ICE FORMATION AND THE DEATH OF PLANT CELLS BY FREEZING

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(WITH ONE FIGURE)

Introduction

The resistance of plant tissues to low temperatures and the causes thereof have been the subject of extensive research by many investigators. Since there have been comprehensive reviews of both phases of the subject written by CHANDLER (**4**, **5**), ROSA (**22**), ÅKERMAN (**1**), MAXIMOV (**15**), LEVITT and SCARTH (**13**), and SCARTH and LEVITT (**23**), this paper refers only to those investigations which are specifically related to the immediate problem.

In all but very few of the previous studies of ice formation in living tissue, the material was frozen by various means, and then after thawing, was examined either grossly or microscopically. What had occurred during freezing was postulated from the appearance of the thawed tissue. Of the few investigators who observed freezing directly, apparently MOLISCH (**17**) was the first to see ice formation as it occurs in living cells. His microscope was put into a specially constructed ice chest with openings to give access to the controls. The apparatus was inconvenient to use, and the temperature could not be regulated, but MOLISCH was able to use it to watch freezing in *Amoebae* and in several plant tissues.

WIEGAND (**26**) investigated the condition of buds during subzero weather. He used a microscope and sectioning apparatus out-of-doors and noted where ice was formed in the buds. Ice was found as a single continuous layer always in the mesophyll of the leaf or scale, never between the scales. The cells on either side were in a state of collapse and often occupied less space than the ice sheet. Ice formation was observed in some species at $-18°$ C., in others not until $-26°$ was reached. This difference seemed to be determined by the size of the cells and the amount of water which they contained. The ice decreased in amount as the temperature rose and finally disappeared at -3.2 to $-2.3°$ C. No injury was noted in buds in which such ice was formed, even after rapid thawing at 21° C.

SCHANDER and SCHAFFNIT (**24**) used a cold chamber mounted on a microscope stage. The chamber was cooled by passing a jet from a tank of compressed CO_2 through ether. By this means the object could be quickly cooled to any temperature down to about $-30°$ C., and the frozen tissue could be observed under the microscope. ÅKERMAN (**1**) used the MOLISCH technique and directly observed the tissues in the frozen condition.

CHAMBERS and HALE (**3**) made microdissection studies of freezing frog muscle, *Amoebae,* and the epidermal cells of red onion. The operations were performed on prechilled material in a cold room at temperatures ranging from 0° to −5° C.

The methods used by these workers are not entirely suitable for observing the process of freezing. The purpose of this investigation was to find some simpler means for directly observing ice formation in plant tissues, and once this method was established, to apply it to some of the problems concerned with the resistance of plants to low temperatures. We wished to determine more certainly, if possible, where and under what conditions ice occurs, and the relation of water content to resistance.

The observations were made with a Spencer binocular microscope of the type with a square stage and inclined oculars. The square stage was necessary because of the design of the cold stage attached to it. Sufficient magnification was obtained with 10×-oculars; oculars of higher power gave an unsatisfactory image. It was essential to replace the 4-mm. objective of the microscope with one having a greater working distance, consequently the objective used was an achromatic model 4-mm., N.A. 0.65, working distance about 0.9 mm. The top lens of the condenser was removed to raise the point of convergence of the rays of light to the level of the mount in the cold stage. The light source was a microscope lamp with a 250-watt filament bulb, a condenser, and a ground glass.

For the actual freezing process, the cold stage and circulatory system described by MASON and ROCHOW (**14**) were used. It was found desirable to make a few modifications for best results with biological materials. The bottom cover glass was sealed in place with LePage's cement rather than vaseline. Celotex was used to insulate the stage since sheet cork of the desired thickness could not easily be obtained at the time the stage was made. As stated by the designers of this apparatus, the operation of the system would have been easier if the channels in the stage had been larger, and the circulatory tubes of correspondingly greater diameter. This would allow for greater flow of liquid, and consequently better temperature control. Ether rather than acetone was the liquid used with the solid carbon dioxide in the Dewar flask. A still better method would involve the use of a mechanical water cooler instead of solid carbon dioxide. The plant material was mounted in paraffin oil on an 18-mm. no. 1 cover glass, and over it was placed a fragment of no. 0 cover glass large enough to cover the specimen. It was found easier to mount the material in this way than between two cover glasses. This mount was placed on a tripod of copper wire in the well of the stage. A 25-mm. cover glass which rested on the flange of the stage was placed over it, leaving an air space underneath. The air space insulated the cover glasses, and the objective conducted heat to the well to such an

extent that the material would not freeze when the objective was in focus. To avoid this difficulty, the designers of the stage recommend that the mount be so made that some of the material to be frozen is in contact with the stage; but with the material used, this was not feasible; therefore, solid carbon dioxide was used to chill the objective. The dry ice was packed into the space between the objective and the stage, above the upper cover glass. A piece of sheet asbestos was fitted over this to prevent the solid carbon dioxide from evaporating too quickly. A dissecting needle could easily be thrust through this layer of dry ice to tap the cover glass and prevent supercooling. The air space between the upper cover glass and the one on which the material was mounted gave adequate insulation against the low temperature induced by the presence of the solid carbon dioxide on the upper cover glass. Whenever this cover glass was broken, however, the mount froze immediately; therefore, no temperature readings were considered valid unless the cover glass remained unbroken throughout the freezing.

