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Short communication

Quantifying liquid water in frozen plant tissues by isothermal calorimetry

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Abstract

An equation to calculate the percentage of water remaining unfrozen at any temperature due to colligative properties of solutions was derived from the freezing point depression equation. The accuracy of the equation was demonstrated with a 0.1 M sucrose solution frozen at temperatures from −0.5 to −6 °C in an isothermal calorimeter. Empirical measurements using latent heat as a measure of the amount of water frozen were within 1% of the expected values calculated from the equation. The extent to which percentages of water freezing in oat crown tissue at varying temperatures follows the expected freezing curve indicates how closely the system follows colligative freezing processes. The freezing curve for non-acclimated crowns followed a colligative freezing pattern more closely than did the curve for crowns from cold-acclimated plants. This suggests that water in crowns from non-acclimated plants may remain unfrozen primarily by colligative means while other mechanisms of keeping water unfrozen are important in cold-acclimated crowns. This may help explain contradictory results of studies that attempt to correlate carbohydrate concentrations with freezing tolerance.
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Keywords: Freezing point depression; Colligative; Cold-acclimation; Oat; Freezing tolerance; Calorimeter

1. Introduction

Calorimetry has been an important tool in the study of different forms of freezing stress because water freezing, that results in tissue damage to biological systems, can be detected thermally (see review by Mazur [1]). Greathouse [2] used calorimetry to measure water transitions in potato and in roots of clover and found considerably more water freezing in non-acclimated tissues than in cold-acclimated tissues; he also provided a review of earlier calorimetry studies in which amounts of free and bound water were measured. Levitt (cited by [3]) used calorimetry to show that more than three times the amount of water remained unfrozen in non-acclimated cabbage as compared to acclimated plants. Tumanov et al. [4] measured unfrozen water in wheat and found that the water retaining power of cells in a plant have a major effect on their frost tolerance. They stated that cells in different organs of the same plant do not retain water to the same extent. Johansson [5] used calorimetry to determine the amount of water freezing in wheat and rye plants, and reported conflicting results between unfrozen water and freezing tolerance. Olien [6,7] found that a shift in latent heat occurred while plants were frozen and attributed this shift to a release of sugar into the apoplast which could have relieved adhesions. Calorimetry was used to demonstrate that pressure caused by an increase in respiratory CO_2 in a closed system induces CO_2 dissolution in water which acts in a colligative manner to reduce the amount of water freezing in oat crowns [8].

Calorimetric experiments with partially frozen systems do not generally provide information as to how unfrozen water is kept in the liquid state. Knowing this could help researchers determine how plants resist various forms of freezing stress. An equation that would determine the percentage of water remaining unfrozen due to colligative properties could allow researchers to help understand stress resistance mechanisms by measuring whether the amount of water freezing in a biological system is following or deviating from a freezing curve based on colligative properties.

2. Experimental

2.1. Plant tissue

Oat (*Avena sativa*, cv Wintok) plants were grown and crown tissue harvested as described elsewhere [9]. Briefly, plants were

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grown for 5 weeks under controlled conditions at 13 °C with a 12 h photoperiod. These were non-acclimated plants. After non-acclimated treatments, plants were transferred to a different chamber at 3 °C with a 10 h photoperiod and grown for 3 weeks. These were cold-acclimated plants. Crown tissue consisted of the bottom 2 cm of the stem after roots and leaves were trimmed.

2.2. Freeze tests/thermal analysis

Water, sucrose solutions (3 g, Fig. 2) and crown tissues (2.2 ± 0.5 g, Figs. 3 and 4) were studied in a Calvet isothermal calorimeter (model MS 80 Setaram, Saint-Cloud, France) inside a small, refrigerated-room at −15 °C. The calorimeter was maintained from −1 to −6 °C by precisely heating the thermopile. It took 24 h for the calorimeter to come to equilibrium once the temperature was changed. At full sensitivity, 1 mV output equaled 17.6 mW.

