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Dehydration of Onion Cells: A Comparison of Freezing vs. Desiccation and Living vs. Dead Cells

By

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Abstract

Measurements were made of the amount of liquid water present in the epidermal cells of onion at various degrees of dehydration caused by slow extracellular freezing and by desiccation. This was achieved by using a pulsed NMR spectrometer during freezing stress and by weighing the epidermal pieces during desiccation. Measurements were made on the extent of cell survival by direct microscopic observation (plasmolysis and protoplasmic streaming).

Onion epidermal cells (*Allium cepa* L. cv. Downing Yellow Globe) were found to survive freezing temperatures as low as −20°C and an equivalent desiccation stress. This equivalence opposes the reports by others on *Hordeum vulgare* and on *Solanum* sp. of greater injury by freezing than by an equivalent dehydration due to desiccation. The discrepancy has been explained in terms of the limitations of the conductivity method used by those authors to evaluate the injury.

The freezing and desiccation curves correspond to the equation:

$$L_t = L_0 \Delta t_m / t + L_u$$

where L_t and L_0 are the amounts of liquid water at temperature t and 0°C respectively. Δt_m is the freezing point depression of the cell sap and L_u is the amount of liquid water which does not freeze.

These results demonstrate that the dehydration of onion cells during both freezing and desiccation duplicates the dehydration of ordinary aqueous solutions. This was equally true for living and dead cells, and suggests that the negative turgor invoked by others is not significantly involved in the dehydration of living *Allium cepa* epidermis cells. An explanation is proposed for these contradictory results.

Introduction

Slow cooling of plant tissue is known to cause extracellular freezing provided that supercooling is prevented. As the stress progresses the ice grows outside the cell by withdrawing water from all parts of the cell, but mostly from the vacuole. This type of freezing causes cell contraction and subjects the cell to a dehydration stress. It has, therefore, been proposed that a freezing stress is parallel in many ways to a direct desiccation due to a water stress. Considerable evidence has been presented in the literature showing a striking similarity between freeze-induced dehydration and desiccation with regard to the relative tolerance by plants of these low temperatures and water stresses (Kanwisher 1957, Scarth 1941, Biebl 1956, Samygin and Matveeva 1961). A detailed discussion of this relation was given by Levitt (1972), who pointed out that most plants resist this type of injury by tolerance of dehydration.

Olien (1971), using leaf tissue of hardened plants of *Hordeum vulgare* concluded that dehydration energy arising from the ratio of the vapor pressure of ice to that of the liquid did not account for freezing injury. He calculated that desiccation requires twice as much dehydration energy compared to freezing to cause 50% of the maximum ionic leakage from the cells. Results of a similar nature were reported by Sukumaran and Weiser (1972) using leaves of two *Solanum* species. They found that the extent of the injury measured as ion leakage caused by slow freezing was greater than that due to equivalent desiccation, particularly in susceptible leaves. On the contrary, Samygin and Livshin (1970) determined the liquid water present in the inner epidermis cells of the scale of *Allium cepa* after subjecting them to various degrees of dehydration by freezing and water stress. They found no difference in the liquid water content of cells exposed to equivalent dehydrative water

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potentials by these two stresses. These results support the earlier view about the similarity of both stresses.

Samygin and Livshin also measured the liquid water retained by dead cells as compared to living cells. They found that at the same level of freeze dehydration and ordinary desiccation, the liquid (freezable) water present in the living cells was much higher than in the dead cells. This they attributed to the negative turgor present in the live cells. From their work they concluded that the injurious effect of the stress was mechanical, thus supporting Iljin's hypothesis (Iljin 1933). In contrast to this, Chen *et al.* (1976) using nuclear magnetic resonance (NMR) technique found that *Solanum* sp. leaf tissue behaves like an ideal solution with respect to the dehydration caused by freezing, and that live and dead cells became dehydrated to a similar degree at the same freezing temperature.

Thus, there are conflicting answers in the literature to the following two questions: (1) Is freeze-induced dehydration any different from evaporative desiccation? (2) Does the living plant behave like an ordinary aqueous solution or is there an additional force present in the cells (negative turgor) which resists dehydration? The present study is an attempt to answer these two questions.

