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Survival of Plant Tissue at Super-Low Temperature VI. Effects of Cooling and Rewarming Rates on Survival

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Summary. The survival rates of the cortical parenchymal cells of mulberry tree were determined as a function of cooling and rewarming rates. When cooling was carried out slowly at 1° to 15° per minute, all of the cells still remained viable even when rewarmed either rapidly or slowly. Survival rates gradually decreased to zero as the cooling rate increased from about 15° to 2000° per minute. In the intermediate cooling rates, when the cells were cooled at the rates lower than 14° per minute, from −2.2° to about −10°, these cells could survive subsequent rapid cooling and rewarming.

However, at cooling rates above 1000° per minute and with rapid rewarming, the effect of cooling rate reversed and survival increased, reaching a maximum at about 200,000° per minute. As the cooling rate increased above 15° per minute, survival rates became increasingly dependent on the rewarming rate, with rapid rewarming becoming less deleterious than slow rewarming.

The temperature range at which damage occurred during rewarming following removal from liquid nitrogen and in which growth rate of ice crystallization was greatest, was −30° to −40°. The survival rates even in the prefrozen cells at −30° decreased considerably by keeping them at −30° for 10 minutes after removal from liquid nitrogen. This fact indicates that intracellular freezable water remains to some degree even in the prefrozen cells at −30°. After removal from liquid nitrogen, all cells retained their viability, when they were passed rapidly through a temperature range between −50° and −2.5° within about 2 seconds, namely at the rates greater than 1000° per minute.

These observations are explained in terms of the size of the crystals formed within the cortical cells.

In order to clarify the mechanism of survival at super-low temperatures with rapid cooling and rewarming (4, 5, 6), further studies were made by using the same cortical parenchymal cells of winter twigs of mulberry tree.

In this paper, the effects of the cooling and rewarming rates upon the survival, the temperature range at which the growth rate of intracellular crystallization is greatest, and the speed of rehydration in cells rewarmed rapidly at subzero temperatures, were investigated.

Materials and Methods

Cortical parenchymal 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. Tangential tissue sections (20–30 μ thick, 1–2 mm wide and 2–3 mm long) were cut from the cortical regions of twigs using razor. Ten tissue sections were used in each experiment. The osmotic concentrations of the parenchymal cells were determined in a balanced salt solution by the usual plasmolytic method. This value was 1.4 M. Winter twigs of white birch (*Betula tauschii* Koidz.) and willow (*Salix sachalinensis* Fr. Schm.) were also used.

Tissue sections were mounted between cover glasses (18 × 18 mm) with 0.05 ml water and cooled in 1 of several ways. The frozen cells were rewarmed, either rapidly in water at 30° (rapid rewarming: 24,000° per min) or slowly in air at 0° (slow rewarming: 160° per min). In order to obtain the greatest cooling rate, an unmounted tissue section was held with a forceps and was immersed directly into an isopentane bath at −150° or into liquid nitrogen.

The temperature was determined with 0.1 mm copper-constantan thermocouple and was recorded by a oscilloscope. The cooling rate of tissue sections mounted with water between cover glasses was determined by putting a thermocouple between these cover glasses. It is, however, very difficult to determine the exact cooling rate of an unmounted

tissue section held with small forceps. Accordingly, the cooling rate was determined by inserting a thermocouple between 2 tissue sections sticked with a binding agent. Therefore, the cooling rate determined by this method may be much lower than that obtained in an unmounted tissue section held with a forceps. The cooling rate was represented as the time required for the temperature to fall from about −5° to within 5° of the final bath temperature, unless otherwise noted. Rewarming rate was also represented as the time required for the temperature to rise from the freezing bath temperature to a few degrees below melting point (about −2.2°) of the tissue.

To dehydrate the cells, tissue sections or twigs were frozen at −5° and then cooled slowly to various temperatures (3).

