Studies on the Frost-Hardiness of Woody Plants*

I. The causal relation between sugar content and frost-hardiness

by

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I. Introduction

It has long been well known that frost-hardiness in woody plants, as a rule, shows remarkable periodicities through the cycle of a year. Many hardy plants, more or less increase their frost-hardiness from summer to winter. In summer the twigs of poplar and willow cannot withstand freezing at −5°C for a short time; while in winter they can survive even a temperature as low as −30°C for a long time[1]. In natural and artificial[2] frost-hardening, the increase of frost-hardiness has been ascertained to be associated with the fluctuations in the content of various substances, such as sugar[3,7], polyhydric alcohol[8,10], protein[11,15], glycoprotein[16,17], amino-acid[18], peptide[18], organic acids[19] and inorganic salts[19], etc.

MÜLLER-THURGAU (1882) demonstrated first that there was a positive correlation between sugar content and frost-hardiness[20]. This problem has been investigated with various plant materials, and a parallelism between the two factors has generally been accepted by most investigators. However, more recently it has been maintained by some workers[11,17,19,21,23] that the increase of sugar content in cells is not the main cause of that of their frost-hardiness; a question has arisen as to the role of sugars in increasing forst-hardiness. These workers have chiefly intended to find out a correlation between the variations of frost-hardiness and sugar content in the process of both natural and artificial frost-hardening. As a matter of fact, it has been impossible to make clear any causal relation between the two factors, since it is impossible to distinguish the effects of low temperature itself and that of increase in sugar concentration in frost-hardening process, especially in artificial frost-hardening. Moreover, no direct evidence has yet been shown that in a cell the increase of sugar content alone results in rise of the frost-hardiness. This may be due to the difficulties in artificially introducing an appreciable amount of sugar into a plant cell. LEVITT (1959) recently reported that hardy cabbage leaf cells showed definite increase in frost-hardiness when they were infiltrated with 0.5 M hexose solution by means of a vaccum desiccator[25]. His observed increase in hardiness agrees very well with the calculated value on the basis of a purely osmotic effect. However, such an increase in the frost-

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hardiness may be considered as the effect of increase of sugar content both in cells and in intercellular space, because of course in the method of vaccum infiltration, sugar is introduced into the intercellular space as well as into the cell interior. Accordingly, to distinguish these two factors, it becomes necessary to introduce sugar into the cell interior without altering the state of intercellular space.

The purpose of the present study is to make clear the causal relation between frost-hardiness and sugar content and to ascertain the mechanism of the protective action of sugar against frost injury in plant cells.

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II. Materials and Methods

1) *Materials*

Many woody plants having various degrees of frost resistance were used. The complete list of materials is as follows:

Mulberry tree	*Morus bombycis* KOIDZ. var. *takinokawa*	Twig
White birch	*Betula tauschii* KOIDZ.	Twig
Willow	*Salix Koriyanagi* KIMURA	Twig
Poplar	*Populus nigra* L. var. *italica* MUENCHH.	Twig
Larch tree	*Larix leptolepis* MURRAY	Twig
Rose	*Rosa pendulina* L.	Twig
Mountain ash	*Sorbus commixta* HEDLUND	Twig
Platanus	*Platanus orientalis* L.	Twig
Jezo spruce	*Picea glehnii* MAST	Leaf
Apple	*Malus pumila* MILL var. *yamatonishiki*	Twig
Pine	*Pinus pentaphylla* MAYR.	Leaf
Todo fir	*Abies Mayriana* MIYABE et KUDO	Leaf
Yew tree	*Taxus cuspidata* SIEB. et ZUCC.	Leaf
Rhododendron	*Rhododendron fauriae* FRANCH var. *roseum* NAKAI	Leaf
Evergreen spindle tree	*Euonymus radicans* SIEB.	Leaf
Pomegranate	*Punica granatum* L.	Twig
Laurel tree	*Laurus nobills* L.	Leaf
Tea plant	*Thea sinensis* L.	Leaf
Gardenia	*Gardenia jasminoides* ELLIS. var. *grandifloria* NAKAI	Leaf

As some of the above-listed trees are unable to overwinter in Sapporo, such relatively tender plants as pomegranate, laurel, tea plant and gradenia were sent to Sapporo from Yamagata, Shizuoka and Nagoya in late January. The leaves or twigs of these plants were used for experiments after they had been chilled at 0°C for 10 days.

In the case of the twig of mulberry tree, only pieces (0.6～0.7 cm in diameter, 1.5 cm in length) cut from the same segment in one year old twig were used in the

same series of experiments. In gardenia leaf, the paired leaves, located at the top of the twigs growing out from the same root were used in the same series of experiments. Moreover, only the matured leaf in fall having the capacity to withstand intercellular freezing was used.

2) *Method of freezing and thawing.*

In the mulberry tree, small twig pieces (in diameter from about 0.6 to 0.8 cm, in length from about 1.5 to 2.0 cm) were placed in glass dish (diameter 3 cm, depth 1 cm) and were cooled in the thermostat at −5°C. After 30 minutes, they were inoculated with ice and then were gradually transfered at one hour intervals to the thermostats of the various graded temperatures from −5°C to −30°C at intervals of 5°C. After the twig pieces were cooled to a desired temperature, they were kept in the thermostats for a certain length of time, and then were slowly thawed in air at 0°C.

In gradenia leaf, the leaves wetted with water were placed in petri dish (diameter 15 cm, depth 4 cm), and then were cooled slowly down to the desired temperature by the similar procedure in twig pieces after seeding with ice at −5°C. After having been kept in desired temperature for a given time, they were slowly thawed at 0°C.

3) *The method of testing survival.*

At a definite period of time after thawing, thin tangential sections were made from the cortical layer of the twig piece. The percentage survival of parenchyma cells in the pieces was determined by both vital staining test with neutral red and plasmolysis test[26]. Intactness of twig as a whole cannot be judged on the basis of plasmolysis test in parenchyma cells immediately after or even many days after thawing, because inner cortex, pith ray, and pith periclinal tissue of a twig are less resistant to freezing than the paraenchyma cells of its cortex[27]. In order to demonstrate the intactness of a twig as a whole, it was planted in moist sand or in water to test its capacity to continue normal development. The grade of frost hardiness of parenchyma cells was represented either by the lowest temperature at which almost all cells survived freezing for a definite period of time or by the percentage survival of cells in the sections frozen and thawed.

In the case of leaves of gardenia, the grade of frost injury was determined by the degree of browning of treated leaf at 6 to 12 hrs after thawing. To determine the lowest tolerable temperature at which the leaf can withstand freezing with little damage, several leaf pieces from the same leaf were frozen at various temperatures for the same period.

4) *The method of frost-hardening*[28].

Twig pieces cut from the same segment in the same twig were wrapped with sheet vynyl to prevent desiccation and then were exposed to 0°C or other low temperatures for various periods.

5) *Measurement of osmotic concentration.*

A number of thin sections were sliced from cortical tissue in twig using a sharp

blade of an old fashioned hand razor. In gradenia leaf, however, instead of cortical cells, spongy or palisade mesophyll cells were used. By the usual plasmolytical method, the osmotic concentration of the cells was determined in a balanced salts solution ($NaCl : CaCl_2 = 9 : 1$).

6) *The method artificially to introduce various substances into cells.*

The petiole of a gardenia leaf was put in a hypotonic solution of various substances at 20°~25°C for various periods ranging from 16 hrs to 48 hrs. As a control, the petiole was put in water under the same conditions. The leaf which suffered toxic effect due to the introduced substances is easily distinguished from intact one by the degree of browning at the surface of the leaf. Even when any browning could not be found, several cross sections were taken from the leaf and the state of the cells was examined under microscope. Only normal leaves showing no sign of injury were used in the following experiments. The osmotic concentration of the leaf after treatment with various substances, was determined by the method of plasmolysis and thereby the amount of introduced substance was determined. In order to investigate the protective action of solutes against frost injury, the following substances were used: sugars: rhamnose, xylose, fructose, glucose, sucrose, maltose, lactose, raffinose; polyhydric alcohols: mannitol, sorbitol, ethylene glycol, glycerol, diethylene glycol, propylene glycol, triethylene glycol; other organic compounds: urea, glycine, acetamide; and inorganic compounds: NaCl, KCl, NH_4Cl, $CaCl_2$.

7) *Quantitative analysis of carbohydrate.*

Sample of 1 g fresh bark from one year old twig of mulberry tree was ground in 20 ml of distilled water with quartz sand and then centrifuged. The remaining precipitate was resuspended in 20 ml of distilled water and centrifuged; the supernatant obtained was combined with the first extract. Using this extract, the writer made determination of reducing sugar by HANES' method[29]. In order to dtermine non-reducing sugar content, the extract was preliminarily hydrolyzed with 0.1% HCl at 100°C for 10 minutes. Except for the study on the seasonal variations of carbohydrate in the twig bark of mulberry tree (Fig. 1), all the carbohydrate determinations were done by the colorimetric method as mentioned below. The protein components of the extract were precipitated with trichloroacetic acid; then the supernatant was obtained by centrifuging, and the precipitated protein was washed with 10% solution of trichloroacetic acid. The supernatant thus obtained was used for the determination of sugar by the colorimetric method using anthrone reagent[30]. The total carbohydrate content was expressed as glucose equivalent to wet or dry weight of tissue.

The residue from the homogenate of bark with water was thoroughly washed with alcohol and then with ether, and air dried. Starch in the dried residue was extracted[31] with cold perchloric acid and analysed colorimetrically use being made of the anthrone reagent. The identifications of various sugars were made by means of paper chromatography. Sample of about 1 g fresh weight from the bark or leaf of

one year old twig was ground in 20 ml of 80% ethanol with quartz sand, and clear extract was obtained by centrifuging. The remaining precipitate was resuspended in 20 ml of 80% ethanol and centrifuged; the supernatant obtained was combined with the first extract. After the evaporation of ethanol under reduced pressure, distilled water was added to the residuel to bring the total volume to about 1 ml. After washing throughly with ether, this aqueous solution was completely dried on a warm water bath. The dried residue was resuspended in 0.5 ml of distilled water. The extract thus obtained was used for paper chromatographic analysis. An aliquot of the extract was developed on WHATMAN No. 1 filter paper, using a solvent containing butanol, acetic acid and water (4 : 1 : 2). Development was repeated six times with the same developmental solution in ascending direction. After development had been completed, the paper was dried and used for the detection of spots. Benzidine reagent[32] was employed for the detection of all sugars whilst diphenylamine reagent[33] was used to identify ketose. Various sugars were eluted respectively from the filter paper with 20 ml of distilled water. The quantity of the sugar contained in the eluate was determined by the colorimetric method with anthrone reagent. When the sugar in the eluate was very small in amount, the eluate was concentrated up to 1～5 ml under reduced pressure previous to the determination. The content of various sugars was expressed respectively as percent of each to fresh tissue weight, but only the content of stachyose was expressed as raffinose equivalent.