Abbreviation: NMR, nuclear magnetic resonance.

Materials and Methods

Onions used in this study were *Allium cepa* L. cv. Downing Yellow Globe and were grown at the University of Minnesota Experimental Station. No herbicide or growth inhibitor, such as maleic hydrazide, was applied.

Preparation of the inner (adaxial) *epidermal layer*

The upper and lower quarters of the onion were discarded and the middle portion was used. The third healthy scale (counting inwards from the outermost fleshy scale) was selected and the inner epidermis used in this study was peeled off (carefully avoiding bending) using a pair of forceps.

Freeze-induced dehydration (NMR study)

About 100 mg of onion epidermal layer was inserted into a 5 mm weighed NMR tube. This tube was weighed again in order to determine the fresh weight of the tissue. It was then inserted into a pulsed NMR spectrometer. The amount of liquid water present in the partially frozen epidermal tissue was calculated by measuring the initial free induction decay signal following a 90 degree pulse. In order to avoid

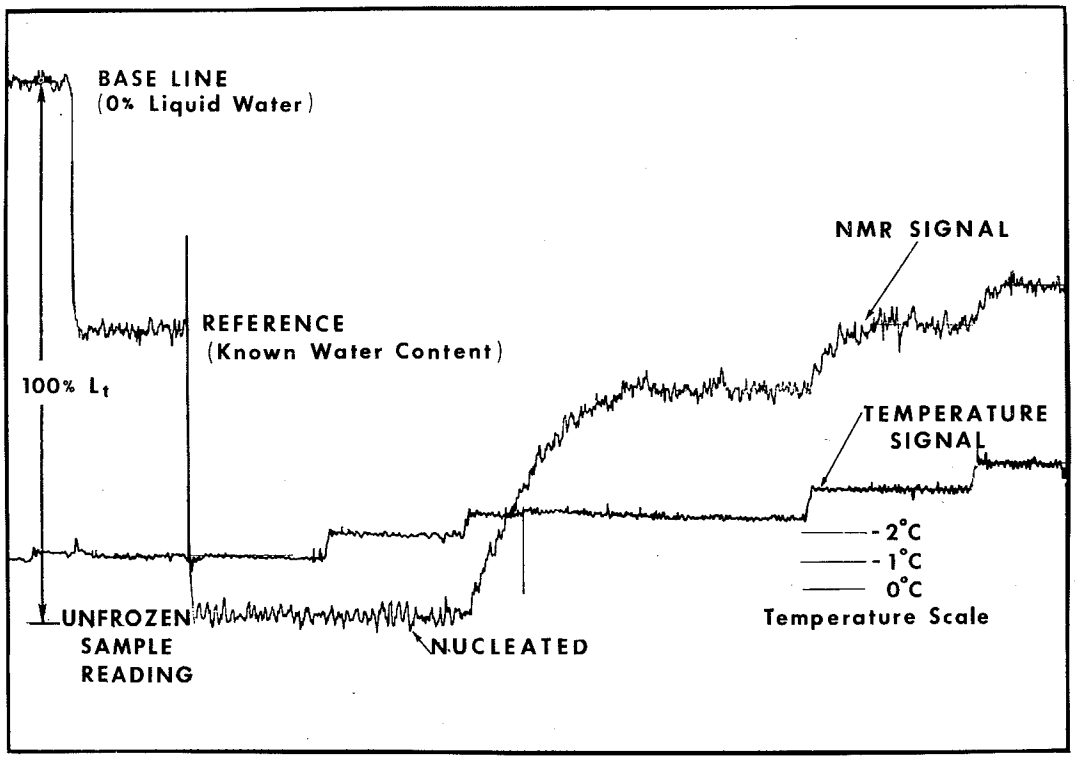

Figure 1. *Measurement of liquid water content in partially frozen onion epidermal cells.* Output of NMR signal and corresponding freezing temperature is shown.

error in the signal due to the ice present in the tissue, measurements were made at 20 μs delay (for details on the theoretical background for this application of NMR, see Burke *et al.* 1975).

