THE FROST-HARDENING MECHANISM OF PLANT CELLS[1]

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(WITH THREE FIGURES)

Introduction

In the foregoing papers of this series (**25, 26**) we have given an account of our more intensive researches on the physiology of cold resistance, approached always through a study of living cells. We now describe a number of lesser excursions into the same field, and combine with this a survey of the whole problem of hardening in the light of those changes which have been found to accompany it. The relation of proved hardening changes to the mechanism of resistance must remain hypothetical unless we know the type of injury which has to be resisted. This, however, is still a problem, and evidently a complex one. The immediate cause of death is not always the same. Sometimes it is only indirectly related to temperature, as in soil heaving, smothering by ice, and physiological drought. Often there is a time factor which would seem to involve a mechanism different from that responsible for immediate killing.

Though we confine our attention to the more direct and immediate action of frost, the problem is still complicated because, as we shall see, the mode of injury varies with conditions, such as the rate of freezing or the rate of thawing, and also with the type of plant. Very tender plants are killed merely by chilling to temperatures which are still above the freezing point of their juices, or even above 0° C., but most plants of temperate regions suffer no harm unless ice forms in their tissues, and they may be supercooled with impunity. The well-known resistance of dry seeds and spores to the extreme cold produced by liquid air or liquid hydrogen shows that low temperature *per se* is not fatal. There is also some evidence, though rather indirect, that the amount of injury to a particular tissue is more or less proportional to the amount of ice formed in it (**1, 31**). Whatever the mechanism of frost injury, apparently any change which reduces or prevents ice-formation will have a hardening effect, and certain theories of hardening are based entirely on this type of resistance. But tissues do freeze, and the major problem before us is how hardening enables a plant to endure an amount of freezing that is fatal in the unhardened state. It is this problem that depends on the mechanism of injury for its solution, and it will therefore be discussed in relation to theories of the same. These fall naturally into two main groups: Those that regard injury as an effect

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of dehydration of the cells; and those that regard it as mechanical. It is convenient, therefore, to classify hardening changes, according to the type of resistance which they seem to offer, into the following categories:

I. Resistance to formation of ice.
II. Resistance to dehydration effects of ice formation.
III. Resistance to mechanical effects of freezing and of thawing.

I. Resistance to formation of ice in tissues

The factors tending to prevent or reduce freezing which are found to become more active with hardening are supercooling, depression of the freezing point, and reduction in the amount of free or freezable water.

Supercooling

Supercooling is always observed when tissues are exposed to freezing temperatures, and has been found (at least in insects) to be greater in hardened than in unhardened tissues (**40**). Generally, however, the maximum extent of the supercooling is but a few degrees, and its duration brief. In such cases it can act only as a first line of defense. But cases of greater and more prolonged supercooling—or pseudo-supercooling—are also on record. Wiegand (**47**) found that in some trees the buds are not frozen at −18° C., and in a smaller number even at −26° C., more than 20° below the freezing point of their cell sap. Lewis and Tuttle (**27**) found that living leaves of *Pyrola* froze at a temperature 28.5° C. lower than that required to freeze dead leaves. Iljin (**21**) says that on account of supercooling, leaves of evergreens, such as *Hedera helix,* often resist temperatures reaching below −20° C. in Central Europe.

In a plant, the conditions for supercooling are well fulfilled as regards breaking up of the liquid mass into separate droplets and capillary columns; but it would seem to be essential for the continuation of the state that the cells be isolated by ice-proof barriers which will prevent spread of crystallization beyond any locus in which it may chance to originate. For this to be maintained there must be very little freezable water in the walls.

In this connection it is significant that *reduction of water content* (which practical men call "maturing") is a regular feature of hardening. The percentage reduction is not usually great, but a small reduction of cell volume can greatly reduce the turgor pressure of the cells and correspondingly greatly increase their suction tension, which of course reduces the water in their walls. Thus, the water which remains in the walls is far below saturation point, and its menisci are retracted into the ultra-microscopic pores, a condition which tends to prevent ice formation. Also, it is known that wilting increases frost resistance, and the presence of free intercellular moisture decreases it. But hardy plants, and animals also, do not

depend upon supercooling for their protection. At a sufficiently low temperature, ice always forms, except in dry objects like seeds, and tissues may be frozen till brittle without fatal effect. In such cases, toleration rather than prevention of freezing is the mode of resistance.

Depression of freezing point: osmotic value

The importance of depression of the freezing point in relation to frost resistance lies not so much in the lowering of the temperature at which ice begins to form—because the difference is only a few degrees at most—as in the reduction in the amount of ice formed, or the increase in the amount of water unfrozen at any temperature below the freezing point. A twice-molar concentration of sugar has a freezing point only 3.25° C. lower than a quarter-molar, but at – 15° C. the amount of ice formed is 75 per cent. and 97 per cent. respectively, and the unfrozen water is 25 per cent. and 3 per cent. of the total water. Since at equilibrium "suction tension," and therefore the freezing point, must be the same in every part of a cell—vacuole, protoplasm, and cell wall—the osmotic value of the cells measured plasmolytically tells us the freezing point of the whole tissue. Experience supports theory in this regard. Wiegand's observations prove that ice, though its locus is intercellular, only begins to develop at the freezing point of the cell sap and completely melts at the same temperature. In the following discussion, therefore, we shall refer to the osmotic value or osmotic pressure of the cells rather than to the freezing point of the tissue.

In our attempt to find a correlation between hardiness and osmotic pressure (Levitt and Scarth, **25**), ten species and more varieties were tested. The two tender herbaceous species (sunflower and castor bean) were incapable either of becoming frost resistant or of increasing in osmotic pressure when exposed to "hardening" temperatures. The two semi-hardy herbaceous species (cabbage and clover) showed an increase of 20 to 30 per cent. in osmotic pressure as a result of hardening. The six hardy woody plants possessed maximum winter osmotic pressures up to 400 per cent. greater than their minimum in spring. Among these, the highest concentration was found in the hardiest species, *Caragana,* and it also reached its maximum earlier in the season than did the others. Of the four apple varieties, one of the two hardy ones showed the highest winter osmotic pressure, whereas the other was no different from the two more tender varieties. However, both reached their maxima earlier in the season than the tender varieties.

In short, our results reveal wide seasonal changes in the osmotic pressure, and support the general principle of a correlation between osmotic pressure and hardiness; but they also show that the correlation is not close nor invariable. In particular, they indicate that osmotic pressure rises to a high

value in many woody plants before there is actual need of hardening, and may remain constant during the subsequent period of falling temperature. Unfortunately, we have as yet no tests of the actual hardiness of the plants during this period. An example of the yearly cycle, that of *Hydrangea paniculata,* is shown graphically in figure 1.

