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# FROST AND CHILLING INJURY TO GROWING PLANTS

H. F. Mayland and J. W. Cary

Snake River Conservation Research Center,
Soil and Water Conservation Research Division,
Agricultural Research Service,
U.S. Department of Agriculture, Kimberly, Idaho

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## I. Introduction

Freezing injury in plants represents a major economic loss to agriculture. Reingold (1960) reports crop losses in the United States resulting from cold weather as follows:

| Crop | Percentage loss |
|---|---|
| Almonds | 10 |
| Apples | 8 |
| Citrus | 8 |
| Stone fruits | 10 |
| Cereals | 3–4 |
| Strawberries | 30–40 |
| Grapes | 10 |

Another survey of the crop-freeze problem was conducted in the contiguous United States for the period 1963 to 1968 (Prestwich, L., "Freeze Damage to Crops," unpublished research work, U.S.S. Agr. Chem., 1969). He found that production of an estimated 3.6 million acres of cropland was destroyed annually by freezing, and that lost production was valued at 341 million dollars per year (Table I).

TABLE I
Average Annual Crop Freezing Losses for Years 1963–1968, Continental United States[a]

| | Freezing losses | | | |
|---|---|---|---|---|
| | | | Loss relative to all crop losses | |
| Crop | Acres (millions) | Dollars (millions) | % of acreage | % of value |
| Fruits | 0.45 (15)[b] | 215 (12.0) | 12 | 63 |
| Vegetables | 0.30 (6) | 58 (2.5) | 8 | 17 |
| Field crops | 2.90 (1) | 68 (0.4) | 79 | 20 |

[a] Prestwick, L., unpublished research work, U.S.S. Agr.-Chem. (1969).
[b] Data in parentheses are percentage of total crop acres or dollar value lost.

While the above data represent losses resulting from ice-induced injuries, there may also be crop production losses caused by low temperature which go unnoticed and are thus unaccounted for. For example, Kuraishi *et al.* (1968) reported that unhardened pea plants were killed at −3°C without ice formation. In addition to losses that are directly attributable to ice formation in plants, there are other yield-reducing factors that may be attributed indirectly to cold temperatures. Plants such as cotton, peanuts, and other tropical species may be permanently injured by cool temperatures of 0 to +10°C (Sellschop and Salmon, 1928). Majumder and Leopold (1967) have reported that callose plugs form in or along the phloem sieve tubes and that this contributes to the low temperature responses of some species. Xylem elements of fruit trees may be permanently occluded by exposure to freezing temperatures (Daniell and Crosby, 1968). Restricted water movement resulting from xylem vessel occlusions limits tree growth and fruit yield and plays a role in peach tree decline.

Early research on freezing phenomena in plants centered on plant selection and classification according to their ability to become cold

hardy and survive freezing temperatures. Recent work has centered on the differentiation between plants with or without the ability to harden.

Perhaps the most fascinating problems are yet to be encountered in the study of cold stress and freezing in nonhardy plants. This includes the varying ability of plants to survive cold temperatures, as during the seedling establishment of corn, beans, and sorghum or during vegetative growth of legumes and pollination and flowering of horticultural plants and small grains. These so-called "nonhardy" plants have, therefore, been subdivided into tender and resistant types in various geographical areas. For example, beans, corn, and peach blossoms in temperate climates may be considered as "tender" crops, while peas, lettuce, and sugarbeets are more cold resistant, although none of these plants are thought of as having the ability to become cold hardy, as do winter wheat and many perennials.

Research on the conditions associated with plant adaptation to cold temperatures has been carried on for nearly a century, and excellent discussions of cold hardiness may be found elsewhere (Levitt, 1956, 1966b, 1967). Smith (1968) summarized the inability of past cold-hardiness studies to satisfactorily associate changes in plant constituents with frost tolerance. He reported that, "... although differences in chemical changes during cold-hardening exist among species, there is still a question as to whether these alterations in plant metabolism are intimately involved in the development of frost hardiness or whether they are merely associated changes." Recent approaches using biochemical techniques are providing definitive evidence of an enzyme system (peroxidase isozyme components) showing major response to cold temperature stresses by plant tissues capable of cold-hardening (McCown *et al.*, 1969a,b).

When hardened plant material is cooled slowly, ice first forms in the extracellular space (Levitt, 1956). The equilibrium vapor pressure of ice is less than that of pure liquid water at any given temperature below 0°C. Thus as the water in the extracellular space freezes, the chemical potential falls below that of the cell sap, and water diffuses from the cells through the semipermeable membrane. The cells become freely permeable, perhaps because of rupture of the plasma membrane by ice crystals when intracellularly frozen, or simply from disruption of the normal structure of the plasma membrane.

Protoplasm may be injured by freezing in two ways—dehydration and mechanical strain. Within certain limits, dehydration is injurious only in conjunction with mechanical strain because dehydration increases the consistency of the protoplasm. The protoplasm is thus more brittle and more liable to rupture under the action of the deforming stress. Super-

imposed upon the above types of injury is the action of concentrated electrolytes within the cell. When dehydration exceeds a certain limit, the increased consistency becomes irreversible and must be regarded as a form of coagulation. Such coagulation is frequently an irreversible colloidal change. Cell death may not result immediately upon coagulation, but eventually membranes rupture and other macromolecules are irreversibly denatured and freezing injury results. This picture of the freezing process, however, does not explain the mechanics of injury nor does it describe cold stress phenomena.

Ice formation and freezing injury in plants have been previously reviewed (Luyet and Gehenio, 1940). Levitt (1956, 1967) has published extensive reviews of factors associated with cold hardiness of plants. Redistribution of water in winter cereals and the subsequent effect of freezing stresses on plant survival were reviewed by Olien (1967a). Idle and Hudson (1968) and Scarth (1944) presented a limited discussion on chilling injury and the physical effects occurring during ice formation in plants. Mazur (1969) discussed concepts, experimental approaches and results of tissue preservation by freezing and relates these to botanical oriented freezing studies. The discussion presented here concentrates on the effects of low-termperature stress on cell membranes and other macromolecules in the cell and relates these to the overall plant response to chilling or frost injury.

## II. Physicochemical Principles of Protein Structure

### A. Structural Requirements

Proteins must be flexible to accomplish their biochemical functions associated with conformational changes. Protein flexibility is provided by weakening or strengthening of intramolecular bonds that maintain secondary and tertiary structure. When temperatures decrease, macromolecules become excessively rigid or brittle, and thus inactive.

The primary structure of proteins is chemical valence bonding in a sequence of amino acids and disulfide bonds. The secondary structure is the polypeptide-chain configuration (series of amino acids) yielding H-bonding between peptide, N—H, and C=O groups. Tertiary structure is the pattern of packing of the secondary structures.

### B. Bonding

#### *1. Types*

Kauzmann (1959) lists seven types of intramolecular bonds that might

be expected to influence the polypeptide chain configuration. These are:

a. Hydrogen bonds between peptide linkages
b. Hydrophobic bonds
c. Salt linkages (ion pair bonds) and other electrostatic forces
d. Hydrogen bonds other than those between peptide links
e. Stabilization by electron delocalization
f. Dispersion forces (London forces), protein chemist's term of secondary bonding
g. Disulfide groups and other cross linkages

H-bonds and hydrophobic bonds are likely to have the most important functions because of the relatively large number of peptide and hydrophobic groups in nearly all proteins. The H-bond is suited to play an important role in physiological processes because of the small bond energy (Table II) and small activation energy involved in its formation and rupture (Pauling, 1960). Many protein properties depend on configurations present in localized regions of the molecule, and these configurations might be determined by some less abundant types of bonds. It is not really safe to say that any of the bonds are "less important than others" except that salt linkages are not prominent contributors to the stability of proteins (Kauzmann, 1959; Matsubara, 1967).

