Plant Physiol. (1989) 90, 1121–1128
0032-0889/89/90/1121/08/$01.00/0

Received for publication July 25, 1988
and in revised form March 3, 1989

Relationship between Thermal Transitions and Freezing Injury in Pea and Soybean Seeds

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ABSTRACT

In an attempt to correlate freezable water with freezing injury, the thermal behavior of pea (*Pisum sativum* L.) and soybean (*Glycine max* L. Merr) seed parts at different moisture contents were compared with survival of the seeds when exposed to low temperatures. Thermal transitions between −150 and 10°C were studied using differential scanning calorimetry. In pea, reduction of germinability, after exposure of seeds to temperatures between −18 and −180°C, occurred at a constant moisture content (about 0.33 gram H_2O/gram dry weight) regardless of the temperature; this moisture level was above that at which freezable water was first detectable by differential scanning calorimetry (0.26 gram H_2O/gram dry weight). In contrast, damage to soybean seeds was observed at progressively lower moisture contents (from 0.33 to 0.20 gram H_2O/gram dry weight) when the temperature was decreased from −18°C to −50°C. At −18 and −30°C, moisture contents at which damage to soybean seeds was evident were above that at which freezable water was first detectable (0.23 gram H_2O/gram dry weight). However, at −50, −80, and −180°C, damage was evident even when freezable water was not detectable. The data suggest that, while the quantity of water is important in the expression of freezing injury, the presence of freezable water does not account for the damage.

The formation of intracellular ice has often been cited as a lethal event during low temperature treatments of plants (reviewed in refs. 4 and 15). This hypothesis is based primarily on the coincidence of exotherms detected by DTA[1] with the limit of survival at low temperatures. The formation of intracellular ice is intimately related to the water content of the tissue (reviewed in ref. 15). At a threshold moisture level, freezable water is present and organisms may not survive exposure to subzero temperatures because ice is formed intracellularly (2, 3, 5, 10, 11, 14, 16, 19, 23, 24).

The hypothesis that intracellular ice formation is responsible for lethality of cold treatments cannot be tested directly in many biological tissues because the removal of the freezable water usually results in death of the tissue via desiccation damage. Seeds provide an ideal opportunity to test the hypothesis because freezable water can be removed with no apparent damage to the tissue. By sequentially adding water to dried, viable tissue and then exposing the tissue to subzero temperatures, we can determine if the presence of freezable water does indeed correlate with damage to the tissue. This type of experiment has been conducted using pollen (5), wheat grains (16, 24) and lettuce achenes (10), and it was learned that water contents at which ice was detectable (0.36, 0.40 and 0.26 g/g for pollen, wheat, and lettuce, respectively) roughly coincided with water contents at which resistance to freezing temperatures was lost. However, reductions in viability were first observed when pollen moistures of about 0.30 g/g were exposed to −25°C, and ice crystals were not detectable by x-ray diffraction (5).

Freezable water is believed to be present in proteins and phospholipids at approximately 0.25 to 0.50 g/g—roughly the water content at which there is sufficient water to fully hydrate the macromolecule (12, 13). The occurrence of freezable water in tissues as detected by NMR, DTA, DSC, or x-ray crystallography ranges from about 0.20 g/g for yeast (28), several recalcitrant seeds (3), cereal leaves (9), and freeze dried strawberries (20) to about 0.50 g/g for silver maple seed (3). The range of moisture contents at which freezable water is detectable is largely dependent on the chemical composition of the material: low values are characteristic of substances with a high lipid contents while high values are characteristic of hydrophilic starch compositions.

In these experiments, thermal transitions detected by DSC of pea and soybean seeds at different moisture levels are compared with survival of seeds after they have been exposed to a series of subzero temperatures. Estimations of the presence of freezable water were accomplished by measuring the energy of melting transitions, values which are directly proportional to the quantity of freezable water. By comparing the germination of seeds at different moisture contents with the presence of freezable water, it is concluded that the formation of ice is not necessarily fatal, and that transitions occurring in other tissue components may also play a role in the expression of freezing injury.

MATERIALS AND METHODS

Soybean (*Glycine max* L. Merr), cv Williams' 82 (Dewine Seeds), and pea (*Pisum sativum* L.), cv Alaska (Burpee Seeds), were used to study the relationship between moisture content, thermal transitions, and low temperature sensitivity.

Moisture Equilibration and Determination

To achieve moisture contents between 0.03 and 0.23 g/g, whole seeds or isolated axes and cotyledons of pea and soy-

[1] Abbreviations: DTA, differential thermal analysis; DSC, differential scanning calorimetry; g/g, g H_2O/g dry weight.

bean were equilibrated at various relative humidities using saturated salt solutions (18, 27). Moisture levels between 0.14 and 0.50 g/g were obtained by adding known quantities of water to preweighed seed samples. Moisture contents for germination assays were determined by measuring the fresh and dry weights of 3 g aliquots of the hydrated seed. For leakage and DSC studies, fresh weights of the samples were made prior to measurements and dry weights were determined afterward. Dry weights were determined by heating samples at 95°C for 5 d. Moisture contents are expressed on a dry weight basis.

