

# Units of Freezing of Deep Supercooled Water in Woody Xylem<sup>1</sup>

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## ABSTRACT

The low temperature exotherms (LTE) of 1-year-old twigs of Haralson apple (*Malus pumila* Mill.), shagbark hickory (*Carya ovata* [Mill.] K. Koch), green ash (*Fraxinus pennsylvanica* Marsh), honey locust (*Gleditsia triacanthos* L.), American chestnut (*Castanea dentata* [Marsh] Borkh.), and red oak (*Quercus rubra* L.) were determined by differential thermal analysis (DTA). In one type of experiment freezing during a DTA experiment was halted for up to 2.5 hours after part of the supercooled water had frozen at temperatures between  $-25$  and  $-42$  C. Upon resumption of cooling the freezing started within 2 C of the stopping temperature. In a second type of experiment living and dead cells were microscopically observed in the same ray after partial freezing in the DTA apparatus. In another experiment, the LTE persisted even after tangential and radial sectioning of the twig to 0.13 millimeters. In a final experiment the LTE of a single multiseriate ray of red oak had the same shape as the LTE of wood with many uniseriate rays.

These experiments confirm that the deep supercooled water in woody xylem or pith freezes in numerous independent events over a span of as much as 20 C. The units which freeze in an event are single cells or small groups of cells. Ice grows very slowly if at all from these units, and water moves very slowly from unfrozen cells to frozen ones. Deep supercooling of ray parenchyma does not require an intact ray.

different than what is often observed in other tissues. In many experiments there is a delay between the freezing of successive cells (1, 11). For example, in one microscopic field of cucumber fruit after the first cell was nucleated, adjacent cells froze at intervals of 0.15–4.5 s averaging 0.85 s (2).

It is unclear under what conditions all cells will freeze when a single cell is nucleated in a body of contiguous cells. In most work, a tissue slice or epidermal strip is embedded in extracellular ice. This places most cells in contact with ice and encourages migration of water out of the cells to the ice (16). In experiments using rapid cooling, it is not possible to separate whether incremental freezing is achieved by the passage of time or by lower temperature. Thermal gradients created by microscopic illumination further complicate interpretation (2). In a *Tradescantia* hair, from which water probably cannot escape through the cuticle, once freezing commences all cells freeze in a sequential chain (1).

Because of the differences between the observed sequence of freezing in hickory rays and that in slices of other parenchymatous tissue the present study was initiated to further examine: (a) if the ray must be intact in order to deep supercool; (b) if the supercooled water throughout the xylem freezes as a single event delayed only by transient barriers, or if it freezes as many disconnected events; and (c) if all cells in the intact ray freeze simultaneously or if barriers to freezing exist within rays.

## MATERIALS AND METHODS

The four types of experiments involved in the study will be treated separately.

### EXPERIMENTS ON INTERRUPTED FREEZING

**Plant Material.** Periodically between October 27, 1978, and March 15, 1979, 1-year-old branches, 30–100 cm long, were removed from 3- to 6-m tall trees of Haralson apple (*Malus pumila* Mill.), shagbark hickory (*Carya ovata* [Mill.] K. Koch), honey locust (*Gleditsia triacanthos* L.), green ash (*Fraxinus pennsylvanica* Marsh), and American chestnut (*Castanea dentata* [Marsh.] Borkh.). The trees were located in St. Paul, Minnesota, and at the University of Minnesota Landscape Arboretum, Chaska. Following collection, all branches unless otherwise noted were stored dark, moist, and well aerated at  $+5$  C. The twigs were analyzed between early December 1978 and mid-April 1979.

**DTA<sup>3</sup>.** The temperature at which the twigs froze were determined by a DTA system similar to one previously described (13). Matching stem pieces 20–30 mm long and 4–7 mm in diameter were cut from the branch avoiding the tip and basal 60 mm. Unless otherwise stated, bark was removed and pith was left intact. The stem piece was wrapped in a small container made with aluminum foil. One junction of a two junction differential thermocouple touched the twig; the second junction (reference) was placed in a similarly sized aluminum container filled with

