

Freezing Injury in Onion Bulb Cells

I. EVALUATION OF THE CONDUCTIVITY METHOD AND ANALYSIS OF ION AND SUGAR EFFLUX FROM INJURED CELLS¹

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ABSTRACT

Onion (*Allium cepa* L.) bulbs were frozen to -4 and -11 C and kept frozen for up to 12 days. After slow thawing, a 2.5-cm square from a bulb scale was transferred to 25 ml deionized H₂O. After shaking for standard times, measurements were made on the effusate and on the effused cells. The results obtained were as follows.

Even when the scale tissue was completely infiltrated, and when up to 85% of the ions had diffused out, all of the cells were still alive, as revealed by cytoplasmic streaming and ability to plasmolyze. The osmotic concentration of the cell sap, as measured plasmolytically, decreased in parallel to the rise in conductivity of the effusate. The K⁺ content of the effusate, plus its assumed counterion, accounted for only 20% of the total solutes, but for 100% of the conductivity. A large part of the nonelectrolytes in the remaining 80% of the solutes was sugars.

The increased cell injury and infiltration in the -11 C treatment, relative to the -4 C and control (unfrozen) treatments, were paralleled by increases in conductivity, K⁺ content, sugar content, and pH of the effusate. In spite of the 100% infiltration of the tissue and the large increase in conductivity of the effusate following freezing, no increase in permeability of the cells to water could be detected.

The above observations may indicate that freezing or thawing involves a disruption of the active transport system before the cells reveal any injury microscopically.

Whereas ion uptake in plants receives considerable attention, the release of solutes from the plant into the environment is less investigated. Considerable amounts of solutes are lost after cell damage and cell death. Often exosmosis is considered to indicate irreversible damage, disregarding clear evidence of recovery of cells from ion leakage (7).

After early measurements of electric conductivity of an effusate (a solution of substances removed from plant tissue by immersion in water) by Heald (3), Dexter *et al.* (1) developed a conductivity method for measuring frost hardness of plants (see 5). This method has been used successfully, particularly for testing relative varietal resistance. The basic assumption is that the greater the injury of the living tissue, the greater the efflux of ions from the thawed cells. These authors clearly recognized that exosmosis occurs not only in dead but also in injured cells, however, without distinguishing reversible damage.

In spite of the general adoption of the conductivity method, there have been no attempts to investigate the basic nature of the freeze-induced efflux of ions. In recent literature, the assumption seems to have been made that the efflux is mainly, if not solely, from dead cells, and therefore one measures the freezing injury by determining the percentage of cells killed (6, 11). This may have been based on analogy with tissue cultures and cell (*e.g.* red blood cells or bacteria) suspensions in which actual counts of living and dead cells are made after freezing. No allowance has been made for the contribution of reversibly and irreversibly injured cells, and exosmosis from uninjured cells has rarely been measured (14).

It was the purpose of this investigation to analyze closer the contribution of injured cells to the conductivity of the effusate. When the effusate originates only from damaged, but living cells, the conductivity method measures the average injury of the cells and may conceivably be an excellent tool for investigating details of the mechanism of freeze injury and resistance.

MATERIALS AND METHODS

PLANT MATERIAL

Downing Yellow Globe onions (*Allium cepa* L.) were used in the present study. The bulbs were grown at the University of Minnesota Agricultural Experiment Station without application of herbicides or sprout inhibitors.

FREEZING AND THAWING OF ONION BULBS

Medium size onion bulbs weighing about 130 g were selected. They were transferred to cardboard boxes which were then placed inside the freezers maintained at -4 ± 0.5 C or -11 ± 0.5 C. From preliminary freezing tests at a graded series of temperatures, it was found that these two resulted in no injury and in moderate injury, respectively. The onion bulbs were kept frozen up to 12 days. After a desired length of time, frozen bulbs were taken out of the freezers and transferred to an ice box for thawing. A continuous record of the temperature inside the bulb was obtained using copper-constantan thermocouple inserted into the middle of the bulb and coupled to a recorder. The details of the freezing and thawing behavior of these onion bulbs have been presented elsewhere (9).

OBSERVATIONS ON THAWED ONIONS

As soon as thawing was complete, one-third of the bulb was removed by means of two longitudinal incisions. The third and fourth scales from the outside of this portion were used immediately for the investigations below. The remaining two-thirds of the bulb was stored at 3 ± 0.5 C with a relative humidity close to

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100% to minimize water loss. It was kept for further observations of injury (8).