Germination Assays

Seeds at various moisture contents were exposed to 5°C (control), −18°C, −30°C, −50°C, −80°C, and −180°C for 16 h and then allowed to warm to room temperature for 4 h before planting. Seeds given the −18 and −80°C treatments were placed in freezers. Seeds cooled to −30 and −50°C were placed in methanol baths cooled to the appropriate temperature. Seeds cooled to −180°C were placed in the vapor phase of cryovats filled with liquid nitrogen. The cooling rate of seeds given the −18 to −80°C treatments was measured by a thermocouple imbedded into a seed and was between 12 and 15°C/min. Seeds exposed to −180°C were cooled at about 45°C/min. Warming rates were generally about 12°C/min. After the temperature treatments, the seeds were rolled in paper towels and germinated at 25°C for 96 h. Vigor is expressed by a germination index: percent germination × radicle length. Each experiment consisted of 15 to 25 moisture treatments per temperature with 25 seeds per treatment. The experiment was repeated twice for both soybean and pea seeds and the results combined. The threshold moisture content, above which seed survival was limited, was determined as the point at which germination was significantly (>1 SE) lower than the control germination (seeds given a 5°C treatment).

Leakage Assays

To understand how moisture content affected the temperature sensitivity of different parts of the seed, leakage of electrolytes from soybean axes and cotyledons was measured after exposure of the tissue to −18 or −80°C. Axes and cotyledons were hydrated as described above and then exposed to −18 or −80°C for 8 h. To avoid leakage from imbibitional damage, the seed parts were humidified at 100% RH for 16 h prior to soaking. The rate of leakage within the first hour of soaking was measured using an ASAC-1000 conductivity meter (Applied Intelligent Systems, Inc.). Five replicates of five axes and six replicates of three cotyledons were used for each moisture and temperature treatment.

Calorimetery

Thermal transitions were measured using a Perkin Elmer DSC-4. Whole axes or slices of cotyledons were equilibrated to different moisture contents and then loaded into aluminum sample pans. Because heating runs are not subject to the artifacts of supercooling, only enthalpic responses are reported here. Samples were cooled at 10°C/min to −150°C. Heating curves were recorded as samples were warmed at 10°C/min from −150 to 20°C. After the DSC measurements, the pans were punctured and dry weights were determined as described above. Generally, about 20 mg dw of material were used per sample. This allowed adequate detection of thermal events at a sensitivity of 2 mcal/s.

RESULTS

When pea and soybean seeds were exposed to low temperatures, the survival of the seeds depended dramatically on the moisture content (Figs. 1 and 2). At the lowest hydration levels, survival of soybean is limited by low temperatures. However, at moistures between 0.05 and 0.18 g/g, both pea and soybean were relatively unaffected by low temperatures. In pea, germination was depressed by cold when moistures were above about 0.33 g/g. At higher moisture levels, survival was significantly reduced at all temperatures studied between −18 and −180°C (Fig. 1). In soybean, germination was similarly depressed by −18 and −30°C treatments at moistures above about 0.32 g/g (Fig. 2); however, at −50, −80 and −180°C (Fig. 2), the threshold moisture content was about 0.20 g/g. Thus, soybean and pea seeds have similar threshold moistures when exposed to temperatures less negative than −30°C, but there is a differential response in sensitivity when seeds are exposed to temperatures of −50°C and lower.

To determine whether different parts of the seed had similar sensitivities to low temperatures, cotyledons and axes were isolated from soybean seeds, equilibrated to different moisture contents, and then exposed to −18 and −80°C. In this experiment, leakage rates were used to evaluate damage. Cotyledons, with moisture contents greater than 0.38 g/g, showed high rates of leakage when exposed to −18°C, but the threshold moisture level changed to 0.20 g/g upon exposure to −80°C (Fig. 3). Leakage rates were also high from cotyledons if moisture contents were less than 0.12 g/g. This may be interpreted as an effect of imbibitional damage rather than a direct effect of exposure to low temperatures. Leakage rates were high when axes at moisture contents greater than 0.36 g/g were exposed to either −18 or −80°C (Fig. 3).

DSC thermograms were used to correlate thermal transitions of seed material with vigor assays. In pea cotyledons (Fig. 4), no transitions were observable if moisture contents were less than 0.24 g/g (0.13 g/g shown in Fig. 4, data from cotyledons at 0.05, 0.08, 0.16, and 0.22 g/g not shown). At 0.24 g/g, a small but broad endothermic event was detected at −35°C. As the seed moisture contents increased above 0.27 g/g, endothermic events occurred at between −23°C and −18°C and progressively increased in size and sharpness. If the endothermic events were due to the melting of water, a straight line is expected when the size of the endotherm is plotted against the moisture content. Such a plot for pea cotyledons is shown in Figure 5. The slope of this line is 239 J/g H_2O (R^2 = 0.974) and the x axis intercept, representing the limit of freezable water, is 0.256 g/g.