8) *Quantitative analysis of polyhydric alcohol.*

An aliquot of the extract obtained by the above procedure was developed on filter paper use being made of the same solvent. For mannitol and sorbitol unlike for glycerol, the development was repeated six times with the same solution in accending direction. For the detection of spots, ammoniacal silver nitrate[34] was employed. Polyhydric alcohols were eluted separately with 20 ml of distilled water from the filter paper. When obtained alcohols are very small in amount, the eluate was concentrated up to 1 ml under reduced pressure. The quantity of the alcohol contained in the eluate was determined by the colorimetric method based upon the periodate oxidation and subsequent colour reaction with chromotropic acid reagent[35]. As erithritol content was proved to be negligible in many materials used in preliminary experiment, this alcohol was not subjected to analysis. When chromotropic acid reagent was employed, sugars also showed colour reaction; but the colour reaction for sugars was different from, and far less sensitive than, that of polyhydric alcohol. Even when development was made six times repeatedly as mentioned above, it is difficult completely to separate sorbitol from mannitol by means of paper chromatography especially in the presence of a large quantity of mannitol, because the latter tends to make tailing. In this experiment, however, it is enough to determine the combined content of mannitol and sorbitol. In the present paper, hereafter, the sum of the amount of mannitol and sorbitol is expressed as sugar alcohol content. The content

of polyhydric alcohols was expressed respectively as percent to fresh weight of tissue.

9) *Determination of soluble protein.*

Sample of 1 g fresh bark from one year old twig of mulberry tree was ground in 15 ml of distilled water with quartz sand, and then was centrifuged. The residue was re-extracted with another 15 ml of distilled water, and the supernatant was combined with the first extracet. Trichloroacetic acid was added to form a 10% solution to total extract, and the precipitated proteins were centrifuged and washed with 10% trichloroacetic acid. Nitrogen determination was made by the method of LEVY and PALMER[36]. When the bark samples were extracted with either water or 0.5 M sodium chloride solution, no difference in the amount of nitrogen between the two extracts was found. It may be said therefore that most of the soluble proteins in the bark of mulberry tree are extractable with water.

III. Results

1) *Seasonal variations in sugar content, osmotic concentration and frost-hardiness.*

Using the cortical tissue of a mulberry twig, the seasonal variations of the frost-

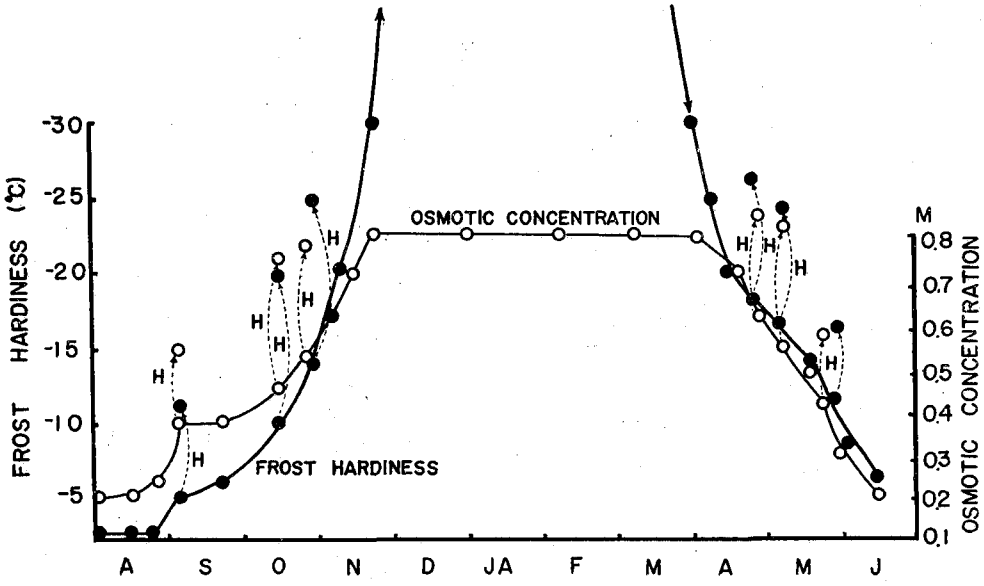


Fig. 1. Seasonal variations in frost-hardiness and osmotic concentration in the parenchyma cells of twig bark of mulberry tree.
Arrows with H indicate the variations of osmotic concentration and frost-hardiness in the parenchyma cells after artificial hardening at 0°C for 10 days. Only twig pieces cut from the same segment in the same twig were used in hardening test. The grade of frost-hardiness of the parenchyma cells is represented by the lowest temperature at which almost all the cells could withstand freezing for 4 hours. Osmotic concentration is indicated as equivalent of molar solution of sodium chloride.

hardiness and the sugar content in both natural and artificial frost-hardening were investigated. The results obtained are summarized in Figs. 1 and 2 where the variations of sucrose content, osmotic concentration and frost-hardiness after frost-hardening at 0°C for 10 days are also presented. From December to late February, the cells could withstand freezing without injury even when exposed to an extremely low temperature[8)7)], consequently, the exact grade of frost-hardiness could not be determined accurately.

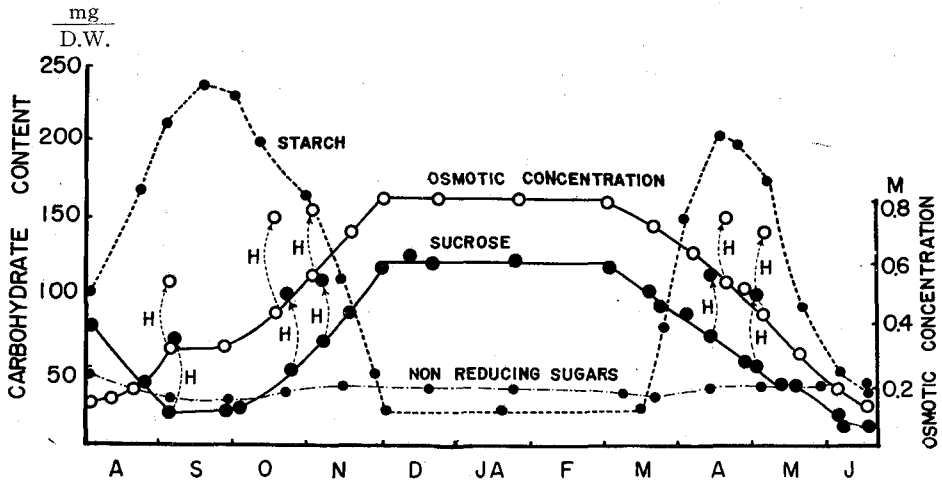


Fig. 2. Seasonal variations in carbohydrate content and osmotic concentration in the parenchyma cells of twig bark of mulberry tree.

Arrows with H indicate variations of sucrose content and osmotic concentration in the parenchyma cells of the twig after artificial hardening at 0°C for 10 days. Carbohydrate content is expressed as mg of glucose per dry weight of twig bark. Osmotic concentration is indicated as equivalent of molar solution of sodium chloride.

In parenchyma cells, from early September to late May, the existence was observed of an intimate correlation among the sucrose content, the osmotic concentration and the degree of the frost-hardiness. Before middle August, when the twig was still growing, the cortical cells thereof were neither frost hardy nor able to increase their frost-hardiness, even if they were hardened by means of cold treatment. Between late August and early September, various remarkable changes took place in cortical cells in association with the maturation of twigs: decrease both of water content (per dry weight: from 3.5 to 1.6), and of activity of the cambium cells (cambium cell layers: from 10 to 5), increase in starch content, rise in both osmotic concentration (from 0.25 M to 0.35 M) and frost-hardiness. In spite of the decrease of sugar content as expressed by proportion of dry tissue weight from late August to early September, increase of osmotic concentration in the parenchyma cells took place. This may be explained as a consequence of the concomitant decrease of water content in the cells.

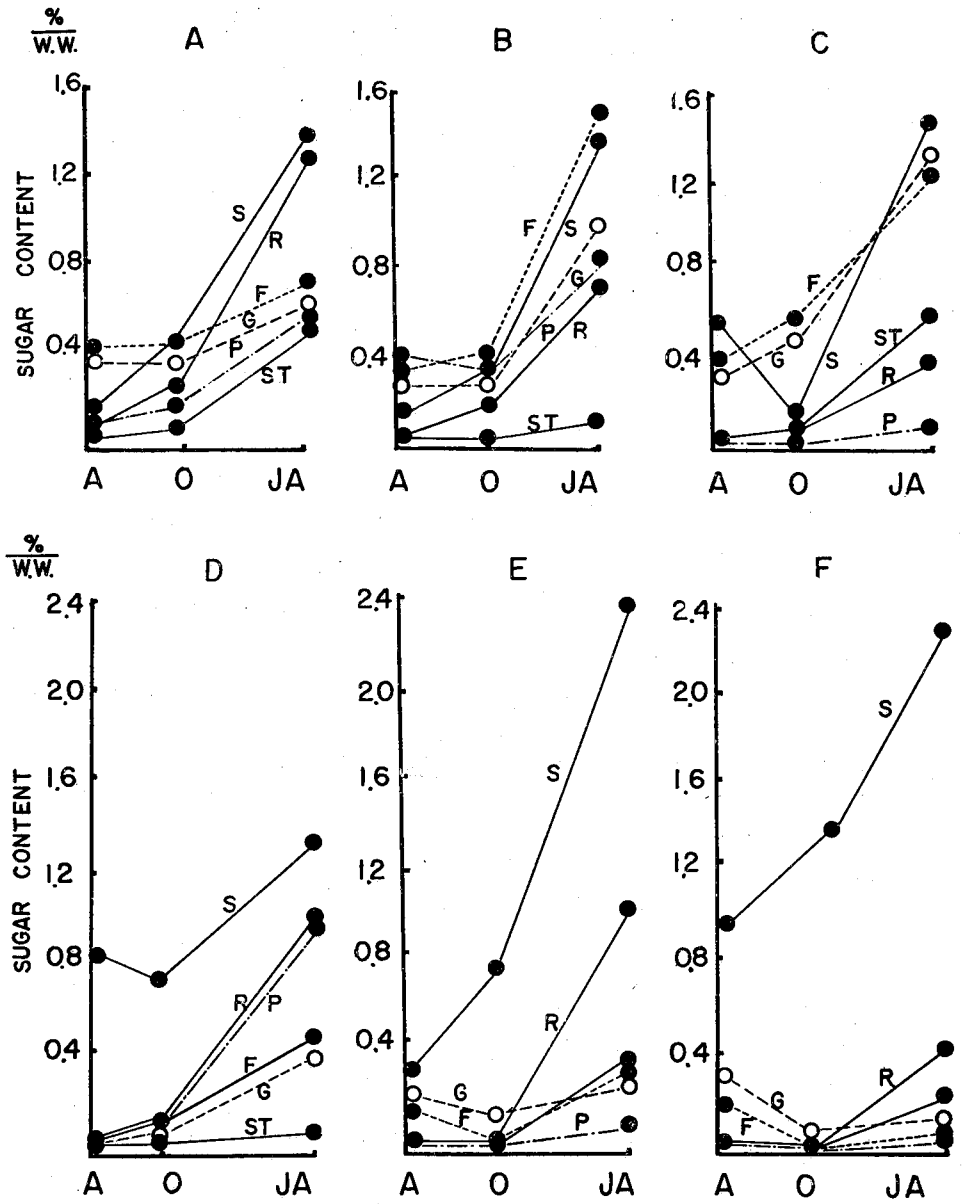


Fig. 3. Seasonal variations in sugars contained in twig bark of various woody plants. A *Betula tauschii*, B *Rosa pendulina*, C *Larix leptolepis*, D *Salix koriyanagi*, E *Populus nigra*, F *Morus bombycis*.

Ordinate indicates sugar content expressed as percentage per wet weight of twig bark. The letters in the figure represent the sugars: S (sucrose), G (glucose), F (fructose), R (raffinose), P (pentoses), ST (stachyose).