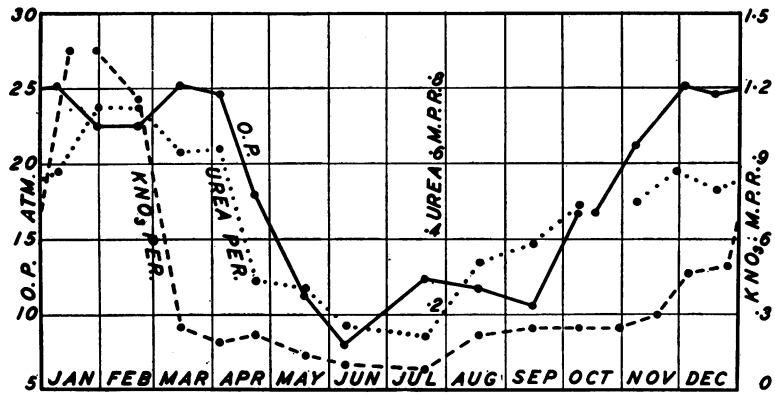

FIG. 1. Seasonal changes in osmotic pressure and urea permeability (Oct. 1934–Oct. 1935) and in KNO_3 permeability (Jan. 1935–Jan. 1936) of cortical cells of *Hydrangea paniculata.*

The depression of the death point, or temperature at which plants are quickly killed, is relatively far greater than the depression of the freezing point of their saps in "natural" hardening, while it is only equal to it in the hardening produced by artificially increasing the sap concentration. ÅKERMAN found that when the osmotic value of the cabbage cells was increased from the equivalent of 0.4 M to 1.0 M sugar, by allowing the leaves to take up erythrol, the death point was lowered from $-2°$ to $-5°$ C. The change in each is 150 per cent., but we find in natural hardening of the same cells that 150 per cent. lowering of the death point is attended by only 20 per cent. increase of osmotic pressure.

REDUCTION IN FREE WATER

The smaller the proportion of freezable water in the cell, the smaller the amount of potential ice. Furthermore, if free water be synonymous with solvent water, its reduction will raise the osmotic pressure and reduce freezing in that way. The percentage of free water may be lessened in three ways: (*a*) loss of water; (*b*) increase of solids; and (*c*) binding of water.

Loss of water in hardening is usually slight and has already been discussed. Increase of solids must of necessity depend upon nutrition and storage, and cannot result from temperature effect alone; but it may be of great importance in frost resistance. The binding of water requires some discussion.

One of the commonest statements in recent literature on winter hardiness is that an increase in cold resistance is associated with an increase in hydrophilic colloid, or, in other words, a greater proportion of "bound water." The evidence for this statement in the work of ROSA, GORTNER, NEWTON and others, though based on different methods of estimation, is all open to criticism and is opposed by some recent findings (MEYER, **32**; MARTIN, **30**; LEBEDINCEV, **23**). Some authorities, such as A. V. HILL, even assert that there is no bound (in the sense of non-solvent) water even in animal tissues, where we should expect a higher ratio than in plants. When GROLLMAN'S formula (**16**), which makes due allowance for hydration of sugar, is used to calculate bound water in wheat juice from GORTNER'S cryoscopic measurements, the percentage of bound water is found to have a quite small and sometimes negative value. The negative value is explaind by GORTNER (**14**) as due to preferential adsorption of solute.

There are several objections to estimation of bound water on plant juices, among them being difficulties of sampling and possible change, with death, in the hydrophilic properties of the colloids.

With living cells it is not possible to measure bound water directly, but, more to the point, free or solvent water can be determined accurately, and from this, if desired, some estimation of bound water may also be reached. As details of methods and results have been given in a preceding paper (LEVITT and SCARTH, **25**) a summary will suffice here.

If the cell is simply a pure solution surrounded by an osmotic membrane, then it will obey BOYLE'S law, so that $P \propto \frac{1}{v}$. If, however, there is an appreciable quantity of non-soluble solids or of non-solvent (*i.e.*, "bound") water, then this relation will not hold. The formula $P \propto \frac{1}{v-x}$ must then be used, x representing that fraction of the cell volume occupied by non-soluble solids and/or non-solvent water.

In determining which of these interpretations of x is valid, an indirect method must be used. Thus, if x is constant for all values of P, then it represents non-soluble solids, since no change in pressure can alter their volume. If, on the other hand, x varies inversely with P, then it is at least partially composed of non-solvent water, since bound and free water are in equilibrium.

Thus, an estimation of x was found to be useful in determining the relationship between bound water and hardiness. In both hardened and unhardened cells of cabbage $x = 0$ and there is, therefore, no appreciable amount either of non-solvent water or of non-soluble solids in the sap. In cortical cells of *Catalpa* and *Liriodendron,* however, x is large—about 40 per cent. in hardened and 25 per cent. in dehardened twigs. Since it varies with osmotic pressure, x must be partially composed of bound water. Figure 2 allows a

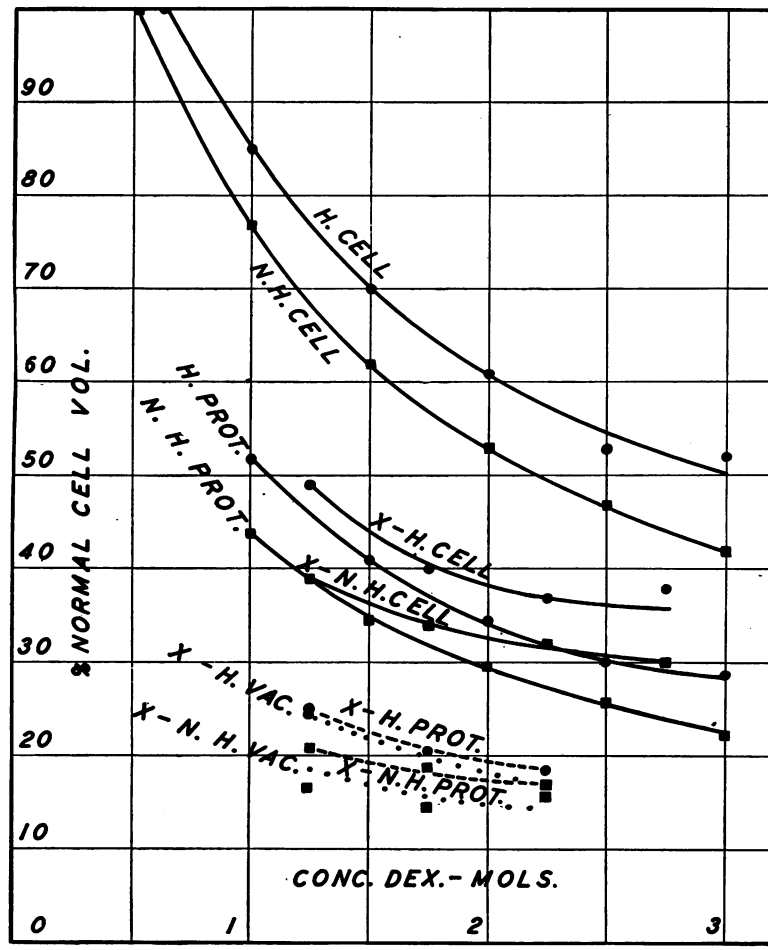

FIG. 2. Change in volume and in non-osmotically active portion (x) of *Catalpa* cortical cells (as well as of protoplasm and vacuole separately) with change in concentration of plasmolyte. The volume of the vacuole is shown by the difference between that of the whole cell and of the protoplasm.

comparison to be made between hardened and dehardened cells as regards the cell volume and the proportion of the cell occupied by x in various concentrations of plasmolyte. The osmotic pressures at normal volume differed only slightly, being 14 atm. in the partially dehardened, and 18 in the hardened; the curves of cell volume tend to diverge in higher concentrations, due to the effect of x. Greater divergence is shown when dehardening is more complete (25). X is proportionally larger in the vacuole than in the protoplasm, and it is here that most of the difference in x between hardened and dehardened cells resides.