Exact determinations of temperature were not considered essential for this phase of the investigation. An alcohol-filled, low temperature thermometer reading to $-50°$ C. was inserted in the thermometer opening in the cold stage. This thermometer was calibrated by observing the temperature at which distilled water froze when placed between cover glasses in the same way as the plant tissues were mounted. This temperature was found to agree within 2° C. with the temperature as determined by immersing the thermometer in melting ice. There was less error when the temperature change was gradual than when it was rapid. Since the difference in temperatures was always greater than the experimental error, this method was considered sufficiently accurate. For more precise temperature readings it would be desirable to use a thermocouple instead of a thermometer.

To insure a uniform water content in experiments where wheat seeds were used, lots of 100 seeds were placed in 2-oz. bottles with a known volume of water. These bottles were then fitted securely with wooden wedges into holes in a large wooden wheel which was rotated slowly with a small electric motor. The rotation kept the grains constantly rolling over so as to bring about a uniform distribution of water. The seeds were weighed when air dry before the water was added to them, and then again after they had been rotated with the water for a given length of time. Some of the highest percentages of water determined may not have been accurate, since in some of these cases not all the water was absorbed.

For gross freezing, a General Electric water cooler was used. Alcohol, in which the bottles containing the seeds were suspended, was placed in the compartment designed for water, and the temperature was regulated in the usual manner. After treatment, the seeds were germinated in sterilized petri dishes containing layers of moist paper toweling.

Observations

For those studies involving microscopic observations, isolated cells of *Sedum acre* L., stamen hairs of *Zebrina pendula* Schnizl., and prothallia of *Polypodium aureum* L. with large irregular plastids were used.

Ice formation in cells of *Sedum acre* L.

The cells of the *Sedum* were obtained as follows: A leaf, split with a dissecting needle, was placed in a drop of paraffin oil on a slide and pressed gently under a cover glass. Then when the cover glass was lifted and the large fragments of the leaf were removed, isolated mesophyll cells, each surrounded by a thin film of cell sap released from the vacuoles of crushed cells, remained in the oil. These cells froze readily, but the thin film of sap froze more quickly than the cell contents and concealed the behavior of the latter.

Ice formation in *Zebrina* stamen hairs

The *Zebrina* stamen hairs were mounted directly in paraffin oil. The streaming, which at room temperature was fairly rapid, gradually became slower as the temperature dropped, and stopped completely at 0° to −2° C. In some instances Brownian movement of the granules began when streaming ceased; it persisted for a few seconds and then stopped. That this was not observed in all cases is probably attributable to the speed at which the temperature was lowered. No visible disintegration of the cells was evident after streaming had stopped. Mature cells froze when the temperature reached −7° or −8° C.; immature cells were more resistant, remaining unfrozen to a temperature of −17° C. These younger cells seemed to have a very high osmotic concentration which apparently decreases rapidly as the cells mature. These younger cells, moreover, showed larger granules and a greater concentration of anthocyanin.

As the freezing point was approached, it was necessary to tap the cover glass with a needle in order to prevent excessive undercooling. Formation of ice crystals always occurred first outside the field of vision, and as each cell froze, beginning at the end toward the base of the stamen hair, it inoculated the one next to it until in rapid sequence all of the cells in each hair had frozen. Each cell as it froze became filled with a closely packed mass of granular ice crystals, and was thereby rendered opaque. Differences in the freezing of the cell parts could not be observed. When cells frozen in this manner were thawed slowly, the cell sap escaped, since the plasma membrane had become more permeable, and the sap collected as droplets in the surrounding paraffin oil. When this occurred, the cells became shrunken and the cell walls very irregular. The cytoplasm assumed a very granular appearance and became stained purple by the vacuolar pigment. The nucleus was much distended and glassy in appearance and more conspicuous than

in the living cell. The appearance of cells after more rapid thawing was similar to that just described; however, in these cells, the cell retained more of its normal semipermeable character, since less of the cell sap escaped, and the cell walls had more nearly their usual contours.

ICE FORMATION IN *Polypodium* PROTHALLIA

The *Polypodium* cells, like those of *Zebrina,* showed excessive undercooling unless the cover glass was tapped with a needle. Ice formation took place in these cells in two ways; one of these may be described as "wave front advance" freezing, and the other as "single cell" freezing. In the first type, inoculation occurred outside of the field of the microscope, and from the first crystals seen, the ice front advanced in a series of localized waves, until the entire visible portion of the field had become frozen. The crystals formed under these conditions were compact and granular. In the "single cell" type of freezing, isolated individual cells froze here and there across the field until all the visible ones were frozen. In some of these, a slight quivering of the cell contents took place just as inoculation occurred, giving the impression that the protoplast had a jelly-like consistency. The ice crystals formed in some cells were compact and granular, like those of the "wave front advance" type. The crystals in other cells were needle-like and developed from one or more sides of the cell. These crystals elongated rapidly until they extended across the cell, giving the latter a striated appearance.