Unfrozen water, and sucrose solutions that were supercooled from −1 to −6 °C were induced to freeze with ice crystals adhering to the end of a narrow-gauge wire (guitar string) inserted into the core of the calorimeter where samples were located. Heat generated from inserting the wire was below limits of detection for settings used in these experiments.

As the sample froze, release of latent heat was recorded on a strip chart recorder and areas under curves were measured with a handheld planimeter. The average of three measurements (less than 3% variation was observed between measurements) was used in all calculations. A standard curve with varying amounts of water indicated a linear relationship between g of water and curve area, up to the largest peak area measured, with a correlation coefficient of 0.999. This standard curve was used to quantify total energy in all subsequent measurements.

3. Calculation

3.1. Equation to determine percentage of water remaining unfrozen

To confirm the accuracy of freezing curves obtained by calorimetry, the familiar freezing point depression equation $\Delta T = -1.86\,\mathrm{m}$, where m is molality, was expanded and solved for percentage of water remaining unfrozen as a function of molality and equilibrium temperature. While this equation is valid for any solute, it is only valid for dilute solutions (0.1 m or below). The freezing point depression must be empirically determined for concentrated solutions particularly those above 1 m. An important assumption of colligative properties is that the solute is not present in the frozen solvent ([10], p. 228). As water freezes, the solute moves into the unfrozen liquid which eventually concentrates to a point which prevents the unfrozen solution from freezing at a particular temperature. One can use this assumption and rearrange the equation to solve for amount of water remaining liquid at a specific temperature. Beginning with

$$\Delta T = \frac{-(1.86\,\mathrm{K\,m^{-1}})(\text{g solute})1000}{(\text{g water})(\text{molecular wt. of solute})} = \frac{-1.86(\text{mol solute})1000}{\text{g water}} \quad (1)$$

Rearranging:

$$\text{g water} = \frac{-1.86(\text{mol solute})1000}{\Delta T} \quad (2)$$

One hundred grams of a 0.1 m solution will contain 0.01 mol of solute. The question can then be asked: what portion of a 100 g, 0.1 m solution will contain all the solute at a particular temperature below the freezing temperature? That portion of the solution will be unfrozen. The equation then becomes:

$$\%\,\text{unfrozen water} = \frac{-1.86(0.01\,\text{mol})1000}{\Delta T} \quad (3)$$

So for any 0.1 m solution at any temperature below 0 °C (but above the eutectic point):

$$\%\,\text{unfrozen water} = \frac{-18.6}{\Delta T} \quad (4)$$

So, for example, at −2 °C, 9.3% of a 0.1 m sucrose solution would be expected to remain unfrozen.

3.2. Calculation of expected unfrozen liquid in acclimated and non-acclimated oats

Assuming colligative effects are determining the percent of water remaining unfrozen, the average moles of solute in 100 g of plant solution was calculated from Eq. (4) at −1, −2, and −3 °C. For non-acclimated crowns, 0.43 m and for acclimated crowns 0.62 m, was used to calculate the expected percentage of water remaining unfrozen (Fig. 4).

4. Results and discussion

4.1. Thermal patterns in water and sucrose at three freezing temperatures

Potential errors and assumptions involved in calorimetrically determining the amount of water that froze using latent heat measurements were discussed previously [11]. By calibrating the system with water, changes in heat capacity of water as it froze were taken into account. Also, when measuring amount of water freezing in crowns, other systems generating or absorbing heat, were assumed to be minimal in comparison to that generated by water freezing [11]. This is a similar assumption that must be made in the use of infrared video thermography (IVT [12]) to determine freezing patterns in plants. However, IVT can identify which specific tissue the heat originated from while calorimetry can only determine change in total heat, albeit at a higher precision than IVT.

