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Cold Resistance and Injury in Woody Plants

Knowledge of hardy plant adaptations to freezing stress may help us to reduce winter damage.

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Only 7.6 percent of the earth's land surface is cultivated by man. Most of the other 92.4 percent is too cold, too hot, too dry, too salty, too rocky, or too steep. Of these stresses, drought and cold are acknowledged to be the two most important factors limiting the distribution of plants (*1*). Freezing damage to native vegetation and crop plants is a ubiquitous problem of major economic significance even in subtropical regions. Among the more commonly recognized types of freezing injury are sunscald and frost splitting of tree trunks, winter burn on conifer foliage, blackheart in stems of trees and shrubs, soil-heaving damage and crown kill of winter cereals and herbaceous perennials, die-back in citrus, midwinter kill of dormant flower buds, and spring and autumn frost damage of tender annuals, flowers, and fruits.

Some woody plants are extremely resistant to freezing stress and men have wondered for centuries how trees and shrubs of the temperate zones survive the winter to produce leaves and bloom each spring. Early scholars speculated that trees, like animals, possessed a "vital heat" that prevented them from freezing (*2*). Tests have shown, however, that water does freeze in hardy plants during the winter and that such plants can survive freezing in liquid nitrogen at — 196°C (*3*).

Attempts to attenuate freezing injury to marginally hardy crop plants have generally involved breeding adapted varieties, modifying the weather, providing mechanical protection, attempting to slow down autumn growth without restricting photosynthesis, and luck. Although these approaches have been partially successful, it is tempting to think that we will ultimately find the means to physiologically manipulate plants to resist or avoid freezing damage.

For this reason, there is considerable research interest in the survival mechanisms of the hardy plants which are uniquely well adapted to resist extreme cold. In the ensuing discussion I have described my interpretation of the current status of knowledge on freezing injury and resistance in such species. In so doing I have relied heavily on research examples from our laboratory. This choice is based on my familiarity with the work, and is not meant to minimize the validity of other equally relevant examples or opinions.

Woody Plant Hardiness Studies

As any experienced researcher might expect, the problem of studying freezing resistance is not so simple as it first appears. Not only do different woody species vary considerably in their inherent capacity to resist freezing stress, but the resistance of individual plants changes dramatically during the year. A plant which is killed at temperatures just slightly below freezing in the summer may survive — 196°C in the winter. Acclimation or hardening are the terms used to describe this change from a susceptible (tender) to a resistant (hardy) condition.

A further complication is the difference in hardiness exhibited by adjacent tissues or parts of a plant (*4*). In stems, for example, the living cells in the wood (xylem parenchyma and pith) are often several degrees less resistant in midwinter than are neighboring cells in the bark tissues (cambium, phloem, cortex, and epidermis) (*5*). Such differences can result in localized damage as in the case of blackheart injury, an oxidative browning of wood cells which is commonly observed in stems following a severe winter (*6*).

Various plant organs also differ in hardiness. Leaves of deciduous plants acclimate little and abscise. Roots are consistently less resistant to freezing than are overwintering stems (*7*). The extent of acclimation in roots is probably closely related to the soil temperature since stems below ground are no more resistant than roots (*8, 9*), and exposed roots are capable of becoming as resistant as aboveground stems (*10*).

Although these variations in resistance complicate discussions of whole-plant hardiness, the resistance of the regenerative tissue of the stem is particularly vital to survival of the plant. For this reason hardiness evaluations are commonly focused on the regenerative cambium region and adjacent phloem tissue in the living bark.

The possibility of physiologically regulating the hardiness of plants is particularly intriguing because relatively minor modifications would be of considerable significance. For example, freezing losses could be greatly reduced if it were possible to (i) delay spring bloom for a week on apricots and peaches; (ii) induce hardiness a week or two earlier in the autumn on woody ornamentals; (iii) increase the level of

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resistance in midwinter on citrus by only a few degrees; and (iv) prolong the dormancy of plants that begin to grow too early in the spring. Unfortunately we neither understand how plants are killed by freezing nor how some plants acclimate. The answers to these two basic questions are needed to provide logical bases for developing techniques for physiologically manipulating plants to reduce freezing damage.

How Does Freezing Kill Plants?

There has been considerable research on the preservation of animal and microbial cells by freezing, and the results of these studies have provided much of the foundation for current views of freezing injury in plants (*11*). Unfortunately, both the study techniques and the intrinsic cell properties of such cells have little in common with the freezing of plant cells in nature. For example, woody plants have a relatively large mass, rigid cell walls, and the ability to acclimate whereas sperm, red blood cells, and microorganisms do not. In addition, the rapid freezing rates and the chemical cryoprotective agents used in most freeze-preservation research have no parallels in nature. Even when biological cryoprotectants like sugars are studied, they are most often supplied at unnaturally high concentrations which cause severe injury or death of plant cells (*12*). In contrast to recent reviews which emphasized tender plant injury (*11, 13*), I have attempted to describe the unique features of freezing injury to hardy plants.

The influence of freezing rate. When woody plants are actively growing, death of stem tissues occurs at the moment of freezing. Since woody tissues exhibit only a minor depression of the freezing point, this means that the killing point, which ranges from about $-2°$ to $-8°$C, is determined largely by the amount of supercooling (*14, 15*). When stem sections are frozen slowly, the heat released by crystallization warms the sample, and equilibrium freezing continues at the slightly depressed freezing point (usually $-0.3°$ to $-1.0°$C). In the autumn when woody stems acclimate, neither the extent of supercooling nor the amount of freezing-point depression change significantly (*14*), but death no longer occurs at the moment of freezing. When plants are fully acclimated in midwinter the tissues of the living bark of numerous hardy species have been found to withstand cooling to $-196°$C if the freezing rate to $-30°$C is relatively slow (*3, 16*).