Cell Viability. Sections of the inner epidermis, outer epidermis, and scale parenchyma were observed under the light microscope. The presence of protoplasmic streaming indicated that a cell was alive. The sections were then transferred to a hypertonic mannitol solution. Plasmolysis indicated that the cell membranes were intact, a further indication of life.

Permeability to Water. The inner epidermis was removed and floated on spring water before use for measurements of water permeability and osmotic value of the cell sap. The permeability of the epidermal cells to water was determined by using a specially designed chamber consisting of two pieces cut from a solid Plexiglas rod 4.4 cm in diameter. An epidermal disc 1.4 cm in diameter was soaked in tritiated H₂O (500 μ Ci/ml) for about 1 hr. This was then transferred to the chamber where it was held flat between the male and female joints of the chamber. The disc was then washed on the noncuticular side with ordinary water at a constant flow rate maintained by a reservoir consisting of an inverted Mariotte flask. The washing liquid was collected at 5-sec intervals. From the activity in the washing solution, a curve was plotted for tritium efflux on semilogarithm paper. Permeability was calculated in the usual way after separating different exponentials from the main curve (10).

Osmotic Potential. The osmotic potential of the cell sap was determined by the method of incipient plasmolysis. Sections from the scale parenchyma were transferred to a series of mannitol solutions. The solution that induced incipient plasmolysis was considered equal in osmotic potential to that of the cell sap.

Efflux of Ions from Scale Tissue. From the third and fourth scales of the thawed onion bulbs, pieces were cut 2.5 cm², weighed, and transferred each to 25 ml of deionized H₂O contained in 250-ml flasks. These were then shaken for 30 min in an automatic shaker. Electrical conductance of the effusate was measured in the vial by means of a standard conductivity bridge. These samples were again shaken in the same effusate for about 30 min and then for about 110 min. At each time, electrical conductance was measured. At the end, the effusate was decanted into vials and quickly frozen for later determination of pH, K⁺ content, total sugars, and total solutes. In some samples the cells were killed to obtain a reference value for the conductivity of a tissue with all cells dead. These samples were submerged for 2 min into liquid N₂ after the third conductivity measurement, placed into the same vial containing the previous effusate, after warming up, and shaken for 30 more min. Subsequently the conductivity was measured.

K⁺ and Ca²⁺ Content in Effusate. The effusate was analyzed for K⁺ and Ca²⁺ content by the atomic absorption spectrum method (Perkin-Elmer model 303).

Total Osmolarity of Effusate. Using a freezing point depression osmometer (Advanced Digimatic Osmometer, model 3D), total osmolarity of the effusate was determined.

Total Sugars in Effusate. Amount of total sugars was deter-

mined by phenol-sulfuric acid assay (2). The results were expressed in terms of D-glucose.

RESULTS

MICROSCOPIC OBSERVATIONS OF CELLS

At the time of the conductivity measurements, a sample from the same bulb scale was used for observations of the cells from the inner and outer epidermis and the scale parenchyma. In general it was found that infiltration of the scale tissue (*i.e.* filling of the intercellular spaces by aqueous liquid) and conductivity of the effusate increased as the freezing temperature was lowered. Although the infiltration of the scale tissue increased from 0 to 100% and up to 85% of the total ions leaked out to the effusate as the freezing temperature was lowered to -11 C, all of the cells plasmolyzed in hypertonic mannitol solution and most exhibited protoplasmic streaming. As the leakage of ions from the scale tissue increased, a decrease in the osmotic value of the cell sap (incipient plasmolysis value) was found (Table I). This experiment was repeated 10 times. Similar results were obtained each time.

Leakage of ions from killed cells was also measured and compared to leakage from live cells. The per cent leakage compared to killed cells was 34% for unfrozen bulbs, 42% for bulbs frozen to -4 C, and 76% for bulbs frozen to -11 C. Clearly leakage of ions from the tissue was not related to death of the cells, for all of the cells in all three treatments were alive even though up to 76% of the ions had leaked out. Thirty-four per cent of the total ions leaked out even from the unfrozen bulb scales, which would be regarded as equivalent to 34% of the cells dead in the scale tissue, on the basis of the usual interpretation of the conductivity method.