In all cases, the chloroplasts froze a few seconds after the other parts of the cell had been obscured by ice. This delay indicates the presence of a membrane around the plastid which may allow greater undercooling to occur. The plastid probably also has a higher osmotic concentration. Some differences seemed to exist between the time of freezing of the vacuolar sap and that of the cytoplasm, the latter freezing first; but the order of freezing could not be determined definitely with the apparatus used. The relative time of freezing of the nucleus remains uncertain.

When the temperature was raised, the crystals of ice melted gradually and formed drops of water. These drops fused and the protoplasts again became visible. The appearance of these protoplasts was comparable to that of the stamen hairs after thawing: the cytoplasm was granular and the outline of the plastids irregular. The plasma membrane likewise became more permeable, as evidenced by the droplets of cell sap accumulated in the oil outside. The cell walls retained their normal contours. No difference was observed between slowly and rapidly thawed cells.

INFLUENCE OF SUCROSE AS A PROTECTIVE AGENT

Experiments were performed with sections of the epidermis of red cabbage which had been kept in the cold room at 5° C. for several months and

presumably were in a hardened condition. Strips of epidermis were put into small test tubes, and the tubes were then immersed in an alcohol bath cooled to – 10° C. After the tubes had remained at this temperature for 10 minutes, chilled sucrose solutions of varying concentrations were poured gradually down the sides of the tubes. The sections were gradually brought to room temperature over a period of 2 hours and then examined under the microscope. Most of the cells showed some degree of plasmolysis, but there was no correlation between the extent of the plasmolysis and concentration of the thawing solution. When the sucrose solution was gradually replaced with distilled water, the protoplast disintegrated, the red pigment diffusing into the bathing solution. Even if the sucrose solution was diluted gradually over a period of an hour, in no case did the protoplast remain intact. This suggests that since the tubes in which the strips of epidermis were frozen were at room temperature when the experiment began, the cells became somewhat plasmolyzed by withdrawal of water during the first 2 or 3 minutes that the tubes were in the freezing bath. Then when they reached the temperature of the bath, or some critical point above this temperature, ice was quickly formed inside the protoplast and the cells retained the appearance they had at the time of freezing. The addition of the sucrose had no protective action in delaying deplasmolysis of the protoplast, but it did prevent the immediate disintegration of the cytoplasmic membranes which occurred in tissue thawed in air or water. The evidence suggests that the cells were dead when the sucrose solutions were added, and the "protective" action was purely mechanical.

To check these observations, the experiment was repeated. The test tubes used, however, were first chilled to the temperature of the bath before the strips of epidermis were introduced. The results were the same except that there was no pseudo-plasmolysis. The protoplasts were killed before any water was withdrawn from them.

Similar experiments were performed freezing the tissue in water and in solutions of sucrose ranging from 0.5 M to 4 M. At – 10° C., the water, 0.5, 1, and 2 M sucrose solutions appeared completely solidified. Ice crystals were formed in the two stronger solutions (3 and 4 M), but there was still a small amount of unfrozen solution present. If the solutions were cooled to the temperature of the bath before the epidermal strips were put into them, no plasmolysis occurred. But if the tissue remained in the solutions for a few minutes before being chilled, plasmolysis took place. When these sections were thawed gradually, and then the sucrose was replaced with distilled water, the protoplast swelled rapidly and the protoplasmic membranes disintegrated. Even when the dilution occurred gradually, the plasma membrane became disorganized and the cell sap diffused out into the bathing solution. In a few strips of epidermis, some two or three very

small cells deplasmolyzed completely and were apparently alive, but when they were plasmolyzed again, they did not survive. There is some doubt as to whether ice was actually formed in these cells, since they were very much smaller than the surrounding cells which did not survive, but even these smaller cells did not survive after the second plasmolysis.

ILJIN (9) found that similar strips of red cabbage epidermis were protected by thawing in sugar solutions. He suggests that the solution absorbs the water which is lost from the cells when the temperature is lowered and prevents its rapid imbibition by the protoplast when thawing takes place. And if the rate of expansion of the cytoplasm is more nearly equal to that of the cell wall, rupture of the plasma membranes and subsequent death of the cell does not occur. ILJIN's experiments may be criticized because the deplasmolysis which was used as the criterion of life was not carried to completion. That the protoplast would expand partially was considered sufficient proof that the cell was alive. The studies of CHAMBERS and HÖFLER (2) with isolated tonoplasts have shown that this membrane can be made to expand and contract with solutions of various concentrations even outside the cell and entirely free from protoplasm. CHAMBERS and HALE (3) describe frozen epidermal cells of onion bulb scales in which the cytoplasm and nucleus had distintegrated as a result of freezing, yet the tonoplast or vacuolar membrane remained intact and showed deplasmolysis on thawing. The evidence presented by ILJIN is not entirely convincing that the cells were still alive after being thawed in the sucrose solutions.

