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Vitrification in Plants as a Natural Form of Cryoprotection

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A small group of woody plants from the far northern hemisphere can, while in the dormant state, tolerate freezing and thawing to and from any subzero temperature at rates <30°C/hr. In addition, the hardiest of them can tolerate cooling and warming between −20°C and any colder temperature at virtually any combination of rates subsequent to cooling to −20°C at rates <5°C/hr. We term this latter capability "quench hardiness." I and my colleagues have shown that the limits of this quench hardiness can be closely correlated to the stability of intracellular glasses formed during the slow cooling of hardy tissues in the presence of extracellular ice. In this paper, I briefly review the evidence for intracellular glass formation and present data indicating that major components of the glass forming solutions are raffinose and stachyose. Evidence from differential scanning calorimetry that sugar-binding soluble proteins are also important is presented. Finally, I correlate data from our work with that of other workers to make the case that, even when most of a cytoplasmic solution is vitrified, fluid microdomains remain which can lead to long-term biodegradation during storage at high subzero temperatures.

Eighteen years ago, Drs. Sakai and Yoshida (10) published a paper purporting to show that the crucial event in the development of extreme cold hardiness in the living wood of northern arboreal plants is a steep increase in cytoplasmic free sugar during autumnal hardening. In the same issue of *Cryobiology*, Siminovitch and his colleagues (13) presented extensive data explaining the phenomenon on the basis of a sharp increase in soluble proteins in the cytoplasm. Both investigations agreed that both sugar and protein increased markedly during hardening.

Until recently there has been no model capable of resolving these differing points of view. Advances in calorimetric measurement techniques via differential scanning calorimetry (DSC) and freeze-etch electron microscopy have allowed us to examine the data from the standpoint of a problem in solution physics (5). Taken this way there is a striking correlation between intracellular aqueous glass stability and mortality and this allows the construction of a hypothesis resolving the protein vs sugar controversy.

Put simply, a glass is a liquid with the viscosity of a solid. Aqueous solutions of many different solutes can form glasses. Thus it is not particularly surprising that a plant which utilizes glass formation as a cryoprotective strategy may use a wide range of protein–sugar combinations to obtain the same general *physical* effect. Furthermore, glass formation confers only *very-low*-temperature protection. If a subtropical plant produces large amounts of sugars, but is killed during freezing at −6°C, intracellular glass formation at −35°C is of no cryoprotective importance. Glass formation is a complex nonequilibrium phenomenon and multiple transitions can occur in the same well-mixed solution due to the formation of microdomains as small as 2 nm in radius. As a result slow degradation of cryopreserved tissue can be expected even at storage temperatures at which most of the cytoplasm is in a frozen glassy state (8, 17).

Our investigations have established that at least three different glass transitions can be identified in hardy *Populus* wood (5). All of these occur at temperatures

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≤ -30°C. The hardened wood has very high concentrations of free sugars, especially raffinose and stachyose (3, 10). It will be demonstrated here that these sugars display aqueous glass transitions that correlate with the highest temperature transitions in the living wood. It will also be shown that the addition of salts and soluble protein to these aqueous sugar solutions cause them to undergo glass transitions at temperatures lower than those of sugars and water alone.

I present data that imply that the specialized proteins of the hardy poplar appear to bind raffinose, stachyose, and KCl and it is these complexes which may be the high-temperature glass-forming component of the living cells.

A case will be made for sugars and specialized proteins acting in unison to largely glassify the quench-hardy poplar cells at temperatures below -30°C. In addition, it will be asserted that this leaves some fluid proteins in microdomains showing lower glass transitions. These are then able to denature, perhaps due to free radical accumulation during long storage at temperatures above -80°C (2, 4, 8, 12, 14–16). Long-term loss of viability in quench-hardy twigs stored at low temperatures has been observed by our group and others (8). Finally, measurements of the calorimetric behavior and drought resistance of *Populus* will be compared to those made on siberian birch by Krasavtsev and his colleagues (6, 18). The birch appears to be truly drought hardy at subzero temperatures, the poplar is not. It will be shown that these differences are still consistent with intracellular glass formation in the birch.

EVIDENCE FOR NATURAL GLASS FORMATION

The evidence for natural glass formation in *Populus* is mainly calorimetric, buttressed by freeze-etch electron microscope data (5). I stress the data accumulated by use of differential scanning calorimetry (DSC) in this paper. The use of the fast scanning rates increases the sensitivity of DSC to low-energy events at the expense of accuracy on the temperature scale. Events labeled with the same letter–number combination represent the same events. The *b*'s are exothermic events, the *a*'s are glass transitions. To understand these complex events, we must first appreciate what is happening to the intracellular solutions at various subzero temperatures. During the annealing process at high subzero temperatures the cellular contents are completely fluid and come into equilibrium fairly rapidly with extracellular ice. However, the diffusion path to the ice is best measured in micrometers not nanometers. Thus cooling at ≥ 100°C/min tends to "trap" the cells at their annealed composition. If they are annealed at a high temperature they will contain relatively dilute aqueous solutions after fast cooling; the lower the temperature of annealing the more concentrated the cell sap. Since the magnitude of the glass transition is known to be roughly proportional to the mol H_2O/mol glass-forming solute (1), the more dilute solution should display glass transitions of greater magnitude. Furthermore, the more molecules of H_2O per molecule of glass-forming solute the less viscous the solution at a given temperature. Thus, the material annealed at a higher temperature and then quickly cooled will display equivalent glass transitions at lower temperatures upon warming. A final measure of the validity of the descriptions of the observed events is the size of the exotherms. Since a less concentrated solution will in general display more ice formation upon warming (devitrification), the increasing size of exotherms in warming records from material annealed at higher temperatures is also consistent with the interpretation of these events as devitrifications following glass transitions.

The most obvious complication in these records is the fact that there are multiple glass transition–devitrifications. This could be due to the complex anatomy of

the wood or, alternatively, microdomains in the primary intracellular solutions allowing multiple events in the same compartment. That the latter is a distinct possibility has been demonstrated by work in our lab (17) on artificial cryopreservative solutions.

