

# Freezing Injury in Onion Bulb Cells

## II. POST-THAWING INJURY OR RECOVERY<sup>1</sup>

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### ABSTRACT

Onion (*Allium cepa* L.) bulbs were subjected for 12 days to either a moderate freeze ( $-4$  C) or a severe freeze ( $-11$  C). They were then thawed slowly over ice. During 7 to 12 days following the thaw, the injury progressed with time in the severely frozen bulbs, but appeared completely repaired in the moderately frozen bulbs. This was shown by the following post-thawing changes.

Infiltration of the intercellular spaces increased from 80 to 90% to 100% after the severe freeze, and decreased from 30 to 50% to zero after the moderate freeze. All of the cells were alive immediately after thawing whether the freeze was moderate or severe. Corresponding to the infiltration results 7 to 12 days later, many to most were dead following the severe freeze, all were alive following the moderate freeze.

The conductivity of the effusate from pieces of bulb tissue increased after the severe freezing, and decreased after the moderate freezing. The concentration of  $K^+$ , total solutes, and sugars in the effusate paralleled the conductivity changes. Neither the pH of the effusate nor the permeability of the cells (as long as cells were living) to water was changed following either the severe or the moderate freezes. Some treatments of the thawed tissue following the severe freeze halted the progress of injury.

The above results indicate that the semipermeable properties of the cell are uninjured but that the ion and sugar transport mechanism is damaged by freezing. Most likely the primary injury is to the active transport mechanism involved in their transport. It must be concluded that the final injury following freezing and thawing cannot be evaluated from the degree of infiltration or the conductivity of the effusate immediately after thawing, since injury may progress or recede following the thawing.

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In agreement with earlier results obtained with other plants (6), our recent work with onion (*Allium cepa* L.) bulbs led us to suspect that freezing injury may occur at three moments: during freezing, during thawing, or after thawing (8). This injury results in the leakage of ions and sugars from the cells. The exosmosis was found to originate not from dead cells, since all of the cells were still alive on thawing. The damaged, but still living cells, afford us the opportunity to investigate the initial injurious events in frozen cells. The following investigation attempts to do this and to determine which treatments enhance the injury and which induce it to recede.

### MATERIALS AND METHODS

The materials and methods used in this investigation were the

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same as described earlier (8). Additional methods used were as follows.

**Observations on Thawed Onions.** As soon as thawing was complete, one-third of the bulb was removed and the third and fourth fleshy scales counting from the outside of this portion were used immediately for the experiments such as cell viability, permeability of cells to water, electrical conductivity, and pH of the effusate and also analysis of amounts of total solutes and sugar in the effusate. The experiments were repeated on the stored (at  $3 \pm 0.5$  C and 100% relative humidity) two-thirds of the bulb, 7 and 12 days after thawing. At each time one-third of the bulb was used. Visual observations on the extent of infiltration of the tissue were also made.

**Post-thawing Treatments of Injured Tissue.** In bulbs frozen to  $-11$  C the injury increased with time after thawing (Table I). An attempt was made to halt the progress of the injury by specific treatments after thawing. The scale pieces after decanting the effusate were rinsed with deionized  $H_2O$  several times to remove most of the solutes from extracellular space. A part of these scale pieces was then transferred to spring water and other parts to 0.01 M KCl, 0.01 M  $CaCl_2$ , and 0.01 M mixture of KCl and  $CaCl_2$  solution (9 parts of 1 M KCl + 1 part of 1 M  $CaCl_2$  were diluted). These pieces, contained in Petri dishes, were then stored at 3 C and observed for cell survival up to 15 days.

### RESULTS

#### CHANGES IN INFILTRATION OF SCALE TISSUE WITH TIME AFTER THAWING

Depending on the temperature to which the onion bulbs were frozen, the infiltration of the scale tissue increased or decreased with time after thawing (Table I). Although the two bulbs frozen to  $-11$  C differed slightly in the initial extent of infiltration of their scale tissue (90% and 80%, respectively), both of the bulbs became completely infiltrated 7 days after thawing, and remained unchanged even 12 days after thawing. The bulbs frozen to  $-4$  C, on the contrary, were 30% infiltrated in one onion and 50% infiltrated in another and the infiltration disappeared 7 days after thawing. These bulb scales resembled the unfrozen ( $+3$  control) bulb scales and remained this way 12 days after thawing.