Seasonal changes are also significant. Early in May, x drops to half its winter value in both *Catalpa* and *Liriodendron* cells, and since this happens before any appreciable growth, it cannot be due to metabolic utilization of non-soluble solids. Nor can it be caused by a simple hydrolysis of the latter to soluble substances, for in this case a rise in osmotic pressure would result, whereas the reverse occurs. Thus the only possible cause of the seasonal drop in x at this period is a decrease in bound water. A further diminution of x occurs later in the season, but this may be due to the development of new growth.

Briefly then, determinations of the non-osmotically active fraction of the cells of woody plants reveals the existence of bound water which occurs in greater quantity in hardened than in unhardened cells, and which partly accounts for the very high winter osmotic pressures possessed by the former. A semi-hardy herbaceous plant, however, was found to have no measurable amount of bound water either in the hardened or unhardened condition.

The importance of increase of non-solvent space in reducing freezing may be illustrated by a calculation given in a previous paper, which shows that, whereas in non-hardened *Catalpa* 75 per cent. of the cell volume is converted into ice at $-6°$ C., in hardened tissue this amount is probably never reached at any temperature. In view also of the fact that $-6°$ C. is about the critical temperature for unhardened cells (95 per cent. were killed in six hours), the extremely low temperature that the hardened cells endure is not surprising.

Important though this factor may be in extreme resistance, it is not likely to be the only one. Moderate hardening is produced in cabbage without it, and more than can be explained by the osmotic increase. We look, therefore, to other theories of resistance.

II. Resistance to physico-chemical effects of dehydration

Reduction in the amount of solvent water as a consequence of ice formation results in concentration of the cell sap, which many authors, from GORKE (**13**) and LIDFORSS (**29**) onward, have regarded as producing death of the cell by virtue of a toxic effect. More precisely, the mechanism is often pictured as a direct flocculation or even salting out of the protoplasmic colloids by coagulating substances, principally electrolytes present in the sap. There are many theories as to the means by which hardening tends to obviate this result. Protective changes have been detected or assumed, both in the composition of the sap or aqueous phase and in the protoplasmic colloids themselves.

It would be highly desirable to know what happens in the aqueous phase of the protoplasm, as distinct both from the vacuolar sap and from its own colloids, but this is difficult. In dealing with press juices, we may separate

the coagulated colloids from a liquid which, in varying but usually very small part, is derived from the protoplasm but is mainly vacuolar sap. In studying living cells, we may measure properties of vacuole and protoplasm separately but cannot distinguish the phases of the latter.

Since in our own research the approach to the problem is the cytological one, we shall classify the changes according as they appear in the vacuolar sap or in the protoplasm, respectively.

Changes in the cell sap

If freezing kills cells through toxic concentration of the sap, resistance to frost injury might be increased, either by reduction in the amount of toxic substances present or by increase in protective substances.

The agents to which the toxic action has been ascribed are electrolytes in general—in virtue of their precipitating action on colloids—and acid in particular. Protective action has been ascribed principally to sugars as inhibitors of protein coagulation. We shall consider these possibilities in turn.

(a) Concentration of electrolytes.—It has been tacitly assumed by most investigators that any increase in the concentration of the cell sap which occurs on hardening is due to an increase solely of organic solutes. Determinations of electrolytes, when these have been made, have generally failed to show any change (Dixon and Atkins, **9**; Lewis and Tuttle, **27**; Newton, **37**). Recently, however, Dexter (**5, 7**) demonstrated a definite decrease in electrolytes amounting to 50 per cent. (per gm. dry matter) during the hardening period of wheat seedlings, but no change in alfalfa. Greathouse and Stuart (**15**) also have reported a decrease in red clover. We have yet to learn if this is a widespread phenomenon among plants. Dexter himself is of the opinion that the removal of salts by diffusion or any other process does not afford any protection to the plant or its sap. Our own results show that both hardened and unhardened cells can be made to take up very large amounts of KNO_3 without suffering injury. Also the evidence of ash content, even for wheat seedlings (Newton **37**) is against any change in concentration of inorganic salts. The decrease must be in organic electrolytes.

(b) Hydrogen ion concentration.—Schnader and Schaffnit (**45**) put forward the hypothesis, also advocated by Harvey (**17**), that on concentration of the cell sap the H-ions first reach toxic limits and that frost injury is really acid injury. In support of this, Zacharowa (**48**) states that the more acid tissues in a plant die first. Most of the facts, however, are opposed to the theory of acid injury.

Thus, hardening is not accompanied by any significant change in the pH of tissue juices, as was proved by Rosa (**42**), Bakke *et al.* (**2**), Newton

(**36**), DEXTER *et al.* (**8**), and DOYLE and CLINCH (**10**). Our own results show a very slight tendency to reduction of acidity in the sap—a pH change of at most 0.2—in hardened cabbage seedlings. This was found also by DEXTER (**6**), KESSLER (**22**), and GREATHOUSE and STUART (**15**). In no case except some of KESSLER's results is the change sufficient to offer appreciable protection. Since, however, it is possible that pH differences might exist in the respective cytoplasms, unaccompanied by corresponding differences in the sap, we approached the problem from another angle and tried the effect of changing the pH of the cells.

Following SCARTH's (**44**) method of altering the pH of living cells, unhardened cabbage seedlings enclosed in bell jars were exposed to the vapor of varying concentrations of ammonia and acetic acid. Solutions of indicators as well as living leaves of *Zebrina pendula* included in the bell jar showed that the series of pH's obtained probably ranged between about 4.4 and 7.5. While still exposed to the vapor, the plants were subjected to frost in a cold chamber. The temperature used (about $-5°$ C.) was sufficient to inflict severe injury on unhardened seedlings, but insufficient to cause much damage to hardened ones. In all cases, both the treated and the control plants suffered between 90 and 100 per cent. injury. KESSLER, allowing plants to take up urea to increase the cellular pH, also found no change in resistance.

Mention may also be made of experiments with expressed juice. First, as found by DEXTER (**6**) and independently by ourselves (LEVITT, **24**), the juice of hardened plants of cabbage is no better buffered and consequently offers no greater opposition to increase of acidity than that of unhardened plants. Secondly, the proteins in the juice of hardened tissue, instead of being more stable toward acid, are more completely precipitated on the acid side of the iso-electric zone than in the unhardened juice. This last point anticipates the discussion of protoplasmic changes, but is mentioned now to complete our argument against the theory of acid injury.

(c) SUGAR CONCENTRATION.—LIDFORSS (**28, 29**) put forward the theory that the sugars, increase of which is responsible for practically all of the osmotic change in hardening, afford a more important protection than mere osmotic action; namely, a specific protection against coagulation of the protoplasmic colloids, analogous to, but perhaps greater than, the protection which they afford to proteins *in vitro* (NEWTON and BROWN, **38**). Whether protoplasm after hardening shows any greater stability, under the action of coagulating agents, will be discussed in the next section. Meanwhile, we may judge the protection theory on the basis of the correlation between sugar concentration and hardiness.

Dependence of some plants upon carbohydrate concentration for hardening seems to follow from the fact that, in the seedling stage when there is no

carbohydrate reserve, they are unable to harden properly if kept in the dark (TUMANOV, **46**; DEXTER, **4**).

On the other hand, as LIDFORSS himself admits, many plants (such as beet and sugar cane) with high sugar content are killed by light frosts, while others (such as mosses and bacteria) with little sugar are highly resistant.