The viability of cells was determined by the vital staining test using neutral red and the plasmolysis test. Plasmolysis and deplasmolysis were repeated twice with a 2 fold isotonic balanced salt solution and water. As a result of the treatment, normally stained and plasmolysed cells were regarded as normal ones and survival rate was expressed as percentage of normal cells in 10 tissue sections. Cortical parenchymal cells of mulberry tree are very favorable to determining the percentage of survival, because unlike many other trees such as willow, poplar, conifers, etc., when 1 cell layer of the cortical tissue is sliced, all of the cells in a tissue section remain alive.

Results

Effects of Cooling and Rewarming Rates on Survival. To investigate the effect of rewarming rate upon survival of cells cooled slowly, tissue sections mounted between cover glasses with water were frozen at −5° and then gradually cooled down to −10°, −30°, −50°, and −70°, respectively, in air at the rate of about 14° per minute. Also, tissue sections cooled down to −30° were immersed in liquid nitrogen. These tissue sections frozen at various temperatures were then rewarmed either slowly in air at 0° or rapidly in water at 30°. All of the cells rewarmed either slowly or rapidly from various temperatures survived.

To clarify the effects of cooling and rewarming rates on the survival of cells cooled in different rates, tissue sections mounted or unmounted were cooled down to −75° at indicated cooling rates and rewarmed slowly or rapidly. The survival rates of the cortical cells as a function of cooling and rewarming rates are summarized in figure 1. When cooling is carried out slowly at 1° to 10° per minute, all of the cells remain viable even when rewarmed either rapidly or slowly. Then the survival rates gradually decrease to zero as the cooling rate increases from about 50° to 2000° per minute. However, at cooling rate above 10,000° per minute

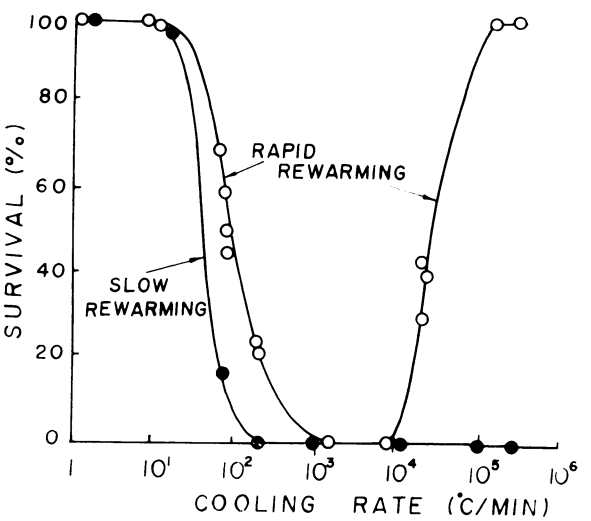

FIG. 1. Survival of cortical cells cooled to −75° at indicated rates on the abscissa and rewarmed either rapidly or slowly. In slow cooling rates, tissue sections mounted with water between cover glasses were cooled with various methods. In the cooling rates higher than 100,000° per minute, an unmounted tissue section held with small forceps were rapidly immersed in liquid nitrogen or liquid isopentane. Rapid rewarming: in water at 30°. Slow rewarming: in air at 0°.

and with rapid rewarming, the effects reverse and survival rates rise, reaching a maximum at 200,000° per minute. When tissue sections are passed rapidly through the temperature range from freezing point of the tissue to −55° within 0.045 seconds, all retain their viability with subsequent rapid rewarming (fig 2). As the cooling rate rises above 15° per minute, survival rates become increasingly dependent on the rewarming rate, with rapid rewarming becoming less deleterious than slow. In slow rewarming, the survival still remain the minimum even at the highest cooling rate used unlike rapid rewarming.

At intermediate cooling rates, the continued drop in survival as cooling rate rises towards 2000° per minute is observed. In these cooling rates, the intracellular water will not be able to dehydrate sufficiently rapidly to maintain vapor pressure equilibrium with the external ice, and so the cell contents will become increasingly supercooled, and they will eventually freeze intracellularly.

