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BARLEY PATTERNS OF RESPONSE TO FREEZING STRESS
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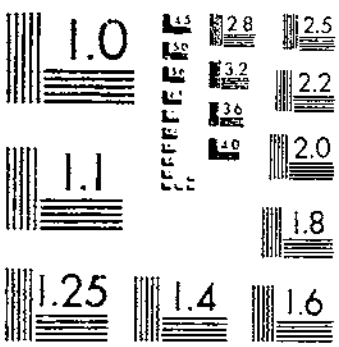

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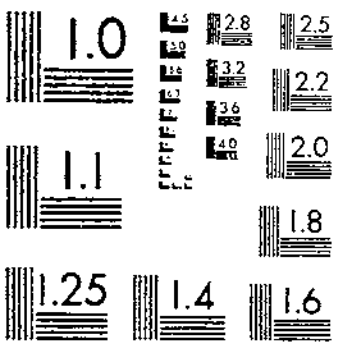

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Barley:
Patterns of Response to Freezing Stress

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Abstract

Several types of stress develop as water freezes in plant tissues. Each stress arises from a distinct form of energy and these have been analyzed thermodynamically. The stresses also have been distinguished by differences in the means by which crystallization energy is dissipated, the temperature range in which each stress becomes injurious, and the histological pattern of injury in the plant.

Heritable traits affect water transition paterns induced by freezing and modify the stress energies that develop as the temperature decreases. Other traits affect the pattern of injury and the ability of the plant to recover. Winter hardiness is a complex property of a genotype involving many genetically determined metabolic systems. Classification of genotypes with respect to specific heritable traits may aid in selection of parental lines for plants breeding, and tests in which specific forms of stress are induced may aid in selection of progeny adapted to particular environments.

A basis for laboratory analysis of plant genotypes to evaluate traits that affect freezing stress or plant response is discussed. An analysis of 'Hudson' barley (*Hordeum vulgare* L.) is illustrated graphically. Comparative data for lines from the U.S. Department of Agriculture world collections of barley and wheat are being compiled from field, nursery, controlled environment, and laboratory tests. We are especially interested in finding cultivars that are uniquely adapted to specific environments or that have distinctive traits that might affect stress, response, or recovery.

Key words: freeze stress, winter hardiness, cultivar analysis, barley, wheat.

Barley: Patterns of Response to Freezing Stress

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Introduction

Because winter hardiness is a complex trait, genetically and physiologically, a systematic approach is needed to understand and manipulate the variables that affect survival. Winter hardiness involves general adaptation to a specific environment as well as various forms of frost hardiness and, therefore, depends on obtaining an optimum combination from a large number of genes. Increases in hardiness beyond present limits require finding new genes in plants from world collections or from related species and incorporating them into the gene pools used to develop commercial cultivars. Such transfers of genetic and physiological information can be done more directly where simply inherited component traits have been identified (*7, 20, 22*).[2]

Freezing stresses basically involve water transitions. Water is a complexly and dynamically structured component of plants. Its physical properties and distribution are affected by the polymer systems of the tissue as well as by various classes of solutes. Water transitions of freezing begin with crystallization, but this new phase competes with all other phases of water association and causes a sequence of transitions to occur until the system again is at equilibrium (*8*).

Analysis of energy relations in freezing is a key in identifying traits that involve water and its interactions with plant substances. Energy relations can be evaluated from analysis of (a) water transitions, (b) freezing kinetics, (c) crystal growth patterns, (d) thermal transitions, and (e) stress vectors. The analytical results can be coordinated to derive a more precise description of stress components.

Patterns of water transition, stress energies, and survival are discussed as criteria for uniquely characterizing specific plant tissues with respect to winter hardiness.

Review of Stress Analysis

Intensity of freezing refers to the amount of energy for ice crystal growth that acts in a specific time interval and region of a tissue (*16*). Because water tends to supercool, ice crystal growth at temperatures of only a few degrees below the freezing point does not occur spontaneously, but as an extension of exogenous ice. This nucleating ice that initiates freezing in winter cereals usually forms in the soil (*13*). Under high relative humidity with radiation cooling, nucleating ice can form on leaf surfaces. These crystals grow as fine irregular structures through the plant tissue at a rate of about 10 centimeters per second (*23*). Primary crystal growth in leaves seems to occur most freely between the epidermis and mesophyll.

