The Freezing Process of Plant Cell*

by

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I. Introduction

From the structural aspect of cell physiology as well as from the view point of the prevention of frost injury, the process of the freezing of living plant cells is one of the most interesting subjects for biologists. The freezing in a cell indicates the reorientation of water molecule accompanied with many phenomena favourable to the study of cell physiology, such as the formation of elongated crystal, the concentration of aqueous solution, dehydration from a water holding system and the displacement of water to the locus where the freezing occurs. Therefore the behavior of the cell constituents in the freezing process may make available some clues to the analysis of the properties of water as a continuous phase in protoplasmic structure.

Although many investigations on the freezing of plant tissue have been made, the methods used seem to be not entirely suitable for observing the process of freezing. Most of the reports published on the subject, so far as the writer is aware, were on the descriptions only of frozen cells, in other words, they were observations on the cells at an advanced stage of the freezing process. Among the few exceptions, Luyet and Gibbs have reported in detail the continuous changes of the cell interior after a rapid freezing of the onion epidermis (*40*). Siminovitch and Scarth have described some of the freezing process of frost hardy plant cells (*56*). Onoda has observed a rapid freezing of *Spirogyra, Elodea, Physcomitrium* and a rather slow freezing of epidermal strips of *Tradescantia, Rhoeo* and *Zebrina* under the "Molish microscope" (*46*). Chambers and Hale inoculated ice seed in Amoeba at a very low degree of supercooling (*20*). With only this method one can observe some of the early steps of the freezing process of living cell. Despite all these excellent studies, there yet exist many unknown important phenomena notably on the process of intracellular freezing. Using the method almost similar to that of Chambers and Hale, the present writer has for many years directly pursued under the microscope,

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On this subject, some preliminary papers have already been published in Japanese (*6, 7, 8, 10*). The present work was supported in part by A Grant in Aid for Fundamental Scientific Research from the Ministry of Education.

the actual freezing process of various kinds of plant cells in a number of plant species. In this paper, a detailed description is given of the processes of freezing and thawing in common plant cells, with a number of photo-micrograph illustrations. Hower, the various individual cases of cell freezing as already stated by many other investigators will not be refered to, except those which are specifically related to the immediate problem, since there are available detailed comprehensive reviews on the subject written by J. LEVITT (*35*, *36*). The main purpose of the present work is to observe more certainly where, when, and how freezing occurs in the cell. As to the properties of the cell surface correlating with the frost resistance some experimental results have also been briefly referred to.

Before going further the writer wishes to express his most cordial gratitude for the constant encouragement and the many helpful suggestions given by Prof. Kiyoshi AOKI. Thanks are also due to Prof. Jungo YOSHIDA for many valuable criticisms.

II. Material and Method

In the present investigation a considerable number of plants of various degrees of cold resistance were used, usually in natural condition but sometimes in *hardened* or *dehardened* one. The complete list of the plants used is as follows.*

Woody Plants

		tissue used
A white fir	*Abies sachalinensis* var. *Schmidtii* TATEWAKI	leaf
Japanese yew	*Acer pictum* THUNB.	leaf
Japanese chestnut	*Castanea crenata* SIEB. et ZUCC.	twig
Mulberry tree	*Morus bombycis* KOIDZUMI	twig
Apple	*Malus pumila* var. *domestica* SCHNEIDER	twig, fruit
A crab-apple	*Malus Toringo* SIEB.	twig
Sand pear tree	*Pyrus montana* NAKAI	twig
A Japanese wild cherry	*Prunus Sargentii* REHDER	twig
Panicle Hydrangea	*Hydrangea paniculata* SIEB.	twig
An evergreen spindle-tree	*Euonymus Fortunei* var. *radicans* REHDER	leaf
A maple	*Acer mono* var. *eupictum* NAKAI	twig
	Cornus controversa HEMSLEY	twig
Red-berried elder	*Sambucus Buergeriana* BLUME	twig

Herbaceous Plant

Table beet	*Beta vulgaris* var. *Rapa* DUMORT.	petiole, succulent root

* To avoid complexity of description, only some of the freezing processes most commonly found in normal plant cells shall be mentioned in detail, though all of these listed plants were actually subjected to freezing under the microscope.

		tissue used
Spinach	*Spinacia oleracea* L.	leaf
Pussley	*Portulaca oleracea* L.	stolon
A rape	*Brassica campestris* subsp. *Napus* HOOK. f. et ANDERS.	stem, leaf
	Brassica chinensis L.	Midrib of leaf
Chinese cabbage	*B. pekinensis* RUPR.	Midrib of leaf
Turnip	*B. campestris* subsp. *Rapa* HOOK. f. et ANDERS	leaf, succulent root
Cabbage	*B. oleracea* L.	leaf
Radish	*Raphanus sativus* L.	leaf, succulent root
Garden pea	*Pisum sativum* L.	pea
Catiang	*Vigna sinensis* var. *Catiang* (BURM.) T. ITO	beanpod
Carrot	*Daucus Carota* var. *sativa* DC.	succulent root
Himalayan primrose	*Primula denticulata* SMITH	flower
Tomato	*Lycopersicon esculentum* MILL.	fruit
Potato	*Solanum tuberosum* L.	leef, stem, tuber
Watermelon	*Citrullus vulgaris* SCHRAD.	fruit
Cucumber	*Cucumis sativus* L.	fruit
Oriental pickling melon	*Cucumis melo* var. *Conomon* MAKINO	fruit
Pumpkin	*Cucurbita moschata* var. *toonas* MAKINO	fruit
Cosmos	*Cosmos bipinnatus* CAVANILLES	flower
Jerusalem artichoke	*Helianthus tuberosus* L.	tuber
Dandelion	*Taraxacum platycarpum* DAHLST.	flower, leaf
	Elodea densa CASPARY	leaf
	Tradescantia virginica L.	flower
	Rhoeo discolor HANCE	leaf
Onion	*Allium Cepa* L.	scale leaf
Welsh onion	*Allium fistulosum* L.	leaf
	Algae	
	Nitella sp.	
	Hydrodictyon reticulatum (L) LAGERH.	
	Spirogyra sp.	
	Slime mould	
	Physarum polycephalum SCHW.	plasmodium

In the vascular plants the tissues most frequently used were the cortical layer from the twig, petiole, fruit and root. As evergreen leaves, white fir, Japanese yew

and a spindle-tree were investigated in winter. As flowers, the petals of primrose in early spring, of cosmos in autumn and of dandelion from spring till early winter served as material. In some herbaceous plants, the naturally isolated cells in fully ripened fruit and the artificially isolated protoplasts of parenchymatous or epidermal cells were also employed. The tissues were usually sectioned with a hand razor in thin tangential sections and blotted gently to remove the sap which flowed out on cutting. They were then placed on a cover slip in a hanging drop of paraffin oil or silicone oil*. A part of these sections was used to determine the concentration of the incipient plasmolysis of the cell. In the case of slime mould, a bit of naked "plasmodial sheet" with some of the "reticulum" (*19*) was cooled on the moistened surface of cover slip without oil, because the protoplasmic surface of this organism is very sensitive to contact with oil.

The mortality of the cells frozen and thawed was determined by means of vital staining, plasmolysis and the following deplasmolysis. A solution of balanced salts (NaCl : CaCl :: 9 : 1) or of sucrose was used as plasmolyzing agent and for vital staining neutral red dissolved in slightly hypotonic sugar solution was employed.

The observations were made with an ordinary microscope converted into a phase-contrast one by a special device (see *26*)**. This modified form of phase-contrast microscope was very convenient to manipulate the cells in the hanging drop. This microscope and a micromanipulating apparatus together with some heating devices and a thermocouple were set up in a small double walled room in which the observations were carried out. This small room with a heating device was constructed in one of the cold rooms of our institute in which air temperature was continuously kept at about −10°C or −25°C as occasion demanded.

The cover slips with the hanging drops were carried to the cold room, where they were kept in a box at 5°C for varying lengths of time, from five minutes to two hours, before being transferred on the microscope stage on which a small rest

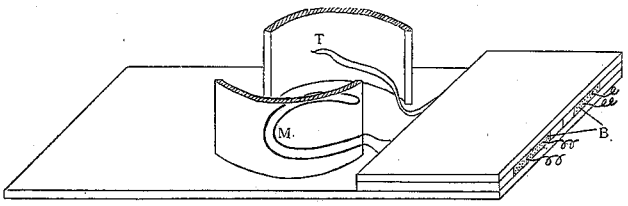

Fig. 1. The glass rest.
T. Tip of thermocouple.
M. Microheater.
B. Bees wax.

* A silicone, DC. 200 (350 cstks) was used. For the hanging drop method, this oil was a better medium in many physical properties than paraffin oil.

** The writer here wishes to express his thanks to Prof. Takeo HORI who has kindly converted the microscope used into a phase-contrast one.

was prepared. As shown in Fig. 1 the rest consisted of a glass slide with two pieces of glass tube split longitudinally, about 1 cm high, fixed 2 cm apart on the slide, each with its inner side faced inward to support the cover slip. In this way the rest was open on both sides to permit the insertion of the various instruments for operation.

The temperature of the tissue slip was read by means of a thermocouple (copper-constantan wire of 0.08 mm diameter) of which the tip was placed as close as possible to the cell under observation (see Pl. XXI, Fig. 91). A microheating device made of a small double u shape of fine nicrome wire, being connected with a battery and sliding resistance, was set up close to the slide of the rest in such a way that the microheater was situated just under the hanging drop. A micropipette or a microneedle mounted on a micromanipulator was inserted under the hanging drop from the left side of the microscope. To maintain a constant temperature or to control the cooling rate of the hanging drop, the microscope together with the accessory apparatus was set in a cubical box which was made of thin celluloid plates, and provided with two windows on the upper side and with a nicrome heater around the inner side of its bottom. The side walls of the box were provided with many holes so that the various screws of the microscope could be handled from the outside of the box. The eyepiece and the shaft of micropipette or microneedle also projected from the box through holes of suitable sizes. At the beginning of the observation the hanging drop was usually maintained at a temperature slightly higher than that of the surrounding air in the celluloid box which was also a few degrees warmer than the cold room. A special adiabatic filter* prepared by the Olympus Co., Tokyo, was interposed between the light source and the microscope. To prevent the condensation of moisture on the lens surface the eyepiece was warmed with a small heater consisting of a spiral nicrome wire around the metallic cylindrical cover. The high heat conductivity of the metal of which the microscope was made, disturbed the good controlling of the temperature of the hanging drop. To lessen this difficulty the draw-tube and the cylindrical cover of the objectives were made of ebonite because of its low heat conductivity as compared to metal. Besides the microscope mentioned above, a polarisation microscope, with some heating devices was also used to identify the ice crystals, however, in the frozen tissue this apparatus was not so effective as had been expected.

To prevent excessive supercooling of the meterials, they were inoculated with an ice-tipped pipette, and then the process of cell freezing was observed at desired temperature and cooling rate. In the most usual cases, however, the material was cooled at a rate of two to four degrees per minute measured between $-1°C$ and $-3°C$, and when cooled down to about $-3°C$ or $-4°C$ ice was seeded on it. For example, the freezing curve of a small tissue section is presented in Fig. 2.

* Olympus Adiabatic Interference Filter.

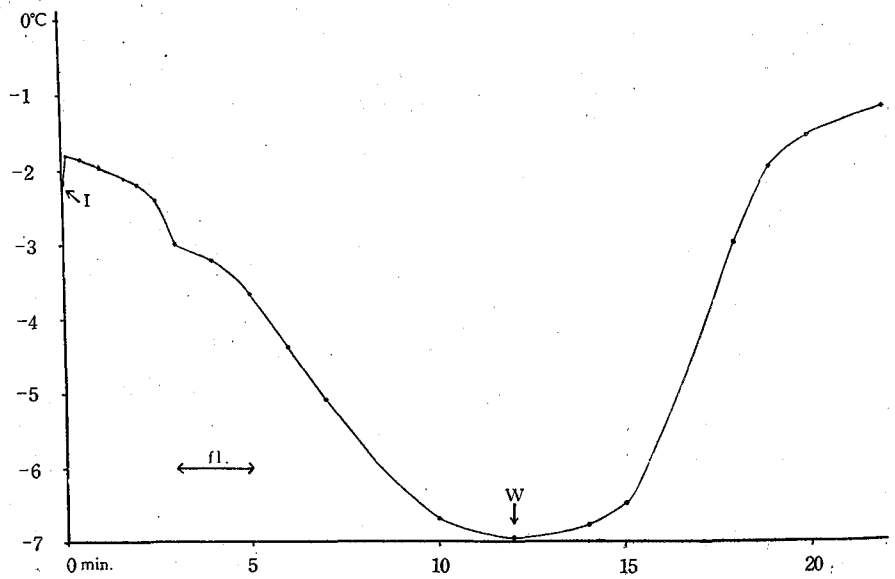

Fig. 2. Time temperature curve in a course of freezing experiment of a tissue slip in oil hanging drop.
(a section of parenchyma of tomato fruit.)
I. The time at which tissue was inoculated, i. e. the freezing of the tissue began from this point.
W. The time from which the rewarming of hanging drop began.
fl. Duration of the freezing period in which "flashing" most frequently occurred in the tissue.

III. Initiation of the Freezing in Plant Tissues

It has long been held that supercooling is a widespread phenomenon among living cells. However, it seems very doubtful whether the phenomenon appears only in living state or not. In cloud physics, a common opinion today is that water drops of a volume comparable to a common plant cell can freeze spontaneously only when cooled down to a temperature below −15°C (*25*, *28*). Besides, JACOBI's detailed work has shown that in the water drops of a diameter less than 0.5 mm the lowest supercooling temperatures in water vapour are always below −33°C (*31*).

ULLRICH and MÄDE have suggested a correlation between the supercooling of the tissue and the freezing point of the intercellular moisture (*58*). Nevertheless, without ice seeding on the tissue, "spontaneous freezing" usually occurs only at a far lower temperature than the freezing point of the tissue fluid. Although the plant tissue slips with wet surface usually freeze more easily than those with dry surface, the main factor of such a readiness for the intiation of freezing is assumably dependent upon the volume of the fluid in which the nucleation of ice usually takes place. In fact, a small slip of plant tissue mounted in oil, whether being alive or

not, can easily remain in unfrozen state at temperatures below −10°C. Such a state is so stable that the tissue is hardly freed from supercooled state by merely mechanical shocks, unless the grade of supercooling is very high. The tissue treated in this way, however, is never vitrified even after having been cooled down to −20°C or more. Against LEVITT's suggestion (see *35*, p. 43), the possibilities for occurrence of the vitrification of plant tissue is hardly conceivable at least at the temperatures above −25°C, provided that it has a natural water content.

Many workers have reported the actual temperatures at which certain kinds of plant tissues freeze. These temperatures, however, seem to vary with the methods used. The temperature at which the "spontaneous freezing" occurs in plant tissue, is dependent upon many factors such as cooling rate, volume, water content and surface conditions of the tissue. Probably the last is the most important. Tissue wet with aqueous solutions of relatively low concentration, is always frozen more easily than that with dry surface. However, some intact flowers in rather cold season, such as Himalayan primrose and late-blooming dandelion, can hardly be frozen even when the dewdrops on their petals have frozen at a temperature of several degrees below their *"freezing temperature"**. A slow lowering of the temperature occasionally favours the supercooling of tissue. This is because of the occurrence of larger frost injury in the tissue cooled very slowly than in that cooled rapidly. When a plant section with cut surfact was cooled very rapidly, say 5° or 10°C per minute from 0°C to −5°C, its supercooled state used to be easily lost at a rather high temperature, say −3°C, and extracellular freezing took place by which none of the *frost-hardy* cells were usually killed within a certain limit of temperatures. On the other hand, when a small tissue slip was cooled slowly, say less than 1°C per minute, and then froze spontaneously at a high degree of supercooling, say −12°C, fatal intracellular freezing occasionaly occurred even in the cells of *hardy* plant as well as of *unhardy* one. In these cases, toleration rather than prevention of freezing is the mode of frost resistance.