Ray cells of certain woody species avoid intracellular freezing by supercooling to temperatures as low as  $-47$  C (3, 6). These rays maintain the supercooled state in the presence of ice in the lumens of adjacent cells. According to George and Burke (5) the exterior surface of the ray is the barrier which prevents nucleation and the intact ray is needed to achieve the deepest supercooling. In its ability to resist inoculation from external ice, the ray resembles epidermis with a heavy cuticle (11) or Rhododendron buds adjacent to frozen stem tissue (7 cited in 14). George and Burke (5) also proposed that no barriers to ice propagation existed between adjacent cells within a ray. In a 0.1-mm section of shagbark hickory cooled at 1 C/min, freezing started at a single point and then spread rapidly from cell to cell throughout the ray. A similar pattern was observed in the cortex of *Catalpa* (16). Burke and George (5) cautioned, however, that the section froze at  $-15$  C whereas the intact stem froze at  $-40$  C. The suggestion that all the cells in a ray froze in rapid succession was supported by scanning calorimetry which showed many sharp exotherms between  $-42$  and  $-47$  C; the size of the larger peaks corresponding to the quantity of water in a single ray (5).

The observation that no barrier exists between cells in a ray is

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<sup>3</sup> Abbreviations: DTA: differential thermal analysis; LTE: low temperature exotherm.

paraffin. Both containers were inserted into wells in an aluminum block. A third independent thermocouple junction was attached directly to the block. A heating coil was wrapped around the block and then the entire block containing the samples and heating coil was wrapped with urethane insulation and placed in a freezer kept near  $-75^{\circ}\text{C}$ . The heating coil was programmed for a cooling rate of  $1 \pm 0.2^{\circ}\text{C}/\text{min}$ . About 20–30 min elapsed from the time the branch was removed from storage until cooling started in DTA.

The thermocouples provided two continuously recorded outputs (Fig. 1). The lower trace is the block temperature which parallels and is within  $1^{\circ}\text{C}$  of the sample temperature except during exotherms. The upper trace shows the difference in temperature between the reference and the twig. The dashed line drawn by the authors is the presumed baseline, when the twig and reference were at the same temperature. When the trace is above this baseline, heat of freezing is being released. The baseline characteristically drifts downward as the sample freezes, perhaps because of changes in the thermal capacity of the twig as water freezes. In Figure 1 the first exotherm began at  $-8^{\circ}\text{C}$ ; it results from the heat released as supercooled extracellular water freezes. The next exotherm began at  $-22.6^{\circ}\text{C}$  and ended below  $-40^{\circ}\text{C}$ . It is the LTE from the heat released when deep supercooled water freezes.

**Treatments.** The treatments were designed to see if different portions of the deep supercooled water nucleated at different temperatures. Each experiment compared a control twig section with a matching treated section. The control section was continuously cooled from ambient temperature to  $-50^{\circ}\text{C}$  at  $1^{\circ}\text{C}/\text{min}$ . The treated section was cooled until some temperature lower than that at which the LTE started but above that at which it finished. Then cooling was stopped and that temperature or a slightly

higher one was maintained for 0–2 h. The sample was then warmed by  $7\text{--}15^{\circ}\text{C}$  at  $1^{\circ}\text{C}/\text{min}$  after which cooling at  $1^{\circ}\text{C}/\text{min}$  to  $-50^{\circ}\text{C}$  was immediately resumed. Table I presents the treatments, and the lower trace in Figure 2 presents one temperature sequence.

For at least two samples of hickory, chestnut and honey locust, DTA analyses were done for pith only, xylem only, and both pith and xylem all from the same branch. The pith was removed with a drill, and about 10% of the pith was left with the xylem. The xylem was removed with a razor and the residual pith sample was up to 8% xylem and ray parenchyma by volume.

#### EXPERIMENTS ON SURGICAL DISRUPTION OF RAYS

On November 18, 1978, 1-year-old branches of red oak (*Quercus rubra* L.), green ash, honey locust, and Haralson apple were collected and stored as described previously until the experiments in mid-February. Debarked matching samples, 25 mm long and 5–9 mm in diameter were cut from internodes and either hand sectioned in one of three ways or left as controls.

a. Control. The controls were intact stem sections or a segment from which one-third, one-half, or two-thirds of the original stem had been removed in a pie-shaped piece.

b. Radial. Samples initially the same size and shape as the control were cut along the stem radius into eight to 24 pieces (Fig. 3). Other samples were cut along the radius to a prescribed average thickness and the pith was omitted.

c. Tangential. Samples initially the same size and shape as the control were cut tangentially so that there were two or three interruptions of the radial axis (Fig. 3). Other samples were dissected to produce tangential sections of a prescribed average thickness.

d. Cross-section. Samples initially the same as controls were cut into cross sections as thin as 0.8 mm.