In the present work some of the strips were frozen in sucrose solutions in the cold stage. If the cells were strongly plasmolyzed before freezing took place, the temperature required for ice formation was much lower than if the cells were not plasmolyzed, which is to be expected in view of the higher osmotic concentration within these plasmolyzed cells, and perhaps because of greater undercooling in this condition. When the cells were very strongly plasmolyzed, ice was not formed at $-10°$ C.

In ILJIN's experiments the temperature was lowered much more slowly than in those here reported. The tissues were kept at some of the higher temperatures for as much as 24 hours. This not only would allow hardening to take place, which would make the cells more resistant to low temperature, but also allowed for plasmolysis which so increased the osmotic concentration of the cells that ice formation was prevented until a very low temperature was reached. This is in agreement with the findings of ÅKERMAN (1).

Experiments such as have just been described were performed with fern prothallia also. The results were comparable.

ICE FORMATION, WATER CONTENT, AND COLD RESISTANCE OF WHEAT

Wheat was chosen for the material to be studied because so many of the previous studies of cold resistance and hardiness have been made with wheat,

and because the seed coats do not interfere with rapid absorption of water. During the winter of 1935–1936 a local commercial grain was used for most of the tests, and the results were checked with Minhardi. During 1936–1937, Minhardi was used altogether, with the exception of one group of experiments for which a non-hardy wheat, Leap, was obtained from the Department of Plant Breeding.

The percentages of germination of the controls was above 95 per cent. in all the wheat used and the seeds were kept in a sealed jar in a cool room to prevent any marked changes either in water content or in vigor. The grains were hand selected for uniform size and were divided into lots containing 100 seeds each. The weight of these seeds before any water was added was considered the "air-dry" weight. The "oven-dry" weight was obtained after drying samples of seeds in a vacuum oven for 288 hours at 90° C. The water content of air-dry grain was determined at intervals for different lots of seed to check the amount of water they contained, so as to be sure that the grain used over a period of time had the same initial water content. Calculated on this oven-dry basis, the air-dry seed contained 10 per cent. of water. For those lots of seed to which water was added, the percentages were computed from the increase in weight of water over the air-dry weight, the latter being considered as 10 per cent. from the previous determinations. The lots of seeds were weighed, put into bottles and a known volume of water added from a burette. The bottles were then stoppered tightly, put into the wheel, and rotated for 48 to 96 hours. In some of the first experiments, this rotation was performed at 25° C., but since at that temperature, seeds containing more than 31 per cent. moisture sprouted, a temperature of 4° C. was used. At the end of the period of rotation, the seeds were weighed again and the average percentage of water in each lot of seed was calculated. Seeds of each class according to their water content were then exposed to a temperature of $-20°$ to $-25°$ C. for 5 hours. After treatment they were germinated at 25° C. Counts were made at the end of 36 to 72 hours. The wheat germinated very quickly, and only rarely did those seeds which failed to germinate after 72 hours germinate at all.

There is no essential difference between the shape of the curve of germination with the Minhardi wheat and that of the Leap. These results are shown on the graph (fig. 1).

Sections of the endosperm of the wheat kernels cut freehand with a razor were mounted in mineral oil in the cold stage. No freezing could be observed except in those endosperms which were saturated with water. Since the endosperm could not be used, the embryo was tried. One slice was cut from the tip of the embryo and discarded; the next slice was used. These slices were cut freehand as thin as possible. Most of the sections used were in parts only one cell thick; probably they were between 10 and 20

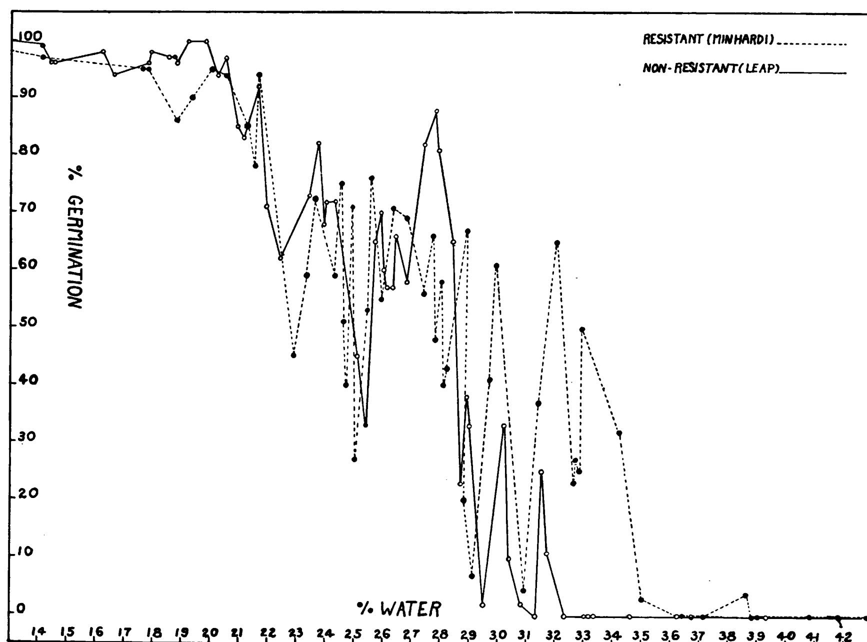

FIG. 1. Comparative behavior in germination of a resistant and a non-resistant variety of wheat at different moisture contents, after subjection to low temperatures (− 20° to − 25° C.) for 5 hours.

