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Freeze-Thaw Injury to Isolated Spinach Protoplasts and Its Simulation at Above Freezing Temperatures[1]

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ABSTRACT

Possibilities to account for the mechanism of freeze-thaw injury to isolated protoplasts of *Spinacia oleracea* L. cv. Winter Bloomsdale were investigated. A freeze-thaw cycle to −3.9 C resulted in 80% lysis of the protoplasts. At −3.9 C, protoplasts are exposed to the equivalent of a 2.1 osmolal solution. Isolated protoplasts behave as ideal osmometers in the range of concentrations tested (0.35 to 2.75 osmolal), arguing against a minimum critical volume as a mechanism of injury. Average protoplast volume after a freeze-thaw cycle was not greatly different than the volume before freezing, arguing against an irreversible influx of solutes while frozen. A wide variety of sugars and sugar alcohols, none of which was freely permeant, were capable of protecting against injury which occurred when protoplasts were frozen in salt solutions. The extent of injury was also dependent upon the type of monovalent ions present, with Li = Na > K = Rb = Cs and Cl ≥ Br > I, in order of decreasing protoplast survival. Osmotic conditions encountered during a freeze-thaw cycle were established at room temperature by exposing protoplasts to high salt concentrations and then diluting the osmoticum. Injury occurred only after dilution of the osmoticum and was correlated with the expansion of the plasma membrane. Injury observed in frozen-thawed protoplasts was correlated with the increase in surface area the plasma membrane should have undergone during thawing, supporting the contention that contraction of the plasma membrane during freezing and its expansion during thawing are two interacting lesions which cause protoplast lysis during a freeze-thaw cycle.

Research concerned with freezing injury in plants and work on microorganisms and animal tissues have essentially been two distinct fields of study and little effort has been made to "... incorporate the results into more effective concepts of freezing injury" (12). Direct comparisons between the two systems have not and should not have been made since the freezing processes of the two systems are quite disparate. With the possible exception of algae, the environment of plant cells frozen *in vivo* is considerably different from that of single mammalian cells frozen *in vitro*. Although the latter are suspended in isotonic aqueous solutions, the former are osmotically stable in H_2O due, in large part, to pressure developed by constraints on the cell membrane by a relatively rigid cell wall. Ice, which surrounds single cells when suspensions are frozen (16), forms only in isolated regions of some plant tissues (6), presumably due to the sparsity of extracellular water available for nucleation and crystallization and/or the location of favorable nucleation sites (7). These disparities between *in vivo* and *in vitro* studies preclude a comparison between the two systems.

Isolated plant protoplasts provide a system in which responses of plant cells to a freeze-thaw cycle can be directly compared with those of mammalian cells experiencing similar environments. It should be possible to determine whether the mechanisms of freeze-thaw-induced lysis of isolated plant protoplasts is similar to that of frozen-thawed mammalian cells. Singh (22) reported that protoplasts isolated from acclimated rye seedlings survive freezing better than protoplasts obtained from nonacclimated rye, suggesting that protoplasts may also be useful in studies of the mechanism of acclimation in plants.

Numerous hypotheses have been suggested to account for cell lysis after a freeze-thaw cycle. During freezing, water is converted to ice, thereby decreasing the water content and simultaneously increasing the concentration of solutes. Lysis may be the result of some detrimental action of this increased solute concentration (9). If the cell is forced to shrink below a "minimum critical volume" because of the osmolality of the remaining unfrozen solution, lysis could occur (14). Some cells become permeable to salts under strongly hypertonic conditions, such as occurs during freezing (5). On thawing, these "loaded" cells could burst due to the high internal solute concentration (30). These are a few of many theories put forth to describe the primary cause of freeze-thaw injury to cells. This study was conducted to determine whether factors reputed to cause injury to other cell types, primarily mammalian red cells, are also causally related to freeze-thaw injury of isolated spinach protoplasts.

MATERIALS AND METHODS

Protoplast Preparation. Protoplasts were obtained from leaves of greenhouse-grown *Spinacia oleracea* L. cv. Winter Bloomsdale. The lower cuticle and epidermis were manually stripped from leaves before the cells on the remaining leaf were plasmolyzed by placing them in 0.6 M mannitol for about 30 min. Cell walls were enzymically removed in a solution of 0.6 M mannitol, 0.3% (w/v) potassium dextran sulfate, 0.5% Macerase (Calbiochem), and 1.5% Cellulysin (Calbiochem) (after ref. 1). This solution was adjusted to pH 5.5 and clarified by centrifugation at 1,500*g* for 10 min before adding the plasmolyzed leaf tissue. Incubation occurred on a rotary shaker (37 rpm) at 33 C in the dark for 3 to 5 hr. The resulting protoplast suspension was filtered through two layers of Miracloth to remove cell debris, and protoplasts were pelleted at 30*g* for 10 min. Further purification was achieved by layering the protoplasts above a cushion of 20% (w/w) sucrose. Protoplasts were collected at the sucrose-mannitol interface after centrifugation at 110*g* for 10 min. Protoplasts were washed at least three times in the respective osmotica before freezing or other treatments.

Freezing Procedure and Viability Determinations. Half-ml suspensions of protoplasts in covered test tubes, typically containing 10^4 to 10^5 protoplasts/ml, were placed directly in a stirred methanol-water bath at −3.9 C. No significant injury resulted from this sudden supercooling. All samples were equilibrated at this temperature for at least 15 min. During this time, most of the

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protoplasts had settled to the bottom of the test tube. Ice formation was initiated by introducing a small ice crystal (grown from condensed water vapor at about −40 C) into each tube. The maximum rate of cooling measured after the initial release of latent heat was about 20 C/hr. The temperature was either held constant or was slowly lowered (5.6 C/hr) to −6.7 C. Protoplasts were held at this temperature for at least 30 min before thawing in a stirred methanol-water bath at 4 C at a rate of about 100 C/hr. All samples were held at 4 C until survival determinations were made. Protoplast survival was determined by their appearance (19), their apparent intactness, and the ability to exclude Evans blue dye (final concentration of 0.1%, w/v). Intact and presumably viable protoplasts were counted on a hemocytometer (0.2-mm depth). The volume analyzed to determine protoplast density was generally 3.2 μl and typically contained 10^2 to 10^3 protoplasts.