TABLE II
Bonding Energies in Kilocalories per Mole[a]

| Bond | Experimental | Calculated |
|---|---|---|
| C—C | 68 | 64 |
| C—N | 65 | 53 |
| C—S | 52 | 57 |
| S—S | 48.5 | 50 |
| H-bond | – | Generally 2–10 |
| Hydrophobic | – | Less than 3 |

[a] The value of hydrophobic bonding energy is a function of the nonpolar groups involved and also temperature, decreasing with a reduction in temperature (Nemethy and Scheraga, 1962b). Other data are from Levitt (1962 Copyright © 1962 Academic Press, New York).

### 2. *Hydrogen Bonding between Peptide Links*

Recent research on protein hydration has demonstrated the close interaction between the hydration shell surrounding protein molecules and the physicochemical properties of the proteins themselves (Bernal, 1965). The hydration shell consists of several layers of water molecules

in an icelike sheath surrounding and linking the protein molecules (Bernal, 1965; Nemethy and Scheraga, 1962a,b). Structure is considered essential for maintaining protein properties and functions. Any alteration of this water structure would result in changes in both the secondary and tertiary protein structures and would be defined as denaturation (Kauzmann, 1959). Such changes prevent proteins from functioning properly because of steric incompatibilities with coenzymes.

### *3. Hydrogen Bonding Other Than Those between Peptide Linkages*

Examples of H-bonding apart from peptide linkages in proteins include carboxylate ion to the phenolic hydroxyl of tyrosine, carboxylate and hydroxyl of threonine or serine and the carboxylate ion and the thiol group of cysteine (Kauzmann, 1959). The energy of this H-bond type is much less than that of the H-bond between two peptide groups. Nonpeptide H-bonds may modify properties of dissociable groups. However, it does not seem likely that nonpeptide H-bonds make a major contribution to the stability of native proteins.

### *4. Hydrophobic Bonding*

The role of the hydrophobic bonds or hydrophobic regions of protein molecules (Fig. 1) has received increasing attention in recent years. Nonpolar side-chain groups of protein molecules modify the water structure in their neighborhood in the direction of greater "crystallinity" (Shikama, 1965b). Nemethy and Scheraga (1962b) consider the hydrophobic bond formation in a protein to consist of two processes: (1) two or more nonpolar side chains which are surrounded by water come into contact, and (2) thereby decrease the total number of the water molecules around them. Hydrophobic bonds play a unique role in stabilizing

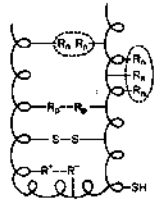


FIG. 1. Schematic representation of a protein molecule, especially showing interactions between side-chain R groups in an aqueous solution. The $R_p$ and $R_n$ represent polar side-chain R groups and nonpolar side-chain groups, respectively. In this model the hydrophobic bonds are pictured with a lattice-ordered layer of water around them, as shown by broken lines.

native protein conformation since these bonds are a function of the water structure around the nonpolar group (Shikama, 1965b; Nethey and Scheraga, 1962b).

Nonpolar amino acids constitute 35 to 45% of proteins (Shikama, 1965b). Examples of these nonpolar side chains are: the methyl group of alanine, the isopropyl group of valine, the isobutyl group of leucine, the *sec*-butyl group of isoleucine, the benzyl group of phenylalanine, and the methyl mercaptan group of methionine (Shikama, 1965b). These nonpolar side chains have a low affinity for water. The polypeptide chain configuration in proteins, which brings large numbers of these groups into contact with each other, removes them from the aqueous phase. This configuration is more stable than others, all other things being equal (Kauzmann, 1959).

### 5. *Disulfide Bonds*

Disulfide bonds (SS) consist of the intramolecular cross linkages by cystine or phosphodiester links. When this type of bond is located in the macromolecular chain, it is impossible for the chain to fold into less stable configurations (Kauzmann, 1959).

### 6. *Other Bonding Types*

The effect of electrolytes and nonelectrolytes will probably depend on the degree to which they cause reorientation of the structured water surrounding the macromolecule. Small, strongly polar molecules, having strong hydrogen bonding characteristics, may break down the highly structured water envelope. Binding of small organic molecules may have strong binding affinity on the inside of the protein helix. Urea molecules, for example, are bound to peptide bonds which normally would be buried within the protein molecule, but protein becomes denatured following the bonding change resulting from the action of the urea molecule (Kauzmann, 1959). Some ions may help to stabilize the protein structure and protect it against denaturation caused by other agents (Boyer *et al.*, 1946a,b).

## C. Inactivation and Denaturation

The overall integrity of protein structure depends on both apolar (hydrophobic) and polar (H-bonding) interactions. Changes in the bonding may induce changes in the protein molecule which result in denaturation and loss of activity. Denaturation, although having a number of definitions, will be used here as "a process(es) in which the spacial

arrangement of the polypeptide chains within the molecule change from that typical of the native protein to a more disordered arrangement" (Kauzmann, 1959). Denaturation may occur when H-bonding is broken, or when hydrophobic bonds are displaced. Bello (1966) has shown that hydrophobic denaturants are effective in disrupting deoxyribonucleic acid (DNA) structure.

The hydrophobic bond is of prime importance in the stabilization of the native protein conformation at normal physiological temperatures. As the temperature is lowered, however, hydrophobic effects become weaker and hydrogen bonds more stable. The effects expected may be: (1) denaturation resulting from disruption of hydrophobic regions, (2) structure stabilization resulting from hydrogen bond stabilization, or (3) denaturation and formation of a new hydrogen-bonded conformation (Bello, 1966) or disulfide bridge (Levitt, 1966b).

An example of the latter is Kavanau's hypothesis (see Langridge, 1963). He proposes that some enzyme inactivation, such as phosphatase and peroxidase at low temperatures (ca. − 10°C), is attributable to an increase in intramolecular H-bonding so that active centers lose their specific configuration. Stability may also result from disulfide bonds or cystine bridges which are found in some heat-stable enzymes. The heat-stable enzyme thermolysin does not have cystine bridges but must obtain its stability from hydrophobic interaction and perhaps, in addition, metal chelation (Matsubara, 1967).

Sulfhydryl (SH) and disulfide (SS) groups help maintain the primary structure of proteins and control of the enzyme activity. Since changes in the steric conformation of proteins may be affected by freezing and thawing (Levitt, 1966a), it follows that these groups may also be involved in the physiological processes that accompany the changes in water activity (Tappel, 1966). Measurements of the SH and SS contents of plants before and after freezing have indicated a conversion of protein SH and SS when the freezing resulted in killing, but not when the plants survived uninjured (Levitt, 1962). Similar results were obtained with injury by heating. On the other hand, when plants of different hardiness were compared, a positive correlation was found between SH content and resistance to freezing injury. Plants incapable of hardening at low temperatures also showed a marked increase in SH at hardening temperatures, but only if permitted to wilt (Levitt *et al.*, 1961).

Levitt (1962) therefore proposed a hypothesis which assumes that ice forms extracellularly when a plant is frozen and the water that separates the protoplasmic proteins moves to these extracellular ice loci, thus causing the cell to dehydrate. At a certain degree of dehydration, which

varies with the plant resistance to freezing injury, the SH and SS groups of adjacent protein molecules would approach one another closely enough to permit chemical reactions to occur (see Levitt, 1962). The reaction could be of two kinds: an oxidation of two SH groups to SS, or an SH ⇄ SS interchange reaction. In each case the result would be an intermolecular SS bond.