The problem arises that in the cells cooled to −70° at those intermediate cooling rates and subsequently rewarmed rapidly, death occurs at any temperature range during the process of cooling. Therefore, the cortical cells were mounted between cover glasses with water and cooled to −70° at different cooling rates from 14° to 1000° per minute. Cooling was also broken at −10°, −20° and −30° respectively, in the course of cooling to −70°, by rapid immersion in water at 40°. In this

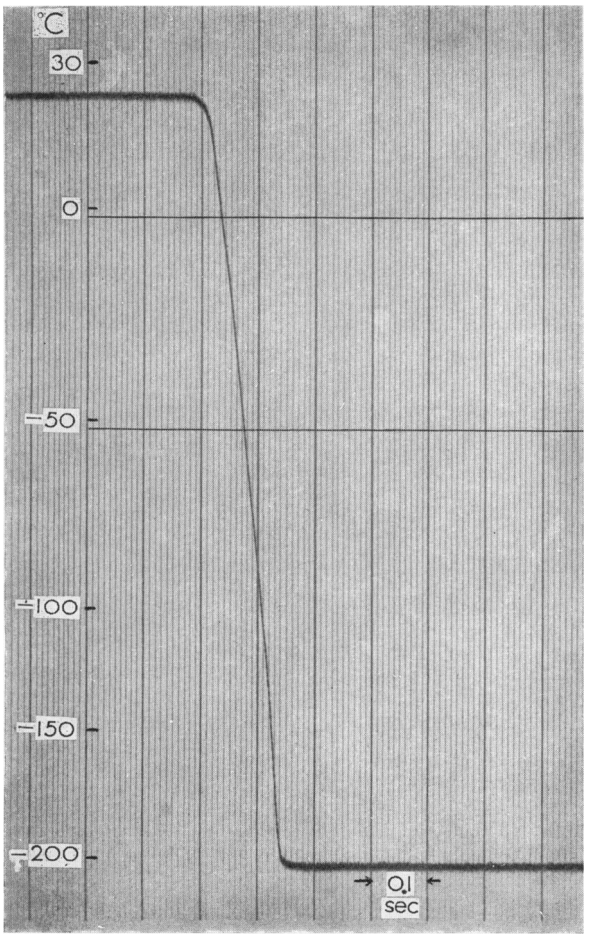

FIG. 2. Oscillogram of a tissue section immersed rapidly into liquid nitrogen. An unmounted tissue section held with small forceps was rapidly immersed into liquid nitrogen. In this case, the time required for the temperature to fall from —2.5 to —55° was within 0.045 seconds. The temperature for the tissue section was determined with 0.1 mm copper-constantan thermocouple and recorded with a oscilloscope.

experiment, the process of cooling was followed with an oscilloscope. In addition, immediately after cooling to −20° and −30° at various rates, tissue sections were rapidly immersed into liquid nitrogen and then they were rapidly rewarmed in water at 40°. In each curve A, B, C and D in figure 3, the times necessary to cool from freezing point (−2.2°) to −20° are 1, 2, 7 and 57 seconds, respectively. Numerals noted alongside the individual curves in figure 3 represent survival rates of the cells in tissue sections which were broken cooling at the indicated temperatures. Numerals noted under arrows indicate the survival rates of the cells in tissue sections immersed into liquid nitrogen from the individual temperatures. In cooling curves A (1000°/min) and B (420°/min), survival rates abruptly decrease at the temperature range from −10° to −30°, especially to −20°.