In this way, the cells of the twig which had matured to a certain degree became frost-hardy and could be further hardened by chilling. Unlike sucrose the content of reducing sugar remained nearly constant throughout the year. Therefore, it may have no relation to the frost-hardiness in the mulberry tree.

2) *Seasonal variations of sugars.*

Seasonal variations of various sugars contained in twig bark of various woody plants were studied by means of paper chromatography; the results are given as graphs in Fig. 3.

These figures show that seasonal variations of the sugars in twig bark differ considerably according to plants, and that sucrose content alone remarkably increases from October to January in all plants here used.

3) *Changes in sugar content, osmotic concentration and frost-hardiness in artificial frost-hardening.*

To clarify the relation between frost-hardiness and osmotic concentration in parenchyma cells, twig pieces of mulberry tree were artificially hardened under various temperature conditions. Within the range of experiments it was found that the increase in frost-hardiness was proportional to the rise in osmotic concentration (Table 1).

Table 1. The effect of hardening temperature on the increase of frost-hardiness. (Oct. 1).

Material: Mulberry tree. Frost-hardening period: 10 days.

Freezing temperature (°C) for 24 hours	Temperature of frost-hardening (°C)			
	15°	10°	5°	0°
− 10°	10**	20	50	100
− 15°	0	5	10	100
− 20°	0	0	0	25
Osmotic concentration (M)*	0.45	0.48	0.53	0.65

* Indicated as equivalent of molar solution of sodium chloride.

** The percentage survival of parenchyma cells.

The contents of water and inorganic salts[19] in the cells did not vary during the period of artificial frost-hardening. It follows, therefore, that the rise in osmotic concentration in the cells is caused by increase in the sugar content. When the twigs were hardened at a definit temperature of 0°C the effectiveness of the hardening increased with the duration of the hardening period within the limit of 10 days; there seems to be a parallelism between the osmotic concentration and the sugar content (Table 2).

Table 2. The effect of hardening period in increasing frost-hardiness in cortical cells. (Oct. 5).
Material: Mulberry tree. Hardening temperature: 0°C.

		Length of frost-hardening period (day)			
		0	5	10	20
Osmotic concentration (M)*		0.45	0.63	0.68	0.68
Sugar content**		95.0	130.0	142.0	140.0
Soluble protein***		2.48	2.11	2.27	2.21
Grade of frost-hardiness	at −10°C for 24 hrs	20****	60	100	100
	at −20°C for 4 hrs	5	50	70	70

* Indicated as equivalent of molar solution of sodium chloride.
** Represented as mg of glucose equivalent per dry weight of twig bark.
*** Represented as percentage of nitrogen per dry weight of twig bark.
**** Percentage survival of cortical cells.

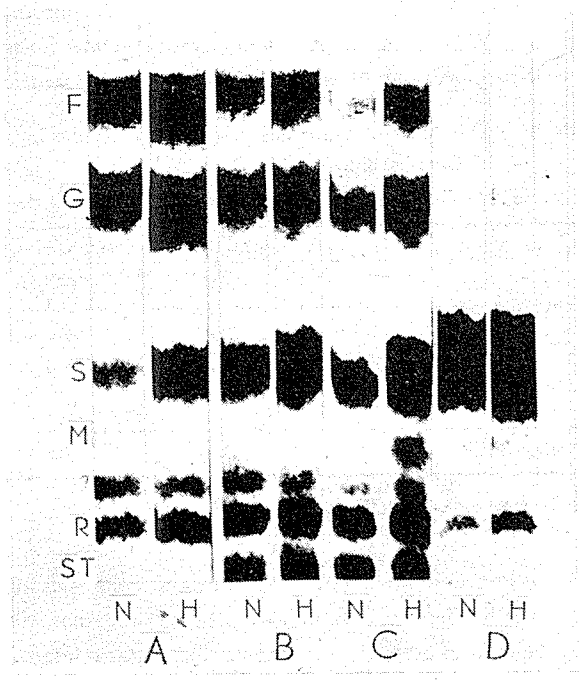


Fig. 4. The paper chromatograms of sugars in extracts from twig barks before (N) and after (H) frost-hardening. Frost-hardening was made at 0°C for 14 days beginning on Oct. 5.
Material; A *Larix leptolopis*, B *Rosa pendulina*, C *Betula tauschii*, D *Morus bombycis*.
Detection of spot: benzidine reagent.
F (fructose), G (glucose), S (sucrose), M (maltose), R (raffinose), ST (stachyose), ? unidentified sugar.

4) *Variations of sugar content in artificial frost-hardening.*

Twigs from several woody plant species were chilled at 0°C for 10 days in early October, when the artificial hardening was remarkably effective. In the twigs hardened in this way the variations of sugar content were analyzed by means of paper chromatography. Fig. 4 illustrates the chromatograms of sugars in hardened and control barks of four different species.

These chromatograms revealed an apparent increase in sugar contents after frost-hardening, especially in sucrose. In Table 3 the variations in amount of sugars in twig barks from seven woody plant species after hardening are tabulated. It shows that the variations of each sugar differ according to species. The amount of increase in the total sugar content as a result of the treatment in all bark samples reaches about 50 to 100% as much per wet weight of that in the untreated twig bark; there is a marked increase in content of sucrose, glucose and fructose.

Table 3. Variations of sugar content in twig bark of woody plants after artificial frost-hardening.

Kind of sugar	Species						
	Rosa pendurina	*Populus nigra*	*Salix koriyanagi*	*Larix leptolepis*	*Betula tauschii*	*Morus bombycis*	*Malus pumila*
Stachyose	3.4*	3.4	0.8	0	8.6	0.2	0.4
Raffinose	7.7	12.3	2.3	3.1	20.0	0	0
Maltose	0	4.6	2.7	0.7	6.7	0	5.3
Sucrose	39.7	68.4	53.4	43.2	25.0	85.1	24.1
Glucose	17.6	6.9	6.5	33.0	16.3	5.7	30.9
Fructose	20.6	4.4	3.3	20.0	19.0	6.7	27.1
Pentoses	11.0	0	31.0	0	4.4	0.3	12.2
Increment of sugar (%)	0.98**	1.66	0.96	1.10	1.53	1.74	1.00
	53.8 ***	112.0	53.5	75.4	62.0	93.6	70.0

Frost-hardening was made at 0°C for 10 days from October 5.

* The percentage of each sugar in relation to the total amount of sugar increased as a result of frost-hardening.

** The percentage of the sugar content increased in hardenen bark per wet weight of twig bark.

*** The percentage of the sugar content increased in the hardened bark in relation to that in normal bark.

5) *The difference in frost-hardiness and sugar content between south side and north side bark on the same twig.*

After middle March, a rise of temperature in the south side bark of a twig due to direct sunshine resulted in a conversion of sugar to starch in the bark cells, while in the north side of the same twig, sugar ramained still unchanged. In this case, too,

there was a parallel correlation between the frost-hardiness and the sugar concentration in parenchyma cells. Moreover, it was found that most of starch contained in the bark on the south side was converted to sucrose by chilling at 0°C for 10 days: such disappearance of starch was attended by concomitant rise in the frost-resistance and in the sugar content as well (Table 4.)

Table 4. Difference in frost-hardiness and carbohydrate content between south side and north side bark in the same twig in early spring (March 20th). Material: Mulberry tree.

		South side	North side	South side **** (after hardening)
I_2-IK reaction		++++*	+~++	++
Starch **		180 mg	100 mg	120 mg
Reducing sugar **		46 »	43 »	44 »
Sucrose **		90 »	150 »	130 »
Grade of frost-hardiness	Freezing at −30°C for 24 hrs.	10***	80	60
	Freezing at −20°C for 4 hrs.	100	100	100

* Number of plus signs indicates the relative amount of starch as estimated by microscopic examination in I_2-IK treated sections.
++++: very dense, ++: medium, +: slight.

** Carbohydrate content is represented as mg of glucose equivalent per dry weight of twig bark.

*** Percentage survival of the parenchyma cells in twig pieces.

**** The frost-hardened twig at 0°C for 10 days.

6) *Relation of polyhydric alcohol to frost-hardiness.*

It has been reported[8,9] in some hardy plants that polyhydric alcohols, especially mannitol and sorbitol are contained in large amount. Accordingly, it seems necessary to ascertain whether or not there is a parallel correlation between the content of polyhydric alcohol and the frost-hardiness. To clarify this problem the variations of polyhydric alcohol content during artificial frost-hardening in twig barks of 7 species of woody plants were studied quantitatively by means of paper chromatography. The results obtained are summarized in Table 5, where the sugar content determined in the same twig bark is compared with total polyhydric alcohol content. The increase in polyhydric alcohol content in artificially hardened twig bark did not exceed 0.1% of the fresh tissue weight in many species, while that in sugar content ranged from about 1.0 to 1.7% per fresh weight in many species. The increase in polyhydric alcohol content in apple and larch tree attained about 10 and 15% of that in the sugar content, but in other species it did not amount to as much as 10%. It may, therefore, be safely said that polyhydric alcohols play little part in increasing frost-hardiness during artificial frost-hardening.

Table 5. The variations of polyhydric alcohol and sugar content in frost-hardening. Frost-hardening was done at 0°C for 10 days from October 5.

Species of woody plant	Polyhydric alcohol*			Sugar*			
	Control	Hardening	Increment ΔP	Control	Hardening	Increment ΔS	$\frac{\Delta P}{\Delta S} \times 100$
Salix koriyanagi	0.15	0.21	0.06	1.80	2.76	0.96	6.3
Betula tauschii	0.24	0.28	0.04	1.40	2.93	1.53	2.6
Populus nigra	0.14	0.16	0.02	1.48	3.14	1.66	1.2
Larix leptolepis	0.28	0.45	0.17	1.46	2.56	1.10	15.0
Morus bombycis	0.06	0.07	0.01	1.86	3.60	1.74	5.8
Rosa pendulina	0.21	0.23	0.02	1.82	2.80	0.98	2.0
Malus pumila	0.75	0.85	0.10	1.33	2.33	1.00	10.0

* The amounts of polyhydric alcohol and sugar are expressed as percentage of mannitol and glucose equivalent respectively per wet weight of twig bark.

In Table 6 the contents of polyhydric alcohols in twig bark or in leaf of 19 species of woody plants in winter are given. The content of glycerol ranged from about 0.1 to 0.5% per wet weight in the tea (leaf), climbing spindle tree (leaf) and platanus (twig bark), while in the other 16 species, its content was very small. Furthermore, during hardening both seasonal and artificial, glycerol content showed only slight changes, not significant. Therefore, the conclusion is that no direct relation seems to exist between frost-hardiness and glycerol content. Sorbitol content amounted to about 1% in apple tree (twig bark), gardenia (leaf), pomegranate (twig bark) and mountain ash (twig bark), but its content in the other 15 species was very small. In winter, the content of mannitol and sorbitol in many species increased from twofold to threefold as much as that in August, but in mulberry tree they declined to a half of the summer value. The total content of polyhydric alcohols in the pomegranate (bark), gardenia (leaf), apple tree (bark) and mountain ash (bark) in winter ranged from about 40 to 50% of total sugar content, but in no other species did it reach even to 10% (Table 6). Furthermore, in many species, no positive correlation between the amount of polyhydric alcohol and the frost-hardiness was observed.