In soybean cotyledons, a broad endotherm was observed at between −43 and −32°C when moisture contents were between 0.048 and 0.21 g/g (Fig. 6). The temperature of the onset of the endotherm (Fig. 7) and also the energy of the transition (Fig. 8) increased as the moisture content increased through this moisture regime. As cotyledon slices were hydrated to moisture contents greater than 0.23 g/g, the nature

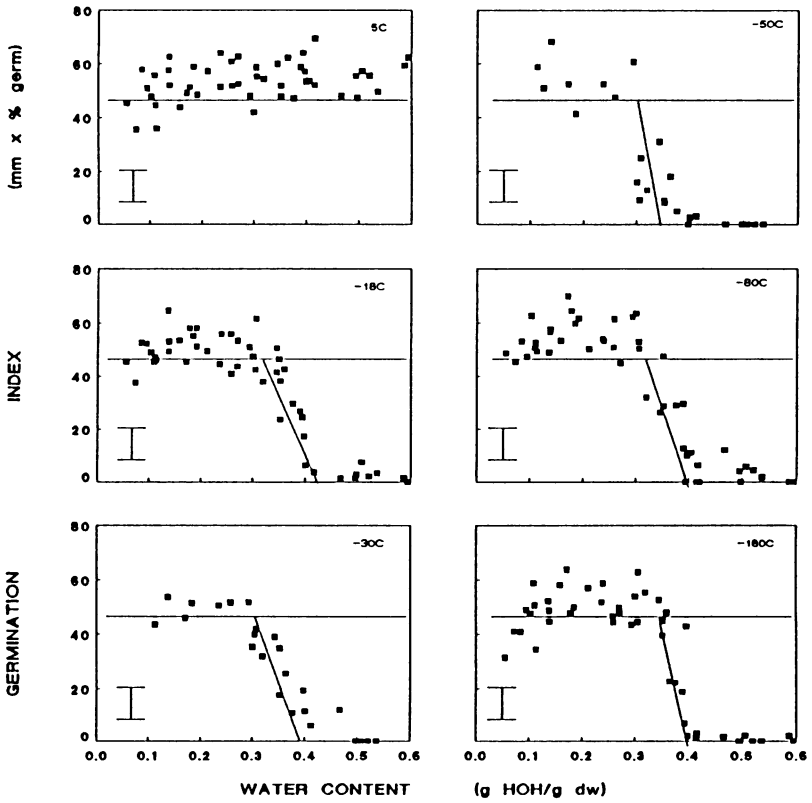

Figure 1. Viability of pea seeds exposed to subzero temperatures at different moisture contents. The error bars represent the maximum standard error of the mean axis length for the given temperature treatment. The horizontal line represents the mean minus 1 SE of the control treatment (5°C). Diagonal lines are drawn as an aid to the eye.

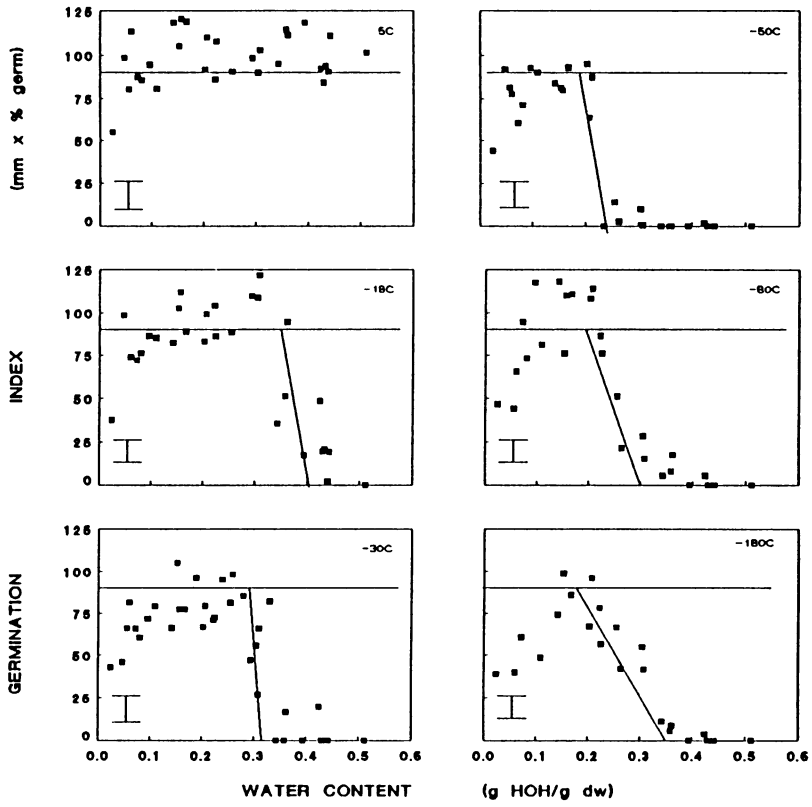

Figure 2. Viability of soybean seeds exposed to subzero temperatures at different moisture contents. The error bars represent the maximum standard error of the mean axis length for the given temperature treatment. The horizontal line represents the mean minus 1 SE of the control treatment (5°C). Diagonal lines are drawn as an aid to the eye.