Also, a single species or individual plant may increase the sugar content of its tissue without any increase of hardiness, as shown in the following results.

An experiment of our own with a tender type of plant may be mentioned first. Sunflower plants were grown in the greenhouse for 90 days. The osmotic pressure was low at first (10.6 atm.), later increased (12.4 atm.), and finally, as the flower bud formed, reached its peak (14.3 atm.). Yet neither this increase in osmotic pressure, nor exposure to low temperature, enabled them to assume any frost resistance. The plant is tender at all stages.

Artificial increase of sugar concentration does not produce hardening, even in plants which are capable of it. DEXTER (**6**) showed that when cut leaves of cabbage were set in sugar solution, the osmotic pressure of their cells increased considerably but their frost resistance was not appreciably altered. KESSLER (**22**) obtained similar results, using evergreen plants and glycerine solutions.

From the discussion of osmotic pressure in a previous section, it appears that a correlation between sugar concentration and hardiness is common, but we see from the above that plants may be hardy without high sugar content, and also that increase of sugars apart from other change affords no protection beyond its osmotic effect.

CHANGES IN THE PROTOPLASM

On the theory that death results from dehydration of the protoplasm, the defense mechanism might be either an increase of resistance to the process of dehydration or a decrease of sensitivity to its effects. We shall call these protoplasmic properties hydrophily, and stability, respectively.

(a) HYDROPHILY.—Protoplasm being surrounded by a semi-permeable membrane, its attraction for water is osmotic, but osmotic pressure in turn may be profoundly affected by binding of water on its colloids. This latter phenomenon has been investigated mainly on extracted juice, involving coagulation of the protoplasm, which may obscure any change in the hydrophily of the colloids produced by hardening. Even negative results, therefore, would not disprove the hypothesis that hardening is associated with greater hydrophily of the protoplasm. The question can be settled only by a study of living cells, and we have attempted this in two ways: (1) by comparing the average volume occupied by the protoplasm in hard-

ened and unhardened tissue; and (2) by estimating the bound water from variation of non-solvent space in relation to degree of plasmolysis.

(1) *Comparison of the average volume occupied by the protoplasm in hardened and unhardened tissue.*—Since the total pressure of the protoplasm, in whatever ratio it is divided between swelling pressure and osmotic pressure, must equal the total pressure in the vacuole, an increase in the hydrophily of the protoplasm must result in transfer of water from the vacuole into it until equilibrium is again established. An increase in the volume of the cytoplasmic layer would be the visible result. Of course, changes in the osmotic pressure of the sap must be taken into account, but, allowing for this, measurement of the thickness of the cytoplasm before and after hardening should help to settle the problem of whether its hydrophily changes or not.

We first attempted to make the measurements of the protoplasmic caps in epidermal cells of bulb scales of *Allium cepa* which does not form starch. The result, if there were no complications, pointed to a *decrease of hydrophily in the protoplasm on hardening* (tables I and II).

TABLE I

TOTAL CROSS-SECTIONAL AREA (SQ. μ) OF BOTH PROTOPLASM CAPS (AVERAGE OF 10 CELLS) FROM PLANIMETER MEASUREMENTS OF CAMERA LUCIDA DRAWINGS

ONION NO.	DAYS AT 25° C. (PREVIOUS TO HARDENING)	UNHARDENED	HARDENED	CHANGE
1	7	300	306	+ 6
2	"	303	281	- 22
3	14	376	338	- 38
4	"	477	370	-107
5	28	376	314	- 62
				Average, - 45

TABLE II

COMPARISON OF 10-CELL SAMPLES

ONION NO.	SAMPLE a	SAMPLE b	DIVERGENCE
1	309	319	10
2	370	389	19
3	284	296	12
			Average, 14

The volume of such caps, however, is affected by other factors than volume of the whole cytoplasm—factors such as surface tension of the vacuole, and vacuolar contraction. The result is therefore indecisive.

More satisfactory determinations of volume were made on the cortical cells of apple twigs. These cells possess very bulky protoplasm, occupying about half the cross-sectional area of each cell. They contain no starch in the hardened state, but an abundance of it when in the naturally unhardened condition. Artificial dehardening of branches taken into the laboratory, however, is accomplished before any starch appears, and it was during a two-week dehardening period of this kind that the measurements were made. The average of four branches shows a practically constant volume of protoplasm during this period of dehardening (table III). Since osmotic pressure fell during the same time from 25.5 to 19 atm.—about 25 per cent. reduction—the pressure in the protoplasm must have fallen *pari passu,* indicating, if due to colloidal change, a slight decrease of hydrophily, or conversely a *slight increase of hydrophily (though not of hydration) with hardening.* On the other hand, the change may be purely osmotic.

TABLE III

TWIGS TAKEN INDOORS FEBRUARY 11, 1935. EACH VALUE IS THE AVERAGE OF 10 CELLS. AREAS OBTAINED FROM PLANIMETER MEASUREMENTS OF CAMERA-LUCIDA DRAWINGS

TIME IN LABORATORY	PERCENTAGE OF CELL AREA OCCUPIED BY PROTOPLASM				
	ALEXANDER	WEALTHY	WOLF RIVER	PATTEN GREENING	AVERAGE
days	%	%	%	%	%
0	47.7	45.2	53.1	47.7	48.4
1	46.3	42.2	53.0	57.4	49.7
3	50.5	46.3	49.5	51.7	49.5
7	52.6	51.1	54.4	49.6	51.6
14	46.7	41.3	50.8	57.6	49.1

Still another comparison was made, using the cortical cells of *Catalpa.* In this case the proportion of protoplasm was found to decrease definitely on dehardening, but the beginnings of growth introduced a complicating factor (fig. 2).

While these results seem to preclude any very great or general increase in protoplasmic volume with hardening, such as the theory of protoplasmic resistance to dehydration would demand, they do not reveal how much of the increase of osmotic value which does occur is due to colloidal change. The next method gives more definite results on this problem.

(2) *Non-solvent space and bound water in the protoplasm.*—It has already been shown that the non-solvent space in hardened cortex cells is

larger than in unhardened cells, and that most of the increase occurs in the vacuole. The protoplasm also possesses a large proportion of non-solvent space—about 50 per cent. of its volume when in equilibrium with a molar dextrose solution, both in hardened and dehardened cells. This is about the same proportion as in the vacuole of dehardened cells, but only two-thirds of that in the vacuole of hardened cells.

Since in the results with *Catalpa,* as shown in figure 2, the volume, both of the protoplasm as a whole and of its non-solvent fraction, is greater in the hardened cells, it follows that the protoplasmic colloids are more hydrophilic.

But even the absence of an increase in the volume of the protoplasm with hardening would not preclude a possible increase of hydration or hydrophilic quality, because of the complication that the dry weight of the protoplasm may diminish. In absence of photosynthesis, there must be a reserve of insoluble material in the cell, which, during the hardening process, is transformed into osmotically active substance and, since simultaneously with the osmotic increase the non-solvent space in the vacuole increases greatly while in the protoplasm it shows an increase only when calculated in relation to the normal cell volume, the diminution of solids is more likely to be in the latter. Commonly, starch is the visible substrate, and that of course is stored in the plastids; but in the starch-free cells, on which our experiments were mostly made, some intermediate reserve carbohydrate or fat may be present in a state of dispersion in the cytoplasm also.