A typical chart output of an NMR signal is shown in Figure 1. The base line reading is the NMR signal with empty tube, reference reading is a sample with known water content and the sample output is the signal from the onion epidermis put into the NMR tube. The distance between the sample and base line is proportional to the liquid water present in the sample. Fraction or percentage of liquid water present in the partially frozen sample was calculated by taking the distance between base line and unfrozen sample as 100% liquid water.

The epidermal tissue was cooled by circulating temperature-controlled nitrogen gas around the NMR tube. To initiate extracellular freezing, the tissue was nucleated between −1°C and −2°C (by contact with a wire dipped before into liquid nitrogen) and kept at that temperature until no further ice formation occurred (leveling off of NMR signal). Thereafter, the temperature was lowered in steps of 1°C to about −5°C and held at each step until no additional freezing could be detected (after about 30 to 45 min). The temperature was then dropped to about −7.5, −10, −15, −20 and −40°C, holding it at each step until equilibrium was attained. The sample was then thawed and oven dried to determine the dry weight. The NMR signal was also measured on the dry sample. The amount of liquid water (L_t) present in partially frozen tissue at any sub-freezing temperature (t) has been found to follow the relationship:

$$L_t = L_0 \Delta t_m / t + L_u$$

where L_0 is the amount of liquid water present in the unfrozen sample, Δt_m is the freezing point depression of the tissue liquid, and L_u is the unfreezable water present in the tissue at any of the freezing temperatures used (Gusta *et al.* 1975). From the plot of L_t/L_0 vs. $1/t$, Δt_m was calculated.

Water stress-induced dehydration

Weighed pieces of epidermal tissue were dehydrated to a constant weight at 20°C on platforms about 3 cm above NaCl solutions of various concentrations in covered containers (7 × 10 cm; height 5 cm). A record was kept of the weight of the tissue with time after exposure to the dehydrating stress; 20 to 40 h were needed to reach final weight. From the weights it was possible to calculate the amount of liquid water present in the tissue at vapor pressure equilibrium with each NaCl solution compared to its original amount. For comparison with freeze-induced dehydration the concentrations of the NaCl solutions were expressed as equivalent freezing point depressions in °C.

Osmotic potential

The osmotic potential of the cell sap was determined by the method of incipient plasmolysis. Sections of the epidermal strips from the same bulb scale as used in the dehydration experiments were transferred to a series of mannitol solutions. The solution that induced incipient plasmolysis in about 50% of the cells in the field of view was selected as equal in osmotic potential to that of the cell sap.

Cell survival

Freezing stress: About 1 cm² pieces of epidermal layer were transferred to test tubes lined with wet filter paper. These test tubes were then transferred to a cooling bath containing an ethylene glycol base antifreeze solution (Freezite-antifreeze and summer coolant, W. H. Barker Oil Co., Minneapolis, Minnesota, U.S.A.). The temperature was lowered at the rate of 1°C per h. The epidermis pieces were nucleated to form ice crystals at −2°C using snow flakes. When a desired temperature was reached, the test tube was removed and buried in ice contained in a styrofoam box, which was held at 0°C. After about 12 h, microscopic observations were made of the epidermis to determine the survival of the cells. Protoplasmic streaming and occurrence of plasmolysis in a hypertonic solution of mannitol were used as criteria of life. Bulb scales exposed to temperatures as low as −21°C were studied.

Water stress: About 1 cm² pieces of epidermal layer were exposed in air in a closed container above various concentrations of NaCl solutions. Two methods were used for producing various degrees of desiccation stresses.

Method 1. Water stresses equivalent to cryostresses of −2, −5, −7.5, −10, −15 and −20°C (approximately −25, −64, −98, −129, −194 and −257 bars) were produced by transferring the epidermal pieces directly to atmospheres (relative humidities) above NaCl solutions with freezing points corresponding to the above. They were left there for 20, 30, and 40 h. Two replications were used for each time. After the desired time of exposure to the stress was reached, the epidermal pieces were exposed to air above distilled water (100% humidity) for 12 h. The epidermal pieces were then transferred to plasmolyzing concentrations of mannitol solutions and observed for cell survival.