Figure 1 shows 50°C/min warming of initially living quench-hardy *Populus* stored at −70°C, loaded in the calorimeter at −50°C, annealed for 10 min at −10°C, and then cooled at 100°C/min to −160°/C before warming at 10°C/min (dashed curve) vs the same sample cooled at 100°C/min after 10 min at −5°/C and subsequently warmed at 10°C/min (solid curve). Note that multiple endotherms ("ascending peaks") indicate glass transitions followed by devitrifications ("valleys"). The pattern is as described above. Equivalent glass transitions in the −10°C annealed wood are smaller and at a higher temperature than those seen when the wood is annealed at −5°C. The lowest glass transition–devitrification (a_3, b_3) does not follow this pattern. It disappears on the trace of −5°C annealed wood. This is probably due to homogeneous nucleation in this microdomain during cooling from a temperature as high as −5°C. Thus the microdomain would actually be more concentrated because of ice formation during cooling than in the −10°C annealed sample. This record is an example of those that led to the "three glass model" (5) of microdomains in the intracellular solution that glassify at different temperatures during cooling. Our estimate of these temperatures from these records is necessarily approximate, especially for the low-temperature events observable only at high scan rates. We estimate the transitions to be at about −30, −45, and −80°C.

To establish the actual temperature of the

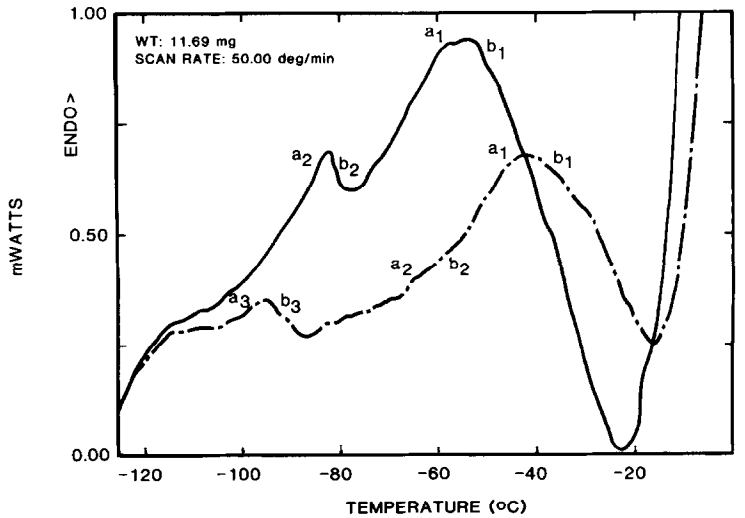


FIG. 1. The dashed curve represents warming after 100°C/min cooling of a quench-hardy sample annealed at −10°C for 10 min. The *a*'s represent glass transitions, the *b*'s devitrifications. The identities of each set of transitions can be determined by annealing at various temperatures and examining the systematic change in position and amplitude of the transitions. The solid curve is warming of the same sample after annealing at −5°C followed by quick cooling. Note that in the more dilute (−5°C annealed) sample the events are of larger magnitude and at lower temperature. Nevertheless, subtle pitfalls abound such as the disappearance of events a_3,b_3. They may represent domains that homogeneously nucleate during cooling from equilibrium at −5°C. Thus they are *more* concentrated and their thermal trace is buried in the record at higher temperature. The most important message in these records is that there are several clear glass transitions and they behave as expected if the plant cells are acting as osmometers. Figure reproduced with permission of The American Society of Plant Physiologists.

transitions accurately one must make measurements at a lower scan rate. Figure 2 shows a comparison of fully hardy material warmed at 10°C/min after 3°C/hr cooling to −70°C (dashed curve) vs the same sample warmed after quick cooling from −20°C (solid curve). Figure 3 shows the same comparison in another fully hardy sample but the warming rate is 1°C/min. The increase in heat capacity in samples slowly cooled to < −50°C occurs at temperatures between −28 and −35°C. After quick cooling from −20°C a distinct increase in glass transition size and lowering of temperature by about 10°C in identifiable events can be seen even at 1°C/min. Thus the highest glass transition can be estimated to be at about −30°C at ambient rates of cooling and warming. The lower energy events, a_2 and a_3 of Fig. 1, are not visible at these scanning rates. Figure 2 also shows clearly that fast cooling after annealing at −20°C does not lead to devitrification upon warming. This is corroborated by electron micrographs (Fig. 4 and 5). In Fig. 4, we see the results of plunging the tissue into liquid nitrogen (LN_2) after annealing at −20°C, rewarming to −27°C for 24 hr, and then replunging into LN_2. The tissue was not injured by this treatment and no evidence of ice formation can be seen in the cytoplasm. In Fig. 5, the situation is dramatically different. Here the tissue was annealed at −15°C, but everything else was done as before. The replica shows many holes where ice formed in the cytoplasm. The tissue mortality was 100%. This result can be predicted from warming thermograms of wood in equilibrium with ice at between −10 and −20°C. They show clear devitrification above −40°C at warming rates as high as 50°C/min. The conclusion is that the resistance of the intracellular glasses to devitrification during warming is crucial to survival. This in turn implies that fast warming after quench cooling will increase survival. We have not systematically studied this in the popular,

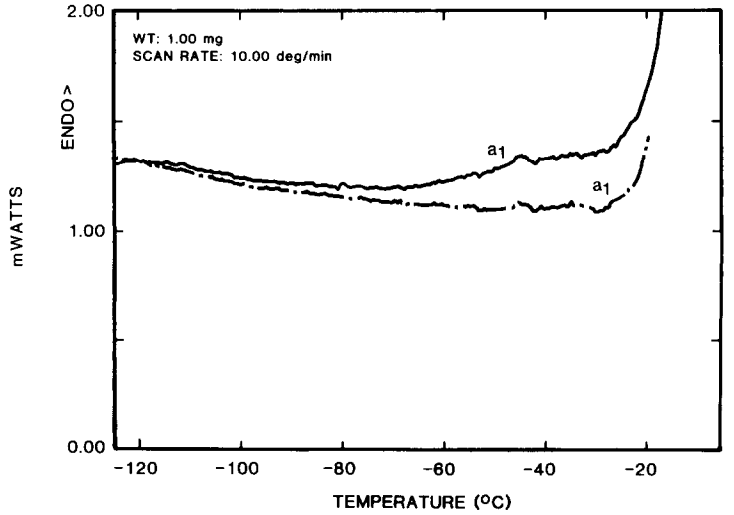


FIG. 2. Slow warming of a maximally quench-hardy sample of about 10 mg (the exact weight was not recorded; default weight of 1.00 mg). The dashed line represents 10°C/min warming after 3°C/hr cooling to −70°C. The solid line represents 10°C/min warming after 10 min annealing at −20°C and 100°C/min cooling to −160°C. Note the great increase in magnitude and drop in the temperature of onset of event a_1. Note also that the glass transition is not followed by a devitrification exotherm, indicating that all microdomains were stabilized against homogeneous nucleation by the time the solution had equilibrated with ice at −20°C. Figure reproduced with the permission of The American Society of Plant Physiologists.