Determination of Osmolality. Osmolality was calculated by the conversion of molarities of individual species into osmolalities using published values (25). The sum of the individual osmolalities was taken as the osmolality of the solution. These calculations were verified by freezing point determinations of various solutions. When appropriate, the calculation of the osmolality was corrected for the volume displaced by the addition of Evan's blue but not the osmotic value of the dye itself (about 1 mM).

RESULTS AND DISCUSSION

Kinetics of Freezing Injury. Figure 1 demonstrates that protoplasts are injured by freezing to −3.9 C. Sucrose, a well known cryoprotectant for plant (8) and animal (13) cells, served as an effective cryoprotectant in this system. The injury, both with and without sucrose, appeared to be complete as soon as the heat arising from the fusion of water (the "exothermic region") had dissipated and the temperature of the sample returned to that of the surrounding bath. This is in agreement with the rapidity with which a freeze-thaw cycle causes injury in red cells (9). The injury that occurred was attributed to ice formation rather than low temperatures of aging because protoplasts which were supercooled at −3.9 C in corresponding solutions for similar periods of time exhibited no decrease in viability.

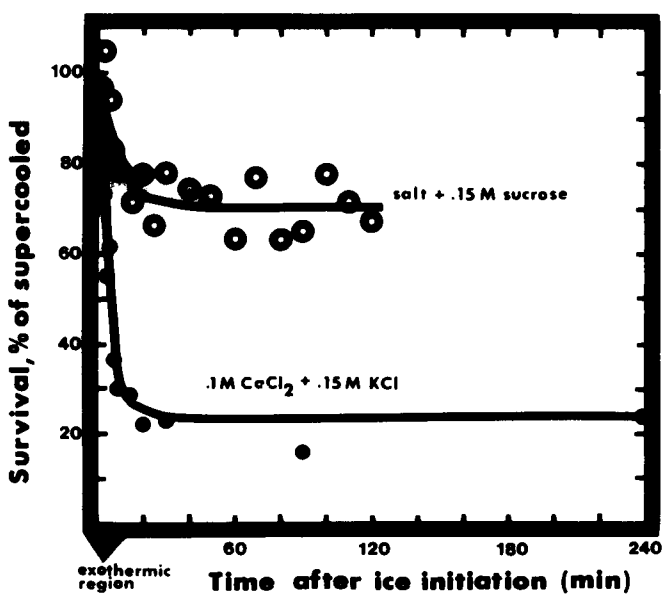

FIG. 1. Time course of protoplast lysis following a freeze-thaw cycle to −3.9 C in 0.1 M $CaCl_2$ + 0.15 M KCl ± 0.15 M sucrose. Survival is a percentage of supercooled controls and was determined as described under "Materials and Methods." "Exothermic region," determined by monitoring the temperature of a sample with a thermocouple, is the time required for reequilibration of the sample temperature with the bath temperature, and was found to be 10 to 12 min.

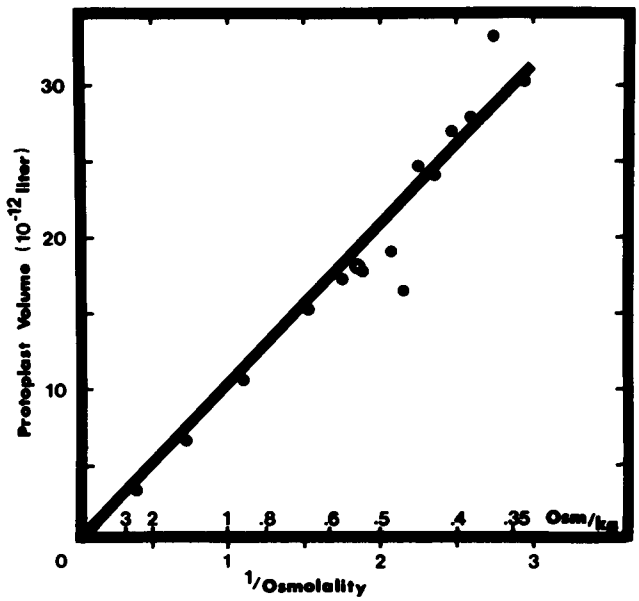

FIG. 2. van't Hoff plot of protoplast volume as a function of external osmolality of equiosmolar $CaCl_2$ + NaCl. Protoplasts were isolated and purified as described under "Materials and Methods." They were subjected to osmolarities of 0.376 to 3.027 (calculated osmolalities of 0.342 to 2.752). Open circle: volume of freshly isolated protoplasts. Remaining protoplasts were exposed to various osmolalities for about 12 hr at 4 C, individually brought to room temperature, 0.05 ml of dye was added to the 0.5-ml suspension of shaken protoplasts, and individual diameters of apparently viable protoplasts were determined with an eyepiece micrometer at a magnification of × 125. Volumes were calculated from the average diameters. Regression coefficient (r = .940) is significant at the 1% level.