If indeed the protoplasm loses solids to the vacuole, and since its own non-solvent space is undiminished or even increased, the bound water element in it must be augmented. In other words, its colloids become more hydrophilic.

On the same condition, since the volume of the whole protoplasm remains the same, its total water content must be higher.

These volume studies are beset with difficulties and cannot be regarded as more than preliminary, but at any rate on the whole they support the theory of an increase in hydrophily of the protoplasm with hardening.

(b) STABILITY.—This property is closely related to the previous one, since the resistance of the protoplasm, or any other colloidal system, to precipitation or salting out, depends largely on the hydrophily of its least stable elements. But an increase in the hydrophilic property of a portion of the solid phase of the protoplasm would not be reflected in a proportional increase in that of the protoplasm as a whole; so that a great increase in protoplasmic stability may attend only a slight increase in its attraction for water, as estimated by the methods described in the previous section. Also, there are probably factors other than hydrophily concerned in such a complex phenomenon as coagulation of protoplasm.

As a possible clue to the relative stability of protoplasm in the hardened and unhardened state, respectively, we may compare its resistance to other coagulation agents than frost. The action of acid and of heat was tried. Hardened and unhardened cabbage plants were exposed together to vapor of a 7.5 per cent. and also of a 10 per cent. solution of acetic acid for 5 hours. All of the plants suffered, but parts of them remained alive. Hardened and unhardened suffered alike.

The heat test was made by dipping leaves into water at 60° C. long enough to produce partial killing. Again the result was the same in hardened and unhardened plants. *There is no indication here, therefore, of protoplasm becoming less sensitive to coagulating agents in general as a result of hardening.*

HARVEY, who is the author of the theory of greater protein stability, bases his hypothesis on tests with expressed juice. He found (**17**) that when the juice of hardened and unhardened cabbage leaves was frozen and centrifuged, chemical analysis revealed that the precipitation of proteins was greater in the latter. MUDRA (**34**) obtained the same result with other plants. HARVEY also found a greater percentage of soluble amino acids in hardened juice, though NEWTON *et al.* (**39**) regard this as an effect of freezing rather than of hardening.

Testing cabbage juice in relation to the H-ion effect, we obtain the contrary result as regards stability. To a series of tubes each containing 10 cc. of tissue extract, various concentrations of 0.1 N HCl were added. The region of complete precipitation was from pH 3.9 to pH 4.4 in the case of both hardened and unhardened plants. On either side of this zone is a region of incomplete precipitation, the limits of which are pH 5.2 and pH 3.9 in the unhardened, whereas in the hardened plants they extend to pH 5.4 and pH 3.5. Beyond this zone there is little or no precipitation on either acid or alkaline side. *The wider pH zone of precipitation in the juice of hardened plants would point to a poorer stability of their colloids.* This experiment goes to offset results from which a more hydrophilic quality of the juice colloids has been inferred. But of course juice is not protoplasm!

Changes in the colloidal stability of a hydrophilic sol are often paralleled by changes in viscosity, since viscosity is directly and profoundly influenced by the hydration of the internal phase. But the so-called viscosity of protoplasm, as it is measured, is rarely true viscosity, and is influenced by aggregation as well as hydration of particles. Viscosity tests and their significance are reserved for a later section of this paper.

Certain facts are at variance with any theory of injury through dehydration; for example, the experience that drought-resistant plants are not always frost-resistant, and the finding of ILJIN that cells of plants which are sensitive both to light frost and to wilting may survive extreme and pro-

longed desiccation, when this is produced by plasmolysis alone or plasmolysis followed by drying. These phenomena point to mechanical factors as the immediate cause of death, both in freezing and wilting, though not exactly the same in the two cases. Moreover, the most conspicuous of the changes so far described—those producing reduction in ice formation—are as applicable to a theory of mechanical as to one of dehydration injury.

III. Resistance to mechanical effects of freezing and thawing

Theories of mechanical injury may be subdivided into two sets: one which ascribes the effect to pressure of ice crystals, and the other to stress set up by displacement of water. In the former case the fatal period will be that of freezing; in the latter it may be either freezing or thawing. An advantage in the study of mechanical as compared with physico-chemical action is that it can be followed microscopically.

Injury through pressure of ice

The view that frost injury is mechanical dates far back. Duhamel and Buffon (**11**) postulated that ice forming inside a cell ruptured the wall by expansion. From direct observation, however, it was proved by Göppert (**12**) that the walls are not ruptured, and by Sachs (**43**), Molisch (**33**) and others that normally ice forms in the intercellular spaces. Later it was shown that with rapid freezing intracellular development of ice does occur (Müller-Thurgau, **35**; Molisch, **33**). This condition is generally fatal to plant cells (Åkerman, **1**; Chambers and Hale, **3**), whereas some animal tissues are said to be less injured by quick than by slow freezing, because the smaller crystals produce less destruction of the tissue. In plant cells, if the vacuole freezes, the compression of the protoplasm between it and the wall, especially if there is a hull of ice outside (Iljin, **19**), is probably sufficient to cause death, even if the protoplasm itself resists freezing. But opinions differ as to the exertion of mechanical pressure by ordinary intercellular ice. Maximov is a leading upholder of the theory that pressure of the ice crystals on the cells induces coagulation of the protoplasm, and he couples with it the suggestion that mere contact of the ice phase with the plasma membrane may be fatal. On the other hand, Schander and Schaffnit (**45**), followed by Iljin, argue that the ice crystals grow into the intercellular spaces and neither press upon the cells nor even touch the protoplasm. Iljin points out that copious intercellular space in a plant is no protection against frost. Yet it has long been known that cells and tissues may be torn apart by the growth of relatively large ice masses (Pfeffer, **41**), and Wiegand's observations of frozen buds and twigs indicate that less obvious pressure is exerted by crystals of smaller and more ordinary dimensions. There is lack of proof, however, that this is a general cause of injury.

The phenomenon with MAXIMOV first quoted in support of the theory, *viz.*, the protective action of external solutions (which reduced the amount of ice), was shown by ÅKERMAN (**1**) and ILJIN (**19**) to depend mainly on plasmolyzing action, and thus pointed the way to an alternative type of mechanical theory.

INJURY THROUGH STRESS SET UP BY DISPLACEMENT OF WATER

WIEGAND (**47**) and others observed that when tissues freeze in the ordinary way the cells shrink, wall and all, as water is withdrawn. HOLLE (**18**) noted the same thing in wilting. This behavior is made possible by liquid cohesion within the cell, and adhesion of protoplasm to wall, there being no external liquid to cause plasmolysis. In proportion to the rigidity of the wall, stresses are set up in the cell by its loss of water, and ILJIN observed that in drying cells the protoplasm might be ruptured within itself or torn from the wall—with fatal effect. This he at first regarded as the regular cause of injury, whether in frost or drought, because, when the protoplast was released from the wall by true plasmolysis, the cells could endure severe desiccation and freezing. Later experiments (**20**) led to a modification of his view, with regard to frost injury at least. He found that application of solutions *after* freezing was also an effective protection, and therefore he assigned injury to the period of thawing. What happens in thawing, according to his observations, is that the walls first absorb water and lift away from the protoplast (pseudoplasmolysis), after which deplasmolysis tends to ensue. He avers that, on account of its high viscosity in the dehydrated state, the protoplast ruptures if deplasmolysis is rapid. Accordingly he argues that the protective function of the solutions bathing the cell is to slow down deplasmolysis. Whether ILJIN'S theory is correct in detail or not, it draws attention to a type of stress to which cells are exposed, and leads us to inquire what kind of changes would enable the cells to avoid mechanical injury under these circumstances.