Net growth of ice requires crystallization energy. At 0°C ice in liquid water is in a dynamic equilibrium with as much free energy for melting as for freezing (about 50 calories per mole for each) (*16*). The half-life of lattice structure at the ice-liquid interface is short, about 10^{-5} seconds (*5, 8*). An excess of free energy for freezing is required for formation of irregular crystals and even more excess free energy is required for growth of ice crystals through plant tissues because of structural features or substances in the plant that must be deformed or altered as the crystal grows (*12, 19*).

Low intensity freezing tests, as they are used to rate hardiness of plants, involve supercooling about 2°C (fig. 5, p. 6). This provides sufficient freezing energy for ice to grow through the plant tissue but not enough

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[2] Italic numbers in parentheses refer to Literature Cited, p. 8.

to destroy membranes and grow into protoplasts of hardened plants. It does provide enough energy for growth of ice into protoplasts of tender plants. No ice formation is tolerated within living protoplasm (*10*). The most universal change that occurs as plants harden is stabilization of the protoplasmic membrane, especially in its effectiveness as a barrier to growth of ice crystals. Hardened barley leaves can survive up to about 5° supercooling (fig. 5).

Water Transition Patterns

The physical properties of all water in the plant are affected by association with solutes and colloidal interfaces (*5, 8, 13*). These water associations are in a dynamic state of turnover and involve structure of water into patterns with a short half-life (10^{-11} sec). Little water is stably bound. Though it may be desirable to identify all phases and association states of water in plants and all changes that accompany freezing, this is so complex and dynamic that generally only three categories have been related to freezing stress: (1) liquid water within the protoplast; (2) liquid in the outer free space, along the cell wall; and (3) ice in the region of the cell wall (*2, 11, 13*).

The primary crystal pattern that develops in tissues that are supercooled 1° or 2° C is fragile and unstable. Secondary crystallization results in stable ice structures that can be studied histologically. Frozen plants can be taken from field nurseries or from a test freezer and sectioned with a microtome in a cold chamber. The sections can be mounted in cold oil that has been stored in contact with ice so that it does not dissolve crystals from the sections. These sections can be examined with a microscope in the cold chamber. Macrocrystal structures can be photographed (fig. 1).

The freezing point of water in plant tissues is not fixed but rather is dependent on its state of organization as a result of association with other plant substances. The freezing point shifts as a function of the amount of water that remains in liquid associations. The various patterns of water redistribution between protoplasmic liquid, cell wall liquid, and ice can be described by exponential equations (*11, 12*).

The amounts of liquid water associated with plant substances cannot be determined by direct histological examination of tissue sections from frozen plants. However, observing diffusion of indicator molecules is possible. Amaranth (trisodium salt of 1-((4 sulfo-1-naphthyl) azo)-2-naphthol-3,6-disulfonic acid) is a red dye that is not taken up by protoplasts so its diffusion is restricted to the outer free space (*1, 9*). It is not toxic. Its negative charge helps prevent adsorption to cell wall polymers. Microdots of dye distributed in a frozen section can be observed to spread by diffusion, and the relative amount of liquid water associated with cell wall substances can be determined from the diffusion rate as a function of temperature (*17*).

Patterns of liquid transition in the outer free space can be evaluated for localized regions within a plant tissue by this method. However, maintenance of a stable condition for many hours is required for each rate determination. A low voltage (about 5 volts per centimeter for barley leaf tissue) will induce electrophoretic mobility of the dye. The mobility data are similar to that obtained by diffusion, but it only requires minutes instead of hours (*20*). Measurements of conductivity can be made instantaneously, and they correlate with diffusion data under special conditions:

- Electrical resistance of only the tissue must be determined so the contact resistance must be low and known.
- Direct current or a square wave alternating current of no more than a few cycles per second is required so the protoplasmic electrolytes do not affect the data.
- Voltage and current must be below a level that affects protoplasts, and
- Amount of electrolyte in the outer free space must be constant.

If the last restriction is not met, the transition pattern will not be reversible with increasing temperature. Release of electrolyte from the protoplasts indicates injury and is the basis of several vitality tests (*3, 10*).

