

Effect of Low Temperature and Calcium on Survival and Membrane Properties of Isolated Winter Wheat Cells¹

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ABSTRACT

Isolated cells obtained by enzymic digestion of young primary leaves of cold-hardened, dark-grown Kharkov winter wheat (*Triticum aestivum* L.) were exposed to various low temperature stresses. The initial uptake of ⁸⁶Rb was generally decreased by increasing concentrations of Ca²⁺, but after longer periods of incubation, the inhibiting effect of high Ca²⁺ levels diminished. Viability of isolated cells suspended in water declined rapidly when ice encased at -1°C, while in the presence of 10 millimolar Ca²⁺ viability declined only gradually over a 5-week period. Ice encasement markedly reduced ⁸⁶Rb uptake prior to a significant decline in cell viability or increased ion efflux. Cell damage increased progressively when the icing temperature was reduced from -1 to -2 and -3°C, but the presence of Ca²⁺ in the suspending medium reduced injury. Cell viability and ion uptake were reduced to a greater extent following slow cooling than after rapid cooling to subfreezing temperatures ranging from -10 to -30°C. The results from this study support the view that an early change in cellular properties due to prolonged ice encasement at -1°C involves the ion transport system, whereas cooling to lower subfreezing temperatures for only a few hours results in more general membrane damage, including loss of semipermeability of the plasma membrane.

Although the molecular mechanisms of cold adaptation and low temperature injury in plants are not fully understood, it is now generally accepted that the plasma membrane plays a central role in these processes (10, 12, 17, 23, 25). Steponkus *et al.* (26, 27) have observed changes in properties of the plasma membrane of winter rye cells and protoplasts during freeze-thaw cycles using cryomicroscopy coupled to a video display. Electron microscopic observations by Singh (21, 22) have shown that irreversible ultrastructural alterations, frequently accompanied by loss of planar bilayer structure of the membrane, are induced during lethal freezing in winter wheat and rye cells. These studies have provided evidence of major structural changes in the plasma membrane during freezing stress. In contrast, during injury due to ice encasement at -1°C, electron microscopic studies have shown that little ultrastructural damage occurs (13). Recent investigations of biochemical changes associated with ice encasement and low temperature flooding suggest rather that cell damage is associated with the accumulation of metabolites of anaerobic respiration, such as ethanol and CO₂, to potentially toxic levels (1, 14). Jackson *et al.* (8) recently demonstrated that ethanol does not reach toxic levels in several plant species during flooding, but their observations are not applicable to ice encase-

ment because ethanol diffuses only very slowly into surrounding ice, and thus accumulates within the plant.

During the past few years, enzymically isolated cells and protoplasts from winter cereals have been used extensively in low temperature stress studies (17, 20, 26, 27). Our earlier studies on changes in membrane properties of isolated winter wheat cells in relation to the anaerobic stress of ice encasement suggested that damage to the ion transport system is an early manifestation of injury due to icing and exposure to ethanol at -1°C (17). This conclusion is consistent with observations of Palta and Li (12) from freezing stress studies on potato and onion.

The effects of Ca²⁺ on many physiological and biochemical events in the cell, including membrane-associated processes such as maintenance of membrane integrity and ion transport, is well established (4, 6). Our preliminary studies revealed that the addition of relatively high concentrations of Ca²⁺ (5-10 mM) to cell suspensions appeared to reduce damage caused by low temperature exposure, and in addition markedly reduced cell clumping, particularly after low temperature injury. The present paper extends our earlier studies (17) on effects of low temperature on membrane properties and describes the role of Ca²⁺ in protecting cells from damage due to low temperature stress.

MATERIALS AND METHODS

Plant Material. Seed of Kharkov 22 MC winter wheat (*Triticum aestivum* L.) was surface sterilized and sown on moist filter paper in glass trays. The trays were placed in the dark at 24°C for 24 h and then cold-acclimated in dark with diurnal temperatures of 2°C/16 h and 0°C/8 h for 6 to 8 weeks.