microns thick. No attempt was made here to find the temperature at which the seeds of different water contents froze. The purpose was to determine as quickly as possible whether seeds within certain water-content ranges would freeze or would not freeze. None of the seeds which contained 24 per cent. or less water would freeze even at temperatures down to − 25° C. In the intermediate group (water content 27 to 28 per cent.) ice was observed in about half the sections but in the upper range (30 to 31 per cent.) all the sections examined showed ice formation. In some of the sections with 27 to 28 per cent. water, in which no ice was formed, apparently some ice formation took place in the intercellular spaces, but it was very difficult to distinguish between air and water in the intercellular spaces; it cannot be stated positively, therefore, whether or not freezing did occur in the intercellular spaces and not in the cells. When the temperature was lowered the cell contents became darker, perhaps because of some colloidal change or of a difference in the refractive index of the water at the low temperature. This darkening occurred regardless of ice formation. Finally, when the temperature became still lower, ice crystals appeared and the sections became opaque. The same precautions were taken to prevent excessive under cooling as were used with the other materials. To shorten the time for these

determinations, this freezing was done at temperatures lower than was necessary for ice formation. It was not as important to find the exact temperature at which ice was formed as it was to determine as rapidly as possible whether or not the embryos would show ice formation at any temperature. The results of the germination tests are shown on the graph. There was a correlation between the water content of the seeds, ice formation as observed in sections of the embryo, and their resistance to freezing. This relation between water content and resistance to freezing is in agreement with the findings of WHITCOMB and SHARP (**25**) for wheat, and of KIESSELBACH and RATCLIFF (**12**) for corn.

ICE FORMATION IN HARDENED AND NON-HARDENED WHEAT LEAVES

Plants of Minhardi wheat were grown in pots until they were about 6 inches high. Some of the pots were then placed in a refrigerator at 8° C. every night for ten nights and kept in the greenhouse during the day, while other pots remained in the greenhouse continuously. As far as possible, the soil moisture was kept the same for both sets of pots. ROSA found that cabbage plants exposed to sunlight and rather high temperatures during the day, and to temperatures close to freezing at night became hardened easily. The wheat plants hardened in this way were darker green, had thicker leaves, and were smaller than the actively growing plants. On one occasion, two pots containing 100 plants each, one set hardened and the other non-hardened, were left outside all night when the temperature reached – 19° C. The hardened plants were not injured, while the non-hardened plants were so injured that only the roots survived. These plants subsequently produced more leaves when they were returned to the greenhouse. Neither the old nor the youngest leaves on the hardened plants were injured. When examined on the freezing microscope, the leaves from the non-hardened plants showed ice formation within the cells at – 10° to – 12° C., while the hardened leaves showed it first at – 25° to – 27° C. The cells were all killed when ice was formed, whether hardened or not. The hardiness was lost in three or four days if the plants were not placed in the refrigerator at night. When the pots were left in the greenhouse for three days, there was almost no difference in the temperature at which ice was formed in the leaves of previously hardened and non-hardened plants.

Discussion

Three hypotheses commmonly offered to explain the death of plants exposed to low temperatures are that this injury is due to drying, to a chemical agency, or to mechanical forces.

The chemical idea was first suggested by MÜLLER-THURGAU (**18, 19**) and strongly supported by MOLISCH (**17**) and GORKE (**8**), the theory being that

death resulted from some chemical action on the protoplasm effected by the removal of water from the protoplasm when ice is formed in the intercellular spaces. MOLISCH supposed the injury was caused by a toxic concentration of the cell sap, the increase in concentration being brought about by the dehydration. According to GORKE, the disintegration was thought to be caused by the precipitation of protective substances from the sap at their eutectic points leaving toxic materials to injure the protoplasm.

MOLISCH found that when filaments of *Spirogyra* were exposed to low temperatures, a crust of ice was formed around each of them. He believed that the water from which this ice was formed came from the interior of the cells, dehydrating them until the concentration of certain substances in the cell sap became so great as to injure the protoplasm. He could see no ice formed inside the cells and concluded, therefore, that death was caused entirely by this dehydrating effect. Our results with the *Sedum* cells have indicated that if a thin aqueous film is present around the cells, this layer of water is converted into ice when the freezing point is reached, and the behavior of the protoplast cannot easily be followed. Undoubtedly such a film was present around the algal filaments. This might explain why MOLISCH observed that the cells were dead after they had been thawed, even though he could see no ice inside them. WIEGAND (**26**) clearly demonstrated the presence of ice within the intercellular spaces of dormant buds, but these buds survived even though the cells adjacent to the ice were so dehydrated that the cell walls were compressed. According to the chemical theory the cells should have been killed by the high concentration of the cell sap. Several investigators have offered rather conclusive evidence that death on freezing is not caused by toxicity resulting from dehydration, and therefore increased concentration, of cell contents.