At a temperature slightly lower than the freezing point of the cell sap, the most effective and perhaps the only method to initiate the freezing of the tissue is the inoculation of ice seed into the freezable liquid adhering to the tissue surface. According to the writer's observation, the vacuole sap, even in the living cells, could be usually frozen at about the freezing point of squeezed cell sap, which value, at least in certain kinds of herbaceous plants, was usually in correspondence to that of a sugar solution approximately isotonic with the concentration of the incipient plasmolysis of the cell. By means of intracellular inoculation, for example, the vacuole sap of parenchymatous cell of *hardy* table beet always froze at a temperature lower than −1.5°C while the freezing point of squeezed cell sap measured by the ordinary method and the concentration of incipient plasmolysis

* In the present paper, the term "freezing temperature" is used only for the meaning of the highest temperature at which the system can be frozen by means of ice seeding.

were −1.43°C and 0.85 molar sucrose respectively.

IV. Extracellular Freezing

The freezing process in plant tissue is strikingly influenced by many factors both external and internal, such as the grade of supercooling, the cooling rate, the hardiness of the cell itself and the amount of freezable liquid moistening the surface of the tissue slips which can elevate the tissue temperature, when it freezes, by the heat of crystallization of ice. In most plant tissues the process of extracellular freezing, however, can be easily and well observed at a temperature slightly lower than the freezing point of the cell sap.

In tissue section at the beginning of the freezing, the ice crystals, in agreement with many earlier observations (*3*, *47*, *51*), were frequently seen to form on the cell wall facing the intercellular space (Pl. III, Fig. 10, Pl. XXXI, Figs. 135, 137). In fact, when these plants had already been subjected to freezing in the open field as well as in the laboratory, a number of ice crystals were generally found in the intercellular space of many evergreen leaves or of various parts of winter annuals. But in vascular plant tissues, as suggested from old times (see *1*), one of the most favourable loci for the growing of ice crystal seems to be in the vessel. In a cold frosty morning, the ice formation in various herbage crops was often found to be mainly confined to the vein; such a remarkable example is represented in Plate I, Fig. 1. At least in herbaceous plants, the darkening which takes place in consequence of the formation of ice crystals was usually observed to occur along the vessels of intact leaves as well as in the sections of the petioles. On the wet surface of a tissue section, the ice frequently grew along the border line of each cell*, particulary on the strips of epidermis.

In some cases, a remarkable extracellular ice formation was observed at the central area on each of the individual cells (Pl. XXXII, Fig. 141). In the cells frozen in this way a pseudoplasmolysis was usually found after a very rapid thawing. Contrary to some statements in the literature (*29*), such pseudoplasmolysis did not always result in a fatal injury to the cell. *Hardy* cortical cells, at least of some woody plants, withstood generally such severe dehydration as well as the following rapid rehydration. Besides, even in some *semihardy* herbaceous plants, the pseudoplasmolysis after an extracellular freezing was not fatal to the cells, within a certain limit of temperature and time of freezing. Plate II, Figs. 5–7 represent an example of such case in the midrib of a winter leaf of dandelion.

When inoculated with ice onto the cell, the liquid moistening the cell surface froze first. Starting from the inoculated point, the freezing rapidly propagated over the whole surface usually with dendritic ice branches. In the case of *nonhardy* plant, the rate of development of such ice crystal soon became very slow unless

* This is probably because of the sufficient existence of easily freezable liquid on that place most of which had not been removed in the preparation of the section.

the cell was irreversibly injured. At this instant, it was a frequent case that ice branches on the cell surface spontaneously divided into many small lumps. The form of ice crystals on these cells, was very rich in variation; it was, for example, dendritic, needle-like, leaf-like or sometimes cubic as shown in Plate III, Fig. 9, Plate XV, Fig. 70 & Pl. XXVI, Fig. 113. Soon after the growing of these crystals ceased, the freezing within the protoplast, i. e. the intracellular freezing always took place even under slow cooling.

When the cell had been once injured by freezing, the rate of growth of extracellular ice became much higher than before and the shape of ice crystal usually had the appearance of a frost column. For instance, the internodial cell of a fresh water algae, *Nitella*, being inoculated with ice onto the cell surface at −0.4°C, suffered an extracellular formation of fine ice crystals (Pl. I, Fig. 2). In such a case most of the frozen cells were scarcely injured at least for several minutes and after thawing, the characteristic streaming of protoplasm was usually observed in the cell. If the cells frozen in this way were cooled further to a temperature lower than −1°C, a distinctly rapid growing of ice column always took place on the cell surface and the cells apparently shrank (Pl. I, Figs. 3, 4). This always resulted in a collapse of the cell surface after thawing, at least in the area in which the rapid ice growing occurred.

On the contrary, the ice formed on the surface of *frost-hardy* cells grew very large, continuously withdrawing water from the cell, and the crystal form in this case was usually irregular as shown in Plate XIV, Fig. 66. These cells frozen in this way, consequently underwent a remarkable dehydration and contraction and, as a rule, none of them froze intracellularly. Sometimes they shrank to such a degree that the two opposite cell walls facing the observer curved inward and eventually came in contact at the centre with each other. In the cells with a coloured vacuole, the centre area of these cells, therefore, became quite light, as the vacuole was squeezed into the periphery of the cell (Pl. XXXI, Fig. 139). Similarly, when the vacuole was squeezed to one side in a frozen cell, such flattening occasionally took place in the opposite side. Upon warming, however, the contracted cells usually absorbed water and expanded as the surrounding ice melted. None of the *hardy* cells, yet with a few exceptions mentioned before, underwent pseudoplasmolysis after thawing; as a rule, they were not injured fatally. However, *unhardy* cells, even after a rather slight degree of freezing, were occasionally observed to be killed, with or without the occurrence of pseudoplasmolsis. The process of injury by extracellular freezing will be described in a later section.

In a slightly supercooled state of cell, the ice formed between the ectoplast and the cell wall was occasionally observed in the course of extracelluler freezing particularly in epidermal cells. In such a case, the ice gradually increassed in amount as the cooling proceeded, withdrawing water from the protoplast, and this resulted in the plasmolysis caused by ice formation or a "frost plasmo-

lysis"*. As a consequence of ice growing, the protoplast decreased in volume frequently being reduced by one-third or sometimes by one-half of its original amount. At any rate in the cells frozen in this way deplasmolysis took place after thawing, with or without fatal injury.

For the observation of this interesting phenomenon the staminal hair of *Tradescantia* was adopted as the most convenient material, though it was also observed in several species of herbaceous or woody plants. When the stamen of *Traescantia* was subjected to freezing the "frost plasmolysis" of the hair cells frequently took place at a temperature usually higher than $-2°C$. At first ice was found at the basal end of a cylindrical cell where the cell was connected with the already frozen cell. Then the ice grew gradually, whereas the protoplast contracted. In the process of the advancing of "frost plasmolysis", the surface of protoplast which was directly in contact with ice appeared to be concave then became plane (Pl. V, Fig. 17). Being maintained for a while at a constant temperature or warmed slightly, this surface gradually became convex (Pl. V, Fig. 18). Although the typical convex form observed in the contour line of plasmolysed cells in ordinary aqueous solutions was scarcely found in "frost plasmolysed" cells, the ice seemed to grow easily along the inner side of the cell wall, because some semisphere ice masses were occasionally formed in the cell along the wall (Pl. V, Fig. 20)**. Besides, there were some cases in which the ice mass also developed in the distal end as well as the basal end of the cell. In addition, a new "frost plasmolysis" sometimes took place in the basal end of the next cell even without any appreciable ice formation at the distal end of the first "frost-plasmolysed" cell. There were some evidences to indicate a tendency of protoplasm to adhere to cell wall. When the "frost-plasmolysed" cell was cooled further to a lower temperature than before, the convex surface of protoplast ajacent to the ice became concave again as the ice crystal grew, a local protrusion being left which adhered to the cell wall. Sometimes, in the ice mass formed between the protoplast and cell wall, a few fine protoplasmic strands were observed (Pl. V, Fig. 19). But in most cases, such ice masses seemed to be quite homogeneous and no detectable structural elements were found. When the protoplast contracted in this way was subjected to freezing, however, a few threads of the coagulated protoplasm or at least some unfreezable cell component was usually found embedded in the ice mass (Pl. V, Figs. 21, 22).

The "frost plasmolysis", when it occurred in the middle part of the cylindrical cell, though it was a rather rare case, often resulted in the complete division of

* Only this phenomenon should be referred to as "frost plasmolysis" by which some authors have often meant a contraction of coagulated protoplast which ensued after freezing and thawing (see *35*, p. 21). For the latter case, in the writer's opinion, the term "frost pseudoplasmolysis" might well be employed.

** This may probably correspond to the phenomenon described by MOLISCH some half century ago in the staminal hair of *Tradescantia*. (*42*)

the protoplast as shown in Plate VI, Figs. 23, 24. After thawing, the divided cell parts expanded to unite with each other and recovered the original appearance of protoplast with active streaming of protoplasm over the whole cytoplasmic layer.

Up to the present the dehydration of a cell caused by the extracellular freezing has long been regarded by some authors as a withdrawal of water due to the osmotic gradient of some outside medium concentrated by the ice formation (*3*, *45*, *54*). In the "frost plasmolysis" as described here, however, no detectable amount of any solution apparently existed between the protoplast and cell wall. If the surface of the protoplast is in direct contact with ice, it is reasonable to suppose that the water can pass through the surface layer of protoplast without any hypertonic outside medium to arrive at the surface of an ice crystal where it freezes. Also in many of the intact tissues, the dehydration caused by extracellular freezing may probably be due to the same reason, though in some cases the existence of some hypertonic outside medium may play a part in the withdrawal of water from the cell interior.

It may be worthy to note that the ice once formed is very unstable in site as well as in size. As DORSAY (*22*) has suggested in the case of frozen peach buds, the water in the tissue always moves before freezing to the already frozen area. Similarly in any frozen tissue in which a thermal gradient exists, the ice gradually migrates to the colder region, for the water molecule moves towards the part of low vapour pressure. Besides, as already noted by some authors (*1*) the vapour pressure on supercooled water surface is always higher than that on ice at the same temperature. For these reasons there are always formed a number of well-developed ice crystals at the colder side in the section experimentally frozen (Pl. II, Fig. 8 & Pl. XXXI, Fig. 138). Also in winter field it has been well known, usually in the succulent roots of various root crops (*18*), that some extraordinarily large crystals of ice being some hundreds times the cell volume were occasionally found in the surface layer of which the temperature must be the lowest, at least in a cooling process.

V. Intracellular Freezing

Observations in the staminal hair of Tradescantia

As indicated by the fact that it was often used by some earlier authorities (*34*, *42*) the full grown staminal hair of *Tradescantia* is very convenient for the observation of intracellular freezing for the following four reasons:

a. The point of inoculation with ice can be restricted to a very small area of the basal end of a cell.

b. Consequently the order of the propagation of cell freezing can always be predicted.

c. The cell surface can be kept completely free from the moisture which disturbs

a good observation of the freezing process in the cell.

d. Owing to the existence of the cuticular envelope no substances in the cell, both frozen and thawed, can easily escape from the cell. Besides, the well known characters of the hair cell making it suitable for cytological observation, such as large size, bright colouring of the cell sap and very clearly detectable streaming of protoplasm, are also of great advantage for experiments such as the present ones.

As with slow cooling, the plasmic streaming in the cell usually ceased at about 0°C.,* and the hairs, even with the filament of stamen, can be cooled down to about −20°C without freezing. At a temperature of −10°C, the hair cells imbedded in a small drop of paraffin oil could be easily kept in supercooled state without any injury for at least six hours. Moreover, they frequently remained unfrozen at the same temperature for several days, though within a full day such a treatment became quite dangerous for the cell.

An inoculation of ice seed was always necessary to congeal the cell, unless the degree of supercooling was considerably high. The filament and the hair could be frozen intracellularly at a temperature lower than −1°C and −1.2°C, respectively. When inoculated onto the cut end of the filament at a moderately supercooled state, say −4°C or −5°C, the cells of the filament froze in rapid succession, then the freezing propagated from the cell at the proximal end of the hair, which was close to the frozen filament, towards the distal end of the hair. The cell once frozen inoculated the cell next to it until all of the cells in a hair froze in succession, with some short time interval between the freezing of each cell. The process of such a rapid cell freezing is typical of so-called "flashing". In the freezing, the ice growing in a cell started at the basal end and proceeded as a dark wave to the distal end in approximately one-half or one-fifth of a second. Owing to this rapid formation of a large number of minute ice crystals throughout the protoplasm and vacuole, the cell interior became intensely opaque and dark (Pl. VII, Fig. 27), and the simultaneous increase of cell volume was easily observed. These crystals rapidly united into larger masses during the first ten seconds or so after their formation, and continued to grow rather slowly. Thus the cell was most dark at the moment of "flashing" and then its opacity progressively decreased so that it became appreciably lighter as time went on (Pl. VIII, Figs. 31–33). After these changes the cytoplasmic layer which had uniformly covered the tonoplast was deformed to an irregular net-like structure as shown in Pl. VII, Fig. 30. At the same time the cell nucleus became distinctly dark (Pl. VII, Fig. 29) and then gradually turned to lighter.

Immediately after "flashing", the cell interior appeared uniformly granular

* MOLISCH has reported the streaming at −2°C in the staminal hair of *T. crassula* (*42*). Assuming from the apparatus used, one supposes that the temperature of the cell itself must have been somewhat higher than the temperature described.

owing mainly to the existence of a large number of irregular droplets of cell sap intermingled with the numerous ice crystals. As the latter increased in size, the former also coalesced into larger droplets. Consequently, after a considerable period of time, a relatively small number of sap drops, together with some unfreezable cellular contents, were found in the ice mass of the frozen cell (Pl. X, Fig. 45). When the succession of the cell "flashing" progressed along the cell chain of a hair, a type of extracellular freezing, as a rule, was observed in each next cell. Namely, at the distal end of a previously frozen cell, a transparent ice mass was always formed withdrawing water from the next cell (Pl. X, Figs. 42–44). This sometimes resulted in a remarkable dehydration and contraction of the latter (Pl. XI, Fig. 48), although the amount of such ice entirely depended upon the length of the time interval between each "flashing". At the temperatures ranging −4°C to −20°C the hair cells behaved upon freezing nearly in the same manner as described above, though the lower the temperature at which the freezing occurred, the shorter was the time interval between each flashing.

If the cell was inoculated at the time of very small degree of supercooling, the process of intracellular freezing could be observed in detail since it froze far more slowly than the case described above. At a temperature of about −1.5°C, the intracellular freezing of a hair cell was not "flashing"; it finished in approximately a second or more. As noted above, a growing of very clear ice mass always took place in the distal end of the cell just frozen, resulting in a projection of the latter into the next unfrozen cell (Pl. IX, Fig. 37). With slightly faster cooling, the ice mass in the projected cell end, though instantaneously, developed rapidly and at the next moment a jet of ice crystals was usually spouted out from the top of this ice mass into the next cell, without any appreciable destruction of the cell wall. Immediately after the freezing of the next cell the projection of the previously frozen cell end disappeared usually within a few minutes (Pl. IX, Figs. 38, 39). These ice crystals now formed in the next cell grew mainly in the cytoplasmic layer towards the distal end, though some of them seemed to be formed between the protoplast and cell wall (Pl. VII, Fig. & 29 Pl. X, Figs. 42–44). In the outside of the tonoplast, consequently, an ice layer constituted of several broad branches was formed, from which a number of newly crystallized ice fronts proceeded inwards. Sometimes, after the ice crystal primarily grown reached the distal end of the cell, it turned backward with divided ice front and proceeded inwards, thus, the freezing in the tonoplast now newly occurred from that end. The pattern of ice in the frozen cell just after such process is represented in Pl. IX, Fig. 40. It was of interest, though it had been expected, that in sap vacuole the ice usually grew distinctly faster than in cytoplasm.

At the instant of freezing, the structures of the cell constituents were entirely destroyed. Being coarsened by dendritic ice crystals, the cytoplasmic layer, though temporarily, had a coarsely granular appearance and then became lighter as the

disintegrated cytoplasm was rapidly concentrated around the developing ice masses. Simultaneously with the primary growing of ice, the nucleus deformed as a very granular mass and soon became more transparent. Fig. 28 in Pl. VII. and Figs. 31, 32 in Pl. VIII., respectively, show the very granular appearance of the nucleus just frozen and its following deformation during the further freezing. The vacuole was divided into many globules of concentrated cell sap irregular in form, because the ice branches grew usually in parallel with each other. The lower the temperature at which the freezing occurred, the more the division of the ice front in the cell. It followed therefore that the freezing at a lower temperature resulted in the formation of a larger number of smaller sap droplets packed in the ice mass. However, the figure of a frozen cell, of course, varied depending upon the mode of distribution of both ice and unfrozen component at the temperature exposed.