Particular details of treatments are given in Figure 3 and Table II. After sectioning, the samples were wrapped in aluminum foil containers and freezing points were determined by DTA with continuous cooling to  $-50^{\circ}\text{C}$  at  $1^{\circ}\text{C}/\text{min}$ . Treatments were compared for their influence on the temperatures at which the LTE started and peaked.

#### EXPERIMENTS ON EXOTHERMS IN A SINGLE RAY

On April 20, 1979, a disk of wood was taken 20 cm from the base of a 10-year-old red oak in Chaska, Minnesota. A 1-year-old branch was collected from the same tree. After storage for 2 days the disk was dissected to produce three samples of wood: one (0.5 mm tangentially  $\times$  9 mm axially  $\times$  22 mm radially) contained one multiseriate ray and no uniseriate rays; a second (1  $\times$  9  $\times$  25 mm) contained both a multi- and many uniseriate rays; and a third (0.5  $\times$  9  $\times$  22 mm) contained only uniseriate rays. These samples contained primarily 1978 and 1977 wood. Immediately after dissection the freezing temperatures were determined by DTA analysis with continuous cooling to  $-50^{\circ}\text{C}$  at  $1^{\circ}\text{C}/\text{min}$ . The 1-year-old branch was similarly cooled.

#### MICROSCOPIC EXAMINATIONS AFTER DTA

One-year-old branches of shagbark hickory and Haralson apple were collected on March 15, 1979, and October 27, 1978, respectively. They were stored as previously described until mid-April when analyzed in DTA. In some experiments twigs were cooled at  $1^{\circ}\text{C}/\text{min}$  to  $-60^{\circ}\text{C}$ . In other experiments four hickory and 10 apple twig pieces were cooled part way into the LTE. Cooling was then stopped and the samples were warmed at  $1^{\circ}\text{C}/\text{min}$  to  $5^{\circ}\text{C}$ . Following DTA, the stems still wrapped in aluminum were stored between 1 and 15 days at  $5^{\circ}\text{C}$ . On removal from storage each stem was hand sectioned radially to include only one intact uniseriate ray in a section. At this time control twigs, which had not been frozen in DTA, were removed from  $5^{\circ}\text{C}$  storage and similarly sectioned. Sections were immediately stained for 3–6 h in 1:10,000

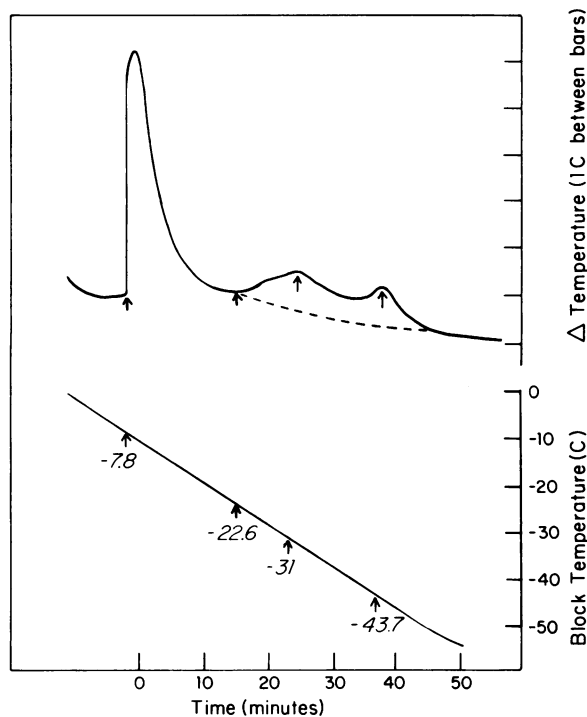


FIG. 1. DTA of Haralson apple (control twig A, Table I). Elapsed time is measured from start of the first exotherm. Lower trace: block temperature, which is within  $1^{\circ}\text{C}$  of the twig when there is no exotherm. Upper trace:  $\Delta$  temperature, was copied directly from the output and is the difference in temperature between the twig and the paraffin reference. Dash baseline is drawn by the authors to represent when the block and twig are presumed to have the same temperature. Baseline was given exaggerated convexity to show more clearly the start of the LTE.