MAXIMOV (**15**), ILJIN (**9**), LEVITT and SCARTH (**13**, II) and CHANDLER and HILDRETH (**6**) support the theory that death at low temperatures is the result of mechanical injury either from ice crystals or from strong dehydration, rather than of chemical injury. ILJIN's suggestion that sometimes the killing is caused by the crushing of the protoplasm between the ice formed in the intercellular spaces and that formed in the vacuole is subject to serious criticism. He states that the concentration of the cell sap is less than that of the protoplasm and, therefore, ice must first be formed within the vacuole. LEVITT and SCARTH (**13**, I) seem to agree with this. They say: "When ice forms inside the cell, its site is the vacuole, not the protoplasm." In a later paper SCARTH and LEVITT (**23**) reiterate that intracellular ice formation, when it occurs, is confined to the sap vacuole. In their earlier paper they refer to CHAMBERS and HALE (**3**) as supporting this, but the latter, in describing the freezing in epidermal cells of the bulb scales of the red onion, cite cases and show photographs indicating ice in the cytoplasm external to

the tonoplast or vacuolar membrane when no ice was visible in the vacuole. In their summary CHAMBERS and HALE say: "The ice sometimes broke the outer membrane of the protoplast but the vacuole or tonoplast remains intact, in which case the tonoplast deplasmolyzes on thawing. Ice was not definitely seen to form within the vacuole but the general impression was obtained that it did so form in some cases."

Theoretical considerations indicate that ice formation would occur most readily outside the wall, followed in order by ice formation outside the outer protoplasmic membrane, then within the cytoplasm, and lastly within the vacuole. The osmotic concentration of the vacuole cannot be less but must be equal to or greater than that of the surrounding cytoplasm, for otherwise water would move to the outer, stronger solution. If the vacuolar membrane exerts any pressure whatever on the contained sap—and the surface tension alone of the membrane must be responsible for some pressure on the contents—this sap must be more concentrated than the bathing solution. If the vacuolar sap were less concentrated than that in the cytoplasm and froze at a higher temperature, it would be comparable to having a cell remaining turgid in a hypertonic solution and freezing at a higher temperature than the bathing solution. This situation could not obtain with a membrane permeable to water as the vacuolar membrane must be. Of course, although the vacuolar sap has a higher concentration and would therefore have a lower freezing point than the cytoplasm, the amount of freezing point lowering must be slight and this alone would not be important in preventing freezing. The combination of a membrane which would interfere with ice inoculation, together with this lower freezing point, may be responsible for ice formation in the cytoplasm first. Furthermore, since ice inoculation is likely to take place from the outside, this would favor ice formation in the cytoplasm before that in the vacuole even if they were at the same concentration. SCHANDER and SCHAFFNIT (**24**) describe ice formation as a thin layer just inside of the cell wall and between the protoplasm and the wall. The observations here reported, as well as those of SCHANDER and SCHAFFNIT, and of CHAMBERS and HALE (**3**), together with the theoretical considerations all indicate that ice formation takes place in the cytoplasm or between the cytoplasm and the wall before it does in the vacuole.

SCARTH and LEVITT (**23**) state that "Since at equilibrium 'suction tension,' and therefore the freezing point, must be the same in every part of a cell—vacuole, protoplasm, the cell wall—the osmotic value of the cells measured plasmolytically tells us the freezing point of the whole tissue." It is true that the suction tension of the cell as a whole and its wall are in equilibrium so that the freezing point at the surface of the cell is determined by the suction tension of the cell. That is, if cell *a* has zero suction tension, as it would have in distilled water, while cell *b* has a suction tension equivalent

to 0.5 M, ice would form at or in the wall of *a* at the freezing point of distilled water, while at the surface of cell *b* it would form only at or below the temperature necessary to freeze a 0.5 M solution, but in both cases the freezing point of the parts within the outer membrane may be very different and will be independent of the suction tension of the cell. For example, the suction tension of the nucleus, the vacuole, or a plastid will be determined not by the suction tension of the cell but by the osmotic concentration of their own immediate bathing media with which they are in equilibrium. As explained above if the vacuolar or plastid membranes exert any pressure on their contents, their osmotic concentrations must be higher and their freezing points lower than the cytoplasm within which they lie. Different parts may therefore have different freezing points and the osmotic concentration of the vacuole does not give an exact measure of the freezing point of the cytoplasm.

The low resistance to freezing observed by ILJIN (**10**) when the intercellular spaces of leaves are filled with water, as compared with normal turgid leaves or wilted leaves, presents a problem which would rarely occur under natural conditions. This free water would greatly favor ice formation in the cell. In those experiments in which ILJIN observed a protective action of sucrose or $CaCl_2$ when frozen tissues were thawed in the solutions, there is no proof that ice was formed within the cells and possibly in some cases not even in the intercellular spaces. He suggests that in most cases death occurs, not when ice formation takes place in the tissues but when the ice melts. The cells would, of course, be dehydrated by the formation of ice in the intercellular spaces. He suggests that when thawing occurs the walls and protoplast rapidly imbibe water and this rapid dilation or unequal swelling of different parts tears the protoplasm, thus resulting in death. If, on the other hand, this rapid or unequal swelling can be reduced by thawing in strong sugar solutions or those of $CaCl_2$ or similar salts of di- or trivalent cations which prevent rapid water absorption, killing is prevented; or the number of cells remaining alive is increased.