Also in plasmolysed cells*, the freezing process observed did not differ significantly from that in normal ones. The contracted protoplast was less resistant to the penetration of ice than was expected. When plasmolysis had occurred only in the distal side of a cylindrical cell, the freezing developed from the basal end of the cell was sometimes prevented at the plasmic membrane which was in contact with the outside solution yet unfrozen. Such a strikingly interesting instance fortunately caught in a photograph is presented in Plate XI, Fig. 46.

In plasmolysed cells subjected to freezing, BUGAEVSKY has reported a "remarkable increase in the permeability of protoplasm" which sometimes resulted in a disappearance of plasmolysis just before ice formation (*17*). In the present observation, however, any expansion of protoplast was not observed prior to the freezing. Indeed in plasmolysed cells, when cooled very slowly, the volume of solution between the protoplast and cell wall remarkably decreased, owing to the dehydration by the growing ice mass in the neighbouring already frozen cell. Consequently the cell together with its wall underwent a remarkable contraction or flattening. The process of such a dehydration is well represented in Plate XI, Figs. 49–52.

When the freezing was very slow, an ice formation limited only to the outer side of the tonoplast was sometimes observed in the cell especially at the proximal end of the hair. Starting from the cell end touched by the frozen filament, the divided crystal fronts of ice grew in parallel with each other under the cell wall and reached the opposite end in a few seconds. Just after the growing of these ice crystals the tonoplast was pushed inward rather rapidly assuming a contraction of vacuole** (Pl. VI, Figs. 25, 26). At the same instant the cytoplasm and nucleus were apparently disintegrated, and the frozen area containing these cell constituents was observed to be always darker than pure ice. Upon contraction, the vacuole

* Only in this case a solution of 0.6 molar solution of KNO_3 was used as plasmolyzing agent.

** For instance, the vacuole shrank to about one-fourth normal size in approximately one minute at −2°C.

usually had a very finely jagged outline with many small concave faces, however, this smoothed in a few minutes. When it was thawed the tonoplast, though it was no longer alive, more or less expanded depending upon the degree of freezing in respect of time and temperature.

Observations in aquatic single cells

When inoculated within a certain range of degrees of supercooling, some aquatic algae were very liable to freeze extracellularly, although they are easily killed by this mode of freezing. Among them *Hydrodictyon* was most resistant to the initiation of freezing inside the cell wall. Upon freezing this alga, whether being alive or not, underwent a remarkable dehydration and contraction. Therefore, the freezing process of their cell interior was usually observed at a few degrees below the freezing point of their sap; *Spirogyra* and *Nitella* were subjected to freezing at −2.5°C and −3.2°C, respectively. The processes observed in their single cells were almost the same as in the case of the "flashing" in *Tradescantia.* As a rule the protoplast of these aquatic plants was so easy to freeze that as soon as the ice was formed at one end of a cylindrical cell, a rapid growing of finely branched ice towards the other end, throughout the protoplasm and vacuole, was always observed. Ice formation between the protoplast and cell wall, i. e. "frost plasmolysis", was never observed in these cells. However, as ONODA has already reported (*46*), vacuole contraction in *Spirogyra* cells due to an intracellular ice formation sometimes occurred. This resulted in a fatal coagulation of the protoplast; after thawing it presented a pseudoplasmolysislike appearance.

In the frozen cells of these algae, the rate of fusion of ice crystals into a larger ice mass as well as of coalescence of droplets of unfreezable cell contents was usually more speedy than in the case of *Tradescantia.* The frozen cells, therefore, became considerably lighter soon after the "flashing." The general orientation of the chloroplast band in *Spirogyra* cells was scarcely changed immediately after the first "flashing" of the cell, though the structure of the band was appreciably disintegrated. A remarkable deformation of the band occurred when the freezing of the cell was repeated. Whereas in the cells of *Nitella*, the characteristic parallel figure of chloroplasts was always destroyed by a single freezing. As a result of extracellular freezing, however, these structures in both algae were easily caused to collapse, provided that a certain amount of water was withdrawn by the ice on the cell surface.

Observations in epidermal cells

From old times, the plant tissue most commonly employed for the investigation of cell freezing, has been epidermis, particularly of monocellular layer. The writer himself also found for the same purpose very suitable materials from among the epidermis of many varieties of onion or welsh onion, though various other kinds of

plants were employed as material. Strips of epidermis from stem, twig or periole as well as from leaf were examined.

As already noted in a previous paper (*13*), it is difficult to seed the intact surface of epidermis with ice crystals if it is kept dry. The inoculation was, therefore, usually performed at a cut end of strip, where a certain amount of freezable liquid exists. The tissue cells were, however, very liable to freeze extracellularly unless the grade of supercooling was considerably high. In a supercooled state, for instance, at a temperature of about five to ten degrees below the "freezing temperature", the cell freezing took the form of a typical "flashing", a darkening originated at one end of the cell and proceeded in a very rapid wave-like fashion to the other end usually in a fraction of a second. Having started from the inoculated point, such "flashing" in tissue cells occurred one by one and propagated all over the strip irregularly, then gradually in rapid succession. At the moment of "flashing", the cell appeared intensely opaque and dark, however, it soon became appreciably lighter. If the temperature at which cells were kept frozen was very low, the high opacity of the cell continued for a while (Pl. XV, Fig. 67). At least for an hour after the "flashing", for example, the cell interior of onion epidermis was quite dark at a temperature lower than −15°C, though various pattern changes had already occurred in the frozen cell.

When somewhat dessicated tissue or the tissue of *hardy* cells was employed, the freezing rate in epidermal cells was retarded and the resultant darkening of the frozen cell was of course lowered. Since there was usually found a greater or less amount of extracellular ice formation in the tissue, the cells under observation must be seeded with ice on the cell surface, although the exact process of inoculation could not be ascertained. As soon as the ice entered at one end of the cell, it grew rapidly with fine dendritic branches, throughout the whole protoplast. The starting of such ice branches, sometimes, took place along one side or on both sides of the cell. But the freezing never occurred at several points in a cell at the same time.

The cell sap was concentrated irregularly between the ice branches and then it divided into a number of droplets. Simultaneously with the congelation of sap vacuole, the cytoplasmic layer was deformed to a net work of usually angular mesh and the nucleus became very granular (Pl. XV, Fig. 68 & Pl. XVI, Fig. 72).

After having frozen most of the epidermal cells behaved in exactly the same way as the frozen cells of *Tradescantia*, so far as the internal changes of the frozen cell were concerned*. However, if the intracellular freezing occurred after a considerable amount of ice had been formed on the cell surface, the numerous irregular droplets of vacuole sap soon became connected with each other; eventually they presented various figures of a coarsely meshed net work, which sometimes exhibited a reticular conformation with many ice globules (Pl. XV, Fig. 71). Also in the case

* Regarding onion epidermis a very detailed description on the pattern changes of frozen cell is to be found in LUYET and GIBBS (*40*).

of plasmolysed cells, the intracellular ice formation occasionally brought about the same result. This seems to be a common phenomenon in the cells dehydrated prior to freezing.

Observation in parenchymatous cells

The intracellular freezing process of living parenchymatous cells is distinctly separable into two types, viz., *flash* and *non-flash* type. The former is a sudden freezing characterized by an instantaneous darkening of the whole cell, whereas the latter is a slow freezing with clearly visible ice growing in the cell. When inoculated at a definite temperature*, a high rate of cooling often causes the freezing of the former type. The difference of these freezing processes is, however, not brought about only by the cooling rate of the material. Upon inoculation, the cells of tomato, melon, cucumber and various other fruits, of onion scale leaf and of fresh bean pod and so on usually froze in *flash* type manner, even if the tissue was cooled slowly**. While at a high cooling rate, say 4°C per minute, the leaf of cabbage, the root of turnip or carrot and the petiole of sugar beet or spinach and so on, usually in summer season, exhibited intracellular freezing of *non-flash* type.

When cells froze in *non-flash* manner, the freezing usually propagated continuously yet with a short time interval between their respective congelation. In such a case, the intracellular freezing started not at a certain point but usually from an area or from one side of the cell surface adjacent to the already frozen cell. From this area, as a rule, several knife-like smooth ice crystals grew in parallel with each other towards the opposite side of the cell, in a period from approximately one-half second to a few seconds. The cell contents were entirely concentrated between these ice crystals as a small number of irregular masses. However, in the cells frozen in this way, the nucleus especially that of *hardy* cells, sometimes remained for a while without any remarkable deformation. As soon as the cell was filled with ice crystals, they rapidly fused with each other resulting in a coalescence of the embedded unfreezable contents into a single mass or a few masses connected with each other. Therefore even just after the freezing, the cells frozen in this way were quite clear and light, contrary to the remarkable darkness in flashed cells (see Pl. IV.).

On the other hand, the process of the "flashing" in parenchymatous cells is essentially similar to that in *Tradescantia* cells. However, according to their morphological and physiological characters, some modifications in freezing process were observed especially in large spheroidal cells of *non-hardy* plant. A typical process

* In the most usual cases tissue section was inoculated at −4°C, however, by the heat of crystallization of ice, the tissue used to be temporarily warmed to approach the freezing point of cell sap.

** 0.5 ~ 1°C per minute near the temperature of inoculation.

of such "flashing" observed generally in the fruit of tomato, cucumber or melon was as follows: after the extracellular ice formation initiated by the inoculation had almost ceased, there was usually a certain time interval*, depending upon the cooling rate, till the "flashing" of cell began. After this interval the "flashing" took place irregularly to one cell after another across the field. At a low temperature, say about five degrees lower than the freezing point of cell sap, the freezing process in a cell was observed only as a wave of opacity that started from the periphery and "faded out" at the center of the cell. Such opacity was caused by the formation of a large number of minute particles, constituted mainly of condensed sap droplets and embedded in an apparently homogeneous ice mass (Pl. XXVIII, Fig. 124). These particles were nearly regular sphere, having sometimes more or less angular contour especially just after the "flashing" (see Pl. XXV, Figs. 109–111). The contour-line of the particles, however, soon became smooth. The diameter of most of the particles was usually smaller than a few micra, though the higher the temperature at which the "flashing" occured, the larger the particle size and also the less the number of particles (see Pl. XXII, XXV & XXVIII). In the cells frozen in this way, even if the cell was kept at a constant temperature, these particles in it gradually fused with each other into larger droplets. At the same time, not a few particles of small size gradually disappeared in the surrounding ice mass. In the case of vitally stained cells, a rapid fading of the colouring of the cell interior was observed on several occasions within a few or some ten minutes after the "flashing" at temperatures ranging −3°C to −8°C.

Closer observations of cell "flashing" at a higher temperature** than in the case just described revealed that the particles of condensed sap droplets, arranged in many lines often parallel with each other (Pl. XXVII Fig. 122), appearred first around the surface of the sap vacuole and then were formed inwards. Consequently the centre of the cell was the last part to be darkened. When the particle formation was completed, the space not occupied by these particles in the vacuole had already been filled with clear ice mass. Simultaneously with the freezing of the vacuole, the cytoplasmic layer froze in the same manner as in the "flashing" of *Tradescantia* cells. In the surface layer of congealed cells, there were found usually many threads or a irregular net work of coagulated protoplasm (Pl. XXV, Fig. 111) which sometimes radiated from the deformed nucleus.

By means of intracellular inoculation with ice, the process of the particle formation could be observed in detail***. Freezing started radially form the inoculated point just like a gentle jet of many fine crystals. They grew around the inner sied of the tonoplast until the whole cell was filled with fine dentritic ice crystals

* This also appears in the freezing curve shown in Fig. 2.

** For instance, −1.5°C and −1°C for the cells of tomato and watermelon respectively.

*** In the large cells of white melon, which have a concentration of incipient plasmolysis of 0.2 M sucrose, the freezing process finished approximately one-half second to three seconds at −1°C to −0.5°C.

which later by rapid fusion became a homogeneous ice mass (Pl. XXI, Fig. 92). Between the ice crystals the cell sap was condensed as many vein-like branches of somewhat dark colour, and then was torn into a large number of minute particles which soon after became spherical, depending upon the temperature at that time.

In a previous paper (*10*), in which the freezing process of this type was first described, it was suggested that the nature of the particles of small sizes, is that of a solid solution and that their surfaces are covered with a thin layer of concentrated colloids. These assumptions were mainly based on the following reasons: there were found neither Brownian movement of granules nor any gas bubbles in the particle. In addition, the particles of small size, occasionally looked as bright as the surrounding ice mass when placed between the nicols of a polarisation microscope. On thawing, especially some of the particles of small size faded away into the surrounding ice mass at a distinctly higher temperature than the melting point of the latter, leaving no liquid but sometimes a very small amount of coagulated substance. However, many later observations on cells fortunately frozen at a high temperature revealed that there were very fine filamentous connections between the particles in the ice mass, as shown in Plate XXVII, Fig. 121, and that the small particles could fuse with each other through this connection as if some particles faded away at their original loci leaving some solid fractions which had been contained in them. Besides, in such a small particle of condensed sap, it was found that neither the Brownian movement of granules nor the gas bubble of distinctly appreciable size appeared until the coalescing particle had reached a certain size. Moreover, a very small liquid droplet tightly packed with ice in every surrounding usually looked rather transparent even under polarized light unless the thickness of the liquid reached a certain degree.

In parallel with the vacuole freezing mentioned above, sometimes formation of ice with dendritic branches was also observed clearly in cytoplasmic layer (Pl. XXVII, Fig.. 119). In most cases, the cytoplasm was rapidly concentrated between ice branches which perhaps developed throughout the sap vacuole and the cytoplasmic layer, nearly in the same manner as in the vacuole sap, although the globules segregated from the concentrated cytoplasm were usually larger than the particles in the frozen vacuole. Consequently, there were found in the surface layer of a frozen cell many globules or branches of smooth contour including coagulated cytoplasm (Pl. XXII, Fig. 98), while the nucleus occasionally remained without any remarkable deformation, yet, showing a somewhat concentrated form within several minutes after "flashing". Such a concentration of the nucleus was probably because of the dehydration usually caused by the growing of underlying ice mass, and sometimes also by the further ice formation on the outside of the cell. Fig. 115 in Plate XXVI shows a distinct extracellular ice formation just above the nucleus. A similar phenomenon had been already observed above the chloroplast band in *Spirogyra* cell also (*46*). Such outside ice formations apparently result from the fact that

these cell constituents are more hard to freeze than the other parts of the cytoplasm.

Sometimes, in the course of the "flashing" of a cell, the freezing of the main part of the sap vacuole prior to that of the cytoplasmic layer was observed. Immediately after the "flashing", the appearance of the nucleus remained entirely unchanged on the instantly darkened sap vacuole (Pl. XXIV, Fig.. 107). In the cells frozen in this way, if thawed soon after freezing, most of the cytoplasm as well as the nucleus was sometimes found to be rather unchanged in appearance, and no apparent formation of net work such as usually resulted from the freezing of the former, was observed. However, both of them were by no means alive and their appearance was no longer turgid. This is shown in Plate XXIV, Fig. 108.

As is well known, the *hardy* cells are very apt to freeze extracellularly, in other words, these cells do not easily freeze intracellularly. Nevertheless, at a sufficient rate of cooling, most of these cells, even of woody plants, also could be frozen in *flash*-type manner. The process of their freezing did not differ significantly from that of epidermal cells. As a rule, the droplets formed in these flashed cells were irregular in size, and when the spherical droplets were formed, as was often the case in *hardy* cells of herbaceous plants, they were always larger in size and far less numerous than in the cases of "flashing" mentioned before. In the *hardy* cells of the petiole of table beet frozen rapidly at about −9°C, for instance, the spherical droplets embedded in ice mass usually have diameters ranging five to ten micra (Pl. IV, Fig. 16).