Table I. Effect on the LTE After Cooling in DTA is Resumed

Initial cooling was stopped after the LTE had started but before it was complete. The control sample was continuously cooled without interruption.

Species	Twig <sup>a</sup>	Treatment				LTE of Treatment Sample		Control	
		Initial Stopping Temp.	→ Holding Temp.	→ High Temp.	→ Final Temp.	Starting Temp. Before Interruption	Starting Temp. After Cooling Resumed	LTE Peak Temp.	
		C							
Apple	A	-30	→ -28 (0.5 h) <sup>b</sup>	→ -17	→ -50	-24	-32	-31	
	B	-31	→ -31 (2 h)	→ -17	→ -50	-22	-32	-25	
	C	-33.6		→ -21	→ -50	-24	-34.4	-27	
	C	-27		→ -18	→ -50	-24	-28	-27	
	D	-42		→ -28	→ -50	-24	-42	-32	
Hickory	E	-25	→ -25 (2 h)	→ -18	→ -50	-22	-27	-25	
	F <sup>c</sup>	-45	→ -44 (0.5 h)	→ -34	→ -60	-44	-46	— <sup>d</sup>	
	Fx	-45	→ -44 (0.5 h)	→ -34	→ -60	-44	-46	—	
	Fp	-45	→ -44 (0.5 h)	→ -34	→ -60	-42	-45	—	
	Gb	-30	→ -28 (0.5 h)	→ -17	→ -60	-27	-31	-32	
	Gx	-30	→ -28 (0.5 h)	→ -17	→ -60	-26	-31	-34	
Chestnut	Gp	-30	→ -28 (0.5 h)	→ -17	→ -60	-23	-31	-33	
	H	-28	→ -27 (1 h)	→ -18	→ -50	-25	-29	—	
	Hx	-28	→ -27 (1 h)	→ -18	→ -50	-24	-30	—	
	Hp	-28	→ -27 (1 h)	→ -18	→ -50	-26	-30	—	
Honey locust	I	-28	→ -4 (0.5 h)	→ -18	→ -50	-24	-29	—	
	J	-29		→ -1	→ -50	-23	-29	—	
	Jx	-29		→ -1	→ -50	-23	-29	—	
Green ash	Jp	-29		→ -1	→ -50	-23	-29	—	
	K	-42		→ -28	→ -50	-28	-43	-35	

<sup>a</sup> Twigs with the same capital letters are matching samples; no notation means xylem and pith but no bark; p means pith only; x means xylem only; b means bark, xylem, and pith.

<sup>b</sup> (0.5 h) means sample was held at indicated temperature for 0.5 h.

<sup>c</sup> The F twig was collected while frozen, stored 30 days at -24 C and analyzed in DTA without being thawed.

<sup>d</sup> No peak observed.

(w/w) neutral red (17). Following staining the sections were placed in 1 molal CaCl<sub>2</sub> for 5 to 25 h. Examined sections contained a single uniseriate ray protected from damage on one side by a single layer of tracheids and on the other side by one to three layers of cells. Totally intact rays were rare, but many had at least one-third their length intact. Cells in several rays per treatment were observed for plasmolysis and vital staining with neutral red, both accepted techniques of viability (12).

## RESULTS AND DISCUSSION

### EXPERIMENTS ON INTERRUPTED FREEZING

Typical results are presented in Table I. They are similar for all five species. The control produced LTE's (Fig. 1) resembling those described elsewhere (6). In some treatments after the LTE was partially complete, cooling was temporarily halted and then resumed. Freezing reappeared within 2 C of the temperature at which cooling had been halted. For example, in sample A of Table I (also shown in Fig. 2), the LTE began at -24 and peaked near -30 C. At -30 C, cooling (Fig. 2) was halted and the block temperature was adjusted to -28 C. After 0.5 h it was raised to -17 C and then cooling was resumed. On recooling the LTE began at -31 C, *i.e.* 2 C lower than the stopping temperature. The interval between the cessation of cooling and the resumption of the LTE was about 1 h. The temperature at which the LTE of the control peaked indicates where within the LTE cooling was halted.