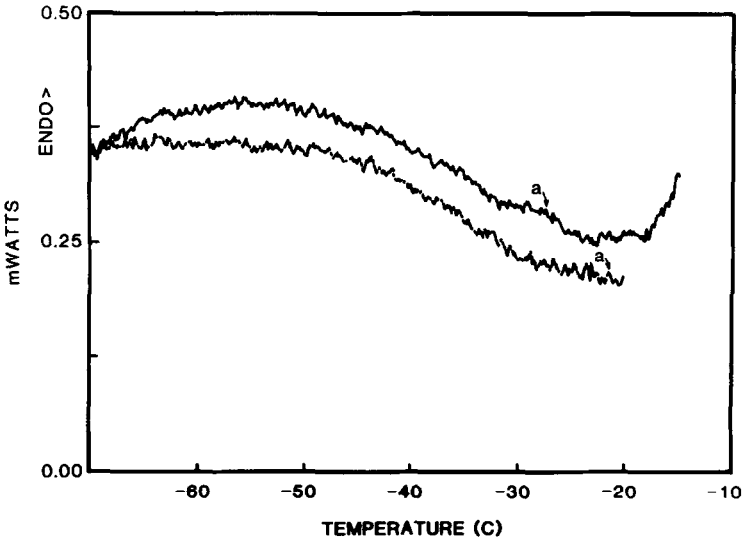


FIG. 3. Very slow warming of maximally quench-hardy *Populus*. Dashed line represents 1°C/min warming of poplar twig cooled at 3°C/hr to −70°C. Solid line represents warming of same sample after annealing at −10°C followed by 100°C/min cooling to −160°C. The a's represent approximate positions of the most important glass transitions. While they are barely above noise, their position near −30°C on the temperature axis and the fact that after annealing at −20°C the transitions become larger and appear at a lower temperature confirm the accuracy of the information developed at 10°C/min.

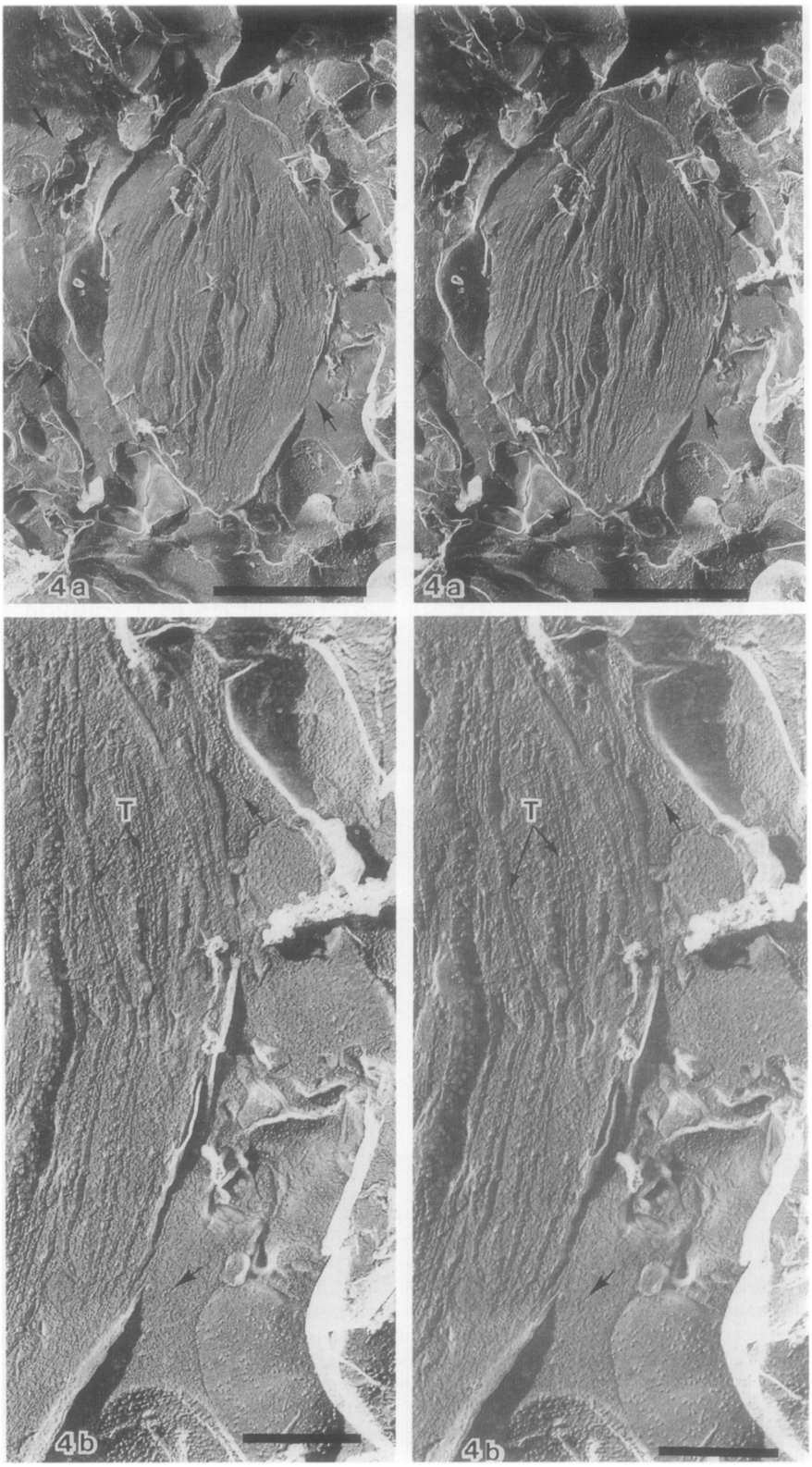
but experiments during dehardening in March clearly have shown that survival after 3°C/hr cooling to −50°C is significantly better when twigs are warmed at 1°C/min than at 3°C/hr.