The cells from thawed onion scales remained alive for several days. All of the bulbs frozen to -4 C recovered completely, and depending upon the length of time frozen at -11 C, many recovered completely and some deteriorated after 7 to 14 days. All of the bulbs which recovered could be regrown and behaved like unfrozen onions.

The osmotic values of the cell sap for the outer and inner epidermis were 400 and 350 mosm, respectively, for bulbs frozen to -11 C and 650 and 490 mosm, respectively, for bulbs frozen to -4 C. This is a decrease in cell sap concentration in -11 C treatment compared to -4 C treatment of about 40% in the outer epidermis and about 30% in the inner epidermis and agrees very well with the 34% increase at -11 C in ions that diffused out (76% versus 42%, Table I) of the cell. In spite of about an 8% difference in the per cent leakage values between -4 C treatment and control, no difference was found between these two samples in the osmotic values of the cell sap. This is probably because when tissue is infiltrated or partially infiltrated (as for the -4 C treatment), the cells have better contact with the effusate and the ions can diffuse out more efficiently. Also, different bulbs were used for each treatment.

Table 1. Effect of prolonged freezing stress on infiltration, conductivity of the effusate, osmotic value of cell sap and viability of onion bulb scale parenchyma cells

Onions were transferred directly to freezing chambers maintained at -4 ± 0.5 C and -11 ± 0.5 C and kept there for 6 days. Observations were made immediately after thawing.

Freezing temp (C)	Infiltration ¹ of the scale tissue (%)	Conductivity ² of the effusate (μ S/g of scale tissue)	Percent leakage compared to killed tissue	Osmotic value of cell sap in mosm		Cell viability
				epidermis outer	inner	
-11	100	152.0	76	400	350	All alive
-4	50	83.2	42	650	490	All alive
+3 (control)	0	66.7	34	630	470	All alive

¹These values are estimated by visual observation.

²Shaking time: 1 hour

Table II. Conductivity of the effusate of frozen and thawed tissue and the permeability of cell membranes to water

From the same scale tissue which was used for conductivity measurements, inner epidermal layer was used for water permeability measurements. Onions were kept frozen up to 12 days.

Freezing temp (C)	Conductivity ² of the effusate ($\mu\text{S/g}$ of scale tissue)	Percent leakage compared to killed tissue (approximate)	Water permeability constant of inner epidermis K_w ($\mu\text{m}\cdot\text{sec}^{-1}$)	Cell survival
-11	99.05 ¹ \pm 12.22	50	2.06 \pm 0.26	All alive
-4	41.57 \pm 12.08	21	1.97 \pm 0.38	All alive
+3 (control)	28.81 \pm 5.83	14	1.76 \pm 0.14	All alive

¹These values are averages of 12 different measurements in 6 separate experiments.

²Shaking time 1/2 hour

Table III. Analysis of the effusate: conductivity compared to K^+ content, Ca^{2+} content, total osmolarity, total sugars and pH of the effusate.

Measurements on conductivity were made after shaking the samples for about 3 hours and the effusate collected was used for analysis. All the values are average of 4 separate measurements and all (except pH) are expressed as per g of scale tissue.

Freezing temp	Conductivity (μS)	K^+ content (mg/liter)	Ca^{2+} content (mg/liter)	Total solutes (mosm/liter)	Total sugars as glucose (mM)	pH
-11	173.0 \pm 12.0	50.6 \pm 1.6	0.45 \pm 0.08	16.2 \pm 1.0	23.3 \pm 3.2	6.45 \pm 0.04
-4	119.2 \pm 16.1	35.2 \pm 5.6	0.40 \pm 0.06	10.7 \pm 1.0	15.1 \pm 1.7	5.99 \pm 0.11
+3 (control)	104.3 \pm 13.8	29.6 \pm 4.9	0.34 \pm 0.04	7.8 \pm 1.3	11.3 \pm 1.7	5.93 \pm 0.06