These results show that the freezing injury, which leads to infiltration of the scale tissue, increased with time after thawing in bulbs frozen to  $-11$  C and decreased with time after thawing in bulbs frozen to  $-4$  C.

#### CHANGES IN CELL SURVIVAL WITH TIME AFTER THAWING

Immediately after thawing, all of the cells were living in all of the onion bulbs frozen to  $-11$  C as well as  $-4$  C (0 day readings, Table I). Seven days after thawing, only 40% and 90% (respectively) of the cells were living in two bulbs frozen to  $-11$  C. However, all of the cells were still living in both of the bulbs

Table I. Changes in the extent of infiltration and cell survival with time after thawing of previously frozen onion bulb scales

Onions were kept frozen at the respective temperatures for 12 days.  
(Note—all these values are within  $\pm 5\%$ ).

Freezing temp (C)	Onion	Infiltration <sup>1</sup> of the scale tissue (%)			Cell Survival (%)		
		Days after thawing			Days after thawing		
		0	7	12	0	7	12
-11	1	90	100	100	100	40	20
	2	80	100	100	100	90	80
-4	3	30	0	0	100	100	100
	4	50	0	0	100	100	100
+3	5	0	0	0	100	100	100
	6	0	0	0	100	100	100

<sup>1</sup>Visually estimated

frozen to  $-4$  C. Twelve days after thawing, the tissues further deteriorated in the bulbs frozen to  $-11$  C. The number of dead cells in the scale tissue continuously increased with time after thawing in bulbs frozen to  $-11$  C, whereas in bulbs frozen to  $-4$  C, all of the cells continued to be normal and alive as in the control bulbs. Cell survival after freezing was not indicated by the initial extent of infiltration of the tissues (Table I).

The two bulbs frozen to  $-11$  C gave markedly different values for cell survival (Table I). Since this freezing temperature ( $-11$  C) and freezing time (12 days) are very close to the stress that produces lethal damage, a slight variation in different bulbs can account for a larger variation among them for cell survival.

#### CHANGES IN ANALYSIS OF EFFUSATE

After shaking pieces of thawed scale tissue in deionized  $H_2O$  for about 3 hr, the effusate was collected and analyzed (8). The average values from four different measurements were plotted as functions of days after thawing (Fig. 1). To standardize the data for better comparison, their relative values were calculated and plotted as a function of days after thawing (Fig. 2). For this calculation the 0 day, as well as control values, were taken as 1, and the respective values for 7 days and 12 days were calculated. Therefore the plots in Figure 2 represent changes with respect to the control as well as with respect to values immediately after thawing (0 day readings).

**Conductivity.** Conductivity of the effusate markedly increased in bulbs frozen to  $-11$  C and decreased noticeably in bulbs frozen to  $-4$  C after 7 days of thawing. After this period, the changes, if any, were small—a slight decrease in both cases (Fig. 1). In the case of the control bulb scales, the conductivity increased slightly with time.

Relative to the control, conductivity increased about 12% in the  $-11$  C treatment 7 days after thawing, and this increase was 5% 12 days after thawing (Fig. 2). However, in the  $-4$  C treatment, conductivity decreased by 12% 7 days after thawing and by 18% 12 days after thawing. The smaller difference between the  $-11$  C and control (12-day readings) may be due to the decreased gradient following the initial loss. In bulbs frozen to  $-4$  C, on the contrary, there occurs a net influx of ions into the cells with time after thawing.

The data shown in Figures 1 and 2 are, as indicated earlier, from the analysis of the effusate collected after 3 hr of shaking time. However, the changes in these values with time after thawing were also affected by the length of the shaking time (Table II). Relative conductivity values in the case of the  $-11$  C treatment 12 days after thawing were 1.32 and 1.13 for 1 hr and 3 hr shaking times, respectively (Table II). This means that the conductivity of the effusate in the  $-11$  C treatment increased by 32% with a shaking time of 1 hr whereas this increase in conductivity was only 13% when the shaking time was 3 hr. Furthermore, in the  $-4$  C treatment where the conductivity of the effusate decreased with time after thawing (Figs. 1 and 2), this decrease was 24% for the 1-hr shaking time and only 11% for the 3-hr shaking time (Table II). Therefore, it is safe to conclude that the changes in various quantities (analyzed in the