Upon adjustment of the temperature, the differential temperature trace ( $\Delta$  temperature) rose (Fig. 2). Such thermal transients, which appear as a rise here, are not caused by freezing but result whenever the cooling or warming rate of the DTA instrument is

altered. These thermal transients mask the freezing which might have occurred after cooling was stopped.

The results presented in Table I suggest that the LTE is the result of many ice nucleations each occurring at different temperatures ranging between the start and end of the LTE. Barriers must exist between the units nucleated at the different temperatures or ice would have propagated throughout the entire sample during the holding periods which lasted from 0.5 to 2 h. Further proof of barriers to ice propagation between units of water with different freezing points was provided by rough quantitative estimates showing that progressively less water froze upon resumption of cooling, when progressively more water was initially frozen.

In Figure 1 the flat, irregular shape of the LTE suggests the integration of many freezing events. The first exotherm in which all supercooled extracellular water froze simultaneously gave a nearly vertical line followed by a short period of freezing and then a trailing-off as heat dissipated from the sample (Fig. 1).

For hickory, chestnut, and honey locust, the LTE's were compared among a twig containing both xylem and pith and matching samples of isolated pith or isolated xylem. Pith, xylem, and intact twigs did not differ either in the shape or in the starting temperatures of the LTE (Table I, twigs F, G, H, and J). They also responded alike to interrupted freezing. Quamme *et al.* (14) found apple pith and xylem had similar LTE properties.

These results confirm by different techniques the conclusion of George and Burke (5) that the LTE of shagbark hickory is composed of numerous freezing events which occur at different temperatures. The experiments extend this conclusion to four additional species: apple, honey locust, chestnut, and green ash. They also show the result applies to both partially cold-hardened

Table II. Effect of Tangential and Radial Sectioning of 1-Year Branches of Red Oak, Green Ash, Honey Locust, and Haralson Apple upon the LTE

Species	Branch	Sectioning Treatment	LTE Temperatures		
			Starting	First Peak	-40 C <sup>d</sup> Peak
				C	
Red oak	A	Control: intact section	-30.9	-39	-44
	A	Radial: eight pieces <sup>a</sup>	-32.3	— <sup>b</sup>	-49
	A	Tangential: 12 pieces (3t × 4r) <sup>c</sup>	-32.3	—	-44
Red oak	B	Control: intact	-29.5	-35	-44
	B	Radial: 24 pieces	-30.9	—	-44
	B	Tangential: 24 pieces (3t × 8r)	-30.9	—	-44
Green ash	C	Control: half section	-17.2	-23	-40
	C	Radial: 16 pieces	-18.5	-25	-40
	C	Tangential: 16 pieces (2t × 8r)	-19.2	-26	-40
Green ash	D	Control: half section	-18.5	-23	-41
	D	Radial: 24 pieces	-20.6	-25	-41
	D	Tangential: 24 pieces (3t × 8r)	-20.6	-25	-41
Green ash	E	Control: half-section	-19.9	-24	-40
	E	Radial: numerous, 0.2 mm thick	-21.3	-24	-40
	E	Tangential: numerous, 0.2 mm thick	-22.6	-26	-40
Honey locust	F	Control: one-third section	-22.6	-37	-44
	F	Radial: 10 piece 0.5 mm	-22.6	—	-44
	F	Tangential: 10 piece 0.5 mm	-22.6	—	-44
Honey locust	FF	Radial: 20 pieces 0.25 mm	-29.5	—	-42
	FF	Tangential: 20 piece 0.25 mm	-29.5	—	-42
	FFF	Radial: 25 pieces 0.13 mm thick	-30.9	—	-42
	FFF	Tangential: 25 pieces 0.13 mm thick	-30.9	—	-41
Apple	H	Control: intact	-24	—	-43
	H	Radial: three pieces 1.2 mm thick	-25.3	—	-44
	H	Tangential: three pieces 1.2 mm thick	-25.3	—	-45

<sup>a</sup> Number of pieces based on complete 360° round section even if only portion of section placed in DTA.

<sup>b</sup> No peak observed.

<sup>c</sup> (3t × 4r) means twig was initially cut radially into four pie-shaped sections, and then each of these was cut tangentially into three pieces (Fig. 3).