When the cell interior was inoculated with ice* at a temperature near the freezing point of the cell sap, the process of intracellular freezing was very interesting. Although the rate of freezing varied, naturally, depending upon the temperature, the freezing in such a cell was usually far slower than all cases noted hitherto in this paper. For one example, a large cylindrical cell of table beet having a diameter and length of about 150×350 micra froze approximately in 10 seconds and 2.5 seconds at −1.5°C and −2.3°C, respectively. From the inoculated point the ice grew radially with a divided, but usually not dendritic, crystal front around the inner side of the tonoplast. In such a growing of ice crystal, the lower the temperature to wich the cell was exposed, the larger the number and the smaller the diameter of the ice branches became. By contrast a higher temperature favored the decreasing of the ramification of ice front and the formation of broad leaf-like, or rarely thin disk-like crystals. Eventually the cell was filled with transparent ice masses irregular in form, and the cell content was concentrated between them. Besides these processes, a strikingly interesting growing of ice in the cell occasionally was observed, although this was also sometimes found in the cells inoculated extracellularly. In such a case, starting from the point of inoculation, the ice with some smooth crystal fronts grew

* Since the cytoplasmic layer in most cells used was very thin, it was hardly possible to manipulate the ice tipped pipette without penetrating the whole layer. Most of the freezing process observed, therefore, was originated by an intravacuolar inoculation.

radially but very irregularly both in rate and in shape, as if it were developing into a delicate structure just like a very dilute jelly. Regarding the details of such an ice growing in some hydrophilic colloid systems including cell constituents, some observations will be reported in another paper.

So far as the present observations were concerned, various parenchymatous cells examined were founed to freeze usually in either one of the freezing processes described above or in some intermediate manner. However, as an exception, when cooled very rapidly, a different kind of cell freezing frequently occurred in the tissue of woody plants, notably in those which had been in a very *hardy* condition. Also in this case, the darkening started from one end of a cell and proceeded as a wave to the other end, but the freezing was confined to the cytoplasmic layer. Therefore, around the unfrozen vacuole a foamy network of coagulated protoplasm was always found soon after the "flashing" (Pl. XXXII, Fig. 144). After thawing, the tonoplast of the cell frozen in this way, as already pointed out by SIMINOVITCH and SCHARTH (*56*), occasionally kept "semi-permeability" to some extent, at least for a short period of time.

Observations in squeezed cell sap

Using the squeezed sap of parenchyma as well as of epidermis, the freezing process of cell sap mounted in silicone oil was observed on many plants of various degrees of *hardiness*. Very thin drops of cell sap, measuring about 30 to 100 micra in thickness, of the various kinds of material froze almost in the same manner.

On the cell sap of tomato fruit, for instance, the process observed at −7°C was as follows; ice formed at the inoculated point rapidly grew in radial directions till the whole drop was filled with dendritic ice crystals; between them the cell sap was condensed. The concentrated sap became at first many rods of dark colour, then these were divided into a large number of minute droplets. Soon after that division, the outline of these droplets turned smooth. Consequently, the frozen mass became closely studded everywhere by minute spherical particles while the space between them was packed with a homogeneous ice mass which had been formed by rapid fusion of the dendritic crystals. From the instant of freezing, the gradual lightening in darkness of the frozen mass was clearly observed. This phenomenon occurs because of the decreasing in number of the droplets due to their coalescence through fine, often invisible, connections. The process of the formation of these spherical droplets as well as of the fused homogeneous ice mass is well represented in Plate XXX. If the sap was subjected to freezing at a temperature close to the freezing point, several smooth ice lobes appeared in the sap. With broad fern-like ice fronts they gradually grew, concentrating the sap between them, and fused with each other rather rapidly. Consequently, some of the condensed sap was trapped in the ice mass as vein-like branches of dark colour. However, contrary to the case of rapid freezing, the branches of trapped sap were rarely divided even after the fusion of

surrounding ice crystals was completed.

VI. Freezing of Cytoplasm

In the egg of sea urchin the present writer has already described the process of the freezing of cytoplasm (*11*). However, attempts to observe in detail the freezing process of plant cytoplasm have not yet been successful. Using the plasmodium of a slime mould, *Physarum polycephalum**, the observation of the freezing of protoplasm was tried. Owing to the remarkable susceptibility of this material to cold, it was hardly possible to maintain the plasmodium in normal state at a subzero temperature without any pathological changes such as blistering and various other types of "syneresis" (see *53*). Even at a temperature above 0°C the plasmodium, at least a certain fraction of it, suffered fatal injury as fully described by GEHENIO and LUYET (*23*). Besides, as a result of cooling, the thin "plasmodial sheet", (*19*) of the plasmodium was usually changed into a thicker layer or sometimes a spherical mass in which the changes in structure were hardly visible.

The surface layer of the plasmodium had a little resistance to the invasion of freezing into the interior, though the degree of the resistance was far lower than in most cell organisms. Being surrounded with ice at −0.2°C, no freezing was observed in any part of the plasmodium**. When it was cooled down to a temperature lower than −0.3°C and placed in contact with ice, a darkening of plasmodium consequent upon freezing occurred at a spot adjacent to the outside ice crystal. From this spot the darkening gradually spread with irregularly curved margin till the whole organism became dark and granular, excepting some areas which soon after blackened more rapidly than the preceding darkening. Whether such peculiar area for freezing corresponded to the "plasma gel" or to the "plasma sol" was uncertain. When the plasmodium was thawed after a short frozen period, an entire collapse in structure always took place soon after thawing. If the frozen plasmodium was kept for a while at a temperature of about −1°C to −2°C, however, a setting of coarse meshed net work assuming a reticular conformation with many small ice globules was occasionally observed. This accords with the description by MOLISCH in a fresh water amoeda (*42*). As a result, this type of structure in coagulated protoplasm was rather stable after thawing.

At a high degree of supercooling, say −7°C, the plasmodium froze as fast as the "flashing" of the cells described above. In such a case, the freezing propagated as a dark wave which transversed throughout the whole organism. Just after the freezing it seemed to be quite dark and finely granular (Pl. XXXIII, Fig. 145). As

* The original culture was kindly furnished by Prof. Noburo KAMIYA of Osaka University, to whom the writer here expresses his thanks.

** According to GEHENIO and LUYET (*23*) the freezing point of the plasmodium of this species is −0.17°C. In the present observation, however, the plasmodium could be frozen only at temperatures below −0.3°C.

observed in the "flashing" of various cells, the fine ice crystals in frozen plasmodium coalesced with one another as time went on. However, the ice fraction was always smaller than the unfrozen one or the coagulated protoplasm, and the rate of pattern change was very low, excepting in the surface layer of the frozen plasmodium, where a separation of ice crystals from the coagulum resulted in a rather rapid formation of irregular protrusions of the coagulum into the surrounding ice mass (Pl. XXXIV, Fig. 151). In the plasmodium frozen rapidly, usually a stable structure of coagulated protoplasm was formed which appeared to be coarsely granular after thawing, and often remained unchanged for a few days even at room temperature (Pl. XXXIII, Fig. 147).

In the young cells of the staminal hair of *Tradescantia*, or in avacuolated cortical cells of some woody plants, the freezing process of cytoplasm was nearly the same as observed in plasmodium. Being frozen rapidly, the young cells of *Tradescantia* without any distinct sap vacuoles instantly became dark and coarsely granular as shown in Plate XII, but not opaque.

In these frozen cells, an onset of network formation was usually observed within about ten minutes, although most of such a pattern hardly remained after thawing. If the cell was inoculated at a high temperature, for instance at −1.5°C to −3°C, the ice with divided branches grew irregularly over all the width of the cylindrical cell until it reached the opposite cell end. The nucleus usually froze, assuming a densely granular appearance, slightly later than the freezing of the surrounding cytoplasm. However in very young cells the ice once had appeared inside the "nuclear membrane", as a result, grew faster in the nucleus than in cytoplasm. Also in the sap vacuole of somewhat older cells, the rate of ice crystallization was far higher than in cytoplasm, although the vacuole was often the last constituent of the cell to freeze. From these observations just mentioned, it is reasonable to suppose that the surface layer of the nucleus as well as the tonoplast could play a role as a barrier against the freezing of their interior, and that in the cytoplasm there existed a fine architecture in which ice crystals could not easily grow.

VII. Intacellular Pattern Changes during the Freezing

As is well known, the most fundamental change occurring upon intracellular freezing is the phase separation, that is, the separation of the cell content into ice and into an unfreezable fraction. Most of such a separation, at least in appearance, is usually finished within some fraction of a second. Nevertheless, for a considerable period after the freezing, the frozen cell was by no means in a stable state. In such a cell, as has been noted many times, coalescence both in the ice crystals and in the concentrated cellular contents always took place, even if the cell was kept at a temperature as low as −20°C (see Pl. XXIX). Besides, the temperature gradient in the material, which could be hardly obviated in the apparatus used, must facilitate the pattern change. Also in frozen cell, the unfreezable fraction tended to concen-

trate to the warmer region in consequence of the migration of ice to the colder one. In addition, the rate of transformation in the frozen mass as well as the rate of freezing was distinctly higher in sap vacuoles than in the cytoplasmic layer. Consequently at the centre of a cell, a large mass of concentrated sap as well as of ice was very easy to develop as shown in Pl. IX, Fig. 39. On the other hand, a water imbibition of the cell prior to the freezing remarkably accelerates the rate of such transformation. Cortical cells of *hardy* table beet, for example, usually freeze in *flash*-type manner after an immersion in tap water. Furthermore, in the cells frozen in this way a very rapid coalescence of numerous sap droplets formed at the time of "flashing" always takes place, resulting in a concentration of the sap as a whole mass or as a few masses even within a few minutes after "flashing" (Pl. III, Fig 11). However, these transformations in frozen cell, which are most active immediately after freeezing, are of course depressed as the temperature is lowered. It, therefore, must be noted that various conditions as mentioned above might be the important factors in determining the type of pattern which results from a cytological fixation after freezing.

The ice portion in a well vacuolated cell of a herbaceous plant was usually far larger than the other phase or the unfrozen fraction and, as a rule, the former was a continuous phase unless the frozen cell was warmed up to a temperature near the freezing point of the cell sap. At any definite temperatur the proportion of these two phases should be, however, principally determined by the amount of easily freezable water in the system. For examiple, when the cells with a considerably high osmotic value, such as previously dehydrated ones or some of the very *hardy* cells, were subjected to freezing, they often produced, soon after freezing, a mesh-work of the unfrozen cell contents, enclosing the ice in its meshes (Pl. XV, Fig. 71).

In frozen cytoplasm, on the other hand, a net-work of the unfreezable fraction was usually formed after the phase separation due to the freezing, whether or not the amount of this fraction was larger than that of ice. In young cells, at least in certain kinds of plants, a structure of denatured protoplasm was very liable to be destroyed and meanwhile an entire separation of protoplasm into both phases took place, in other words, the cell contents other than ice was easily concentrated as a mass. On the other hand, in the plasmodium of slime mould, though its "freezing temperature" was usually higher than that of any plant cell examined, the resulting structure of frozen protoplasm was very stable. These difference in the rate of transformation of frozen figure may probably be due to the difference in amount of easily freezable water in the architecture of denatured protoplasm.

VIII Thawing and Refreezing of the Intracellularly Frozen Cells

Upon thawing, at least at the beginning of the process, the droplets embedded in the frozen mass behaved likewise as in a frozen cell left to itself at a constant

low temperature. The droplets or the particles of concentrated cell contents united with larger ones through the fine connections between them which were often hardly visible under microscope. When these growing droplets got to a certain size, they occasionally elongated in parallel with each other, perhaps along the contact planes of the ice crystals and coalesced upon coming into contact with other droplets (see Pl. XXII). If the rate of warming was slow, such a coalesced drop was very apt to become a sphere. In these drops or globules, the bubbles of dissolved gases were occasionally clearly visible (Pl. XXV, Fig. 112). While these globules gradually grew larger, the surrounding ice mass began to melt evidently. Since there were usually some concentrated substances in the very narrow spaces between the ice masses, the former, when warmed, gradually enlarged their dimensions by the water supply from the melting ice and occasionally also by coalescence with the globules mentioned above. Consequently the frozen mass, with which the cell had been filled, was divided into several masses (Pl. XVI). The liquefaction of these masses, still imbedding some of the drops of condensed sap, usually started from their periphery and proceeded inwards (Pl. XVII). When the edges of these melting masses reached the embedded globules, the latter rapidly diffused into the surrounding melted ice. Simultaneously, every one of the gas bubbles in these globules, instantly disappeared. Thus the melted fluid finally filled the entire cell. As the thawing proceeds, the coagulated protoplasm gradually sank to the bottom of the cell. If the cell was thawed after a short period of freezing, the layer of coagulated cytoplasm was, however, frequently found to lie approximately in the position that it had held in the living state. In the cell frozen very rapidly, such a layer was observed immediately after thawing as a fine network and the nucleus as a densely contracted mesh-work (Pl. XXVI, Fig. 118). In a slowly frozen cell, though it was occasionally deformed, the structure of the nucleus sometimes appeared to be rather intact, but with various abnormalities such as a dark colour and a more sharply defined outline. In the latter case, such conditions of the nuclei in thawed cells may have been brought about by the dehydration or sometimes by the pressure between ice masses at the time of cell freezing, but not by the freezing of the nuclei themselves.

When a cell with coloured vacuole was thawed after having been frozen for only a short time, the colouring of the cell hardly suffered any change, at least during the melting of the ice, but soon it began to fade as the pigment diffused out from the cell into the surrounding medium, leaving the cell wall with the characteristic dead cell wall colouring. However, in the cell with a cuticular outside layer not easily permeable to water, as seen in the grown staminal hair of *Tradescantia*, the colour of the melted sap remained unchanged for often some ten hours or more at room temperature.

After thawing, the appearance of cell wall clearly shows the influence of the mode of freezing. As a rule, rapid freezing induced no changes in the outline of cell wall, while a slow freezing always caused some appreciable deformation in it.

When thawed after a "flashing", for example, the cell wall usually retained its normal appearance. A closer observation, however, revealed that the smooth turgid surface of the normal cell wall as observed before freezing had become slightly flaccid (Pl. XXI). A cell frozen in *non-flash* type manner always possesed a more distorted cell wall after thawing. This is apparently because of a violent migration of water through the cell wall which always was caused by a slow freezing.

If the thawed cell was again subjected to freezing, the mode of freezing can be controlled principally by the cooling rate. With rapid cooling, even if the membrane-like structure of coagulated cytoplasm was entirely absent around the cell wall, the freezing process observed was almost similar to the initial "flashing", indicating that the cell contents had been sufficiently supercooled before the wall was penetrated by any ice crystal. At least in some tissues, such as the staminal hair of *Tradescantia* or the outer epidermis of a scale leaf of onion, the cells could flash repeatedly ten times or more. However, the darkening of frozen cells became faint with the repeated "flashing".

On the other hand, the cells refrozen at a moderate cooling rate behaved in the same manner as those frozen in *non-flash* type manner, although it was difficult to observe the time interval between the freezing of each individual. If the cooling rate was sufficiently slow, however, the thawed cell, when inoculated with ice onto the cell surface, was still resistant to intracellular freezing. Consequently these cells were very apt to freeze extracellularly (Pl. XV, Fig. 69). The ice crystal formed on the surface of such cells often grew very large and, as the cooling proceeded, the rate of its growing sometimes became so high that a gentle jet of ice crystals was observed on the outside cell surface at a few loci where the extracellular ice had attached. After re-thawing, the cell wall was remarkably destroyed at the area where the extraordinary rapid growing of ice had occurred. Besides, there were many indications to suggest that the destruction of the cell wall due to the freezing was mainly caused by the extraordinarily rapid migration of water, but not by the expansion of the cell contents at the time of freezing.

IX. General Discussion

Factors directly affecting the types of freezing

As was fully reviewed by LEVITT (*35*, *36*), there have been numerous observations regarding the freezing pattern in plant cells, in which, however, many contradictions are included; some of them may have been described on the basis of erroneous interpretations. The present observations have shown that the various types of cell freezing can be artificially produced even in cells from the same tissue. It is, therefore, necessary to consider the factors directly influencing the types of cell freezing, although some of them have been already noted in the foregoing sections.

Regarding the position where the ice formation takes place, there are two funda-

mentally different types of cell freezing, namely, extracellular and intracellular freezing. Being different in degree the former type of freezing occurs in every case of cell freezing. Nevertheless, the cell can freeze without any appreciable external ice formation in exseptional cases in which the cell is cooled enough to be frozen spontaneously or is cooled at an extraordinarily high cooling rate. Therefore the intracellular freezing, as a rule, occurs subsequent to the extracellular freezing.

The intracellular freezing has often been mentioned to occur only when a cell is subjected to freezing *in vitro*. As suggested by SIMINOVITCH and SCARTH (*56*), however, even in the intact plants this type of freezing is occasionally found in nature as well as in laboratory.