Method 2. Water stresses greater than the equivalent of −7.5°C were produced in steps. Eighteen epidermal pieces were first transferred to air above a NaCl solution equivalent to −7.5°C (*i.e.* −58 bars) and kept there for 24 h. After this, epidermal pieces in each were transferred to relative humidities above NaCl solutions equivalent to −10, −15, and −20°C, in groups of six each. Fifteen hours after this transfer, two pieces from each treatment were transferred back to the −7.5°C chamber and kept there for 5 h. The epidermal pieces were then transferred to air above distilled water, kept there for 5 h and then finally transferred to hypertonic mannitol solutions. Observations were then made for cell survival. From the remaining four epidermal pieces in these chambers two were taken out after 25 h and the other two after 50 h. These were treated the

Table 1. *Survival of inner epidermal cells of onion scale after exposing them to various degrees of water stress for various periods of time.* Two methods were used for producing stress (see Methods).

Concentration of NaCl solution used, *M*	Water stress expressed as: Relative humidity, %	Pressure potential, bars	Freezing point depression of NaCl solution, °C	Number of cells living after exposure to stress (h) Method 1: 20	30	40	Method 2: 15	25	50
0.58	98.2	−25	−2	All	All	All	All	All	All
1.42	95.2	−64	−5	All	All	All	All	All	All
2.08	92.6	−98	−7.5	All	All	All	All	All	All
2.65	90.1	−129	−10	All	Few	Few	All	All	All
3.65	84.9	−193	−15	Most	Few	None	All	All	All
4.51	80.7	−258	−20	Half	Few	None	All	Most	Few

same way as the first two cuttings and finally observed for cell survival.

Protoplasmic streaming or plasmolysis in a hypertonic mannitol solution was regarded as evidence of life.

Results

Cell survival

Freezing stress: Although there was some variability between different bulbs, about 60 to 80% of the inner epidermal cells, frozen at a cooling rate of 1°C per h survived −20°C. After freezing at −21 or −22°C most cells appeared dead and above −18°C all cells were living. More rapid cooling rates (up to 4°C per h) gave the same results. These experiments were repeated five times.

Water stress: Data on survival of onion epidermal cells exposed to water stress are presented in Table 1. All the cells survived a desiccation stress equivalent to −7.5°C (−98 bars). At stresses greater than −7.5°C survival of the cells was markedly affected by the method used to produce the stress and by the length of the time the cells remained under the stress. With Method 1, all cells survived after 20 h of stress equivalent to −10°C (−129 bars), the majority of these cells survived a stress equivalent to −15°C (−194 bars) and only half survived that equivalent to −20°C (−258 bars). When exposed to a desiccation stress equivalent to or more than −10°C, most of the cells were dead after a stress of 30 h or longer.

Results were somewhat different when the stress was produced in two steps (Method 2). All the cells survived a desiccation stress equivalent to −15°C (−194 bars) for at least 50 h. The majority of the cells survived a desiccation equivalent to −20°C for 25 h, whereas all of them were living when the stress was applied for only 15 h.

It thus appears that the majority of the onion epidermal cells can survive both a gradual desiccation due to a water stress up to the equivlent of a cryostress of −20°C and a dehydration due to an actual freezing temperature as low as −20°C. A gradual production and removal of the stress results in a higher degree of stress tolerance by the cells. This is in agreement with the finding of Iljin (1933).

The onion epidermal cells used by Samygin and Livshin (1970) also survived freezing and water stress both to the same degree of dehydration, but this was only to −10°C. This could be due to a difference in the onions used in their study or in the method used for producing and removing the stress.

Extent of dehydration caused by freezing and water stress

The process of dehydration of epidermal cells caused by either a desiccating or by a freezing stress followed the behavior exhibited by ideal salt solutions. This was found from the plots of fractions of liquid water (L_t/L_0) present in the cells against the reciprocals of the corresponding temperatures $1/t$ (Figure 2, lines A and B). To calculate L_t/L_0 the mean for all experiments for the water content (L_0) of fresh tissue (5.88 g water/g dry weight) was used. These data fit well to a straight line. The correlation coefficient is 0.99 in the case of freezing data and 0.98 in the case of desiccation data.

