Alterations in membrane transport properties by freezing injury in herbaceous plants: Evidence against rupture theory

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Abstract

Leakage of ions from a thawed tissue is a common phenomenon of freezing injury. This leakage is usually assumed to be due to loss of membrane semipermeability or membrane rupture by freezing injury. Freeze injured, yet living, onion (*Allium cepa* L.) epidermal cells were used to study alterations in cell membranes that result in leakage of ions. In spite of a large efflux of ions, freeze injured cells could be plasmolysed and they remained plasmolysed for several days just like the unfrozen control cells. Injured cells also exhibited protoplasmic streaming.

Passive transport of KCl, urea and methyl urea across the cell membranes of injured and control cells was also studied. No difference could be detected for the transport rates of urea and methyl urea between control and injured cells. However, a dramatic increase in the transport rate of KCl was found for the injured cells.

Depending upon the extent of initial freezing injury, an increase or a decrease in injury symptoms was found in the post-thaw period. During the progress of freezing injury, 10 days after thawing, a swelling of the protoplasm was seen in the irreversibly injured cells. In spite of this swelling, these cells could be plasmolysed. It appears that the high amount of K^+ that leaks out into the extracellular water, due to freezing injury, causes protoplasmic swelling by replacing Ca^{2+} in the plasma membrane. We conclude that protoplasmic swelling is a sign of secondary injury.

The results presented in this study show that membrane semipermeability is not completely lost and membrane rupture does not occur during the initial stage of freezing injury. In fact, the cells have the ability to repair damage depending upon the degree of injury. Our results show there are specific alterations in membrane semipermeability (*e.g.*, transport of K^+) which could be repaired completely depending on the degree of injury. These findings suggest that ion leakage due to freezing injury is due to alteration in the membrane proteins and not in the membrane lipids.

Key-words: Freezing injury, ion leakage, plasma membrane, membrane transport, nonelectrolyte permeability, membrane semipermeability, extracellular freezing, protoplasm swelling, onion, potato, herbaceous plants.

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Introduction

In nature, herbaceous plants are usually exposed to slow freezing, which produces extracellular ice formation resulting in dehydration (Levitt 1972, Li and Palta 1978, Palta *et al.* 1977d). After a slow thaw, the first visual sign of a freezing injury is the infiltration of the thawed tissue's intercellular space with water. This gives the tissue a soaked appearance and many times results in loss of turgor and flaccid tissue (Palta *et al.* 1977a). Leakage of ions from the injured tissue that accompanies this infiltration is another common phenomenon of freezing injury (Palta *et al.* 1977b). This leakage of ions from the frozen and thawed tissue and its infiltration with water has been assumed to be due to breakdown or a complete loss of membrane semipermeability (Levitt 1972, Sukumaran and Weiser 1972a). It has been suggested that membrane elasticity could be lost, due to irreversible denaturation of the proteins by freeze induced dehydration (Levitt 1972). This type of alteration would lead to a loss in the integrity of the bilayer and membrane rupture on thawing. In a recent review Steponkus and Wiest (1978) concluded: "After 65 years and several thousand papers on the subject of cold hardiness and freezing injury, it can only be said that freezing results in membrane rupture or loss of semipermeability."

Maximov (1912) pointed out that freezing injury must be due to injury of the plasma membrane. Ever since that time attempts have been made to study the cell membrane in relation to freezing injury and resistance (Levitt 1972). But because of the assumption that freezing results in a complete loss of semipermeability, not much effort has been made to study the nature of injury at the membrane level. Only recently Palta *et al.* (1977b) have shown that in spite of leakage of ions and loss of turgor due to freezing injury, the semipermeability of the cell membrane remained intact. Furthermore, these authors have

demonstrated that depending upon the degree of damage, freezing injury is reversible (Palta *et al.* 1977a,c).