The difference between these two types of freezing is very simple. In extracellular freezing the penetration of ice crystal into the cell interior is certainly prevented at the cell surface, but in intracellular freezing it is not, provided that the ice formation inside the cell only occurs when inoculated from the outside. So-called "frost hardy" cells are characteristically resistant to such a penetration of ice crystals. These cells, however, readily freeze intracellularly under the condition in which a sufficient super-cooling of the cell interior is brought about in the presence of ice on the cell surface. Good examples of such conditions are the freezing under a considerably rapid cooling and the onset of extracellular ice formation after a strong supercooling of the cell. The small size of the material treated occasionally brings the same results as it is at a pretty rapid cooling.

It has been widely held in old literature that the intracellular freezing takes place only as a result of rapid cooling (*42*, *44*, *51*). However, with an intimate contact of the cell surface with ice, *unhardy* cells of certain kinds are very liable to freeze intracellularly even if cooled slowly. Nevertheless, when inoculated at a temperature slightly lower than their "freezing temperatures", even these tender cells freeze extracellularly only. A large volume of the material as well as a great amount of the surrounding sap occasionally causes the same result owing to the large heat capacity and the considerable amount of heat liberated at the freezing.

It is a well-known fact in plant tissues that an infiltration with water remarkably decreases the frost resistance, while an infiltration with some hypertonic solutions increases the resistance (see *35*. pp. 163–164). An explanation for the former fact may be that the cell or at least some parts of it are crushed by the mechanical pressure due to the expansion in volume at the moment of freezing of the water filled in the intercellular space (*30*, *41*). This is, however, by no means the sole factor for a low resistance of infiltrated tissue, since the freezing process in ordinary plant cells must be strongly influenced by the water imbibition as well as by the dehydration prior to the freezing. Results supporting this view were also obtained from a freezing experiment on pollen grain coated with oil (*2*). The present writer's work on some *frost-hardy* herbaceous plants such as table beet, cabbage and turnip

in overwintering stage further proves the validity of this explanation (*9*).

In these plants most cortical tissues of exposed parts above the ground are usually resistant to freezing at −10°C for a full day if prevented from an excessive supercooling at the initiation of the freezing. Using some slender cell chains isolated from the pith of petioles, the capacity of resistance to the penetration of ice crystal into the supercooled cell interior was determined under the microscope. It was found that these cells can withstand the transmission of feezing even when inoculated directly on their surface at a temperature several degrees lower than the freezing point of their cell sap (see Pl. XX). These cells, however, when they have been immersed in a hypotonic solution prior to the freezing, invariably decrease such frost preventing capacity. An immersion of the cell in the sugar solutions of a series of graded concentrations revealed that a concentration of the medium lower than some two-thirds of the isotonic concentration of the original cell sap was evidently effective to cause an appreciable depression in that capacity. It must be noted in such a case that these cells generally freeze in *flash* type but not in *non-flash* type manner. At the same time, the length of immersion in the sugar solutions, of course within limits, seemed to be of little importance, since the immerison in water for only half a minute was effective enough to bring about the intracellular freezing.

It is therefore reasonable to suppose that at least in *hardy* cells a certain volume of water imbibition surely decreases the frost preventing capacity of the cell. In addition, as already demonstrated in a previous paper (*4*), a well-known depression of frost resistance of the tissue of *dehardened* plant can also be explained, though within a limit of cooling rate, by a remarkably increased proportion of the cells undergoing intracellular freezing. In contrast to this, an artificial dehydration of a given cell either with or without plasmolysis increases such resistance capacity.

As a modification of extracellular freezing under certain conditions, there is a phenomenon of striking interest found in various plant cells especially of epidermal origin, that is "frost plasmolysis". Regarding the cause of such a type of ice formation in plant cells, the freezing of a certain amount of water existing already between the protoplast and cell wall, has been noted by some authors (*46*). Although some reports seem to support the concept (*17*, *42*), so far as the present observations are concerned, scarcely any fact has been found to favor this view. As pointed out by LEVITT (see *35*, p. 22), why such water should be introduced between the cell wall and protoplasmic membrane is still a mystery, at least in a healthy cell. As noted in a previous paper (*6*), after a long period of supercooling, say a full day, a partial contraction of denatured protoplast which was resulted from its fatal coagulation, frequently occurred in the hair cells of *Tradescantia* stamen. However, the seeding of ice onto such cells always failed to intiate the "frost plasmolysis", but an intracellular freezing occurred instead. Frost plasmolysis is invariably caused by the growing of ice crystal formed between cell wall and protoplast withdrawing water from the latter. In the cells frozen in this way the fine crystal front of the outside ice should certainly penetrate the cell wall but not the plasmic membrane. Nearly at the "freezing temperature" of various plant cells,

this occasionally takes place under the condition of a very slow cooling after an inoculation with ice. In *hardy* cells a well developed resistance of the protoplasmic surface against freezing is certainly favourable to this modification of extracellular freezing. In fact, the "frost plasmolysis" usually gives rise to no harmful effects in most of such *hardy* cells, those of epidermis of Welsh onion and of table beet, particularly in a cold season. Even in the stamen cells of the summer flower of *Tradescantia*, "frost plasmolysis" at least within a few hours, never prevented after thawing a complete recovery of their normal state with active streaming of protoplasm.

In general, the processes of intracellular freezing can be classified into two types, namely, *flash* and *non-flash* type, although in epidermis the latter type is not so conspicuous as in parenchyma (*7*). The former certainly indicates a sufficiently supercooled state in the cell at the moment of freezing. The latter suggests that the supercooled cell interior has been set free from such unstable state at a temperature near the freezing point of the cell sap. It follows therefore that whether the cell freezes in the manner of *flash* or *non-flash* type is mainly dependent upon the frost preventing properties of the cell surface under the given condition.

It is very interesting to note that, at a certain degree of supercooling, most of the cells of some herbaceous plants, on which the *hardening* is able to be very effective, undergo *non-flash* type freezing, while the cells which freeze in *flash* type manner are usually of *non-* or *low-hardy* plants. In the former case, the tissue is easy to freeze extracellularly unless the cooling is rapid. Nevertheless, under a slightly supercooled condition, cells of most of the *non-hardy* fruits, such as tomato, melon and cucumber, freeze in *non-flash* type usually with or sometimes without intracellular inoculation. In these cells, however, the ice grows radially from a point, but not from a wide area as is observed in the usual case of *non-flash* type freezing. On the other hand, various *hardy* cells of herbaceous plants occasionally freeze in *flash* type, if they have been in a sufficiently supercooled state just at the moment of freezing.

As noted in a previous paper (*6*), the patterns of frozen cell which resulted from the "flashing" can be roughly divided into two subtypes, i. e. "sap drop" and "spherical particle type"*, although some intermediate types can frequently be found, too. In the flashed cells of the former type, one can find numerous droplets of unfrozen fraction embedded in ice mass in irregular form. While in the patterns of cells frozen in the latter type, a number of minute spherical particles are usually observed instead of the irregular droplets. Good examples of both types are shown in Plates VII and XXV. In *non-hardy* herbaceous plants, parenchymatous cells generally flash in "spherical particle type", whereas epidermal cells do in "sap drop type" pattern. *Hardy* cells also, both epidermal and parenchymatous, usually flash in the latter type. Although it is doubtful whether a fundamental difference exists

* This type was referred to as "ice particle type" in the previous reports (*8*, *10*).

between these two types or not, the cell which indicates the frozen pattern of each type seems to differ from others in certain characters. Suitable factors to cause the freezing of "spherical particle type" seem to be a large cell volume, in comparison with its surface area, most space of which is occupied by sap vacuole, and a low concentration of vacuole sap, both in hydrophilic colloids and in crystalloids. The first factor leads to a large amount of heat liberation at the "flashing" compared with the case of flattened or thin cells. It is reasonable to suppose that such a heat libration may contribute to the "spherical particle" formation, for in a frozen cell the sap drops having irregular form just after freezing occasionally become spherical when warmed slightly. The latter factor is probably one of the main elements in the problem. Most *hardy* cells can be easily caused to froze in *flash* type manner by a water imbibition prior to the freezing. The resulting type of frozen cell is usually "sap drop" one, yet is sometimes a "spherical particle" one, especially in the cells of relatively low *hardiness*. In most large parenchymatous cells of tender summer fruits, which characteristically freeze in "spherical particle type", the plasmolysis prior to the freezing usually brings about the "sap drop type" flashing. On the other hand, as often noted in the foregoing sections, in a frozen mass constituted from ice and concentrated fluid drops the spherical form in either fraction seems probably to be more stable than the irregular one. Therefore, the quantity as well as the quality of the hydrophilic colloids contained in the cell sap, by which the surface of these embedded drops in frozen mass must be covered, may be an important factor to bring about such differences in the freezing-figure of fluid fraction.

In a frozen aqueous system the proportion of ice to the other fraction or concentrated contents depends mainly on the amount of easily freezable water at any given temperature of the system. This is the reason for the occurrence of the relatively small smount of ice crystal in the frozen plasmodium of slime mould compared to its high "freezing temperature". Under a given condition, if the amount of such freezable water in the cell is small, many ice globules embedded in fluid fraction occasionaly appear after freezing, as was illustrated by BUGAEVSKY (*17*). From similar figures in some frozen systems MOLISCH appeared to have supposed a simultaneous formation of not a few crystallization centres (*42*). However, according to the results observed in sea urchin egg (*11*), it is almost certain that such ice globules are resulted from the division of ice branches which have usually grown from a single inoculated point.

There have been some discussions on the order of propagation of freezing in cell parts (*57*). It is safely said, however, that when an ice formation has occurred in the protoplasm, whether the congelation of the cytoplasm is completed more rapidly than that of the vacuole is dependent merely upon how readily the ice crystal may penetrate through the tonoplast into the vacuole. The reason is that the ice grows usually far faster in the vacuole sap than in the protoplasm. In most of

the *unhardy* cells the ice seems to develop in the vacuole as soon as the intracellular freezing occurs. Under a suitable condition, the ice occasionally grows at first in the cytoplasm and then penetrates through the tonoplast even in the cell of relatively low hardiness. In very *hardy* cells, the restriction of ice formation to the cytoplasm is certainly explained by the remarkable frost preventing property of the tonoplast.

Some causes of death by freezing

As is well known, frost killing in a number of cells does not always results in fatal injury to the whole tissue affected, in which a certain fraction is occupied by the killed cells. Since the death of the whole tissue is very occasionally brought about by a post-thawing injury (*24*), the process described in this section will be restricted to the cases directly accompanied by the death of the cell itself, during and soon after the freezing.

Intracellular freezing almost invariably causes death. Some reports presenting evidence against this concept are conceivably based on erroneous interpretations. Assuming from the description the harmless ice formation inside the cell observed by SCHANDER and SHAFFNIT (*51*) appears to be a "frost plasmolysis" mentioned above. The maintenance of some vital characters in intracellularly frozen cell has been recorded many times (*20, 40*). The arguments presented seem to be based on the fact that the thawed protoplast or its fraction can yet expand in a surrounding hypotonic medium. This, however, can be explained by a faster inflow of water than the diffusing out of solute from the remains of the protoplast. In fact, upon warming of flashed cells, in which the formation of numerous fine ice crystals has occurred throughout the protoplasm and vacuole, the globules of condensed sap covered with coagulated protoplasm usually expand just after the ice has completely disappeared in the cell (see Pl. XXIII, Fig. 104).

Yet there remains a very rare case in which a harmless intracellular freezing is brought about nearly at the "freezing temperature" of the cell, provided that the ice is inoculated effectively only in the sap vacuole without any ice formation in the cytoplasm. This is possible at least theoretically, since ice seeding in the vacuole is usually easier than in the latter. Judging from an experimental result cited by LUYET and GIBBS, CHAMBERS's skilful technique seems to have enabled a succesful performance of such a difficult procedure.* So far as the present writer's experiments are concerned, however, in a very slightly supercooled cell the ice buds which had developed from the ice tipped pipette were separated with great difficulty from the pipette, at least within the accuracy of the temperature controlling of the apparatus used. It was, consequently, almost impossible to pull the pipette out of the cell leaving any seeds of ice in it. Furthermore at a larger degree of supercooling, the intracellular ice seeding was not succesfull, unless the ice in the cytoplasmic

* Unpublished work cited by LUYET and GIBBS (*40*).

layer had reached a certain amount. Such an ice formation in the cytoplasm invariably gives rise to a fatal injury to the cell.

In most cases, death caused by an intracellular freezing is certainly a result of the freezing of protoplasm itself, although the dehydration or sometimes mechanical pressure due to the ice formation may play some part in the process. From the result obtained in the work with sea urchin egg, the process of the frost killing of cytoplasm is assumably classified into two modes (*14*). One, observed in most of the slowly frozen egg cells, is an intracellular formation of ice crystals of relatively large size by which the cytoplasm is injured mechanically in similar manner to that induced by a careless penetration with glass needle into the cell, namely, a cytolysis accompanied by a vacuolization in the cell. The other sort of frost killing is a characteristic process of injury in a rapid cell freezing or "flashing" by which numerous minute ice crystals are instantly formed at every locus among the elements of the fine architecture of the protoplasm. In this latter case, consequently, a strong dehydratien takes place at an extraordinary rapid rate throughout the whole protoplasm followed by a fatal coagulation of it. Therefore, the protoplasm killed in this way occasionally shows a net-work-like configuration in which the spaces having been filled with developing ice crystals to a certain size can be noted after thawing as a number of empty vacuoles.

Also in plant protoplasm these modes of frost killing seem likewise to occur. In fact, immediately after thawing, a layer exhibiting a fine net work of denatured cytoplasm and a densely perforated nucleus were were occasionally found in a flashed cell in the same positions as they were at the moment of "flashing" (see Pl. XXVI, Fig. 118). Besides, a foamy appearance of the cytoplasmic layer of flashed cells in woody plants also favours the view just mentioned. In slowly frozen cells, an entire collapse of the general configuration of the cytoplasmic layer usually occurs even in the thawing process. The vacuolization of the cytoplasm is, however, hardly observed in the case of slow freezing, probably disturbed by the rapid transformation of ice crystals in common plant cells in which the proportion of easily freezable water is usually far larger than in the sea urchin egg cell.

On frost killing caused by an extracellular freezing, whether the injury occurs during the freezing or the thawing has long been a subject of discussion (see *35*, pp. 29–43). However, it seems reasonable that the principal cause of the killing is the dehydation of protoplasm during the course of the freezing process, though whether or not the mechanism of the injury is entirely similar to that under nomal temperature conditions is still a matter of uncertainty.

In extracellular freezing, when dehydration exceeds a certain limit, the increased consistency of protoplasm becomes irreversible and must be regarded as a type of coagulation, with or without some immediate changes in its appearance (*48*). This interpretation is based mainly on a series of micrurgical experiments carried out at normal temperatures (*37*, *55*). Also in the present observation, the behavior of

some cell consituents under an extracellular freezing yields striking confirmation of this view.

Some of the processes observed are as follows: In most tender cells, there is sometimes found a remarkable change, visible from the outside, of the nucleus as well as the cytoplasmic layer which comes to have a granular appearance even within a few minutes under the freezing (Pl. XXVI, Fig. 116). In the frozen cell with coloured vacuole, a stronger staining of these protoplasmic constituents than before occasionally takes place prior to the change mentioned above. After thawing, a coagulated protoplast with an irregularly contracted outline is occasionally observed in these cells. This perhaps may be a true figure that has been erroneously noted as "frost plasmolysis" by some early authors (see *35*, p. 20). In less injured cells, there are hardly found any appreciable changes in the appearance of the cell parts at least for a short duration of frozen period. However, the cells in some cases exhibit various pathological changes such as gradual darkness, fine or coarse granulation, partial or entirely frothy appearance and occurrence of sharply defined outline. Besides, it is often the case in these cells that no changes are visible soon aften thawing, without complete loss of its "semipermeability". Such a cell is capable of plasmolysing at least immediately after thawing, but a following deplasmolysis even if it is considerably slow, usually leads the cell to a fatal rupture of the surface membrane of its protoplasts. If such cells were left to themselves without any treatment, they sooner or later, used to lose some of their contents which diffuse out into the surrounding medium. Sometimes a rupture of the ectoplast or the surface membrane of their protoplasts is clearly observed in the course of thawing.