Kinetics of freezing

Though conductivity data require control experiments to evaluate transitions in cell wall liquid during freezing, the speed with which data are obtained permits kinetics of freezing to be studied in localized regions of a plant (*12*). The temperature decreases faster than ice can form, and crystallization energy is determined by the displacement of temperature from equilibrium and studied as a function of the freezing rate.

Because, under the conditions described, freezing occurs as an extension of existing crystals, the ice-liquid interface acts as a catalyst and lowers the activation energy of freezing. Substances, such as some araboxylan mucilages that are normal constituents of the cell wall, form a film in the interface as the liquid freezes. They act as freezing inhibitors in the sense that they interfere with transition of liquid to the ice lattice, and greater crystallization energy is required to maintain a freezing rate. Ice structure affected by freezing inhibitors consists of smaller and more irregular crystals, and the distribution of crystals within the plant tissues is altered (*19*). Extracted araboxylans can be rated for kinetics inhibitor activity by a flow-freezing technique to screen plant genotypes (*14*). Inhibitor

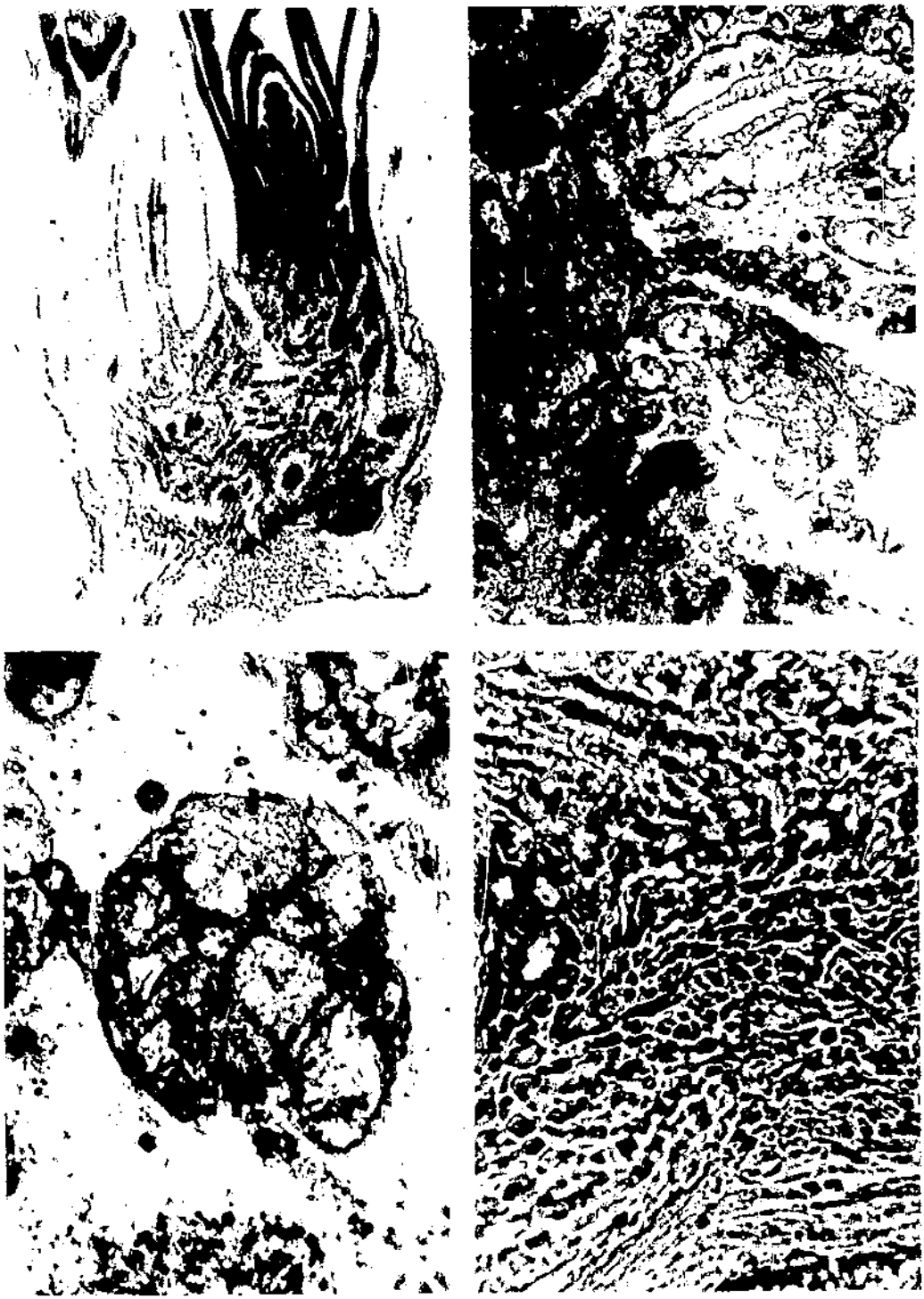

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FIGURE 1.—A histological study of ice in 'Hudson' barley crown tissues sectioned from plants frozen at 78 percent moisture: (Upper left) Longitudinal section of barley crown showing apical meristem of a tiller; central vascular transitional zone with root vascular elements in lower crown; root primordia developing from an axillary meristem in lower left region of the crown. (Upper right) Longitudinal section through region of axillary meristem taken from a frozen barley crown to show the array of larger ice crystals that form in the crown (magnification 5× greater than upper left). (Lower left) Section from the vascular transitional region to show ice structures in a vascular element. A large crystal has ruptured a xylem vessel (magnification 2× greater than upper right). (Lower right) Section through region of axillary meristem to show smaller ice structures associated with partially frost-dehydrated protoplasts (magnification 2× greater than lower left).

activity behaves as a component trait of winter hardiness (22).