In the present study we show that freezing injury does not result in membrane rupture or a complete loss of semipermeability. Using the freeze injured intact cells, an attempt was made to study the nature of membrane alterations that are produced by incipient freezing injury and result in leakage of ions.

Materials and methods

Plant material

Medium sized onion bulbs (*Allium cepa* L. cv. Downing Yellow Globe) weighing about 130 *g* were used in this study. The bulbs were grown at the University of Minnesota Agricultural Experiment Station without the application of herbicides or sprout inhibitors. Seed tubers of potato *Solanum tuberosum* L. cv. Red Pontiac were grown in a greenhouse mix soil (2:1:1 of soil: peat: sand) using plastic pots (20 cm diameter and 22.5 cm deep), under a warm temperature and long day regime (20/15°C, day/night; 14 h daylength), in a growth chamber. These plants, when two months old, were subjected to a cold treatment (5/5°C, day/night; 14 h daylength) for 2 weeks. From these cold treated plants mature leaflets were taken for making a freeze thaw experiment and microscopic observations.

Freezing and thawing

For determination of cell viability a corkborer was used to cut out round 1 cm diameter pieces from the third (counting from outside) scale of the onion bulbs. These pieces were then subjected to freezing at the rate of 1°C/h and thawed over ice. Cell viability measurement and details of the method was the same as given by Palta *et al.* (1977d). Temperatures as low as –21°C were used in these measurements.

For the other experiments onion bulbs were transferred to a card board box, which was then placed inside a freezer maintained at –10 ± 0.5°C. A continuous record of the temperature inside the bulb was obtained using a copper-constanten thermocouple inserted into the middle of the bulb. Under these conditions it took about 30 to 35 h to freeze onion down to –10 ± 0.5°C. Onions were kept frozen for 6 days and transferred to an ice box for thawing. Details of the freezing and thawing behavior of these onion bulbs have been presented elsewhere (Palta *et al.* 1977a).

Excised potato leaflets from the third compound leaf from the top were frozen at the rate of 1°C/h and thawed over ice using the method described by Sukumaran and Weiser (1972b).

Microscopic observations

Light microscopic observations were made using a Reichert microscope, and photographs were taken using a Robot (Recorder 24 ME) camera mounted on the microscope. The inner epidermis of onion bulb scales, which can be peeled off as a single cell layer, was used for this purpose. The intactness of the cell membranes was tested by plasmolyzing these epidermal cells in a hypertonic (0.2 *M*) mannitol solution as well as in a salt solution (mixture of 9 parts of 1 *M* KCl and 1 part of 1 *M* $CaCl_2$ diluted to desired concentration with distilled water). Presence or absence of protoplasmic streaming was also recorded.

Excised potato leaflets, frozen and subsequently thawed, were used for electron microscopic observation. Rectangular leaf strips (about 2 × 5 mm) were made from the central part of the leaflet and were fixed in glutaraldehyde for 3 h at room temperature (about 20°C). These samples were then prepared in the usual way for making observations with transmission electron microscope.

Membrane transport studies

A plasmometric method described by Stadelmann (1966) was used to stury the passive transport of urea, methyl urea and K^+ across cell membranes of onion epidermal cells. The inner epidermal layer was peeled off from the frozen and subsequently thawed onion bulb scales. The epidermal cells were plasmolyzed in hypertonic (0.8 *M*) mannitol solution and allowed to reach osmotic equilibrium. They were then transferred to a perfusion chamber. The chamber was perfused with the solution of a permeable solute (urea, methyl urea or KCl) which was of exactly the same osmotic concentration as the plasmolyzing mannitol solution. The plasmolysed protoplasts expanded in response to passive uptake of the permeable solutes. This expansion was measured directly with a microscope equipped with an eyepiece micrometer. The permeability constant was calculated using the formula given by Stadelmann (1966).