For the observation of these phenomena the artificially isolated protoplast of watermelon was employed, owing to their large size and low *hardiness*. Although they were very easy to freeze intracellularly when in normal tissue section, when the isolated cells were subjected to freezing independently in oil hanging drop they occasionally underwent an extracellular freezing with accompanying remarkable contraction. Upon warming, they gradually expanded as the thawing proceeded, and when the surrounding ice had almost melted their protoplasmic surface frequently exhibited a finely granular or sometimes frothey appearance. Then, at an instant that sometimes arrived several minutes after the thawing, somewhat irregular and rather sudden partial expansions in the contour of the protoplast were observed, as if partial ruptures of ectoplast had occurred, resulting in the fatal collapse of the protoplast with or without any apparent bursting out of cell contents.

All of these items seem to support the theory that, in the protoplasm of a cell frozen extracellularly, the irreversible coagulation due to the freezing takes place during the freezing rather than during the thawing.

On the other hand, the ice pressure on the cell has probably been exaggerated as a cause of frost killing, although it must play some important part in such injury. SCARTH and LEVITT supposed that the frost injury caused by the ice pressurs may be lowered in the tissue of *hardy* plant, for, in these cells, the remarkable permeability to water probably favours an ice formation of small crystals (*50*). This statement, however, seems to be paradoxical, because in an extracellular freezing at a certain cooling rate, the higher rate of water supply from the cell interior usually results in a larger rate of ice crystallization and simultaneously larger heat

liberation at the interface between ice and cell surface. This certainly causes the formation of large size crystals. As is well shown in many photographs in the present paper, the size of ice crystals developed on *hardy* cells is usually far larger than that on *unhardy* ones, provided that the cell is kept healthy.

It must be noted that the isolated protoplasts or cells once deplasmolysed occasionally can stand against the outside ice formation at a certain temperature at which they usually would succumb to the freezing so far as they are left in normal tissue section. For instance, the parenchymatous cells of watermelon which are invariably killed by a feezing at −7°C* within five minutes, could occasionally remain alive after an extracellular freezing at −8°C for ten minutes, if they had been deplasmolysed prior to the freezing, and usually recovered the active streaming of protoplasm. It follows therefore that an ease in separation of protoplast from the cell wall may decrease the frost injury of the cell. The cause of death in such a injury is probably due to being apt to cause the intracellular freezing, for in the treated tissue the occurrence of "flashing" is always far less frequent than in untreated.

As is well known, the time factor in frost injury is also important. Although a certain duration of time is necessary to reduce the temperature of a freezing tissue, such a time factor must be mainly involved in the injury caused by an extracellular freezing, since at a certain cooling rate the intracellular freezing occurs only in the early steps of the freezing process. Being frozen extracellularly at an adequate depth of temperature, plant cells are usually killed after various lengths of time. Cells of very tender plants are killed after a fraction of an hour or even within a few minutes; those of *hardy* herbaceous plants are killed after several hours or a few days; the most *hardy* cells of woody plants sometimes can stand freezing for several months. In the case of relatively short duration of the frozen period, the cause of death might be conceivably attributable, as supposed by many workers, to the dehydration having attained a certain degree.

It is, however, very doubtful whether death after a long period of freezing is also brought about only by the same cause. SCHOLANDER and others recently determined the respiratory gas exchange at graded temperatures down to −20°C in frozen plants and in an insect (*52*). From the results obtained they supposed a potentiality for extremely long survival time even at moderately low temperatures in these organisms. However, there is no reason to believe that the same mode of metabolism which occurs at ordinary temperatures persists in these frozen states. In fact, being kept at a constant low temperature some organisms both frozen and supercooled do not survive so long as expected. This may be assumed due to their metabolic changes in such a cold condition. So far as a preliminary experiment on an over-wintering insect larva** is concerned, there remains some possibilities to

* The final temperature after an inoculation at −2°C.

** This larva can be frozen extracellularly for some 250 days at −20°C without any harmful effect on its further development.

suggest that its metabolism at a temperature below −10°C, with or without freezing, conceivably differs from that at temperatures above 0°C (*15*).

On the mechanisms for the prevention of the penetration of ice crystal into plant cell

On the mechanism of resistance to the actual freezing of plant cell, a number of theories have been expounded. At present, however, a few of them remain tenable as reasonable statements being based on knowledge of some physical properties of protoplasm. A series of works along this line carried on by SCARTH and his two colleagues, LEVITT and SIMINOVITCH, might be evaluated as one of the most convincing contributions (*37*, *48*, *50*, *55*, *56*). In the same period, KESSLER and RUHLAND came to an almost similar conclusion although they held an interpretation opposed to the Canadian worker's in respect to the plasmic viscosity or consistency of *hardy* cells (*32*). The mechanisms stated are that, in a process of *hardening* of plant cells an increase of protoplasmic hydration always causes an increase of plasmic permeability to water and also a decrease in both plasmic consistency and the amount of freezable water, and that all of these are essential factors for the resistance to the freezing both intracellular and extracellular. A large permeability to water is a most important factor to prevent the fatal intracellular freezing and a low consistency of protoplasm as well as a paucity of freezable water is very favourable to avoid the mechanical injury due to an extracellular freezing. In addition, well hydrated protoplasm might effectively decrease the harmful dehydration injury resulting from an outside ice formation. "As the frost increases", LEVITT states," the progressive concentration of the cell sap tends to keep its freezing point below the temperature of the moment as long as wall pressure persists" (see *35*, p. 182). Indeed, the extracellular freezing elevates the osmotic concentration of the cell contents by the extraction of water. However, some duration of time seems to be necessary to depress the freezing point of the inner sap as low as the temperature of the moment. For, by means of intracellular ice seeding, the cells can usually freeze intracellularly within a few minutes after the beginning of the outside ice formation. At a constant temperature of −7°C*, for example, well *hardened* parenchymatous cells of table beet reached a state of equilibrium after five minutes from the beginning of extracellular freezing. In other words more precisely, only after five minutes or more from the beginning of outside ice formation, the ice seeding into the protoplast always fails to form any appreciable amount of intracellular ice crystal at −7°C. It is therefore reasonable that a prevention of the transmission of freezing into the supercooled cell interior is prerequisite to bring about an extracellular freezing unless the cooling is sufficiently slow. It

* Accompanying a temporary elevation of the tissue temperature, (reached a maximum value of −4°C), just after the onset of ice formation due to the rapid crystallization of surrounding sap.

has been shown that the plasma membrane is a sufficient barrier for this purpose (*20*). Besides, from the known facts evidencing the resemblance in structures between the plasmic surface membrane, or ectoplast, and tonoplast (*49*), a similar property in respect to the freezing is also expected in these membranes. Indeed, in the present work, it was demonstrated that not only the ectoplast but also the tonoplast and even the nuclear surface had such frost preventing ability, too.

Regarding the mechanisms of such an important character of plasmic membrane, a fuller explanation may be made. Very recently LUSENA and COOK published a series of works in which they tried to determine the resistance of various membranes and jellies to the penetration of freezing (*38*, *39*). Unfortunately, their apparatus, a model cell assembly, appears by no means suitable for obtaining results upon which to generalize about living cells. They adopted the occurrence of a very short duration of supercooling, say a few minutes, of the solutions in the space separated by a membrane from ice crystal, as a criterion of the frost preventing capacity of the membrane. This may occasionally, if not always, lead to an erroneous interpretation, at least in a certain limit of temperature, because some duration of time is necessary for ice crystals to penetrate through a membrane even a very thin one. They used regenerated cellulose films of a thickness ranging 0.25 mm to 0.76 mm or sometimes used a gelatin membrane of 3 mm thick, while the protoplasmic layer, even a whole cytoplasmic layer, of a thickness in order of one micron is commonly found in plant cells which usually possess a frost preventing ability of a certain degree. In fact, they drew a contradictory conclusion that, in a membrane of low porosity, the permeability to ice crystal decreases when the rate of cooling is high. They supposed an occurrence of discontinuous crystallization of ice in biological materials, but their view point is perhaps impossible at least under natural temperature conditions. Moreover, the assumed formation of "small crystal or amorphous phase" certainly does occur in living cells, if possible, only under an exceptionally supercooled condition which has never been experienced by any living matter other than those in some very special laboratories. The contributions of LUSENA and COOK, however, must be highly appreciated for their increasing of available knowledge for the explanation of frost resistant mechanism in ordinary living cells.

As has been indicated in the foregoing sections, it is almost evident that an ice formation in a cell occurs only when inoculated by the ice crystal on the cell surface. Since a lower temperature always favours more than a higher one an easy penetration of freezing into the cell, an ice crystal formed at a lower temperature must possess some favourable character for this purpose. The present writer's experiments (unpublished) reveal that in every aqueous system from living materials, the lower the temperature at which freezing occurs the more slender become the ice branches which are formed. This is probably explained by the fact that at a rapid cooling, there occurs a stronger temperature gradient at the top of the

crystal front which brings about a far faster crystallization of water at the top than at any other part on the crystal. But in the tissue fluids which are kept at a slightly supercooled state, the ice originates from discoid shaped germ and grows slowly to fern-like crystals. In such a case, an elevation of the temperature is invariably followed by an increase in the width of the leaves of the fern-like crystals. Among the investigators on ice crystallization, it has been a well-known phenomenon that, at a very small degree of supercooling, when water is seeded, it occasionally crystallizes as a thin discoid which sometimes grows to a good size unless it is cooled faster (*2*, *5*).

It is therefore reasonable to suppose that the ice, when it comes into contact with cell surface, may not penetrate into the fine structure of protoplasm or even that of cell wall, if the temperature at the contact interphase is sufficiently high. Since the ice itself, as mentioned in a foregoing section, can withdraw water from the cell interior, new crystallizations successively take place at the interface as the cooling proceeds. In such a case one of the known properties of the *hardy* cell, a high permeability to water, certainly accelerates the rate of the crystallization of ice, resulting in an increase in the rate of temperature elevation at the interface. This interpretation would also supply the reasons for the good frost preventing properties, at a slow cooling, of the cell walls of plant cells both plasmolysed and refrozen or dead. HORIE has demonstrated in *Hydrodictyon* a remarkably low permeability of the cell wall to the solutes of large molecular volume (*27*). The cells of this alga, whether they are alive or not, are also very resistant to the toleration of freezing inside the wall. This fact, too, might support the view just mentioned.

If these explanations are to hold valid, however, an initiation of the extracellutar freezing at and only at a slightly supercooled state of the cell is necessary, for preventing the transmission of freezing into the cell. In nature this is effectively performed by the ice seeding from the hoar frost formed on the plant surface or from the other out-door ice crystals (*13*). In the laboratory, it is now clear that some living membranes can act as a barrier against ice crystals even under a supercooled state of several degrees lower than the "freezing temperature" of the cell. For the analysis of this interesting property of protoplasm, the experimental results cited above (see page 110) on water imbibing *hardy* cells may provide a useful clue. From this imbibition experiment, one may easily suppose a swelling of the membrane in which the water space is so enlarged that the ice crystallization in it is no longer disturbed. This hypothesis, however, must be discarded, because a remarkable "flashing" is usually found in these cells. Since a "flashing" means a well supercooled state of cell interior at the freezing, the water imbibed membrane must certainly act as a sufficient barrier to the penetration of ice at least until such a state of the cell interior has been brought about. LUCENA and COOK claim "When ice crystal formed in solutions at subeutectic concentrations, a solution of lower

feezing point advanced ahead of the ice front and apparently enhanced the resistance of the membrane to crystal penetration" (*38*). This is probable and, in fact, they found a highly concentrated solution of potassium chloride, a test solute, at the membrane. If that is so, in a *hardy* cell why a slight difference of concentration of immersed solutions brings about a remarkable decrease of frost preventing capacity of the membrane is still a mystery. In the petiole parenchyma of a *hardy* table beet, the examined tissue sections always froze extracellularly after having been immersed in a sucrose solution of above 80% isotonic concentration of the cell sap, while in those immersed in 70% insotonic solution, an appreciable frequency of "flashing" was invariably observed (*9*).

On the other hand, ice crystals forming in aqueous solutions rich in hydrophilic colloids, as suggested by KISTLER (*33*), are very apt to become covered with a sheath of dehydrated colloid, and thus to fail to seed the entire solution. Therefore, if a cell has deen covered with such solution, the layer of the condensed solution formed at the moment of freezing may not only retard the propagation of freezing, but effectively conceal the surface of the growing ice front, so that ice crystals can hardly be in direct contact with the cell surface (*12*). Some observations in LUCENA and COOK's recent work also seem to support this view (*39*). As is demonstrated in the present observation, without intimate contact of the cell surface with ice, it is very difficult or perhaps almost impossble to initiate the freezing of the cell interior at least within a limit of temperature. Although little has been known about the existence of such a "protecting fluid" in *hardy* plants, so far as the writer is aware, there is occasionally found an appreciable amount of more or less sticky fluid in some overwintering plant tissues. A reasonable interpretation today is that a bathing of body tissue in blood rich in hydrophilic colloid plays an important role in the frost resistance mechanism of some frost hardy insects in which freezing occurs only at a strikingly high degree of supercooling, −20°C (*16*).

Now it is evident that under certain conditions a membraneous structure acts as a barrier to the penetration of ice crystal, whether it be alive or not. The living condition, however, seems to be more favourable than the lifeless one to increase such ability of the membrane. Even an interface between two aqueous phases, having a certain difference in the content of easily freezable water, may certainly exhibit some of such characters (*38*, *43*). At any rate, problem of the frost preventing mechanism of living membrane might probably remain unsolved until a fuller knowledge is obtained about the protoplasmic structure, especially the structure of the cell surface layer.

Conclusion and Summary

A number of kinds of plant cells of various degrees in frost hardiness were subjected to freezing in a cold chamber, and the process of their freezing at various temperatures and cooling rates was directly observed in thin hanging oil drops under

a special microscope. An excessive supercooling was prevented by means of ice seeding. The temperature was read with a small thermojunction inserted in the hanging drop during the observation.

Plant cells, whether alive or not, can easily remain in unfrozen state at temperatures below −10°C. Without ice seeding on the tissue, "spontaneous freezing" can occur only at a far lower temperature than the freezing point of the tissue fluid. However, the vitrification of plant tissue is hardly conceivable if the natural water content persists. The essence of libration from supercooled state is assumably the great amount of the freezable water on the tissue.

As a rule, an inoculation with ice crystal is a prerequisite for the initiation of freezing in the cell which is not in a considerable supercooled state. By this means, the vacuole sap can be usually frozen at about its freezing point even in the living cell. The cell freezes extracellularly or intracellularly depending upon whether or not the freezing is effectively prevented by the surface layer of protoplasm. So-called frost-hardy cells are characteristically resistant to a penetration of ice crystals. On the surface of these cells ice crystals of a large size usually develop owing to their remarkably high permeability to water, while *unhardy* cells easily freeze intracellularly. If inoculated with ice at a temperature slightly lower than their "freezing temperature", they, too, freeze extracellularly, although they are very liable to be injured by such mode of freezing. When the ice penetrates only the cell wall but not the surface layer of protoplasm, an ice formation occurs between them. In this case water is withdrawn from the cell interior by the ice, and a "frost plasmolysis" frequently results by the growing of the ice mass without any harmful effects. Every condition under which a sufficient supercooling is brought about in the cell in the presence of ice on the cell surfase favours an intracellular freezing.

In general the processes of intracellular freezing can be classified into two types, namely, *flash* and *non-flash* type. The former is a sudden freezing indicating a sufficiently supercooled state of the cell at the moment of freezing; the latter is a slow freezing indicating that the cell interior has been set free from the supercooled state at a temperature near the freezing point of the cell sap. Which type of freezing, *flash* or *non-flash*, occurs in a cell, therefore, depends under the presented condition upon whether or not the cell surface is able to prevent the penetration of freezing until a certain degree of supercooling is brought about in the cell. When the ice formation is effectively prevented at the tonoplast, the intracellular freezing occurs only in cytoplasm but not in sap vacuole. This is often the case in very *hardy* cells. In the cytoplasm the ice usually grows far slower than in sap vacuole.

In the process of *flash* type freezing, or "flashing", the freezing starts at a cell end and proceeds very rapidly as a dark wave to the other end, forming a large number of fine ice crystals throughout the protoplasm and vacuole. In some tender large spheroidal cells, ice crystal grows around the inner side of the cell wall until

the whole cell is filled with fine dendritic ice branches. When subjected to a very slow freezing, some cells freeze in such manner that the ice, branching into several broad fronts, grows mainly in the cytoplasmic layer and then penetrates into the vacuole with the secondary developed ice of slender fronts. In most cases of *non-flash* type freezing, the ice crystals seem to penetrate the cytoplasmic layer very easily and grow toward the cell end concentrating the cell contents between them.