CONDUCTIVITY AS RELATED TO SHAKING TIME

Samples of thawed bulb scales were first shaken for 30 min. After reading the conductivity values, they were read again after shaking for 30 min and then for another 110 min. With the increase in shaking time, the conductivity of the effusate increased markedly (Fig. 1). The relative increase was greatest in the control and lowest in the -11°C treatment bulb tissue. For example, from Figure 1 it can be seen that the 30-min reading for the -11°C treatment was $99\ \mu\text{S}^2$ compared to $23\ \mu\text{S}$ for the control, a ratio of more than four times. The 110-min readings for these two were $167\ \mu\text{S}$ and $90\ \mu\text{S}$, respectively, a ratio of less than two times. In other words, as the shaking time was increased from 30 to 110 min, the conductivity of the effusate increased by 1.7 times in the -11°C treatment, by 2.6 times in the -4°C treatment, and by 3.1 times in the control. The time course of the effusion is comparable to the one found for stem pieces of *Rubus* (12). The difference for the conductivity values between the treatments becomes smaller with increase in shaking time and can be expected to reach a constant value. It is interesting to note that after shaking for 170 min, about 45% of the total ions leaked out of the cells in the control treatment and about 80% in the -11°C treatments. In spite of this, all cells in all treatments were alive.

ANALYSIS OF EFFUSATE

After shaking pieces of thawed scale tissue in deionized H_2O for about 3 hr, the effusate was removed and analyzed. A summary of the data from four such experiments is given in Table III. Here the differences in conductivity values between -11°C and the control are not as great as in Tables I and II. As discussed above, and shown in Figure 1, this is due to the fact that these samples were shaken for about 3 hr.

K^+ and Ca^{2+} Content. The only ions analyzed in the effusate were K^+ and Ca^{2+} . It was found the K^+ was the main cation present, almost 100 times the concentration of Ca^{2+} . This is to be expected since K^+ is the major free cation present in the cell sap. The amount of K^+ present in the effusates was proportional

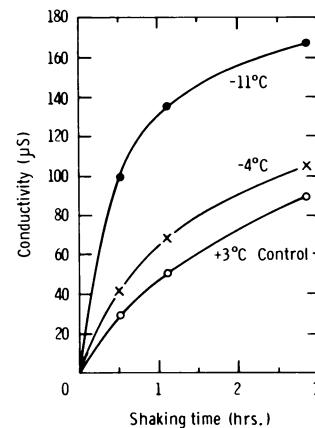


FIG. 1. Changes in conductivity of effusate (calculated as per g of scale tissue) with shaking time.

to the conductivity (Table III). These values account for about 10% of the total solutes (mosm) in the effusates in all of the treatments. Since an equivalent amount of anions must also be present in the effusate, the total of the K^+ plus the counterions accounts for 20% of the total osmolar solutes present in the effusate. Although the amount of Ca^{2+} present in the effusate was very small, it also increased in the -11°C compared to the -4°C and the control treatments.

Total Solutes. In agreement with conductivity and K^+ content, the total of the osmotically effective solutes present in the effusate increased with the lowering of the freezing temperature (Table III). This increase in total solutes was proportionately higher than the increase in conductivity and K^+ content. One possible reason for this is that as the injury to the cell increases, relatively more sugar (and other nonelectrolytes) than ions leak out of the cell.

Total Sugars. Only the total sugars present in the effusate were determined and expressed as glucose. The oligosaccharides are hydrolyzed to monosaccharides in the procedure used to determine the total sugars as glucose. Therefore, if all of the sugar was present in the effusate as sucrose, the true values for total sugar content would be about half the values in Table III.

² Abbreviation: μS : microsiemens.

This explains the impossible higher values for total sugars than for total solutes (which includes the sugars). The relative values in Table III are, however, fully comparable, and again demonstrate that the concentration of the effusate from scales previously frozen at -11 C is about double that of the controls.

pH of Effusate. There was a consistent increase in pH of the effusate with a lowering of the freezing temperature (Table III). This increase became much more pronounced when the data were calculated in terms of H^+ concentration instead of pH. It was found that the H^+ concentration was 0.35, 1.02, and 1.17 μM of the effusate in -11 C , -4 C , and control treatments, respectively. This means a decrease of more than three times in the effusate from the -11 C treatment compared to the control.

