

Cold Hardiness and Deep Supercooling in Xylem of Shagbark Hickory¹

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ABSTRACT

Differential thermal analysis, differential scanning calorimetry, pulsed nuclear magnetic resonance spectroscopy, and low temperature microscopy are utilized to investigate low temperature freezing points or exotherms which occur near -40°C in the xylem of cold-acclimated shagbark hickory (*Carya ovata* L.). Experiments using these methods demonstrate that the low temperature exotherm results from the freezing of cellular water in a manner predicted for supercooled dilute aqueous solutions. Heat release on freezing, nuclear magnetic resonance relaxation times, and freezing and thawing curves for hickory twigs all point to a supercooled fraction in the xylem at subfreezing temperatures. Calorimetric and low temperature microscopic analyses indicate that freezing occurs intracellularly in the xylem ray parenchyma. The supercooled fraction is found to be extremely stable, even at temperatures only slightly above the homogeneous nucleation temperature for water (-38°C). Xylem water is also observed to be resistant to dehydration when exposed to 80% relative humidity at 20°C . D_2O exchange experiments find that only a weak kinetic barrier to water transport exists in the xylem rays of shagbark hickory.

Despite the overwhelming evidence which supports the concept that most frost-resistant plant parts survive low temperature in a frozen condition (16, 27), a few persistent examples of freezing resistance of plant parts by avoidance of freezing have been noted, and recent studies indicate that deep supercooling may play a more important role in winter survival than originally presumed.

Among the first to propose deep supercooling as a freezing injury-avoidance mechanism in plants were Tumanov and Kravtsev (25). In calorimetric studies, they found low temperature exotherms associated with injury in oak, birch, fir, and pine branches. Later, Tumanov *et al.* (26) recorded freezing points between -20 and -30°C in cherry flower buds. Quamme *et al.* (20) have found exotherms near -40°C in midwinter apple twigs which cause injury to the xylem ray parenchyma. In terms of plant materials investigated, two of the most extensive studies of low temperature exotherms have been made by Graham (12) and by George *et al.* (8). In a study of flower buds from a

number of *Rhododendron* varieties, Graham (12) reported that floral primordia did not freeze near -2°C as did the bud scales and stem axis to which they are attached, but in hardy varieties, they froze at temperatures as low as -43°C . Experiments (7, 9) utilizing DTA,⁴ NMR, DSC, and low temperature microscopy have shown that primordia are in fact supercooled at low temperature. Low temperature freezing points have since been observed by Quamme (19) in floral primordia of several *Prunus* species. To examine the possibility of widespread existence of low temperature exotherms in xylem, George *et al.* (8) in 1974 performed DTA experiments during midwinter on the wood of 49 woody species native to North America. Of the 49 species, 25 had freezing exotherms near -40°C which correlated with xylem injury. Each of the species with a low temperature exotherm has a northern range distribution which corresponds to the region in North America where environmental temperatures below -40°C are not commonly observed. Maximum hardiness reported in plant parts of species having low temperature exotherms is usually near -40°C (8, 20). This limit has physical significance since it is in the range of homogeneous nucleation temperatures for aqueous solutions of less than 2 molar concentration (22). Recent work by Burke *et al.* (3) on killing low temperature exotherms in the xylem of shagbark hickory supports the concept of simple supercooling of cellular water in the ray parenchyma to its homogeneous nucleation temperature. Work reported below presents results of extensive analysis of low temperature exotherms in shagbark hickory utilizing DTA, NMR, DSC, and low temperature microscopy.

MATERIALS AND METHODS

Physical analysis of low temperature exotherms in the xylem of shagbark hickory twigs was carried out from September 1974 to August 1975. Certain experiments performed below required analysis on twigs without low temperature exotherms. For these experiments, cold-acclimated red osier dogwood stems were used.

Plant Materials. Stems were collected from trees of shagbark hickory (*Carya ovata* [Mill.] K. Koch) on the St. Paul campus of the University of Minnesota or at the University of Minnesota Landscape Arboretum near Excelsior. One large collection of fully hardy twigs made at the arboretum on February 24, 1975, was stored at -10°C . These materials were used for specific tests noted below which required fully cold-acclimated materials. All other hickory twigs collected were tested immediately or stored at 5°C for a period less than 2 days. Red osier dogwood (*Cornus stolonifera* Michx.) stems were collected in mid-February from

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⁴ Abbreviations: DTA: differential thermal analysis; NMR: nuclear magnetic resonance; DSC: differential scanning calorimetry; T_{exo} : temperature of killing exotherm; ΔQ : heat release.

plantings on the St. Paul campus. These twigs were stored at -10 C until testing.