Young primary leaves (4-5 cm) were harvested, cut into 0.5- to 1-mm cross-sectional pieces with a razor blade, and cells isolated by a modification of the enzymic digestion method of Singh (20) previously described (17). Following digestion in pectolyase and purification in a Percoll gradient, the purified cells were suspended either in 10 mM Ca²⁺ (CaCl₂·2H₂O) or glass distilled H₂O. Cell viability after various treatments was estimated by counting live and dead cells on a hemacytometer. Live cells were identified by their ability to plasmolyze and deplasmolyze in 1 M NaCl and to accumulate neutral red dye in their vacuoles. All glassware and pipettes were rinsed with Sigmacote (Sigma Chemical Co.) to reduce sticking of cells to glass.

Uptake and Efflux of ⁸⁶Rb. Purified cells were suspended in water or 10 mM Ca²⁺ solution to a final volume of 10 times the packed volume following centrifugation at 200g. For uptake experiments, 1-ml aliquots were added to 50-ml Erlenmeyer flasks containing 19 ml of water or various concentrations of Ca²⁺. Following exposure to various stresses (see below), 10 μl of a ⁸⁶RbCl solution, containing 2 μCi of radioactive tracer, were added to each flask, and the flasks were placed on a rotary shaker at 2°C. At various intervals, the sides of the flasks were scraped with a 'rubber policeman' to free adhering cells and triplicate

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0.3-ml aliquots were pipetted onto 3- μ m Millipore filter pads. The cells were washed several times with distilled H₂O at 20°C until no significant radioactivity was detected in the wash. The washed cells and the pad were transferred to scintillation vials containing 10 ml of water and radioactivity in the cells was determined using a Beckman LS 8000 scintillation counter.

Efflux of ⁸⁶Rb was determined by measuring the loss of label from cells labeled prior to stress treatments. Cells were incubated for 2 h at 2°C on a rotary shaker in 10 ml of water or 10 mM Ca²⁺ containing 4 μ Ci of ⁸⁶RbCl. The cells were then washed four times during a 1-h period with water, radioactivity determined in the untreated cells, and the cells were then subjected to various freezing and icing treatments. The flasks were then scraped with a rubber policeman and triplicate 0.3-ml aliquots of cell suspension were removed after 0, 2, and 24 h, washed, and radioactivity determined, as described above.

Ice Encasement Stress. Ice encasement refers to treatments in which isolated cells were totally immersed in water or 10 mM Ca²⁺ and held slightly below the freezing point for extended periods, resulting in anaerobiosis (1), but not in the formation of intracellular ice (27). One-ml aliquots of cells were pipetted onto the surface of 15 ml of frozen water or 10 mM Ca²⁺ solution. After the cell suspension had frozen, 4 ml of appropriate medium were added, and the flasks transferred to freezers at -1, -2, or -3° ± 0.2°C. Noniced controls were maintained in the unfrozen state at -1°C. Samples were removed at various intervals over a 5-week period, thawed, and analyzed for uptake and efflux at 2°C, as described above.

Freezing Stress. Freezing stress refers to treatments in which cells were exposed to various subfreezing temperatures in the range -5 to -30°C for periods of less than 2 h. Aliquots of 1 ml of purified cells were added to 19 ml of water or 10 mM Ca²⁺ in 50-ml Erlenmeyer flasks. For slow cooling treatments, the flasks were placed in programable freezers at 0°C and the temperature decreased 1 to 2°C/h overnight to the required temperature. The cell suspensions were seeded with a small chip of ice at -3°C to prevent supercooling. For fast cooling treatments, flasks were transferred directly to freezers at -5, -10, -20, and -30°C, and were seeded and left at these temperatures for 2 h. The cooling rates observed in these treatments were as follows: -5°C, 2.5°C/h; -10°C, 5°C/h; -20°C, 15°C/h; and -30°C, 30°C/h. The flasks were removed from the freezers and allowed to thaw at 2°C, and uptake and efflux of ⁸⁶Rb were measured at 2°C, as described above.

RESULTS

The rate of ⁸⁶Rb uptake by freshly isolated winter wheat cells was generally decreased by increasing concentrations of Ca²⁺ during the initial stages of uptake (Fig. 1, 1 h). After 6 h, the rate of uptake in the absence of Ca²⁺ declined, whereas uptake in the presence of Ca²⁺ remained relatively constant so that after 24 h, total uptake was similar in all concentrations of Ca²⁺. Approximately 50% of the total available label was taken up by the cells after a 24-h incubation period, and little additional uptake was observed after 48 h. Efflux of ⁸⁶Rb from prelabeled cells was generally unaffected by the presence of various concentrations of Ca²⁺ and about 60 to 70% of the label was retained by the cells after a 48-h efflux period at 2°C (data not shown).

