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Water Permeability and Cold Hardiness of Cortex Cells in *Cornus stolonifera* Michx.—A Preliminary Report[1]

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ABSTRACT

The relationship of freezing resistance to water permeability of cortex cells was studied in stems of red osier dogwood (*Cornus stolonifera* Michx.). Permeability was estimated by determining the diffusion flux of tritiated water from cortex slices previously equilibrated in tritiated water. Energy of activation and diffusion time comparisons of tritiated water flux from living cortex slices and slices killed by immersion in liquid N_2 verified that intact membranes of uninjured cortex cells limited water flux.

Water permeability of living phloem and cortical parenchyma cells increased during the initial (photoperiodically induced) phase of cold acclimation. This accompanied an increase in hardiness from −3 to −12 C. Little if any further increase in permeability was noted during subsequent acclimation to below −65 C.

Permeability measurements on nonhardy cortex samples yielded consistent results, but measurements on samples from hardy twigs were often difficult to reproduce. This unexplained variability precludes specific conclusions, but the tritiated water diffusion flux technique may provide an alternative to traditional plasmolytic techniques in studying water permeability in woody plant tissues.

Intracellular freezing occurs during cooling when water cannot move out of cells to extracellular ice nuclei rapidly enough to avoid supercooling and subsequent spontaneous ice nucleation within the protoplasm. Such intracellular ice formation causes extensive protoplasmic disruption and is always lethal to plant cells (7). In nature, damage from this type of freezing is thought to be the cause of sunscald injury to smooth barked tree species and of winter burn damage to foliage of certain evergreen species (15). Avoidance of intracellular ice formation is essential to the survival of plants, and the permeability of cells to water is likely to be a key factor in avoidance of intracellular freezing.

Most hypotheses of freezing death in plant cells are directed at explaining death caused by slow (extracellular) freezing. All presuppose avoidance of intracellular crystallization and assume that membranes are rather freely permeable to water. Tumanov and Krasavtsev (13), however, proposed that slow freezing death also involves intracellular crystallization; *i.e.*, when membranes restrict movement of water out of cells at low subfreezing temperatures and cause a "second supercooling point," intracellular freezing, and death.

Levitt and Scarth (6) demonstrated that the permeability of cells to an electrolyte (KNO_3) was closely correlated with hardiness. Water permeability showed a similar trend. Cells from cold-acclimated cabbage plants were about twice as permeable to water as those from nonacclimated plants and cells of hardy woody plants were more permeable to water than were cabbage cells (11).

In addition, Sakai (10) found that water permeability in mulberry cortical cells also increased with increasing hardiness from October to January, but that maximum permeability was retained until May even though considerable cold hardiness was lost during that period. This questions whether water permeability is directly related to hardiness, particularly during dehardening.

Although the seasonal changes in cell permeability and hardiness are documented in a few species, the specific timing of permeability changes in relation to environmental stimuli which induce or reduce cold hardiness has not been well characterized. This is due in part to the lack of a simple quantitative procedure for studying water permeability of plant cells in thick pieces of tissue. For individual cells or cell monolayers, the plasmolytic technique yields numerical values for the permeability constant for water (12).

Glinka and Reinhold (3) utilized an isotopic technique to measure water permeability of xylem parenchyma cells from root storage tissue of *Daucus carota* L. The present study was undertaken to evaluate the applicability of the isotopic, THO[5] diffusion, flux method for studying water permeability of woody plant cells, and was undertaken specifically to identify the nature and extent of permeability changes associated with the daylength- and temperature-induced phases of cold acclimation and dehardening of *Cornus stolonifera* cortex cells.

MATERIALS AND METHODS

Plant Culture. Clones of red osier dogwood (*Cornus stolonifera* Michx.) were propagated vegetatively and grown in a greenhouse under a 16-hr photoperiod (natural daylength ex-

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[5] Abbreviations: THO: tritiated water; SD: short day; LD: long day.

tended with mercury vapor lamps) at a day/night temperature regime of 20/15 C for 2 months or more. Uniform plants were then subjected to cold acclimating environments in a controlled environment chamber for various periods prior to hardiness and permeability measurements.