Histological studies of ice structure and distribution within plant tissues are aided by use of low levels of opposition energy (such as sonic, infrared, or microwave radiation) to cause a range of abnormalities. Some stresses of crystal growth are relieved by inducing finer patterns of crystallization. However, adhesion effects can be intensified by bringing more protoplasts into association with ice lattices and coagulated polymers.

Nonequilibrium freezing

Low intensity freezing induces equilibrium water transitions (*11, 13*). This is distinguished from nonequilibrium transitions that occur when the freezing intensity is increased to a level where significant differences develop in the patterns of water transitions, ice structures, killing temperature, or pattern of injury within the plant (table 1).

High intensity freezing occurs in tissues that supercool more than 5° C or in plants at high moisture content that are in contact with such an effective heat sink as frozen soil. The latent heat of transition is dissipated rapidly. The relative hardiness of barley genotypes, especially in segregated breeders' lines, depends on the intensity of freezing.

TABLE 1.—*Forms of freezing stress that affect survival of winter cereals*

	FREEZING (Phase Association)		DESICCATION (Phase Separation)		EXTERNAL ICE
	NON-EQUILIBRIUM	EQUILIBRIUM	OSMOTIC	FROST (VAPOR)	
STRESS ENERGY	ΔG_{TRS}	$U_{ADHESION}$	π	Ψ	METABOLIC
$\Delta G \rightarrow 0$	W_I	ΔE_{LIMIT}	ΔN_L	ΔN_V	——
EFFECTIVE TEMPERATURE	SUPERCOOLED -5C	-10C	SOLUTE DEPENDENT	-20C	>-2C
INJURY	LOWER CROWN	ICE ASSOCIATED	PLASMOLYSIS	WALL SHRINKAGE	UPPER CROWN

NOTE. Stress energies develop as the free energy of water transition is dissipated; (ΔG_{TRS}) Free energy of transition; ($U_{adhesion}$) Potential energy of adhesion that draws ice and hydrophilic plant systems into matrices; (π) Osmotic activity; (Ψ) Water potential. Water redistribution occurs as ice forms until the ΔG_{TRS} is zero. ΔG is dissipated by irreversible work (W_I), shifts in activation limits of transitions (ΔE_{LIMIT}), or shifts in the density functions (ΔN_L = number of molecules in liquid at the ice interface) (ΔN_V = number of water molecules in a gas phase at the ice interface). "Effective temperature" is that which results in injury of hardened 'Hudson' barley leaf tissue frozen under various test conditions in which the different stress energies predominate. Each form of stress causes a unique pattern of injury. The most characteristic feature is indicated.