When tissue sections cooled to −20° at the rates of 420° and 1000° per minute were immersed into liquid nitrogen, almost all of the cells were killed. However, when cooled at a slow rate (70°/min), only a slight decrease in survival was observed during the process of cooling and also half of the cells cooled to −20° at the same rate were alive after immersion into liquid nitrogen. In curve D (14°/min), all of the cells cooled to −70° were alive, but the survival rate in the cells immersed into liquid nitrogen after cooling to −10° was 65. In contrast, tissue sections cooled to −10° at the same cooling rate were held there for 30 seconds, 1 and 3 minutes respectively, before rapid immersion in liquid nitrogen and subsequent rapid rewarming (curve E). All of the cells kept for 3 minutes at −10° were alive. Besides, even in the tissue sections kept at −5° for 5 minutes in frozen state, most of the cells remained alive after immersion in liquid nitrogen and subsequent rapid rewarming.

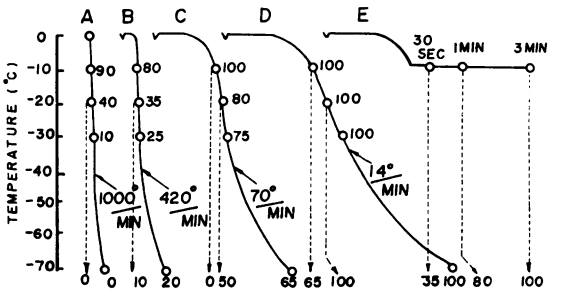

FIG. 3. Survival of cortical cells cooled to —70° at intermediate cooling rates. Cortical cells were mounted between cover glasses with water and were cooled to —70° at the rates noted alongside the individual curves. Numerals noted alongside the individual curves represent survival rates of the cells in tissue sections which were broken cooling at the indicated temperatures by rapid immersion in water at 40°. Numerals under allows indicate survival rates of the cells in tissue sections immersed into liquid nitrogen from various temperatures. In all experiments, rewarming was rapid.

For maintaining their viability after cooling to deep temperatures at the intermediate cooling rates, it is necessary to cool slowly in the frozen state the temperature range between freezing point and about −10°.

Effect of Rewarming Rate on Survival of Cells Immersed in Liquid Nitrogen. To investigate the temperature range at which the damage occurs during rewarming following removal from liquid nitrogen, tissue sections immersed in liquid nitrogen following prefreezing at −10° for 5 minutes were rapidly transferred to isopentane baths at temperatures ranging from −5° to −70° at 5° of temperature intervals and were kept there for 3 seconds, 3, 10, and 60 minutes respectively before rapid rewarming. The results are summarized in figure 4.

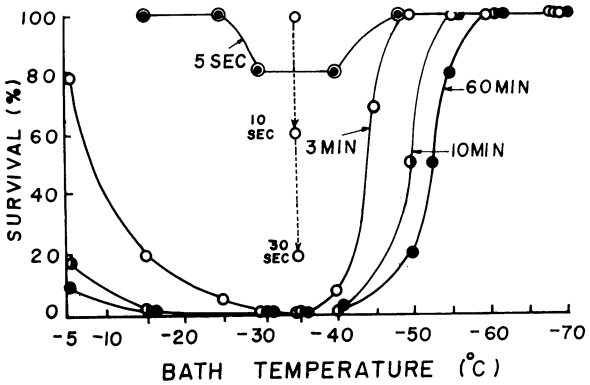

FIG. 4. Survival of prefrozen cells kept for different times at various temperatures following removal from liquid nitrogen. Tissue sections prefrozen at —10° for 5 minutes were immersed into liquid nitrogen. The prefrozen cells were kept for 3 seconds, 3, 10, and 60 minutes in isopentane baths maintained at various temperatures following removal from liquid nitrogen, before being rapidly rewarmed in water at 30°.

The main characteristic of survival is that, in a limited temperature range, the survival abruptly decreases with the rising temperature from −60° to −50°, from −55° to −45° and from −45° to −40° in the cells kept for 60, 10 and 3 minutes respectively. In the cells kept at various temperatures for 5 seconds, only a slight decrease in survival was noted in the temperature range from −40° to −30°. In this experiment, it took about 3 seconds to cool from −60° to −30°. When rewarmed immediately after reaching to −30°, all were alive, while the survival rates of the cells kept at −35° decreased with the length of time, and most of the cells were killed in 30 seconds.