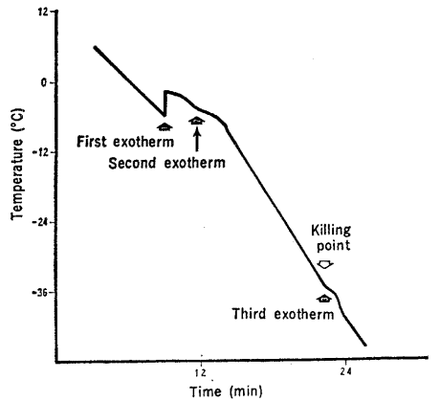

Fig. 1. A typical record of tissue temperature (freezing curve) during the controlled freezing of an acclimated stem section of a semihardy woody species. Exotherms are the points at which the heat of fusion from the freezing of water in the excised stem detectably raises the sample temperature. This figure shows the cooling curve of a stem which initially supercooled to $-6°$C before freezing. The third exotherm has been observed to be the killing point of stem tissues in a number of woody species.

In nature the air temperature rarely decreases more than a few degrees an hour. At such slow rates of freezing, ice forms first outside of the protoplasts of cells where the water is purest. Hardy woody plant cells can survive such extracellular freezing (*2, 17*). When freezing is rapid (10° to 100°C per minute), however, ice crystals form within the protoplasm of plant, animals, and microbial cells (*11, 18*). When such intracellular freezing occurs death almost invariably results. At extremely rapid rates of cooling, cellular water solidifies without the formation of crystalline ice (vitrification). Even nonhardy cells can survive vitrification to extremely low temperatures (*2, 11, 16, 19*). This illustrates that ice and not low temperature is the cause of death (*11*).

There are a few examples of rapid intracellular freezing injury in nature (*20, 21*). Evergreen foliage which is not injured at $-87°$C during slow freezing in the winter is killed at $-10°$C when thawed tissues are frozen rapidly (8° to 10°C per minute) (*21*). Foliage on the southwest sides of evergreen plants in Minnesota were found to cool at these rates on sunny winter days when the sun moved behind a hill or some other obstruction on the southwest horizon (*21*). Sunscald injury, which occurs on south-facing branches of many tree species, is probably also caused by rapid temperature changes which give rise to intracellular freezing.

Since the rate at which water can escape from cells is critical in these situations, factors which increase the permeability of membranes to water should reduce injury. There was a great deal of excitement several years ago when Kuiper reported that decenylsuccinic acid increased the water permeability of plant cell membranes and thereby increased frost resistance (*22*). Subsequent research by other workers has failed to verify either of these purported effects (*23, 24*).

Slow-freezing injury. The remarkable cold resistance attained by some species and the occasional instances of rapid freezing damage are interesting topics of study. Of more general interest, however, are questions related to the injury caused by slow-freezing stress. Many semihardy plants of economic importance are killed at temperatures of $-15°$ to $-45°$C and at rates of cooling which commonly occur in nature.

What happens in the bark of an apple stem in January between $-29°$ and $-30°$C that makes the difference between life and death? Is the destructive influence of ice direct and mechanical (*2, 11, 25*)? Is some type of intracellular crystallization always the cause of death (*17*)? Does the movement of water out of cells to extracellular ice nuclei dehydrate the protoplasm and increase the concentration of ions to a point that protein constituents irreversibly plasmolyze (*24, 26*)? Do destructive disulfide linkages form between sulfhydryl groups of adjacent protein molecules as they approach one another during freeze dehydration (*27, 28*)? Does death result from the removal of shells of hydrogen-bonded water surrounding hydrated enzymes or structural proteins (*29, 30*)? Is destruction of the cell membrane the primary site of injury (*31–33*)? Does the internal compartmentalization of cells break down, resulting in self-destruction from uncontrolled enzymatic activity (*34–36*)? Is ice always the causal factor or can low temperature cause death (*37*)? Is death a rapid and dramatic event (*17, 38*) or a slow process (*11, 39*)?

Evidence bearing on the hypotheses summarized in the preceeding questions is available from calorimetric (*17*)

microscopic (*17, 40*), and freezing-curve studies (*14, 15, 38*) of stem sections. Freezing curves are simply records of tissue temperature during freezing. Calorimetric measurements (*16, 17*) show that there is a slow but continuous freezing of water in hardy stems to temperatures as low as − 60°C. Woody plant cells are known to increase in water permeability during acclimation (*28*), and in hardy tissues the protoplasts and cell membranes appear to offer little resistance to the free passage of water. It has been shown that water moves out of hardy living cells to external ice nuclei almost as freely as it does from dead cells which were killed by steam just prior to freezing (*17*).

By summarizing the results of calorimetric, freezing-curve, and microscopic studies of hardened bark cells, one arrives at the following sequential description of slow-freezing death: Supercooling → Extracellular freezing of water between cells and in the nonliving xylem elements → Rapid propagation of ice throughout the stem resulting in: → A substantial release of heat of fusion (an exotherm) which raises the tissue temperature from the supercooling point (− 2° to − 8°C) to a plateau at the freezing point of the free water in the stem (− 0.3° to − 1.0°C) → Further cooling after the readily available water is frozen → Migration of protoplasmic water out of cells to extracellular ice nuclei in response to the extracellular, aqueous, vapor-pressure deficit → A second distinct exotherm followed by decreasing tissue temperature and a continuous slow movement of cellular water out to extracellular ice nuclei → Shrinkage of the protoplasts, plasmolysis, and concentration of solutes in the cell → Continued slow movement of cellular water out to extracellular ice as the temperature decreases → A calorimetric lag indicating that freezing, or water movement out of the cell, is arrested → A third exotherm → Granulation of the protoplasm → Death. Under certain kinds of light, living cortical cells fluoresce. This fluorescence changes abruptly at the moment of death (*17, 41*).

Two Hypotheses to Explain Death

Russian workers call the death point, and the accompanying heat release, the second supercooling point (*17*). We also observe a distinct exotherm at the moment of injury (Fig. 1) in freezing-curve studies (*14, 38*) which we call the vital water exotherm. I suspect that this lethal exotherm (the third) is the same as Tumanov and Krasavtsev's second supercooling point (*17*). However, our interpretations, as well as the names for this phenomenon, differ as indicated (Table 1).

Table 1. Two hypotheses to explain death. Left, the second-supercooling-point hypothesis of Tumanov and Krasavtsev (*17*); right, the vital water exotherm hypothesis proposed by the author based on work by McLeester *et al.* (*14*) and Graham and Mullin (*38*).