Seasonal Variation in Exotherm Temperature. Shagbark hickory stem sections were collected and evaluated by DTA for low temperature exotherm position throughout the growing and dormant season. Latest season's growth was tested over the entire period, and 2nd and 3rd year stems were tested from February to July, 1975. Stem sections were cut approximately 1.5 cm in length, and the bark was removed. Sections were placed in a small container of aluminum foil and attached to one side of a differential thermocouple so the thermocouple touched the twig. A similar empty container of aluminum foil was attached to the opposite (reference) side of the differential thermocouple. Ambient temperature of the reference side was monitored by a separate thermocouple. The DTA system was essentially that of George *et al.* (9). The cooling rate was $1.0 \pm 0.2\text{ C/min}$. The characteristic temperature of the killing exotherm (T_{exo}) was evaluated by estimating the area of the DTA freezing curve and then dividing by 2 to find the center of the exotherm. The rationale for using this method to determine T_{exo} is discussed below.

DTA and pulsed NMR analysis of both freezing and thawing curves were performed at selected times during the test period. Pulsed NMR experiments were performed on stem sections with bark removed at 20 MHz on a Bruker M-20 pulsed spectrometer with programmable temperature controller. Freezing of tissue water was monitored by recording the free induction decay after the second and succeeding pulses of a 90 degree pulse series, each pulse separated by 1 sec. The free induction decay was measured 6 μsec after the pulse center. After testing, twigs were dried in a vacuum oven at 70 C, and the signal from the dry sample was measured. Correction of the NMR signal for ice and dry sample was made as described by Burke *et al.* (3). Correction for the Boltzmann temperature effect was made by standardizing all measurements to 0 C. Cooling and thawing rate for both DTA and NMR was $1.0 \pm 0.2\text{ C/min}$.

Low Temperature Microscopy. Microscopy was carried out on a Wild M20 microscope with a low temperature stage. The low temperature stage was constructed by placing thin flat Dewars above and below the specimen to be observed to prevent condensation at subfreezing temperatures. Dewars were formed by fusing two quartz microscope slides together and evacuating the air space between them. Thickness of one Dewar is approximately 3 mm. The top Dewar was separated from the lower Dewar by a rectangular plastic frame of 1 cm height. A port at either end of the frame allowed dry N_2 gas to be passed between the Dewars. Temperature of the N_2 gas was controlled automatically using the same system as noted for DTA. Hardy sections of approximately 0.1-mm thickness were mounted on glass coverslips in fluorocarbon liquid (FC-80, Minnesota Mining and Manufacturing Company). The coverslip was then attached to the underside of the Dewar closest to the objective lens of the microscope. To prevent desiccation of the sample by the dry gas, the coverslip was attached to the Dewar by placing a thin band of petroleum jelly around the coverslip edge and then pressing the coverslip lightly against the Dewar surface so as not to allow any direct connection to exist between the sample and dry N_2 gas. Cooling rate for microscopy experiments was $1.0 \pm 0.2\text{ C/min}$.

Exotherm Fine Structure. Small cross-sections of hickory xylem approximately 2 mm in height and 3 mm diameter were sealed in aluminum sample pans. Testing was performed on a Perkin-Elmer DSC-2 scanning calorimeter. Samples were frozen at 0.31 C/min on the highest sensitivity setting of the calorimeter (0.1 mc/sec). This analysis was performed on hardy twigs in mid-December 1974.

Exotherm Heat Release. Exotherm heat release (ΔQ) was measured by DSC for three individual stem sections. The cooling rate was 1.25 C/min and the exotherm temperature was noted.

The materials tested were from the twigs stored at -10 C . Preparation of stem sections for DSC was as described above. Fresh weights of each section were measured on a Cahn DTL electrobalance prior to sealing in the DSC sample pans. After testing, samples were dried in a vacuum oven for at least 2 days at 70 C to allow estimation of total tissue water content. Heat liberated/g water frozen in the exotherm ($\Delta Q/\text{g H}_2\text{O}$) was found by dividing the heat measured from DSC by the fraction of total tissue water freezing between -35 and -55 C . This fraction was determined from separate NMR analysis of unfrozen tissue water at these temperatures in similar hardy twigs collected February 1, 1975. NMR tests were conducted as described above. Results are compared with the predicted value for the heat of fusion (ΔH_{fusion}) of pure water at its homogeneous nucleation temperature (-38 C).

NMR Relaxation Times. T_1 , the spin-lattice relaxation time, and T_2 , the spin-spin relaxation time, of hardy xylem were measured by pulsed NMR using the method described by Burke *et al.* (2) for similar measurements on stems of red osier dogwood. Measurements were made at 0 C and -31.5 C before and after freezing to -75 C . Xylem tested was from a twig stored at -10 C .