Using Eq. (4) (percentage of unfrozen water), the curve of expected percentages of unfrozen water in a 0.1 m solution of

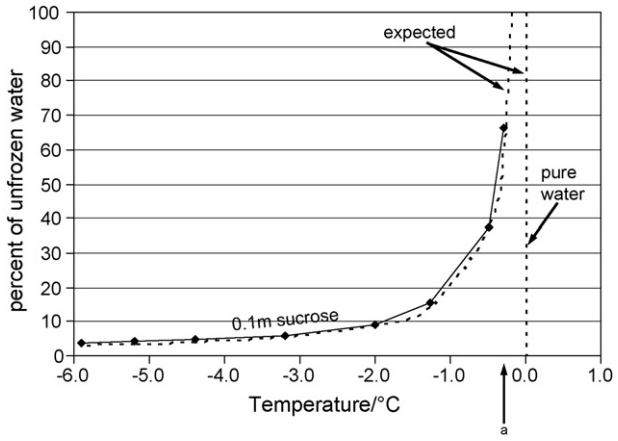

Fig. 1. Percentage of unfrozen water in 0.5 g of a 0.1 m sucrose solution frozen at varying temperatures. The broken line is the expected amount of unfrozen water calculated using the % freezing equation derived from the freezing point depression equation (see Section 3). The freezing point depression of 0.1 m sucrose (−0.186 °C), is shown by "a". Note that in contrast to the sucrose solution, pure water completely freezes at 0 °C, once latent heat is removed, and then follows the 0% unfrozen line to −6 °C.

sucrose was compared to calorimetrically determined results at approximately 1 °C intervals down to −6 °C (Fig. 1). Empirically determined results averaged less than 1% above and below the expected value (Fig. 1) and were less than 1% of Williams and Meryman's [13] similar measurement using 0.1 m KCl, taking into account the dissociation of KCl in solution.

The inability to consistently supercool the solution below −6 °C without it spontaneously freezing prevented us from obtaining results at lower temperatures. However, the nature of the percent freezing equation is such that if the temperature was continuously lowered, the calculated percentage of water remaining unfrozen would approach but never reach 0%. The solution will only freeze completely when the eutectic point of the solute is reached, which for sucrose is approximately −14 °C [14]. At this temperature the amount of unfrozen water deviates from the calculated value and becomes 0% almost immediately (see [13] for an example with KCl).

Not surprisingly, freezing temperature had a dramatic effect on thermal response (Fig. 2) in water and sucrose. As the temperature was lowered, the time it took for samples to completely freeze was correspondingly reduced from about 2.25 h at −1 °C to 1 h at −3 °C (Fig. 2).

The shapes of the curves in Fig. 2 were related to the geometry of the container being frozen as well as the heat released in the initial freeze. Meryman [15] stated that water in a cylinder (the shape of the calorimeter vessel) would rapidly freeze initially, then freeze at a nearly linear rate and finally accelerate to a rapid rate as the center of the cylinder freezes. Heat released in the initial freeze would also reduce the rate of freezing of the remaining liquid. The sequence of freezing described by Meryman [15] resembles the shapes of the water curves in Fig. 2 particularly at −1 °C. When smaller volumes were frozen (not shown) the height of the liquid in the vessel was reduced and the linear portion of the curve just after the initial freeze was not present in either water or sucrose frozen at −2 and −3 °C.