Ice Encasement Stress. The viability of isolated cells suspended in water declined rapidly during ice encasement at -1°C (Fig. 2). After only 3 d, viability was reduced by 50% and by 3 weeks, nearly all the cells were dead. In contrast, viability of cells suspended in 10 mM Ca²⁺ declined only gradually and after ice encasement for 5 weeks, approximately 60% of the cells were still alive. Viability of cell suspensions maintained unfrozen at -1°C declined less than the iced cells in Ca²⁺, and slightly greater survival was observed in the presence than in the absence of

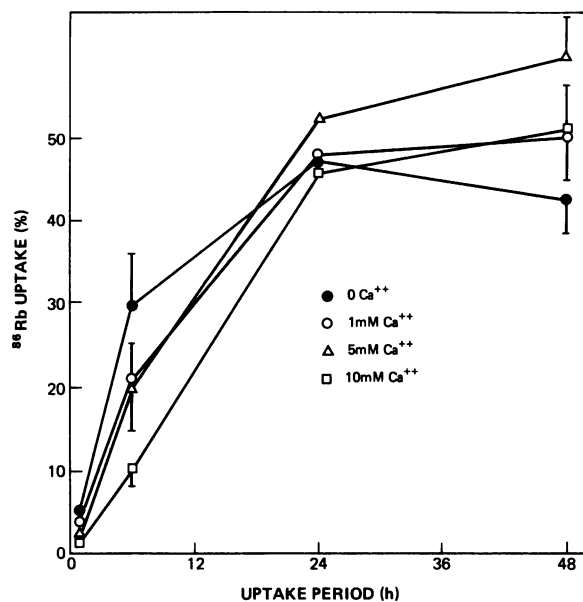


FIG. 1. Uptake of ⁸⁶Rb by winter wheat cells suspended in various concentrations of Ca²⁺. Uptake is expressed as percentage of total available label in the incubation system. Bars, SE. The average SE at 1 h is 0.5%.

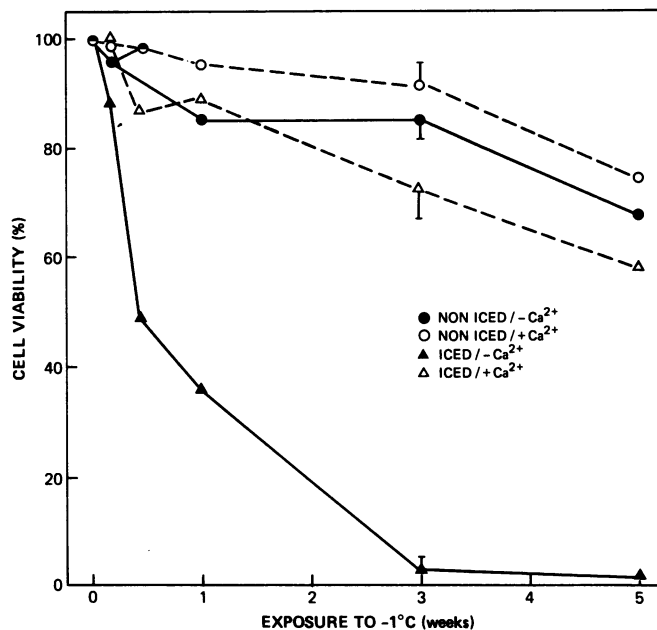


FIG. 2. Viability of winter wheat cells after 24 h following exposure to -1°C (iced and noniced) in presence and absence of 10 mM Ca²⁺. Bars, SE.

Ca²⁺.

Uptake of ⁸⁶Rb by cells suspended in either water or in Ca²⁺ was inhibited severely after ice encasement at -1°C (Fig. 3). The presence of Ca²⁺ during icing slightly enhanced uptake following the early stages of icing, but after 2 to 3 weeks, no further protection was observed. In noniced cells, a marked increase in uptake of label was observed after 1 d at -1°C (Fig. 3). The rate of uptake was greater in cells suspended in water than in Ca²⁺, but uptake continued to increase for about 1 week in cells suspended in Ca²⁺, while a decline in uptake was observed after 1 d in cells suspended in water. Uptake of ⁸⁶Rb by noniced cells declined only gradually between 1 and 5 weeks of treatment, consistent with the gradual decline in cell viability (Fig. 2).