HARDENING CHANGES IN RELATION TO MECHANICAL INJURY

As already mentioned, some of the changes discussed under dehydration injury are just as applicable here. Part of the osmotic increase is almost always due to lowering of moisture content which, while it reduces the amount of ice and of mechanical injury, is itself a stage in dehydration and not a protection against this result. (*Cf.*, however, its possible rôle in supercooling, as described previously.) The binding of water, while actually a disadvantage as regards undue concentration of the sap, may be very useful against mechanical injury.

The supposed protective action of sugars has also been invoked by MAXIMOV in the case of protoplasmic coagulation from mechanical causes.

But there are certain other properties of the protoplasm which must play a part in resistance to mechanical injury such as we have described, and which have been neglected almost entirely in studies of the mechanism of frost resistance. Such are (a) the permeability and (b) the viscosity of the protoplasm.

(a) PERMEABILITY.—In a previous report (LEVITT and SCARTH, **26**) we have demonstrated that the permeability of cells for polar compounds increases remarkably with hardening and that it undergoes a greater change and is better correlated with hardiness than any other character, even osmotic pressure. Towards apolar non-electrolytes, such as urethane and succinimide, there is no measurable change. Towards polar non-electrolytes, such as urea, thiourea, and glycol, which have relatively small molecules, there is a general relation between permeability and hardening, but the seasonal changes do not always run parallel. Towards an electrolyte (KNO_3) the variation is still greater and the correlation with hardiness much closer. Data regarding water permeability are insufficient to allow of an equally general statement, but as far as they go they show the same trend as with other polar compounds. Cabbage cells are about twice as permeable to water when hardened as when not, and those of woody plants show a greater difference. On the membrane theory of permeability we draw the general inference that there is a widening of the aqueous pores of one or both the protoplasmic membranes of the cell.

Our results show that the correlation between permeability and hardiness exists in various types of plants and shows no exceptions. Also the relation is independent of the nature of the cause which induced the hardening. Low temperature, drought, checking of growth, all increase permeability as well as hardiness.

To some extent the relation seems to hold even in the vegetative, unhardened phase of life. Thus, highly cold resistant cells, such as those of bacteria, mosses, and cortex of woody plants, are at all times and seasons unusually permeable to KNO_3 and perhaps to water.

In view of this widespread association of the two conditions and of the fact that a permeability change does not necessarily attend an osmotic change and is therefore not simply incidental to the latter, it seems likely that there is a direct causal connection between permeability and hardiness. This will be discussed later.

(b) VISCOSITY OF PROTOPLASM.—If, as ILJIN finds, injury may occur through tearing of the protoplast in a phase of thawing out, we might expect to find protection through an increase of plasticity or reduction of viscosity in hardened cells. A comparison of the viscosity of protoplasm in the hardened and unhardened state has been made by KESSLER (**22**), who arrived at the conclusion that viscosity increased, not decreased, in hardening. He

tested the cells of various plants in summer and winter by the centrifuge as well as by the plasmolytic method. The centrifuge method in this case is handicapped by the fact that starch is present in the unhardened and absent in the hardened condition of plants. From subsidiary experiments with darkened plants, KESSLER reached the astonishing conclusion that the specific gravity of starch-free plastids is higher than of those with starch—in spite of the fact that starch is much denser than protoplasm in general, while chloroplasts are believed to be rather lipoidal in composition, and were actually found by himself to have a tendency to move *centripetally* in some hardened tissue. If KESSLER's premise as to specific gravity is at fault, his result that chloroplasts in unhardened plants are more easily thrown down than in hardened is no proof of lower viscosity.

The "plasmolysis time" test was applicable only to *Sempervivum* among the plants tested by KESSLER, where it gave a comparable result. Slower rounding up in the hardened cells pointed to a greater viscosity—or rather a stronger adhesion to the cell wall—in the hardened state. The other genera used, *Hedera* and *Saxifraga,* did not allow comparison, because the protoplast rounded up immediately on plasmolysis.

In our own work on cold resistance, the attempt was first made to distinguish a viscosity difference by observing Brownian movement. However, neither streaming nor any appreciable Brownian movement could be detected in hardened *Catalpa* cells. Nevertheless, the plastids and protoplasm seemed to be clumped together at the periphery and mostly the ends of the cells, perhaps indicating a low viscosity which would allow free play to surface tension. In the dehardened cells, on the other hand, streaming was active and Brownian movement was apparent in the currents. Yet, if the dehardened cells were observed immediately after sectioning, no streaming and little or no Brownian movement was discernible. The protoplasm showed much less peripheral clumping than in the hardened cells. Hardened and unhardened cabbage cells both exhibited Brownian movement, but no difference in the activity could be distinguished.

Plasmolysis shape was next observed. Sections of hardened and dehardened *Catalpa* were placed on a slide in an isotonic solution of $CaCl_2$ (0.30 M and 0.18 M, respectively) which was allowed to evaporate in the air. At the end of four hours the cells were strongly plasmolyzed in both, but the shape differed. The hardened cells were well-rounded, the dehardened ones were strongly concave, adhering to the wall in many places (fig. 3).

In the case of cabbage, sections from hardened and unhardened seedlings were placed in twice isotonic dextrose and examined from time to time. Though the difference was not so striking as in the case of *Catalpa* cells, the rate of rounding up appeared somewhat more rapid in the hardened cells (table IV).

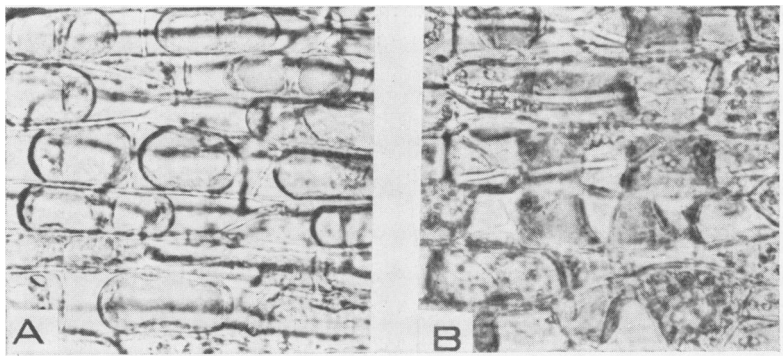

FIG. 3. Plasmolysis shape in hardened (A) and dehardened (B) *Catalpa* cells in isotonic $CaCl_2$ solutions after evaporation in air for six hours.

TABLE IV

RATE OF ROUNDING UP ("PLASMOLYSIS TIME") OF UNHARDENED AND HARDENED CABBAGE CELLS IN TWICE ISOTONIC DEXTROSE. EACH AN AVERAGE OF THREE PLANTS

SAMPLES	NON-HARDENED		HARDENED	
	OSMOTIC PRESSURE	PLASMOLYSIS TIME	OSMOTIC PRESSURE	PLASMOLYSIS TIME
1.	10.0	90	13.6	60
2.	10.6	75	13.6	60
Av.	10.3	82	13.6	60

RESISTANCE TO DEPLASMOLYSIS.—Another mode of experimentation also gave definite and positive results. Sections of hardened and unhardened cabbage plants were compared as regards the ability of the protoplasts to withstand the stretching caused by rapid deplasmolysis. The tendency to injury from this cause generally increases with viscosity of the protoplasm.