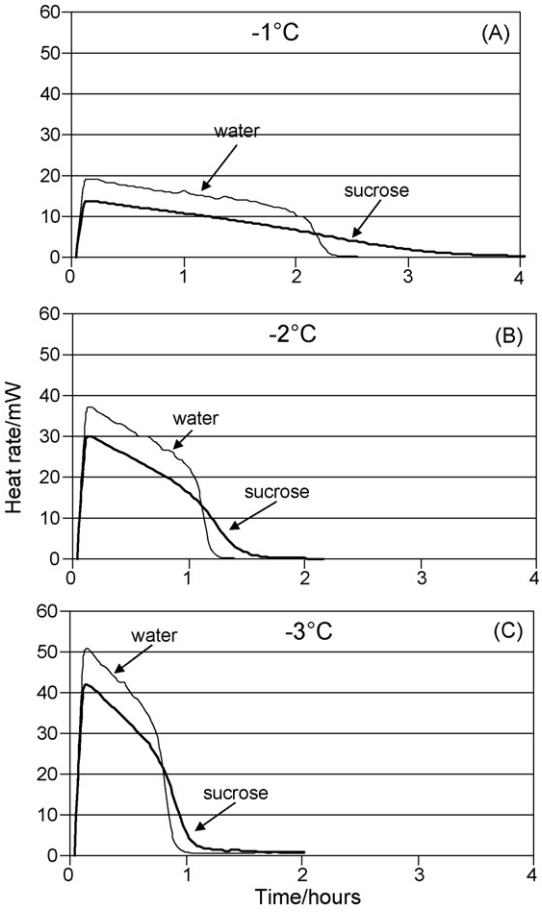

Fig. 2. Thermal output of 3 g of pure water and 0.1 m sucrose frozen at three different temperatures.

The slower freezing rate towards the end of the curve for the sucrose solution (Fig. 2) was a result of sucrose being frozen out of the ice lattice and concentrating in the remaining liquid. As the ice lattice approached the more highly concentrated sucrose, the remaining liquid froze more slowly than it did in the pure water system. The percentage of water that did not freeze at the different temperatures (Fig. 1) was the concentrated sucrose solution at its respective freezing point. The exclusion of a solute from an ice lattice as a solution freezes (until the eutectic point is reached) is a crucial assumption of the freezing point depression equation [10].

4.2. *Freezing in crown tissue*

A noticeably different pattern of freezing was observed when crowns were frozen than was observed when freezing pure water; this reflects the numerous differences between a simple and complex system (Fig. 3). The most obvious effect, besides the general shape of the curve, was a longer time to reach equilibrium. Membrane stability and permeability to water movement [1,14], colligative and matrix effects, as well as pores restricting water freezing [16] are all factors which could have reduced the amount of water freezing in crowns compared to that in pure water. Indeed, "no single event can serve to explain the

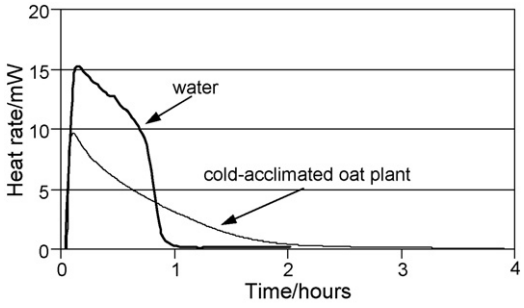

Fig. 3. Thermal output of 0.9 g water and crowns from cold-acclimated oat plants frozen at −3 °C.

increasingly complex phenomena that occur during freezing and thawing" [17].

Differences between the freezing pattern of simple systems and crowns were also evaluated by comparing the percentage of water expected to remain unfrozen in crowns to the actual percentage of water remaining unfrozen at various temperatures (Fig. 4). This comparison is ostensibly a measure of the fit of the empirically determined freezing curve to a hypothetical curve that would be a result of the colligative properties of solutions. Because the molality of the solution remaining unfrozen in crowns could not be accurately measured, the molality was estimated by solving the equation for the average number of moles of solute in 100 g of solution as a function of the percentage of water remaining unfrozen (see Section 3). This is not surprising since numerous other factors besides colligative properties are undoubtedly involved in the percent water remaining unfrozen in crowns. Neither cold-acclimated nor non-acclimated curves followed expected curves as closely as the sucrose solution did (compare Figs. 1 and 4).