Winter hardiness involves a large number of traits and has been found to be complex both genetically and physiologically (*4, 21*). Components involve such nonadaptive traits as anatomical features as well as highly adaptive metabolic changes that occur as plants harden at low temperature (*13*). Relative hardiness of commercial cultivars involves traits that affect the stresses which develop as the temperature is lowered as well as the resistance of tissues to stress (*13*).

Stress Energies

Temperature is not an index of stress but is a measure of exchangeable kinetic energy—molecular motion that prevents potential bond energies from organizing water into a lattice structure. The potential energy is in balance with the kinetic energy at the freezing point. Stresses arise from potential energy interactions that become more effective as the temperature is reduced and draw water and other plant components into injurious configurations (*5, 8*).

With high crystallization energies, ice crystal growth can be destructive if the energy is opposed by tissue structures. Ice in a leaf does little damage when it only lifts the epidermis, but if crystals grow in a crown imbedded in frozen soil, significant injury can occur (*11, 19*). Crystallization energies can be determined from the water transition patterns by using the displacement of temperature, caused by supercooling (type I) or rapid heat transfer (type II), from equilibrium (fig. 6, p. 7).

With low crystallization energies, other interactions become important and effects of ice separated by a gas phase from wet tissue components are different from ice in the interface. If a system has equilibrated with ice, and then the temperature is lowered, crystallization energy develops. If the ice is separated by a gas phase, the crystal grows as long as the wet polymer system can provide water vapor. The free energy is dissipated as the vapor pressure drops. This does not happen in an ice-polymer interface—the free energy is dissipated by shifts in the activation energies of transitions of water from the polymer to ice (*16*). Activation limits of transition are quantitative descriptive traits and can be evaluated by thermal transition patterns.

Thermal transition patterns

The shift in activation energies reduces the latent heat of freezing. The latent heat of freezing, like the freezing point, is a variable function of the amount of liquid remaining (*17*). The net latent heat can be determined by coordinating the water transition data with thermal transition data. Nearly identical samples of plant tissue

can be obtained from the same genotype of barley grown and hardened in a controlled environment.

Thermal transition patterns that correspond to the water transition patterns discussed previously are obtained by microcalorimetry, following the same temperature pattern for both analyses. Coordinating this data gives the net latent heat of transition. It depends on the concentration of solutes and the energy with which liquid water is associated with plant polymer systems. As freezing progresses, the remaining liquid is more tightly bound, and by —10° C, the latent heat of release from a hydrophilic polymer is approximately the same as release from an ice crystal (*17*). Consequently, the net latent heat is small for freezing at this temperature and is similar to that for transfer of water between ice crystals.

The latent heat of association for water with such plant substances as the cell wall polymers can be obtained by subtracting the net latent heat from the latent heat of ice (*17*). The physical properties of water associated with hydrophilic polymers acting in opposition to ice are increasingly affected by interactions as the temperature decreases. Significant deviations exist below —10° C.

Stress vector analysis

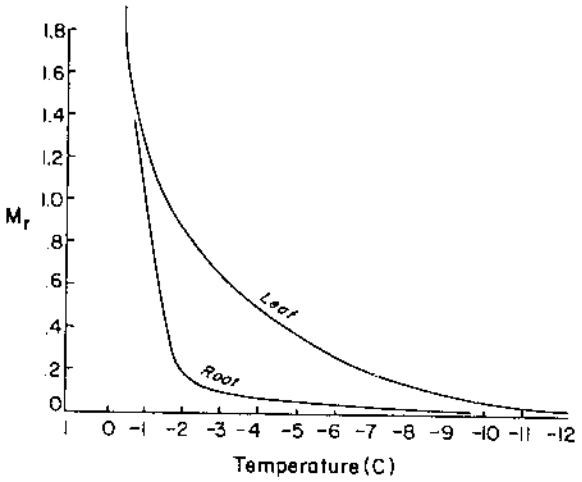
Effects of interaction in complex interfaces of hydrophilic polymers with liquid water and ice can be studied by stress vector analysis (*15*, *17*). Freezing patterns are evaluated for the same polymer system with ice throughout and with ice separated by a vapor phase. In the latter case, temperature and vapor pressure can be manipulated independently. Deviation in the water transition patterns are shown for cellulose in figure 3. Vector analysis helps distinguish matrix interactions that involve stresses from adhesion and interaction between ice and polymers from frost desiccation where ice merely acts independently as a water accumulator. The vapor pressure (or density of water in the gas phase) characterizes interactions of plant systems with liquid water when no interaction occurs.