Figure 1. *Photomicrographs of freeze injured and unfrozen control onion epidermal* (a to e) *and potato leaf parenchymal* (f to h) *cells.* Onions were forzen gradually to –10°C and thawed over ice. Excised potato leaflets were frozen at 1°C/h and thawed over ice. (a) Immediately after thawing, medium used was tap water. (b) Plasmolysis of (a) in 0.8 *M* mannitol solution. (c) Protoplasmic swelling in some irreversibly injured cells 10 days after thawing, medium used was tap water. During the post thaw period onion were stored at 5°C. (d) Plasmolysis of (c) in 0.8 *M* mannitol solution giving a cap on the surface of the protoplast. (e) Cells in (b) on transfer to equimolal KCl solution. Rapid uptake of K^+ leading to separation of plasma membrane from cytoplasm. (f) Unfrozen control cells. (g) Leaf parenchyma cells immediately after thawing, previously frozen to –3.0°C. (h) Cells in (g) frozen to –4°C. CH: chloroplast: CW: Cell wall: EX: Extracellular space: M: mitrochondrion: N: nucleus: PM: plasma membrane: PS: protoplasmic strand: SP: swollen protoplasm: T: thylakoid membrane: TN: tonoplast: V: vacuole. Bar in a to e = 50 μm and in f to h = 2 μm.

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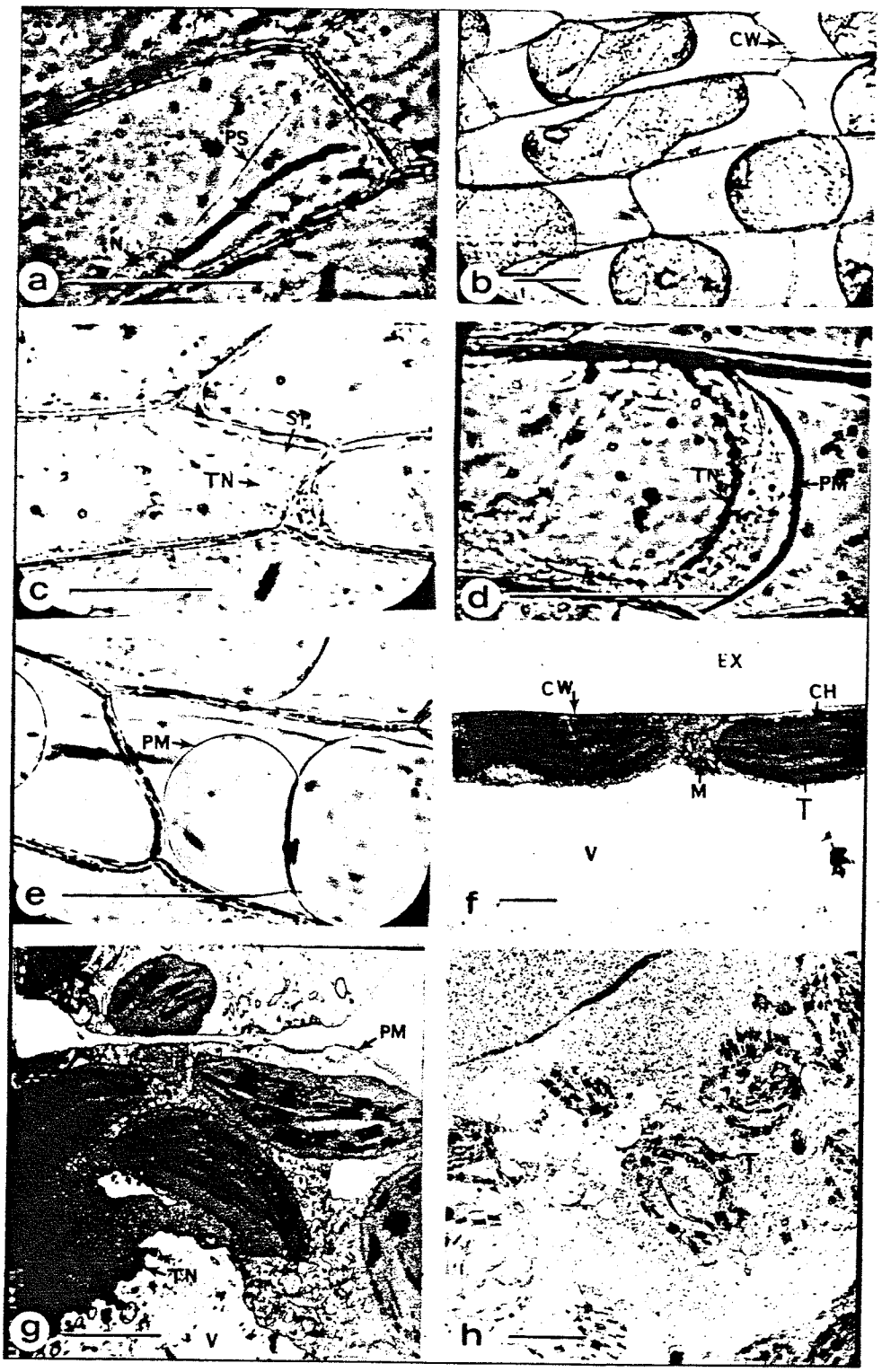