Time Constants for Freezing. It was of interest to measure time constants for freezing (T_f) at temperatures above T_{exo} and to determine ice nucleation rates, J , in hardy hickory xylem. The mathematical details for determining nucleation rates in small droplets from isothermal experiments have been developed (28). Rasmussen and Loper (21) have shown that heat release per time (dq/dt) in isothermal experiments performed on a DSC-2 calorimeter is related to the nucleation rate by the simple relation

$$\ln \frac{dq}{dt} = JVt + (\text{constant})$$

where V equals average volume of a liquid droplet. The time constant for freezing (T_f) is then

$$T_f = \frac{1}{JV}$$

If the assumption is made that freezing at low temperature in hardy hickory wood is from nucleation of water in small volumes (*i.e.* xylem rays or ray cells), then in principle, the above experiment can be used. Hardy samples were prepared for DSC as noted above. The sample temperature was dropped at a cooling rate of 320 C/min from 0 C to one of a series of temperatures ranging from -42 to -46 C . Because of the slow freezing rate for hickory xylem exotherms above -42 C , higher temperature isothermal experiments were performed on the pulsed NMR.

Sample Desiccation. To determine the response of xylem water to desiccation above freezing, 2nd year twigs of hickory and latest seasons stems of red osier dogwood (no low temperature exotherm) were placed in humidity chambers at 20 C. Materials were from twigs stored at -10 C and the bark was removed. Glycerol and water were mixed in the proper proportions to give 40, 50, 60, 70, 80, 90, and 100% relative humidities (15). Chambers were open glass containers of 19 cm diameter and 10 cm height. Samples were placed in perforated aluminum cups and suspended on a wire screen over the solutions. Parafilm was placed over the open surface of the glass container and a glass plate covered the Parafilm. Samples were evaluated after 4, 9, and 15 days by DTA for freezing of water. Sample fresh weights were recorded and twigs were dried at 70 C for at least 2 days in a vacuum oven.

D₂O Exchange. Water exchange in shagbark hickory and red osier dogwood stems was compared by exchanging tissue H_2O for external D_2O vapor (D_2O of 99.7% purity, Aldrich Chemical Co., Inc.). Since the Bruker M-20 pulsed NMR spectrometer does not detect deuterium, it was possible to use NMR to

measure exchange in these experiments. The exchange was monitored by extrapolating the echo amplitude of a Carr-Purcell-Meiboom-Gill pulse sequence (5) to zero time. The extrapolated echo amplitude was measured periodically for 15 days and time constants for exchange (T_e) were calculated. Five ml of D_2O were placed in the bottom of an NMR tube of 9 mm inner diameter and 23 cm length. Second year hickory or 1st year dogwood stems were suspended over the D_2O on a thin glass rod. Materials used were from those stored at -10 C. The top of the NMR tube was sealed with Parafilm. D_2O exchange for hickory was carried out at 80 and 100% relative humidity on twigs without bark. Eighty per cent humidity was produced by mixing NaCl with D_2O in concentration determined from the International Critical Tables (15). For dogwood, D_2O exchange was performed at 100% relative humidity on a stem with the bark removed. After completion of final exchange measurements, DTA analysis was performed on the twigs to evaluate T_{exo} .

RESULTS AND DISCUSSION

Seasonal Variation in T_{exo} . Seasonal variation in T_{exo} is shown in Figure 1. The increase and decrease of xylem hardness are in agreement with similar DTA analysis by Quamme *et al.* (20) on low temperature exotherms in apple twigs. Second and 3rd year xylem tested between February and July, 1975 had exotherm temperatures within 4 C of the latest years' growth in all cases and no specific trends between different years' growth were observed. There was a difference between T_{exo} for 1974 and 1975 wood in late spring and early summer. When the twigs were actively growing (late May), T_{exo} for 1975 wood was near -10.1 C or in the range of supercooling for any twig which is cooled below 0 C and not seeded with ice crystals (16). In contrast, T_{exo} for 1974 wood was -18.9 C.

The freezing curve of shagbark hickory xylem as observed by pulsed NMR shows little change until the temperature range of the low temperature exotherm is approached (3). The NMR freezing curve displays the same seasonal variation as the DTA freezing curve, but provides additional information since it can be used to estimate the total tissue water unfrozen at subfreezing temperatures (2, 13). Figure 2 displays DTA and NMR freezing and thawing curves for a twig collected October 26, 1974. T_{exo} as determined by evaluating the temperature at one-half DTA peak height and correcting for heat of fusion and instrumental heat capacity is -31.6 C, while the thawing point is -1.2 C. This freezing and thawing behavior is not typical of a melting point depression or eutectic phenomenon which are equilibrium processes that have the same freezing and melting points, but charac-

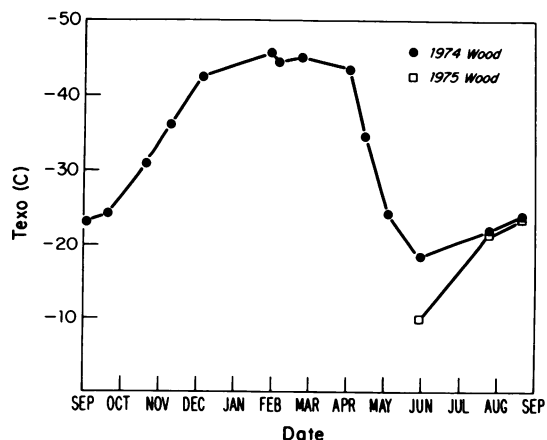


FIG. 1. Seasonal variation in xylem exotherm temperature (T_{exo}) for shagbark hickory.