Two alternative explanations, however, may be offered. Possibly at the lower freezing temperature all of the cells were killed, but the strong salt or sugar solutions prevented complete deplasmolysis, and the cells therefore merely appeared alive. In the experiments with red cabbage here reported, frozen cells appeared alive if thawed in strong sugar solutions but on transference to dilute solutions or water they were found to be dead, whereas tissues which had not been killed by freezing retained their contents when placed in distilled water or dilute solutions. Another alternative is that, on slow freezing, ice had not formed in the cells but between them. Thawing in water or air may have supplied free water which would be available for ice formation in the cells which were undercooled or were at a temperature

low enough to form ice in the cell when water became available either from the melting intercellular ice or from the applied water or dilute solution. ILJIN gives no evidence that ice ever formed in cells that survived. In our own experiments killing occurred invariably when ice formed in the cells and, so far as was determined, only then.

Evidence from many sources, notably ÅKERMAN (**1**), ILJIN (**11**), and CHANDLER and HILDRETH (**6**), has demonstrated that a high osmotic concentration in cells increases their resistance to freezing. That the presence of solutes lowers the freezing point of the cell is obvious but it is not so obvious why there is often no direct relation between the increase in concentration and increased resistance. The failure to observe a direct relation between osmotic concentration and resistance to freezing is probably chiefly attributable to the fact that differences in rates of chilling, in permeability of the cell membrane to water, or differences in ratio of surface to volume of cell, and differences in amount of undercooling in part due to differences in readiness of ice inoculation across the outer membrane, may largely determine whether ice does or does not form in the cells. For example, a slow lowering of the temperature, high permeability to water, large surface relative to volume, and interference of the membrane to ice inoculation will greatly favor ice formation outside of the cells, thus bringing about a gradual increase in osmotic concentration inside the cell, with perhaps complete failure to form ice within the cells at the temperature to which the cells have been subjected. If the surface membrane interferes with inoculation across it, the fact that the ice outside the cell will have a lower vapor pressure than the undercooled solution inside may further favor outward movement of water, an increased osmotic concentration in the cell, and a lessened likelihood of intracellular ice formation. These several factors may explain the fact that a slight increase in osmotic concentration may be associated with greatly increased frost resistance.

Some investigators hold the opinion that hydrophilic colloids play a more important part than true solutes in increasing cold resistance, and suggest that these colloids by binding water prevent its loss from the cell and thus prevent killing by desiccation. ROSA (**22**) and NEWTON (**20, 21**) have consistently found increased bound water in hardened plants and suggest that the increase in bound water is responsible for the increased cold resistance. Many seem to think that the retention of water within the cell by the colloids is in itself significant. It is hard to imagine, however, that such bound water is any more available to the cell than if it were not there at all. The colloidal material itself, on the other hand, may serve as a protective colloid, perhaps preventing precipitation by its peptizing action; or better still, by reducing the free water and increasing the osmotic concentration would thus reduce the likelihood of ice formation.

CHRYSLER (**7**) found that in kelp stems the total amount of bound water increased as the amount of free water increased. If this holds for most plant materials tending to bind water (and there is a fair amount of evidence that it does), one would expect soaked seeds to contain more bound water than dry seeds, but the higher the water content of the seeds at the time of freezing the less is their resistance. With an increase in free water, on the other hand, even though there is an accompanying increase in bound water, there is an increased probability of ice formation and also a decreased resistance. This strongly points to lack of ice formation, not increased bound water, as responsible for resistance and that ice formation, not desiccation, is responsible for the injury. MEYER (**16**) actually found more bound water as well as more free water per gram of dry matter in the leaves of *Pinus rigida* in the summer, when the needles were not cold resistant, than in the winter when they were cold resistant.

Our experiments with hardened and non-hardened wheat leaves showed that free water was available for ice formation in the non-hardened leaves at – 10° to – 12° C. and they were killed at this temperature, but no ice could be observed in the section of hardened leaves until the temperature was lowered to – 25° to – 27° C. The resistance of dry wheat seed to ice formation and to killing, while those with more water formed ice and were killed, is further evidence that killing is not caused by desiccation or absorption of water after desiccation but to ice formation in the cells.

CHANDLER and HILDRETH (**6**) coated pollen of peach and *Amaryllis* with castor oil or cotton-seed oil, placed it on ice at – 15° to – 17° C., and water at 0° C. was slowly dropped on the pollen so that it froze as fast as it was added. If the oil was added to the dry pollen as it came from the anthers the freezing caused no injury, but if the pollen was first moistened no protective effect was noted. This is in agreement with our findings with dry and moistened seeds. It would seem that the oil had prevented absorption of water by the dry pollen and therefore no ice formed in the cells; but the moistened pollen had enough free water to allow for internal ice formation, and therefore killing, at the temperature used. The protective effect of the sugar solution is similar to that observed by ÅKERMAN and others, and is probably caused by an osmotic withdrawal of water and thus by prevention of ice formation within the cells.