THE CHEMISTRY OF GLASS FORMATION

A. Sugars

Several workers (3, 7, 8, 10, 13) have shown that a large amount of free sugar accumulates in the wood of hardy trees. The measured contents in *Populus* are as high as 27% dry wt in some clones with the range being from 17 to 27%. We have measured a soluble fraction with mW <2000 as representing 15 to 20% of the dry weight of the tissue. Higher molecular weight material in our rather crude dialysis experiments appeared to be about 2% of dry weight. Others have also found about 2% dry weight for soluble protein (10). About half of the free sugar in the *Populus* is raffinose and stachyose; a trisaccharide and tetrasaccharide, respectively. We have investi-

FIG. 4. (a) A cross-fractured chloroplast and surrounding organelles and cytoplasm (black arrows) are shown in this maximally quench-hardy sample subject to an initial 3°C/hr cooling to −20°C before plunging into LN_2, followed by 12 hr at −27°C, rapid recooling in LN_2, fracturing, and etching. Note that both the chloroplast and the surrounding cytoplasm appear completely free of evidence of etching. A sample from this tissue lived ater 3°C/hr warming from −70°C storage. Black bar equals 1.0 μm. ×23,000. (b) Enlargement of the sample illustrated in (a). The thylakoid stacks are clearly seen in an undistorted state (T) and the large headed arrows point to the smooth cytoplasm. Despite 12 hr at −27°C and fast recooling, no observable devitrification has taken place. This corresponds well with the lack of a DSC warming exotherm in quench-cooled −20°C equilibrated material (Fig. 2) and the survival of the tissue upon slow thawing. Black bar equals 0.25 μm. ×68,000. Figure reproduced with the permission of The American Society of Plant Physiologists.

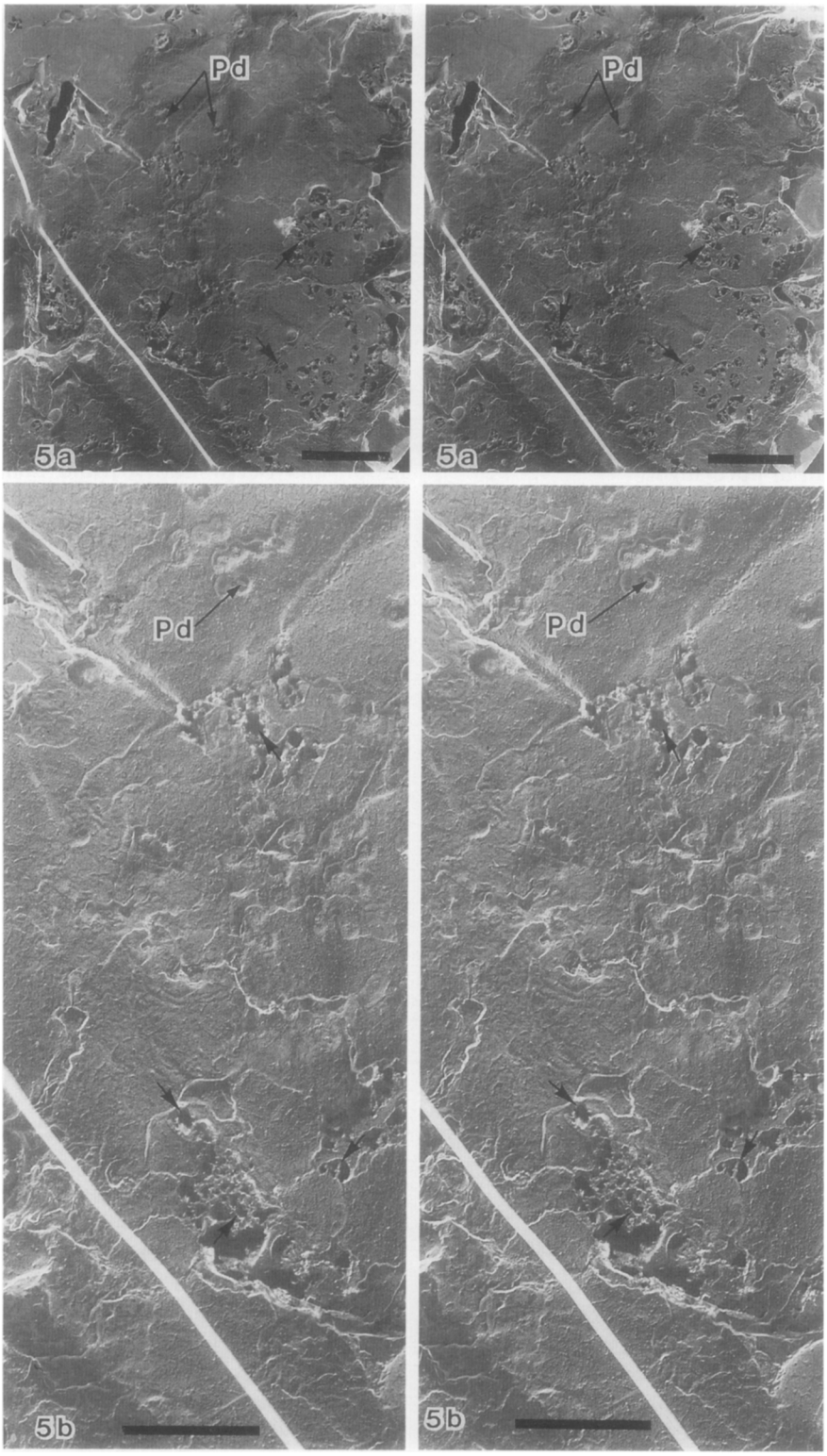

4a
4a
T
T
4b
4b

gated the aqueous glass-forming abilities of these two sugars extensively. Figure 6 shows the warming of a solution of 20% stachyose cooled at 20°C/min to −90°C from the thawed state (dashed curve) vs the same solution cooled to −90°C, rewarmed to −28°C, annealed, and then recooled before being warmed as shown by the solid curve. The endothermic events labeled *a* represent the glass transition of a concentrated aqueous stachyose glass. The event *b*, present only in the record of the unannealed sample, represents the collapse of trapped high-energy states due to fast cooling. Clearly the record so far seems remarkably like the whole twig. What is especially noteworthy about the glass transition in the record of the sample after annealing is that it is in precisely the same temperature range as that in the wood. Event *c* is the excess enthalpy neeeded to loosen low-energy states formed during annealing in the temperature range of high-energy collapse. The event *d* is the ordinary melting of ice in the concentrated sugar solution. Except for the occurrence of events *b* and *c* this record would appear to explain the highest temperature transitions in the wood quite nicely. Unfortunately, it is necessary to examine complex solutions more representative of the cell sap before the role of the sugars in glass formation can be properly understood.