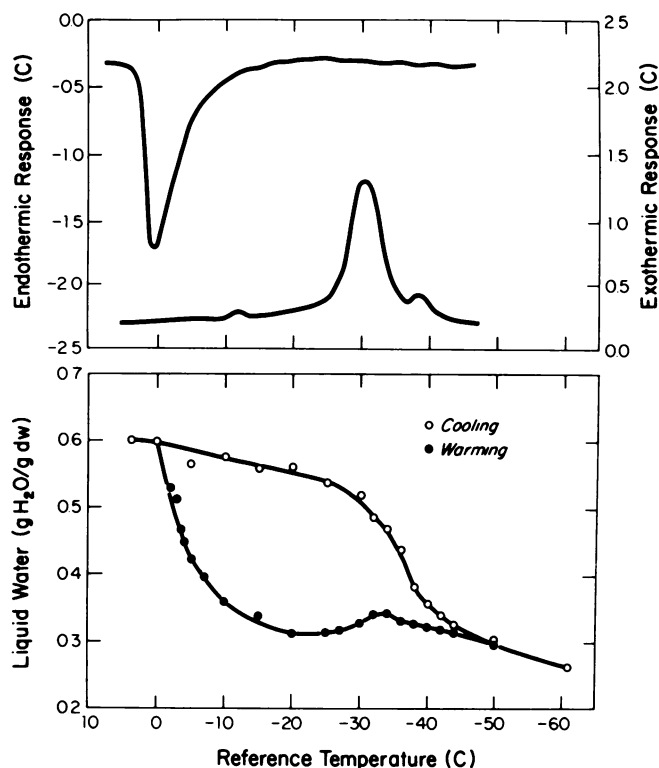


FIG. 2. DTA and NMR freezing and thawing curves for shagbark hickory xylem collected October 26, 1974.

teristic of a "metastable" or supercooled state (11). The NMR thawing curve is also seen to have a transition near -30 C. This may arise from the rapidity of freezing during the low temperature exotherm, which produces unstable ice. The unstable ice then recrystallizes to a more stable ice upon thawing. It was found that the same transition could be produced in dogwood xylem by immersing a twig directly in liquid N_2 , transferring the twig, unthawed, to the pulsed NMR at -60 C, and then thawing at 1.0 C/min. If a similar dogwood stem is cooled and thawed at 1.0 C/min, no transition on thawing is observed. It has been noted elsewhere that rapid cooling of plant tissues generally leads to intracellular freezing of supercooled cellular contents (16). The low temperature exotherm in hickory has been shown to exist in NMR experiments where a twig, stored in a freezer at -10 C for greater than 3 months, was transferred unthawed to the spectrometer and then cooled to below -50 C (3). This evidence indirectly supports the contention that the water freezing at low temperature in hickory xylem is indeed supercooled. It should be noted that other experiments performed by cooling and thawing the hickory twigs in small steps have not always found this thawing transition (3). Cooling and thawing in small steps may allow the unstable ice sufficient time to rearrange itself into more stable ice over a wide range of temperature. The concept of recrystallization has been discussed by Luyet (17) for aqueous solutions, muscle fibers, and onion epidermis which have been frozen at rapid cooling rates.

Low Temperature Microscopy. Cooling 0.1 -mm sections of cold-hardy xylem in fluorocarbon under the light microscope reveals that the predominant observable freezing takes place in the xylem ray parenchyma. Freezing is observed to occur at a single point in the tissue and then spread rapidly from cell to cell. The ray parenchyma are seen to darken suddenly as the tissue water freezes intracellularly and no barrier to ice growth from cell to cell in the xylem rays is observed. The microscopic observations are somewhat clouded by the fact that the cells freeze near -15 C and not near -40 C as found by DTA for

whole twigs. To investigate this difference, sections of approximately 0.1 mm were made and placed in fluorocarbon. These sections were then cooled and observed by DTA. The exotherm was found to have moved from near -40 C to approximately the same temperature as observed in microscopic analysis. That the exotherm changes position is not surprising since xylem rays in hardwoods can have heights much greater than 0.5 mm (18) and thin sectioning will inevitably destroy the integrity of the tissue. Although the microscopic observations of freezing must be viewed with this artifact in mind, they do demonstrate that the intact xylem ray structure is critical to the freezing process.