To clarify the effect of rewarming rate on the survival, the mounted tissue sections between cover glasses with water were prefrozen at −10° for 5 minutes, and were immersed into liquid nitrogen before being transferred to an isopentane bath at −60°. These cells were then rewarmed at various rewarming rates. When these cells were passed rapidly through the temperature range from −55° to −2.5° within about 2.2 seconds (rates greater than 1200° per min), they could retain viability (fig 5).

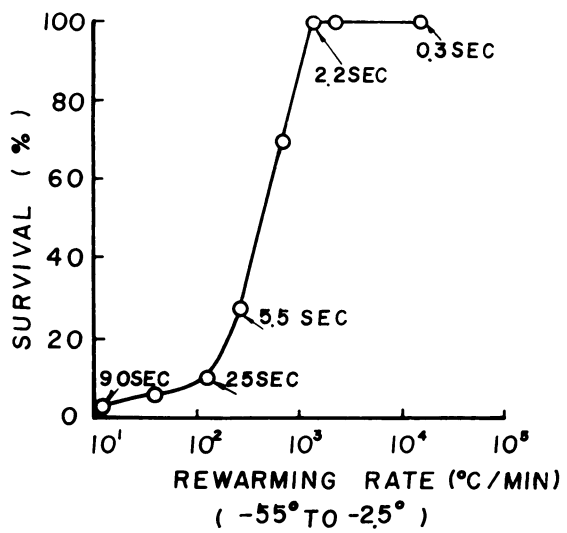

FIG. 5. Effect of rewarming rate upon the survival of the cells immersed in liquid nitrogen. Tissue sections mounted between cover glasses with water were prefrozen at —10° for 5 minutes before immersion in liquid nitrogen and then transferred to isopentane bath at —60°. The tissue sections kept at —60° were rewarmed at various rates. Times noted alongside the curve indicate the time required for the temperature to rise from —55 to —2.5° at various rewarming rates.

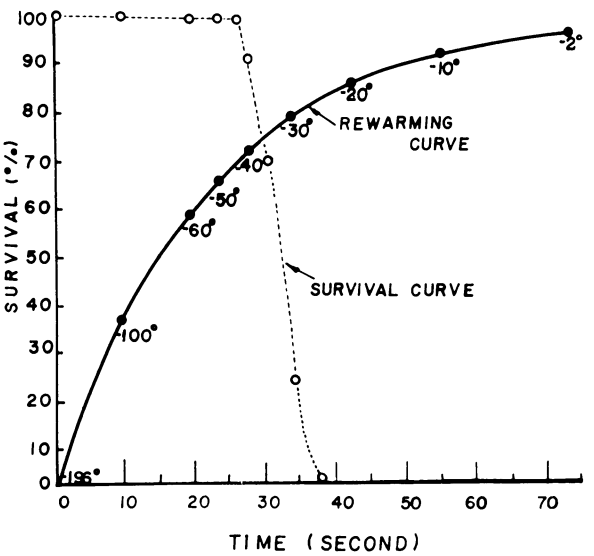

FIG. 6. Temperature range causing damage in the process of rewarming following removal from liquid nitrogen. The cortical cells prefrozen at —10° and immersed into liquid nitrogen were slowly rewarmed (at rate 160° per min) in air at 0°. In the process of rewarming following removal from liquid nitrogen, tissue sections were rapidly rewarmed at various temperatures by immersion into water at 30°. Survival rates of the cells in tissue sections rewarmed rapidly at various temperatures are shown on survival curve.

In order to determine the temperature range at which the cells are damaged during rewarming after removal from liquid nitrogen, another experiment was made. Tissue sections prefrozen at −10° were rewarmed in air at rate 160° per minute after immersion in liquid nitrogen. The tissue sections in the process of rewarming were rapidly rewarmed by immersion into water at 30°, immediately after reaching to various temperatures. As shown in figure 6, in this experiment also, almost all of the cells were killed abruptly at the temperature range between −40° and −30° within 6 seconds.