Environmental Treatments. The controlled environments used to acclimate plants and the stepwise acclimation of dogwood in response to short days and low temperature have been previously discussed in some detail (8, 14). Plants were exposed to the following sequence of environmental regimes: (*a*) a SD (8 hr) warm temperature (20/15 C, day/night) treatment for 4 weeks to induce the initial phase of acclimation to −10 C; (*b*) a SD (8 hr) cool temperature (15/5 C) treatment for 2 weeks to promote further acclimation to −20 C; and (*c*) exposure to −5 C for 1 hr, on successive nights, to trigger the frost-induced stage of acclimation to hardiness levels below −65 C; (*d*) in addition, potted plants were collected from the field during the winter, packed in snow-filled plastic bags and stored at −12 C. These plants were hardly below −75 C. Field plants were also brought into the laboratory and dehardened by thawing overnight at 5 C, then exposed to 21 C for various periods of time before permeability and hardiness testing.

Hardiness Measurements. The hardiness of excised twig sections was assessed as previously described (8) by controlled freezing tests on triplicate samples. However, only cortex tissue was evaluated. Hardiness was expressed as the lowest survival temperature; *i.e.*, the lowest test temperature which cortex tissues survived without injury. Cells were evaluated for injury under the microscope.

Permeability Measurements. Cortex strips, 1 to 2 mm wide and 10 to 15 mm long, were hand-sectioned with a razor blade and over a 1-hr period rinsed three times in 0.1 M sucrose. During this period the intercellular spaces became saturated with solution. The cortex strips consisted of phloem and cortical parenchyma cells at about a 30:70 ratio, respectively. Sucrose was found to be necessary in the equilibration solution after sectioning to prevent rupture of the plasmalemma upon its initial contact with water (8).

Excess moisture was blotted from the uniform tissue slices weighing 520 ± 30 mg. Slices were then immersed for 2 to 3 hr in 4 ml of aerated 0.1 M sucrose solution containing 20 μc/ml of THO at 15 C. Preliminary studies revealed that longer incubation times in THO (up to 7 hr) did not alter results. Additional slices were killed by freezing in liquid nitrogen (−196 C) immediately after sectioning, then handled the same as living samples to provide a basis for comparison of permeability measurements between treatments.

THO exchange between THO-equilibrated tissue slices and fresh 0.1 M sucrose solutions was measured by counting the radioactivity which diffused out of slices after successive incubations in 0.1 M sucrose. THO-equilibrated slices were placed in 10 × 21 mm vials containing 10 ml of 0.1 M sucrose and were continuously stirred with a stream of air bubbles. After exactly 1 or 2 min, depending upon the activity of the sample, the solution was decanted then replaced with 10 ml of fresh sucrose solution. This process was repeated 10 times and after each transfer, excess solution adhering to the glass was removed by suction.

One-milliliter aliquots from each of the 10 successive incubations were transferred to 10 ml of scintillation fluid (Aquasol, commercial preparation from New England Nuclear) and counted in a liquid scintillation spectrometer. The counts per minute from successive incubations were plotted on semilog paper from which the slope and half-time ($t_{1/2}$) rate constant were calculated. The $t_{1/2}$ rate constant was calculated from the slope of the slow diffusional component using the formula $t_{1/2}$ = 0.693/ln (slope) as outlined by Brey (1). Data from tender material were used for Arrhenius plots.

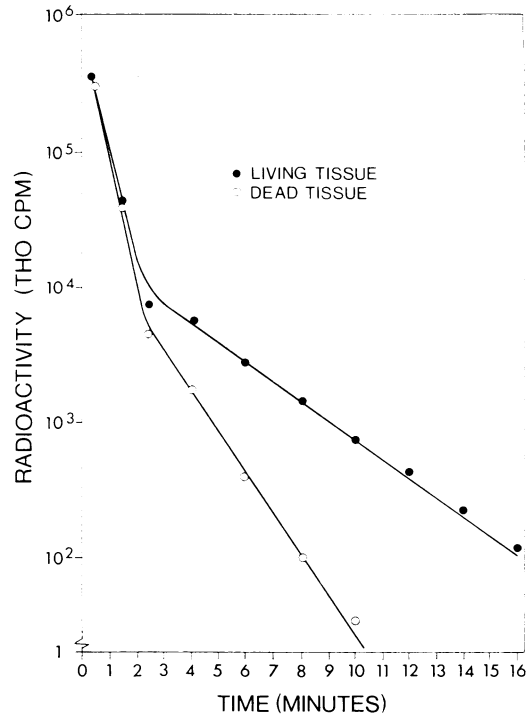

FIG. 1. Time-dependent loss of tritiated water from the contents of living and dead cortex cells of tender *Cornus stolonifera* stems hardy to −5 C. Measurements were made at 15 C.