In the cells frozen in *flash* type manner, there are found a large number of droplets; the space between them is packed with a homogeneous ice mass. The process of the formation of such droplets is as follows. Being condensed between numerous slender ice branches, the concentrated cell contents is separated from ice as many zigzag rods and then they are divided into a number of minute droplets, as the dendritic ice branches rapidly fuse with each other. Regarding the form of these liquid drops trapped in the ice mass, the resultant pattern of a flashed cell falls roughly into two subtypes, viz., "sap drop-" and "sperical particle-type". The quantity as well as the quality of the contained hydrophilic colloids of cell sap, by which the surface of these trapped drops is certainly covered, may play an important part in giving rise to such differences in the figure of the fluid fraction of a frozen cell. So far as the cell wall tension persists, the "flashing" can be repeated many times, provided that the cell interior can be sufficiently supercooled prior to the freezing.

Because of the invariable occurence of the freezing in protoplasm, intracellular freezing is always fatal to the cell. The process of injury in slowly frozen cytoplasm is assumably a cytolysis due to mechanical tears by an ice formation in it; death in rapidly frozen cytoplasm is probably caused by the extraordinarily strong dehydration, due to the rapid formation of minute ice crystals at every locus between the fine architecture of protoplasm. The principal cause of killing by extracellular freezing seems to be a dehydration injury, i. e. an irreversible coagulation, brought about during the freezing but not during the thawing. However, death in plant cells after a long period of freezing may probably be attributable to some changes in their metabolism under a certain low temperature.

That frost killing occurs in the plant tissue previously infiltrated with water more easily than in untreated tissue is explained by the easier occurrence of intracellular freezing rather than by the pressure of ice mass formed in the intercellular space. At least in hardy cells a water imbibition to a certain degree remarkably decreases the preventing ability of the cell to the penetration of ice crystal. It was demonstrated that not only the ectoplast but also the tonoplast and even the nuclear surface, at any rate, acted as a barrier to the penetration of ice crystal into the supercooled interior. The large size of ice crystal front is one of the outstanding factors which enables these membranes to prevent a ready passage through them. This must occur effectively in *hardy* cells, because their wellknown character of high permeability to water certainly accelerates the rate of the crystallization of

ice, therefore, an increase in the temperature elevation at the ice-cell interface results. Since water freezes only as a discoid or a fern-like crystal with wide leaf at a slightly supercooled state, the crystal fronts of ice formed at such interface are certainly too large to penetrate into the fine structure of the protoplasmic membrane or sometimes even that of the cell wall.

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

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P. S. After the present paper was prepared summary of a lecture on "Die Frosthärtung als zellphysiologisches Problem", given by Prof. H. ULLRICH at a meeting held in faculty of science of Tokyo University in March of 1955, has come to hand through the kindness of Prof. S. HATTORI of Tokyo University. This work deals with an increase in amount of glycoproteide found in frost hardy cells by which the rate of ice crystallization may be lowered and, therefore, the capacity of supercooling of cell content may be increased.

Plate I.—Extracellular Freezing of Intact Plant.

Fig. 1. Frozen cabbage at about —3°C in winter field, showing the darkening of vein part owing to the inside ice formation. (× 1/4; only this photograph was taken at natural condition).

Figs. 2–4. Internodial cell of *Nitella*. (× 100; exposure 1/25 sec.)

Fig. 2. Crystallization at the cell surface at —0.6°C, one minute after the inoculation with ice.

Fig. 3. Same field at —1°C, 3minutes after the inoculation.

Fig. 4. Same field at —0.8°C, 5 minutes after the inoculation.

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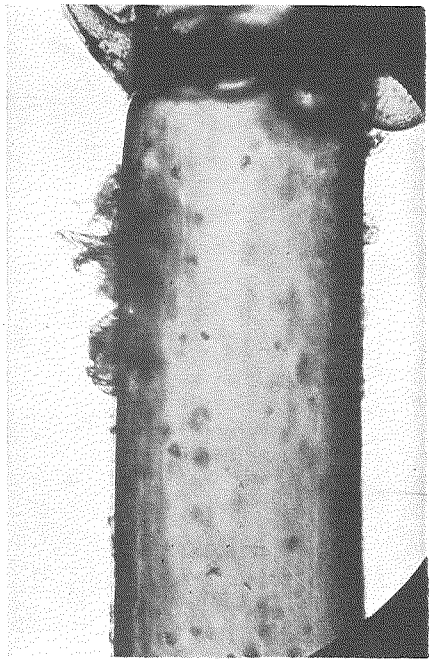

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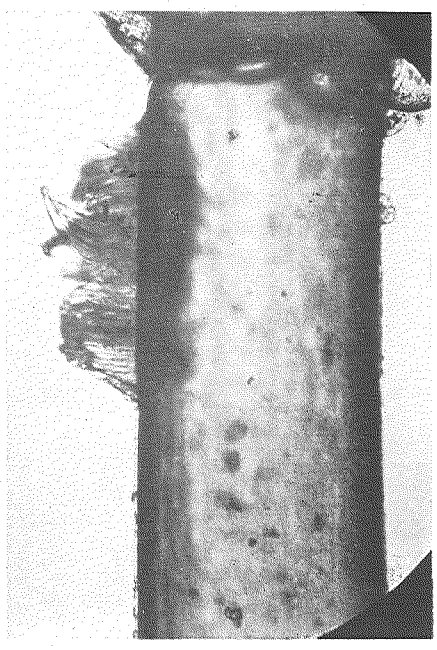

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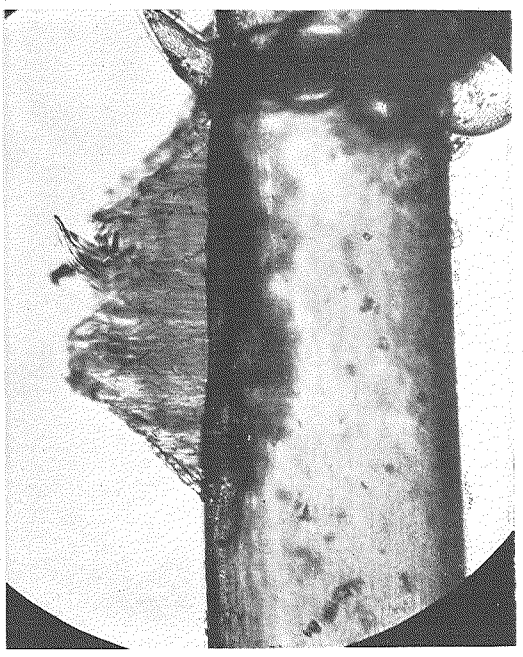

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Plate II.—Extracellular Freezing of Tissue Section.

Figs. 5–7. Vitally stained cells of pith in petiole of dandelion. (× 315; exposure 1/10 sec.)

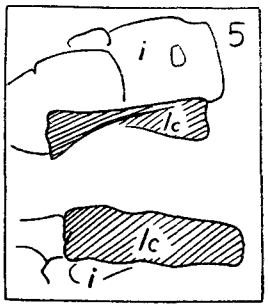
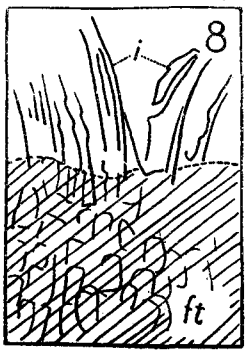


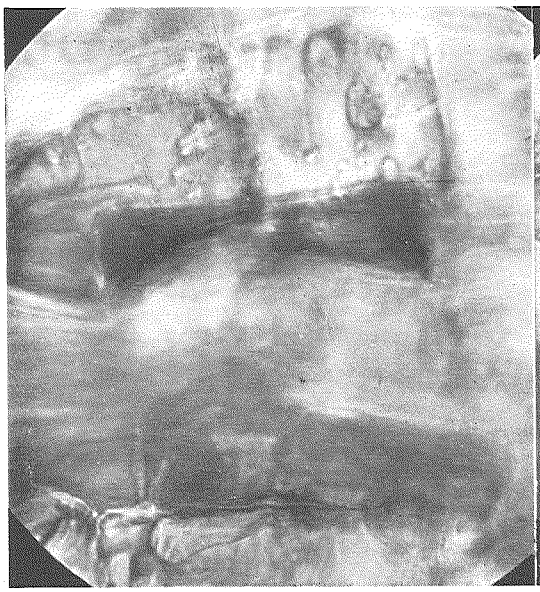
Fig. 5. Frozen tissue at −3.4°C, 9 minutes after the inoculation with ice.
i, ice crystal; lc, living cell.

Fig. 6. Same cells warmed to −0.8°C, 18 minutes after the inoculation.

Fig. 7. Same cells just thawed at −0.1°C, 21 minutes after the inoculation.

Fig. 8. Frozen tissue of potato sprout 5 minutes after the inoculation at −3°C, showing the remarkable ice formation at the cut end located in colder side. (× 100; exposure 1/100 sec.)
ft, frozen tissue; i, ice crystal.





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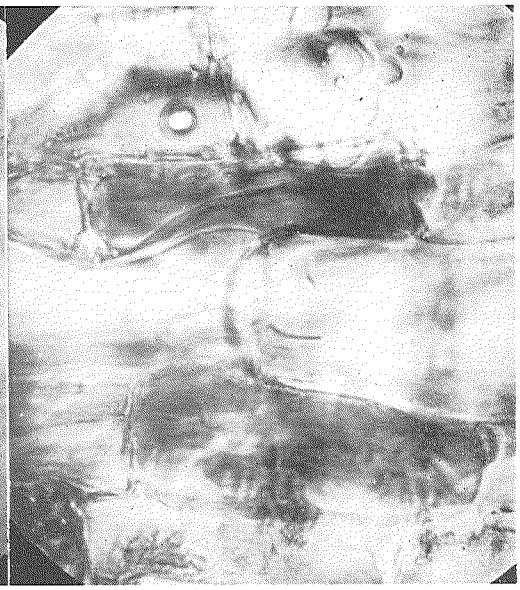

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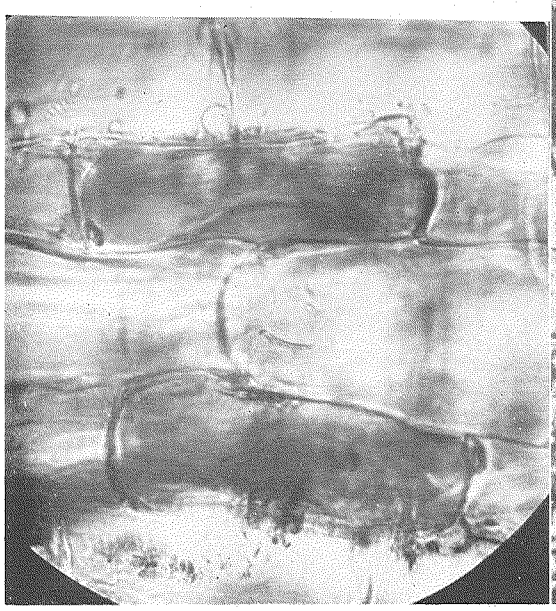

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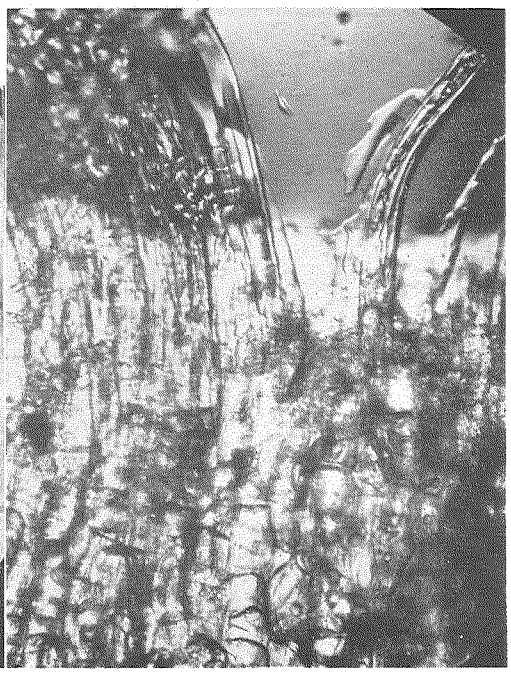

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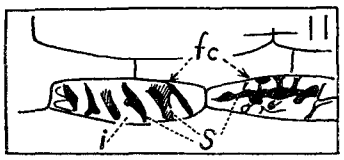
Plate III.—Freezing of Parenchymatous Tissue Section.

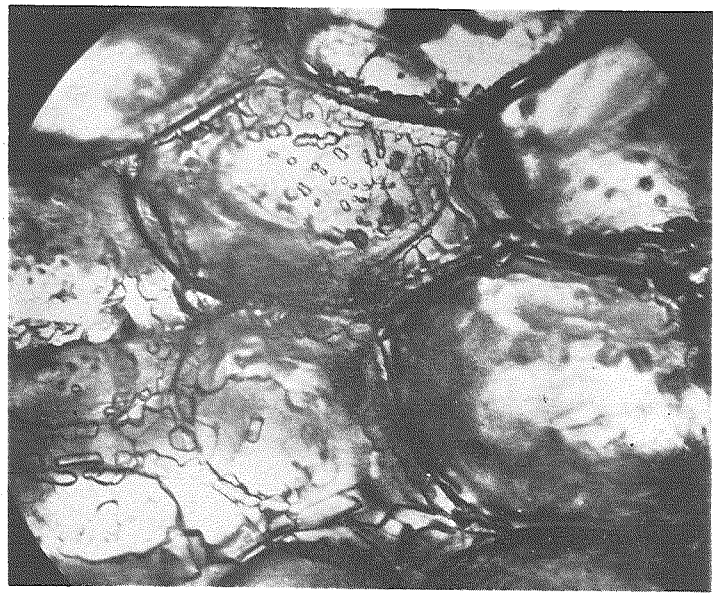
(× 100; exposure 1/25 sec.)

Fig. 9. Extracellular freezing in the fruit tissue of water melon at —3°C, 2 minutes after the inoculation with ice.

Fig. 10. Extracellular freezing of the cortical tissue of petiole of *hardy* table beet at —5.4°C, 19 minutes after the inoculation, showing the remarkable ice formation in the intercellular space.

Fig. 11. Cortical tissue of petiole of *hardy* table beet frozen after a water immersion, 2 minutes after the "flashing" of the cells at —4.8°C, showing the cell sap concentrated into a few masses embedded in ice mass in frozen cells. fc, frozen cell; i, ice; s. condensed cell sap.





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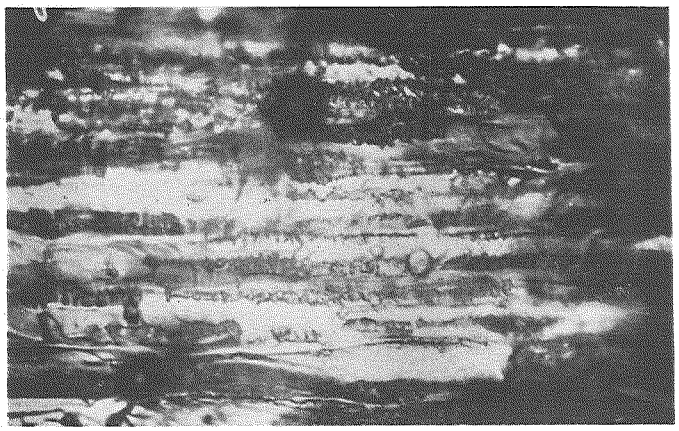

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Plate IV.—Intracellular Freezing of Non-flash Type.

Fig. 12. Pith cells of the stem of summer radish frozen at —5°C. (× 230; exposure 1/25 sec.)
fc, frozen cell; i, ice crystal.

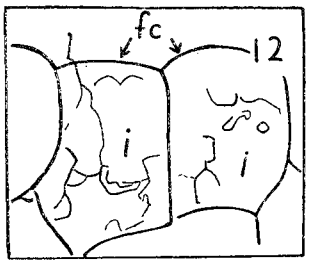


Fig. 13. Cortical tissue of the petiole of *unhardy* rape frozen at —5°C. (× 100; exposure 1/100 sec.)