From the regression lines A and B drawn in Figure 2, the amount of liquid water present in these epidermal cells, at various degrees of dehydration, was calculated and is presented in Table 2. The amount of bound (unfreezable) water (intercepts in Figure 2) seemed much higher in the frozen sample than in the desiccated sample; therefore, for proper comparison these values were subtracted and the resulting values are given in Table 2. The liquid water content of the dehydrated samples was lower in samples subjected to water stress than in samples subjected to equivalent freezing stress. However, this difference becomes smaller and smaller with increasing intensity of stress (*e.g.* only 1.5% of the original amount at −20°C, Table 2). This also is clear from the greater slope of the regression line for the freezing stress than for the water stress (Figure 2).

The difference in the value of bound (unfreezable) water (Figure 2, intercepts) in frozen epidermal cells and in cells subjected to desiccation may reflect the difficulty in defining

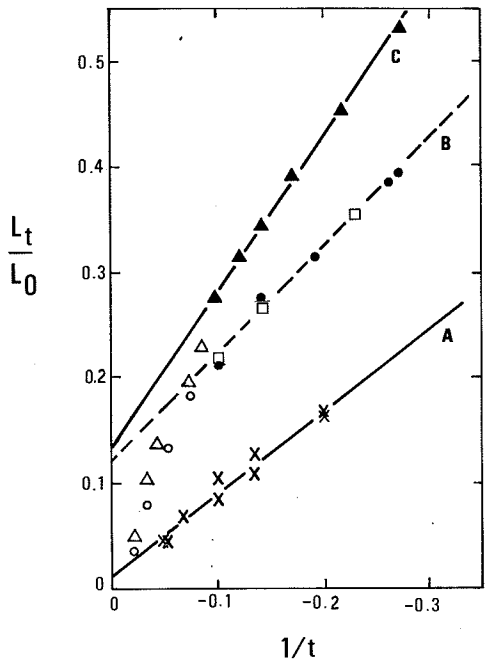

Figure 2 (i) *Comparison of the dehydration behavior of onion epidermal cells under desiccation* (line A) *and freezing* (line B) *stress.* Fractions of liquid water (L_t/L_0), present in onion epidermal cells, are plotted against the reciprocals of the corresponding temperatures ($t°C^{-1}$). t is the freezing point depression of the NaCl solutions used for producing desiccation. (□) represent the readings of L_t/L_0 against $1/t$ when the same cells were killed by quick freezing down to −40°C. (ii) *Effect of the cell sap concentration* (osmotic ground value) *on freezing behavior of onion epidermal cells of different osmotic ground values* (0.50 osmolal line B and 0.76 osmolal line C). (iii) Points with open circles (○) and triangles (△) represent the measurements below −10°C for the experiments represented by lines B and C correspondingly. These points have not been taken into consideration for making regression lines (for explanation, see text). (iv) Regression equation: Line A: $L_t/L_0 = 0.02 - 0.76/t$, Line B: $L_t/L_0 = 0.12 - 1.03/t$, Line C: $L_t/L_0 = 0.13 - 1.48/t$.

Table 2. *Amounts of liquid water retained by the epidermal cells at various degrees of dehydration.* These values have been calculated from the regression lines of Figure 2. Values for bound water have been subtracted in both stresses.

Equivalent dehydrating stress		Liquid water content of epidermal cells, % of original amount	
°C	Bars	Water stress	Freezing stress
−5	−64	15.2	20.7
−7.5	−98	10.1	13.8
−10	−129	7.6	10.3
−15	−193	5.1	6.9
−20	−258	3.7	5.2

bound water. In the freezing experiment ice forms in the extracellular spaces which contain cell wall. At the interface between the ice and cell wall a fraction of water may, and probably does exist which will not form the usual crystalline ice structure. The NMR properties of such water have been measured (Hsi *et al.* 1977). This interfacial water has the NMR properties of a viscous liquid. Such a fraction will be identified as unfreezable water by NMR methods. The situation is substantially different in the dehydration experiments where no ice or ice-cell wall interfaces exist.