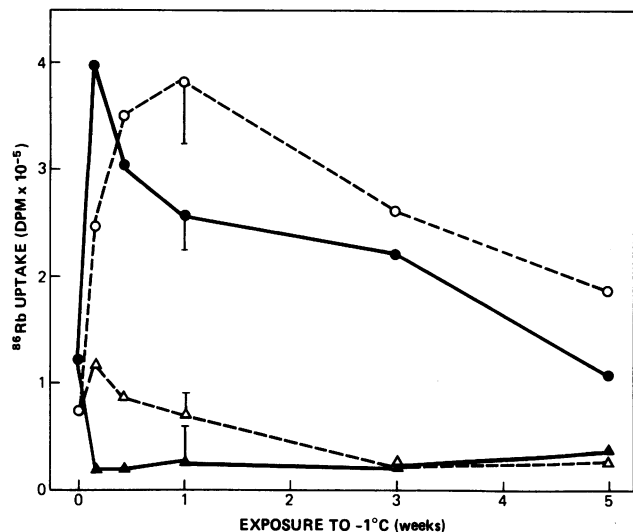


FIG. 3. Uptake of ^{86}Rb by winter wheat cells during a 1-h period immediately following exposure to -1°C in presence and absence of 10 mM Ca^{2+} . Legend as in Figure 2. Bars, SE.

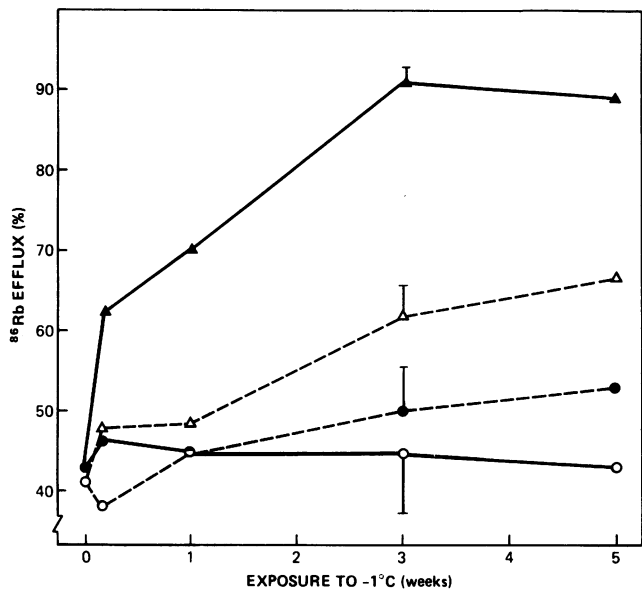


FIG. 4. Efflux of ^{86}Rb from prelabeled winter wheat cells after 2 h following exposure to -1°C in presence and absence of 10 mM Ca^{2+} . Legend as in Figure 2. Efflux is expressed as percentage of total label retained following labeling and washing of the cells. Bars, SE.

Approximately 40 to 50% of preloaded label diffused from all treatments in a 2-h period at low temperature (Fig. 4, time 0). No further significant efflux was observed from noniced cells even after 5 weeks at -1°C . However, an abrupt increase in efflux of label was observed from cells suspended in water and iced at -1°C for only 1 d, and by 3 weeks of icing, 90% of the label was lost in 2 h. In contrast, efflux of ^{86}Rb from cells iced in 10 mM Ca^{2+} occurred gradually and in cells iced for 5 weeks, about 65% of the label diffused from the thawed cells in 2 h.

Damage due to ice encasement increased significantly with only a slight decrease in icing temperature (Table I). Viability of cells declined when the icing temperature was lowered from -1°C to -2 and -3°C , and damage was slightly more severe in the absence than in the presence of Ca^{2+} in the suspending medium. Uptake of ^{86}Rb by cells iced at the lower temperature also was reduced markedly, and consistent with the viability

results, the reduction in uptake was greater in cells suspended in water than in Ca^{2+} . Efflux of ^{86}Rb from prelabeled cells also increased appreciably at the lower icing temperature, but the magnitude of change was not as great as was observed with ion uptake.