Osmometric Behavior of Isolated Protoplasts. Meryman and co-workers (14, 28) have speculated that the attainment of a minimum critical volume is responsible for freezing injury to red blood cells and spinach thylakoid vesicles. This hypothesis suggests that either there exists some volume beyond which the cell cannot shrink without injury occurring or there exists a physical minimum volume which results in a pressure differential across the plasma membrane. The former possibility is difficult to test directly whereas the latter is amenable to experimental ascertainment. Substantial injury occurred to protoplasts which underwent a freeze-thaw cycle to −3.9 C (Fig. 1). At −3.9 C the system is composed of ice and a solution in equilibrium with the ice which has a calculated osmolality of about 2.1 (11). If injury which occurred following a freeze-thaw cycle to −3.9 C was due to the attainment of a physical minimum volume, this volume should have been achieved at 2.1 osmolal. Spinach protoplasts behave as osmometers in solutions between 0.35 and 2.75 osmol ($CaCl_2$ + NaCl)/kg H_2O, the latter concentration being higher than that experienced by protoplasts frozen to −3.9 C (Fig. 2). There is no evidence in Figure 2 suggesting that protoplasts even resist shrinkage at relatively high osmolalities. It is doubtful that attainment of a physical minimum volume could account for freeze-thaw-induced lysis of spinach protoplasts. Evidence for its existence is weak even with red blood cells. Replotting red cell data of Farrant and Woolgar (4), who measured the amount of intracellular water isotopically, reveals a linear (r = 0.998) van't Hoff plot for concentrations of NaCl up to 3.55 osmolal (27). Most work suggesting the existence of a minimum critical volume has been done using a hematocrit, apparently without correcting for the volume of the extracellular solution (14, 28), or by using indirect calculations on measurements of whole plant cells (29). Hemolysis measurements of red cells were made only after a "sudden return to isotonic solution" (15). Other data to be reported here are also not consistent with the concept of injury being associated with the attainment of a physical minimum volume.

Cryoprotection. To investigate other possible mechanisms of injury to frozen-thawed protoplasts, a general characterization of the system is required. Knowledge of the qualitative and quantitative specificity of cryoprotectants is necessary before an analogy between frozen-thawed protoplasts and other cell types can be drawn. In addition, the effect of the qualitative nature of electrolytes on the injury to protoplasts could aid in establishing the mechanism of injury.

Table I demonstrates that a wide variety of sugars and sugar alcohols were capable of protecting protoplasts against the lysis experienced when frozen in salt alone. Ficoll, a long chain polymer of sucrose with a mol wt of about 400,000, was unable to protect against injury at the concentration used. A controversy currently exists as to whether or not compounds must permeate the cell to be protective (compare refs. 10 and 13). If the sugars were not permeant, the volume of protoplasts in $CaCl_2$ + KCl plus 0.3 M sugar (assuming 0.3 M to be roughly equal to 0.3 osmolal) should be about 63.6% (calculated from Fig. 2) of their volume in salt alone, or 9.1 picoliters. The values obtained, ranging from 6.9 to 10.7, are within experimental variability of this expected value and would suggest that the plasma membrane of protoplasts is not freely permeable to any of the sugars. However, only a very gross uptake of sugars could be deduced from volume measurements. For instance, if the protoplasts had accumulated sufficient sugar to raise the internal concentration by 0.1 M, their calculated volume would be 10.5 picoliters, which is also within the range of experimental observations. Whether the protection afforded by sugars is due to permeation cannot be determined from experiments in which cell volume is used as the criterion for determining permeability.

Cryoprotection has long been thought to be due to a colligative reduction in the extracellular and/or intracellular solute concentration attained in the frozen state (10, 11). The conclusion drawn from Table I, that sugars are not freely permeant and yet act as cryoprotectants, argues against the premise that sugars colligatively reduce the intracellular solute concentration and therefore prevent lysis. The premise that cryoprotection occurs by the reduction of extracellular salt concentrations was tested by the experiment shown in Figure 3. Prior to freezing the protoplasts were suspended in 0.1 M $CaCl_2$ + 0.15 M KCl plus various concentrations of either sucrose or equimolar glucose + fructose. Alternatively, both the sucrose and salt concentrations were varied in an attempt to keep the total osmolality of the solution relatively constant. The results presented in Figure 3A suggest that at least for sucrose and equimolar glucose + fructose, protection appears to be correlated with the concentration of hydroxyl groups, as has been observed with rapidly frozen human erythrocytes (2). When the sucrose concentration was varied and the total osmolality of the solution held relatively constant, survival was not as high (*e.g.* at any given concentration of hydroxyl groups) as when the initial salt concentration was held relatively constant and the osmolality of the suspending medium was increased by the addition of sugar. The protection afforded by sucrose, and probably other sugars as well, is due neither to a colligative reduction of the extracellular salt concentration nor to an effect related solely to the concentration of sugar hydroxyl groups. Figure 3B represents the same data plotted as a function of the total osmolality of the suspending solution (determined by freezing point depression, similar results (rms deviation = 0.042 osmolal) were obtained when osmolality was determined with a dewpoint hygrometer). It can be seen that survival after all treatments can be adequately described by the total osmolality of the suspending medium, with higher survival occurring at higher osmolalities. We will return to this observation later.

Table I. Effect of Sugars on the Survival of Spinach Protoplasts

Solutions containing protoplasts were either supercooled or frozen to -6.7 for 12 hrs. Volume measurements were made of supercooled controls as described in Figure 2. Survival is expressed as a percentage of supercooled controls.

Osmoticum: Addition	0.1 M $CaCl_2$+0.15 M KCl Volume (pliters)	% Survival Frozen	% Survival Supercooled[1]	0.1 M $CaCl_2$+0.15 M NaCl % Survival Frozen	% Survival Supercooled[1]
None	14.3	8	56	13	61
0.3 M methyl-α-D-glucose	10.7	21	89	38	54
0.3 M glucose	8.3	25	50	47	53
0.3 M fructose	10.7	23	52	52	37
0.15 M glucose + 0.15 M fructose	n.d.[2]	26	58	47	37
0.3 M mannose	6.9	30	61	44	62
0.3 M mannitol	7.6	39	56	53	63
0.3 M sucrose	9.8	32	76	49	60
5% (w/v) Ficoll	n.d.	n.d.	n.d.	13	67
LSD .05		38	19	24	16

[1] Number of protoplasts surviving supercooling as a percentage of the freshly isolated number. Survival values are means of 3 experiments except methyl-α-D-glucose in $CaCl_2$+KCl and Ficoll where n = 2.