Second supercooling point	Vital water exotherm
During freezing a point is reached when the free movement of water out of the cell to extracellular ice becomes restricted by the protoplast and/or plasma membrane.	During freezing a point is reached when all readily available water has been frozen extracellularly and only "vital" water remains in the protoplasm.
Water is trapped in the protoplasm where it temporarily supercools as the temperature continues to decrease.	As the temperature continues to decrease vital water is pulled away from protoplasmic constituents to the extracellular ice.
Intracellular nucleation occurs suddenly resulting in death.	This sets off a chain reaction of denaturation, additional vital water release, and death.

What we have hypothesized, in effect, is that a point is reached during the freezing process when the only water left in the protoplasm is "vital water"—the water which is intimately associated with protoplasmic constituents (*42*) and necessary for life. We interpret the freezing lag prior to death (*17*) to indicate that all the readily available water has frozen, and not that the cell membranes have suddenly become more restrictive to the outward flow of water.

This viewpoint is circumstantially supported by the results of recent research which emphasized the ordered and intimate association of water molecules with biological macromolecules (*19, 43–51*). It is generally accepted that water influences macromolecular configuration (*47*). For example, nucleoproteins and lipoproteins incorporate water which serves both a structural and functional role (*46, 52, 53*). Nucleic acids (*49*) and proteins (*54, 55*) are known to bind quantities of water amounting to 30 to 50 percent of their weight. The removal of water from some proteins causes a complete destruction of their specific configuration. In a fibrillar protein like collagen, water is thought to be a structural element of the protein helix as well as a necessary stabilizing agent (*48*). Structured water apparently also plays a material role in stabilizing the surface of red blood cells (*51*), and the removal of this water during freeze preservation is thought to be a key factor in the loss of viability (*56*).

Hydration is the term which describes water that is under the influence of a macromolecule. Since "influence" is usually described in terms of mobility, the less mobile structured water around cell components is often called bound water (*49*). The binding forces between water and biological macromolecules are of two major kinds—hydrogen bonds between polar groups and water molecules (*43, 45, 47, 52*), and hydrophobic bonds between water molecules and nonpolar residues (*44, 47, 50, 52*). Both kinds of interactions stabilize macromolecules (*47*), but recent research (*47, 50*) supports the view that the hydrophobic bonding of water into so-called "icebergs" (*52*) plays the more decisive role in preserving the structure of native macromolecules (*47*) and in determining their physiochemical properties (*50*). In proteins the nonpolar residues of amino acids like alanine, valine, leucine, cystine, methionine, and phenylalanine provide surfaces which can participate in this type of hydrophobic bonding.

The possible relationships of water binding to plant hardiness are particularly intriguing because the structure of water changes with temperature. A number of these changes will be discussed in the section of this article dealing with acclimation.

Our vital water hypothesis does not preclude the idea that resistance is related to high water permeability of cell membranes since lethal intracellular freezing must still be avoided, but survival would ultimately depend upon the tenacity of water binding to protoplasmic constituents in quasi-crystalline configurations (*19, 43, 57, 58*), or upon the extent to which bound water is replaced by other protective molecules (*29*).

By contrast the second-supercooling-point hypothesis (*17*) suggests that death is a direct result of mechanical disruption of the protoplasm by intracellular ice (*11*). In this case, dehydra-

tion is not considered to be injurious since Tumanov and Krasavtsev (*17*) suggest that death would not occur if the membranes continued to permit the free passage of water. This idea is attractive since it is generally agreed that intracellular freezing is lethal. It seems reasonable that temporary intracellular supercooling can occur in frozen tissues because cell membranes appear to be effective nucleation barriers (*11*). This hypothesis also fits nicely with concepts of compartmentalization of different water fractions in complex organs during freezing (*13*). It does, however, require that a rather abrupt change must occur in the water permeability of the protoplast and plasma membrane at low temperatures. There is, as yet, no direct evidence that such changes occur.

Following the presentation of their second-supercooling-point hypothesis (*17*), Tumanov and Krasavtsev (*59*) suggested further that hardy cells may contain protective substances in the protoplasm which inhibit intracellular crystallization. Similar ideas have been proposed by researchers studying herbaceous plants. A heat-stable protein or nucleoprotein from spinach leaves has been found to protect spinach chloroplast membranes from freezing (*30*). Olien (*60*) has found that polysaccharide polymers extracted from cereal grain plants interfere with ice crystallization and appear to be related to resistance.

Although neither hypothesis resolves the confusion about injury to woody plant cells, the main point made by both is that death occurs as a result of a specific freezing event. On this basis, the gradual concentration of solutes, dehydration of protoplasm, formation of intermolecular linkages, and other continuous nonexothermic freezing phenomena cannot directly account for the sudden and dramatic crystallization associated with death in woody tissues. We still need to determine whether the exotherm at the moment of death is the cause or the result of injury. The immediate loss of vital fluorescence observed at the killing point (*17, 41*), indicates that the exotherm and death are very closely, if not causally, related. The loss of vital fluorescence also supports the concept that death in hardy woody tissues is a sudden process which is not associated with thawing.

In mammalian cells, it is thought that freezing death results when electrolytes reach critical concentrations as cells lose water during freezing (*26*). Hardy woody plant cells however are extremely resistant to dehydration (*16, 17*), and the applicability of this hypothesis to hardy woody tissues has been questioned (*17, 28*). Arguments have been reviewed by Mazur (*11*).

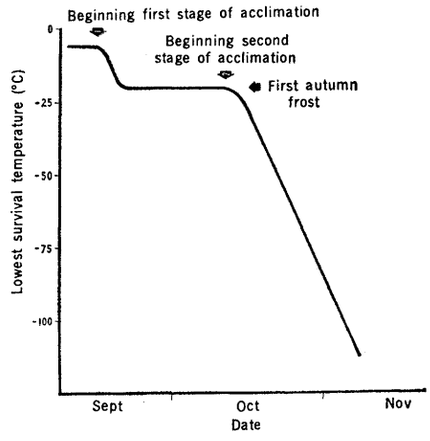

Fig. 2. A typical seasonal pattern of cold resistance in the living bark of *Cornus stolonifera* stems in Minnesota. In nature, acclimation in this hardy shrub and in a number of other woody species proceeds in two distinct stages as shown. The beginning of the second stage of acclimation characteristically coincides with the first autumn frost.