Exotherm Fine Structure. Microscopic observations showed that a xylem ray parenchyma cell was approximately 40×10^{-4} cm \times 20×10^{-4} cm \times 20×10^{-4} cm or 1.6×10^{-8} cm³. Heat release by the spontaneous freezing of water in a volume this size is roughly equal to the noise level of the DSC-2 calorimeter. Cooling at a rate of 0.31 C/min shows that the volumes of water freezing in the low temperature exotherm are of the order of 100 cells of 1.6×10^{-8} cm³ each (Fig. 3). Results of this experiment support the microscopic finding that freezing occurs in the xylem rays and no barrier exists from cell to cell in the ray. The whole ray freezes in a rapid manner after ice nucleation, and each ray or small groups of rays are nucleated independently of each other. These data also suggest that 50% of the xylem ray cells are killed at the midpoint of the low temperature exotherm, giving justification to the method used for determining T_{exo} . The finding that freezing in the wood occurs in small volumes of about 100 cells is used below for calculating ice nucleation rates (J).

Exotherm Heat Release. It was found from NMR freezing curves that 43% of total tissue water froze between -35 C and -55 C. Utilizing this figure, the $\Delta Q/g$ H₂O for the low temperature exotherm evaluated calorimetrically was found to equal 51 ± 3 cal/g H₂O for the three xylem sections tested. T_{exo} for the three sections was -46 ± 2 C. The predicted heat of fusion for supercooled water at its homogeneous nucleation temperature (-38 C) is 61.1 cal/g (23). $\Delta Q/g$ H₂O for the hickory low temperature exotherm is, therefore, in good agreement with this value. It should be noted here that T_{exo} for fully acclimated hickory xylem is approximately 6 to 8 C lower than the homogeneous nucleation temperature for pure water. Rasmussen and MacKenzie (22) have shown that the homogeneous nucleation temperature in aqueous solutions is lowered approximately twice the melting point depression. The melting point depression observed by DTA for hardy shagbark hickory is found to be near -2 C, so a 4 C lowering of T_{exo} can be predicted based on these data. Within the limits of the experiments described here for living wood, this would seem to account for most of the depression in T_{exo} compared with the homogeneous nucleation temper-

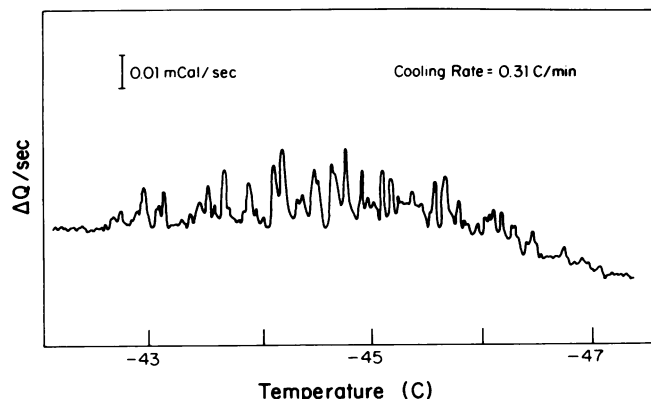


FIG. 3. Exotherm fine structure as revealed by calorimetric analysis. A large peak roughly equals 100 ray parenchyma cells (1.6×10^{-6} cm³) freezing spontaneously.

ature of pure water. Levitt (16) observes that higher plants always have melting point depressions from 0 to -4 C. Burke *et al.* (3) note that this would place an approximate limit of -38 to -47 C on low temperature exotherms in plant tissues. George *et al.* (8) found exotherms in this range for 25 fully cold-acclimated hardwoods of the Eastern deciduous forest. In other experiments (3), ethanol has been added to fully acclimated shagbark hickory wood depressing the melting point to -7 C while depressing T_{exo} to -52 C, a value close to the -54 C predicted by Rasmussen and MacKenzie's data for aqueous solutions (22). These analyses all lend credence to the concept of supercooling in the living xylem of hickory and also a large number of other hardwoods.

NMR Relaxation Times. Pulsed NMR measurements of T_1 and T_2 made on fully acclimated hickory stems at 0 C and -31.5 C before and after freezing to -75 C are given in Table I. Before cooling to -75 C, both T_1 and T_2 demonstrated a fast and slow component, whereas after cooling to -75 C, only T_1 could be resolved into two components. For T_1 , the slow component comprises about 70% of the tissue water, while for T_2 , the slow component accounts for approximately 60% of tissue water. Acclimated stems of red osier dogwood have been shown to display slow and fast components for both T_1 and T_2 (2). The reader is referred to Burke *et al.* (3) for a discussion of the reasons for multicomponent NMR relaxation times in biological systems. T_2 and the slow T_1 components measured at 0 C decrease after freezing to -75 C. Since xylem ray parenchyma cells are injured by freezing of water near -40 C, the decrease in relaxation times is an indication of injury to the parenchyma. Similar decreases in NMR relaxation times have been shown for azalea floral primordia which are injured by freezing of supercooled water at low temperatures (7, 9). Before cooling to -75 C, the slow components of both T_1 and T_2 demonstrated a weak temperature dependence as the wood was cooled from 0 to -31.5 C. Both components decreased to approximately 75% of their original value. T_1 relaxation time of D₂O has been shown to decrease by greater than 10-fold when supercooled to -35 C (14). In addition, the self-diffusion coefficient of liquid water decreases by over five times when supercooled to -31 C (10). This reduction in self-diffusion coefficient acts directly to reduce the T_2 relaxation time (5). The decreases in relaxation times for shagbark hickory xylem as a function of temperature are, therefore, expected. At -75 C, no T_2 relaxation times could be measured, indicating that both slow and fast water components at 0 and -31.5 C had frozen. The behavior of T_1 and T_2 relaxation times for water in hickory xylem at 0 and -31.5 C is similar to that found experimentally for pure supercooled water.