[2] n.d. = not determined.

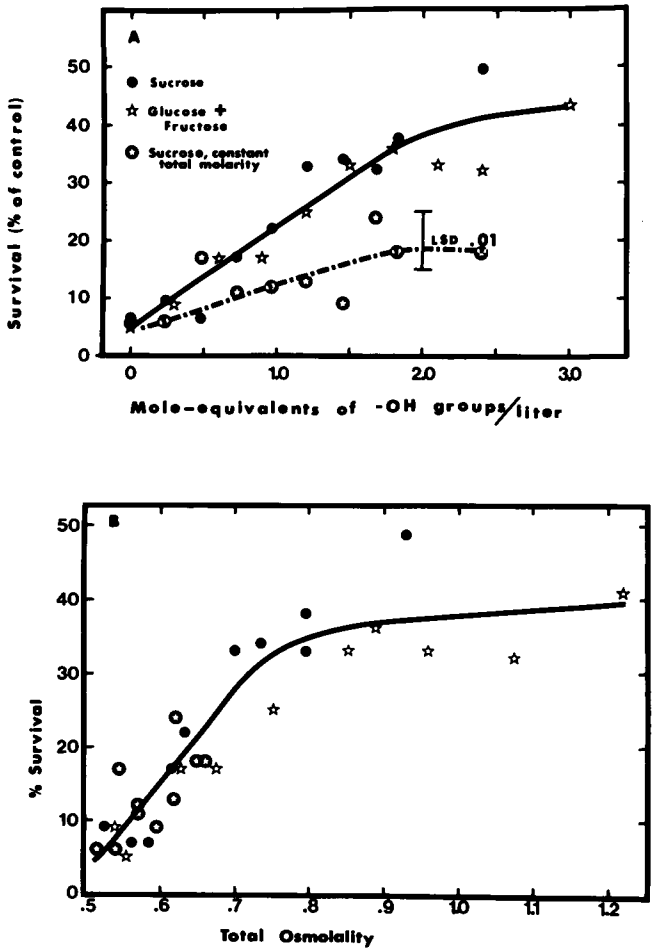

FIG. 3. Survival of protoplasts (as a percentage of supercooled controls) subjected to a freeze-thaw cycle to −6.7 for 12 hr in either 0.1 M $CaCl_2$ + 0.15 M KCl and varying concentrations of sucrose or equimolar glucose + fructose or in varying concentrations of salt (ranging from 0.1 M $CaCl_2$ + 0.15 M KCl to 0.05 M $CaCl_2$ + 0.075 M KCl) and sucrose (ranging from 0 to 0.3 M) designed to keep the osmolality relatively constant. A: survival as a function of the number of sugar hydroxyl groups; B: survival as a function of the over-all osmolality of the suspending osmoticum.

Effect of Monovalent Ions on Freeze-Thaw-induced Lysis. In suggesting salt toxicity as a mechanism of injury, Lovelock (9) demonstrated that injury of red cells was higher when frozen in LiI than in NaCl. Table I demonstrates that freeze-thaw injury to protoplasts was greater when K^+ was the monovalent cation in the osmoticum even though K^+ had no apparent detrimental effect on survival of the supercooled controls. Differential effects of various monovalent cations and anions on the survival of frozen-thawed protoplasts in the presence of sucrose were further investigated (Table II). In order of decreasing protoplast survival,

Table II. Effect of Monovalent Electrolytes on the Extent of Freeze-thaw Injury to Protoplasts

Protoplasts were isolated as described in Materials and Methods. They were transferred from the sucrose cushion directly into 0.1 M $CaCl_2$ + 0.15 M 1:1 salt with a pasteur pipette. They were washed (110 g, 10 min) in these solutions twice before sucrose was added. Protoplasts were frozen to -3.9 C for 40 to 45 min in an osmoticum of 0.1 M $CaCl_2$, 0.3 M sucrose and 0.15 M 1:1 salt as indicated. Survival is expressed as a % of the supercooled controls. Values represent the mean ± SD (n=4). Volume measurements of supercooled controls were made as described in Figure 2.

1:1 salt[1]	% Survival	Volume (pliters)
LiCl	83 ± 6	11.0
NaCl	81 ± 6	11.4
NaBr	70 ± 6	12.1
NaI	43 ± 7	8.4
KCl	56 ± 6	14.5
RbCl	58 ± 7	10.1
CsCl	47 ± 8	10.3

[1] A 1:1 salt is composed of two oppositely charged monovalent ions.

cations are ranked Li = Na > K = Rb = Cs and anions are ranked Cl ≥ Br > I. There appears to be no correlation between the extent of injury and the volume of supercooled controls in the various salts. Since this suggests that there are no gross differences in the permeability of protoplasts to the various salts, the differential injury was probably not due to a differential intracellular salt concentration in the various osmotica. An unequivocal interpretation of Table II is not possible since different anions and cations may affect the activity of sucrose differently (18). We have found no significant difference between the freezing points of equiosmolar solutions containing sucrose, $CaCl_2$ and either LiCl, NaCl, or KCl, suggesting that this is not a probable reason for the differences in the amount of injury.

Since a wide variety of sugars were cryoprotective to protoplasts (Table I), with little if any specificity, and since the specific monovalent ions in the osmoticum affected the amount of injury (Tables I and II), salt toxicity as a mechanism of injury as suggested by Lovelock (9) was thought to be a feasible hypothesis of injury to test.