Permeability Ratio. The length of the diffusion path influenced the half-time rate constant ($t_{1/2}$) of THO exchange. This gave rise to variability between comparisons of tender unhardened slices of cortex, which could be peeled readily from the stem at the cambium layer, and acclimated cortex samples, which adhered tightly to the xylem. Thus it was necessary to measure THO flux from killed samples in each treatment to correct for diffusional pathlength differences. Hence the $t_{1/2}$ rate constant from each hardy or tender living sample was divided by the $t_{1/2}$ rate constant from its corresponding killed mate. This was termed the permeability ratio. The lower the permeability ratio, the more permeable were the cell membranes to water.

RESULTS

Water Flux through Living Membranes. Kohn and Dainty (4) suggested that the "unstirred layer" of water and various extracellular resistances can reduce THO diffusion from cells and prevent accurate estimates of membrane water permeability. In beet root and artichoke discs they demonstrated that the extracellular resistances were rate-limiting, thereby preventing measurements of membrane permeability. In this study, preliminary experiments were conducted on living and dead cortex slices to establish the degree to which membranes limited water flux in living cortex slices of red osier dogwood.

The plots of THO water flux from dead and living cortex strips verified that membranes limited water flux. The plots from living and dead cortex slices were biphasic curves as shown in Figure 1. The fast component of the flux curve was due mainly to the THO adhering to the surface of the tissue and inside the vial. The straight line nature of the slower component from these curves represents a single first-order rate

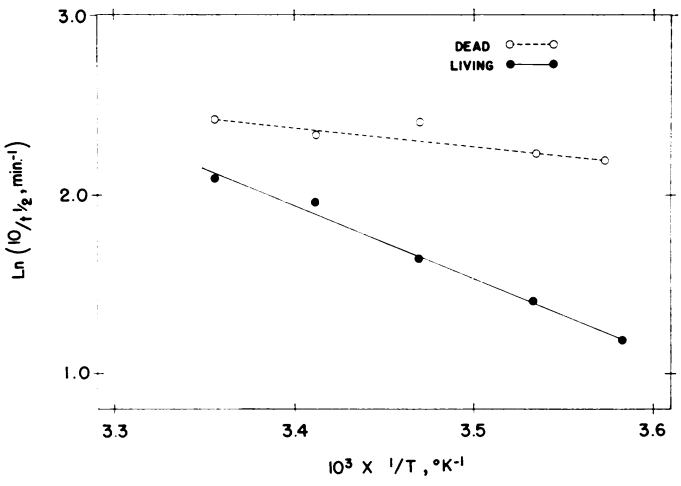

FIG. 2. Arrhenius plot of $t_{1/2}$ for tritiated water flux for living and dead cortex slices from tender *Cornus stolonifera* stems.

Table I. *Water Permeability and Hardiness of Living Cortex Cells in Cornus stolonifera Grown at Various Photoperiod and Temperature Regimes*

Data represent the mean and standard deviation of five plants in each treatment.

Treatment	Permeability Ratio ($t_{1/2}$ alive/$t_{1/2}$ dead)	Hardiness (Lowest Survival Temp.)
		C
Greenhouse control 20/15 C at 16-hr daylength	2.03 ± 0.11	−3
4 weeks at 20/15 C and 8-hr daylength	1.42 ± 0.11	−12
4 weeks at 20/15 C plus 2 weeks at at 15/5 C and 8-hr daylength	1.45 ± 0.13	−22

process. The slope of THO flux from dead strips where membranes had been destroyed was always greater, indicating faster diffusion rates. In Figure 1, for example, the $t_{1/2}$ for living cortex was 2.12 min, while for dead cortex, $t_{1/2}$ was 0.96 min. This gave a permeability ratio of 2.28.

The influence of membranes on water permeability was also evident in comparisons of the temperature dependence of THO flux in living and dead strips. This is apparent in Figure 2 where Arrhenius plots of the energy of activation for THO exchange are shown. Between 6 and 25 C, the relationship was linear. The energy of activation for THO exchange in dead samples was 2.14 kcal/mole which is similar to that reported in lobster nerve (9) where water diffuses through aqueous pores. In living cortex samples the energy of activation was 4-fold higher (8.05 kcal/mole), suggesting that membranes in living tissue influence water flux.

Water Permeability Changes during Acclimation. *Cornus stolonifera* acclimates effectively in a number of stages (14). In this study, water permeability and hardiness were compared with greenhouse-grown plants (20/15 C, day/night temperatures) under LD (16 hr), with plants grown 4 weeks under 8-hr photoperiod at 20/15 C and with plants grown an additional 2 weeks at 15/5 C.