The close agreement of the non-acclimated, crown freezing curve with the expected curve (Fig. 4) indicates that the

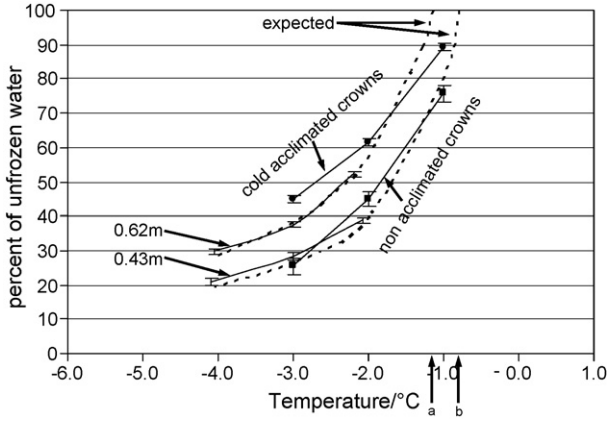

Fig. 4. Percentage of unfrozen water in oat crowns. The broken line is the expected amount of unfrozen water calculated using the % freezing equation derived from the freezing point depression equation (see Section 3). The expected freezing point depression of each treatment is indicated by "a" or "b". The lines labeled 0.62 and 0.43 m are sucrose solutions that were compared to the expected % unfrozen water lines at −2, −3 and −4 °C. The bars above and below data points are the least significant difference at a probability of 0.05. Note that the curve for crowns from acclimated plants deviates from the expected curve to a greater extent than the curve from non-acclimated crowns.

amount of water remaining unfrozen in crowns followed a pattern that was similar to water freezing in a pure sucrose solution. While some water may remain unfrozen in crowns for colligative reasons, water sequestering within cells is likely the major factor that restricts freezing. Since there are numerous means that plants use to maintain the integrity of membranes (and prevent intracellular water from freezing) it is noteworthy that the actual freezing curve follows the expected curve as closely as it does. Apparently, non-acclimated crowns have little or no ability to resist intracellular freezing so that colligative effects become the major factor determining the amount of water remaining unfrozen.

In contrast to crowns from non-acclimated plants, crowns from cold-acclimated plants deviated significantly from the expected curve (Fig. 4). While carbohydrate changes were not measured in this study, it has long been recognized that a major effect of cold-acclimation is accumulation of carbohydrates, especially in crown tissue. The results shown in Fig. 4 suggest that, despite carbohydrate accumulation during cold-acclimation [19] and a lower percentage of water freezing the means of reduction in the percentage of water freezing was likely not colligative.

If solutes are excluded from an advancing ice lattice as crowns freeze, like they are in pure solutions [10], the liquid solution into which the solutes move will eventually reach a concentration that will not freeze at a particular temperature (as long as that temperature is above the eutectic point). These regions of concentrated solution could become barriers to advancing ice and prevent further freeze damage. Putative barriers were observed in oat crowns that were recovering from freezing [9].

4.3. Conclusion

These results underscore the difficulty of determining cause and effect between carbohydrates and freezing tolerance. In biological systems, water is always in solution and is in various kinds of associations with hydrophilic compounds. Carbohydrates as well as other solutes are strong hydrogen bonders and can very efficiently bind water [1,15] and prevent freezing. However, species such as sugar cane contain extreme concentrations of sugars but are very winter tender. So how carbohydrates are involved in preventing water from freezing and in turn preventing injury in frozen tissues is a complicated matter. Adding to the ambiguity is the fact that water is in a gradient in which its physical properties such as freezing point and fluidity are altered by their distance from hydrophilic colloids up to a few molecular diameters [18]. Each variation in the interaction of water with plant components will effect whether or not that component will be disrupted when frozen, and would have a cascading effect on individual cells, tissues and ultimately the whole plant. The amount of water remaining unfrozen in crowns from cold-acclimated plants is probably determined by more complex means such as water permeability changes of cells, by up and down regulation of aquaporins [20] and the increase in various non-colligative cryoprotectants during cold-acclimation [14,21] within various tissues of the crown. More research is needed to determine if barriers to freezing [9,22] impact total

amount of water freezing and how, or if, this process is controlled genetically.

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