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Results

Cell viability

Most cells were found to be dead immediately after thawing, when freezing temperatures were –20 and –4°C for onion epidermal and potato leaf parenchyma cells respectively. The freezing injury (ionic leakage, soaking of tissue, flaccidness, *etc.*) was repaired completely in 2–3 days for potato leaves frozen to –2.5°C. In onion epidermal cells, also, all these symptoms of injury were reversible depending upon the degree of injury, which depended upon the length of the time the onions were kept frozen at –10°C. During the progress of irreversible injury, all cells did not die simultaneously in a given tissue, rather the death occurred over a period of days.

Membrane semipermeability of freeze injured cells

Freeze injured and subsequently thawed onion epidermal cells were observed directly under the light microscope. In spite of the large efflux of ions and sugars from freeze injured epidermal cells (Palta *et al.* 1977b), infiltration of tissue with water and loss of turgor (Palta *et al.* 1977a), the cells appeared normal under a light microscope (Figure 1a). These injured cells could be plasmolyzed (Figure 1b) in a hypertonic mannitol solution (0.8 *M*) as well as in a salt solution (mixture of 9 parts of 1 *M* KCl and 1 part of 1 *M* $CaCl_2$ diluted to 0.8 osmol concentration), and they remained plamolyzed for several days just like the unfrozen control cells.

Electron microscopic observation of freeze injured cells

An electron photomicrograph of freeze injured potato leaf parenchyma cells is shown in Figure 1g. In spite of the large efflux of ions from these cells, loss of turgor and infiltration of tissue intercellular space with water, the cell membranes were intact, and no visible change in the mitochondria or chloroplast was detected (Figure 1g). The first sign of cytological alteration observed was swelling of the protoplasm (Figure 1g). For comparison, electron micrographs of unfrozen control and dead cells are shown in Figures 1f and 1h respectively.

Membrane transport properties of freeze injured cells

Figure 2 shows the relative rates of passive transport of KCl, urea and methyl urea across the freeze injured as well as unfrozen epidermal cell membranes. The unfrozen control cells did not take up K^+ during the 2 h they were followed, and remained plasmolyzed to a constant volume (length of the protoplast remained constant). On the other hand, the injured cells started expanding immediately on transfer to KCl solution and gradually deplasmolyzed completely. The rate of K^+ uptake varied from 1.2 to 41.9 × 10^{-1} μm s^{-1} for different cells. This was