Freezing-Curve Studies

Before leaving the subject of injury, some further discussion of the technique of freezing-curve analysis in relation to hardiness research seems appropriate. Records of tissue temperature during freezing (freezing curves) indicate points at which substantial water crystallization occurs because exotherms are created when the latent heat of fusion temporarily warms the sample. This simple but little-used technique is essentially a poor man's calorimetry in the qualitative sense. By recording freezing curves, we have found that florets in dormant flower buds exhibit a single distinct exotherm in midwinter. The florets are killed at the moment this freezing point is reached. You will recall that stem tissues are not killed until the third freezing point (Fig. 1). In contrast to that in stems, flower bud hardiness is apparently based entirely on avoidance of freezing. The killing temperature of florets can therefore be predicted simply by recording the temperature of the freezing point (*38*) Dormant florets of some deciduous azaleas have been observed to avoid crystallization down to −43°C in midwinter (*38*).

Similarly, observations of the third (vital water) exotherm in semihardy stems permits direct observation of the killing point. Unfortunately, this exotherm is inconspicuous in some species unless freezing is relatively rapid (1.5° to 2.0°C per minute). Increased resolution can be attained by measuring potential differences between two thermocouple junctions (*61*) when one is placed in the tissue and one in the surrounding air. If death from drought is caused by a loss of vital water, it may also be possible to predict drought resistance by examining freezing curves.

Membrane destruction and injury? When a stem section is frozen and killed and then thawed and refrozen, the second freezing curve is hyperbolic and reveals only a single exotherm (*15*). If death did not result from the first freezing cycle, the freezing curves from both cycles resemble the curve shown in Fig. 1. The disappearance of multiple exotherms in the second freezing cycle provides a rapid means for determining viability. It also provides a basis for speculating about the cellular site of injury. For example, the fact that multiple exotherms are a unique property of living tissues (*14, 15, 61–64*), suggests that there are either barriers to the movement of water from the cell to extracellular ice, or that there is some kind of a tissue-by-tissue compartmentalization of the freezing process (*15*). In either case the rapid loss of multiple exotherms in dead stems makes it tempting to consider that cell membrane destruction is the primary site of freezing injury. Electrolytic tests for freezing injury which are based on the loss of selective permeability (*65, 66*), and the flaccid, water-soaked appearance of tender plants on the morning following a killing frost indirectly support this conclusion. In such cases, however, there is a time lag between freezing and the measurement or observation of loss of selective permeability. This then does not preclude the possibility that membrane destruction is a secondary effect of injury.

Several other kinds of evidence weaken my confidence in the idea that membrane destruction is the immediate and direct result of freezing. At low (1 kilohertz), but not at high (100 kilohertz), frequencies the electrical admittance (current passing

through a sample divided by applied voltage) of a tissue is thought to be closely related to the integrity of the cell membranes. As one would expect, stem sections killed by chloroform fumes show a rapid and substantial change in admittance at low frequencies. Stem sections killed by freezing, however, show no immediate change in admittance upon thawing (*39*). There is also evidence that factors which preserve internal compartmentalization (specifically lysosome integrity) reduce frost injury (*34, 36*). Rapid destruction of nucleic acid components by ribonucleases has also been observed after freezing and during freeze drying (*35*). These findings suggest that destruction of the outer cell membrane (plasma membrane) may not be the primary site of freezing injury.

In summary, freezing in hardy woody stems is a discontinuous process and injury is associated with a distinct freezing point. Two explanations which take these observations into account are the concepts of a second supercooling point and the freezing of vital water. Membranes may be the primary site of freezing damage in hardy plant cells, but the evidence is not fully convincing.

How Do Plants Become Resistant?

Hardy woody species characteristically undergo a series of changes in the autumn which enable them to withstand freezing stress. Figure 2 shows the typical two-stage pattern of cold acclimation which is found in woody plants native to the temperate zones (*17, 67–69*).

When several climatic races of a single species are grown at one location, there are marked differences in the timing of acclimation but the overall patterns of hardiness development are similar. Figure 3 diagrammatically illustrates the acclimation patterns of three climatic races of a hardy woody shrub (*Cornus stolonifera* Michx.) grown at St. Paul, Minnesota. More than 25 races were collected from widespread locations in North America (*70*), and they all became very hardy (— 196°C) by midwinter in Minnesota. In spite of this, plants native to coastal regions with mild climates were often partially killed back by fall and early winter frosts in Minnesota because they did not acclimate soon enough or fast enough (*70*). For example, a clone

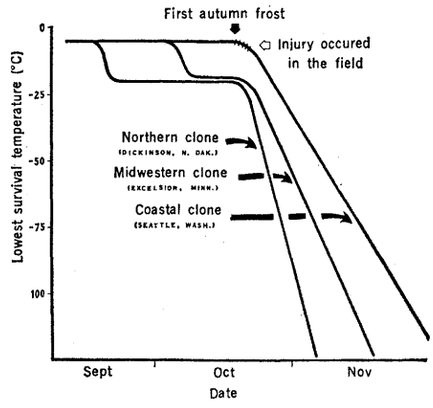

Fig. 3. Typical seasonal patterns of cold resistance in the living bark of three climatic races of *Cornus stolonifera*. The acclimation curves shown are for clones from North Dakota, Minnesota, and Washington grown in the field in Minnesota. Races from regions with mild climates and long growing seasons acclimate later and more slowly than clones from regions with severe climates and short growing seasons. Over 25 clones which have been collected from widespread locations in North America became resistant to —196°C by midwinter in Minnesota.

from Seattle, Washington, did not become hardy to — 13°C until 17 October, 32 days later than a clone from Dickinson, North Dakota, which became hardy to — 33°C 50 days sooner than the Washington clone (*71*).

Many of our cultivated trees and shrubs are grown in locations remote from their parental sites of origin. It is not unlikely that many species possess the ability to resist severe freezing stress like *Cornus stolonifera*, but lack the proper timing of acclimation to survive. One possible example is flowering dogwood which becomes hardy to — 65°C in midwinter (*72*) and yet fails to survive in regions where the minimum temperatures are considerably higher.