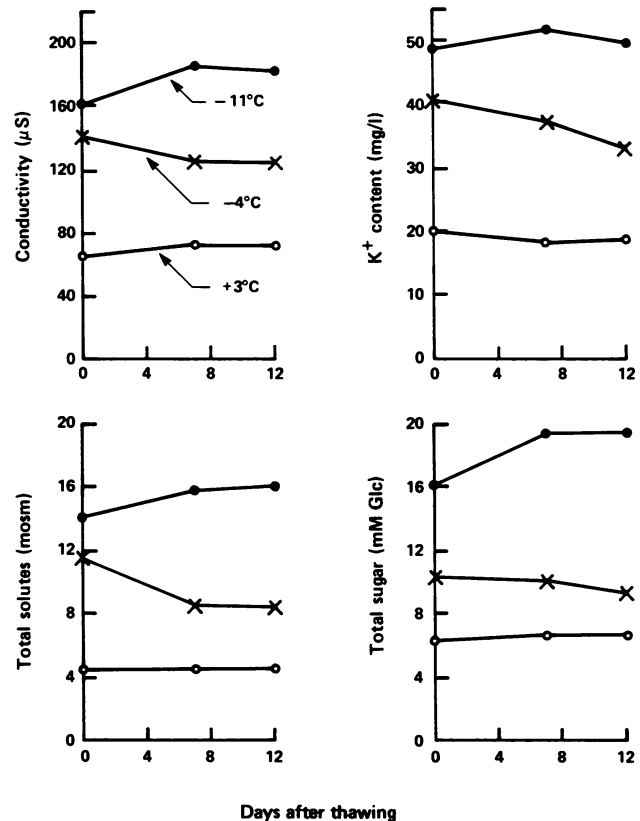


Fig. 1. Changes with time after thawing, in conductivity,  $\text{K}^+$  content, total solutes, and total sugar of the 3-hr effusate from a thawed piece of bulb scale (previously frozen to various temperatures).

effusate) with time after thawing (Figs. 1 and 2) would be more than two times if the effusate samples were collected after shaking the bulb scales with deionized  $H_2O$  for 1 hr. This explains why only an 11% decrease in conductivity was recorded during 12 days after the moderate freeze ( $-4$  C) although the cells recovered fully, as indicated by disappearance of the infiltration.

**$\text{K}^+$  Content.** Changes in the  $\text{K}^+$  content of the effusate with time after thawing were very similar to the conductivity changes (Fig. 2). This is to be expected because the  $\text{K}^+$  along with an unknown counterion accounts for essentially all of the conductivity of the effusate (8). A continuous decrease in  $\text{K}^+$  content of the effusate with time after thawing in bulbs frozen to  $-4$  C (Figs. 1 and 2) indicates a continuous net influx of  $\text{K}^+$  from the intercellular spaces into the cells. Conversely, in bulbs frozen to  $-11$  C a net efflux of  $\text{K}^+$  continues after thawing. However, 12 days after thawing there is a slight decrease in this net efflux of  $\text{K}^+$  (Figs. 1 and 2). As mentioned above, this may be due to a decreased gradient.

**Total Solutes.** In bulbs frozen to  $-11$  C, total solutes in the

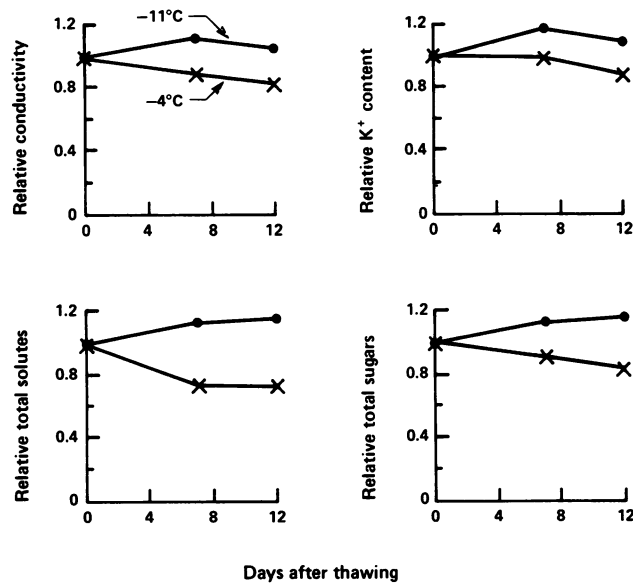


FIG. 2. Changes with time after thawing, in relative conductivity,  $K^+$  content, total solutes, and total sugars (expressed as glucose) of the 3-hr effusate from a thawed piece of bulb scale (previously frozen to various temperatures). Here, values have been standardized with respect to control (taken as 1) as well as with respect to time after thawing (0 day reading taken as 1).