Simulation of Freezing Injury. In preliminary experiments it was immediately apparent that exposure to high salt (1.8 osmolar) for up to 1 hr had no obvious deleterious effect on protoplasts. Injury was manifested only after dilution from high salt concentrations. Lovelock (9) has similarly shown that 23% of red cells exposed to 3.6 M NaCl were hemolyzed, whereas hemolysis increased to 80% when the red cells were diluted to 0.15 M NaCl. The amount of injury to red cells occurring after dilution could account for 80 to 100% of the lysis after a freeze-thaw cycle (9). This type of evidence gave rise to the "posthypertonic hemolysis" theory in which it was proposed that cells took up electrolytes in hypertonic solutions and burst upon return to isotonic conditions (30) due to the fact that the cell exceeded a maximum tolerable volume.

To assess the possibility that cell lysis was due to a large influx of solutes in the frozen state, volumes of protoplasts were determined after a freeze-thaw cycle. Table III demonstrates that the volume of protoplasts surviving a freeze-thaw cycle in 0.1 M $CaCl_2$ + 0.15 M NaCl both in the presence and absence of cryoprotectants appears to be somewhat larger than the volume of unfrozen controls. However, as will be demonstrated later, a large amount of the injury incurred during a freeze-thaw cycle is correlated with the increase in surface area of the plasma membrane during thawing. At −6.7 C the protoplasts are exposed to a solution of 3.6 osmolal in which, assuming they behave as spherical osmometers at this concentration and temperature, the protoplasts had a volume of 830 μm^3 (calculated from Fig. 2) and thus a surface area of 427 μm^2 and would have undergone an increase in surface area of 2,527, 2,670 or 2,830 μm^2 during thawing if their final volumes were 15.1, 16.3, and 17.6 picoliters, respectively. The difference in volume between 16.3 and 17.6 picoliters, if it is real, represents only a 6% difference in the surface area expansion during thawing, suggesting that an influx of solutes could account for only a negligible amount of increased lysis during a freeze-thaw cycle.

To determine whether a "loading" of protoplasts occurs upon exposure to hypertonic salt solutions at room temperature, protoplast volumes were determined in various hypertonic solutions before and after various extents of dilution from these solutions (Fig. 4). Had an irreversible influx of electrolytes occurred, protoplast volumes would deviate from a linear van't Hoff plot. If more salt were taken up by protoplasts exposed to 1.6 osmolal solutions than when exposed to 1.0 osmolal, the volume of the former, following dilution to 0.8 osmolal, would be greater than that of the latter, which was also diluted to 0.8 osmolal. Figure 4 demonstrates that this is not the case. The linearity of Figure 4, regardless of the hypertonic salt concentration or the extent of dilution from these concentrations, demonstrates that a loading of all protoplasts, and subsequent bursting of some upon dilution of the osmoticum, cannot explain the lysis observed following dilution.

A substantial amount of work with red cells suggests that injury is in some way associated with the swelling of the cell in hypotonic solutions or after return from hypertonic conditions (9, 17, 30). Red cells are thought to possess a maximum critical volume (17). Being concave discoids initially, they are able to double in volume with little or no change in surface area (3). Plant protoplasts, on the other hand, are spherical and must increase in surface area during swelling. Whether protoplast lysis is associated with the attainment of a maximum volume can be determined by observing survival as a function of plasma membrane surface area (Fig. 5). Protoplasts were isolated in 0.54 osmolal salt and either forced to

Table III. Protoplast Volume Before and After A Freeze-Thaw Cycle

Protoplasts suspended in 0.1 M $CaCl_2$+0.15 M NaCl in the presence or absence of various additives were frozen to -6.7 C for 12 hrs. Volume measurements were made as described in Figure 2.

Addition	Volume, pliters[1] Unfrozen (4C)	After a Freeze-thaw Cycle
None	15.1	17.6
0.3M Glucose	7.6	9.6
0.3M Mannose	9.7	10.3
0.3M Mannitol	8.9	12.0

[1] The average volume of the freshly isolated controls was 16.3 pliters.

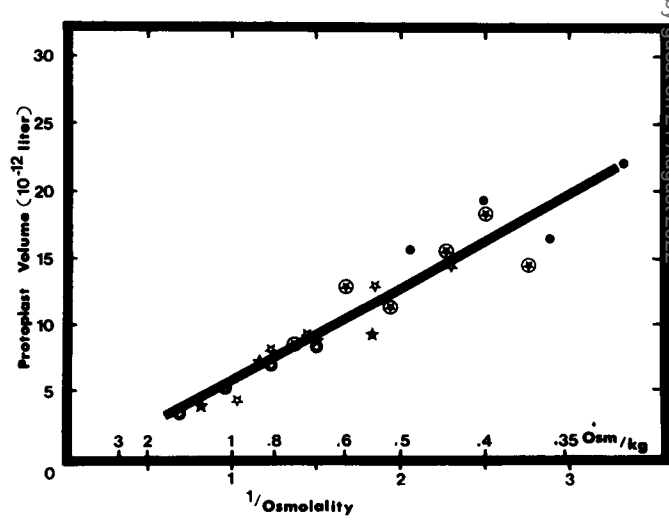

FIG. 4. van't Hoff plot of protoplast volume after 10-min contraction in salt (equiosmolar $CaCl_2$ + NaCl) followed by immediate addition of H_2O to induce protoplast expansion and obtain final osmolalities shown in figure. Contraction was achieved in salt concentrations of 0.536 osmolal (●), 0.803 osmolal (⊛), 1.071 osmolal (◉), 1.338 osmolal (★), and 1.607 osmolal (○). Volumes and osmolalities were determined as in Figure 2. Regression coefficient, (r = .951) is significant at the 1% level.