From these observations it seems evident that in both natural and artificial frost-hardening, polyhydric alcohol plays little part in increasing frost-hardiness in most woody plants. However, in some species whose polyhydric alcohol content is about 40% of the total sugar content, such as the gardenia, apple tree, mountain ash and pomegranate, polyhydric alcohol may have some part in increasing the frost-hardiness.

Table 6. The polyhydric alcohol contents in the twig bark or leaf in winter represented as the percentage per wet weight of sample.

Species of woody plant	Mannitol	Sorbitol	Glycerol	Total polyhydric alcohol content (P)	Total* sugar content (S)	$100\times\frac{P}{S}$	Frost-hardiness ℃
Betula tauschii	0.23	0.05	0.07	0.35	5.23	6.7	−30**
Populus nigra	0.14	0.05	0.02	0.21	4.69	4.5	〃
Sarix koriyanagi	0.20	0.06	0.08	0.34	5.40	6.3	〃
Larix leptolepis	0.40	0.09	0.03	0.52	5.29	9.8	〃
Taxus cuspidata	0.16	0.10	0.06	0.32	4.27	7.5	〃
Pinus pentaphylla	0.13	0.03	0.06	0.22	3.97	5.6	−28
Rosa pendulina	0.35	0.06	0.04	0.45	5.26	8.6	〃
Rhododendron fauriae	0.18	0.10	0.05	0.33	4.65	7.7	〃
Abies mayriana	0.11	0.04	0.08	0.23	4.00	5.8	〃
Morus bombycis	0.07	0.03	0.04	0.14	4.29	3.3	−25
Malus pumila	0.47	1.15	0.03	1.65	3.15	52.1	〃
Platanus orientalis	0.33	0.08	0.13	0.54	4.77	8.8	〃
Euonymus radicans	0.14	0.20	0.15	0.49	3.82	12.8	〃
Punica granatum	0.50	0.91	0.05	1.46	3.56	41.0	−15
Laurus nobillus	0.25	0.45	0.03	0.73	3.61	20.0	〃
Thea sinensis	0.08	0.05	0.12	0.25	3.33	7.5	〃
Gardenia jasminoides	0.80	0.59	0.02	1.41	2.54	55.5	−12
Picea glehnii	0.07	0.06	0.04	0.17	2.89	5.9	—
Sorbus commixta	0.35	1.05	0.02	1.42	3.48	40.9	—

* Percentage of total sugar expressed as glucose equivalent per wet weight of sample.
** Minimum temperature at which the twig as a whole or leaf can withstand freezing for a full day.

7) *The relation of soluble protein to frost-hardiness.*

Data on seasonal variation of soluble protein content in the twig bark of mulberry tree are graphed in Fig. 5. The variation was roughly proportional to that in the frost-hardiness. However, the soluble protein content attained to maximum about one month before the onset of natural frost-hardening: from early November to December the frost-hardiness in cortex cells increased remarkably with little increase in soluble protein. Moreover, in artificial hardening in late September and middle May, the remarkable increase in frost-hardiness of the bark cells was not accompanied by any increase in the soluble protein. These findings seem to show that the increase of frost-hardiness cannot be explained only by increase in the amount of soluble protein. Especially, in artificial hardening, the amount of soluble protein in bark cells of mulberry tree is not causally related to the increase of their frost-hardiness.

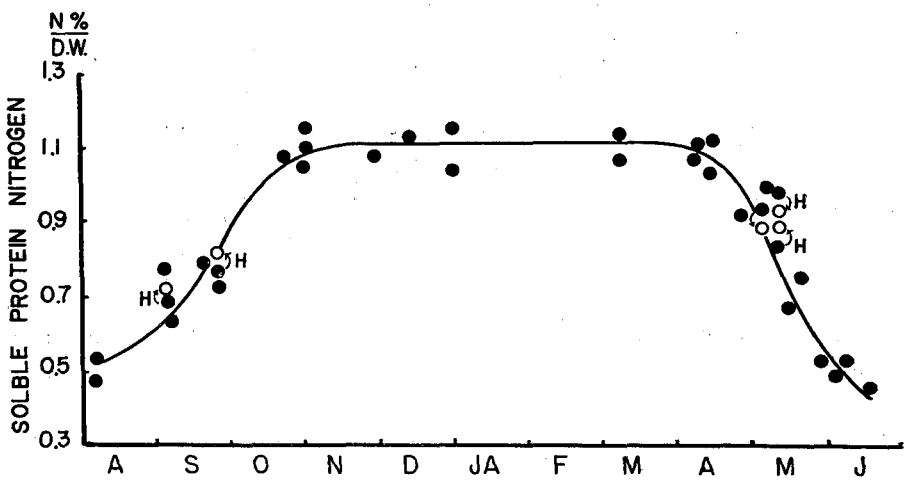


Fig. 5. Seasonal variation of soluble protein content in twig bark in mulberry tree.

The amount of soluble protein is expressed as percentage of soluble protein nitrogen per dry weight of bark. Frost-hardening was done at 0°C for 10 days. Arrows with H indicate the variation of soluble protein after hardening at 0°C for 10 days. Only twig pieces cut from the same segment in the same twig were used in hardening test.

8) *The role of low temperature in artificial frost-hardening.*

To clarify the causal relationship between frost-hardiness and sugar content at the time of artificial hardening, it seems necessary to distinguish the effect of low temperature itself and that of increase in sucrose concentration in a cell. This was successfully performed by examining twig pieces in spring desiccated at room temperature.

When twig pieces cut from a mulberry tree in April were gradually desiccated at room temperature (about 20°C) for 2 or 3 days, considerable changes took place in the cortical tissues: decrease in water content (12% per wet weight) and in starch

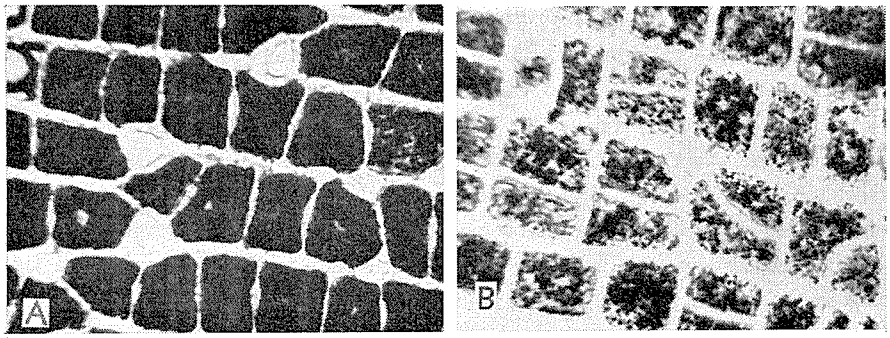


Fig. 6. Decrease in starch in cortical cells caused by desiccation.
Material: mulberry tree. (April. 20).
A: Parenchyma cells of normal twig pieces, in which starch granules are densely packed.
B: Parenchyma cells of twig pieces desiccated for 2 days at about 20°C.
Starch granules were detected by means of I_2-IK reagent.

Table 7. Decrease in starch content in cortical cells caused by desiccation. (April 20).
Material: mulberry tree. Method of desiccation: for 2 days in air at 15~20°C.

	Starch content			I_2-IK reaction	
	Control	Desiccation	Decrement (%)	Control	Desiccation
No. 1	8.1*	5.5	32.3	+++**	+−±
No. 2	5.5	4.1	25.0	++	+−±

* The amount of starch is represented as the percentage of glucose equivalent per dry weight of bark tissue.

** Number of plus signs indicates the relative amount of starch as estimated by microscopic examination of I_2-IK treated section.
+++ = very dense, ++ = medium, + = slight, − = no starch.

Table 8. Increase in sugar content caused by desiccation. (April 20).
Material: Twig piece of mulberry tree.
Desiccation: for 2 days in air at 15°~20°C.

	Sugar content* (per dry weight)			Sugar content* (per wet weight)		
	Control	Desiccation	Increment (%)	Control	Desiccation	Increment (%)
No. 1	13.2	19.5	47.5	5.3	9.6	81.0
No. 2	10.3	15.7	52.0	3.9	7.1	82.0
No. 3	8.8	12.3	42.0	2.9	5.4	86.4

* The amount of sugar is represented as percentage of glucose equivalent per dry weight or wet weight of bark tissues.

content (Table 7, Fig. 6), increase in sucrose (Table 8) and in osmotic concentration (from 0.35 to 0.53 M) (Table 9).

In this case, the rise of osmotic concentration calculated on the basis of decrease of water due to desiccation was equivalent to about 0.07 M. When these twig pieces were frozen at −10°C and −20°C for 24 hours, the percentage survival of the parenchyma cells was 100 and 70 respectively, while in the control it was 25 and 0 respectively (Table 9). The cells when desiccated at room temperature showed greater increase in frost-hardiness as well as in sucrose concentration than in the cells in normal twig pieces chilled at 0°C for 10 days (Table 9).

Table 9. Increase in frost-hardiness and osmotic concentration resultant from desiccation or chilling. (April. 20).
Material: Pieces of a mulberry twig.

Treatment	Freezing temperature			Osmotic ** concentration (M)
	−10°C	−20°C	−30°C	
Control	25 *	0	0	0.35
Desiccation for 2 days at 15° ~20°C	100	70	30	0.50–0.55
Hardening at 0°C for 10 days	100	40	10	0.45–0.47

* The percentage survival of parenchyma cells.
** Indicated as equivalent of molar solution of sodium chloride.

Table 10. Frost-hardening of twig pieces having no starch granules in parenchyma cells. (Oct. 5). Frost-hardening was done at 0°C for 10 days.
Material: Pieces of a mulberry twig.

	Osmotic concentration (M)	Frost-hardiness (at −20°C for 6 hrs)
Control	0.48–0.52*	35**
Hardening (twig piece having no starch)	0.50–0.52	40
Hardening (twig piece having starch)	0.65	100

* Indicated as equivalent of molar solution of sodium chloride.
** percentage survival.

Pieces of a mulberry twig upon which artificial hardening had been effective were kept for about 20 days at 15°C without desiccation. In this way they were allowed to use up the starch granules in the parenchyma cells. If these pieces were chilled for 10 days at 0°C, the parenchyma cells showed no increase in frost-hardiness nor

in sugar content (Table 10).

Table 2 demonstrates that when the twig pieces were hardened at 0°C, the increase of frost-hardiness in the parenchyma cells was closely parallel to that of the sugar content, but that if chilling resulted in no increase of the sugar content, the parenchyma cells could not be hardened further by chilling. Accordingly, it is obvious that to increase the frost-resistance in twig cells, low temperature itself has no direct effect, but that increase in sugar concentration produced by low temperature treatment may be a primary factor.

9) *Increase of frost-hardiness resulting from artificial introduction of sugar into cells.*

In order to increase the sugar content in leaf cells, petioles of matured gardenia leaves in September and October were put in hypotonic glucose solution at 20°~25°C for 16~24 hrs. The petiole of a control leaf was similarly put in water. The osmotic concentration of the spongy cells or the palisade mesophyll cells in gardenia leaf before the treatment ranged from 1.4 to 1.5 M. In September the matured leaf could withstand freezing at −5°C at least for about 16 hrs. Osmotic concentration, sugar content and degree of frost-resistance in the leaf cells treated with glucose solution or in control are shown in Table 11.

Table 11. Effect of glucose uptake on frost-hardiness in gardenia leaf cells. (Oct. 10).