First the cells were plasmolyzed for 15 to 20 minutes in twice isotonic $CaCl_2$ and then transferred to distilled water. To determine the number of cells surviving deplasmolysis, the sections were once more transferred to the plasmolyzing solution. The results are presented in table V.

Tender plants always showed sensitivity to deplasmolysis. Thus, *Cordyline* petiole cells almost all burst when transferred to distilled water from twice isotonic $CaCl_2$. In the case of others (tomato, bean), it was found impossible to determine urea permeability, since even this slow deplasmolysis proved fatal.

Hardened and dehardened *Catalpa* cells were then tested, but in their case more severe treatment was necessary to cause injury. Treatments and results are given in table VI.

TABLE V

COMPARISON OF DEPLASMOLYSIS INJURY IN HARDENED AND UNHARDENED CABBAGE CELLS. PLASMOLYZED IN TWICE ISOTONIC $CaCl_2$; DEPLASMOLYZED IN DISTILLED WATER

CONDITION OF PLANT	OSMOTIC PRESSURE (M $CaCl_2$)	PERCENTAGE OF SURVIVING CELLS	
		IN EPIDERMIS AND CHLORENCHYMA	IN PITH
Non-hardened	0.16	trace	0
5-day hardened	0.23	most	0
Non-hardened	0.17	few	0
10-day hardened	0.25	all	many

TABLE VI

COMPARISON OF DEPLASMOLYSIS INJURY IN HARDENED AND DEHARDENED *CATALPA* CELLS

CONDITION OF PLANT	TIME IN PLASMOLYTE (MIN.)	NUMBER OF SURVIVING CELLS
	(a) PLASMOLYZED IN 9 PARTS 2M NaCl: 1 PART 2M $CaCl_2$. DEPLASMOLYZED IN DISTILLED WATER	
Dehardened	60	few
Hardened	360	all
	(b) PLASMOLYZED IN 3M NaCl. DEPLASMOLYZED IN DISTILLED WATER	
Dehardened	15	none
Hardened	15	almost all

Further evidence was obtained indicating that this difference in ability to withstand deplasmolysis injury is even more marked at low temperatures. Thus a much less severe treatment than the above, namely a transfer from twice isotonic to half isotonic dextrose, caused no injury to either at room temperature. Even at 0° C., this procedure had no harmful effect on hardened cells but was fatal to the unhardened, due to the increased viscosity in the latter, which was apparently such as to more than counteract the decreased protoplasmal stress resulting from the reduced deplasmolysis rate.

It is thus evident that hardened cells are more resistant to the ill effects of deplasmolysis than are unhardened cells, and this in spite of the more rapid rate in the former.

Discussion

Reviewing the changes said to be associated with hardening, we have seen that some are not well established and that no single one is adequate to explain the whole phenomenon. Our own research on living cells confirms the importance of certain changes in the sap and brings to light others in the protoplasm. The significance and relative importance of these is difficult

to evaluate from lack of knowledge of the exact mode or modes of injury. The nature of the protection is inferred mainly from the nature of the hardening change.

A wide range of protection is possible with those changes that reduce freezing. Of these, supercooling is not fundamental because hardy tissues survive severe freezing; theoretically, supercooling may even be harmful by leading to intracellular ice development during the quick freezing which follows its breakdown. A rise in osmotic pressure is generally well correlated with hardiness, but in all true hardening it fails to explain more than a fraction of the increased resistance.

Enhancing the osmotic effect in woody plants is a marked increased in "non-solvent space" in the sap vacuole. Part of this is due to insoluble solid—apparently hydrophilic colloid—and part to bound water. This change causes the osmotic pressure to mount more rapidly as the cell contracts and sets a higher limit to the minimum volume which can be reached through water loss. It is calculated that hardened *Catalpa* cells lose no more water through freezing at the lowest possible temperature than the unhardened cells do at only $-6°$ C.

The gelatinous condition of the sap in tree cells may serve another purpose. In ordinary herbaceous plants, intracellular freezing—when it occurs—is located in the sap vacuole. In other words, this is the vulnerable part of the cell as regards the invasion of ice, and such invasion is usually fatal. But in hardy tree cells the vacuole is as rich in hydrophilic colloid as the protoplasm and probably equally protected from freezing even when the eutectic point of the sap is reached.

Important though these factors may be in resistance to extreme cold, they are not the full explanation of hardiness. For example, in the non-hardy state, tree cells still have much colloid in their sap, but they are then more sensitive to frost than hardened cabbage cells which have no colloid and about the same osmotic pressure.

Other changes in the sap have not been shown to be of any great significance. Of those which are supposed to offset the danger of salting out through concentration of the sap, a reduction, with hardening, of the total electrolytes has so far been proved in one or two cases only and disproved in others. Also, in spite of a permeability increase on hardening, there is no evidence of exosmosis from the cells in winter time. Moreover, a high concentration of salt (KNO_3) has been produced inside cells without injury. As regards the theory of toxic acidity of the sap, a reduction of H-ion concentration in hardening has been found in several cases, but only to a slight degree, while artificial alteration of the acidity of the sap is without effect on frost resistance.

The view that the sugars, which are responsible for most of the osmotic change, also exert a specific protection against coagulation of the protoplasm

is countered by the fact that increase of sugar may take place naturally or be produced artificially without a true hardening effect.

As regards changes in the protoplasm, a widely held view is that hardening is accompanied by an *increase of hydrophilic colloid* and consequently of resistance to freezing, to dehydration, and to coagulation.

In attempting to investigate this problem with living cells, in which alone the hydrophilic property of protoplasm is normal, we have seen that in cases where the protoplasm does not swell appreciably in hardening, it may nevertheless be more hydrated if, as seems probable, its insoluble solids diminish. Where swelling does occur the conclusion is more definite.

Evidence regarding changes in the *stability* of the protoplasmic colloids is not decisive. Contradictory results have been obtained with press juices, and with living cells we find that hardening confers no increase of resistance to the action of acid or heat, agents which coagulate proteins.

Altogether, the possibility that frost injury is a physico-chemical effect of dehydration is opposed by the fact that tender cells may often be deprived of water by other means than frost without ill effect. The alternative is mechanical injury. Whatever the precise nature of this injury, the sap changes which tend to reduce the amount of freezing ought to afford some protection; but as we have seen these are not enough.

It would seem that in addition the protoplasm must become more resistant to the mechanical action. Apart from the evidence of a possible colloidal change, which we have just discussed, proof of two very pronounced protoplasmic changes has been educed in the course of our work, *viz.*, an increase in permeability and what may for convenience be termed a fall of viscosity. Let us see if these two hardening changes can be fitted into a scheme of protection against such types of mechanical injury as are known to occur. At least three modes of mechanical injury have been recorded by various authors from direct observation of cells.

INTRACELLULAR FREEZING.—Ice formation within the cell has sometimes been noted and though confined to the vacuole the result is nearly always fatal. The mechanism of injury in this case may be, as ILJIN suggests, compression of the protoplasm between the freezing and expanding sap on the inside and the cell wall or a rigid hull of ice on the outside, or it may also be laceration of the vacuolar membrane and other structures by the ice crystals.