<sup>d</sup> The -40 C peak refers to the peak which characteristically occurs near this temperature just before the end of the LTE.

(sample G) and totally cold-hardened (sample F) hickory, and to pith parenchyma as well as xylem parenchyma.

#### EXPERIMENTS ON SURGICAL DISRUPTION OF THE RAYS

The major finding of these experiments is that tangential cutting of wood did not importantly alter the LTE (Table II, Fig. 3). Since over 80% of the rays were cut twice in such modest surgical treatments as branch A (tangential) or branch D (tangential) (Table II), and all rays were cut numerous times by treatments such as F (tangential), it follows that intact rays are not required for normal supercooling to occur.

The tangential cuts did lower the starting temperature of the LTE; e.g. from -29.5 to -30.9 C in branch B (Fig. 3, Table II). The tangential cuts also altered the shape of the LTE so that less water froze just after the start of the LTE. In Figure 3, this is illustrated by the lower heat release between -31 and -35 C for the tangential compared to the control sample. However, the radial cut treatments which left most of the rays intact produced the same changes in LTE as tangential cuts. This indicates that the changes in the LTE were caused not by the cutting of the rays, but by the cutting of the twig. Cross-sectional slicing which reduced stems from 25 mm lengths to lengths between 0.8 and 12 mm similarly lowered the LTE. Regardless of the orientation of cutting, the LTE tended to start at lower temperatures as the xylem was sliced thinner (Table II). The reason for the temperature shift is unknown; desiccation may be responsible.

#### EXPERIMENTS ON LTE IN A SINGLE RAY

A single multiseriate ray or red oak produced an LTE with the same starting point and shape as a section of wood with only uniseriate rays (Fig. 4). The longitudinal parenchyma constituted too small a percentage of total living cell volume to influence the results.

#### MICROSCOPIC EXAMINATION AFTER DTA

Cells were considered alive when stained with neutral red. Most stained cells were also plasmolyzed. Unstained cells were considered dead.

In apple and hickory twigs which had not been frozen during DTA, almost all ray cells were stained. All ray cells were unstained and unplasmolyzed in stems frozen to -60 C. In stems frozen to a temperature between the start and conclusion of the LTE, some cells within a ray were alive and others dead (Fig. 5). Some intracellular ice existed in each twig for at least 20 min.

#### CONCLUSIONS

The experiments in interrupted freezing confirm for rays and extend to pith the previous conclusion that the xylem parenchyma freeze over a range of temperatures (5). In the experiments of George and Burke (5) the sample was continuously cooled and the effects of temperature could have been confounded with the need for time to propagate ice. In the 2-h holding treatment,

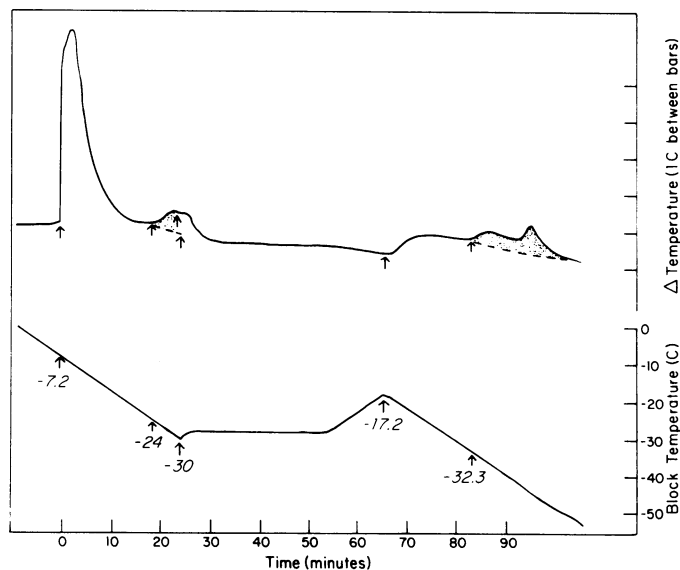


FIG. 2. DTA trace of Haralson apple treatment twig A in Table I. Details are as in Figure 1 but with the interrupted freezing cycle explained in the text. Shading indicates temperature rise from freezing of deep supercooled water. Unshaded areas above the baseline are thermal transients produced when cooling or heating rate is changed.