CONDUCTIVITY AS RELATED TO K^+ CONTENT

The major cation present in the effusate was found to be K^+ . An equivalent amount of anion must also be present in the effusate. Assuming that this anion is Cl^- , we can calculate the conductance of the KCl solution. From these calculations it was found that the amount of K^+ and its corresponding anion present in the effusate accounted for almost all of the conductivity of this effusate (the standard value used here was $0.02\text{ N KCl} = 2.77\text{ mS}\cdot\text{cm}^{-1}$; the cell constant for the conductivity cell was 1). To determine how well conductivity and K^+ content were related, a plot was made of all data from 12 separate measurements for each treatment (Fig. 2). A very high correlation was found between the two (correlation coefficient = 0.98). The value of the intercept was very close to zero, again indicating that as the K^+ content in the effusate decreased to zero the conductivity also dropped to zero. This confirmed our earlier calculations which showed that the K^+ along with an anion accounted for all of the conductivity of the effusate. This was true for the high as well as the low values, *i.e.* for all of the treatments.

K^+ CONTENT AS RELATED TO TOTAL SOLUTES

From the measured K^+ content as mg/l in the effusate, the mM K^+ concentration was calculated. K^+ accounted for only about 8% of the total solutes in the case of the -11 C treatment and only about 10% in the control treatment (Table III data). The same samples of effusate from which the data for Figure 2 were determined were analyzed for total solutes. A plot of the K^+ content as against total solutes in the effusate is given in Figure 3. A good fit (although not as close as for K^+ content as against conductivity) was obtained to a straight line (correlation coefficient = 0.93). A more careful examination of Figure 3 reveals that the points fit the line better for values of K^+ content below

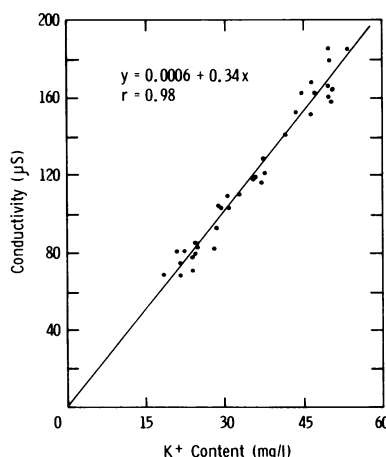


FIG. 2. Relationship between K^+ content and conductivity of effusate (calculated as per g of scale tissue).

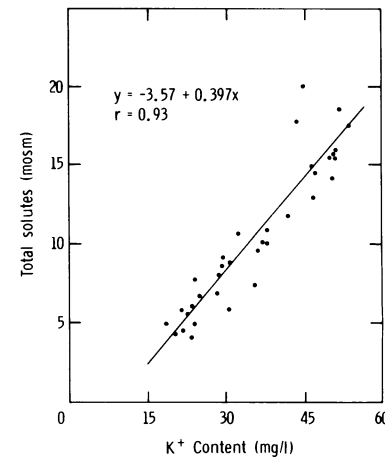


FIG. 3. Relationship between K^+ content and total solutes in effusate (calculated as per g of scale tissue).

40 mg/l . Conversely, the data points for K^+ content above 40 mg/l are much more spread out. In this upper range there seems to be a greater increase in total solutes relative to K^+ content. This range of values corresponds mostly to the -11 C treatment. This indicates that at higher levels of injury relatively higher amounts of nonelectrolytes leak out of the cells than electrolytes. Inversely it can be expected that at a low degree of drainage a relatively higher efflux of ions occurs. This is consistent with the data presented in Table III.

K^+ CONTENT AS RELATED TO H^+ CONTENT

From the data presented in Table III, it was found that the K^+ concentration in the effusate varied from about 1.3 to 0.8 mM whereas the H^+ concentration (calculated from pH values) varied from 0.4 to 4 μM . This means that H^+ concentration in the effusate was about 1,000 times smaller than K^+ concentration. To see if there was any relationship between the two, data from the same 36 samples as those used for Figures 2 and 3 were plotted (Fig. 4). The large scatter in the data indicates a poor relationship (correlation coefficient = -0.52). Yet there seems to be a general trend: as the K^+ content of the effusate increased, there was a decrease in H^+ concentration. This would seem to imply an exchange between the H^+ and K^+ with a K^+ efflux associated with an H^+ influx. However, the H^+ decrease is very small compared to the K^+ increase. This may be partly due to the buffering effect of the effusate.