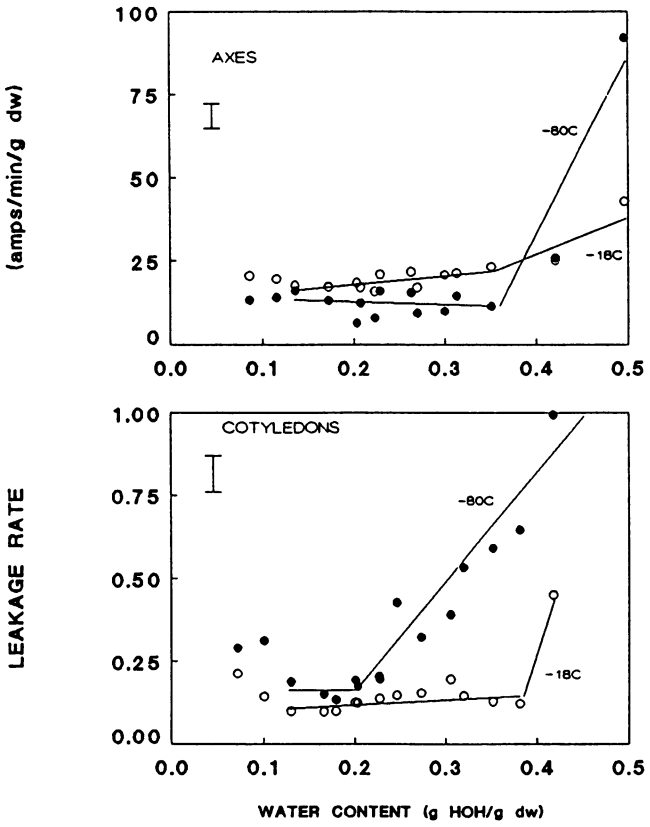

Figure 3. Rates of electrolyte leakage from soybean axes and cotyledons when seeds with different moisture contents were exposed to −18 (open circles) or −80°C (closed circles). The error bars represent the maximum standard error of the mean leakage rate. This value was ±2.4 and 0.005 A/min/g dw for axes and cotyledons, respectively. The lines drawn are the least squares best fit for the data with moisture contents above 0.12 g/g. Points of intersection of the lines are calculated to be 0.38 and 0.20 g/g for soybean cotyledons at −18 and −80°C, respectively and 0.35 and 0.36 g/g for axes at −18 and −80°C, respectively.

of the endotherm changed: a sharp second peak was apparent at about −20 to −12°C (the temperature increased as the moisture increased) and several pretransitions were observable from −90 to −50°C (Fig. 6). The plot of enthalpy of transitions between −40 and 0°C against water content was linear in the moisture range of 0.23 and 0.43 g/g with a slope of 417 J/g H_2O (R^2 = 0.955) (Fig. 8). Another curve, with a slope of 48.3 J/g H_2O, was drawn for water contents below 0.225 g/g (R^2 = 0.654). The point of intersection between the best fit lines drawn for moistures between 0.05 and 0.21 g/g and between 0.23 and 0.43 g/g was at 0.225 g/g. This represents the moisture content at which freezable water is present.

Thermograms of soybean axes also changed as the moisture content changed. At moisture contents between 0.09 and 0.22 g/g, there were two peaks evident with onset temperatures of −93°C and −48°C (Fig. 9). As the moisture content was increased to 0.23 g/g or greater, a more complicated thermogram resulted: the structure and temperature of the existing peaks changed showing several pretransitions and a broad endotherm. A sharp peak at −18°C became increasingly apparent as the axes were hydrated further. The line resulting from the best fit of enthalpy of transitions vs moisture contents between 0.24 g/g and 0.39 g/g had a slope of 250 J/g H_2O (R^2 = 0.967) (Fig. 10). The x intercept from this line is 0.248 g/g, indicating the moisture level at which freezable water is present.

At moisture contents of about 0.30 (pea), 0.25 (soybean cotyledon) and 0.27 g/g (soybean axes) or greater, large exothermic transitions were noted in tissues prior to the melting endotherm (Figs. 4, 6, and 9). The exothermic pretransitions could be eliminated from the DSC scan by annealing tissue that had been cooled to −150°C at 10°C/min at −25°C, recooling to −150°C, and finally rewarming through the endotherm (data not shown). The ability to eliminate transitions by the annealing process indicates that glasses formed in the aqueous phase. Therefore, the exothermic pretransitions were probably devitrification events caused by the melting of aqueous glasses and the consequent freezing of water at low temperatures. The extent of devitrification could be manipulated by cooling rates: cooling at 1°C/min almost eliminated the pretransitions (data not shown).

To study further the thermal characteristics of soybean cotyledons, DSC scans of soybean oil, purchased from Sigma Chemical Company, were performed (Fig. 11). The resulting thermogram shows several endothermic peaks above −40°C and a devitrification event at about −75°C. The multipeaked characteristics of the oil thermogram do not resemble the characteristics of dry soybean cotyledons (*i.e.* 0.08 g/g in Fig. 6) unless the soybean oil sample was annealed at −65°C, recooled to −150°C, and then warmed at 10°C/min through the lipid transition (data not presented). The energy of the melting endotherm of soybean oil was 71.4 J/g oil.

DISCUSSION

These experiments were conducted to compare the thermal behavior of seed tissues at different moisture contents with sensitivity of seeds to low temperature stresses. The thermal behavior of seed parts were examined using DSC warming thermograms. Depending on the seed tissue and moisture level, melting transitions of lipids and water were detected.