Table II. Changes in relative conductivity of the effusate with time after thawing as affected by shaking time

Each value is an average of 4 separate measurements.

Freezing temp (C)	Relative conductivity of the effusate 12 days after thawing (zero day readings taken as 1)	
	Shaking Time	
	1 hour	3 hours
-11	1.32 ± 0.22	1.13 ± 0.04
-4	0.76 ± 0.03	0.89 ± 0.02
+3	1.08 ± 0.19	1.08 ± 0.08
(Control)		

effusate increased continuously with time after thawing. However, most of this increase came in the first 7 days after thawing (Fig. 1). Conversely, total solutes in the effusate decreased continuously after thawing in bulbs frozen to  $-4$  C and again most of this decrease came in the first 7 days after thawing (Fig. 1). In the control bulbs the total solutes in the effusate did not change at all with time.

Figure 2 shows that the changes in total solutes (in the effusate) after thawing were much more pronounced than the  $K^+$  content and conductivity changes in the effusate. This is to be expected, because  $K^+$  along with a counterion accounts for only 20% of the total solutes in the effusate (8). The increase and decrease of the total solutes in the effusate accompany the increase and decrease in the infiltration of the tissue (Table I).

**Total Sugars.** Total sugars in the effusate, in the case of the  $-11$  C treatment, increased after thawing in a manner similar to that of the total solutes (Fig. 1). In the case of the  $-4$  C treatment they decreased continuously with time after thawing, but this decrease was not as marked as for total solutes (Fig. 1). Unfortunately, the analysis for total sugar in the effusate samples was made 6 months after they were collected. Although these samples were kept frozen during storage, they developed a small amount of fungus growth. This resulted in a high variability in sugar content of these samples. Nevertheless, there was a decrease in total sugars with time after thawing in the case of the  $-4$  C treatment and an increase with time after thawing in the case of the  $-11$  C treatment (Fig. 1).

**pH.** There was no significant change in the pH of the effusate with time after thawing in any treatment (Table III). This is probably due to the buffering effect. The pH values of the effusate from the bulbs frozen to  $-11$  C were consistently higher than those of the  $-4$  C treatment and the control-treated bulbs (Table III).

#### PERMEABILITY OF CELL MEMBRANES TO WATER

Water permeability measurements were made on discs of inner epidermis. The water permeability values remained about  $2 \mu\text{m} \cdot \text{sec}^{-1}$  as long as all of the cells in the epidermal layer were observed under the microscope to be living. Only after the cells died was an increase in water permeability found.

#### REMEDIAL TREATMENT OF INJURED TISSUE

**Washing Out Solutes from Extracellular Space.** Normally injured tissue (frozen to  $-11$  C) became infiltrated with water (Table I) and appeared to have lost turgor. This loss of turgor is the result of the high concentration of solutes present in the extracellular space. With the above treatment in spring water, as the solutes were washed out, the tissues regained turgor and became quite normal in about 1 day. All of the cells survived in the scale tissue treated in this way compared to the increasing cell death with time in the untreated tissue (Table I). This treatment was tried at six different times and each time the survival of the cells was markedly increased. Observations on the treated tissue were made up to 15 days after treatment.

**Transferring Tissue to KCl and  $\text{CaCl}_2$  Solutions.** In general, treatments with 0.01 M  $\text{CaCl}_2$  or 0.01 M mixed KCl and  $\text{CaCl}_2$  solution increased cell survival significantly, whereas 0.01 M KCl did not improve cell survival over the untreated cells. These results, however, are preliminary in nature. The extent of the survival also depended upon the severity of the injury. Only when the injury was severe enough but not too severe (most of the cells dying within a few days after thawing) did this treatment improve cell survival compared to the 0.01 M KCl treatment and the control, untreated cells.