Activation limits that must be overcome for water transitions to occur are affected in complex interfaces (*16*). Deviations in activation energies of transition characterize interactions of plant systems with liquid water as ice; plant polymers are drawn into closely associated interacting systems. The activation limits can be determined when the density function has been evaluated by vector analysis and the latent heat calculated by coordination of thermal and mass water transition patterns (*17*).

This interpretation of freezing stress data was derived by partition of chemical potential based on frequency distribution of exchangeable kinetic energy over the activation limits (*16*). It provides an expanded expression of transitional energy that consistently interrelates descriptive parameters (such as activation energy and density functions) of plant components that interact as freezing progresses. Screening tests have been developed for plant traits that affect these biophysical parameters of freezing stress (*18*). This expression of transitional energy also can be used to quantitatively evaluate and distinguish various forms of stress energy (table 1).

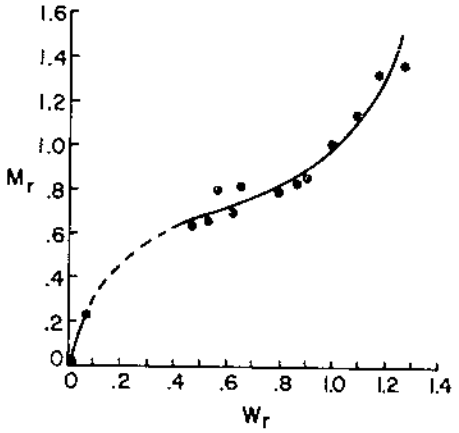
The energy of adhesion is determined by the decrease in the activation energy of melting caused by competitive interactions of equilibrium freezing (*17*, *18*). Because only the activation energy of melting shifts significantly as a function of temperature with equilibrium freezing, the adhesion energy approximately equals the shift in latent heat (*17*, *18*). Stable adhesions develop between —3° and —4° C as the adhesion energy becomes greater than the free energy of melting.

Ice crystals also organize solutes in the intercellular liquid by excluding them from the lattice and depositing them in planes between crystals or in tubules within crystals (*12*, *14*). Polymers such as the araboxylans tend to form a film on ice crystal surfaces and also can adhere to the protoplasmic membrane. Sublimation of the ice causes contraction of the polymer. This combination of events has been observed to be grossly destructive causing strands of protoplasm to be pulled out through breaks in the cell wall. It is a form of injury caused by ice in freeze desiccation that differs from drought injury. Cryoprotectants such as glycerol and sucrose tend to prevent adhesions of ice and polymer with the protoplast.



M_r Relative Content of Liquid Water in The Outer Free Space

FIGURE 2.—Relative content of liquid water along the cell walls (M_r) at equilibrium with ice in hardened 'Hudson' barley leaves and roots. The freezing point shifts as a function of the liquid content (average of five replications) (*11*).



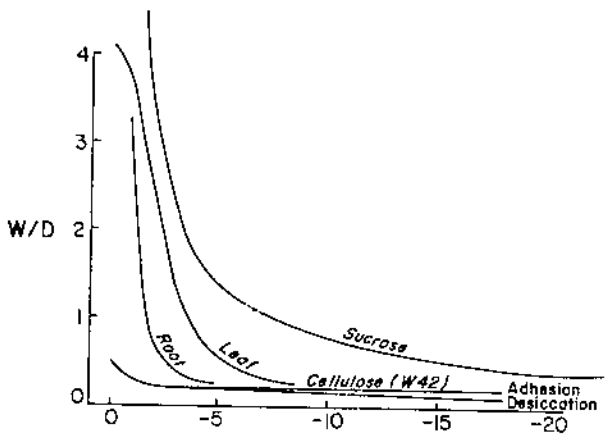
M_r Relative Content of Liquid Water in The Outer Free Space
W_r Relative Content of Total Liquid Water

FIGURE 4.—Relative content of liquid water along the cell walls (M_r) at equilibrium with ice in leaves of hardened 'Hudson' barley as a function of the relative content of total liquid water.