As a next step we examined the effects of KCl on aqueous stachyose. Figure 7 shows warming records of a concentrated stachyose–KCl solution. The solution was formulated by drying a 10% stachyose–0.2 *M* KCl (1.5%) solution until a final composition containing only 6% of the original water was reached. In the *Populus* about 10% of the intracellular water remains in solution at −20°C, so this is a reasonably close approximation to the *in vivo* state. This material was then cooled at 100°C/min to −160°C, rewarmed to −40°C for 10 min, recooled, and rewarmed. The low temperature limiting glass transition is now at about −50°C, considerably below that in pure aqueous stachyose. It is also biphasic with the higher temperature transition only a few degrees below that in pure aqueous stachyose.

What happens when stachyose and raffinose are mixed? Figure 8 shows two warming curves of a solution initially 21.5% (w/w) raffinose and 21.5% (w/w) stachyose. The solid curve shows the thermal behavior after quick cooling to −160°C, rewarming to −24°C, and then recooling to −160°C. Once again we see evidence of a glass transition at *a*, loss of high-energy states at *b*, completion of the glass transition at *c*, and the ordinary melting of ice at *d*. When the process is repeated but annealing is at −32°C, in the high-energy collapse region, the excess enthalpy of glass melting (event *c*) increases markedly as expected. It is clear than that the two sugars alone do not interfere with each other's formation of a high-temperature glass.

FIG. 5. (a) Maximally quench-hardy *Populus* cooled at 3°C/hr to −15°C, plunged into LN_2, (1200°C/min cooling), reinserted into a cooling bath at −27°C (10°C/min warming), held for 12 hr, and then put in dry ice for storage (1°C/min cooling). This sample was fractured under LN_2, held at −110°C during evacuation, and then etched at −90°C for 2 min. The P-face (PF) of the plasma membranes is shown as well as the associated plasmadesmata (Pd). The large-headed arrows point to clearly defined clusters of holes, the remains of ice crystals of several hundred nanometers mean radius. Black bar equals 1.0 μm ×12,900. (b) An enlargement of (a) showing P-face (PF) of plasma membrane, plasmadesmata (Pd), and clusters of holes representing intracellular ice crystals (large arrows). Black bar equals 0.5 μm ×38,900. Figure reproduced with the permission of The American Society of Plant Physiologists.


Pd
Pd
5a
5a
Pd
Pd
5b
5b

If one adds protein to raffinose, the result is similar to the effect KCl has on the stachyose solutions. Figure 9 compares limiting glass transitions for raffinose (solid curve) versus raffinose plus human serum albumin (HSA) at a weight percentage concentration one-tenth that of the raffinose (dashed curve). Once again the glass transition has been lowered 10 to 15°C. If one starts with 10% stachyose, 10% raffinose, and 2% HSA and anneals at low temperature one gets a result like the solid curve in Fig. 10. The dashed curve is the limiting glass behavior of raffinose and HSA with no stachyose. The T_g for both systems is lower than in pure sugar.

The DSC studies of sugar solutions lead to several conclusions. The most important is that when ordinary soluble proteins and salts are added to the sugar solutions they decrease T_g significantly below the value *in vivo*. Another important conclusion is that to attain the maximally stable high-temperature glass in mixed sugar–salt or sugar–protein solutions requires annealing in the high-energy state collapse–devitrification region just below the maximum temperature of glass transition. This is illustrated in Fig. 11. This is in contrast to the behavior of the tissue as illustrated in Fig. 2. The tissue shows no exothermic behavor previous to the peak of the glass transition despite the fact that the diffusion path to ice crystals is much longer in the tissue. We have no explanation for this except to say that it is another indication of the greater complexity of the *in vivo* glasses.

CONSEQUENCES OF MULTIPLE GLASS TRANSITIONS

Sagisaka (8) has recently published an extensive study showing that long-term storage of *Populus* at a temperature of $-10°$ leads to deterioration of the wood in about 1 year. This is in agreement with our data which indicate a 6–8 month limit to storage of *Populus balsamifera* at subzero temperatures between -20 and -80°C. Sagisaka presented good evidence that the crucial factor limiting long-term survival was the inactivation of peroxidases perhaps coupled to their loss of reducing substrates. All of his detailed studies were done at -10°C. At that temperature, the cell sap is completely fluid. Thus one cannot make the argument that glass formation is protective at those temperatures. As the temperature falls, the length of time one can store grows shorter for many very hardy plants. Sakai reported very short limiting values, 10 days at -30°C, for one species of poplar (9), but our data are inconsistent with that. Nevertheless, our three glass model (5) predicts that deterioration can occur due to fluid anatomical subunits and/or general microdomains in the intracellular fluid at temperatures above the lowest glass transition. Full protection for a period >1 year would not be expected until a temperature 10°C or so below the lowest glass transition. For *P. balsamifera*, this appears to be <-100°C, and may be <-120°C.

The model implies that as storage temperatures are lowered below -30°C, fewer lesions should occur. This is because as the storage temperature is decreased below $-30°$ domains liquid at higher temperatures become glasses and thus are resistant to free radical damage as well as various forms of autocatalytic degradation. Since the model postulates major glass transitions in the wood at ~ -30, ~ -40, and ~ -80°C, it is only below about -100°C that resistance to slow degradative processes would be expected to be complete. At temperatures between -100 and -30°C, there would be fewer fluid compartments the lower the temperature. This leads to the notion of fewer lesions at lower temperatures. This might not lengthen storage time per se, but it might make it easier to counteract the effects of the remaining fluidity as the storage temperature is lowered. Certainly Sagisaka's data imply that preloading the tissue with an inert gas such as N_2 might have a significant positive effect. Such an effect for a tender plant