Time Constants for Freezing. The calculated nucleation rates

Table I. T_1 , spin-lattice relaxation time, and T_2 , spin-spin relaxation time, for shagbark hickory before and after freezing.

CONDITIONS	T_1		T_2	
	slow	fast	slow	fast
	msec			
BEFORE COOLING TO -75 C				
0 C	60	3	43	2
-31.5 C	40	3	35	2
AFTER COOLING TO -75 C				
0 C	33	4	7	
-31.5 C	34	3	4	

(J) and experimentally observed time constants for freezing (T_f) in hickory xylem are shown in Figure 4. Fletcher (6) has discussed the details of homogeneous nucleation in water. J is related to temperature by the relation

$$J = 10^{27} e^{-\Delta G^*/RT}$$

where ΔG^* is the total free energy change in forming an ice-like cluster of critical radius necessary to promote formation of macroscopic ice. ΔG^* is a function of $1/\Delta T^2$ where ΔT is the degree of supercooling. In tests reported here, sample temperatures were subtracted from the melting point depression of the tissue (-2 C) to arrive at a value for ΔT . The value of the pre-exponential factor is an indication of homogeneous or heterogeneous nucleation. If the value is greater than 10^{27} , homogeneous nucleation is assumed (6). For hickory xylem, the pre-exponential factor is $10^{13 \pm 1.4}$, indicating that nucleation is heterogeneous at temperatures above the homogeneous nucleation point. Rasmussen and MacKenzie (24) have reported a lower value for the pre-exponential factor in yeast cells. The interpretation of this result is that a rather inefficient heterogeneous nucleating surface is the main nucleating agent in the temperature region. However, as the sample temperature approaches -40 C, homo-

geneous nucleation becomes the predominant ice nucleation mechanism. The freezing observed for hickory xylem above -38 C is, therefore, intermediate between homogeneous nucleation for pure water and the weak heterogeneous nucleation found for yeast cells. T_f for freezing is seen to change by nearly seven orders of magnitude from -32 to -42 C for hickory xylem. As indicated above, the number of cells freezing per nucleation was approximately 100 ($1.6 \times 10^{-6} \text{ cm}^3$) and this volume was used in the calculations of J. The freezing rate at -32 C is very slow and the time required for substantial freezing of xylem water is much longer than the time any one hickory tree would be exposed to this low temperature in winter. The fraction of water unfrozen at low temperature is, therefore, in a relatively stable state at temperatures only a few degrees above -40 C.

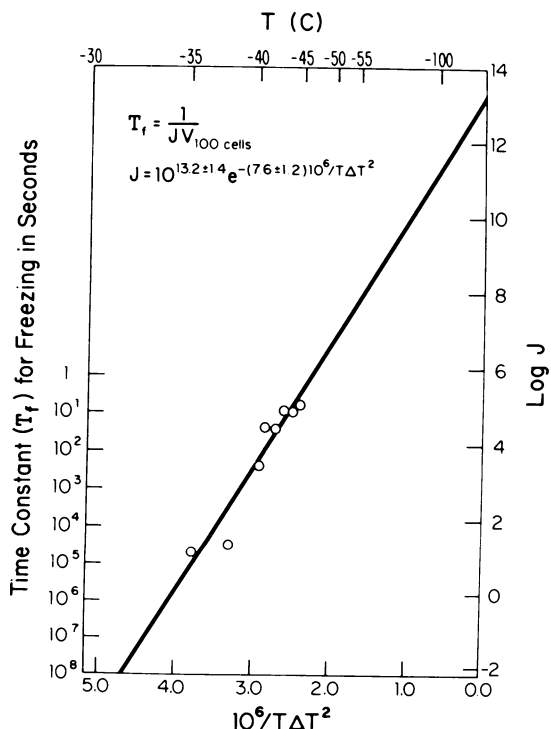


FIG. 4. Time constants for freezing (T_f) and nucleation rates (J) at temperatures approaching -46 C as determined by DSC and NMR. The relation between J and T_f is shown. $V_{100} \text{ cells} = 1.6 \times 10^{-6} \text{ cm}^3$.