Let us go back for a moment and take a closer look at the environmental stimuli which regulate acclimation. One of the first things that a hardiness researcher looks for are procedures for inducing acclimation at will under controlled conditions. Anyone who has worked with hardy woody species soon becomes aware that this is not possible because of cyclic internal (endogenous) factors. During the spring flush of growth plants will not acclimate fully regardless of the regimes of photoperiod and temperature which can be provided in controlled environment chambers (*69, 73*). Equally interesting is the observation that plants acclimate to some extent at the end of the growth cycle in the autumn even when they are subjected to supposedly noninductive long daylengths and high temperatures in a greenhouse (*67*). These kinds of results suggest that there are hardiness rhythms (*67, 74*) associated with the cessation of growth and the physiological age of the plant, or with seasonal environmental stimuli other than temperature or photoperiod; perhaps seasonal changes in the spectral composition of sunlight.

Following the spring flush of growth, and before the onset of the endogenous autumn hardiness rhythm, growing woody plants can be induced to acclimate in a manner which is reasonably typical of the hardiness stages which occur in nature. A period of short days and warm temperatures followed by a period of low temperatures usually gives optimum acclimation in deciduous species.

The First Stage of Acclimation

In nature the first stage of acclimation appears to be induced by short days (*17, 67–69, 74*). Decreasing photoperiods cause growth cessation in many northern deciduous species by triggering the onset of rest period. Rest period and quiescence are terms which describe the two phases of the dormant period in plants. Dormant-quiescent plants will begin to grow under favorable environmental conditions whereas dormant-resting plants will not. The internal factors which prevent growth during the rest period are overcome by exposure to a certain number of degree-days of chilling temperature within a critical range. The temperatures which most effectively satisfy the chilling requirements of many species are just a few degrees above freezing. The key factor in the induction of acclimation, however, appears to be growth cessation rather than rest-period induction because low temperatures can stop growth and bring about acclimation without inducing rest (*67*).

Horticulturists are interested in prolonging the rest period of plants in order to delay spring growth and thereby avoid damage from frosts (*6*). This is a logical approach but, to date, attempts to prolong rest period in nature have met with only limited success. It has also been hypothesized that rest period may prevent the loss of hardiness (dehardening) which can occur

in midwinter during periods of unseasonably warm weather (*75*). Unfortunately, this does not appear to be true. Resting raspberry canes have been found to deharden in response to high temperatures more rapidly than equally hardy canes which were quiescent (*76*).

Studies of modified plants. Recently a great deal has been learned about the environmental control of cold acclimation by (i) dividing plants with light or temperature barriers (split plant studies) so that branches of a single plant may be simultaneously exposed to different environments (*12, 67, 77, 78*), (ii) by partially or completely defoliating plants at different times during acclimation (*12, 67, 78*), (iii) by removing a band of bark around a stem (girdling) to interrupt the transport of substances in the phloem (*12, 78*), and (iv) by studying the hardiness of grafted plants composed of genotypes which differ in their ability to acclimate (*12*).

The major results of these studies indicate that: (*a*) growth cessation is a prerequisite to cold acclimation in woody plants; (*b*) plants severely depleted in photosynthetic reserves cannot acclimate; (*c*) leaves are the site of perception of the short-day stimulus which initiates the first stage of acclimation; (*d*) low temperature inhibits the short-day induced phase of acclimation; (*e*) long-day induced leaves are the source of a translocatable factor(s) which inhibits cold acclimation; (*f*) short-day induced leaves are the source of a translocatable factor(s) which promotes acclimation; (*g*) the hardiness-promoting factor moves from the leaves to overwintering stems through the bark; (*h*) the hardiness-promoting factor from the leaves of a hardy genotype can enhance the acclimation of a branch of a less hardy genotype when the two are grafted together; (*i*) frost triggers the second stage of acclimation; (*j*) the frost-induced phase of acclimation does not involve translocatable factors; and (*k*) plants exposed to long days and frost will eventually become fully hardy, but plants exposed to short days and relatively high temperatures only reach the first stage of acclimation (Fig. 2).

The observation that the hardiness-promoting factor from a hardy species can increase the hardiness of a less hardy type (See *h* above) may be particularly important (*12*). If the hardiness-promoting factor can be isolated and identified, it may ultimately be possible to chemically program the onset of hardiness in species which have the inherent capacity to acclimate but the wrong timing to survive in certain climates (*70, 72*).

Short days probably function as an early warning system in nature. The first stage of acclimation appears to involve two distinct events, growth cessation and the initiation of the metabolic changes which facilitate the plant's response to low temperatures during the second stage of acclimation. Although the increase in resistance during the first stage of acclimation is relatively minor, it may be very significant since just a few degrees of resistance can make the difference between life and death.

The hardiness-promoting factor. Inasmuch as the nature of the short-day-induced hardiness-promoting factor is still a mystery, its elucidation deserves concentrated research attention. We know that hardiness is promoted to some extent by treatments which promote growth cessation and the accumulation of an adequate supply of photosynthate, particularly in plants which have a tendency to continue growing in the fall. A major question is whether the translocatable hardiness-promoting factor will prove to be a growth inhibitor which indirectly influences hardiness by stopping growth (*79*), a simple sugar (*80*), or a regulatory substance (hormone) which plays a direct role in mobilizing the metabolic machinery responsible for the first stage of acclimation.

Does the promoting factor merely stop growth? It seems possible that the hardiness-promoting factor may play an indirect role in acclimation by simply slowing or stopping growth. We know, for example, that actively growing woody plants do not acclimate and that chemical treatments, which stop growth, sometimes increase hardiness to a limited extent (*20, 81, 82*). These observations, however, cannot begin to account for the wide spectrum of metabolic changes which occur during acclimation when growth has stopped. The major realignment of metabolism following growth cessation in woody plants (see ensuing discussion) suggests that the promoting factor plays an active regulatory role and that the synthetic processes associated with acclimation cannot begin to function until growth processes stop. It is interesting that this is not the case in a relatively hardy, but nonwoody, plant like cabbage where the physiological processes responsible for acclimation function more effectively when plants are growing than when they are dormant (*83*).