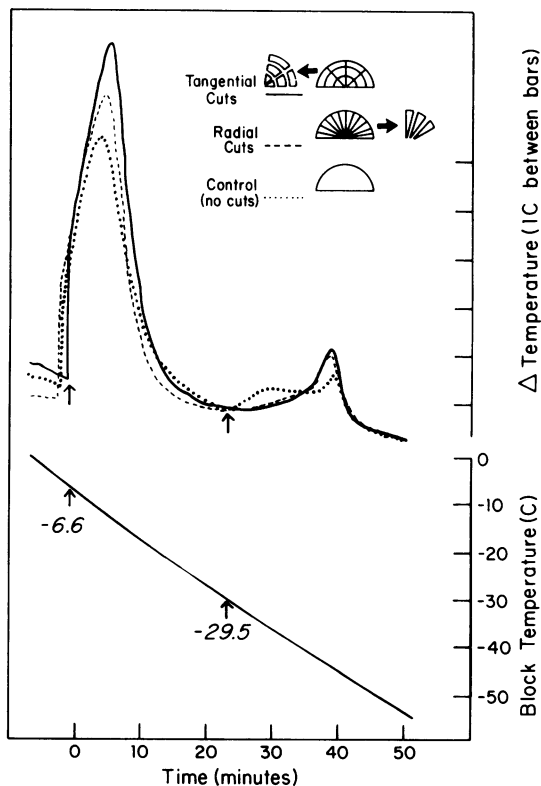


FIG. 3. Changes in the LTE produced by sectioning half cyclinders of red oak twigs 3 mm in radius and 25 mm long (branch B, Table II). Treatments are diagrammatically indicated in the inset and explained in Table I. Other details are as in Figure 1.

ample time was given for ice to spread.

The result as summarized below suggest that the intact ray is not prerequisite for deep supercooling. The summarized results also suggest that all of the cells in a ray do not freeze simultaneously but instead freeze as individuals or in small groups.

a. Cutting of the ray had minimal effect on the LTE of red oak,

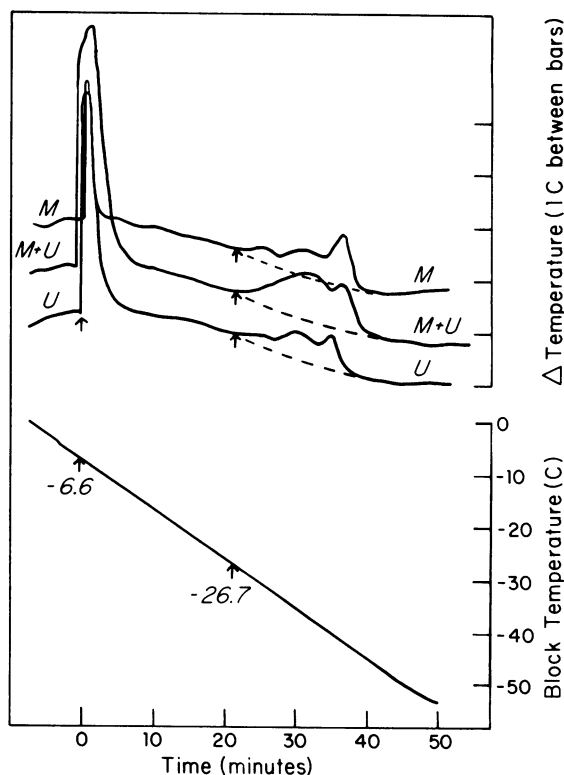


FIG. 4. LTE of a single multiseriate ray (M); a single multiseriate ray plus many uniseriate rays (M + U); and many uniseriate rays (U). Rays were from the most recent 2 years of wood of a 10-year-old red oak. Other details are as in Figure 1.

green ash, apple, and honey locust. Cutting would destroy any isolation barrier inherent in the outer boundary of an intact ray and would put extracellular ice in contact with the upper or lower surfaces of interior ray cells. The maintenance of the LTE in cut rays means any barrier preventing nucleation can be operative for the individual ray cell.

b. Microscopic examination of hickory and apple wood in which only part of the deep supercooled water had been frozen showed adjacent living and dead cells within a single ray (Fig. 5).

c. A slice of red oak wood containing one multiseriate ray and no uniseriate rays produced the same broad shape LTE as a slice with many uniseriate and no multiseriate rays (Fig. 4). Were the ray the smallest unit freezing, the single multiseriate ray would have produced a sharp spiked LTE.

d. The LTE of isolated pith is also composed of numerous freezing events which occur over the same span of temperatures as the xylem (Table I). Since the pith is completely parenchymatous, the barrier to ice propagation must be between parenchyma cells.