A deviation from linearity between L_t/L_0 and $1/t$ was observed for temperatures below −10°C. Points with open circles and triangles (○, △, Figure 2) represents the measurements below −10°C. These points have not been taken into consideration for making regression lines B and C. This nonlinear behavior of NMR free induction decay signal at low temperatures is probably caused by the very high proportion of ice present (about 80% of the original freezable water) in the sample. Under there conditions relaxation time T_2 becomes very short and T_1 becomes longer (see Hsi *et al.* 1977, Beall *et al.* 1976). This introduces another difficulty in NMR measurements of water content in partially frozen tissues. These complications, obviously, do not affect the proportionality of L_t/L_0 with $1/t$ for the linear part (see Figure 2).

Osmotic value of the cell sap vs. Δt_m

The osmotic value of the epidermal cell sap as determined by incipient plasmolysis was found to be 0.50 osmol. This corresponds to a freezing point depression (Δt_m) of about 0.93°C. Another independent way to get this value is to determine the slope of the regression lines in Figure 2,

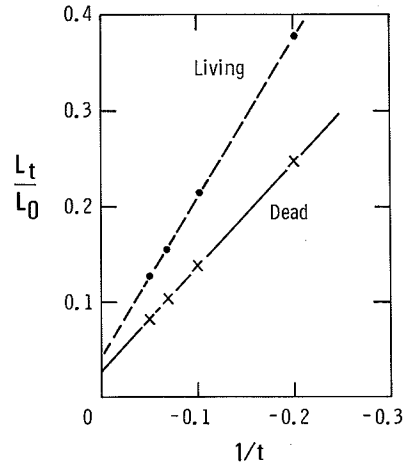

Figure 3. *Fractions of liquid water content (L_t/L_0) in living and dead onion epidermal cells at various degrees of desiccation.* Degree of desiccation is expressed as inverse of the freezing point depression of NaCl solution. Data from Samygin and Livshin (1970).

which is the same as Δt_m (Burke *et al.* 1975). The slopes for the freezing (line B) and desiccation (line A) curves in Figure 2, were found to be 1.03 and 0.76°C (corresponding to 0.52 and 0.41 osmol for the osmotic value of the cell sap), respectively. Therefore, the Δt_m calculated from the plots of L_t/L_0 vs. $1/t$ was lower for desiccation and higher for freeze-induced dehydration. These variations could be due to the different conditions in these two types of experiments. In desiccation experiments the samples were exposed to corresponding relative humidities at 20°C (see Materials and Methods) for more than 1 day. In this case metabolic activities could be expected to be considerably different than at subfreezing temperatures in the freezing experiment which was terminated in several hours. Thus changes in cell sap concentration may develop during desiccation stress.

The relationship between the slope of the lines A and B in Figure 2 and the osmotic values of the cell sap was tested by artificially increasing the cell sap concentration. This was achieved by transferring epidermis pieces into a 0.3 *M* urea solution and leaving them in this solution for 4 h. After that time the concentration of the cell sap (determined quickly by incipient plasmolysis) increased from 0.50 to 0.76 osmol. The freezing curve of this epidermis with increased cell sap concentration is shown in Figure 2 as line C. The amount of unfreezable liquid water (intercepts of lines B and C in Figure 2) increased from 12 to 13% of the original amount, an increase of about 9%. However, the slope of the regression line increased from 1.03 to 1.48°C (corresponding to 0.52 and 0.73 osmol), an increase of about 50%. Here again, the data fit very well to a regression line with a correlation coefficient of 0.99, indicating that the freezing behavior of onion epidermal cells is similar to that of an ideal solution. A similar freezing behavior was found for leaves of *Solanum tuberosum* (Chen *et al.* 1976) and *Triticum vulgare* (Gusta *et al.* 1975).

Liquid water retained (L_t) in partially frozen living and dead cells

The onion epidermal pieces in which L_t was determined at various subfreezing temperatures were cooled down to −40°C and warmed up quickly to about −2°C. This rapid cooling and thawing kills the cells. With these pieces of dead epidermal cells L_t/L_0 was determined at various subfreezing temperatures. These values of L_t/L_0 are plotted in Figure 2 (□) and were found very close to the original L_t/L_0 values for living cells. This experiment was repeated three times with similar results.

Discussion

The results indicate that the onion epidermal cells are equally tolerant of dehydration by freezing as by desiccation. This agrees with the findings of Samygin and Livshin (1970). This also supports the earlier view discussed by Levitt (1972) that most plants survive either a gradual freezing or a water stress by tolerance of dehydration. However, our results are contrary to the finding of Olien (1971) and Sukumaran and Weiser (1972).