Is the promoting factor a sugar? Circumstantial evidence points to the possibility that sugar may be the translocatable promoting factor: for example, (i) leaf disks of cabbage (*2, 84*) or leaves of gardenia (*85, 86*) infused with sugar increase in frost resistance; (ii) plants cannot acclimate when they are depleted in photosynthetic substrate (*12, 13*); (iii) sugar protects the enzyme systems associated with oxidative phosphorylation in isolated spinach chloroplasts subjected to freezing (*30*); (iv) there are in vitro sugar-protein interactions which may occur in vivo and protect freezing-sensitive proteins (*87*); and (v) there is an extensive amount of correlative data which indicates that starch is converted to sugar in plant tissues exposed to low temperatures in the autumn (*1, 11, 13, 88*).

The idea that sugar is the factor which promotes the first stage of acclimation is unconvincing to me in spite of the collective weight or this evidence. Some amount of sugar or other metabolic substrate is undoubtedly necessary for acclimation, but beyond this, I feel that sugar is not the factor which induces acclimation in hardy woody plants. The basis for this statement is simple. I know of no experimental evidence which shows that applying sugars to hardy woody plant tissues before, during, or after acclimation has increased their resistance. We have been singularly unsuccessful in enhancing the acclimation of *Cornus stolonifera* by prolonged applications of various concentrations of glucose and sucrose (*12*). Further, one pair of short-day-induced leaves can promote acclimation in a large branch (*88*). This qualitative type of response would not be expected if the translocatable hardiness-promoting factor were a photosynthetic end product whose synthesis was quantitatively dependent upon leaf area.

I do not mean to imply that the short-term protective effects of sugar on cereal coleoptiles, spinach chloroplasts, cabbage leaf disks, yeast, algae, or red blood cells are unreal. Rather I suggest that the factors causing freezing death in these tender cells are probably different from those in hardy woody cells.

Siminovitch has stated that increased hardiness in black locust trees is more likely to be due to the disappearance of starch than to the increase in sugars in the autumn (*74*). Sakai has been unable to correlate sugar content with freezing resistance in hardy poplar trees, and he has concluded that there is a basic difference in the response of hardy and tender cells to sugars (*86*).

Is the promoting factor a regulatory hormone? We are left with the idea that the hardiness-promoting factor may be a hormone or complex of hormones which play a specific and direct role in initiating acclimation in woody plants (*12, 67, 78*). This idea is appealing because cold acclimation is just as important to the survival of a species as are many other vital processes (like flowering) for which intricate control systems have evolved.

During the first stage of acclimation, short days, as detected by a biological clock in the leaves, probably function to stop growth and set into motion the metabolic changes which ultimately lead to freezing resistance (*89*). It seems likely that the short-day message is transmitted via a translocatable hormone. The large array of metabolic changes involved in acclimation implies that this hormone exerts its regulatory function on nucleic acid transcription. It has been demonstrated that a common plant hormone (auxin) acts in this manner to increase the rate of synthesis of ribonucleic acids by making an increased portion of the genetic information available for transcription (*90*).

The Second Stage of Acclimation

The second stage of acclimation in nature is apparently induced by low temperatures; In fact, frost often appears to be the triggering stimulus (*17, 67, 69*) (Figs. 2 and 3). Because of this, it is tempting to conclude that the second stage of acclimation is a physical, rather than a metabolic, process. Such a conclusion is probably unwarranted because a number of enzyme-mediated reactions are known to be induced by low temperature, and there is ample opportunity for subsequent metabolic activity during the warm sunny days in autumn which alternate with frosty (inductive) nights.

I am intrigued by the possibility that the second stage of acclimation may involve a reorientation of macromolecules into stable forms which can resist severe dehydration. Proteins with hydrophobic residues are known to be particularly temperature sensitive (*91*). It has been proposed that there may be a temperature-reversible regulation of hydration which accounts for the conversion of proteins from a polymerized to a depolymerized configuration at low temperatures (*53*). In the polymerized state, the protein is biologically active and sensitive to stress whereas depolymerized molecules are inactive but stress resistant (*53*). During the latter phases of the frost-induced stage of acclimation when hardy plants become very resistant, tissue hydration decreases, and there is little metabolic activity. One wonders whether some of the delicate macromolecular and cellular structures associated with active growth are disassembled, packaged, and shelved for the winter. The situation could be roughly analogous to what happens during the final stages of ripening in dry seeds.

There are several ways in which this might occur. Particularly interesting is the work by Lauffer (*52*) on the temperature-dependent reversible reactions of a specific tobacco mosaic virus (TMV) protein. He has found that this protein aggregates at room temperature and disaggregates near 0°C. The process is readily reversible. Water is bound during disaggregation and released upon aggregation. He suggests that water is bound by depolymerized proteins at low temperatures since hydrophobic surfaces and ionizable groups become exposed and bind water via the "iceberg" or hydrogen-bonding routes, respectively.

Both types of water binding are known to respond to temperature changes but in different ways. At room temperature hydrophobic structuring of water is weak, but the number of ordered water molecules in the vicinity of a nonpolar hydrophobic surface increases with decreasing temperature (*44*). Hydrogen bonds in macromolecules, on the other hand, are broken at low temperatures but there is an increased probability of water-to-water hydrogen bonding (*47*).

Lauffer suggests that the stability of the aggregated and disaggregated forms of TMV protein (at room temperature and near 0°C, respectively) can be accounted for simply by the necessity that exposed surfaces bind water. This seems reasonable since the binding of water at low temperature will be accompanied by a decrease in free energy. At high temperatures, the depolymerization of aggregated protein requires an increase in free energy (*52*).