Treatment	Osmotic concentration in cells after treatment (M)	Changes in osmotic concentration in cells after treatment	Sugar content in leaves*	Percentage survival after freezing for 24 hrs.	
				at −10°C	at −15°C
Water (control)	1.40 M	−0.1 M (decrease)	1.8**	0	0
Glucose solution (0.7 M)	2.40 M	+1.0 M (increase)	3.6	100	80

* Exclusive of nerve portion.

** Represented as the percentage of glucose equivalent per wet weight of leaf.

After the treatment, the leaf cells showed considerable increase in sugar content, with concomitant remarkable rise in their frost-hardiness. Analysis revealed that only about 25 per cent of the sugar which penetrated into the cells was converted into sugar alcohol (Fig. 7). It may therefore be reasonable to suppose that the increase of sugar content in the leaf cells treated with glucose solution directly results in rise of their frost-hardiness.

In the next experiment, to vary the sugar content in leaf cells, the petioles of leaves were put in glucose solution of various concentrations. After that treatment, these leaves were frozen at different temperatures under the same conditions. The result of tests indicates that the higher the concentration of sugar introduced into leaf cells, the greater the increase in frost hardiness (Figs. 7, 8).

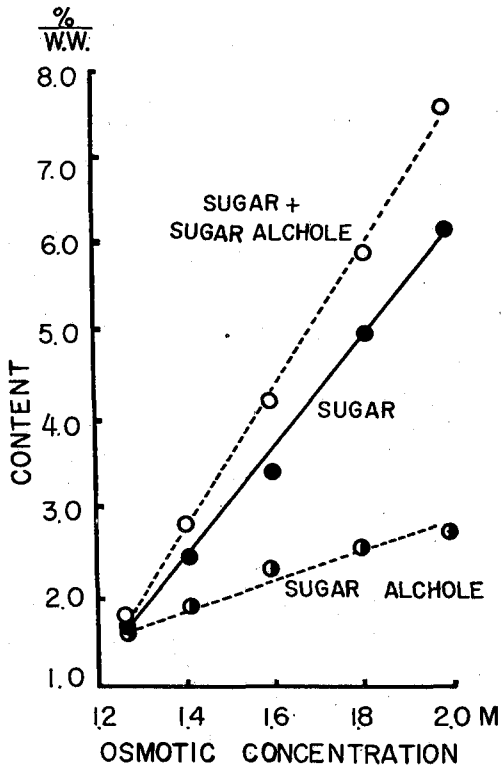


Fig. 7. Relationship between sugar content and osmotic concentration in gardenia leaf cells in which the sugar content was varied by putting the petiole in glucose solution of different concentrations. (September 5).
The sugar content is represented as percentage of glucose equivalent per wet weight of leaf.

Furthermore, some sugars other than glucose, such as xylose, rhamnose, fructose, galactose, sucrose, maltose, lactose and raffinose, were also found to penetrate leaf cells under similar conditions, with marked resultant protective action against frost injury in gardenia leaf cells. In order to compare the garde of protective action of various sugars against frost injury, sugars were introduced into leaf cells at the same osmotic concentration. To ascertain whether these sugars actually penetrated into leaf cells, the osmotic concentration and the sugar content in a treated leaf were examined (Table 12).

At the same time, the amounts of both sugar and sugar alcohol in a leaf were quantitatively investigated by the method of paper chromatography, in order to make clear what amount, if any, of penetrated sugar still remained unchanged in the cells. The chromatograms of leaves treated with sugars are reproduced in Figs. 9-A, B., showing the conversion occurs with some sugars after penetration into leaf cells.

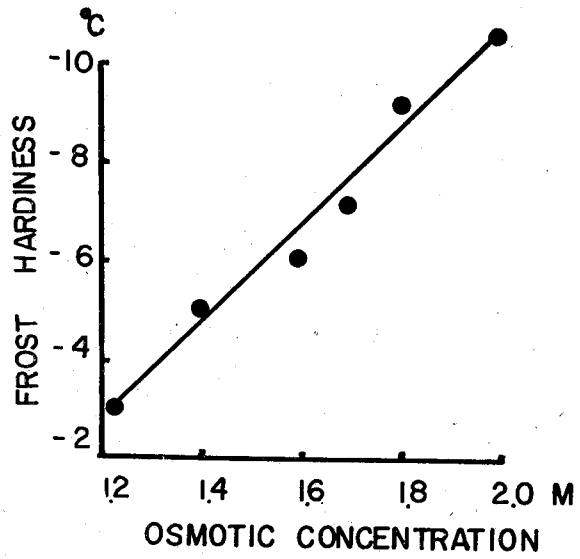


Fig. 8. Relationship between the osmotic concentration and the frost-hardiness in the leaf cells in which glucose was artificially introduced. (September 5).
The grade of frost-hardiness is expressed as minimum temperature at which the leaf as a whole can withstand freezing for 16 hrs without injury.

Table 12. Amount of sugar content in the gardenia leaves treated with various sugar solution.

Solutions (M)	Osmotic concentration (M)	Total* sugar content	Total* sugar alcohol
Water (control)	1.40	1.41	1.89
Xylose (0.7)	2.40	4.22	2.14
Glucose (0.7)	2.50	4.58	2.85
Sucrose (0.7)	2.40	5.46	2.92
Raffinose (0.7)	2.30	5.87	2.71

* Total sugar content and total sugaralcohol content are represented as the percentage of glucose and mannitol equivalent respectively per wet weight of leaf.

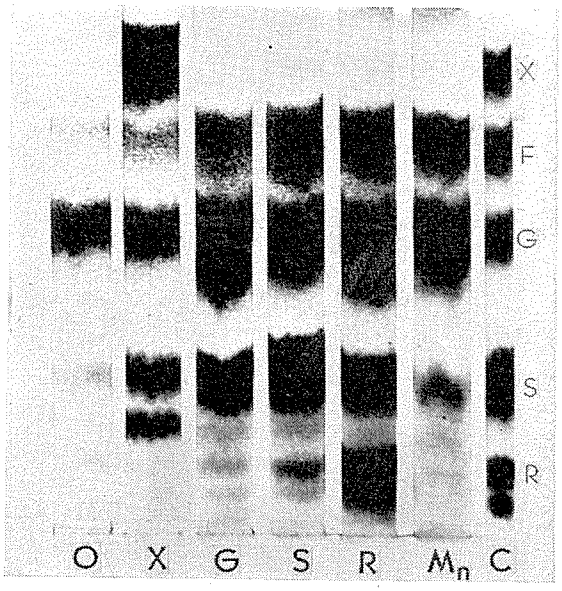
It was also found that 50～70 percent of the amount of the sugars which penetrated into the leaf cells still remained unchanged, except for maltose, which largely converted into other sugars after penetration. Moreover, in gardenia leaf, some penetrated sugars were partially converted into sugar alcohols. In short, 45～60 percent of each sugar penetrated into the leaves still remained unchanged in the leaf cells (Fig. 10).

Fig. 9-A.

Chromatograms of sugars in extracts from gardenia leaves treated with various different sugar solutions. The chromatogram of the leaf treated with mannitol (Mn) solution is also presented for comparison.

Solvent of development: butanol, acetic acid, water (4 : 1 : 2)
Development: 7 times in ascending direction.
Detection of spot: benzidine reagent.

Each of the letters under the chromatogram, O, X, G, S, R and Mn indicates the chromatogram of sugars in the leaf treated with water (control), xylose, glucose, sucrose, raffinose, and mannitol solution respectively.
C: X (xylose), F (fructose), G (glucose), S (sucrose), R (raffinose) as marker.



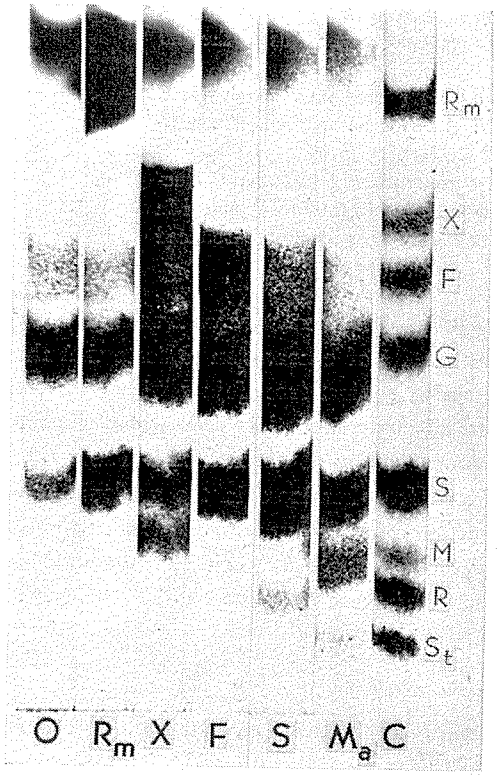


Fig. 9-B.

Chomatograms of sugars in extracts from gardenia leaves treated with various different sugar solutions.

Each of the letters under the chromatogram, O, Rm, X, F, S, and Ma indicates the chromatogram of sugars in the leaf treated with water, rhamnose, xylose, fructose, sucrose and maltose respectively.

C: Rm (rhamnose), X (xylose), F (fructose), G (glucose), S (sucrose), M (maltose), R (raffinose) and St (stachyose) as marker.

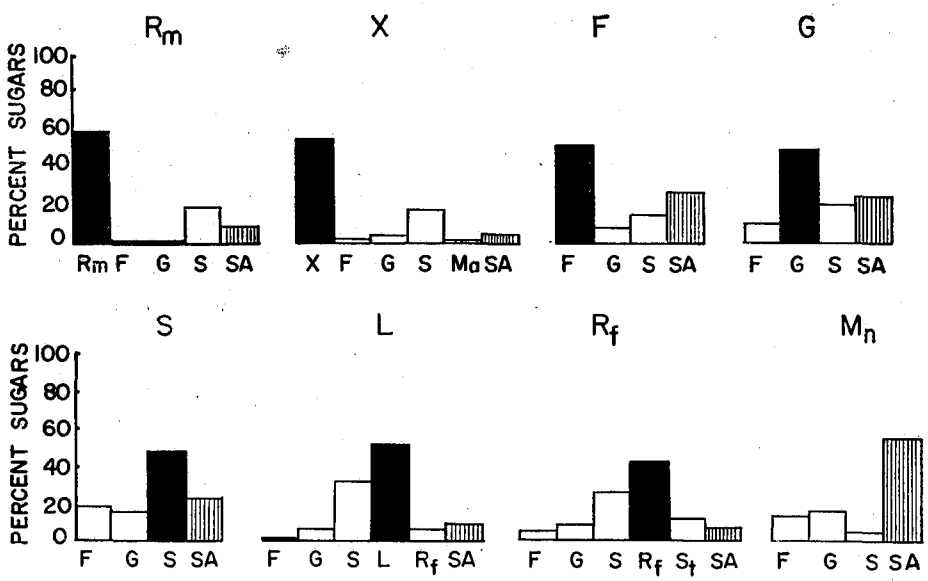


Fig. 10. The conversion of sugars after penetration into gardenia leaf cells to other sugars and sugar alcohol.
Ordinate indicates percent of each sugar to the total amount of the sugar and the sugar alcohol increased in the cells of the leaf treated with sugar solution.
Abscissa represents the kind of sugar contained in the leaf. Rm: rhamnose, F: fructose, G: glucose, S: sucrose, Rf: raffinose, St: stachyose, SA: sugar alcohol.
The letters above the figure, Rm (rhamnose), X (xylose), F (fructose), G (glucose), S (sucrose), L (lactose), Rf (raffinose) and Mn (mannitol) represent the sugar and the sugar alcohol introduced into the leaf respectively.