Amount of Freezable Water Remaining in Cells After Prefreezing at Various Temperatures. Nearly the same results as depicted in figure 4, were obtained in the prefrozen cells at −20°, except that the survival rates of the cells kept at the temperatures above −20° were much higher than those of the cells prefrozen at −10°. Even in the cells prefrozen at −20°, almost all of the cells were killed after exposure, within 1 minute, to the temperature range from −30° to −40° following removal from liquid nitrogen, before being rewarmed rapidly. These findings may be interpreted to indicate that freezable water still remains to some degree in those cells after prefreezing at −20° and the intracellular crystallization nuclei formed in the course of a rapid cooling to the temperature of liquid nitrogen probably grow and cause damage during a subsequent rewarming. It may be therefore considered that sufficiently dehydrated cells by extracellular freezing are not damaged by rapid immersion into liquid nitrogen and by subsequent exposure to −30° for 60 minutes following removal from liquid nitrogen.

To strengthen this notion and to investigate the effect of rewarming conditions upon the survival rates of cells prefrozen at various temperatures, tissue sections mounted between cover glasses with water were frozen at −5°, and were then slowly cooled to various temperatures. They were kept there for 10 minutes and then immersed into liquid nitrogen before being rewarmed at different conditions. The results obtained are summarized in figure 7. When rewarmed rapidly by immersion into water at 40°, all of the cells prefrozen at the temperatures below −10° could survive immersion in liquid nitrogen, and also when rewarmed slowly in air at 0°, almost all of the cells prefrozen at the temperatures below −25° retained their viability. However, when kept at −30° for 60 minutes following removal from liquid nitrogen, before being rewarmed rapidly by immersion in water, the survival rates of cells prefrozen at the temperatures above −30°, decreased considerably than those of the cells rewarmed slowly in air at 0°. However, in the prefrozen cells at the temperatures below −45°, almost all of the cells still retained their viability even after keeping at −30° for 60 minutes following removal from liquid nitrogen. From these findings, it seems reasonable to consider that almost all of the freezable water in cells will be withdrawn by extracellular freezing down to about −45°.

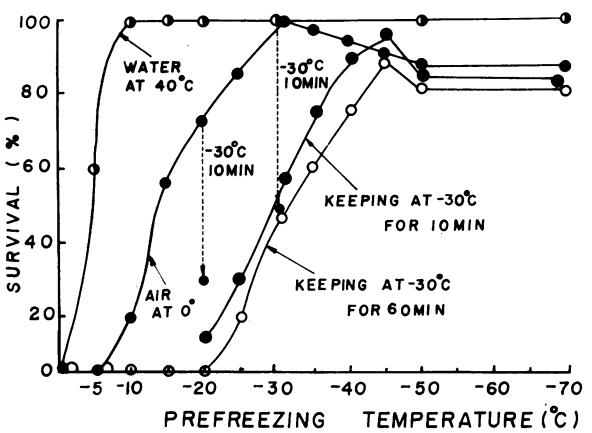

FIG. 7. Effect of rewarming conditions upon the survival cells immersed in liquid nitrogen after prefreezing at various temperatures. The tissue sections mounted between cover glasses with 0.05 ml water were prefrozen at various temperatures for 10 minutes. Then, these prefrozen cells were rewarmed at various conditions after removal from liquid nitrogen. Rewarming conditions: in water at 40°, in air at 0° and in water at 40° after exposure to —30° for 10 or 60 minutes following removal from liquid nitrogen. Broken lines indicate the decrease in survival of the cells in the tissue sections kept at —30° for 10 minutes after removal from liquid nitrogen.