During these treatments, after 4 weeks of 8-hr photoperiods, plants acclimated to −12 C. Exposure to cooler temperatures (15/5 C) for 2 additional weeks induced SD grown plants to harden to −22 C. These combinations of environment and the extent of hardiness attained were essentially comparable to what occurs in nature during autumn when dogwood plants begin to acclimate (to about −20 C) in response to short days and cool nights alternating with relatively warm days (14).

As shown in Table I, there was a marked increase in water permeability of the cortex of SD plants during 4 weeks at 20/15 C while the tissue acclimated to −12 C. The permeability ratio of SD-treated tissue was 1.42 compared to 2.03 for the LD tender (−3 C) greenhouse material. Although an additional 2 weeks at 15/5 C effectively increased the hardiness of SD plants to −22 C, further change in water permeability was not apparent.

Effect of Frost on Water Permeability. Exposures of SD-induced plants to noninjurious frost is known to induce a rapid increase in hardiness (14). In this study when SD-induced plants (prehardened to −40 C) were epxosed to a −5 C frost for 1 to 6 successive nights, hardiness increased so rapidly that the −50 and −65 C minimum test temperatures, in subsequent hardiness evaluation, did not cause injury. As shown in Table II, however, there was no marked change in water permeability of cortex cells during this frost-induced phase of acclimation.

Water Permeability Changes during Dehardening. When hardy stems were dehardened by exposure to 21 C, the water permeability of cortex cells appeared to decrease; however, the large variability in dehardened samples prevented meaningful differences from being attained. Table III presents the permeability ratios of samples from plants in the process of dehardening from below −70 C to −35 C and to −5 C. The extremely hardy cortex had a permeability ratio of 1.31, whereas plants dehardened to −35 C after 5 days and −5 C after 14 days had permeability ratios of 1.53 and 1.66, respectively.

Table II. *Water Permeability and Hardiness of Living Cortex Cells in Cornus stolonifera Hardened to −40 C then Hardened Further via Exposure to −5 C Frosts*

Data represent the mean and standard deviations of four plants in each treatment.

Treatment	Permeability Ratio ($t_{1/2}$ alive/$t_{1/2}$ dead)	Hardiness (Lowest Survival Temp.)
		C
Before frost	1.50 ± 0.28	−40
After 1 frost	1.61 ± 0.21	below −50
After 6 frosts	1.47 ± 0.08	below −65

Table III. *Water Permeability and Hardiness of Cortex Cells in Cornus stolonifera from Field-grown Plants Hardy below −70 C and Dehardened at 21 C for 5 or 14 Days*

Data represent the mean and standard deviation of four plants in each treatment.

Treatment	Permeability Ratio ($t_{1/2}$ alive/$t_{1/2}$ dead)	Hardiness (Lowest Survival Temp.)
		C
Hardy control	1.31 ± 0.05	below −70
Dehardened 5 days	1.58 ± 0.27	−35
Dehardened 14 days	1.66 ± 0.25	−5

DISCUSSION

The THO technique proved to be simple and straightforward, but was not without complications. Water permeability ratios of 2.03 ± 0.11 were obtained with tender greenhouse-grown plants. However, variability in samples from plants acclimated in growth chambers were considerably increased, making it difficult to establish whether relatively small differences (1.80 *versus* 1.30) in permeability ratios between partially hardy and very hardy stem sections were significant.

Other factors may interact to influence variability in water permeability measurements. For example, in subepidermal cells of *Pisum sativum* stems, the water permeability responses to red and far red light are influenced by the time of day and stage of growth. Water permeability may also be increased by ABA (2). However, water-stressed (drought-hardened) *Pisum sativum* cells have very low water permeability (5) even though ABA may increase up to 40-fold (16, 17). Hence other factors, still unknown, can influence the water permeability of plant cell membranes.

The main advantage of the technique is that it is rapid and simple and provides data based on the average response of a large number of cells. However, it may prove useful for directly measuring the changes in permeability of the lipid portion of membranes via studies with radioisotopes like ^{14}C-methyl urea or other lipophilic substances which permeate the membrane more slowly.

No studies yet have unequivocally identified the primary site of injury or the role which water permeability of membranes play in the acclimation process. The data substantiate the results of earlier research which indicated that cell water permeability increases as plants become hardy. However, in cortex cells from *Cornus stolonifera*, this relationship was clear only during the very early SD-induced phase of acclimation. Additional studies are clearly needed to further our understanding of membrane involvement in both injury and acclimation. The THO flux technique will probably be a useful tool in those endeavors where an alternative to the plasmometric method is desirable.

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