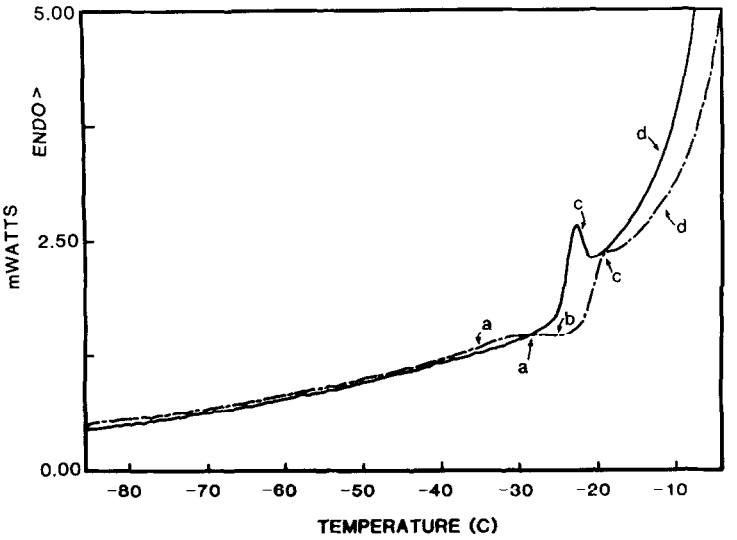


FIG. 6. Twenty percent stachyose was cooled at 20°C/min to −90°C and then warmed at 10°C/min as shown by the dashed curve. The sample was then recooled rapidly to −90°C, warmed to −28°C, annealed 10 min, and finally recooled at ≥100°C/min to −90°C before warming at 10°C/min as shown by the solid curve. The beginning of the limiting glass transition is at *a* on the dashed curve and 10 to 15°C lower at *a* on the solid curve. This is due to the collapse of high energy states and devitrification as shown by *b* on the solid curve. Areas labeled *d* are the equilibrium melt of ice.

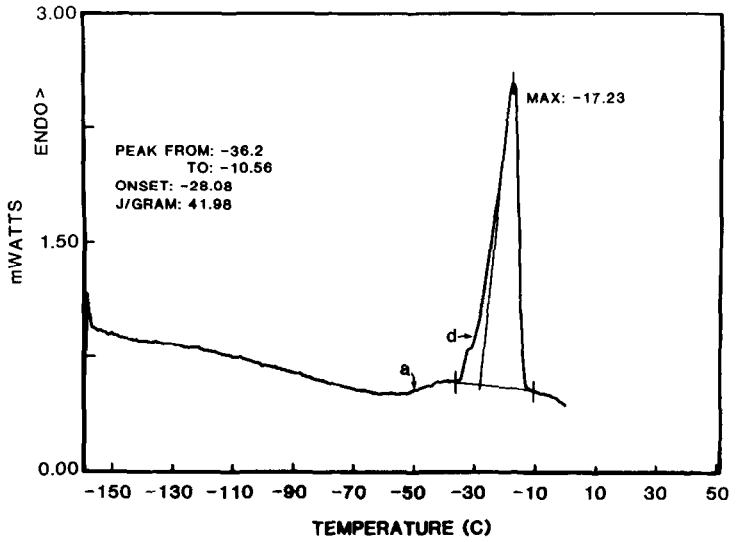


FIG. 7. A solution containing by weight 0.94 mg H_2O, 0.23 mg KCl, and 1.5 mg stachyose, formed by drying a 0.2 *M* KCl–10% stachyose solution. Cooled to −160°C, annealed at −40°C, recooled, and warmed at 10°C/min. The large endotherm starting at *d* indicates that about one-third of the 0.93 mg of water was ice. Thus the estimated final solute composition is about 75% solute (w/w). This is quite close to the estimated composition of the poplar in equilibrium with ice at −20°C, and the glass transition of the cells in such circumstances is in this temperature range (−70 to −40°C, see Fig. 2). The difference is that the cells lose further water when annealed at temperatures below −20°C and are capable of forming a more concentrated glass by loss of H_2O to ice.

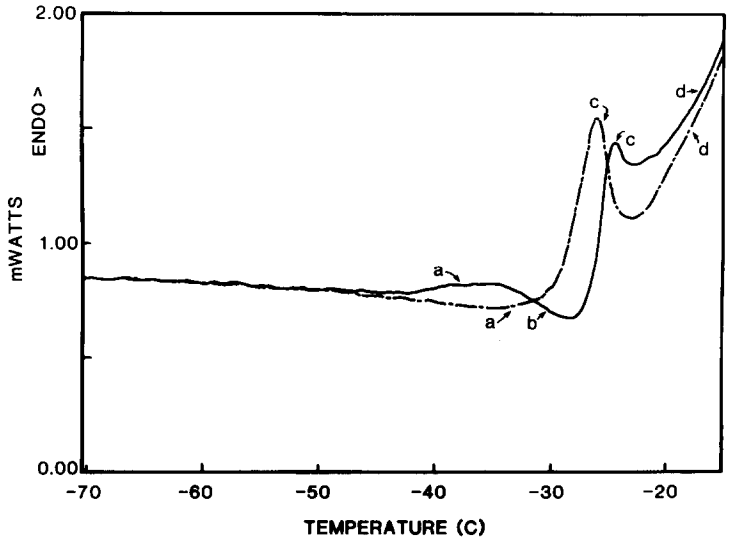


FIG. 8. A concentrated solution of 22.5% stachyose, 22.5% raffinose, 55% H_2O w/w was frozen to −160°C, warmed to −24°C, annealed 15 min, and then cooled at 100°C/min to −160°C. The solid curve represents warming after this treatment. The dashed curve represents the same sample after being annealed at −32°C before recooling. The higher T_g of the dashed curve corresponds well to the slowly cooled cellular solutions, implying the latter's behavior is primarily that of a sugar–water interaction.

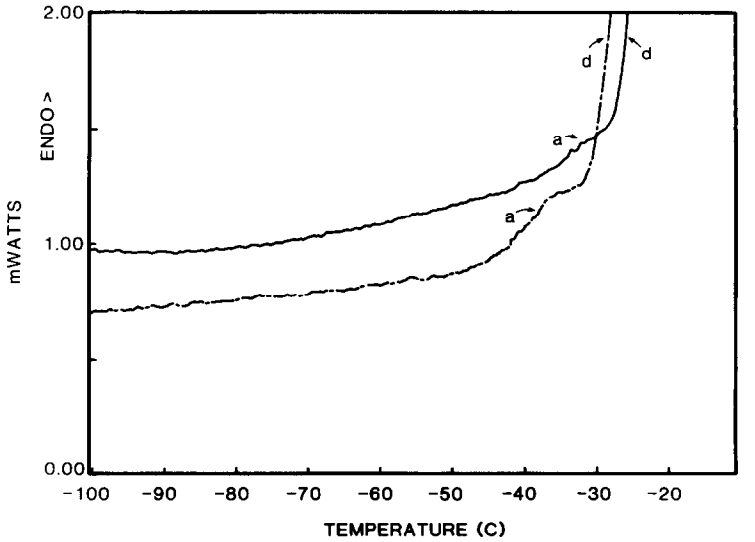


FIG. 9. The solid curve represents warming at 10°C/min of raffinose in water, annealed so that a limiting glass has formed. The event at *a* shows the onset of the glass transition, at *d* the final stage of the glass transition. The dashed curve shows the 10°C/min warming of a similarly treated solution in which human serum albumin was added 1:10 to the raffinose. The glass transition of the albumin–sugar solution is at least 10°C lower than pure raffinose.