Sample Desiccation. Desiccation of freezable water in the xylem of hickory and dogwood after 15 days is shown in Figure 5. Exotherm areas/g dry weight are divided by exotherm area/g dry weight at 100% relative humidity to determine relative values. Red osier dogwood is an extremely cold-hardy species which survives subfreezing temperatures as low as -196 C by tolerating extracellular ice formation and associated cellular dehydration (2). Dogwood xylem has a freezing exotherm near -2 C, but this freezing causes no injury to the living cells in cold-acclimated plants. At 90% relative humidity, essentially all freezable water, detectable by DTA, was removed from dogwood xylem after 4 days. For shagbark hickory, a relative humidity of 70% was required before relative exotherm area was reduced substantially. At 80% relative humidity, exotherm area decreased slightly after 4 days, but thereafter remained constant and exhibited little change even after 30 days. Exotherm temperature was also found to vary with relative humidity, approaching the limiting values shown in Figure 5 after 15 days. Decreases in tissue hydration allow the tissues to supercool to lower temperatures. Total tissue water/g dry weight for hickory has a slight plateau in the region of 80% relative humidity (Fig. 5). Dogwood is observed to dehydrate in a continuous fashion. The plateau region in hickory is apparently associated with the water freezing in the low temperature exotherm.

Supercooled xylem water must resist a desiccation stress from the water potential difference between supercooled water and ice. The ice may be outside of the plant or in other plant parts. Apple twigs which have low temperature exotherms in the xylem are observed to have ice in the bark at temperatures near -2 C (20). From data for water vapor pressure over ice and over supercooled water (D. H. Rasmussen, personal communication), it was possible to calculate desiccation energies produced by low temperature which are equivalent to those produced in the relative humidity chambers at 20 C. The results are indicated in Figure 5. The limit of low temperature desiccation in terms of reduction in relative exotherm area is near -40 C. It seems then

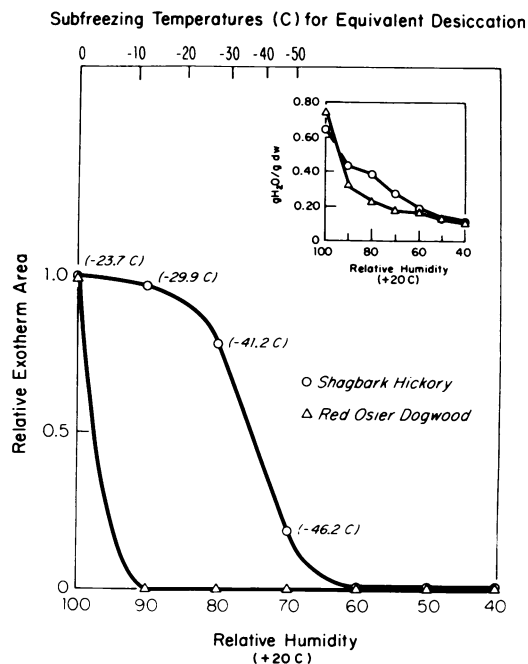


FIG. 5. Relative DTA exotherm areas and g tissue water/g dry weight ($\text{g H}_2\text{O/g dw}$) for shagbark hickory and red osier dogwood after 15 days exposure to various relative humidities at 20 C. Exotherm temperatures for hickory are given in parentheses. Dogwood has a high temperature exotherm near -8 C at 100%. Subfreezing temperatures required to give similar desiccation energies as produced in the relative humidity chambers are displayed on the top axis.

that the ability of cold-hardy hickory xylem to resist desiccation at 80% relative humidity is integrally related to the existence of stable supercooled systems near -40 C. If this were not the case, tissue dehydration would occur via sublimation from the supercooled cellular water to the ice in other plant parts or to ice outside the plant. Red osier dogwood, an extremely hardy species, which survives cellular dehydration during extracellular ice formation, does not resist dehydration.

D₂O Exchange. D₂O exchange experiments were performed to test whether supercooled water in xylem ray parenchyma is separated from ice elsewhere in the plant by a barrier which is impermeable to water transport. This would be an alternate explanation to the simple thermodynamic one which would be that the water potential of the cell sap is reduced to that of ice at subfreezing temperatures. Results of D₂O exchange experiments seem to discount the existence of an impermeable or kinetic barrier to water transport from the xylem rays (Table II). T_e values for xylem water of hickory at 80 and 100% relative humidities are of the same order of magnitude as T_e for dogwood. Dogwood, as shown above, has little capacity to resist tissue dehydration. Therefore, the geometry of the wood appears to be the important factor in D₂O exchange. In addition, time constants measured for exchange of H₂O at 20 C are much shorter than the lifetime of the supercooled state at temperatures above -35 C (Fig. 4) or for the xylem water observed in desiccation experiments at 80% relative humidity. T_e values reported here are similar to time constants observed for exchange of amide protons on folded groups in proteins with solvent water (4, 29). T_{exo} values for hickory xylem in the 80 and 100% D₂O experiments were -34.8 and -27.6 C, respectively. The homogeneous nucleation temperature for pure D₂O is -35 C (23).