Endotherms from the lipid component of soybean cotyledons and axes were detected at all moisture levels. In soybean cotyledons, the enthalpy of lipid transitions changed with the moisture content (Fig. 8). The positive slope of the regression line calculated for the transition enthalpy of soybean cotyledons at moistures between 0.05 and 0.21 g/g (Fig. 8) suggests that the transition energy of soybean lipids increases with water content by a factor of about 48 J/g H_2O. If the line is extrapolated to the y-intercept, an enthalpy of 7.1 J/g dry weight is given. Assuming that soybean cotyledons contain about 22% of their dry weight as lipid, 7.1 J/g dry weight equals 32.3 J/g lipid. This value is lower than the 71.4 J/g lipid determined as the melting enthalpy of soybean oil purchased from Sigma Chemicals. The enthalpy at the point of intersection of the two lines in Figure 8 is about 17 J/g dry weight or 77 J/mg lipid, a value that corresponds well with the standard. As cotyledons are hydrated from 0.05 to 0.21 g/g, the transition temperature is increased (Fig. 7). This is exactly opposite of the trend observed for extracted phospho-

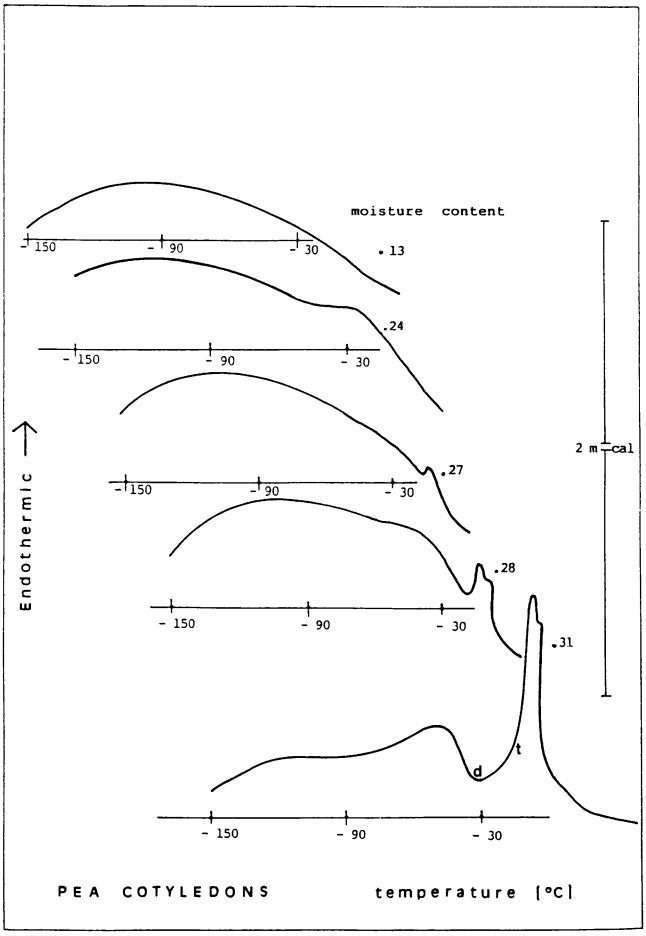

Figure 4. Heating thermograms of slices of pea cotyledons at different moisture contents. Samples were cooled at 10°C/min to −150°C, then warmed at 10°C/min. In the bottom thermogram, the "d" and "t" represent the devitrification and main transition events, respectively.

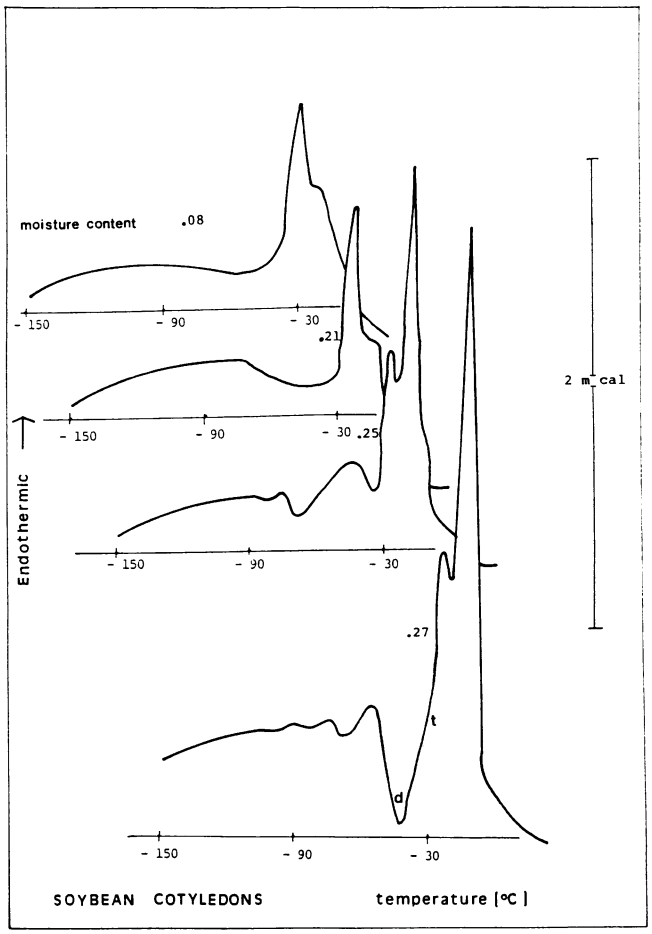

Figure 6. Heating thermograms of slices of soybean cotyledons at different moisture contents. Samples were cooled at 10°C/min to −150°C, then warmed at 10°C/min. In the bottom thermogram, the "d" and "t" represent the devitrification and main transition events, respectively.