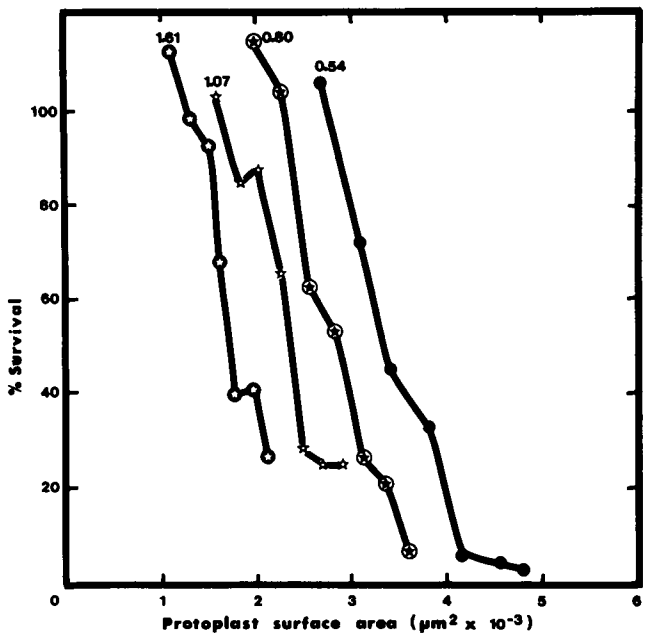

FIG. 5. Dependence of protoplast survival on surface area of protoplast after osmotic manipulation of suspending medium, with various extents of dilution after exposure to different high salt concentrations for 10 min. Survival is plotted as a function of surface area of protoplasts when contracted in varying salt concentrations and then expanded to varying extents by dilution of the osmoticum. Numbers above each line represent highest osmolality to which protoplasts were exposed before dilution. Symbols are the same as in Figure 4. Surface areas were calculated from volumes given by least squares line in Figure 4.

expand by dilution of the osmoticum or contracted in several hypertonic concentrations before dilution of the osmoticum. Figure 5 demonstrates that 50% of the protoplasts exposed to 0.54 osmolal salt and diluted to varying extents had lysed before they reached a surface area of about 3,400 μm^2. On the other hand, 50% of the protoplasts contracted in 1.61 osmolal salt before dilution lysed before they had reattained 1,700 μm^2, a surface area less than that which they possessed in 0.54 osmolal salt. Protoplasts which had been contracted to intermediate values underwent lysis when they were expanded to intermediate surface areas. These data demonstrate that protoplasts do not possess a single maximum surface area at which lysis occurs. Rather, the surface area at which lysis occurs is decreased if protoplasts have previously been subjected to contraction. Figure 6A demonstrates protoplast survival as a function of the increase in surface area of the plasma membrane during dilution. Lysis appears to be correlated with the absolute magnitude of the change in surface area of the plasma membrane of the protoplast, regardless of the other conditions to which they were exposed. For instance, 50% survival occurred when protoplasts incurred a change in surface area of about 900 μm^2 (Fig. 6A) regardless of whether they were initially exposed to 1.61 osmolal and diluted to 0.81 osmolal or exposed to 0.54 osmolal followed by dilution to 0.36 osmolal. When survival is plotted as a function of the volume change (Fig. 6B), it becomes apparent that the cellular increase in volume at which lysis of 50% of the protoplasts occurs varies with the degree of contraction. While volume is the proper parameter to use for characterizing the osmometric behavior of cells, lysis resulting from this osmometric behavior is best described by the increase in surface area of the plasma membrane during expansion. Although it is possible that lysis as a function of volume increment is confounded with some other factor associated with the concentration of the hypertonic osmoticum and/or the concentration of the osmoticum following dilution, a simpler interpretation of Figure 6 is that lysis is related to the increase in surface area of the plasma membrane caused by osmotically induced swelling. For this hypothesis to be true, some contraction-induced alteration in the plasma membrane must occur such that the final surface area which the protoplast can achieve is limited.

Since only volumes of protoplasts surviving various treatments were measured, several assumptions must be made during the interpretation of Figures 4, 5 and 6. These assumptions include: (*a*) a relatively homogeneous population of cells was sampled; (*b*) protoplasts which lysed were, before lysis, representative of the osmometric behavior of the entire population; and (*c*) all cells experienced a uniform stress and underwent an equal strain. If the first assumption was not true, *i.e.* if the population of cells was not homogeneous in its sensitivity to a stress, then a correlation between a stress and resulting injury would not be affected, although the quantitative nature of the relationship would not necessarily be representative of any unique portion of the population. Assumptions (*b*) and (*c*) are somewhat analogous and their validation will be required before the observed correlation (Fig. 6A) can be considered as a cause and effect relationship.

Relevance of Simulation Experiments to Freeze-Thaw Injury. Figure 6A demonstrates a correlation between protoplast survival and the mean increase in surface area of the plasma membrane. This correlation allows the comparison of injury due to osmotic manipulation at room temperature with injury following a freeze-thaw cycle. If the mechanism of injury incurred during a freeze-thaw cycle was qualitatively similar to injury incurred by expansion of the protoplast at room temperature, survival of protoplasts after a freeze-thaw cycle should be positively correlated with the concentration of the suspending osmoticum. The higher the initial salt concentration, the less the protoplasts will have to swell during thawing. Protoplasts were frozen to −3.9 C in different initial salt concentrations. As shown in Table IV, the survival of protoplasts increased as the initial salt concentration was increased. The constancy of the number of protoplasts surviving supercooling in different salt concentrations demonstrates that the high survival of protoplasts frozen in high initial salt concentrations was not due to an artifact arising from a decreased survival of supercooled controls under high salt conditions.