'Hudson' Barley Leaf— Reference for Stress Analysis and Response

The data obtained in a study of leaf tissue from hardened plants of 'Hudson' barley (*Hordeum vulgare* L.) are presented in figures 2-5. Hudson barley plants were grown from seed in sand culture with a Hoagland's nutrient solution for 6 weeks at 15° C in a plant growth chamber. They were hardened for 3 weeks at 2°. Only young leaves were used, and the transition patterns in these tissues were similar to those in the apical region of the crown (*11*, *13*). Transition patterns for cellulose and sucrose are shown for comparison (*20*, *24*). The shape of these transition patterns depends somewhat on the relation between ice and the liquid system.

Ice finely dispersed throughout the system raises the pattern slightly, whereas the pattern is depressed when ice is separated from the liquid containing system. Competitive interactions between ice and hydrophilic



W/D Total Liquid Water Per Gram of Dry Matter

FIGURE 3.—Total liquid water per gram of dry matter (W/D) in frozen leaves and roots of hardened 'Hudson' barley (average of five replications) compared with sucrose and ice adhesion vs. desiccation of cellulose (*17*, *20*, *24*).

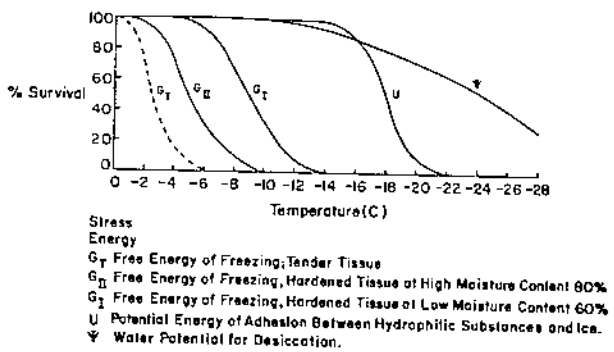


FIGURE 5.—Response of hardened leaf tissue of 'Hudson' barley to freezing stresses. Survival evaluated from percent of electrolyte retained (*3*). Data for free energy of freezing (G_T, G_I, and G_{II}) are the averages of 10 replications. Data for potential energy of adhesion (U) and water potential (ψ) were taken from "A comparison of freezing and desiccation as stress vectors" (*15*).

substances result in shifts in the activation limits for water transitions where adhesion matrices develop; only vapor pressure equilibrium is involved where the phases are separate (*16, 17*).

Figure 4 shows the relation between liquid water content of the outer free space and total liquid. These data were used to cross check the equilibrium transition patterns found as functions of temperature (figs. 2 and 3). Sudden changes in permeability of the protoplasmic membrane, caused by shock or injury, were avoided by slowly adjusting the moisture content of intact plants from 1.2 to 4.5 gram of water per gram of dry matter. The moisture contents of detached tissues were carefully adjusted through smaller ranges as water contents were assayed. Sudden release of protoplasmic contents causes the pattern in figure 4 to rise. This also is prominent in freezing patterns of tender tissues. Drifting patterns caused by more gradual shifts characterize some freezing processes (*13*).

The response of 'Hudson' leaf tissue to nonequilibrium freezing is presented in figure 5. The response to equilibrium freezing after a minimum nonequilibrium freeze also is presented. Equilibrium freezing must always start with a nonequilibrium freeze to provide energy for crystal growth along the cell walls so that phase association can be established. For studies of equilibrium freezing, this initial effect must not be injurious. The LD_{50} for frost desiccation, where the phases come to vapor pressure equilibrium while separated, was −25° C (*15*).

The relative liquid content in the outer free space and the relative total liquid content at equilibrium with ice throughout the system (phase association) are shown in figure 6. The supercooling required to induce 50 percent survival when freezing occurs at 4 and at 1.2 gram total water per gram dry matter also are illustrated with their nonequilibrium transition patterns. Major differences between transition patterns occur simultaneously in different tissues of a single plant. Large differences also occur between transition patterns that involve the protoplasmic liquid compared with the liquid of the outer free space. Small differences exist in the transition patterns that are determined by whether the ice is associated with other phases of water in the tissue or is separated from them. Generally, the more abrupt the transition, the more severe nonequilibrium stresses are apt to develop.