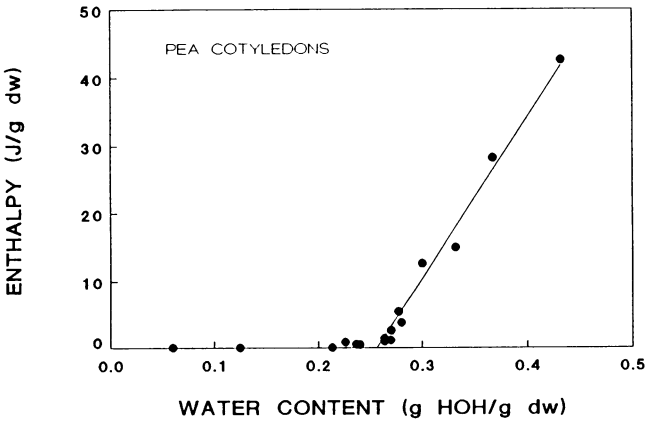

Figure 5. Effect of water content on the enthalpy of the transition in pea cotyledons. Transition enthalpies were determined by measuring the area under the peak from endotherms similar to those shown in Figure 4. The line drawn is the least squares best fit for the data (R^2 = 0.975; P < 0.00001).

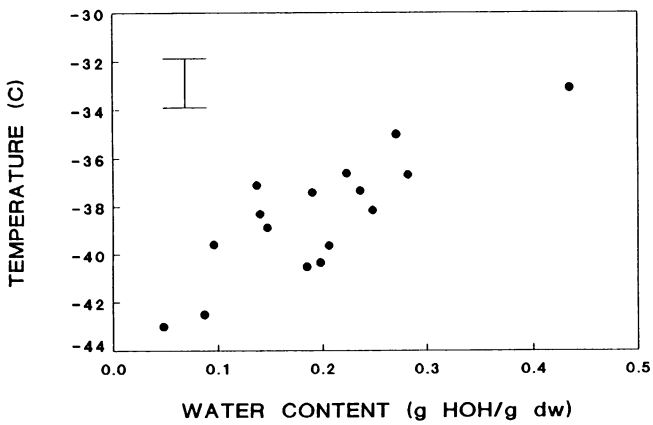

Figure 7. Effect of water content on the temperature of the onset of the major transition in soybean cotyledons. Onset temperatures were determined from endotherms such as those shown in Figure 6. The error bars represent the maximum standard error of the mean of three replicates (about ±1.1°C).

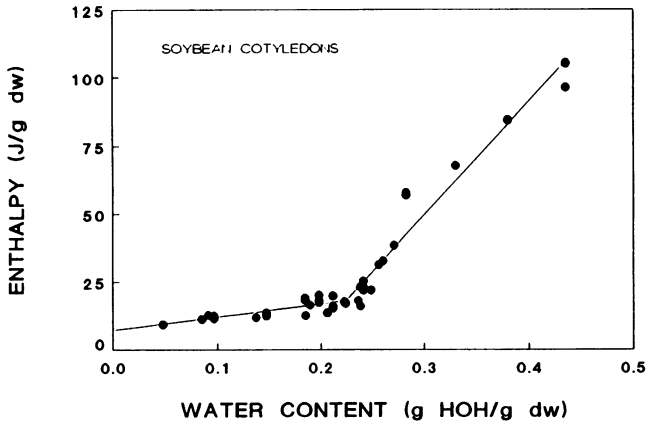

Figure 8. Effect of water content on the enthalpy of the transition in soybean cotyledons. Transition enthalpies were determined by measuring the area under the peak from endotherms similar to those shown in Figure 6. The lines drawn are the least squares best fit for the data ($R^2 = 0.654$, $P < 0.00001$ for moistures between 0.05 and 0.21 g/g; $R^2 = 0.955$, $P < 0.00001$ for moistures between 0.23 and 0.43 g/g).

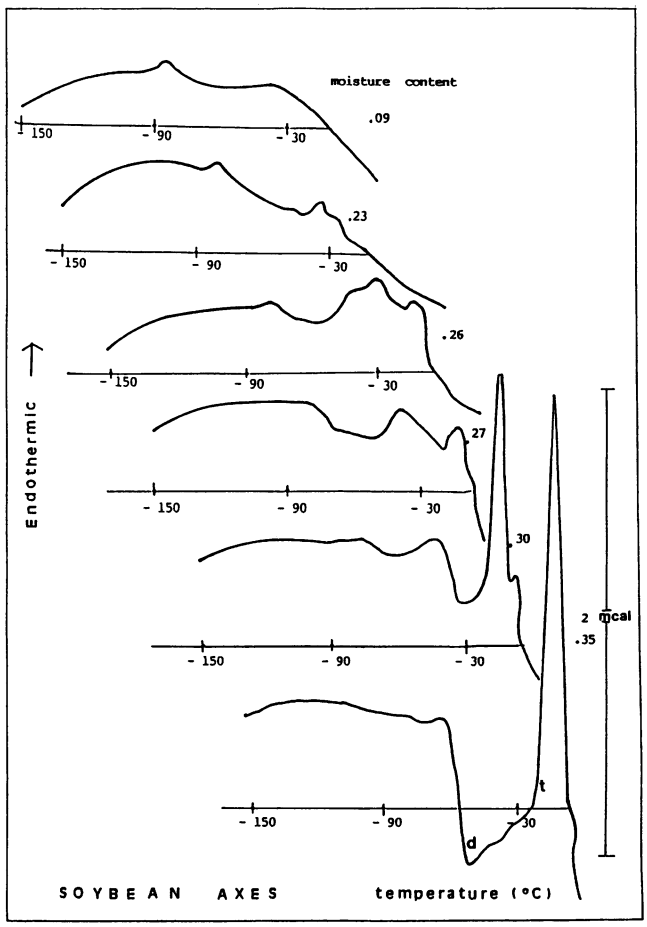

Figure 9. Heating thermograms of soybean axes at different moisture contents. Samples were cooled at 10°C/min to −150°C, then warmed at 10°C/min. In the bottom thermogram, the "d" and "t" represent the devitrification and main transition events, respectively.