Freezing Stress. Exposure of cell suspensions to decreasing subfreezing temperatures gradually reduced cell viability (Fig. 5). Survival was reduced to a greater extent by slow cooling over 24 h than by rapid cooling over a 2-h period, and the presence of Ca^{2+} in the suspending medium generally enhanced survival. The protective effect of Ca^{2+} was more pronounced when cells were cooled rapidly, but only for 2 h, than when the temperature was reduced slowly over 24 h. For example, at -20°C nearly 40% of cells survived fast cooling and 20% slow cooling in the presence of Ca^{2+} , whereas less than 5% of cells remained viable after either slow or rapid cooling in the absence of Ca^{2+} . When the temperature was reduced to -30°C , nearly all cells were killed, regardless of treatment conditions.

Uptake of ^{86}Rb by cells suspended either in presence or absence of Ca^{2+} , and cooled slowly was markedly reduced even at -5°C (Fig. 6). In contrast, uptake by cells rapidly cooled to -10°C in either the presence or absence of Ca^{2+} was not inhibited. At -20°C , severe inhibition of uptake was observed by all cells except those rapidly cooled in the presence of Ca^{2+} , and at -30°C uptake of label had declined to extremely low levels in cells from all treatments.

Efflux of ^{86}Rb from cells labeled prior to exposure to freezing stress increased with decrease in freezing temperature (Fig. 7). At -10°C , efflux was significantly higher in cells frozen slowly than in those rapidly frozen, consistent with the higher level of damage observed in cells at this temperature. Efflux from cells at -20°C was lowest in cells suspended in 10 mM Ca^{2+} and frozen rapidly. Nearly all of the label diffused from cells frozen slowly or rapidly to -30°C in the presence or absence of Ca^{2+} .

DISCUSSION

The results obtained in this study demonstrate the general beneficial role of relatively high concentrations of Ca^{2+} in isolated winter wheat cell suspension systems, either in the presence or absence of external stresses. Although Ca^{2+} is usually considered to be important in cell-to-cell adhesions in intact tissues, the presence of added Ca^{2+} in cell suspensions reduced cell clumping and adherence to glassware, thus facilitating both the physical manipulation of the cells and determination of cell numbers in experimental treatments. This apparent discrepancy may be due to the removal of Ca pectate from the cell wall during enzymic digestion. The subsequent addition of Ca^{2+} to these cell suspensions may facilitate cross-linking of exposed ends of carbohydrate chains, thus reducing the possibility of intercell linkages upon cell-to-cell contact.

The general protective effect of Ca^{2+} observed when cells are exposed to low temperature stresses is consistent with its postulated beneficial role in many cellular processes (4–7). Cell viability was significantly enhanced by the presence of Ca^{2+} in cell suspensions exposed to both ice encasement and freezing stresses, resulting in higher levels of ion uptake and lower ion efflux than observed in the absence of Ca^{2+} . The observed protective effect of Ca^{2+} was considerably more pronounced during ice encasement stress than during either slow or fast cooling. This probably results from the fact that ice encasement at -1 to -3°C is a less severe and very different form of stress, involving an anaerobic component, than freezing to temperatures in the -10° to -30°C range for only a few hours, where even the presence of Ca^{2+} cannot prevent serious damage to the cells. The mechanism whereby Ca^{2+} reduces stress-induced damage to isolated cells is not understood, but this is not unexpected since the mechanism(s) of low temperature stress injury has itself not yet been

Table I. *Effect of Icing Temperature on Cell Viability and ⁸⁶Rb Uptake and Efflux*
 Each value represents an average ± SE of three determinations from each of two separate experiments.

Treatment	Ca ²⁺ mM	Viability in 24 h ^a	⁸⁶ Rb Accumulated in 24 h ^b %	⁸⁶ Rb Efflux in 24 h ^b
Control	0	100	28.7 ± 3.2	42.1 ± 2.1
	10	100	35.2 ± 3.5	50.2 ± 7.1
-1°C/7 d noniced	0	78.9 ± 6.2	49.8 ± 5.4	55.0 ± 11.0
	10	97.5 ± 1.5	54.1 ± 10.2	50.2 ± 4.5
-1°C/7 d iced	0	72.0 ± 5.1	4.6 ± 0.6	67.0 ± 8.7
	10	86.7 ± 4.0	44.5 ± 4.1	53.5 ± 5.4
-2°C/7 d iced	0	56.5 ± 1.9	3.5 ± 0.4	77.0 ± 10.7
	10	63.2 ± 3.5	8.3 ± 0.8	86.5 ± 11.2
-3°C/7 d iced	0	46.5 ± 3.2	2.2 ± 0.2	83.5 ± 10.8
	10	50.0 ± 5.8	4.6 ± 0.9	77.5 ± 6.9

^a Viability determined 24 h after removal from treatments. ^b Uptake values represent the percentage of total available label accumulated by the cells while efflux values represent the percentage of label which diffused from the cells following indicated treatments.