Since the nature of freeze-thaw-induced injury appeared to be

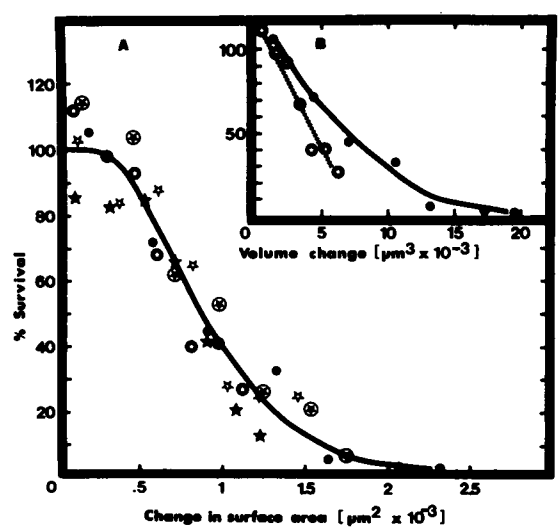

FIG. 6. Dependence of protoplast survival on increase in surface area or volume of protoplast. Conditions were as listed in legends of Figures 4 and 5. A: survival as a function of increase in surface area of plasma membrane during dilution of osmoticum (expansion of protoplast). Symbols, which indicate salt concentration in which the protoplasts were contracted, are the same as in Figures 4 and 5. Survival appears to be independent of the high salt concentration. Insert (B) demonstrates that this is not the case when protoplast survival is plotted as a function of change in volume.

Table IV. Effect of Initial Salt Concentration on Survival of Frozen-thawed Protoplasts

Protoplasts were frozen to -3.9 C for about 12 hrs. Survival is expressed as a percentage of supercooled controls. Values in parentheses indicate the number of protoplasts in each supercooled sample (protoplasts per $3x10^{-5}$ ml).

Osmoticum $CaCl_2$ (M)	Osmoticum NaCl (M)	Osmolality (OSM)	Volume[1] (μm^3)	Change in surface area[2] (μm^2)	% Survival Observed	% Survival Expected[3]
0.10	0.15	0.486	13090	1922	11 (475)	5
0.14	0.21	0.682	8950	1320	36 (522)	22
0.18	0.27	0.877	6650	946	44 (461)	43
0.22	0.33	1.073	5190	685	81 (436)	69
0.26	0.39	1.273	4170	487	90 (511)	86
0.30	0.45	1.459	3460	340	85 (472)	95

[1]Volume calculated from Figure 4.

[2]At -3.9 the unfrozen portion of the solution has an osmolality of 2.1. Therefore, protoplast volume was 1990 μm^3 and surface area was 765 μm^2.

[3]These values represent the expected % survival of protoplasts exposed to the same change in plasma membrane surface area at room temperature (from Figure 6). The change in surface area the frozen-thawed protoplasts underwent was assumed to be equal to that which can be calculated from Figure 4 (see text).

qualitatively accounted for by the simulation experiments, a quantitative comparison should indicate what proportion of freeze-thaw injury can be accounted for by the osmometric behavior of the protoplast. The extent of injury one would expect to protoplasts subjected to a freeze-thaw cycle, but caused solely by the increase in surface area of the plasma membrane during thawing, was calculated as follows. At −3.9 C the protoplasts are subjected to an osmolality of 2.1. Therefore, their mean volume can be approximated by the regression equation of Figure 4:

$$-1.36 + 7.03\frac{1}{P} = V \quad (1)$$

where P = the osmolality of the external solution and V = protoplast volume in picoliters. For $P = 2.1$, $V = 1.99$ picoliters so that in the partially frozen solution the protoplasts should have had a mean surface area of 765 μm^2. If these protoplasts were initially suspended in, *e.g.* 0.18 M $CaCl_2$ + 0.27 M NaCl (total osmolality = 0.877 after the addition of Evan's blue dye), they would return to a volume of 6.65 picoliters and have a surface area of 1,711 μm^2 after thawing. Thus, the protoplasts were subjected to a change in surface area of 946 μm^2 during the freeze-thaw cycle. From Figure 6A one observes that 43% of the protoplasts which underwent this change in surface area at room temperature survived. By this calculation in survival values of protoplasts frozen in the presence of varying salt concentrations as listed under the "Expected" column were obtained. The agreement between the extent of injury predicted in this manner and the observed amount of injury after a freeze-thaw cycle in varying concentrations of $CaCl_2$ + NaCl (Table IV) is quite good. It seems that the simulation experiments do mimic injury during a freeze-thaw cycle.

As discussed above, the simulation experiments suggest that the amount of lysis after a freeze-thaw cycle to a given temperature should decrease as the osmolality to which the protoplasts are exposed is increased. This can be an explanation for the observed cryoprotective activity of various sugars (Figs. 1 and 3; Table I). Ficoll was not a very good cryoprotectant (Table I), and is osmotically rather inert due to its large mol wt. Figure 3B demonstrates that survival is related to the osmolality to which the protoplasts were exposed after thawing, and apparently independent of the ionic strength of the osmoticum. The possibility exists that cryoprotection of isolated plant protoplasts by various sugars is simply an osmotic phenomenon, limiting the extent to which the protoplasts must expand after thawing.

Injury after a freeze-thaw cycle is maximal and complete only when the formation of ice, as indicated by the "exothermic region," is completed (Fig. 1). This indicates that injury is maximal only when the protoplasts have shrunken to the extent dictated by the osmolality of a frozen solution of that temperature, and then expanded during thawing to a surface area dictated by the osmolality of the suspending osmoticum. Maximal injury occurred only when the protoplasts were thawed following the completion of ice formation, *i.e.* when the protoplasts were presumably in osmotic equilibrium with the external solution. This observation suggests that injury during a freeze-thaw cycle can be kinetically accounted for by the contraction- and expansion-induced responses of isolated protoplasts.