From the observations here reported and the experiments of other workers the balance of evidence seems strongly in favor of the theory that death of plant tissues at freezing temperatures is brought about by mechanical injury due to ice formation within the cell. Anything that will decrease the amount of free water present will increase resistance to these low temperatures since ice is less likely to form within the cells. We have in no case found any cells to remain alive after ice once formed within them.

SCHANDER and SCHAFFNIT (**24**) and ÅKERMAN (**1**), however, cite cases in which they observed cells occasionally to remain alive after a small amount of ice was formed within them. By avoiding undercooling and cautiously lowering the temperature SCHANDER and SCHAFFNIT observed a thin layer of ice to form on the inner side of the cell wall at about – 5° C. If the tissue was then thawed the cells seemed alive as tested by plasmolysis with glycerine. If the temperature was further lowered the layer of ice grew thicker and the cells were killed. ÅKERMAN found that epidermal cells of red cabbage would withstand a small amount of internal ice formation without killing at – 5.2° C., but further lowering to – 7° or – 8° C. resulted in death. He found that any ice formation in cells of *Rhoeo* resulted in death. These cases of intracellular ice formation without killing were exceptional. They seem to demonstrate that ice can form in cells without killing but it is also conceivable that the ice formed between the wall and the cytoplasm, or that the injury was so slight that only the outer layer of cytoplasm was injured and that the tonoplast or vacuolar membrane was still intact and was responsible for the osmotic responses. CHAMBERS and HALE (**3**) observed ice formation in and disintegration of the cytoplasm and nucleus without destruction of the tonoplast membrane. It seems then that ice formation within the cytoplasm is almost certain to result in death and perhaps always so. Many claim that ice formation outside of the cell is usually responsible for the killing by freezing because of its dehydrating effect, but although one would expect such dehydration we know of no conclusive evidence that ice formation outside of the cell alone ever causes killing.

The observations of LEVITT and SCARTH (**13**, II) that cells hardened against freezing are much more permeable to water, strongly support the interpretation here given. As the temperature of the hardened cells is lowered the water would readily move through the surface membrane and form ice outside of the cell and therefore cause no injury. For the non-hardened cells free water would move out much more slowly and ice formation would be more likely to occur within the cell and result in its death. The conditions that reduce the likelihood of ice formation within the cell all seem to favor resistance to freezing. These are low amounts of free water within the cell, high osmotic concentration, high permeability of the membranes to water, small size of cell (high surface relative to volume), slow rate of temperature fall, and a membrane that favors undercooling by preventing inoculation across it.

Summary

1. A technique is described for direct microscopical observation of the freezing of living cells. When water or sap is present on the outer surface of cells the formation of an ice layer outside of the cells obscures what is

happening in the cells. Freezing in cells without this water film was observed when mounted in paraffin oil.

2. As the temperature of the cells of stamen hairs of *Zebrina pendula* was lowered, streaming became slower and finally ceased. Occasionally Brownian movement was visible for a few seconds after streaming stopped. Then ice formation took place at about $-7°$ C., appearing first in the basal cells and advancing across the field to the tip. Ice crystals were uniformly granular.

3. In the prothallia of *Polypodium aureum* granular crystals were usually produced, but occasionally needle like crystals were formed. Freezing occurred either cell by cell, or a wave of crystallization swept across the field. Insofar as could be determined the order of freezing agrees with the theoretical considerations; ice was formed first in the cytoplasm, then in the vacuole, and lastly in the plastids. The behavior of the nucleus could not be determined.

4. On thawing, the surface membrane was found to have lost its semipermeable character, allowing droplets of cell sap to collect in the mounting medium; the cytoplasm became granular and disintegrated; the nucleus assumed a glassy appearance.

5. Curves giving data on germination show that seeds of both the wheat varieties, Minhardi and Leap, were more resistant to freezing temperatures when the water content was low. Microscopic freezing tests of sections from the embryos of seeds containing varying amounts of water showed ice formation in seeds with high water and poor germination, and lack of ice in those with low water and good germination.

6. Leaves from plants of Minhardi wheat which had not been hardened showed ice formation at $-10°$ to $-12°$ C., whereas hardened leaves resisted ice formation down to $-25°$ C. Death always resulted if ice was formed in the cells.

7. Sucrose solutions did not protect strips of red cabbage epidermis from the injurious action of ice within the protoplast. If the cells were somewhat plasmolyzed by the solution before freezing began, a lower temperature was required for ice formation.

8. The evidence here presented and the observations of other workers strongly suggest that the death of plant tissues at freezing temperatures is caused by mechanical injury resulting from ice formation within the cells. Anything which will decrease the amount of free water within the cell at the time of freezing is likely to decrease the possibilities of ice formation within the cytoplasm and thus increase the resistance of the cell to freezing temperatures.

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