#### DISCUSSION

**Increase or Decrease of Injury after Thawing.** It has long been known that the extent of injury to plants kept at 0 to 5 C for 24 hr after freezing and thawing may be different than if immediately transferred to room temperature (7). This is usually interpreted to mean that freeze-damaged but still living cells may die at higher temperatures but may recover at 0 to 5 C. This interpretation has not been adequately investigated. The present investigation demonstrates that cell properties actually do change during a 1- to 2-week post-thawing period.

These results also emphasize the need for caution in evaluating freezing injury. Observations of infiltration and conductivity measurements made shortly after thawing cannot be used for evaluating the final injury 1 to 2 weeks later.

**Controlling Factors for Increase or Decrease in Injury during Thawing.** The infiltration of the intercellular spaces on thawing

Table III. Changes in pH value of the effusate with time after thawing of previously frozen onion bulb scales

Each value is an average of 4 separate measurements.

Freezing temp (C)	pH of the effusate		
	days after thawing		
	0	7	12
-11	6.22 ± 0.38	6.04 ± 0.63	6.29 ± 0.14
-4	5.61 ± 0.04	5.62 ± 0.09	5.52 ± 0.09
+3	5.50 ± 0.09	5.71 ± 0.22	5.51 ± 0.21
(Control)			

increases during 1 to 2 weeks after the more severe freeze, and disappears completely after the moderate freeze. Therefore, the more severely injured cells must be unable to reabsorb the solution from the intercellular spaces (and continue to lose solutes), the moderately injured are able to do so. Direct measurements on the effusate support this conclusion; for the conductivity,  $K^+$  and sugar concentration all increase after the severe freeze, all decrease after the moderate freeze. However, these are all measurements of a *net* leakage from the cells into the external solution. If there is still, to a limited extent, active transport going on in such cells, the increased leakage must be due to an excess of efflux over influx, the decreased leakage to an excess of influx over efflux. Since the leakage is from a high concentration inside the cell to a low concentration outside it, the efflux may be considered to be passive diffusion, but the influx must be due to active transport. The increased injury during thawing, as indicated by the net leakage, may result from either an increased efflux due to damage to the semipermeability of the plasmalemma, or a decreased influx due to damage to the active transport system. Conversely, the decreased injury during thawing may result from a recovery of one or the other of these two membrane properties.

This post-thawing increase in injury could be due to lack of oxygen in the infiltrated tissue. Also a high concentration of  $K^+$  present in the extracellular solution of the injured tissue may be further damaging to the cell. The reason for this damage is probably by replacement of  $Ca^{2+}$  in the plasma membrane by  $K^+$  thus making the cell membrane structure weaker (10). A higher concentration of  $Ca^{2+}$  found in the effusate of injured tissue compared to control (8) supports this reasoning.

**Initial Steps in Freezing Injury.** The above results point to either the semipermeability of the cell (plasma) membranes (*i.e.* the phospholipid portion) or the active transport sites as the locus of the initial injurious effect of freezing. Measurements of water permeability, however, showed that no change occurred in the injured cells, indicating that the passive semipermeable properties of the membrane remained unchanged.

Furthermore, the disappearance of the partial infiltration of the intercellular spaces following the moderate freeze is possible

only as a result of active transport of the intercellular solutes into the cells. Therefore, the active transport component of the membrane appears to be the locus of initial freezing injury.

It is now accepted that membrane-located ATPases constitute the ion pumps (9). Several ATPases involved in ion uptake by plant cells have been described (1, 4, 5, 7). It has already been found that freezing may in some way inactivate the ATPases of chloroplasts (2, 3).

Therefore, the plasma membrane ATPases and the mechanism for active sugar transport may conceivably be the loci of the initial freezing injury. This hypothesis would also explain the high  $K^+$  and sugar concentrations in the effusate from the frozen and thawed, but still living cells. If the freezing damaged the  $K^+$ -activated ATPase and the mechanism for active sugar transport, this would decrease the active influx of  $K^+$  and sugars while permitting the passive efflux unaltered or enhanced (the damaged ion pumps and mechanisms for active sugar transport could be visualized as sites for facilitated effusion). This would result in a net efflux of  $K^+$  and sugars.

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