The reason for this contradiction may be partly explained by having a detailed look at the methods used by the authors for evaluating the injury. Olien (1971) and Sukumaran and Weiser (1972) used the conductivity method described by Dexter (1956). There are two problems with this method. First, it is frequently assumed that an increase in conductivity following freezing is a measure of the efflux of ions from dead cells alone (cf. Osterhout 1922). In recent experiments on onion cells all the cells were still alive after the efflux of ions was considerable (Palta *et al.* 1977). Second, the conductivity of the effusate has been found to increase up to two times if the unfrozen tissue is infiltrated with water before shaking (Palta, unpublished). The cause for this increase may be an enhanced diffusion of ions out of the intercellular space and the cell walls, when the tissue is infiltrated before shaking. Also, K^+ ions have been found to diffuse out of the intact cells when they are placed in distilled water (Sutcliffe 1952, p. 65). This point is of great interest in this discussion.

Normally, when a frozen tissue is thawed, the ice in the extracellular space between the cell walls melts, and if some injury has occurred, this water is not rapidly or completely reabsorbed by the cells. This results in a water-soaked or infiltrated tissue. No such infiltration occurs when desiccated tissue is rehydrated. The tissue subjected to water stress does not become infiltrated on release from the stress, because desiccation involves removal of the water from the tissues, rather than storage in the extracellular spaces. Therefore, a frozen and thawed tissue should give a higher conductivity value than a desiccated and rewatered tissue. This may explain why Olien (1971) and Sukumaran and Weiser (1972) recorded a greater injury by a freezing stress compared to an equivalent desiccating stress. In this study, instead of the indirect conductivity method, direct tests for cell viability were used.

Another explanation for the conflicting data by these authors could be that they did not reach equilibrium with the water potential of the desiccating medium. Our experiments show that the kinetics of dehydration by freezing and desiccation are quite different. Desiccation takes several orders of magnitude longer time to reach equilibrium compared to freezing. A nonequilibrated desiccation will tend to produce less injury compared to equilibrated freezing stress.

According to Gusta *et al.* (1975) and Chen *et al.* (1976) plant tissues freeze like ideal solutions and the freezing curve has the form:

$$L_t = L_0 \Delta t_m / t + L_u$$

The data in this study strongly support this finding and show that the desiccation curve for onion epidermal cells

follows the same pattern. This further supports the concept that extracellular freezing produces a dehydration similar to that by desiccation. Samygin and Livshin (1970), however, have argued against the concept that the freezing behavior of plant tissue is a direct function of the solute concentration of the cell sap and presented data which led them to conclude that there is an additional force (negative turgor) present in live cells.

To find an explanation for this conclusion of Samygin and Livshin (1970) we plotted their data (Figure 3). Both the living and the dead cells exactly follow the behavior of an ideal solution. There was a difference of 0.57°C in the slopes of their regression lines, the living cells having the higher slope. When expressed as osmotic value of their cell sap, the concentration in the living cells was 0.89 osmol and in the dead cells 0.59 osmol. Since these authors have not indicated the method used to kill the cells, it is difficult to explain this difference in concentration. Most commonly, cells are killed by dipping the tissue quickly in liquid nitrogen, followed by fast thawing. If Samygin and Livshin (1970) used this method, it is possible that some of the salts diffused out of the cells since the tissue becomes completely infiltrated when killed this way. Since they fully saturate the tissue before running a test, water adhering or condensed on the epidermis would be available as solvent for solutes from the cell sap after cell death. Droplets of this solution could have been lost during manipulation, thus decreasing the amount of solutes in the epidermis.

In conclusion, two statements should be advanced. First, that the freeze-induced dehydration is similar in effect on the cell to direct desiccation and second, that the plant cells being dehydrated do behave like an ordinary aqueous solution. There exists a proportionality between relative amount of unfrozen water and the negative inverse of subfreezing temperature. This relationship is the same for live and dead cells. Hence we do not find any evidence for the presence of an additional water-retaining force in living onion epidermal cells (for instance, negative turgor) which resists dehydration.

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