For a given degree of supercooling or rate of temperature change in a heat sink, faster heat transfer occurs from a unit of freezing tissue with an abrupt pattern because of greater latent heat from the larger number of water molecules involved. At high moisture contents, more freezing energy tends to develop in root

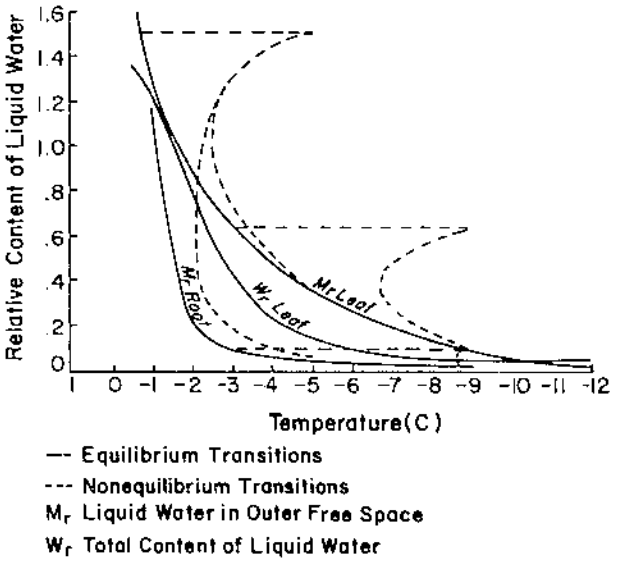


FIGURE 6.—Relative liquid content of outer free space (M_r) and relative total liquid content (W_r) at equilibrium with ice. Also, freezing kinetics patterns in plant tissues with the leaf moisture content at 4 and at 1.2 gram water per gram dry matter. The temperature displacement of the nonequilibrium transition pattern from the equilibrium transition pattern can be used to calculate the energy of crystallization (*12, 16, 17*). Freezing occurs in two phases when it initiates after supercooling. In phase I, the latent heat dominates thermal diffusion and causes the temperature to rise. In phase II, thermal diffusion dominates and the temperature returns to that of the heat sink.

tissues than in leaves. At low moisture contents, more energy tends to develop in leaves.

Freezing of a supercooled system has two phases. In the first phase, the temperature rises above the sink temperature as water freezes rapidly. In the second phase, the temperature returns to the sink temperature while water freezes more slowly with less free energy. The free energy per mole of water is approximately 5.2 times the temperature displacement from equilibrium (*16*). The temperature displacement for the second phase mainly depends on thermal conductivity to the heat sink. The displacement is approximately 15 times the rate of temperature change, though inhibitors of freezing kinetics affect this relationship (*12*). At low moisture contents, equilibrium freezing cannot be studied.

The supercooling required to initiate freezing in the outer free space results in simultaneous injury to the protoplasts. Studying equilibrium freezing in plant tissues is difficult at high moisture content. When the freezing energy per mole is sufficiently high to establish good phase association, a large amount of water may freeze very rapidly. In the laboratory this can be

avoided by regulating thermal conductivity. However, in normal field conditions, the thermal contact with the frozen soil heat sink usually is good and injury accompanies the initial freeze (*13*).

The response of 'Hudson' barley to stress patterns is shown in table 1. The free energy of transition per mole of water required to induce 50 percent survival increases from nonequilibrium freezing on the left to frost desiccation on the right. However, the time in which the energy acts involves seconds for nonequilibrium freezing, minutes for equilibrium freezing, and hours to days for plasmolysis and frost desiccation. So, the intensity required (power per area of cell membrane) is high for nonequilibrium freezing, diminishes for equilibrium freezing, and is least for frost desiccation.

The response curve can be expressed as a linear regression and the slope describes the uniformity of response (*6*, *7*). Probit analysis showed that cells of hardened 'Hudson' barley leaf tissue responded more uniformly to freezing stresses that involved direct interactions with ice than they did to frost desiccation.

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