After treatment with various sugar solutions, the leaves were frozen at −15°C for 16 hrs under identical conditions. The results of observation indicate that these sugars showed nearly the same protective action against frost injury within the limit of concentration at which they exert no bad effect upon the leaf cells when artificially introduced thereinto at the same osmotic concentration of about 2.4 M (Table 13).

It may therefore be likely that the rise of frost-hardiness in the gradenia leaves treated with sugars is chiefly due to the action of the sugars themselves introduced into cells. In this case, the protective action of pentoses such as rhamnose and xylose seems to be slightly greater than that of hexose and disaccharide, whilst that of raffinose is smaller than the latter.

10) *The protective action of polyhydric alcohols.*

In the same way as the case of sugar, various polyhydric alcohols were introduced into gardenia leaf cells and their protective effects were investigated. Ethylen glycol,

Table 13. Degree of protective action of various sugars against frost injury in gardenia leaf. Experiment was made in the period from middle September to late October. Freezing test: at −15°C for 18 hrs. The results of the leaf treated with mannitol are also showed in comparison with those of the sugar.

Experiment number	The kind of sugar (0.7 M)									
	Rhamnose	Xylose	Fructose	Glucose	Sucrose	Maltose	Lactose	Raffinose	Mannitol	Water (control)
No. 1	−	·	·	·	+	·	·	·	·	·
No. 2	±	·	·	·	±	·	·	·	·	·
No. 3	±	·	·	·	+	·	·	·	·	·
No. 4	±	±	+	·	·	+	·	·	·	·
No. 5	·	·	·	·	±	·	±	±	·	++++*
No. 6	·	·	±	±	±	·	·	·	·	·
No. 7	·	−	·	·	·	·	−	·	·	·
No. 8	·	·	−	±	·	·	·	·	·	·
No. 9	·	±	·	+	+	·	·	·	·	·
No. 10	·	±	·	+	·	·	·	·	++	++++
No. 11	·	·	·	−	·	·	·	+	+	·
No. 12	·	·	·	+	·	·	·	·	++	++++
No. 13	·	·	·	±	±	·	·	·	+	++++
No. 14	·	·	·	·	±	·	·	·	+	++++
No. 15	·	·	·	·	·	+	+	+	·	·
No. 16	·	·	·	+	·	·	+	·	·	·
No. 17	·	·	·	+	·	·	·	++	·	·
No. 18	·	·	·	−	·	·	·	±	·	·
No. 19	·	·	·	+	·	·	·	+	·	·

* The degree of frost injury is relatively expressed by plus numbers.
−: Normal. ±: Leaf nerve and its neighborhood are yellow brown and other parts of leaf are normal. +: All the leaf parts are yellow brown. ++: Leaf nerve and its neighborhood are brown and other parts of leaf are yellow brown. +++: Leaf nerve and its neighborhood are black brown and other parts of leaf are brown. ++++: All the leaf parts are black brown.

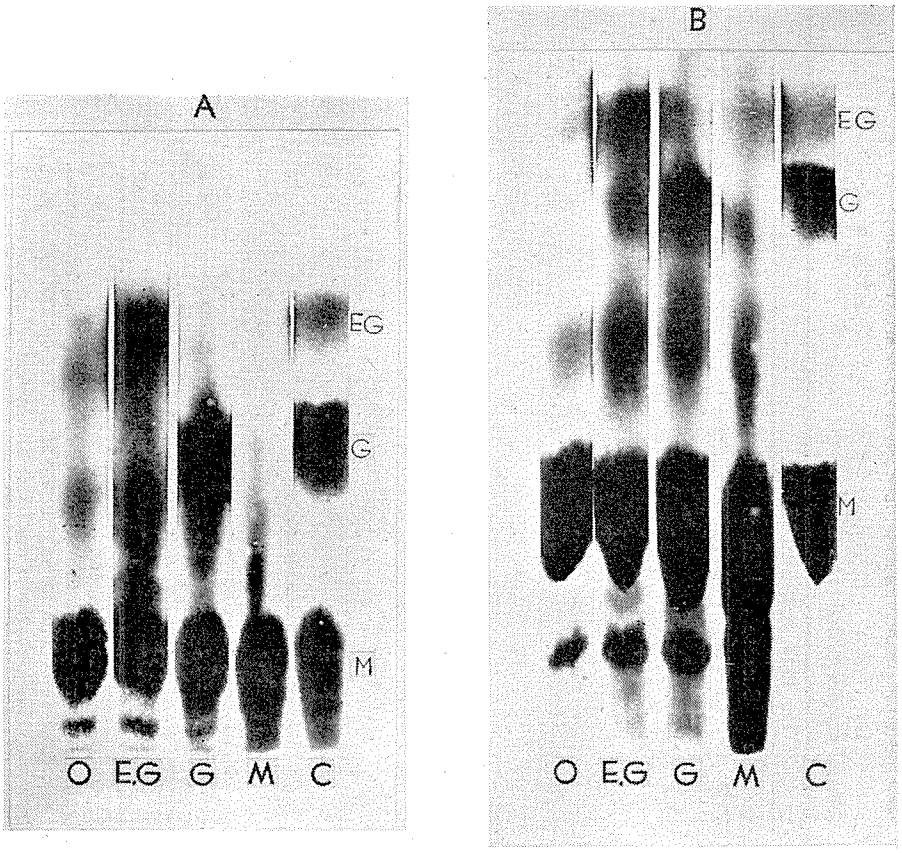


Fig. 11. Chromatograms of polyhydric alcohols in the extracts from gardenia leaves treated with different polyhydric alcohol solutions.
A: once development.
B: four times development.
Solvent of development: butanol, acetic acid, water (4 : 1 : 2).
Detection of spot: Ammoniacal silver nitrate.
Each of the letters below chromatogram, O, E. G, G and M indicates the chromatogram of the extract from the leaf treated with water, ethylene glycol, glycerol and mannitol solution respectively.
C: Ethylene glycol (E. G), Glycerol (G) and Mannitol (M) as maker.
In these chromatograms the spots representing reducing sugars as well as polyhydric alcohols are also detectable.

glycerol, mannitol and sorbitol penetrated in a large amount into the leaf cells. Chromatograms of the extracts from treated leaves ars shown in Fig. 11.

After penetration, 20 to 25 percent of glycerol is converted into sugar and sugar alcohol, and about 40 percent of mannitol converted into various sugars. As ethylene glycol easily diffuses out from the cell interior, and the quantitative method here

employed is not sensitive to this substance, both the osmotic concentration and the amount of ethylene glycol in cells could not be determined. After treatment with polyhydric alcohols the leaves were frozen under identical conditions. The results indicate that all of these polyhydric alcohols have protective action against frost injury (Table 14).

Table 14. Protective action of polyhydric alcohols against frost injury in gardenia leaf cells.

	Test solutions			
	Water (control)	Ethylene glycol (0.7 M)	Glycerol (0.7 M)	Mannitol (0.7 M)
Osmotic concentration (M)	1.46～1.50	—	2.54～2.58	2.50～2.55
Frost-hardiness *	0	100	100	70

* The grade of frost-hardiness is represented by the percentage survival of gardenia leaf cells after freezing at −10°C for 16 hrs.

In this experiment, the protective action of ethylen glycol and glycerol was found to be greater than that of mannitol. No protective action was found with diethylene glycol, propyrene glycol, triethylene glycol and polyethylene glycol which could not penetrate into leaf cells by the method here used.

11) *Protective action of some organic and inorganic compounds against frost injury.*

Acetoamide, urea, glycine, ammonium chloride, potassium chloride, sodium chloride and calcium chlordie were investigated in examination of their protective action against frost injury. These substances were found to penetrate easily into gardenia cells. As urea and sodium chloride exert some toxic effects upon the cells when introduced into the cells in any great amount, their effect to prevent frost injury was small. Potassium chloride, ammonium chloride and glycine showed no preventive effect because of their toxic action upon cells. The toxic effect of calcium chloride was slighter than that of potassium chloride and of ammonium chloride, but its preventive effect was very small too. Among these compounds, only acetoamide showed a considerable protective effect against frost injury.

12) *Degree of ability of various substances to protect gardenia leaf cells against frost injury.*

It has been demonstrated in above sections that various sugars show nearly the same protective action against frost injury when artificially introduced in the same osmotic concentration. Similar relation is also found with glycerol and sucrose (Table 15).

Therefore, this may be a common rule not only among the sugars, but also among other substances. However, the limit of concentration at which solutes can

Table 15. Relationship between osmotic concentration and frost injury in gardenia leaves treated with sucrose or glycerol solution. Experiment was made in the period from middle September to late October. Freezing test: at −15°C for 18 hrs.

Number of experiment	Sucrose		Glycerol	
	Osmotic concentration (M)	Degree of frost injury*	Osmotic concentration (M)	Degree of frost injury*
No. 1	2.4	++	2.3	++
No. 2	2.1	++	2.2	++
No. 3	2.5	±	2.5	+
No. 4	3.0	+	2.9	+
No. 5	3.0	+	2.9	+
No. 6	2.2	++	3.3	±
No. 7	2.1	−	2.3	±～−
No. 8	2.0	+	2.3	−
No. 9	2.6	±	2.8	−
No. 10	2.4	+	2.4	+
No. 11	2.0	+	2.6	−
No. 12	2.3	++	2.3	++
No. 13	2.3	±	2.6	±
Ne. 14	2.3～2.4	++	2.6	++

* The degree of frost injury was relatively expressed by plus numbers.
− : Normal.
± : Leaf nerve and its neighborhood are yellow brown and other parts of leaf are normal.
+ : All the parts of leaf are yellow brown.
++ : Leaf nerve and its neighborhood are brown and other parts of leaf are yellow brown.

be introduced without any toxic effect upon the cells is remarkably different according to the solutes. When the osmotic concentration in normal leaf cells is 1.5 M, the maximum osmotic concentrations above which the toxic effect of the introduced substances takes place are as follows: glycerol: 4.0 M; sugars: mannitol and sorbitol: 2.7～3.0 M; acetoamide: 2.2 M. It will be seen that among the solutes here used glycerol can be introduced into cells at the highest concentration with little injury. Then, to clarify the relation between frost resistance and glycerol content, that alcohol was introduced into leaf cells at various different concentrations. As indicated in Fig. 12, only 20～25 percent of the glycerol which penetrated into cells is converted into sugar and sugar alcohol even when it is in a large amount. Obviously, therefore, that rise in osmotic value in treated leaf cells is chiefly due to the amount of glycerol in the cells.

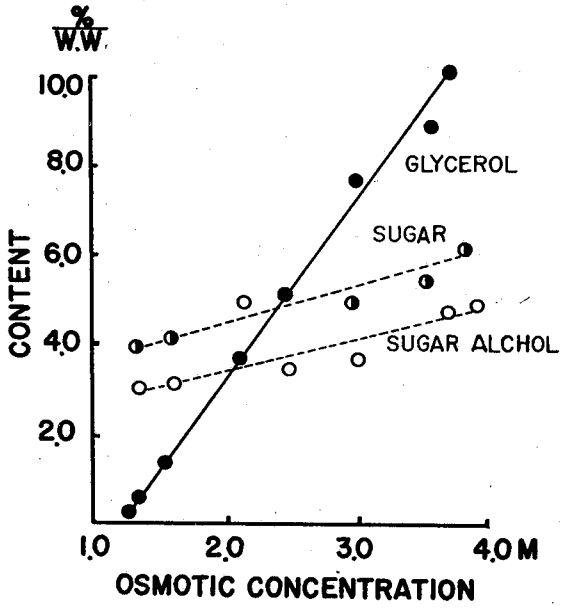


Fig. 12.