If these studies with isolated protoplasts are to be considered relevant to whole plant injury following a freeze-thaw cycle, immersion of leaf sections in a plasmolyzing osmoticum prior to freezing should be less injurious than immersion in water, since tissue immersed in such an osmoticum would not expand to the same extent as tissue immersed in water after thawing. Figure 7 depicts injury (as estimated by the leakage of ninhydrin-reactive compounds) of leaf tissue immersed in solutions of differing initial osmolalities (ranging from 0 to 0.861 osmolal) during a freeze-thaw cycle. These results are qualitatively similar to those of Table IV in that injury was less severe when the cells were exposed to hypertonic salt before the freeze-thaw cycle. Since the leaf pieces, with the lower epidermis removed, were about 0.25 cm^2 and were incubated in the various osmotica for 3 hr, it is reasonable to assume that they approached osmotic equilibrium before being frozen. The large amount of apparent injury in tissues which were frozen and thawed before salt was added indicates that the decreased injury of frozen-thawed leaves in 0.36 osmolar or greater osmotica was not due to an impaired leakage of compounds in high salt conditions. These results are consistent with those of Samygin (20) who has shown that kale stems frozen and thawed in sucrose survived, whereas tissues which were frozen and thawed in sucrose, and then deplasmolyzed in H_2O after thawing, were killed. These results are also consistent with others suggesting a correlation between water content and killing point of a tissue (24).

Discrepancies between the freezing of leaf pieces and protoplasts should not go unnoticed. Although leaves were frozen to a lower temperature (−9.4 C) than protoplasts (−3.9 C), essentially complete protection of leaves was achieved by 0.36 osmolar salt (Fig. 7) whereas about 50% of the isolated protoplasts were injured even when frozen in 0.9 osmolal salt (Table IV). Although several possibilities to account for this discrepancy exist, a likely explanation can be found in geometrical considerations. Protoplasts were selected for these studies because of their spherical shape so that surface areas and volumes could be directly calculated. The

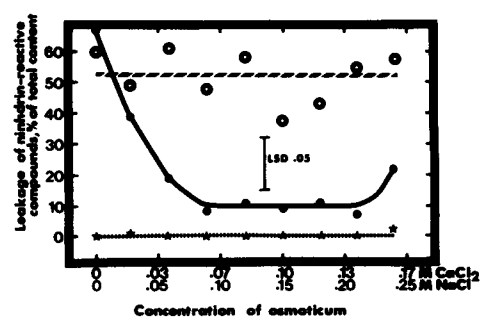

FIG. 7. Leakage of ninhydrin-reactive compounds from leaves following a freeze-thaw cycle to −9.4 C for 1.5 hr. Lower epidermis was stripped from leaves, pieces (about 0.25 cm^2) of which were immediately placed in 10 ml of water or solutions shown in the figure. Leaf pieces were incubated in these solutions at 4 C for 3 hr and shaken at about 100 rpm. They were rinsed in the respective solutions before being frozen and thawed while suspended in 5-ml solutions (●). (○): stripped leaves which were floated on water before freezing. After thawing, 5 ml of the respective solutions were added. (★): treated the same as (●) except that they were held at 4 C instead of being frozen. Ninhydrin analysis was as previously described (32) except that leaves were shaken in 5-ml solutions and 0.2-ml samples were taken for ninhydrin analysis.

geometry which the cell assumes during contraction is of utmost importance in determining the change in surface area of the plasma membrane for any given change in volume of the cell. Inasmuch as plant cells are not generally spherical, the change in surface area of isolated protoplasts may not equal that of the mesophyll cells *in situ* under the same osmotic conditions. For instance, Scarth *et al.* (21) have shown that during plasmolysis cells can contract in either a concave or convex manner. The surface area of the plasma membrane may remain unaltered, or may even increase during concave plasmolysis. Thus, differences in the geometry of intact mesophyll cells and isolated protoplasts could account for much of the quantitative discrepancy observed.

CONCLUSIONS

As early as 1939 Tornava (23) observed protoplast diameters of several plant species at the moment of hypotonic lysis. The general conclusion was that protoplasts burst when the surface area was approximately double the initial surface area, although it was admitted that "... in many experiments [the surface area at the moment of lysis as a percentage of the original surface area] has been 110% or less, and that in several trials, where the protoplast first has been made to contract itself or been plasmolyzed, not even the original surface area has been reached" (23). Scarth *et al.* (21) also described this phenomenon in a qualitative manner: "... the limit [of expansion] varies greatly with such simple treatment as plasmolysis. The point of bursting of cells which have been strongly plasmolyzed beforehand is lower than those weakly plasmolyzed." In addition, they found that cells from acclimated plants withstood a greater amount of deplasmolysis than nonacclimated cells (21), suggesting that the ability to withstand a freeze-thaw cycle may be related to the ability to withstand deplasmolysis. The results presented in the present paper are in complete agreement with these earlier observations (21, 23).

As was stated earlier, Meryman (14, 15) has developed the "minimum critical volume" hypothesis to account for freeze-thaw-induced lysis. Although we do not feel that this hypothesis accurately represents the mechanism of freeze-thaw-induced lysis of spinach protoplasts, it would appear from our results that volume/surface area reduction does lead to some membrane alteration which limits the membrane expansion potential. Although Meryman (15) alluded to the existence of a single critical volume, our results suggest that this contraction-induced alteration is a continuous (or nonresolvable discrete) function of contraction.

In summary, results of this paper demonstrate that protoplasts were injured by a freeze-thaw cycle. Injury was not due to the attainment of a physical minimum critical volume or an uptake of solutes. Many sugars and sugar alcohols were capable of protecting against this injury. The extent of injury was also dependent upon the nature of the monovalent ions in the osmoticum. From simulation experiments conducted at room temperature, survival was found to be directly correlated with the mean value of the absolute magnitude of the change in surface area which the plasma membrane of the protoplast underwent during dilution of the osmoticum. The amount of injury incurred during a freeze-thaw cycle can be quantitatively accounted for by injury that occurs when protoplasts are osmotically induced to contract and expand at room temperature. We suggest that the two major strains on protoplasts associated with a freeze-thaw cycle are a freeze- or contraction-induced membrane alteration which limits the expansion potential of the plasma membrane as well as a thaw- or expansion-induced dissolution of the plasma membrane. These two strains interact during a freeze-thaw cycle and result in lysis of isolated spinach protoplasts.

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