Since the SS bond is covalent, it is far stronger than the hydrogen of hydrophobic bonds (Table II) which are responsible for much of the tertiary structure of the protein. Consequently, when thawing occurs and water reenters the protoplasm, pushing the proteins apart, the newly formed SS bonds remain intact, whereas many of the weaker hydrogen and hydrophobic bonds are broken by the stresses, and protein molecules then unfold or denature. If the intermolecular SS bond forms by SH ⇄ SS interchange, the unfolding could occur during the freezing process since an intramolecular SS bond would be broken. If a sufficient number of intramolecular SS bonds are formed, the unfolding would lead to protein denaturation and cell death.

The above hypothesis seems to fit many natural conditions and provides a useful explanation of injury (Levitt, 1967). A study of desiccation injury in cabbage leaves supports Levitt's sulfhydryl-disulfide hypothesis (Gaff, 1966). Structural protein extracted from cabbage leaves displayed an apparent unfolding at water potentials less than −40 bars. The degree of unfolding increased with increasing disiccation until cell death occurred at −94 bars water potential. Direct evidence is still lacking to support the sulfhydryl hypothesis of freezing injury. Trials to visualize tissue bound SH groups by electron microscopy have given only equivocal results (Pihl and Falkmer, 1968). Addition of SH-containing compounds (i.e., cysteine and glutathione) to chloroplast systems has failed to provide protection against freezing (Heber and Santarius, 1964). Krull (1967), however, reports conclusive evidence that frost resistance in epidermal cells of red cabbage is increased by mercaptoethanol, which alters disulfide content of proteins. Addition of nonpenetrating sugars protected epidermal cells of red cabbage, but no evidence was obtained for the protection of surface SH groups on cell wall membranes by the sugars (Levitt and Haseman, 1964). It was concluded that the protection must, therefore, be internal to the cytoplasmic proteins.

The SH groups of proteins are of considerable chemical interest since they are the most highly reactive of the amino acid side chains. The SH groups have a varying reactivity, which is as yet unexplained except for some broad steric possibilities (Battell *et al.*, 1968). Some proteins do not contain disulfide bridges. One such protein is glycogen phosphorylase,

which can have two sulfhydryl groups per mole of enzyme bound without loss of enzymatic activity. A second class of sulfhydryl groups in the same protein when bound by amperometric titration results in complete loss of enzymatic activity and denaturation. The first two SH groups must be fully exposed on the enzyme surface, allowing the possible disulfide bond formation between phosphorylase monomers, which then results in intermolecular disulfides connecting enzyme molecules into large aggregates. Upon protein denaturation, another class of sulfhydryl groups will be exposed; the number depends upon conditions, but will include as many as 12 more SH groups per mole (Battell *et al.*, 1968).

## D. "Bound" Water

### 1. *Definition*

Current usage in cryobiology loosely defines "bound" water as that which does not freeze (Meryman, 1966). The energy status of this water is shown in Table III. There is little doubt that biochemical systems contain liquid water at subfreezing temperatures, and that the amount of this bound water (Fig. 2) decreases with temperature (Levitt, 1956; Toledo *et al.*, 1968) and/or with molecular denaturation (Pichel, 1965).

TABLE III
Vapor Pressure versus Temperature for Water and Ice and the Corresponding Vapor Pressure Potential of the Water[a]

| Temperature (°C) | Aqueous vapor pressure | | Potential | |
|---|---|---|---|---|
| | Ice (mmHg) | Water (mmHg) | Joules $kg^{-1}$ | −Bars |
| 0 | 4.579 | 4.579 | 0 | 0 |
| −1 | 4.217 | 4.258 | −1213 | 12 |
| −2 | 3.880 | 3.956 | −2426 | 24 |
| −3 | 3.568 | 3.673 | −3620 | 36 |
| −4 | 3.280 | 3.410 | −4827 | 48 |
| −5 | 3.013 | 3.163 | −6012 | 60 |
| −6 | 2.765 | 2.931 | −7188 | 72 |
| −7 | 2.537 | 2.715 | −8326 | 83 |
| −8 | 2.326 | 2.514 | −9465 | 95 |
| −9 | 2.131 | 2.326 | −10,675 | 107 |
| −10 | 1.950 | 2.149 | −11,807 | 118 |
| −15 | 1.241 | 1.436 | −20,861 | 209 |

[a] Assumptions are: atmospheric pressure and ice and water at vapor pressure equilibrium.

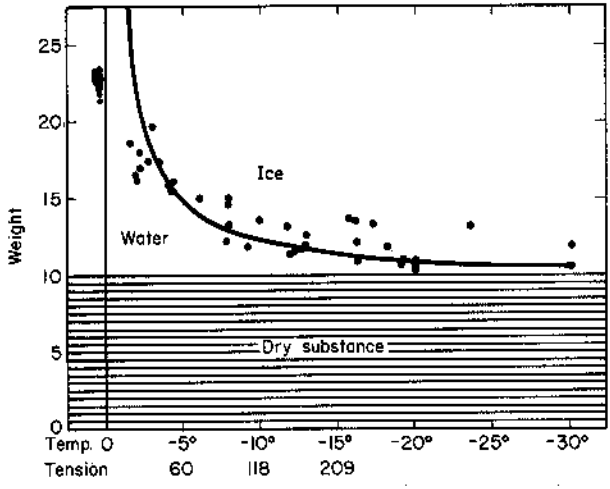


FIG. 2. Progressive ice formation with decreased temperature in the lichen *Cetraria richardsonii*. Temperature in degrees centigrade and tension in bars (from Table III) at the ice–water interface under equilibrium conditions. (From Levitt, 1956 Copyright © 1956 Academic Press, New York.)

### 2. *Experimental Evaluation of "Bound" Water*

Microorganisms maintain about 10% of their total water in a nonfrozen state at −20°C (Mazur, 1966). This 10% residual water in cells is not normal supercooled water, but is water bound to cellular solids by forces of varying strength. Even at nonfreezing temperatures, sharp distinctions cannot be made between wholly "free" water or liquid water which at one extreme is totally unengaged in relationships other than with itself, and the other extreme to totally "bound" water which is active in determining secondary or tertiary macromolecular structure. Some progress in measurement of bound water appears to be possible, utilizing nuclear magnetic resonance (NMR) spectroscopy. Toledo *et al.* (1968) were able to measure the bound water content of wheat flour dough with good precision, for a given temperature, such as −18°C, regardless of total water content. Considerable progress has already been made in defining protein hydration characteristics at freezing temperatures. Kuntz *et al.* (1969) reported the hydration of proteins and nucleic acid solutions at −35°C to be 0.3–0.5 g of water per gram of protein. Nucleic acids were three to five times more hydrated than proteins. It is well to point out that high-resolution NMR spectra analysis shows that the "bound" water is not "icelike" in any literal sense, although it is clearly less mobile than liquid water at the same temperature. There is a remote possibility

that this "bound" water may be related to "anomalous" or "poly water," which is receiving much current attention (Lippincott *et al.*, 1969).

Attempts have been made to differentiate between the physical properties of cytoplasmic protein-water extracts of cold-hardy and nonhardy plants (Brown, J. H., Bula, R. J., and Low, P. F., unpublished information, Purdue University). Essentially no differences were found in the apparent specific heat capacities, ice nucleating abilities, or the amount of water absorbed to the dry protein. Partial specific volumes were similar, but showed increases as plants were exposed to decreasing temperatures.

### 3. *Chemical Potentials*

All the water in plants first supercools and then begins to freeze, generally in the extracellular space, as the temperature is lowered under "equilibrium" conditions (rate $\leq$ 1°C per minute). The liquid water remaining within the cell is subjected to a lesser change in chemical potential than that surrounding the ice crystal outside the cell (Table III).