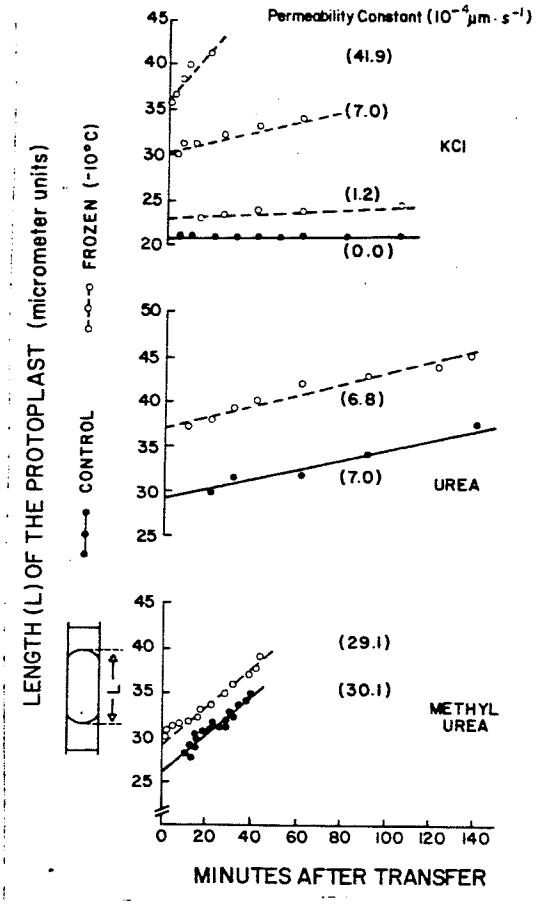

Figure 2. *Expansion of plasmolyzed protoplasts in response to passive uptake of KCl, urea and methyl urea.* Cells were plasmolyzed in 0.8 *M* mannitol (like Figure 1b) and were transferred at zero time to equiosmolar solutions of the permeating solute. In the absence of osmotic gradients, the expansion of the protoplast is solely due to a passive transport of the permeable solute from outside to inside the cell which results in a passive uptake of water by the cell (see Palta *et al.* 1977c for details). The protoplast expansion (length, L) was measured directly with a microscope equipped with an eyepiece micrometer. Here, 1 micrometer unit = 6.25 μm.

expected since the extent of injury to different cells in the same tissue varies.

A dramatic increase in K^+ permeability could be observed in some irreversibly injured cells. These cells, plasmolyzed in mannitol solution, showed separation of the plasma membrane from the cytoplasm within a minute when transferred to equiosmolar KCl solution. This led to formation of round caps on the surface of the plasmolyzed protoplasts (Figure 1e). The reason for this seems to be a rapid transport of K^+ through the plasma membrane followed by a simultaneous uptake of water. Unfrozen controls, as well as reversibly injured cells, did not show such formation of caps.

Although freeze injury resulted in a gradual increase in the rate of K^+ transport across the cell membranes, no

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changes in these rates were detected for nonelectrolytes such as urea and methyl urea (Figure 2). These experiments were repeated four times and similar results were obtained each time.

Swelling of the protoplasm during progress of freezing injury

During the post thaw period, depending upon the extent of initial freezing injury, an increase or decrease in injury symptoms (ion efflux, infiltration of the tissue) was reported recently (Palta *et al.* 1977c). Thus, after thawing, an injured but living cell may have the ability to recover completely (reversible injury) or may eventually die (irreversible injury). Ten days after thawing some irreversibly injured cell showed a swelling of the protoplasm during the progression of injury (Figure 1c). Onions after thawing were left at 5°C for making such observations. As is indicated in Figure 1c this swelling was quite apparent in the nonplasmolysed state. In spite of the swelling of the protoplasm, these cells could be plasmolyzed in hypertonic mannitol solution just like the unfrozen control or reversibly injured cells. As these irreversibly injured cells equilibrate to the hypertonic medium, a distinct swelling of the protoplasm could still be observed (Figure 1d).