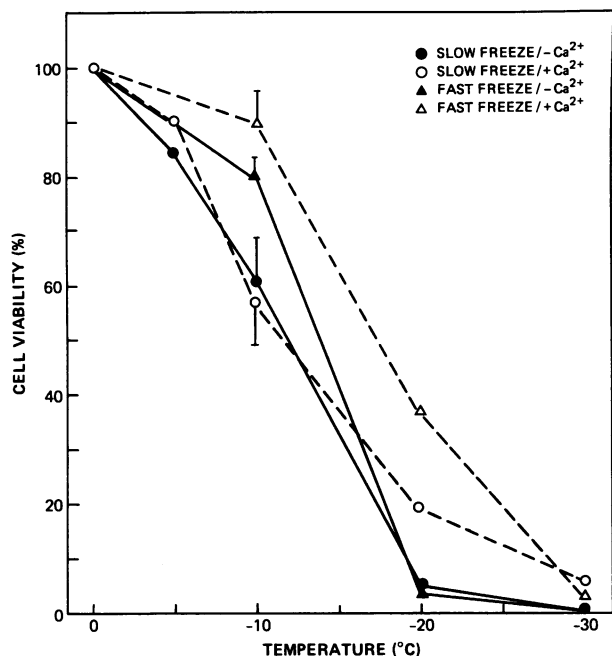


FIG. 5. Viability of winter wheat cells after 24 h following slow and rapid cooling stress in presence and absence of 10 mM Ca²⁺. Bars, SE.

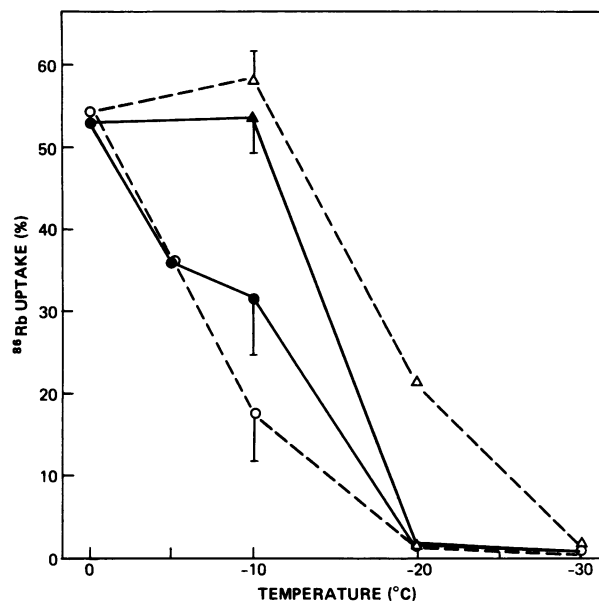


FIG. 6. Uptake of ⁸⁶Rb by winter wheat cells after 24 h following exposure to slow and rapid cooling in presence and absence of 10 mM Ca²⁺. Legend as in Figure 5. Bars, SE.

elucidated. However, the frequent observations that Ca²⁺ plays an important role in membrane-associated processes, such as maintenance of membrane integrity, reduction of ion leakage, uptake of ions and amino acids, and maintenance of configuration of enzyme binding sites in cells and plant tissues (3-7, 18, 24, 28) provides numerous possibilities for amelioration of the negative effects of low temperature stresses.

The initial low rate of ion uptake and inhibition of uptake by high levels of Ca²⁺ observed in unstressed cells are generally in accord with results obtained with other systems (2, 7, 11, 19). Low rates of lysine uptake by cultured tobacco cells have been attributed to some form of reversible damage induced by experimental procedures (7). The marked increase in ion uptake by wheat cells observed in the present study after a few hours also suggests recovery from a depressed level of uptake induced by the stress of cell isolation and purification procedures. Stimula-

tion of K⁺ and amino acid uptake by 0.5 to 1 mM Ca²⁺ has been reported (2, 7, 24), with maximum rates of uptake observed after several hours incubation with Ca²⁺, while uptake is reduced in the presence of either higher or lower concentrations of Ca²⁺.