Dehydration of cellular protoplasm occurs during freezing in response to gradients in water energy. The vapor pressure gradient caused by extracellular freezing may be used to estimate the driving potential for water flow only if temperature and electrical gradients are negligible. As ice crystals grow in an aqueous solution, the solutes tend to be largely excluded from the crystal, and thus they concentrate in the solution. If specific ions are present in the solution, particularly $F^-$ and $NH_4^+$, ($F^-$ and $NH_4^+$ are highly toxic and generally not present in plants) a preferential trapping of ions in the crystal can occur, resulting in potentials of 20–30 V or more between the crystal and the solution (LeFebre, 1967). While this has not been measured in plants, it could conceivably enter into the reactions that take place in the bound water and membrane regions during freezing. Since freezing releases heat, it is also possible that signifficant thermal gradients develop across cell walls and membranes.

The technique of atomizing microorganism cells in $O_2$-free atmospheres of known relative humidity has been used to study "bound" water. Organisms thus exposed rapidly lose 90–95% of their total water content, but the remainder is less easily lost. Webb (1965), using this aerosolization method, reported that the death rate was directly related to the amount of "bound" water removed from these cells (Fig. 3). Thermodynamic analysis of the death rates obtained during two periods (0 to 1 hour and 1 to 5 hours) and a wide range of temperatures indicated that death results from a tightening of molecular structures and is associated with

relatively small activation energies (Webb, 1965). Very few deaths occur at above 70% relative humidity (RH), (corresponding to water potential of −130 bars at 20°C or a temperature effect of −10°C), but a sudden increase in the cell's sensitivity occurs as the RH is lowered further.

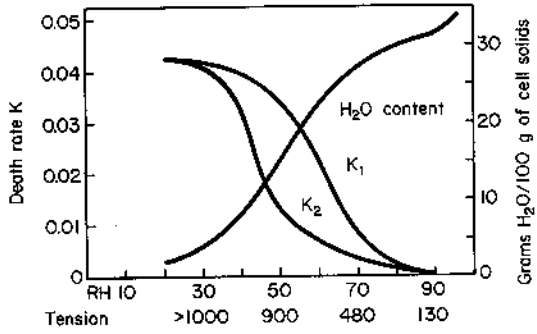


FIG. 3. The effect of relative humidity (RH) on the water content and death rates of *Serratia marcescens*. Death rate $K = \ln N_t/N_0$ with $K_1$ representing the time interval between 0 and 1 hour, while $K_2$ represents interval of 1 to 5 hours. *S. marcescens* ordinarily has 400 g of water per 100 g of solids. Data were taken at 25°C. Tension (water potential) is in bars, as taken from Table III. (From S. Webb, 1965, "Bound Water in Biological Integrity," Thomas Springfield, Ill. with permission.)

## III. Cold Lability of Enzymes

### A. *In Vitro* EVIDENCE

The main factor contributing to protein denaturation by freezing and thawing is the change in water structure around the native protein molecule during freezing and thawing. Shikama (1965b) has shown that there is a critical temperature region in which catalase and myosin are denatured during freezing and thawing. Denaturation begins at −12°C for catalase and −20°C for myosin. The double-stranded helical structure of DNA is not broken down by freezing (0 to −192°C) and thawing (Shikama, 1965a). Infrared spectroscopy of DNA, however, showed that structural changes occurred in the molecule which corresponded to the water activity where microorganism viability was lost (Webb, 1965). X-ray analysis of the water remaining on the macromolecule suggested that water reorientation also occurred (Webb, 1965). Although there may be several different DNA enzyme to water interactions, Cox (1968) has suggested that loss of the water layers from the DNA molecule produces a biologically inactive moiety by semireversible formation of a hydrate. Some enzymes are not inactivated by freezing and thawing. Two of these

enzymes are invertase and sucrose phosphorylase (Barskaya and Vichurina, 1966). Glycogen phosphorylase *b*, in contrast to phosphorylase *a*, loses its enzymatic activity at 0°C (Graves *et al.*, 1965). Pyruvate carboxylase is rapidly inactivated by exposure to low temperature, but the enzyme inactivation is at least partially reversible by rewarming (Scrutton and Utter, 1964).

Heber (1967) and Heber and Santarius (1964) considered dehydration of the adenosine triphosphate (ATP) synthesis system by freezing as responsible for its inactivation. This may occur above −8°C (Borzhkovskaya and Khrabrova, 1966), but dehydration may be more complete at −18° to −25°C (Ivanova and Semikhatova, 1966). Removal of functional water from the membrane system to the growing ice crystals apparently leads to the uncoupling of the phosphorylatory system from electron transport in the case of photosynthetic phosphorylation and, in other cases, to related effects (Heber and Santarius, 1964).

### B. *In Vivo* Evidence

The *in vitro* evidence for cold lability of enzymes is further supported by Ng (1969), who concluded that the decrease in cell yield of *Escherichia coli* with decreasing growth temperature resulted from the uncoupling of energy production from energy utilization. Stewart and Guinn (1969) observed a decrease in ATP with chilling of cotton seedlings at 5°C and concluded that oxidative and photophosphorylation were more sensitive to low temperature inhibition than systems that use ATP. The close association of both enzyme and membrane sensitivity to low temperature is reinforced here. The inner mitochondrial membrane contains the entire electron transfer chain as well as the enzymes of oxidative phosphorylation (Green and Tzagoloff, 1966). Kuiper (1969a) postulated that membrane ATPase is sensitive to denaturation by freezing. He (Kuiper, 1967) reported that potato ATPase was cold labile except when treated with compounds such as $10^{-3}$ *M* 1,5-difluoro-2,4-dinitrobenzene, which was found to increase water permeability of bean root cell membranes and to afford considerable protection of bean plants against freezing damage. Pullman *et al.*, (1960) also reported ATPase to be cold labile and inactivated at temperatures of 4°C. McCarty and Racker (1966), in searching for coupling factors for photophosphorylation, reported cold lability at ATPase activity. This loss of activity at 0°C was accelerated by salts and was pH dependent. Cyclic photophosphorylation of intact and broken chloroplasts isolated from frozen and unfrozen leaves of winter wheat and spinach was examined by Heber and Santarius (1967). Living and frost-killed leaves were supplied with radioactive sucrose, and in both

cases this sucrose was converted into a number of organic compounds including organic acids. It was concluded that the destruction resulting from freezing of the phosphorylation reactants (which provide the energy necessary to maintain life) takes place *in vitro* and *in vivo*.

Freezing and/or freeze-drying is a common method for the long-term preservation of animal viruses and may be a satisfactory method for such plant viruses as tobacco mosaic, southern bean mosaic, tomato bushy stunt, and others (Kaper and Siberg, 1969). Turnip yellow mosaic virus, however, is structurally injured by *in vitro* freezing of its water solutions and is completely degraded into its RNA, which remains intact, and its protein component, which becomes predominatly fragmented (Kaper and Siberg, 1969).

The temperature at which an enzyme is denatured by heat can be significantly increased for certain enzymes if they are preconditioned by exposure to increasingly higher temperatures. Similarly, conditioning of bean plants (*Phaseolus acutifolius*, var. Tepary Buff) to cool temperatures tended to increase the heat stability of the extracted malic dehydrogenase (Kinbacher, E. J., unpublished, University of Nebraska).

Very strong contrasts to enzyme denaturation at subzero temperatures may be found in nonequilibrium freezing (lowering of temperature at rates in excess of 10–100 centigrade degrees per minute) studies of single cells as well as of higher plants (Doebbler *et al.*, 1966).

## IV. Membrane Composition and Permeability

### A. Description

Cellular membranes must also be considered in any discussion of freezing injury in plants. Nearly all cells killed by freezing and thawing show membrane damage (Mazur, 1966). Water movement from the cell to the extracellular space during slow freezing was previously discussed. This freezing process does not always kill the plant. Figure 4 illustrates the rate at which the supercooled water in plant cells (yeast) would be expected to equilibrate with the external frozen water by dehydration of the cellular constituents (Mazur, 1966).