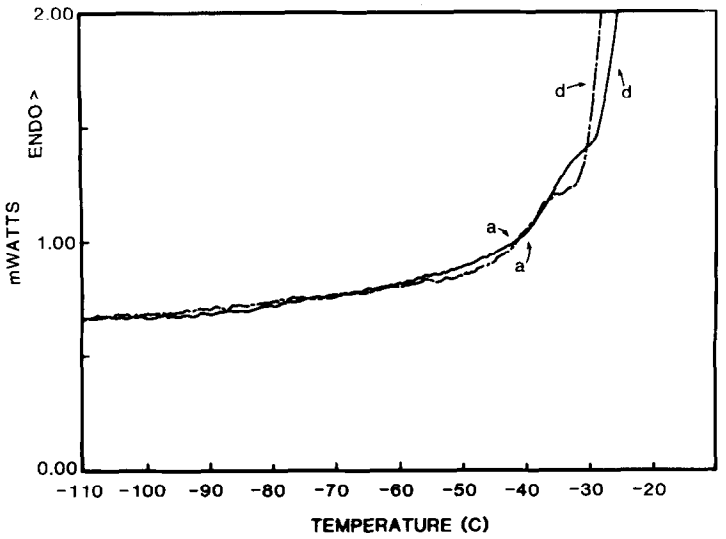


FIG. 10. A comparison of 1:5:5 human serum albumin:raffinose:stachyose, annealed to produce a limiting glass (solid curve), vs 1:10 human serum albumin:raffinose (dashed curve). Warming in both cases was 10°C/min and the increased complexity of the dual sugar solution seems to have shifted the onset of the glass transition, *a*, to a slightly lower temperature. Events labeled *d* are the final stage of the glass transition.

(Haworthia) was reported earlier by Siegel *et al.* (12), but no extensive body of evidence exists to indicate exactly what portion of very-low-temperature damage is due to long-term accumulation of free radicals. However, significance of the problem of free radical accumulation has been well established (2, 4, 14, 15, 16).

When we first considered what might be happening in the *Populus* cells that allows them to resist LN_2 temperatures, one of the alternative hypotheses to glass formation was the ability to withstand massive dehydration. This would be similar to the resurrection mosses (11), which can dry to 1% (w/w) H_2O without damage. It is clear from our records that that is not the strategy of *P. balsamifera*. In fact, repeated attempts to dry *Populus* at -6 and -20°C gave the same result: at total water contents less than 0.6 g H_2O/g dry wt, recovery upon thawing fell rapidly to zero. At -20°C, approximately 10% of the initial intracellular water remains in the fluid state. The rest is extracellular ice. Thus the 6- to 12-month storage time at -20 or -70°C seems inconsistent with the sensitivity of the wood to drying. Our only explanation of this dilemma is that the cells remain dehydrated as they thaw when previously dried at subzero temperatures. Drying takes weeks at such temperatures so rehydration during thawing will be very low as well. Thus even 3°C/hr warming on ice would cause the cells to warm in the plasmolyzed state. This appears to be quite damaging.

These results are significantly different from those reported by Krasavtsev and his colleagues (6, 18). They freeze dried quench-hardy *Betula verrucosa* (birch) at -10°C to 8.5% H_2O (percentage dry wt) and found no damage upon rehydration. To compare these results to ours, we proceed as follows. We note from the Russian data that 0.32 g/g dry wt water remains unfrozen at -10°C under normal conditions. The initial intracellular water content was about 1.0 g/g dry wt.

Thus 70% of cellular water has been removed from the cell to ice. After drying only 8.5% of the initial water remains, so all the remaining water must be intracel-

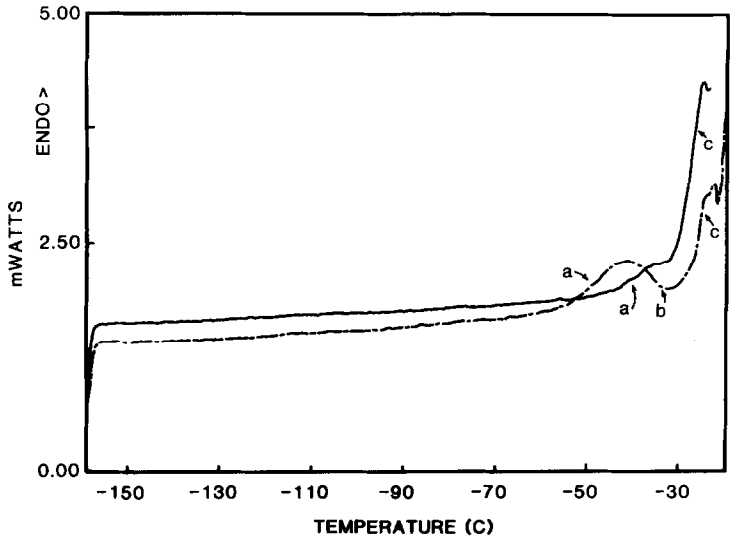


FIG. 11. This figure illustrates the need to anneal the sugar solutions in the high-energy state collapse–devitrification temperature range to achieve a maximal glass transition. The solution was 10:1 raffinose:human serum albumin. The dashed curve represents 10°C/min warming of the solution after cooling to −160°C, rewarming to −20°C for 15 min, and then recooling at 100°C/min to −160°C. *a* is the beginning of the glass transition, *b* is high energy state collapse and devitrification, *c* is the beginning of the melting of ice. The solid curve represents the same sequence of events but the annealing was done at −28°C, in the exothermic region. Note that *a*, the beginning of the glass melt, now occurs at a higher temperature, but *c* has moved to a lower temperature relative to the sample annealed at −20°C. This implies that the bulk of the energy in the exotherm came from devitrification, as opposed to high-energy-state collapse in the glass itself. If high-energy-state collapse were the major contributing factor, the enthalpic overshoot at the end of the glassy melt would occur at a temperature higher than the peak of the glass transition in the sample annealed at a higher temperature. Nevertheless, the increase in overshoot size (as in Fig. 8) after annealing in the exothermic region implies that both processes are at work. It may seem puzzling that devitrification would occur in a solution already containing large quantities of ice. This can be explained by homogeneous nucleation in the fluid layers between the ice crystals during cooling at temperatures above T_g but well below the temperature of maximum crystal ice growth rate. This effect is probably exacerbated by solute gradients in the glassified regions between the ice crystals. The wood does not seem to display this instability.