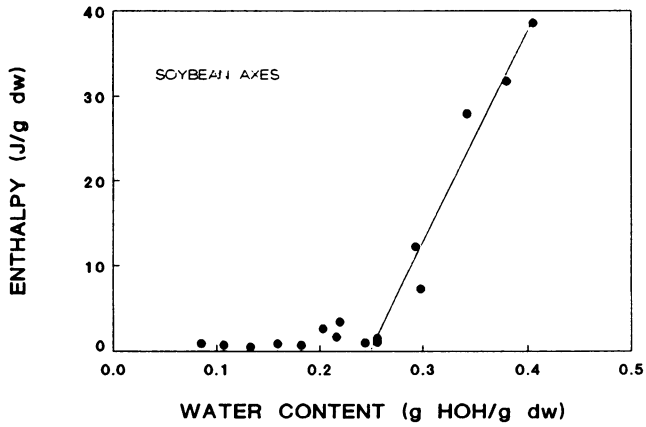

Figure 10. Effect of water content on the enthalpy of the transition in soybean axes. Transition enthalpies were determined by measuring the area under the peak from endotherms similar to those shown in Figure 9. The line drawn is the least squares best fit for the data ($R^2 = 0.967$; $P < 0.00002$).

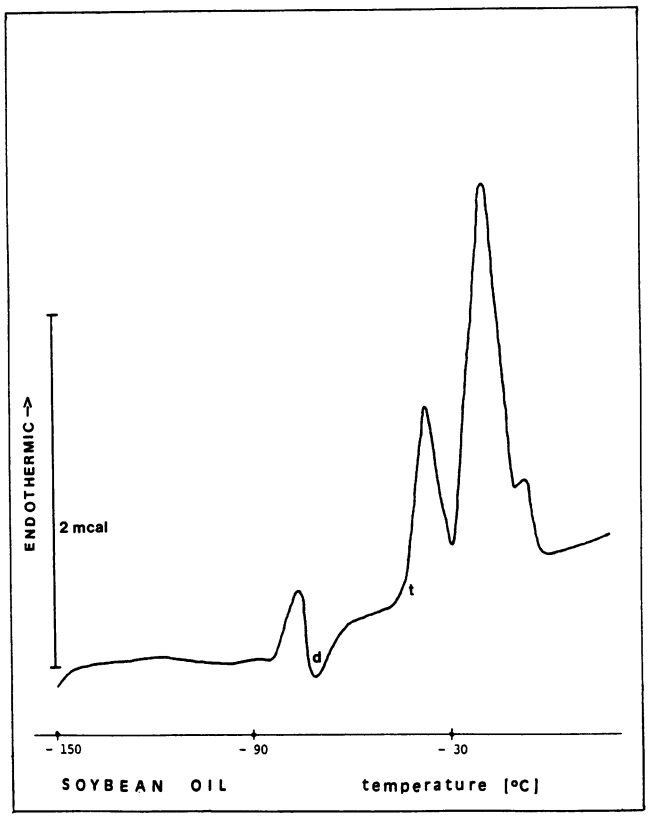

Figure 11. Heating thermogram of soybean oil purchased from Sigma Chemical Co. The 5 mg sample was cooled at 10°C/min to −150°C, then warmed at 10°C/min. The "d" and "t" represent the devitrification and main transition events, respectively.

lipids (6, 13). A trend toward increasing enthalpy and onset temperature of melting endotherms is typically found when impure samples are purified. Thus, the changes observed in the lipid component of the cotyledon tissue with hydration may indicate a "purification" or consolidation of the lipid *in*

vivo. Based on calorimetric and ultrastructural studies, it was suggested that at moisture contents of about 0.21 g/g, lipid droplets coalesce due to the onset of hydrophobic interactions (26).

As the moisture content of the seed tissues increased further, large changes in the warming thermograms were observed (Figs. 4, 6, and 9). In soybean cotyledons, the shoulder at −20°C became peaked. In soybean axes and pea cotyledons a peak at about −20°C was observable. These new transitions are interpreted as the melting of water. The limit of freezable water was determined by regressing the energy of the melting transition with the moisture content. This technique assumes that the energy of the endotherm is directly proportional to the amount of material undergoing the transition. By extrapolating the regression line to the x intercept where the enthalpy of melting is 0, we can determine the limit of freezable water. Using similar methods, Ladbrooke and Chapman (13) found the limit of freezable water in serine, phosphatidylethanolamine and natural lecithin mixtures to be about 0.0, 0.18, and 0.30 g/g, respectively. Recently, Roos (20) examined the thermal behavior of strawberries and found that a moisture content of 0.214 g/g was the point at which ice was found to melt. Values for the limit of freezable water in soybean and pea tissues ranged from 0.22 to 0.26 g/g and correspond well to values reported earlier for other tissues (3, 9, 10, 20, 28).