In addition to water movement across cell walls, we must consider water movement across organelle membranes, such as for mitochondria and chromosomes. Some correlation has been found between structural alteration of certain organelle membranes and associated enzyme activity as a function of freezing rate (Sherman and Kim, 1967). These authors pointed out that there are differences in the reaction and resistance of various organelles to ice formation and dissolution in and around them.

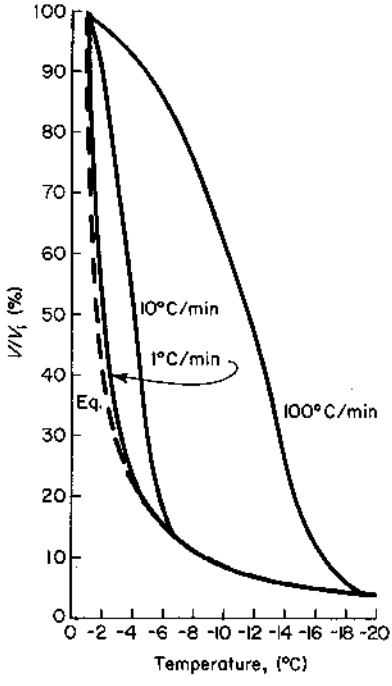


FIG. 4. Calculated percentages of supercooled intracellular water remaining at various temperatures in yeast cells cooled at indicated rates. Where $V$ = volume of supercooled water in cell, and $V_1$ = initial water in cell. The dashed line represents equilibrium conditions. (From Mazur, 1966 Copyright © 1966 Academic Press, New York.)

Thus, the damage at the cell membrane surface may cause a decrease in the capacity of the living protoplasmic membrane to serve as a barrier against ice inoculation into the cell. Freezing injury in nonhardy plants has been observed to result from a disruption of the diffusion barrier by intracellular ice formation and subsequent mechanical rupture which exposes cellular contents to the freezing site (Olien, 1961). Sakai and Yoshida (1968) concluded that freezing injury in cabbage cells resulted from disruption of the plasma membrane which is a very important structural component of the cell.

Electron microscopy shows the cell membrane to be highly ordered. In almost all plant cells this membrane consists of a layered material approximately 75 Å thick. Two dark electron-dense areas, each being about 25 Å, are separated by a light layer. Stein (1967) represents the membrane schematically as a sandwich containing a bimolecular lipid center with polar groups on the exterior side. The paraffinic lipid sectors are bonded

primarily by hydrophobic bonding (Green and Tzagoloff, 1966) to polypeptide chains or mucoproteins which make direct contact with the aqueous exterior or interior of the membrane system.

### B. Composition

Chemical analyses show high concentrations of phospholipids, cholesterol, and protein. The lipid composition is species-dependent. The protein mass may be two to three times that of the lipids. Many of these proteins are enzymes, such as ATPase and acetylcholinesterase. The phospholipids are composed of a large hydrophilic phosphate ester grouping. It is expected that the hydrophilic groups of the phospholipid will be preferentially situated in the aqueous interface and the hydrophobic fatty acid chains will interlock with one another. The lipid composition may determine the membrane permeability (Christophersen, 1967). This is supported not only by an increase in the fatty acid content in hardened plants, but also by a preferential accumulation of polyunsaturated fatty acids, especially linoleic and linolenic fatty acids (Gerloff *et al.*, 1966). Insects and microorganisms, in addition to higher plants, contain increased proportions of unsaturated fatty acids, or more highly unsaturated fatty acids, if they are grown at low temperatures (Chapman, 1967).

This is further supported by Kuiper (1969b), who reported that applying galactolipids to fruit flower buds increased resistance of flowers to freezing as tested 2–3 days later. Application of other lipid types resulted in decreased resistance. This relationship demonstrates the importance of lipids for water transport across membranes and for membrane stability against freezing. Siminovitch *et al.* (1968) reported increases in polar lipids (principally phospholipids) and lipoproteins without changes in total lipids in living bark cells of the black locust tree during the development of extreme freezing resistance.

Damage to the lipoproteins occurs when the last traces of water are removed as ice so that the lipoprotein complexes are brought into actual contact with one another (Keltz and Lovelock, 1955; Lovelock, 1957). Such disruption of the membranes may allow nucleation of the supercooled water within the cell. Heber (1967) attributed the uncoupling of phosphorylation from electron transport (Section III, B) to damage of chloroplast membranes resulting from freezing.

Within the cell protoplasm are numerous bodies which are also enclosed within membranes. Chloroplast membranes are frost sensitive (Heber and Ernst, 1967). Increase in activity of some mitochondrial enzymes and all those of lysosomes is found when these organelles in

animal cells are disrupted as in freezing and thawing (Tappel, 1966). Plant cell microbodies, if similar to animal cell lysosomes (Frederick *et al.*, 1968), are cell organelles containing families of hydrolytic enzymes in a nonreactive state. The lysosome membrane is a complex one consisting of a unit phospholipid-protein and associated protein. The membrane complex can be made permeable or can be disrupted by freezing and thawing. After release, lysosomal enzymes initiate catabolic reactions which could rapidly lead to considerable disorganization within the cell (Tappel, 1966). Because of their high latency and content of hydrolytic enzymes of broad specificity, the lysosomes appear to be the most important cell structure involved in the freezing injury (Tappel, 1966).

### C. Permeability

Olien (1965, 1967b) extracted water-soluble, cell wall carbohydrate polymers from tissues of winter cereals. He reported that polymers isolated from cold-hardy tissue interact with the ice–liquid interface, resulting in less perfectly structured ice. The polymers had little effect on the freezing temperature, but interfered with the liquid $\rightleftarrows$ solid reaction as a competitive inhibitor. Similar findings have been reported by Trumanov and Krasavtsev (1966).

It has been observed (Cary and Mayland, unpublished) that ice may form and melt in the leaves of such plants as peas (*Pisum sativum*), lettuce (*Lactuca sativa*), and sugarbeets (*Beta vulgaris*) without causing visible damage if temperatures do not drop below $-5°C$ and the freezing time is not longer than 5 or 6 hours. Increases in membrane permeability accompany the cold-hardening process (Levitt, 1956). Plants like sugarbeets, peas, and lettuce may be protected from ice injury by highly water-permeable membranes, or by some polysaccharide ice–interface reaction as suggested by Olien (1965, 1967b). It is possible that permeable membranes allow particular polysaccharides to move onto the extracellular surfaces where they can interact with growing ice crystals. Hassid (1969), in his review of polysaccharide biosynthesis in plants, emphasizes the further importance of the plasma membrane as a source of cell wall building materials.

## V. Protection from Freezing

### A. Evidence of Chemical Effects

Protection against freezing damage has been obtained by microclimate modification. Adding water via surface or sprinkler systems has

been successful in some cases because of the great heat capacity of water. High-expansion foams which blanket plants, providing insulation against temperature changes, are being developed for use on low growing crops.

Some data show that protection against freezing damage may be achieved by use of chemicals. Within the group of compounds generally classified as growth retardants are several which may protect against chilling (Tolbert, 1961) and freezing injury. One such compound, 2-chloroethyl trimethylammonium chloride (CCC, also Cycocel) has provided at least limited protection against freezing damage, as well as protection against drought (Shafer and Wayne, 1967). Treatment with CCC increased freezing resistance in cabbage, one-year-old pear trees, tomatoes, and wheat (Shafer and Wayne, 1967; Michniewicz and Kentzer, 1965; Wunsche, 1966). Similar treatment with CCC increased winter hardiness of cabbage (Marth, 1965), and wheat (Toman and Mitchell, 1968). The compound 1,5-difluoro-2,4-dinitrobenzene gave protection to young bean plants against an 8-hour freezing period at −3°C (Kuiper, 1967).