Relationship between glycerol content and osmotic concentration in the cells of gardenia leaf treated with different concentrations of glycerol.

Glycerol content is represented as the percentage of glycerol per wet weight of leaf.

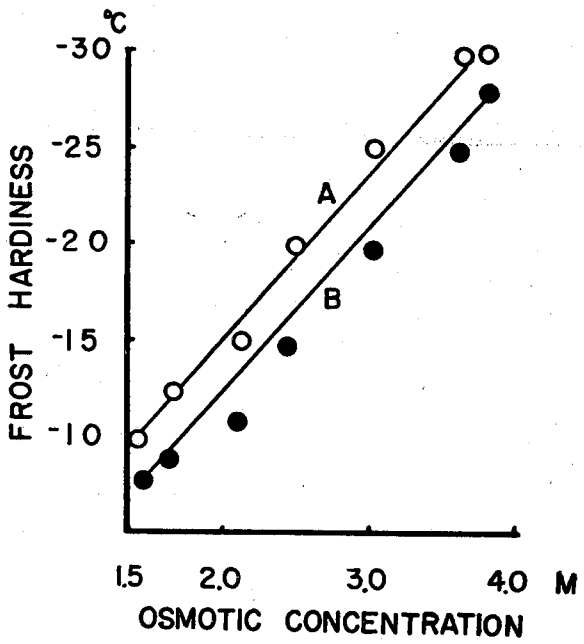


Fig. 13.

Relationship between osmotic concentration and frost-hardiness in the cells of gardenia leaf treated with different concentration of glycerol.

The grade of the frost-hardiness is expressed as minimum temperature at which leaf can withstand freezing.

A: freezing for 4 hrs.

B: freezing for 16 hrs.

In this case too, linear relation was found between the frost-hardiness and the glycerol content in gardenia cells (Fig. 13).

The results above presented indicate that the grade of the protective action of various sustances against frost injury depends upon the amount in which they can be introduced into the cells without any damage, at least in non electrolites. The same relation seems to exist in electrolites, too. The degree of the ability of various substances to protect gardenia leaf cells against frost injury may be arranged as follows:

glycerol > rhamnose, glucose, fructose > raffinose
ethylene glycol > xylose, sorbitol, sucrose, lactose > mannitol > acetoamide > urea
> Water > glycine.

NaCl > $CaCl_2$ > Water > KCl, NH_4Cl.

IV. Discussion

1) *The causal relation between frost-hardiness and sugar content.*

Recently, some workers such as PARKER[5,14], HEBER[7], HARADA *et al*[39], JEREMIAS[6], *etc.* have demonstrated a positive relation between sugar content and frost-hardiness. However, some earlier reports[17,19,22,23,40] could not find out a parallelism between sugar content and frost hardiness. This may be chiefly due to some unsuitable experimental procedure and erroneous considerations as follows:

1. Sugar content was expressed as per dry weight[22].
2. The determinations of frost-resistance and sugar content were not made in the same tissue[40]: for example, the determination of sugar content was made in the cortical tissue in twig and that of frost-hardiness was made in twig as a whole.
3. The degree of frost hardiness was determined only by the value of dehydration resistance in a hypertonic salts solution[11,22].
4. Some authors[19,24,40] did not take into consideration the following facts:

a. In considering the relation of sugar content to frost-hardiness in different stages of the same plant, the increase of the frost-hardiness is not explained only by the increase of the sugar content[19,40].

b. Unlike the case in different varieties of the same species, there is little parallelism between the sugar content and the frost-hardiness in the tisssues of different plant species[19,24,40].

Based on the results of the present experiments some considerations will be offered about these problems. When sugar content is expressed as amount per dry weight, a parallel correlation between sugar content and frost-hardiness in mulberry tree is not always found out. From late August to early September the sugar in cells converts into starch, resulting in decline in the content when expressed as per dry weight of tissue cells. At the same time a remarkable decrease in water content in the cells occurs[52]. Consequently, in spite of decrease of sugar content when expressed as per dry weight, the osmotic concentration or sugar content expressed as per wet weignt

rises in proportion to the increase in the frost-hardiness. Accordingly, the increase of frost-hardiness in this case cannot be explained only by the amount of sugar expressed as per dry weight. Therefore, in considering the relation between frost-hardiness and sugar content in natural frost-hardening, it seems necessary for considering to depend upon the sugar concentration in cells, but not the absolute quantity of sugar contained in them.

Using about 30 species of woody plants living in nature from subtropical to polar regions, the relation between the osmotic concentration and the frost-hardiness among the different species in winter has been studied[41]. The results revealed that the woody plants showing great degree of frost-hadiness had generally a high osmotic concentration, but not *vice versa*. Some subtropical plants, such as the persimmon, gardenia, tangerine, *etc.* showed high osmotic concentration, although their frost-hardiness was considerably small. Consequently, there seems little parallelism between the values of osmotic concentration and the frost-hardiness among the different plant species. However, among the valieties of any one species there is an intimate correlation between these two factors[40,42,44,45]

In hardy cells, the frost-hardiness necessarily rises with increase of the sugar content, but unhardy cells such as those in the bulbs and tubers of gladiolus[46], dahlia and potato[47], do not increase their frost hardiness as a result of exposure to low temperature[48], although sugar content in the cells does remarkably increase. This fact shows that if cells and tissues are not in hardy state, the increase of sugars in the cells can result in no or little rise of their frost-hardiness. The cortical cells of the growing twig of woody plants are not frost hardy and can not increase their frost-hardiness even as a result of exposure to 0°C for ten days[49-51], although the sugar content in the cells does considerably increase. Soon after the growth of twig ceased, the cortical cells of the twig undergo various considerable change[52]: decrease of both water content and activity of the cambium cells; increase of both starch granules and osmotic concentration. Without these changes, the cortical cells can be neither frost-hardy nor hardened further by chilling. After late September, resultant from hardening the twig at 0°C for 10 days, the osmotic concentration of parenchyma cells of the twig cortex in mulberry tree, reaches nearly to its maximum which can be usually found in winter[52]. However, in late October, these cells become much more hardy than those hardened similarly at late September, even though their osmotic concentration attain the same value[52]. This fact shows that the ratio of the increase of frost-hardiness per unit increase of sugar in cells is remarkably different in the developmental stages of twig, whether the increase of sugar is made by artificial frost-hardening or by artificial introduction of sugar into the cells. Accordingly, in different stages of the same plant, the degree of frost-hardiness, cannot be explained only by the amount of sugar content in the cells, unlike the relation between these two factors in the cells of the same stage. From these findings, it is reasonable to emphasize, therefore, that in discussing the relation of sugar content to frost-hardiness,

the stage and the conditions of plant tissue as well as their sugar content must be necessarily taken into consideration.

SIMINOVITCH[21] reported that a high starch content in cells would make them more susceptible to frost injury because of the fact that the starch granules serve to enhance stress and distorted effects upon the protoplasm as engendered by dehydration during the freezing process. However, as mentioned above, from late August to early September starch granules in cells remarkably increase in quantity but the protein content is nearly invariable, whereas the frost-hardiness in this season increases rather than decreases. It is obvious, therefore, that the increase of the frost-hardiness associated with the decrease of starch granules from fall to winter is not due to the exclusion of a detrimental effect exerted by starch granules as supposed by Siminovitch, but is caused by the increase of sugar concentration.

The relation between frost-hardiness and dehydration resistance will be considered later. As a criterion of frost-hardiness, SIMINOVITCH[53] used the dehydration test, based on a determination of the percentage survival of cells after dehydration in hypertonic salts solution. He has noted a positive correlation between the content of water soluble protein and the degree of dehydration resistance in parenchyma cells of black locust tree[11-13,53]. According to him, evidence so far available did not suggest any participation of soluble carbohydrate in the mechanism of dehydration resistance. However, it has been revealed[4,54] that the value of dehydration resistance is considerably affected by the amount of sugar contained in cells. Accordingly, the increase of dehydration resistance cannot be explained only by the amount of water soluble protein.

The dehydration resistance test[53] was employed by SIMINOVITCH and BRIGGS to know the capacity of cells to withstand dehydration by extracellular freezing. Accordingly, the application of this test to unhardy cells such as cells in growing twig is not suitable. In the dehydration test, with hypertonic balanced salt solutions as the plasmolyte, the test is practicable only in the hypertonic solution below saturated concentration. For this reason, SIMINOVITCH[11,13] detected little variation in the grade of dehydration resistance of the twig cells during the period from November to January when their frost-hardiness increased to its maximum level. Moreover, the salts solution employed in dehydration test is in itself toxic. Consequently the injury observed in plasmolyzed cells especially in the high concentration is more than the injury expected on the basis of dehydration alone[54]. Based on these consideration, it may be said that the employment of value of dehydration resistance as a measure of the frost resistance capacity involves much risk. Accordingly, for the determination of the degree of frost-hardiness, it is necessary to investigate the cells directly by freezing test.

As mentioned previously, some investigators[5-7,39,55] have recently shown a positive relation between the increase of sugar content and that of frost-hardiness, but there was presented no evidence to show that the observed relation was causal. Recently, however, LEVITT (1959) found that the infiltration of hexose into frost hardy cabbage

leaves resulted in an increased frost-hardiness equal to the value calculated on basis of a purely osmotic effect[25]. Under his method, it seems highly difficult to determine whether the increase of frost-hardiness is due to the increase in sugar content of cell interior or of intercellular space, because in his method of infiltration, the sugar may be introduced into the intercellular space as well as into the cells. The present author has reported[56,57] that the cells of hardy cabbage leaf frozen in water could withstand freezing at −15°C for 16 hours, whereas, these cells when frozen in glucose solution were able to survive even freezing at −70°C for a full day. It follows, therefore, that when the cabbage cells were subjected to freezing in media containing sugar, the cells were not injured even by deep freezing though cabbage cells were themselves inpermeable to sugars. Accordingly, the conclusion can not be drawn from the data alone presented by Levitt that the increase of glucose within the cells resulted in that of their frost-hardiness. In the present experiments, it has been demonstrated that the increase in frost-hardiness of hardy cells is closely proportional to that in their sugar concentration, and that in artificial hardening, low temperature itself has no direct effect in increasing frost-hardiness, but the increase of sugar in cells may be a primary factor in that of the frost-hardiness. Moreover, the gardenia leaves when sugar was introduced into cell interior without altering the state of intercellular space, showed considerable rise in their frost-hardiness. Therefore, it is obvious that the increase of sugar in hardy cells directly causes increase of their frost-hardiness. Accordingly, the causal relation between sugar and frost-hardiness was first confirmed by the present experiments.

2) *Some protective substances against frost injury contained in woody plants.*

It was demonstrated in a foregoing section that the protective action against frost injury in gardenia leaf was not unique to sugar, but other substances, too, exhibited protective action against frost injury if they could easily penetrate into the leaf cells in great amount without toxicity. In woody plant cells substances which are most effective in preventing frost injury were found to be sugar and polyhydric alcohol. In general, polyhydric alcohol is contained in very small quantity, but in some woody plants in winter such as gardenia, apple tree, mountainash and pomegranate, the alcohol content amounts to about 40 percent of the total sugar content. Accordingly, in these woody plants having considerable amount of polyhydric alcohol, the alcohol is considered to play some part in increasing their frost-hardiness.