CONDUCTIVITY AS RELATED TO PERMEABILITY OF MEMBRANES TO WATER

The -11 C treatment produced a 3- to 4-fold increase in the conductivity of the effusate compared to the control (Table II). In contrast, the permeability of the cells to water, measured on discs of inner epidermis taken from the same bulb tissue, did not change significantly. The water permeability constant (K_w) was 2.06, 1.97, and 1.76 $\mu\text{m}\cdot\text{sec}^{-1}$ for the -11 C , -4 C , and control treatments, respectively (Table II). Although the average values indicate an apparent slight increase in water permeability with the lowering of the freezing temperature, the variability within a treatment was higher than the differences among the treatments. Thus the freezing treatment of -11 C resulted in a net efflux of 50% of the ions from the living cells without a significant increase in cell permeability to water.

DISCUSSION

Viability. The cells in a frozen and thawed onion scale were alive even when 50% of the mobile ions were lost on the basis of the conductivity. These cells showed normal cytoplasmic stream-

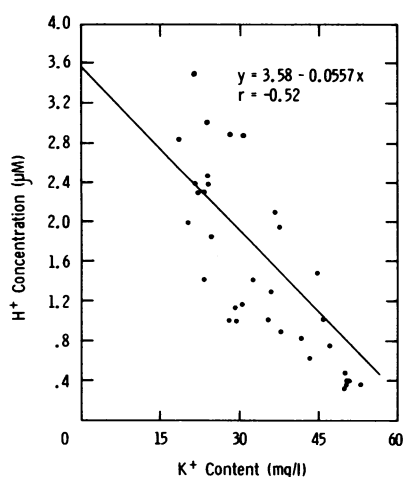


FIG. 4. Relationship between K^+ and H^+ concentration of effusate (calculated as per g of scale tissue).

ing, plasmolyzed normally, and revealed no observable damage under the optical microscope. Therefore the conductivity method does not measure the percentage of the cells killed, at least in the onion epidermis. Rather, the conductivity indicates the efflux of ions from still living cells and the excess above control value reveals the degree of microscopically unobservable injury to these living cells. This conclusion is supported by the simultaneous marked decrease in the cell sap concentration of the same cells, determined plasmolytically.

Ionic Composition of Effusate. Since it was generally assumed in the past that increase in conductivity of effusate following freezing and thawing was simply due to the efflux of all ions from completely killed cells, no analyses of effusate were made. In the effusate of *A. cepa* bulb scales K^+ ions plus counterions accounted for almost all of the increase in conductivity. There are two possible explanations. (a) Freezing increased passive permeability of cells to K^+ and possibly to counterion(s). (b) Freezing inactivated the active uptake mechanisms for K^+ and possibly the counterion(s). In the former case, efflux would be increased; in the latter, influx would be decreased leading to a net efflux. The high amounts of K^+ leaving the cell make the first alternative more probable.

Nonelectrolyte Efflux. The conductivity (in terms of K^+ and its counterion) accounted for only 20% of the total solutes leaked from the living but freeze-injured cells. Thus about 80% leakage from cells was of nonelectrolytes. The conductivity method, therefore, measures only part of the total leakage from cells. The most common nonelectrolytes present in plant cells are sugars. Direct measurements showed that freezing increased

the leakage of sugars to about the same degree as it increased the leakage of K^+ . The same two explanations can be given as for the increased K^+ efflux. These data agree well with the earlier findings on loss of sugars from bulb scale tissue of *A. cepa* (4, 13).

Passive Permeability. In earlier work it was generally assumed that the efflux of ions was due to the loss of semipermeability or increase in passive permeability of killed cells. Since the injured yet living cells could be plasmolyzed, the present study proves the first assumption invalid, at least for the onion bulb scale tissue. The latter, however, has to be tested experimentally. Although still alive, the cells may have suffered an injurious increase in passive permeability. Measurements revealed only a slight and insignificant increase in cell permeability to water. Water moves through the phospholipid bilayer part of the membrane in the usually accepted way by solution diffusion. It is unlikely that such increase in passive permeability could account for the large amounts of sugars and K^+ ions found to be released by the cell.

As an alternate hypothesis, the large ion and sugar efflux and the almost unchanged water permeability may be best explained on the basis of the complex structure of the plasma membrane and the tonoplast, and by assuming that the intrinsic membrane proteins controlling K^+ and sugar transport are damaged. It is generally thought that these passage routes are not available for water. This agrees well with insignificant change in permeability of cells to water observed in the present study.

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