Similar responses have been reported for *N,N*-dimethylamino succinamic acid (B-nine, B995, and Alar). Significant increases in cold temperature tolerance were not observed after spraying tomato transplants with Alar (Hillyer and Brunaugh, 1969). Using this chemical resulted in more flowers on apple and cherry trees, and greater number of sweet corn ears (Cathey, 1964). CCC and B-nine, however, are relatively long lived (months to one year) and so may be undesirable for short-term protection of tender plants. The chemicals discussed here are generally classed as growth regulators. Their effect on flowering and final crop yield has not been fully evaluated. Some preliminary work suggests that snap bean yield (Sanders and Nylund, 1969) can be reduced and pea yield (Maurer *et al.*, 1969) can be increased in some cases by applying B995 or CCC. Another chemical, N-decenylsuccinic acid, applied 4 hours before initiation of freezing temperatures, has been shown to prevent apple blossom injury when exposed to −6°C for 2 hours (Hilborn, 1967). Applying this compound at any time before the 4-hour prefreeze interval was ineffective. Earlier studies with this same fatty acid (Kuiper, 1964b) showed that the compound induces freezing resistance in young bean plants. When the fatty acid was sprayed on flowering peach, apple, and pear trees, most of the flowers resisted freezing injury at −6°C.

Inducing frost resistance in strawberry flowers by application of decenylsuccinic acid and a few of its monoamides was reported by Kuiper (1967). Flower survival was: control, 8%; decenylsuccinic acid, 10%; decenyl-*N,N*-dimethylsuccinamic acid, 30%; and decenyl-*N,N*-di-

methylsuccinichydrazide, 40%. There is experimental evidence that decenylsuccinic acid is incorporated into the lipid layers of the cytoplasmic membrane, where it raises the membrane permeability to water where only the viscosity effect is observed (Kuiper, 1964b). The beneficial effects of decenylsuccinic acid found by Kuiper have been challenged. Newman and Kramer (1966), attempting to duplicate Kuiper's findings (1964a,b), found that roots of intact bean plants are killed by exposure to $10^{-3}$ *M* decenylsuccinic acid. They concluded that this chemical acted as a metabolic inhibitor and that increases in water permeability resulted from root injury.

Heber and Ernst (1967) isolated a high-molecular protein (possibly a nucleoprotein) from chloroplasts of hardy spinach leaves which was effective in protecting chloroplast membranes from frost injury. This isolated protein was also heat stable against 90°C for 2 minutes. Dycus (1969) observed less injury by high and low temperatures after spraying plants with zinc-containing compounds, but not copper or iron. He also isolated a subcellular particle from the tomato plant which seemed to be associated with zinc content and low temperature tolerance. Zinc ions are powerful inhibitors of ribonuclease (RNase destroys RNA) and could therefore influence protein synthesis (Hanson and Fairley, 1967). Since zinc concentrations in the plant are inversely related to RNase activity (Kessler, 1961), additional zinc would be helpful in controlling the activity of this hydrolytic enzyme, which might be released from plant cell microbodies (Section IV, B) during cold temperature stress. Zinc as well as boron and manganese may increase protoplasmic viscosity (Shkol'nik and Natanson, 1953) and, therefore, increase freezing resistance.

DeVries and Wohlschlag (1969) have isolated a glycoprotein from an Antarctic fish which was responsible for 30% of the freezing-point depression of the fish's serum. A more critical look at these substances and related compounds might provide opportunities for control of frost susceptibility in plants.

## B. Mechanisms of Freeze-Injury Protection

### *1. Bond Protection*

Although little direct evidence is available on the nature of freezing processes, it is suspected that, aside from direct mechanical rupture of ice crystals, lipoprotein alteration of membranes may be a primary cause of freezing injury (Heber and Santarius, 1964). Water removal during freezing may lead to lipoprotein injury. Structure alteration (denaturation)

could then be caused by hydrogen bond breakage allowing lipoprotein interaction. This is supported by the fact that hydroxyl-containing compounds such as sugars act as protective substances against the inactivation of the phosphorylation process. Heber and Santarius (1964) explain the protective action of sugars and other compounds by their ability to retain or substitute for water via hydrogen bonding in proteins sensitive to dehydration. Sugars provide protective action against freezing injury in cabbage cells (Sakai, 1962). Apparently this protection is a surface phenomenon which prevents removal of the surface water layer from protoplasts by the dehydrating action associated with freezing and thawing. In simple cells, inositol (benzene ring surrounded by 6—OH) gives some degree of protection from stress such as freezing or radiation. It is suggested that the protection afforded by this chemical results from its ability to protect H-bonding (Webb, 1965) or possibly in substituting for the water structure.

Sokolowski *et al.* (1969), however, discounted the suggestion that inositol takes the place of water in maintaining the stability of desiccated cells. They suggested that the observed inositol effect may result from a conformational change in the protein brought about by inositol binding at positions adjacent to the reaction site.

A large number of cryoprotective compounds (chemicals which preserve cellular integrity at subzero temperatures) have been evaluated for their effectiveness in protecting simple cell systems. Although these cyroprotective compounds may be diverse, some generalizations may be made even though the mechanisms of protection may not be completely explained. These generalizations go far toward correlating cryoprotective activity with molecular structure (Doebbler, 1966). After examining the molecular structure of known cryoprotective solutes, it is apparent that all are capable of some degree of hydrogen bonding (Doebbler *et al.*, 1966). Some association of the cryoprotective agents with the cell membrane appears to also take place. Steric and electrostatic properties of protective additives perhaps act via effects on adsorption which can also influence the recoverability of frozen cells (Rowe, 1966). An interesting further generalization with regard to hydrogen bonding is the similarity in types of compounds that afford cryoprotection and those that protect microorganisms against drying or radiation, or which protect proteins against thermal denaturation (Doebbler, 1966; Webb, 1965).

### 2. *Membrane Effects*

Rowe (1966) has suggested that cryoprotective compounds interact directly or indirectly with the cell membranes to stabilize the water–

lipid–protein complex tertiary structure. It is at the cellular membrane level that biological integrity appears to be insulted by freezing (Livne, 1969), and it is at the membrane level that biochemical understanding of cryoprotection must be sought. A correlation between mole equivalents of potential hydrogen bonding sites provided by a solute and protection of some simple cell systems during freezing has been reported (Doebbler, 1966). There is a question, though, as to how quantitative this method would really be because of the variation in hydrogen bonding energies.

Jung *et al.* (1967) found that applying certain purines and pyrimidines enhanced the development or maintenance of cold hardiness. Hardy plant varities contained greater amounts of DNA and ribonucleic acid (RNA) in the water-soluble trichloroacetic acid (TCA)-precipitable protein fraction than those of less hardy varieties during the development and maintenance of cold hardiness. In addition, the content of these constituents was increased by exposing the plants to low temperatures at a short photoperiod. The metabolic processes were altered by the chemical treatments in a manner that made the TCA-fraction of the nonhardy plants more nearly like that of untreated plants of a hardy variety. This supports the conclusions of others (Siminovitch *et al.*, 1962) that water-soluble protein content is related to development and maintenance of cold hardiness.

Thermostability of human and bovine serum albumin has been increased when the protein was combined with fatty acids and related compounds (Boyer *et al.*, 1946b). The protective action of the fatty acid ion increased with chain length up to $C_{12}$, but maximum stabilization at high concentrations was obtained with $C_7$ and $C_8$. Native proteins were protected against heat denaturation by fatty acids which prevented viscosity increases in heated solutions. In another paper, Boyer *et al.* (1946a) reported that low fatty acid concentrations prevented an increase in viscosity due to denaturation by urea or guanidine. The action of the fatty acid anions appears to result from their combination with certain groups or areas of the molecule and is probably the result of the combination of the anion with both the positive and the nonpolar portions of the protein.