Although the results do not support the conclusion of George and Burke (5) that the ray is the unit of freezing, they do not refute their two lines of evidence.

The irregular pattern of freezing observed microscopically could be attributed either to barriers which prevent ice growth from cell to cell or to water migration from unfrozen cells to ice in frozen cells or to both. Rates of water movement within rays or between pith cells appear to be slow. Interrupting freezing at  $-31^{\circ}\text{C}$  for up to 2 h did not desiccate unfrozen cells enough to greatly alter their freezing temperatures. However, the conclusion is tentative since thermal transients prevented measurement of freezing during the holding period.

The barrier which prevented cell to cell propagation of ice is not known. For other tissues it has been proposed that the cell

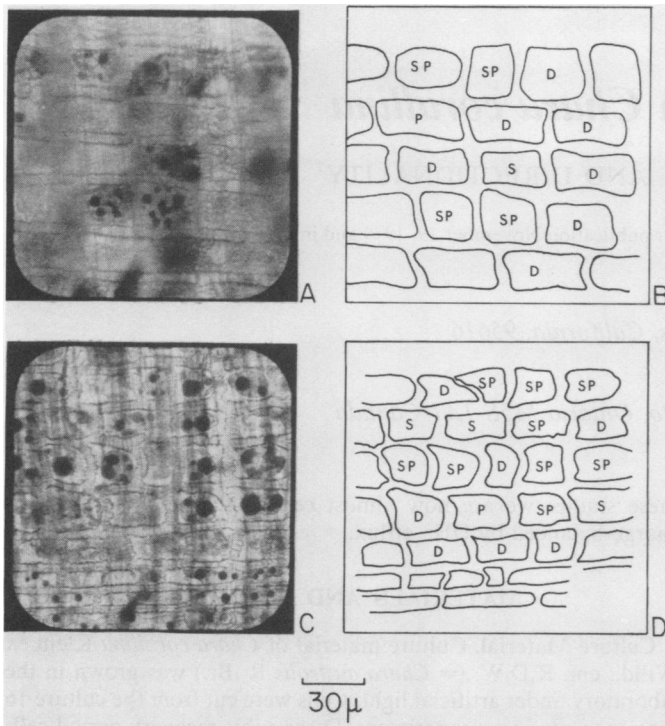


FIG. 5. A: radial view of uniseriate ray of shagbark hickory. LTE began at  $-28^{\circ}\text{C}$  and cooling in DTA was stopped at  $-28.8^{\circ}\text{C}$ . B: a tracing of Figure 5A showing the location of cell walls and classifying cells which are stained with neutral red (S), plasmolyzed (P), both (SP), neither stained nor plasmolyzed (D). C: radial view of uniseriate ray of Haralson apple. The stem had been cooled at  $-26^{\circ}\text{C}$  in DTA. LTE began at  $-23^{\circ}\text{C}$  and probably would have ended about  $-40^{\circ}\text{C}$ . D: a tracing of Figure 5C with notations explained previously. Photograph shows more unstained uniplasmolyzed cells (D) than were typical for the treatment.

membrane(s) provides the resistance (4, 8). Mazur (9, 10) concluded that below  $-5$  to  $-15^{\circ}\text{C}$ , the cell membrane loses its ability to prevent nucleation from extracellular ice and that the freezing of intracellular water generally requires the presence of extracellular ice. The membrane may not be the key barrier which prevents extracellular ice from nucleating pith and ray cells when

they deep supercool to below  $-30^{\circ}\text{C}$ . One evidence against a membrane barrier is that xylem still has an exotherm at  $-40^{\circ}\text{C}$  after being freeze-dried (13) or freeze killed. In xylem and pith which are deep supercooled there is no evidence that the heterogenous nucleation is from ice. Intracellular nucleators such as have been hypothesized for yeast (15) could be responsible for nucleation.

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