The results obtained from the ice encasement and the slow and rapid cooling experiments suggest that a different mechanism may be involved in cellular injury due to different low temperature stresses. The rapid decline in ion uptake before the decrease in cell viability during ice encasement, similar to that previously reported (15, 17), demonstrates that the ion transport system of these cells is an early target of icing-induced injury. The increase in ion efflux is delayed until visible cell injury occurs indicating that general membrane damage, sufficient to induce a loss in membrane semipermeability, does not accompany the marked decline in transport properties of the cells. In contrast, freezing stress induces more general damage to the cells, as evidenced by a correlation among reduced ion uptake, increased efflux, and a

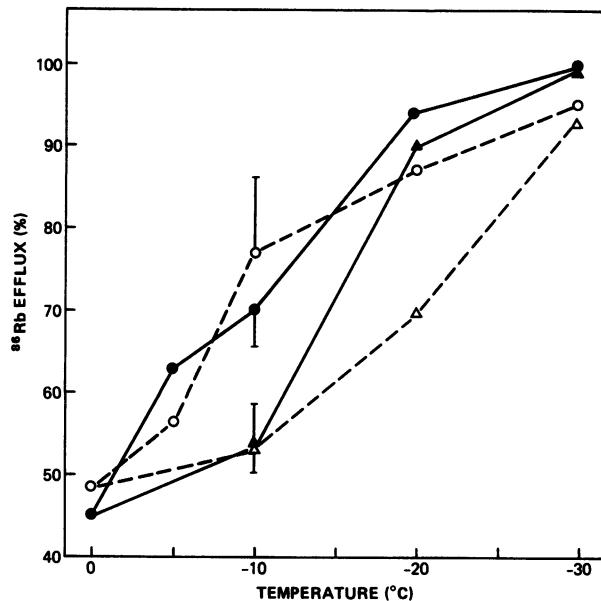


FIG. 7. Efflux of ^{86}Rb from pre-labeled winter wheat cells after 24 h following slow and rapid cooling stress in presence and absence of 10 mM Ca^{2+} . Legend as in Figure 5. Bars, SE.

loss of cell viability. Furthermore, previous electron microscopic studies showed that freezing stress induces severe ultrastructural damage in winter cereals (13, 21), whereas ice encasement induces a proliferation of ER membranes, but does not seriously disrupt cellular organization (13). These observations support the view that an early manifestation of damage due to icing results from damage to the ion transport system induced by accumulated anaerobic metabolites, whereas freezing stress results, in general, from physical disruption of cellular membranes, simultaneously affecting both uptake and efflux.

The reason for enhanced survival of cells cooled rapidly compared to cells cooled slowly was not pursued in this study. With whole plants, it is generally accepted that rapid cooling is more detrimental to survival than slow cooling to the same temperature (9). It appears, therefore, that for free cells, the 'rapid' cooling rates employed in these experiments were sufficiently slow for the cells to dehydrate and attain osmotic equilibrium, thus preventing injury from intracellular ice formation. It is probable, then, that greater survival was observed following rapid cooling simply because the cells were maintained at low subfreezing temperatures for much shorter periods of time, since it has been established that increased duration of exposure of plants to sublethal freezing stress reduces survival (16).

The results obtained in this study have clearly demonstrated the general protective effect of Ca^{2+} on isolated winter wheat cells exposed to low temperature stresses. It is apparent from other experiments in this laboratory (data not shown) that a similar protective effect against ice encasement is not elicited by either Mg^{2+} or La^{3+} , providing further evidence of a specific effect of Ca^{2+} on membrane function. Evidence also was obtained to support the view that freezing damage results in general deterioration of membrane properties, including loss of semi-permeability, whereas initial injury from ice encasement is restricted to the ion transport system of the cell. The precise mechanism(s) of ice encasement injury is still unknown, and further experiments are currently in progress in an attempt to

determine whether inhibition of ion uptake results from a reduction of energy charge below that required for active ion uptake, or from damage to plasma membrane ATPase(s) involved in this process.

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