Discussion

Freezing injury and membrane rupture

During the past 45 years, the most commonly used quantitative procedure for measuring freezing injury has been the conductivity method developed by Dexter *et al.* (1932). In this method leakage of ions from injured tissue is compared with leakage from completely killed tissue (by boiling or immersing in liquid N_2). A 50% leakage of ions is usually assumed to mean that 50% of the cells are killed and, therefore, leak all of their solutes into the surrounding medium. This concept was probably developed by an analogy with tissue cultures, and cell suspensions, (*e.g.* red blood cells or bacteria) in which counts of living and dead cells are made after freezing. Based on such observations freezing injury has been proposed to result in a complete loss of membrane semipermeability or membrane rupture (Levitt 1972, Sukumaran and Weiser 1972a,b, Steponkus and Wiest 1978).

Recently Palta *et al.* (1977b) found that in spite of 50% or more leakage of total ions and loss of turgor, the onion epidermal cells exhibited protoplasmic streaming and could be vitally stained. They further showed that the membranes of these injured cells had the same water permeability values as the control. From these results they concluded that membrane semipermeability was intact and that the leakage of ions, as measured by the conductivity test, was due to leakage of ions from injured yet alive cells. The results of the present study further support this conclusion. In spite of reversible or irreversible freezing injury, onion epidermal cells could be plasmolysed and remained plasmolysed in mannitol or balanced salt solution, just like the unfrozen control cells (Figure 1b). This means freezing injury did not result in membrane rupture or a complete loss in semipermeability. However, our results (Figure 2) indicate that leakage of ions, which is primarily K^+ (Palta *et al.* 1977b), is due to increased passive permeability of K^+ across the cell membranes. This means that the membrane has lost semipermeability to K^+.

In appears, therefore, that freezing injury results in specific alteration of membrane semipermeability rather than a complete loss of semipermeability as has been assured in the past. Depending on the degree of injury, leakage of ions, loss of turgor and infiltration of thawed tissue was found to be completely reversible (Palta *et al.* 1977c). Thus this specific loss of semipermeability for instance to K^+ observed in the present study could be temporary, and the cell may be able to repair such loss completely.

Nature of membrane alterations

It appears from the previous discussion that membrane rupture or a complete loss of semipermeability is an oversimplified qualitative statement of the end result of an irreversible freezing injury. This concept does not explain reversible freezing injury (a complete recovery) and provides no insight into the primary events of injury. Because of general belief in this concept in the past, no attempt was made to study the nature of a primary freezing injury at the membrane level. The results of the present study and some recent findings (Palta *et al.* 1977a,b,c) provide experimental evidence that freezing causes quantitative alterations in the cell membranes, leading to increased efflux of ions and sugars from the cells, loss of turgor, and infiltration of the tissue with water.

The permeability of nonelectrolytes has long been known as a direct function of their lipid solubility, *i.e.*, the oil/water distribution coefficient (Collander and Bärlund 1933). A change in the nonelectrolyte permeability, therefore, should reflect the physical status of the lipid portion of the membrane. Urea and methyl urea used in this study varied considerably in their lipid solubilities ($K_{oil/water}$ of 15×10^{-5} and 44×10^{-5} respectively). Absence of any change in the permeability constants of these nonelectrolytes showed that the lipid portion of cell membranes is intact and probably unaltered by freezing injury. This conclusion is further strengthened by the finding that water permeability constants also remain unchanged during freezing injury (Palta *et al.* 1977b).

The fact that there was a gradual increase in K^+ permeability and no change in nonelectrolyte permeability (Figure 2) suggests that the membrane proteins, possibly the intrinsic membrane proteins that participate in ion transport (Singer 1975), are altered during the initial

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stages of freezing injury. Due to the alterations, these intrinsic proteins when inactivated may serve as channels for passive ion transport, giving increased K^+ permeability values (Figures 1e and 2). Evidence for the existence of such proteins comes, for instance, from studies with gramicidin A, a simple channel (ionic pathway) forming molecule (Kolb *et al.* 1975). A large passive efflux of ions (reports on injured cells; Palta *et al.* 1977b), can also occur through these channels in the direction of the concentration gradient (vacuole to the extracellular solution).