The slopes of the enthalpy vs moisture content curves in Figures 5, 8, and 10 are equal to the heat of fusion of the water in the tissue. The heat of fusion of pure water is 333 J/g water. Ladbrooke and Chapman (13) reported values of about 296, 363, and 431 J/g water for serine, phosphatidylethanolamine and lecitin mixtures, respectively. Roos (20) reported a value of 374 J/g water for freeze dried strawberries. Heat of fusion values calculated in this paper for water in pea cotyledon, soybean axes and soybean cotyledon are 239, 250, and 417 J/g, respectively. While statistical comparisons of the slopes showed that 239 and 250 were not significantly different at $P = 0.10$, all the slopes were significantly different from the theoretical slope of 333 at $P < 0.005$. The physiological significance, if any, for the discrepancies between the energies of water transitions in seed tissues and that of pure water is not known.

The moisture contents determined as the limits to freezable water coincide well with the moisture contents described as the onset of region 3 of water binding determined by sorption isotherms: between 0.20 and 0.24 for soybean cotyledons, between 0.24 and 0.26 g/g for soybean axes, and 0.26 g/g for pea cotyledon (27). Type 3 water associates with macromolecular surfaces via multimolecular sorption (7) or as bridges over hydrophobic groups (21), and it is suggested that the third level of hydration marks the beginning of hydrophobic interactions (27). The data presented in this report suggest that at least some of the type 3 water is susceptible to freezing.

In pea, reductions in germination of seeds exposed to −18°C or lower are not observed until the seed moisture content exceeds 0.33 g/g (Fig. 1). Similarly, in soybean axes, increased leakage of tissue exposed to freezing temperatures is not observed until the level exceeds 0.35 g/g (Fig. 3). In these instances ice formation occurs at lower moisture levels than the moisture levels at which damage is detected. This observation holds for soybean seeds and cotyledons when exposed to −18°C or −30°C (Fig. 2) where germination is not affected unless moisture levels exceed about 0.32 g/g. However, upon exposure to temperatures of −50°C and lower, damage to soybean seeds and cotyledons was observed at moisture levels at or lower than where freezable water is present (Figs. 2 and 3). Threshold moisture levels for lettuce seeds change from about 0.25 g/g at −18°C to about 0.16 at temperatures below −70°C (10, 19). The threshold moisture level for sesame seeds exposed to −196°C is about 0.13 g/g (23).

Comparisons of the presence of freezable water with damage to seed tissues exposed to subzero temperatures do not support the hypothesis that the formation of intracellular ice is the lethal event in freezing injury. In most instances, ice formation is observed at moisture contents lower than where freezing damage occurs. Since the seeds had been equilibrated to various moisture levels and freezing rates were relatively rapid, it is likely that the ice formation observed was intracellular. Further evidence that the cytoplasm was hydrated comes from observations that mitochondrial respiration is possible at water contents representing the limits of freezable water (26).

It is suggested that although water within the third level of hydration is freezable, the ice crystals that form are too small to cause significant damage. For example, organelles from mammalian cells maintain their structure if ice crystals are less than 0.05 μm (22). However, in most instances, it has been reported that small crystals recrystalize into larger crystals capable of causing damage (1, 8, 17), and thus Levitt (15) concluded that intracellular ice formation was always lethal. If the ice that formed during freezing of seed tissues did not cause damage because crystal size was too small, then we must conclude that, unlike in tissues studied previously, the ice crystals that formed when moisture contents were less than about 0.33 g/g were unable to coalesce. At higher moisture levels where free water is present (≥0.33 g/g) (25, 27), the ice crystals that formed were large enough to cause death.

The question then arises as to why, at relatively low moisture contents, soybean cotyledons are killed at temperatures below −50°C and, under similar circumstances, other tissues experience no detrimental effects. Since freezable water is observed at moisture levels believed to be where water condenses over hydrophobic moieties (21, 27), it is suggested that the onset of the hydrophobic interaction is in some way responsible for the differences in freezing susceptibility between soybean cotyledons and soybean axes or pea seeds. The difference in freezing susceptibility in soybean seeds occurs between −30 and −50°C, within the temperature range at which the lipids undergo a transition (Fig. 7). Perhaps then, the thermal transitions in the lipid component of the tissue is responsible for the differential sensitivities of soybean and pea to low temperatures. It is hypothesized that the interaction between the lipid and water somehow enabled ice crystals to form that were large enough to cause lethal damage.

This paper addresses the question of whether the formation of intracellular ice causes freezing damage in tissues. Evidence presented here neither proves nor disproves the theory; however, it does offer some refinements: (a) freezable water may be present with no damage to seeds, (b) freezable water may also be present in minute quantities and cause devastating effects on seed viability. Lipid transitions may be involved in

the latter phenomenon. It is speculated that triglycerides interact with water and this interaction can trigger a sensitivity to freezing damage. Thus, water content is intimately involved in extent of "freezing" injury, but at low levels of hydration the *freezing* of water does not itself account for freezing injury.

ACKNOWLEDGMENTS

Appreciation is expressed to Drs. A. C. Leopold and S. Sowa for their helpful comments on the manuscript.

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