Protective action against freezing damage in higher plants has been evaluated from a standpoint of membrane permeability. Kuiper (1967) studied the effect of surface active chemicals as regulators of plant growth and membrane permeability. Several compounds, including the decenylsuccinic acid groups, were tested for their effects on water permeability of bean roots and growth retardation of young bean plants. In each group the effectiveness increased by increasing the number of carbon atoms.

There appears to be a definite effect of the hydrocarbon chain length of the surface active chemical on both permeability and resistance to freezing. These surface active chemicals probably affect permeability of the plasma membrane and its resistance to freezing by incorporating the molecules into the lipid layers of the plasma membrane. Charged lipids occurring in the plasma membrane may contribute in the same way to the permeability characteristics and the freezing resistance of the membrane. Kuiper (1969b) also reported that when galactolipids were added to the root environment, an increase in water transport through the plant was observed. Applying this lipid to fruit flower buds increased resistance to flower freezing as tested 2 or 3 days later. The results demonstrate the relation of lipids to water transport across membranes and to membrane stability against freezing.

Dimethyl sulfoxide (DMSO), which is a dipolar aprotic solvent with a high dielectric constant and a tendency to accept rather than donate protons, has been used as a carrier for many compounds used in cryoprotective studies. DMSO has been found to prevent loss of respiratory control and to decrease inefficiency of oxidative phosphorolation of plant mitochondria stored in liquid nitrogen (Dickinson *et al.*, 1967). The mechanism by which DMSO protects some biological membranes against freezing damage is not known and, in fact, its beneficial effect of altering the permeability characteristics has been disputed in some studies (Chang and Simon, 1968). They, instead, attribute the *in vivo* effects of DMSO primarily to its ability to alter enzyme reaction rates. The native form of biopolymers is surrounded by the ordered arrays of water molecules. Substitution or removal of the biopolymer's hydration sheath by DMSO would be expected to alter the protein configuration (Chang and Simon, 1968). It is possible, therefore, that at low concentrations DMSO permits a protein molecule, such as RNA, to assume a more open, less hydrogen-bonded configuration.

DMSO is an excellent fat solvent and has been shown to remove some fatty acids from the bacterial membrane (Adams, 1967), and membrane porosity may, therefore, increase (Ghajar and Harmon, 1968). This increase in permeability in cell membranes by DMSO may be similar to natural changes occurring during the cold-hardening processes.

The protection afforded to plasma lipoproteins by polyhydroxyl compounds against damage by freezing or drying has a parallel in the case of some simple cells (Keltz and Lovelock, 1955). The mechanism of the damage caused by freezing and drying has some points of similarity to the picture of temporary collision complexes occurring in the exchange of lipids between lipoprotein complexes. The difference lies in the avail-

ability of water molecules to interact with an exposed group in the parent lipoprotein complexes in solution. In the frozen system, the ice lattice may draw water molecues away from the lipoprotein complexes, disrupting the structure of the complexes. It is noteworthy that all the molecule species that protect against damage by freezing or drying are themselves rich in hydroxy groups (Keltz and Lovelock, 1955). Possibly their presence offers the lipoprotein complexes some alternative molecules to associate with in the place of water molecules that have become unavailable. In the presence of either water or some other molecule-containing hydroxyl groups, the lipoprotein complex may rearrange to allow some internal compensation and assume a configuration which returns to the original structure when water is readmitted to the system.

### C. Undercooling and Nucleation

Even though the temperature is below the freezing point of plant water, ice crystals may not form. The solution must first be nucleated. The nucleation of undercooled liquid water is not well understood. Pure water may cool below −30°C without forming ice. Elaborate preparations are required to demonstrate this, since even the slightest foreign particle may cause nucleation (Dorsey, 1948). The initiation of ice crystal formation is evidently a surface interface reaction. Davis and Blair (1969) have presented data suggesting that the presence of strain energy in suspended particles may enhance their ability to cause nucleation. It is not known whether or not lattice strain energies could be important in ice nucleation in plant tissue. This may be involved in the increased undercooling which occurs with faster cooling rates in some plants (Cary and Mayland, unpublished). Mechanical shock does not cause nucleation in either bulk solution or plant tissue (Dorsey, 1948; Kitaura, 1967).

It has been established that ice forms preferentially in the extracellular spaces. The pressure of the water in the extracellular spaces may be important. Dorsey (1948) has stated that the spontaneous (undercooled) freezing temperature of solutions tends to parallel decreases in the true freezing point induced by the addition of salt. The freezing point may also be decreased by raising the pressure (Evans, 1967). If pressure affects the spontaneous undercooling in a similar way, nucleation should occur first in the extracellular spaces where liquid pressures are generally less than those inside the cells. An ice crystal is also an excellent nucleator. This nucleation may occur as ice from the atmosphere settles on the plant surface, contacting a continuous liquid film leading to the extracellular spaces and thus causing nucleation of the water in these areas.

Single (1964) found that wheat plants could be stored in a dry (low humidity) chamber at −3 to −5°C almost indefinitely without the formation of ice crystals. Yet he felt this would not occur in the field since crystals are present in the air which could initiate freezing of undercooled water in the plant upon contact with the leaf. When following the progression of ice formation in his wheat plants, Single noted varying resistance to freezing in different plant parts, the rate of advance of ice being sharply reduced at internodes compared to leaves. It is known that capillaries and type of solute affect both the rate of growth and the shape of the ice crystals (Pruppacher, 1967a,b).

Beans (*Phaseolus vulgaris*, var. Pinto and Sanilac), corn (*Zea mays*), and tomatoes (*Lycopersicon esculentum*) were exposed to temperatures ranging from −2 to −3°C for various lengths of time (Mayland and Cary, 1969). When the relative humidity was less than 100%, the plants undercooled and ice crystals did not form for several hours. Eventually some plants did begin to freeze at random. When ice crystals were allowed to come in contact with the leaves, undercooling stopped and ice began to form in the tissues resulting in death of the tissue from mechanical cell rupture. In these experiments, it appeared that nucleation occurs on the surface if the atmospheric dew point is reached. Otherwise, nucleation occurs inside the plant tissue and factors within the plant may exert some influence on the spontaneous nucleation temperature. Results supporting this hypothesis have also been reported by Kitaura (1967) and Modlibowska (1962).

Once nucleation has occurred, the ice phase spreads rapidly (i.e., with velocities of up to 1 cm/sec) through the conductive tissue, so long as the temperature of the tissue is below the freezing temperature of the solution it contains. The rate of ice nucleation from the conductive elements into the surrounding cellular tissue depends on the initial energy of the water in the plant, at least in the case of beans (Cary and Mayland, unpublished). When the energy of the plant water is high (−6 to −8 bars), the spread of ice is rapid throughout the leaves and results in death. If the plant-water energy is lower (−12 to −15 bars in the case of beans), the ice spreading rate is less by at least an order of magnitude, resulting in bean leaf damage of the type shown by Young and Peynado (1967) for citrus leaves.

The energy level of plant water is also related to the anatomical characteristic of leaf surfaces. It has been shown (Cary and Mayland, unpublished) that undercooled water in corn seedlings with a water potential of −18 bars is not nucleated by ice crystals on the leaf surface. Corn seedlings with higher potentials (−8 bars) are easily nucleated by exterior ice crystals. While the nature of this barrier to nucleation is not under-

stood, some aspects of the problem concerning nucleation sites have been discussed by Salt (1963).