Rapid Cooling and Rewarming of Twigs Prefrozen at −10°. Some experiments were made with rapid cooling and rewarming for maintaining the viability of twigs after immersion into liquid nitrogen. It was demonstrated in previous reports (3) that prefreezing temperature which is effective in maintaining the viability of twigs of willow (*Salix sachalinensis*) following immersion in liquid nitrogen was −15°, provided that they were kept at −30° for 3 hours after removal from liquid nitrogen, before being rewarmed slowly in air at 0°. It was also confirmed that the twigs frozen at the temperatures below −15° were killed by rapid thawing by a direct immersion in water at 30°, while these twigs frozen at the temperatures above −15° were not damaged by the same rapid thawing. Therefore, in the twigs dehydrated partially at −5° and −10°, freezable water still remains to some degree in these cells. Also, these twigs are not damaged by rapid thawing.

Twig pieces (10 cm in length, 0.4 cm in diameter) of willow were tied with a thread and prefrozen at −5° or −10° for 3 hours. These prefrozen twigs were immersed in liquid nitrogen and then rewarmed rapidly in water at 40°. To determine the intactness of these twig pieces, they were planted in water for 2 months. The prefrozen twigs at −5° became brown soon after thawing and the prefrozen twigs at −10° still remained normal for 2 weeks, but gradually became abnormal. In contrast to the findings of Krasavtsev (7, 8), all of the treated twigs perished. The same result was also obtained with the winter twigs of white birch (10 cm in length, 0.4 cm in diameter).

Speed of Rehydration in Frozen State. To know roughly the speed at which frozen cells are rehydrated as temperature ascends, some experiments were made by using winter twigs of white birch. As mentioned above, the prefreezing temperature which is effective for maintaining the viability of twigs of white birch after immersion in liquid nitrogen is −15°. Therefore, if the cells

in the twigs have been rehydrated fully during rewarming from −30° to −10°, these twigs will be killed by immersion in liquid nitrogen.

To check this problem, the frozen twigs at −5° were gradually cooled down to −30° and kept there for 16 hours. These frozen twigs were immersed into isopentane bath at −10° and were kept there for 30 seconds, 1, 3, and 10 minutes respectively. These twigs were then immersed into liquid nitrogen, before being rewarmed slowly in air at 0°. 2 of 4 twigs kept at −10° for 30 seconds developed their leaves, while all of these twigs kept at −10° for 1, 3, and 10 minutes respectively, showed no sign of the development. This fact indicates that when those twigs were rewarmed from −30° to −10°, the cells in those twigs could be rehydrated within 1 minute even in frozen state.

Discussion

Tissue sections mounted between cover glasses with water could withstand slow freezing to any temperature above −196°. These cells frozen at any temperature could also survive subsequent rapid or slow rewarming although cells from less hardy plant are well known to be sensitive to a rapid thawing. However, as shown in previous papers (4, 5), when tissue sections mounted in water were rapidly frozen by direct immersion from room temperature into isopentane baths below −20°, all of the cells were destroyed by intracellular freezing, irrespective of the rewarming rates. Besides, unlike human red blood cells, a critical temperature range does not exist in the cortical cells at least within 24 hours. From these facts it is reasonable to consider that the destruction of cortical cells may be caused only by intracellular freezing.

For these reasons, this material is favorable to consider the mechanism of injury on the basis of survival rate, and also this simplifies the interpretation of the results.

Mazur (1, 2) calculated percentage of intracellular water remaining in yeast cells as a function of temperature and cooling velocity. The cells cooled slowly at the rate lower than 10° per minute will nearly remain at equilibrium with the external ice by dehydration and will reach equilibrium when the temperature reaches −4° in the rate 1° per minute or −8° in the rate 10° per minute. On the other hand, the water content of the cells cooled at 10° per minute or more is markedly greater than the equilibrium water content, and is extensively supercooled.

In the material used here, survival rates also decrease as cooling rate rises from about 50° to 2000° per minute, reaching nearly zero at the cooling rate 1000° per minute. The continued drop in survival as the cooling rate rises towards 2000° per minute presumably reflects that an increasing proportion of the intracellular water results in an increasing proportion of the cells frozen intracellularly. The following facts also seem to support this consideration.