Metabolic changes during acclimation. The metabolic changes associated with the acclimation of overwintering stems have been the subject of much research (*1, 11*). Although some of this research has been focused on changes during the first stage of acclimation, much of it has been directed at changes associated with the second stage. In both cases dramatic correlative changes have been found in almost every group of compounds examined. For example, during acclimation in *Cornus stolonifera* we have found changes in total protein content (*73, 92–94*), specific proteins (*95*), lipid unsaturation (*7*), tissue hydration (*96*), translocatable hardiness-promoting and -inhibiting factors (*12, 67, 88*), starch (*92*), sugars (*92, 94*), nonvolatile organic acids (*44, 93*), free (*92*) and bound amino acids (*73*), organic and inorganic phosphorous (*93*), total ribonucleic acids (*95, 97*), DNA (*97*), and transfer and ribosomal ribonucleic acids (*35, 97*). Similar changes have been observed in other hardy species (*1, 2, 11*). Most recently, research attention has been focused on the changes in proteins (*74, 94, 95, 98, 99*), specific enzymes (*29–31, 99*), nucleic acids (*35, 74, 97, 100*), sugars (*30, 80, 85–87, 93, 101, 102*), polysaccharides (*13, 60*), and membranes (*60, 74, 86, 99*) associated with acclimation.

Studies of metabolic changes in plants during the autumn have given rise to several hypotheses and many questions about acclimation. It has been suggested that: total acclimation results from several independent physiological events whose effects are additive (*74*); acclimation involves a sequence of processes which are mutually dependent (*89*); resistance is related to an inhibition of intermolecular disulfide linkages which form between protein sulfhydryl groups as the cells become dehydrated during freezing (*27*); sugars replace water in forming protective shells around sensitive proteins (*30, 31*); acclimation involves an augmentation of protoplasm and lipid transformations (*74, 99*); new peroxidase isozymes synthesized in the autumn alter the protoplasm and unsaturated fatty acid components of membranes (*99*); temperature-sensitive cellular components assume more stable configurations (*91*);

a specific protein is synthesized which interacts directly with other cell components to protect them from freezing stress (*30*); reduced hydration of the protoplasm in the autumn increases resistance by reducing the amount of free water available for destructive ice-crystal formation (*96*); acclimation may involve phase changes of protoplasmic water (*19, 43, 52*); resistance is determined by the increased water permeability of membranes in the autumn which permits cellular water to escape to extracellular ice nuclei (*2, 11, 17*); and that acclimation depends upon factors which increase the resistance of protoplasmic constituents to salt denaturation during freezing (*26*).

The studies of metabolic changes associated with cold acclimation provide results which can be interpreted to support almost any and all hypotheses which have been proposed. In spite of this, one point emerges from the confusion. Clearly, many physiological changes are taking place in plants at a time of the year when one would expect the metabolic machinery to grind to a halt as plants become dormant. The array and magnitude of the metabolic adjustments strongly support arguments that acclimation is an active process and not just something that happens when growth stops.

Beyond this, it is unlikely that the results of descriptive studies of metabolic changes in the autumn will explain acclimation. I expect that breakthroughs in our understanding of acclimation will result from studies of cellular properties such as permeability and the status of protoplasmic water. Unfortunately, research on these phenomena requires biophysical approaches that most plant scientists are not equipped, either by training or inclination, to undertake.

The Third Stage of Acclimation

There appears to be a third stage of acclimation in hardy woody species which is induced by low temperatures (−30° to −50°C) (*17*). Prolonged exposure to temperatures in this range can ultimately cause hardened twigs to attain a state of hardiness which may not commonly be attained in nature. This kind of hardiness is quickly lost.