A comparison of sugar in bark or leaf taken from 18 species of woody plants revealed[58,59] no significant qualitative variation in sugars: chromatograms of sugar from bark samples or leaves in winter showed the presence of stachyose, raffinose, maltose, sucrose, glucose, fructose, xylose, ribose, rhamnose, *etc.* The proportion of content of various sugars in bark and leaf showed considerable variations in the 18 different species studied. However, there seems little intimate correlation between the rate of sugar composition in bark or leaf samples and their frost-hardiness[58,59].

A positive relation of raffinose and stachyose to frost-hardiness has been stated by many workers[3,5,6,58,60-62]. PARKER[5,62] reported that raffinose gradually increased in autumn as cold-hardening proceeded, and that during winter it was kept at a fairly constant level; in spring, however, it declined sharply when hardiness of plant had largely disappeared; in contrast, sucrose seemed to have no relation to hardiness changes. The present author's experimental evidence[58] obtained with twig bark of mulberry tree indicated that with the deceleration of the growth process of the twig, raffinose appeared in middle August and stachyose, too, in middle October. They increased towards the colder months, but showed a marked decline in spring; stachyose was not detectable in late April and raffinose not in late May. However, even in winter the content of these two sugars does not amount to 40 percent of the total sugar content in any of the twigs or leaves in the 18 species[59]. Besides, in an artificial frost-hardening, the increase of raffinose is far less than that of sucrose, glucose and fructose, In addition, the protective action of raffinose against frost injury in gardenia leaf has been shown to be much less than that of sucrose and glucose. From these facts, it may be unlikely that raffinose and stachyose play any more important part in enhancing frost-hardiness than other sugars, such as sucrose and glucose in various species of woody plants.

3) *The relation of soluble proteins to frost-hardiness.*

Using the twig bark of black locust, SIMINOVITCH[11,13,21,22] investigated the behavior of water soluble proteins and sugars in relation to frost-hardiness; he made clear that the increase in frost-hardiness was closely proportional to the increasing amount of water solublu protein, but not to that of sugar content. However, he obtained no direct evidence showing a causal relation between the two factors. As was shown in the above paragraph, in the twig bark of mulberry tree, a rough parallelism exists between the degree of frost-hardiness and the amount of soluble protein. In seasonal variations, however, it is impossible to distinguish the effect of the increase of soluble protein upon frost-hardiness from that of sugar because of the lack of suitable method to bring about the soluble protein increase without any rise of sugar content. Therefore, it seems highly difficult from these studies to clarify whether a causal relation actually does exist between these two factors.

The author has found that, whenever the artificial frost-hardining in the cortical cells is effective, the increase in frost-hardiness is closely proportional to that in their sugar content, but the increase in frost-hardiness is not accompanied by any increase in their soluble protein, provided that they are artificially chilled. Moreover, from early November to December, the frost-hardiness of cortical cells rises remarkably, in spite of a slight increase in soluble proteins. As stated before, after late September, by hardening the twig at 0°C for 10 days the osmotic concentration of parenchyma cells of the twig cortex in mulberry tree is brought up to nearly its maximum; that maximum is usually found in winter. However, in late October, these cells become

more hardy than those hardened similarly in late September, even though their osmotic concentrations attain nearly the same value[52]. Also, from late September to late October, the amount of soluble proteins remarkably increases. These facts show that the greater the amount of the soluble protein in the cells, the higher becomes the ratio of increase of the frost-hardiness per unit increase of the sugar content. The accumulation of the soluble protein in cells seems to be prerequisite to accomplishing the natural frost-hardening in woody plants. It may be, therefore, reasonable to say that the increase of suger in hardy cells directly results in that of their frost-hardiness without accompanying any change in their soluble protein content. On the other hand, the increase of soluble protein content in cells is not directly connected with any change of their frost-hardiness, though it promotes the hardening effect caused by the increasing sugar and polyhydric alcohol.

Some authors[16,17] reported a parallel relation of glycoprotein to the frost-hardiness. As the quantitative method for estimation of glycoprotein used by them was not suitable, it is doubtful whether there was any parallelism between the two factors. Recently, HEBER[16] made an exact analysis of the amount of glycoprotein in winter wheat, and demonstrated that in seasonal variation there was no parallelism between the content and frost-hardiness. He showed, moreover, that increase in the sucrose concentration was closely proportional to that in the frost-hardiness, but the latter was accompanied with no increase in the content of glycoprotein and soluble protein[15].

4) *The mechanism of protection of sugar against frost injury.*

As the twig cells and gardenia leaf cells in the present experiment were in hardy state and were slowly cooled after inoculation with ice at −5°C, it may well be considered that the frost injury in these cells was brought about by the intense dehydration caused by extracellular freezing[63]. Accordingly, only the mechanism of the injury due to extracellular freezing will now be discussed.

It was demonstrated above that the protective action against frost injury in gardenia leaf cells was not unique to sugar; but other substances which can easily penetrate into the leaf cells and are not toxic even when they penetrate in great amount also showed a protective action against frost injury. In such a case, when various substances were artificially introduced into leaf cells to secure the same osmotic concentration, these substances showed nearly the same protective action within the limits of concentration up to which they exerted no bad effect upon cells. Accordingly, it is obvious that the grade of protective action of these various substances against frost injury depends upon the amount to which they can be introduced into cells without any damage,

In plant cells, LIDFORSS[64] first asserted that damage during freezing was caused by increase in concentration of electrolytes within the cells. This "salt injury theory" is still supported by some authors working with animal[65-67] and plant[15,68] cells. Contrary to these, MAXIMOV[69-71] was of the opinion that the increase of salt concen-

tration in a cell caused by freezing could not be a primary factor in the frost injury. Besides, some workers[63,72-76] maintain that frost-injury is due to the injurious mechanical stress resulting from the dehydration of the cell on freezing and from its subsequent rehydration on thawing. The author[56,57] presented some evidence that frost injury was not explained by the increase of salt concentration in cells; hardy cabbage cells frozen in water were killed by freezing at −15°C, when frozen in hypotonic glucose solution, these cells could withstand even freezing at −70°C. Moreover, it was ascertained that cabbage cells were themselves inpermeable to glucose. From these facts it seems evident that the increase of salt concentration in cells due to freezing cannot be a primary factor in increasing frost injury to plant cells, and that the frost injury is caused by the injurious effects arising in the surface layer of protoplast of cells due to the removal of their water on freezing.

Most of the theories concerning the frost injury in cells deal with damage in cells subjected to freezing and thawing in media containing various concentrations of solutes. Accordingly, the protective effect against frost injury in such cells is supposed to depend upon the quality and the concentration of the surrounding media used rather than upon the amount of protective substance contained in cell interior. In the cells of intact leaves and twigs here used, as a rule, no fluids are found in their intercellular spaces, and there seems no need to consider various effects caused by the surrounding media in considering the mechanism of their frost injury.

As mentioned above, cabbage leaf cells could withstand even freezing at both −30°C and −70°C, provided that they are frozen and thawed in glucose solution. The osmotic concentration (0.7 M) of the cabbage leaf cells is about half of that in many woody plant cells in winter. On the other hand, when intact cabbage leaves in which various concentrations of glycerol had been introduced into the cells were frozen in air, only the leaf petiol having the increased osmotic concentration of threefold the normal isotonicity could withstand freezing at −30°C[41]. Accordingly, when the cells are subjected to freezing in sugar solutions, it seems unnecessary to give special attention to the amount of the protective substances contained in cells; when the cells are frozen in air as in the case of intact leaves or twigs, a high intracellular concentration of some protective solutes is essential to prevent frost injury. It seems, therefore, that for the analysis of the mechanism of the frost injury of plant cells, the method in which cells and tissues are subjected to freezing must be taken into consideration. At the present time, however, the mechanism of the protective action of solutes in the cells when they are frozen without any media outside is not yet clear.

Summary

To make clear the causal relation between frost-hardiness and sugar content in plant cells, some experiments were made.

In artificial frost-hardening of the parenchyma cells in cortex of mulberry tree, the lower the temperature at which the treatment is made, the greater the effectiveness

of the hardening treatment becomes. At a definite temperature, a relatively longer period of cold treatment is more effective than shorter ones within a certain limit.

In all these experiments, the increase in frost-hardiness of parenchyma cells is closely proportional to that in their sucrose concentration. But the former is not accompanied by any increase in soluble protein contents of the cells provided that they are artificially chilled.

It becomes necessary, however, to distinguish the effect of low temperature itself and that of the increase in sucrose concentration in considering the frost-hardening process in plant cells as a result of artificial chilling. When pieces of twig cut from a mulberry tree in April were gradually desiccated at room temperature (about 20°C) for 2–3 days, considerably significant changes took place in the cortical tissues; namely, decrease both in water content and in starch content, increase both in sucrose and in osmotic concentration. The cells desiccated in this way showed greater increase in frost-hardiness as well as in sucrose concentration than the parenchyma cells in normal twig pieces in the same season. Pieces of twig in which artificial frost-hardening had become effective were kept for about 20 days at 15°C without desiccation so that the starch granules in the parenchyma cells might be used up. Afterwards, these pieces were chilled for 10 days at 0°C; in the parenchyma cells in these pieces, however, no increase was found either in frost-hardiness or in sucrose concentration. In this way even at a relatively high temperature, the increase in sucrose concentration in the parenchyma cells also resulted in rise of their frost-resistance. On the other hand, whenever chilling in parenchyma cells resulted in no increase in their sucrose concentration, they could not be hardened further by chilling. These results seem to indicate that, in artificial hardening, low temperature itself has no direct effect on the increase in frost-hardiness, but the increase of sucrose concentration may be a primary factor in frost-hardiness.

Furthermore, some experiments were made to find out whether the increase of sugar concentration in plant cells directly results in an increase of their frost-hardiness or not. In order to increase the sugar content in a cell, petioles of gardenia leaves in September and October were put into hypotonic glucose solution (1.4 M) at 25°C for 24 hrs. At the same time, the petiole of another leaf was put in water under the same conditons. The osmotic value of the original leaf cells was about 1.4 M. After treatment, the sugar content in the gardenia leaf cells amounted to twice as much as that of control, with concomitant considerable rise in their frost-hardiness. It is concluded therefore that the increase of sugar in cells directly results in that of their frost-hardiness.

Some other sugars besides glucose, such as xylose, rhamnose, sucrose, lactose and raffinose also brought about a considerable increase in the frost-hardiness as well as in sugar content when they were introduced into leaf cells. In addition, some polyhydric alcohols and acetoamide, too, showed a protective action against frost injury. These results suggested that the protective action against frost injury in gardenia leaf

cells was not unique to sugar, but other substances which could easily penetrate into the leaf cells and were not toxic even when they had penetrated in great amount could also exhibit a protective action against frost injury. When artificially introduced into leaf cells at the same osmotic concentration, these substances showed nearly the same protective action against frost injury within the limits of concentration at which they exert no bed effect upon cells. The grade of protective action of various substances against frost injury depends upon the amount to which they can be introduced into cells without doing damage.

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"Low Temp. Sci." in the following is the abbreviation for "Low Temperature Science", a scientific publication written in Japanese with English summary, issued by the Institute of Low Temperature Science, Hokkaido University, Sapporo, Japan.

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