2 or at any low temperatures will probably grow during the subsequent slow rewarming and will damage the cells, although there has been no direct evidence of this in the experiments reported in this paper.

It has been demonstrated in many insects and plants that more than 90 % of the total water content, or nearly all of the freezable water crystallizes

at −30° (14–16). These results show that the amount of unfrozen water remaining in cells, as determined by the calorimetric method, decreases gradually and continuously with decreasing temperatures, especially below −15°. Therefore, it is nearly impossible to determine by this method the temperature at which the freezable water is withdrawn by extracellular freezing. However, this temperature can be roughly determined from the curves of the survival value in cells immersed in liquid nitrogen following prefreezing at various temperatures (10–12).

Literature Cited

1. Gehenio, P. M., G. L. Rapatz, and B. J. Luyet. 1963. Effects of freezing velocities in causing or preventing hemolysis. Biodynamica 9: 77–82.
2. Goet, A. and S. S. Goet. 1938. Death by devitrification in yeast cells. Biodynamica 43: 1–8.
3. Lovelock, J. E. 1954. The protective action of neutral solutes against haemolysis by freezing and thawing. Biochem. J. 56: 265–70.
4. Luyet, B. J. 1937. The vitrification of organic colloids and of protoplasm. Biodynamica 29: 1–14.
5. Luyet, B. J. and P. M. Gehenio. 1938. The survival of moss vitrified in liquid air and its relation to water content. Biodynamica 42: 1–7.
6. Luyet, B. J. and P. M. Gehenio. 1954. Effect of the rewarming velocity on the survival of embryonic tissues frozen after treatment in ethylene glycol. Biodynamica 7: 213–23.
7. Luyet, B. and G. Rapatz. 1958. Patterns of ice formation in some aqueous solutions. Biodynamica 8: 1–68.
8. Luyet, B. 1960. On various phase transitions occurring in aqueous solutions at low temperatures. Ann. N. Y. Acad. Sci. 85: 549–69.
9. Rapatz, G. and B. Luyet. 1963. Effects of cooling rates on the preservation of erythrocytes in frozen glycerolated blood. Biodynamica 9: 125–36.
10. Sakai, A. 1956. Survival of plant tissue at superlow temperatures. Low Temp. Sci. Ser. B, 14: 17–23.
11. Sakai, A. 1958. Survival of plant tissue at superlow temperatures. II. Low Temp. Sci. Ser. B, 16: 41–53.
12. Sakai, A. 1960. Survival of the twig of woody plants at −196°. Nature 185: 392–94.
13. Sakai, A. 1965. Survival of plant tissue at superlow temperatures. III. Relation between effective prefreezing temperatures and degree of frost hardiness. Plant Physiol. 40: 882–87.
14. Scholander, P. F., W. Flagg, R. J. Hock, and L. Irving. 1953. Studies on the physiology of frozen plants and animals in arctic. J. Cell. Comp. Physiol. 42: 1–56.
15. Shinozaki, J. 1962. Amount of ice formed in the prepupa of slug Moth and its periodicity. Contrib. Inst. Low Temp. Sci. Ser. B, 12: 1–52.
16. Tranquillini, W. and K. Holzer. 1958. Über das Gefrien und Auftau von Coniferennädeln. Ber. Deut. Botan. Ges. 71: 143–56.