lular. If the *Populus*, with about 25% initial intracellular solute mass, were to dry down to 8.5% of the initial H_2O that would be equivalent to a 75% w/w intracellular solution. This can be compared to the intracellular solution composition at −20°C wherein the estimated composition is about 72% solute. The latter material has a maximum glass transition between −40 and −50°C. Thus our conclusion is that even if the birch is a somewhat better glass former than the poplar, it is unlikely that the tissue could resist desiccation to 8.5% H_2O content by intracellular glass formation at −10°C. This is important because it means that we cannot assume that the birch uses a different method of cryoprotection because its desiccation resistance is so much better than the poplar's. Tumanov and Krasavtsev (18) also reported that water continued to freeze out between −30 and −100°C in the hardy birch. This amounted to 5% of the dry weight of the tissue. If this were the poplar that would mean that about 50% of the remaining cellular water at −20°C is freezable. However, they measured 0.23 g/g dry wt unfrozen water in the birch at −20°C, whereas we measured 0.1

g unfrozen H_2O/g dry wt in the poplar. Thus the birch would seem to have a significantly higher intracellular H_2O content than the poplar at high subzero temperatures. However, the final inert intracellular H_2O content at $-100°C$ is about 7% of dry weight in the birch and must be almost the same in the poplar. In light of these facts, we should examine the glass-forming behavior of the birch to determine whether it correlates with the limits of the birch's quench hardiness.

CONCLUSION

We have demonstrated that at least one very-cold-resistant plant forms high-temperature intracellular glasses as a protection from intracellular ice formation at temperatures below $-20°C$. This strategy also limits intracellular water loss to significantly less than that experienced by completely (and truly) drought-hardy plants such as some of the mosses. We have provided evidence that the central player in the drama is intracellular sugar, but we feel that preliminary evidence favors the view that protein–sugar complexes are the primary glass-forming solutes. We note that some siberian trees appear to possess much greater subzero drought hardiness than *P. balsamifera*. It is still unclear how they accomplish this and how that relates to their glass-forming characteristics. Finally, I wish to stress our view that liquid microdomains in a largely glassified intracellular solution pose difficult barriers to long-term cryopreservation at temperature $> -100°C$. This may be largely due to unrestrained free radical activity in these fluid regions even at low temperatures.

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REFERENCES

1. Angell, C. A., and Tucker, J. C. Heat capacity changes in glass-forming aqueous solutions and the glass transition in vitreous water. *J. Phys. Chem.* **84**, 268–272 (1980).
2. Armitage, W. J., Matthes, G., and Pegg, D. E. Seleno-DL-methionine reduces freezing injury in hearts protected with ethanediol. *Cryobiology* **18**, 370–377 (1981).
3. Fege, A. S., and Brown, G. N. Carbohydrate distribution in dormant populus shoots and hardwood cuttings. *Forest Sci.* **30**(4) 999–1010 (1984).
4. Heckly, R. J., and Quay, J. Adventitious chemistry at reduced water activities: Free radicals and polyhydroxy agents. *Cryobiology* **20**, 613–624 (1983).
5. Hirsh, A. G., Williams, R. J., and Meryman, H. T. A novel method of natural cryoprotection. *Plant Physiol.* **79**, 41–56 (1985).
6. Krasavtsev, O. A. Frost hardening of woody plants at temperatures below zero. *In* "Cellular Injury and Resistance in Freeezing Organisms" (E. Asalina, Ed.) Proceedings, International Conference on Low Temperature Science II. Conference on Cryobiology Proceedings, Vol. II. Inst. of Low Temp. Science, Sapporo, Japan, 1967.
7. Parker, J. Seasonal changes in cold resistance and free sugars of some hardwood tree barks. *Forest Sci.* **8**, 255–262 (1962).
8. Sagisaka, S. Injuries of cold acclimatized poplar twigs resulting from enzyme inactivation and substrate depression during frozen storage at ambient temperature for a long period. *Plant Cell Physiol.* **26**(6), 1135–1145 (1985).
9. Sakai, A. The effect of temperatures on the maintenance of the frost hardiness. *Low Temp. Sci. Ser. B* **14**, 1–6 (1956).
10. Sakai, A., and Yoshida, S. The role of sugar and related compounds in variations of freezing resistance. *Cryobiology* **5**, 160–174 (1968).
11. Schonbeck, M. W., and Bewley, J. D. Responses of the moss tortula ruralis to desiccation treatments. I. Effects of minimum water content and rates of dehydration and rehydration. *Canad. J. Bot.* **59**, 2698–2706 (1981).
12. Siegel, S. M., Speitel, T., and Stoecker, R. Life in earth extreme environments: A study of cryobiotic potentialities. *Cryobiology* **6**, 160–181 (1969).
13. Siminovitch, D., Rheaume, B., Pomeroy, K., and Lepage, M. Phospholipid, protein, and nucleic acid increases in protoplasm and membrane

structures associated with development of extreme freezing resistance in black locust tree cells. *Cryobiology* **5**, 202–225 (1968).
14. Swartz, H. M. Effect of oxygen on freezing damage: II. Physical-chemical effects. *Cryobiology* **8**, 255–264 (1971).
15. Swartz, H. M. Effect of oxygen on freezing damage: III Modification by β-mercaptoethylamine. *Cryobiology* **8**, 543–549 (1971).
16. Symons, M. C. R. Radiation processes in frozen aqueous systems. *Ultramicroscopy* **10**, 97–104 (1982).
17. Takahashi, T., and Hirsh, A. Calorimetric studies of the state of water in deeply frozen human monocytes. *Biophy. J.* **47**, 373–380 (1985).
18. Tumanov, I. I., and Krasavtsev, O. A. Hardening of northern woody plants by temperatures below zero. *Fiziol. Rast.* **6**, 663–673 (1959).