CONCLUSION

Results of experiments reported here give substantial support to the contention that shagbark hickory contains a supercooled water fraction at subfreezing temperature (3). This fraction of water is seen to vary considerably in freezing point with seasonal changes. However, the exotherm is never found below -46 C or below the homogeneous nucleation temperatures predicted for aqueous solutions of less than 4 molar concentration (22). Heat release during the low temperature exotherm. NMR relaxation time behavior, and freezing and thawing curves for the cold-hardy twigs investigated all give results consistent with those expected for supercooled dilute aqueous solutions. Results of DSC and low temperature microscopy indicate the low temperature freezing in shagbark hickory occurs intracellularly in the xylem rays. Freezing time constants determine that the supercooled xylem water is stable at low temperatures for long periods

of time. Desiccation experiments reveal that hickory xylem can avoid dehydration for long periods at relative humidities near 80%. This dehydration resistance is necessary to allow maintenance of supercooled tissue water at temperatures approaching -40 C. Analysis of water exchange in hickory xylem suggests that no substantial barrier to water transport exists. This leads to the conclusion that the water potential of the xylem ray parenchyma cells is reduced by some means at subfreezing temperatures so ice outside the xylem rays and the cellular water can be in thermodynamic equilibrium. At -40 C, the water potential of supercooled cells would have to be lowered to about -400 bars in order for the cells to be in vapor equilibrium with ice.

These results can be understood in terms of a simple surface chemical effect, the "ink bottle" pore effect (1). Ink bottle pores are represented in Figure 6. The stability of the liquid to desiccation will depend on the radius of curvature of the meniscus according to the Kelvin equation (1). For example, with the highly curved meniscus at point A, the system is in equilibrium at 80% relative humidity; however, if the relative humidity is lowered even minutely, then due to evaporation, the meniscus is drawn through the capillary (point A with a curved meniscus) to the body of the "ink bottle" pore (point B with a flat meniscus). Because of the change from a curved to an almost flat meniscus and the resultant increase in vapor pressure predicted by the Kelvin equation, the remaining liquid in the pore evaporates. Such "ink bottle" pores are often used in accounting for desorption curves like those in Figure 5. The water potential of the pores depends on the radius of the largest capillary, about -400 bars for Figure 6. If more than one capillary is present per pore, only the one with the largest radius need be considered.

How cells of living wood could be placed under so great a hydrostatic tension as -400 bars is open to debate. Xylem in shagbark hickory can withstand compressions of this order (18), so possibly the structural integrity is present which could stand the tension. Such considerations as these suggest that the xylem ray parenchyma of cold-acclimated hickory are covered by a blanket of cellulose which has capillaries of the order of 100 Å diameter and not larger. In order that the cellular water remain under hydrostatic tension, nucleation of vapor cavitations must not occur. This absence of vapor cavitations may be related to the ability of the same solutions to supercool to the homogeneous nucleation temperature without ice nucleations. Certainly, partitioning of xylem parenchyma into isolated rays would prevent propagation of ice or a vapor cavitation and thus confine injury from a single nucleation to one ray. Even though these considerations are highly speculative and the exact anatomical and morphological details which allow supercooling are not known, it is evident that supercooling does play a major role in the freezing resistance of shagbark hickory.

Table II. Time constants for exchange (T_e) of tissue H₂O for external D₂O vapor in shagbark hickory and red osier dogwood as measured by pulsed NMR.

Species	Relative Humidity (D ₂ O Vapor)	T_e hr
	%	
SHAGBARK HICKORY (no bark)	100	135 ± 24
	80	126 ± 26
RED OSIER DOGWOOD (no bark)	100	96 ± 5

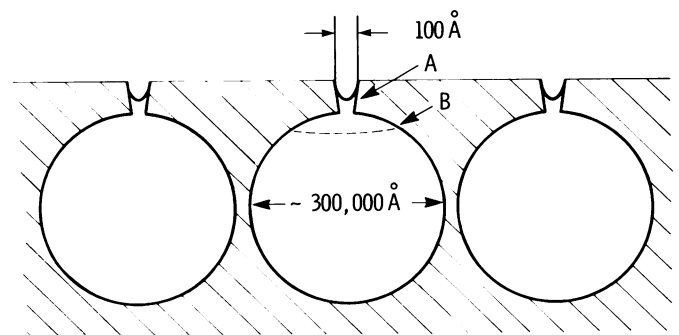


FIG. 6. "Ink bottle" pores having volumes approximately equal to 100 ray parenchyma cells. The relative humidity calculated for the meniscus at point A is 80% and at point B is slightly below 100%. Such pores would not dehydrate significantly until the relative humidity was dropped to below 80%.

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