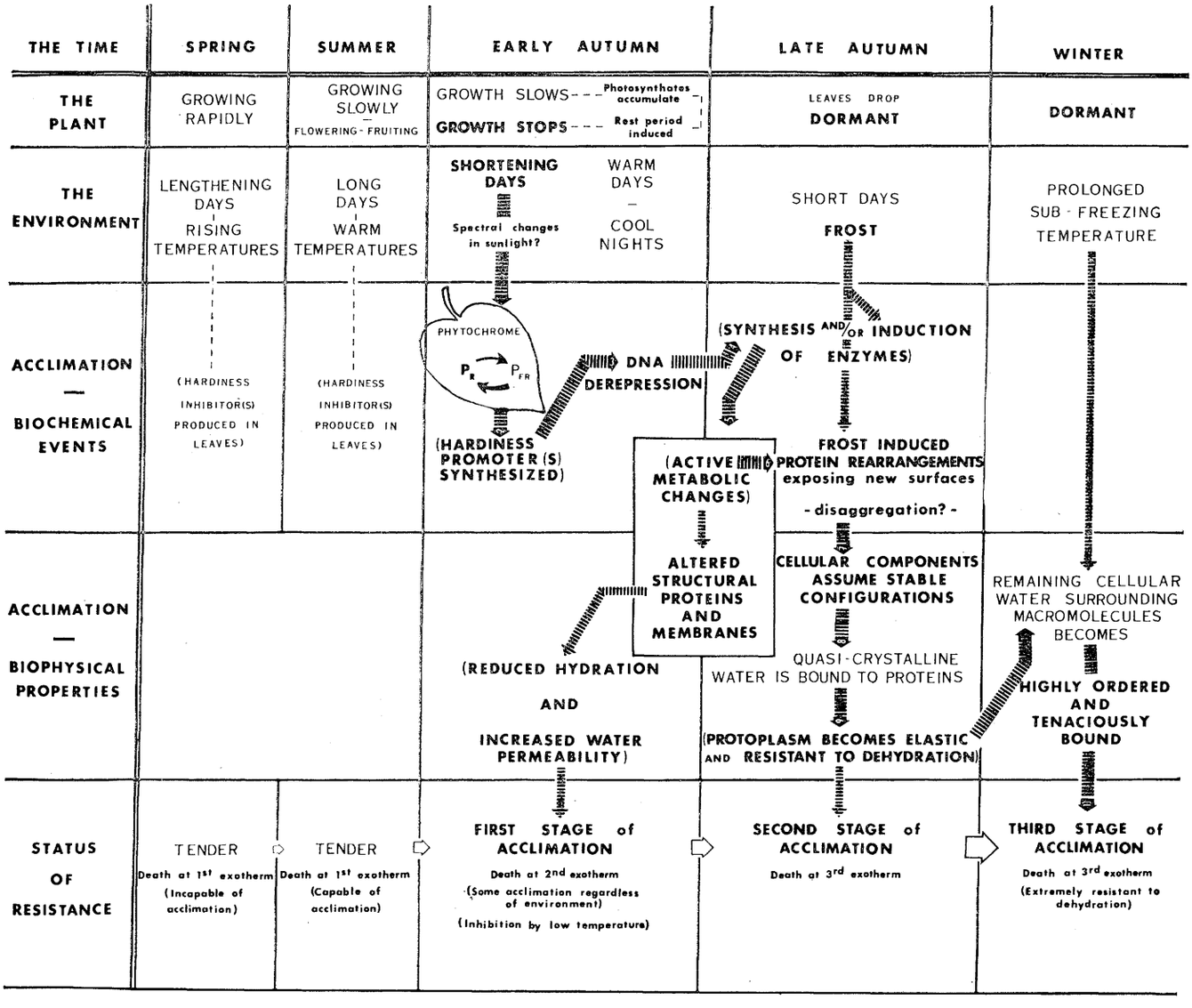

Fig. 4. A hypothesis to explain cold acclimation in hardy woody plants. Numbered arrows indicate the hypothetical sequence of events resulting in the most efficient and complete acclimation. Arrows without numbers identify sequential relationships and alternate acclimation pathways. Parentheses denote events which can be observed experimentally.

Hardy stems thawed for as little as 6 hours decrease in resistance from −195° to −45°C (*17*). The rapid fluctuations in midwinter hardiness in hardy stems (*67*) and buds (*6, 103*) may reflect changes in this phase of resistance. Such fluctuations seem to be largely dependent upon the temperature of the preceding day.

It has been proposed that the third phase of acclimation is a physical process associated with the reduced intermolecular distances and thermal motion of molecules in frozen cells (*17*). Tumanov and Krasavtsev do not think that it is related to frost dehydration because the amount of unfrozen water may change as little as 1 percent during prolonged exposure to temperatures which can induce a substantial increase in hardiness (*17*). This seems to contradict their speculation that reduced intermolecular distances are involved.

I am attracted to the idea that the third phase of acclimation may be related to the amount and degree of orientation of quasi-crystalline water in the cell; for example, prolonged low temperatures may increase the sphere of influence of hydrophobic molecular surfaces over surrounding water by creating a higher degree of order among molecules (*44*). This could increase the tenacity of binding as well as the proportion of bound water and thereby increase dehydration resistance (vital water hypothesis) or reduce the amount of water available for destructive crystallization (second supercooling point hypothesis) or both. The enlarged sphere of influence would be quickly lost upon warming when thermal activity increases and thawed extracellular water reinvades the cell.

Calorimetric techniques can be used to measure the tenacity of hydrophobic water binding, and infrared spectroscopy and nuclear magnetic resonance techniques can be used to estimate changes in hydrogen bonding near macromolecules (*49*). It would be interesting to see these techniques applied to studying the status of water in woody plant cells during freezing and acclimation.

Summary

It is interesting that plants can eventually acclimate fully in response to low temperatures in the absence of inductive photoperiods (*67*). This suggests that there is more than one route to resistance or more than one ignition key to start the machinery. In either case, the ability of plants to acclimate in response to more than one environmental stimulus provides adaptive flexibility which enhances survival potential.

Although the information at hand does little to actually explain acclimation in woody plants, it does favor the idea that ultimate resistance is the product of several distinct processes. It seems likely that the first stage of acclimation is an active metabolic process; that the second stage is metabolic, physical or both; and that the third stage is a physical phenomenon.

In Fig. 4, I have presented my views on the sequence of events which may occur in nature during acclimation. The diagram summarizes the preceding discussion on the environmental regulation and physiological implementation of cold acclimation in adapted woody plant species.

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Integrated Incentives for Fertility Control

Wider use of material incentives should make family planning programs more effective.

Lenni W. Kangas

Most of the experience and much of the discussion about the role of material incentives in population and family planning programs have regarded the usefulness of such payments in an unnecessarily restrictive way. Financial incentives have been given, for example, to acceptors of contraceptive methods, to personnel who provide family planning services, or to agent-recruiters who bring clients to service centers. Occasionally, a mix of these rewards is offered (as in India, Pakistan, and the United Arab Republic), but these rewards apply exclusively to events leading to and ending with the initial provision of contraceptive methods. Group and community rewards are conspicuously absent from both present action programs and the majority of proposals to date. Also missing is a system of meshing these rewards with incentives to individuals who adopt and continue contraceptive practices.

Despite limited world experience in the use of incentive rewards to make family planning programs more effective, the innovation has engendered a great deal of speculative discussion and even considerable controversy. Some regard an appropriate incentive system as a near-panacea for circumventing the difficulties and frustrations confronting organized efforts to bring down birthrates in developing countries. Moreover, many of the proposals that have surfaced recently strongly reflect the desire to discover the "one best way" or single kind of payment that will prove to be the determining factor in reducing human fertility.

Critics and skeptics, on the other hand, often view incentive-payment systems as Machiavellian mixtures of bribery and coercion, particularly if they are to be applied with sophisticated popularization techniques to an unsophisticated, tradition-oriented peasant population. Ethical questions have been raised as to whether it is proper to interfere so blatantly (or commercially) in the sacred arena of human reproduction, where, these critics maintain, voluntary and individual freedom of choice should remain paramount. Projected costs, vaguely or explicitly justified with axioms suggesting that an averted birth is equal to one to two times the per capita income, impress real-life planners and policy makers as exorbitant regardless of the presumed logic of the economic analysis.

In this article, I examine some of the characteristics of current programs and proposals that embody incentive schemes and go on to suggest an enlarged, adaptable framework for a more comprehensive approach to employing material incentives in fertility control in less developed countries.

Incentives, as used in this discussion, will refer to the direct (or indirect) payment of money or material goods and services to members of the target population and to service personnel or larger groups in the community (or both) in return for a desired practice supportive of lowered fertility. The frame of reference is both economic and psychological, and the subsequent discussion deals with mechanisms whereby economic and psychological motivations are linked and reinforce one another.

Present Incentive Programs

Present incentive programs operate almost exclusively on the individual level with regard both to recipients and to providers, although some incentives for group performance are offered to providers. In India, a man presenting himself for a vasectomy receives a nominal monetary reimbursement to compensate for time lost from gainful labor, for personal inconvenience, and (unofficially) as an inducement to undergo the operation. In many countries,

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