At any rate, the condition for internal freezing is that the temperature of the cell sap fall below its freezing point. Ice first starts to form on the cell walls outside the cells, where it normally grows at the expense of water which diffuses from cells. If this keeps pace with the fall of temperature, the resulting increase in its concentration will prevent the sap from freezing, but with a sudden drop of temperature or sudden crystallization as a result of supercooling, the rate at which water can pass out of the cell may be the

limiting factor in deciding whether or not ice will penetrate. Here, then, is a condition when high water permeability may mean safety to the cell.

INTERCELLULAR ICE MASSES.—It is frequently found that when plants which are not hardy are exposed to temperatures slightly below freezing point, large ice masses develop locally in the tissues. These in their growth crush the cells in the neighborhood and may even tear the tissues apart. Since the size of crystals is a function of rate of crystallization, macrocrystalline ice is naturally found only when very gradual freezing takes place, but the more moderate aggregates which develop under other conditions may also caused injury to the cells in contact with them. Conceivably, the rate of crystallization may sometimes depend upon the rate at which water can pass out of the cells, so that the higher the cell permeability to water the smaller the crystals and the less the danger of injury from pressure. It must be admitted, however, that the rate of exosmosis of water from even non-hardy cells is such that this hypothesis of protection is less plausible than that applied to internal freezing.

THAWING.—ILJIN, backed by his experiments, has revived SACHS'S hypothesis of death during thawing. His results cannot controvert the finding of many authors that death may occur in freezing, but they do seem to prove that with the use of salt or sugar solutions, frozen tissues which are still alive may be saved from dying at the stage of thawing out.

Danger would seem to attend both phases of thawing, namely, pseudoplasmolysis, and deplasmolysis. During the former, as ILJIN showed, the water released by thawing is taken up by the cell wall faster than by the protoplast. The former extends quickly to its normal size, while the latter remains contracted for a time. As in true plasmolysis, injury is liable to occur here under certain conditions. If the protoplast adheres to the wall in places, its plasma membrane tends to become disorganized through stretching, or if the external membrane allows water to pass more freely than the vacuolar membrane, the cytoplasm swells and becomes vacuolated. These mishaps in thawing would be avoided if the permeability of the protoplast to water approached that of the wall, and if the permeability of the tonoplast also equalled that of the ectoplast. In other words, increased permeability of the protoplasm, and especially of the tonoplast layer, which normally seems to be the less permeable (HÖFLER), would tend to protect the cell from mechanical injury of this kind. Wall and protoplast would extend together without any pseudoplasmolysis.

According to ILJIN'S later observations, however, the main damage is caused by the rapid stretching in deplasmolysis. The rate of expansion would only be exaggerated by greater permeability to water, but apparently a compensating change in the physical properties of the protoplasm is another feature of the hardening process.

The cells become much more resistant to injury by rapid deplasmolysis

after true plasmolysis, and we may assume that the same would be the case after pseudoplasmolysis in thawing.

The difference between hardened and unhardened cells in this respect is greater at the low temperature at which thawing out occurs than at room temperature. It is probably the result of a lower viscosity of the protoplasm in hardened plants and also a smaller effect of temperature upon its viscosity.

PLASMOLYSIS DUE TO FREEZING.—That cells do plasmolyze sometimes on freezing with formation of ice between the wall and the protoplast is a matter of observation (*e.g.*, CHAMBERS and HALL, 3), but unlike the previous conditions cited, the injury here is hypothetical. Injury in ordinary plasmolysis is common when the protoplasm is highly viscid and adhering to the wall. Low temperature tends to make protoplasm more viscous and hence more liable to injury of this type. As we have seen, the behavior of hardened cells on plasmolysis is exactly such as to reduce this danger. Another advantage of smoother plasmolysis is that strands of protoplasm are less liable to be pinched off by the investing ice.

COLLOIDAL CHANGES IN HARDENING.—The different behavior of hardened cells, both in deplasmolysis and plasmolysis, is such as may be explained by a lowering of viscosity, either of the protoplasm as a whole or at least of its superficial layer. In a visco-elastic material like protoplasm, such a change in consistency is due to gel → sol transformation rather than the true viscosity reduction of a Newtonian liquid. Increased hydration of particles which should increase *true* viscosity may tend towards solation and reduction of *apparent* viscosity. If this should be accompanied by increased hydration of the protoplasm *en masse,* the effect is still more likely to follow.

These are the colloidal changes which our volumetric experiments and other observations have led us to regard as probable. They are also the changes which increase of permeability points to—as regards the plasma membranes at least.

The chemical nature of the colloids involved does not enter into our work, but inasmuch as the osmotic changes are largely produced by carbohydrate transformation, it is likely that the hydrophilic colloid which occupies so much of the sap of hardy tree cells belongs to the same category. This would explain the simultaneous fluctuation of osmotic and colloid substance. The parallel variation of protoplasmic viscosity, and especially permeability, suggests that proteins and lipoids undergo the same type of change as the carbohydrates.

The linked series of changes associated with hardiness as we have described them may be summarized as follows:

1. Complicated hydrolytic breakdown of carbohydrates increases the osmotic pressure of the cell and also in the hardier plants the non-solvent space in the vacuole at the expense of starch and perhaps of other reserves held in the cytoplasm.

2. Due to similar changes in the protoplasmic colloids, the whole cytoplasm, probably, and the plasmic membranes, almost certainly, become more hydrated.

3. As a consequence of this change, the viscosity of the protoplasm is lowered.

4. Because of the change in the membranes in particular, cell permeability is increased.

If all of these changes are causally connected one with another, the correlation of each and all of them with frost resistance would be found, though only one might play a part in it. We have theorized as to the possible rôle of each, but a satisfactory demonstration requires more knowledge of the mechanisms both of injury and of resistance than we yet possess.

Summary

1. Cell changes in hardening are reviewed and theories of the mechanism of frost resistance are discussed. The following are features of hardened as compared with unhardened cells, according to our own results with the plants named:
   - Resistance to injury by deplasmolysis is greater.
   - Rate of rounding up in plasmolysis is greater (*Catalpa,* cabbage, etc.).
   - (Two differences which may point to lower "viscosity.")
   - The relative volume of protoplasm and vacuole changes but little if at all (apple cortex, onion).
   - Resistance to acid and to heat does not change (cabbage).
2. Also the following are features which have been reported in a previous paper:
   - The osmotic pressure is increased, especially in the hardier woody plants (many woody and herbaceous species).
   - Non-solvent space (= colloids and bound water), if present in measurable amount, increases markedly (*Catalpa* and *Liriodendron*), but the increase is greater in the vacuole than in the protoplasm (*Catalpa*).
   - Non-solvent space is practically absent in cabbage, hardened as well as unhardened.
   - Permeability to polar compounds increases, and the more the increase the hardier the plant (many woody and herbaceous species).
3. The press juice of hardened cabbage plants shows:
   - Precipitation of colloids over a wider zone in the pH scale.
   - H-ion concentration slightly lower.
   - Buffering capacity unchanged.
4. Artificial change in the H-ion concentration of the sap in life does not affect hardiness (cabbage).

5. The most pronounced and unmistakable changes in the protoplasm are increased permeability and lowered viscosity; in the vacuole, increased osmotic pressure and (in trees) non-solvent space. Ways are suggested in which all of these protect the cells against mechanical injury due to frost.

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