As is clear from the data presented in Figures 1e and 2 the increase in passive K^+ permeability following freezing injury was related to the degree of injury. For example, in different injured cells the K^+ permeability values varied from 1.2 to 41.9 $\times$ 10^{-4} $\mu m\ s^{-1}$. Furthermore, in some cells there was such a dramatic increase that on transfer to KCl solution, the protoplast developed swollen caps within a few seconds (Figure 1e). These observations indicate that injury to membrane proteins (discussed above) was quantitatively graded, which can be visualized in terms of various degrees of denaturation of transport proteins. This explanation supports the results of Palta *et al.* (1977c), who found that a complete recovery after the injury depended upon the degree of initial injury. The injured cell may have the ability to repair partially denatured proteins or resynthesize and reincorporate these proteins into the membrane during recovery. This line of argument is strengthened by reports on recovery of solute uptake from gas shock by the cell suspension of *Acer pseudoplatanus* (Thoiron *et al.* 1979, 1980). The results of these studies suggest that the inability of the cells to absorb solutes following a gas shock is due to the alteration or release of membrane macromolecules engaged in transport.

Thus the results of the present study show that ion leakage due to freezing injury is most likely due to specific alterations (*e.g.* K^+ transport) in the membrane transport properties and not due to a complete loss in membrane semipermeability or membrane rupture. These alterations, our results suggest, are probably in the membrane proteins and not in the membrane lipids.

Secondary injury: Swelling of protoplasm

Many plant cells, when placed in a hypertonic solution of an alkali salt (*e.g.* potassium nitrate or potassium chloride), show a normal plasmolysis at first, but after several hours the protoplast swells and gives what is known as cap plasmolysis (Höfler 1928, Stadelmann 1966). Sometimes the protoplasm volume can increase 50–100 times the original volume (Stadelmann 1966). This swelling of the protoplasm has been thought to be the result of an increased plasma membrane permeability to the alkali ions (Stadelmann 1966). It is believed that when cells are left in K^+ solution for a long time the plasma membrane becomes highly permeable to K^+, which results in accumulation of K^+ in the protoplasm. Because of the build up of K^+ concentration, the protoplasm takes up water that gives rise to cap plasmolysis.

In the present study we found swelling of the protoplasm in irreversibly injured cells only when they were left at 50°C for 10 days after thawing (Figure 1c). We know that in irreversibly injured cells the ion leakage increases continuously during the post thaw period (Palta *et al.* 1977c). These ions, with K^+ as the main cation (Palta *et al.* 1977b), accumulate in the extracellular water. It is possible that the high concentration of K^+ that builds up in the extracellular water produces swelling of the protoplasm similar to that observed by earlier workers in alkali salt solution (Höfler 1928, Stadelmann 1966). During the post thaw period a small but increasing amount of Ca^{2+} efflux was reported for the irreversibly injured cells (Palta *et al.* 1977b,c). This increased efflux of Ca^{2+} could be caused by replacing Ca^{2+} in the plasma membrane with K^+ that builds up in the extracellular water. Since Ca^{2+} is important for membrane stability, replacing it with K^+ could weaken the membrane structure. It is possible that the protoplasmic swelling, which is observed in cells exposed to hypertonic alkali salt solutions (Höfler 1928) and during the progress of injury in irreversibly freeze injured cells (Figure 1c), is due to replacing Ca^{2+} in the membrane by alkali cations like K^+.

Protoplasmic swelling was only observed in the irreversibly injured cells. It appears therefore that the swelling of the protoplasm, during the progress of injury, is a sign of secondary injury and may explain in part why irreversibly injured tissue eventually dies. In spite of protoplasmic swelling, the cells could be plasmolysed (Figure 1d), which indicates that even these cells have not completely lost their semipermeability and that membrane rupture has not occurred. A complete loss of semipermeability or membrane rupture are probably one of the last events taking place during the progress of injury that eventually results in cell death.

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