Even at the injurious cooling rates pointed above, if the cells are cooled sufficiently slowly to maintain vapor pressure equilibrium with the external ice, or are kept at −5° or −10° in frozen state for 3 minutes, a subsequent rapid cooling to deep temperatures has no effect on the survival of the cells, provided that they are rewarmed rapidly.

At cooling rates above about 10,000° per minute and with rapid rewarming, the effect reverses and survival rates rise abruptly with the increasing cooling rate until at the highest cooling rate of 200,000° per minute. These observations are explained in terms of crystal size (4, 5). As the cooling rate rises, the ice crystals that form within some of the cells begin to be small or imperfect enough to be innocuous, hence, when rewarming is carried out rapidly, they melt before they have time to grow, and most of the cells still remain viable. However, when rewarming is carried out slowly, they grow to damaging size and kill the cells. At intermediate cooling rates, survival rates remain low even with rapid rewarming used. Presumably, this means that the crystals that form in most of the cells during cooling are large enough to be immediately damaging.

Recently, some direct evidences supporting these considerations were obtained with an electron microscope (6). When immersed rapidly into isopentane baths below −60° or into liquid nitrogen, the intracellular ice cavities left after freeze-substitution of the ice particles formed during the initial rapid cooling, could not be found in these cells. Further, the tissue sections immersed in liquid nitrogen were rapidly transferred to isopentane baths at temperatures ranging from −70° to −10° before rapid rewarming. No damage was observed in the cells kept at temperatures below −50° for 10 minutes or below −60° for 16 hours. No ice cavities were also found in these cells. However, above −45°, especially above −30°, all of the cells were completely destroyed even when exposed only for 1 minute, and many ice cavities were observed throughout the cells. To determine the damaging size of intracellular ice crystals, further studies are being made. The mechanism of growth of intracellular nuclei or crystallite is not yet clear.

If any freezable water still remains in cells following prefreezing, these cells will be damaged during a subsequent slow rewarming in air. Survival rates of cells prefrozen at various temperatures are affected remarkably by rewarming rates after removal from liquid nitrogen. It is therefore impossible to know, on the basis of the survival rate alone, the temperature at which almost all of the freezable water in cells has been withdrawn. However, it may be possible to know roughly this temperature on the basis of survival curves, pro-

vided that these cells immersed in liquid nitrogen are rewarmed very slowly by keeping at −30° for 60 minutes. This temperature lies around −45° in the cortical cells used. To check the validy of this method, some trials are being made with electron microscope and with usual calorimetric method. However, at deep temperatures, it seems to be difficult to know the exact temperature with the later, especially in the cortical cells unlike twigs pieces.

In a previous report (3) it was demonstrated that plant tissues which can survive freezing at approximately −70° are not injured, even when exposed to extremely low temperatures, and that the prefreezing temperatures necessary for maintaining viability after immersion into liquid nitrogen differed considerably in the temperature range from −15° to −30°, according to the degree of frost hardiness. However, the range was recently revised from −13° to approximately −70° as a result of further extensive researches. It was also confirmed that the greater the frost hardiness, the higher the effective prefreezing temperature (3). Therefore, the effective prefreezing temperature may be used as a reliable indication of the degree of frost hardiness in extremely hardy plants which can withstand freezing below −70°. To determine the effective prefreezing temperature, twigs were prefrozen at temperatures between −10° and −70° and then immersed in liquid nitrogen. These twigs were slowly rewarmed in air at −30° for 3 hours and then were rewarmed at 0°. Under this rewarming condition, if freezable water still remains to some degree in cells before being immersed in liquid nitrogen, these cells may be destroyed during slow rewarming. It may be reasonable to consider that the cells prefrozen at the temperatures which are effective in maintaining viability after immersion into liquid nitrogen has scarcely any freezable water in the cells, and that the greater the frost hardiness, the higher the temperature of withdrawal of intracellular freezable water.

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