Kaku and Salt (1968) concluded that the freezing temperature of conifer needles increased ultimately as the number and quality of favorable nucleation sites increased. Hudson and Brustkern (1965) observed permeability differences in young moss leaves of different ages and that the permeability increased with hardening and with age. Upon exposure to freezing temperatures, cells supercooled until a wave of intracellular freezing was initiated at −8°C in some leaves. These authors observed the freezing wave progressing from one cell to another and suggested that this probably occurred via the plasmodesmata. They further observed in very young leaves that the freezing did not start spontaneously, but was initiated by inoculation through the imperfectly developed cell walls at the apices of the leaves at approximately −4°C.

As previously noted, some compounds provide freeze-injury protection to tender plants. Protection may result from changes in the membrane which render it less susceptible to rupture by ice crystals. The benefit may come about from permeability changes allowing rapid water transmission out of the cell to external ice crystals, thus reducing the chance of nucleation inside the cell. An alternative explanation, which has received little attention, is that these compounds may increase the stability of undercooled water (1) by changing the surface properties of the leaf so that ice crystals on the surface are not able to initiate nucleation in the extracellular spaces, or (2) by increasing membrane permeability allowing solutes from the cell to leak into the extracellular spaces, thus causing the fluid to be more stable to undercooling, or (3) by directly affecting the nucleation temperature of water in the plant. The reports on urea effects on frost tolerance fit into this possibility. Occasionally, spraying with urea has been credited with decreasing damage during mild freezes (van der Boon and Tanczos, 1964). However, as pointed out earlier, urea is very undesirable as far as protein denaturation in an ice crystal system is concerned. Urea strongly affects the molecular structure of water, and so it is possible that it could lower the nucleation temperatures and prevent ice crystal formation during a mild freeze, yet increase damage to the cell constituents if ice crystals do appear.

### D. Chilling Injury

It is known that temperatures well above freezing cause injury to many tropical plants. From the preceding sections it is not difficult to see how

a shift in hydrogen and hydrophobic bonds caused by cool temperatures could result in an irreversible change in specific enzymes. Moreover, it is possible that such changes occur in temperate climate plants during cool periods causing changes in growth that go unnoticed or unexplained.

Protoplasmic streaming used as an index in cellular heat injury in plants may also be used to differentiate between cold-sensitive and cold-insensitive plants. The differentiation is made on the ability of protoplasmic streaming to continue in cells exposed to cold temperatures. In some chill-sensitive plants, protoplasmic streaming stops suddenly when the tissue temperature drops to 10°C, although streaming continues in cells from chill-resistant plants almost to 0°C (Langridge and McWilliam, 1967).

Lyons *et al.* (1964) studied the physiochemical nature of mitochondrial membranes of chilling-sensitive and chilling-resistant plants. The mitochrondria of chilling-resistant species had the greatest capacity for swelling and the greatest degree of unsaturation of the membrane fatty acids. This degree of unsaturation is directly related to membrane flexibility. The swelling and contraction of mitochondria are closely correlated to oxidative phosphorylation. The phosphorylative system, which is dependent on membrane associated enzymes, could be disrupted by membrane inflexibility, and the available ATP supply could be reduced.

Low temperatures, 0–10°C, are known to promote callose plug formation in the conductive tissue of beans (Majumder and Leopold, 1967). Another example of chilling effects is the marked change which occurs in corn growth as a result of cool root temperatures (Walker, 1967). Stewart and Guinn (1969) have reported an extensive decrease in the ATP levels of cotton seedlings resulting from 2 days of chilling at 5°C. Kuraishi *et al.* (1968) have shown that even peas *(Pisum sativum)*, commonly thought to tolerate some cold stress, may show biochemical changes as a result of chilling. Chilling of germinating cotton seed reduced plant height, delayed fruiting, and reduced fiber quality in direct relation to cold exposure time (Christiansen and Thomas, 1969). Garden bean (*Phaseolus vulgaris*, var. Sanilac) seedlings exposed to −1°C for 4 hours had delayed fruiting and maturity dates (Mayland, unpublished). Potato leaves may be injured in varying degrees of severity depending upon leaf maturity, relative maturity of leaf sections, position of leaf, temperature, and other factors (Hooker, 1968). Rainbow flint corn seedlings are sensitive to chilling injury up to 5.5 C (Bramlage, W. J., unpublished, University of Massachusetts). Chilling stress effects on plants appears to offer new and challenging frontiers for plant physiologists.

## VI. Conclusions

The freezing temperature of plant material is first of all determined by the chemical potential of the plant water. The freezing point drops approximately 1°C for each 12 bars equivalent negative pressure (in the range of 0 to −10°C). Ice does not form spontaneously as the temperature drops to the freezing point of the plant water. Rather, the solution tends to undercool to variable and somewhat unpredictable temperatures. When nucleation does occur, it will generally be in the extracellular space.

The chemical potential of ice is less than that of liquid water at the same temperature. Water will consequently move from the cell to the ice lens on the outside so that the cell dehydrates as the lens grows. If the cooling rate is fast or if the cell has a low permeability, the dehydration of the cell will be too slow to maintain a stable supercooled solution and ice crystals will appear inside the cell. Ice crystals inside the cell increase the chances of injury.

As soon as ice appears, the plant begins to undergo desiccation due to the decreasing volume of the liquid phase. The resulting stress increases by approximately 12 bars effective negative pressure for each degree below 0°C. Thus, as the temperatures decrease, the water stress may become great enough to cause significant chemical damage through the disruption of bonds. This can lead to membrane changes and protein denaturation. Stress from desiccation does not develop with decreasing temperature when the solution undercools without forming the ice phase, though some bonding in large molecules may still be rearranged.

When tender plants show immediate frost injury from temperatures not lower than −3 or −4°C, the damage is caused primarily by ruptured cell membranes. Some nonhardy plants such as peas and lettuce can survive temperatures of −3 or −4°C for a few hours, even though ice is present in the plant tissue. Other plants, such as beans and corn, may survive similar freezing conditions only if the plant water undercools without the spread of ice through the tissue. Under these conditions the dew point of the atmosphere and the water content of the plants are important factors.

As the temperature decreases below −8 or −10°C, the chances of the ice phase being absent are small and plants which survive these conditions with the accompanying stress of −80 to −100 bars become those in the cold-hardy group. These are known to tolerate the growth of ice crystals in the intercellular cavities. When injury occurs in these plants, it is more apt to be from chemical bonding changes resulting from desiccation. Freezing and survival of cold-hardy plants has received much attention. Further progress in this area rests mainly in the realm of biochemistry,

particularly with respect to composition and bonding in lipids and proteins.

Causes of cold injury in some plants, aside from the mechanical forms of injury induced by ice formation, may not be separable from some of the effects of enzyme inactivation observed during freezing conditions. Here, the *in vitro* studies of Heber and Santarius (1964) and Young (1969) on the sensitivity to freezing by enzymes of the election transport system of photosynthesis may provide further clues to chilling and freeze injury in plants. The *in vivo* studies of Stewart and Guinn (1969), which showed a decrease in ATP of seedlings chilled at 5°C, certainly give support to the biochemical approach of studying low temperature stresses in plants. Future studies of the temperature sensitivity of the oxidative- and photophosphorylation system of plants should help describe injuries resulting from exposure to either cold or freezing temperatures.

Freezing of nonhardy plants has received little study in spite of great economic importance. It is possible that significant advances may now be made in this area. Five areas in particular need to be studied:

a. The chemical control of cell membrane permeability

b. The identification and control of polysaccharides or other molecules which interact with the ice–water interface

c. The internal control of nucleation of undercooled plant solutions

d. The surface properties that prevent ice particles in the atmosphere from nucleating water in the plant

e. Identification of low temperature-sensitive links in the electron transport system and evaluation of such links to determine opportunities for genetic alteration.

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