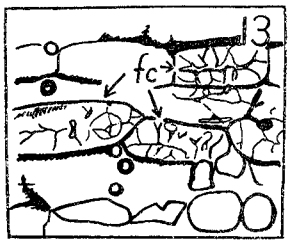


Fig. 14. Cortical tissue of the petiole of *unhardy* table beet frozen at —4°C. (× 100; exposure 1/25 sec.)
fc, frozen cell; s, condenced cell sap.

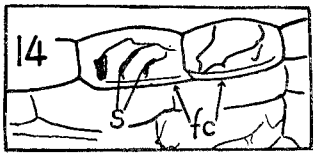
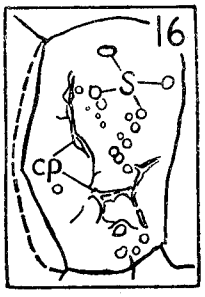
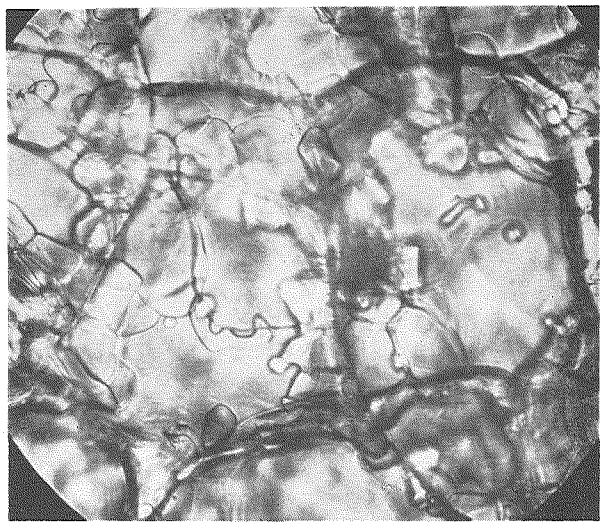


Fig. 15. Pith cells of the petiole of dehardened table beet frozen at —6°C. (× 315, exposure 1/10 sec.)

Fig. 16. Cortical cell of the petiole of *hardy* table beet rapidly frozen at —9°C, showing the spherical sap drops in frozen cell. cf. Pl. XXII. (× 230; exposure 1/10 sec.)
cp, coagulated protoplasm; s, drop of cell sap.





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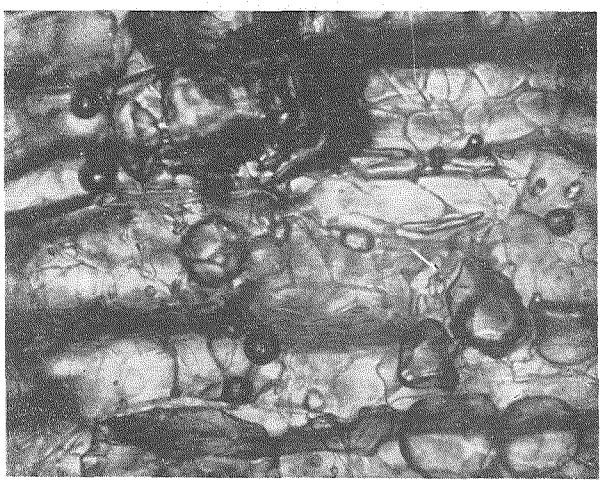

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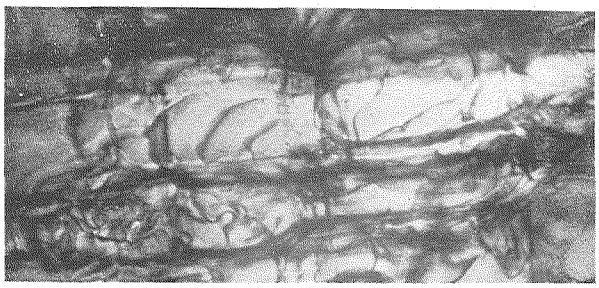

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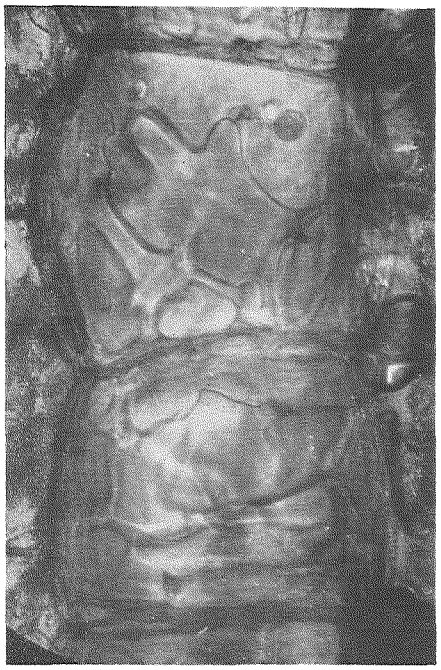

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Plate V.—"Frost plasmolysis" in the Cells of Staminal Hair of *Tradescantia.*

Fig. 17. A cell kept at −2.2°C for 15 minutes after the beginning of "frost plasmolysis". (× 315 : exposure 1/10 sec.)

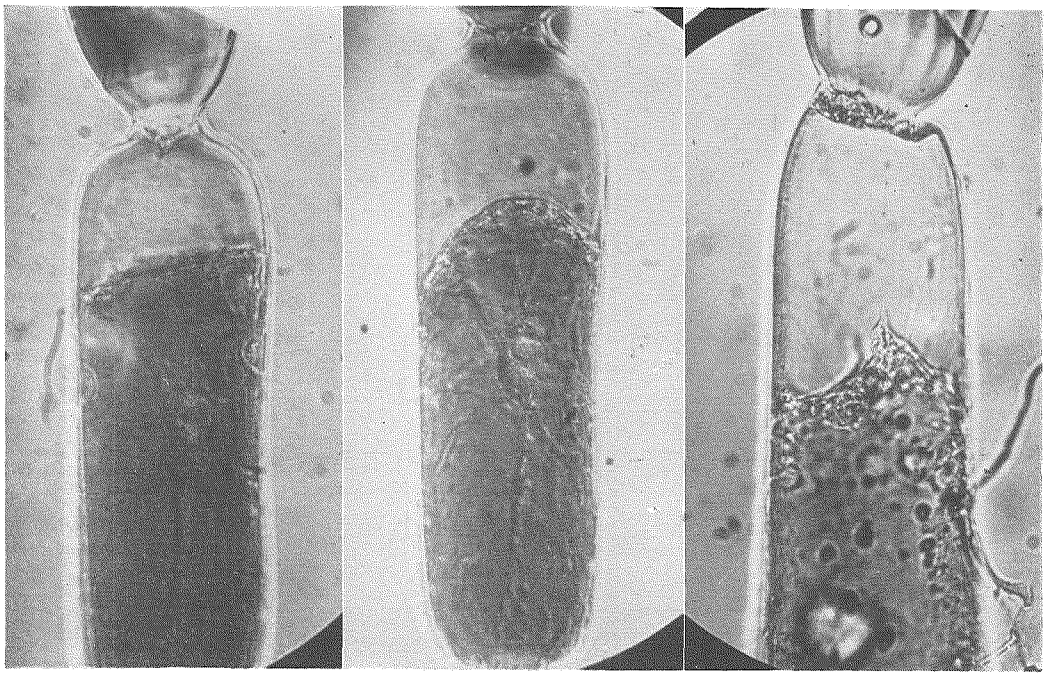
Fig. 18. Same cell 45 minutes later at −2.2°C. (× 315; exposure 1/10 sec.)

Fig. 19. "Frost plasmolysis" at −1.4°C, showing protoplasmic strands in homogeneous ice mass. (× 440; exposure 1/10 sec.)

Fig. 20. An early stage of "frost plasmolysis" at −1.4°C, showing semi-spherical ice mass inside the cell wall. (× 400; exposure 1/5 sec.)

Fig. 21. "Frost plasmolysis" at −1.9°C. (× 315; exposure 1/10 sec.)

Fig. 22. Same cell 3 minutes later, just frozen intracellularly at −2.1°C, showing the instant change in figure of ice mass at cell end. (× 315; exposure 1/10 sec.)



17 18 19

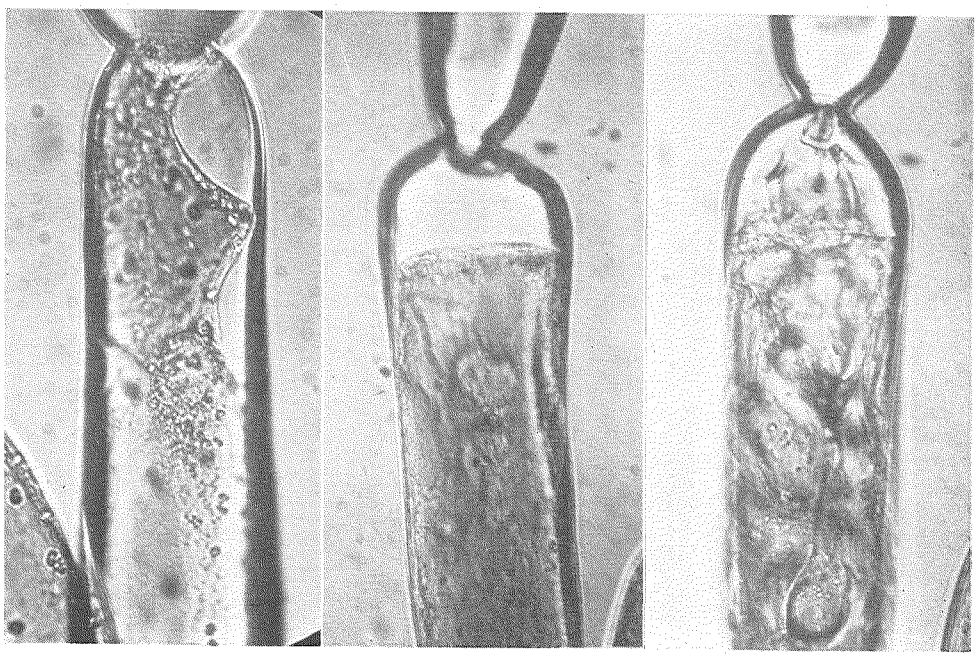


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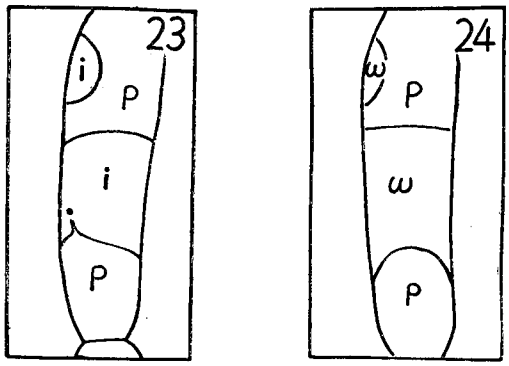
Plate VI.—Staminal Hair of *Tradescantia.*



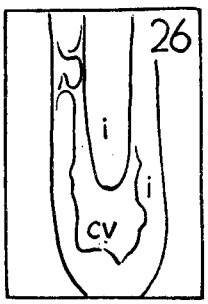
i, ice;
p, protoplast;
w, water.

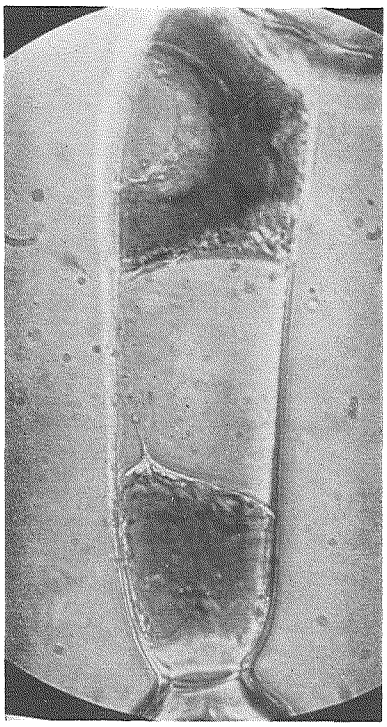
Fig. 23. Division of protoplast, due to "frost plasmolysis" having occurred in the middle part of cylindrical cell, at —1.9°C, 180 minutes after the inoculation with ice. (× 315; exposure 1/10 sec.)

Fig. 24. Same cell at 1°C, 2 minutes after thawing, showing the deplasmolysis of the divided protoplasts. (× 315; exposure 1/10 sec.)

Fig. 25. Cells frozen intracellularly at —1.4°C, 9 minutes after the inoculation, showing the contraction of the vacuole. (× 100; exposure 1/25 sec.)

Fig. 26. Contraction of the vacuole in a frozen cell at —1.9°C, 5.5 minutes after the inoculation. (×440; exposure 1/5 sec.) cv, contracted vacuole; i, ice crystal.





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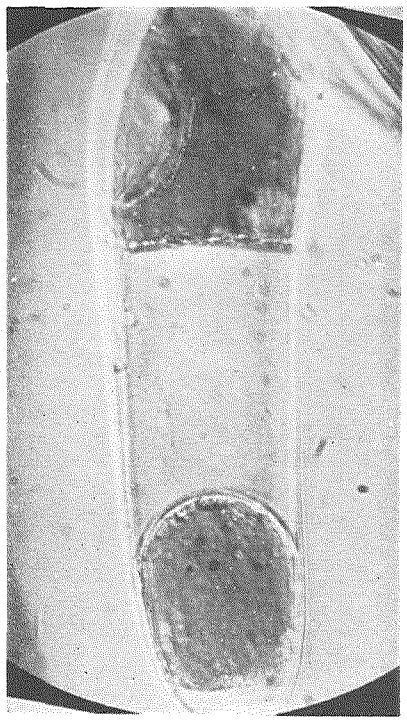

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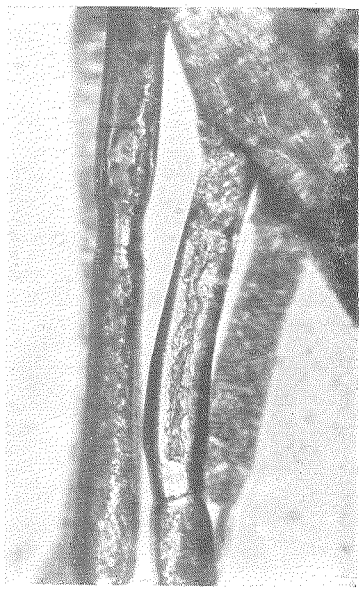

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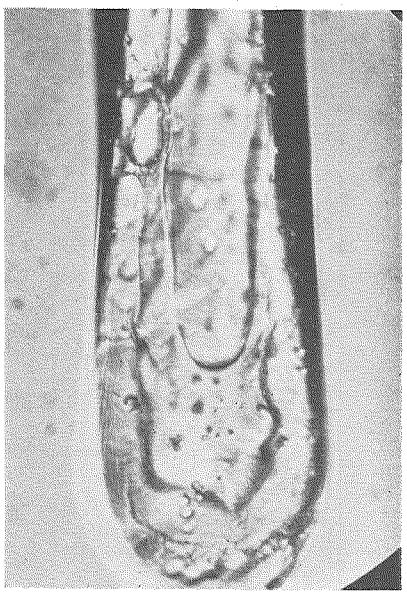

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Plate VII.—"Flashing" in the Cells of Staminal Hair of *Tradescantia.*

Fig. 27. "Flashing" at —10°C, a good example of "sap-drop type". (× 230; exposure 1/10 sec.)

Fig. 28. Detail of Fig. 27, 5 minutes later. (× 440; exposure 1/2 sec.)
cp, coagulated protoplasm;
fn, frozen nucleus;
s, droplet of condensed sap.

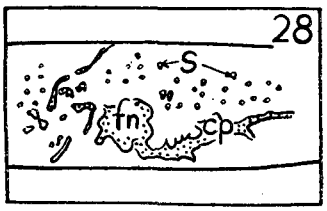


Fig. 29. "Flashing" at —6°C, showing the slender ice branches having grown in the cytoplasmic layer. (× 230; exposure 1/25 sec.)
fn, frozen nucleus;
ib, ice branch.

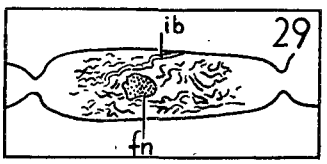
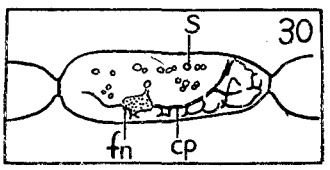
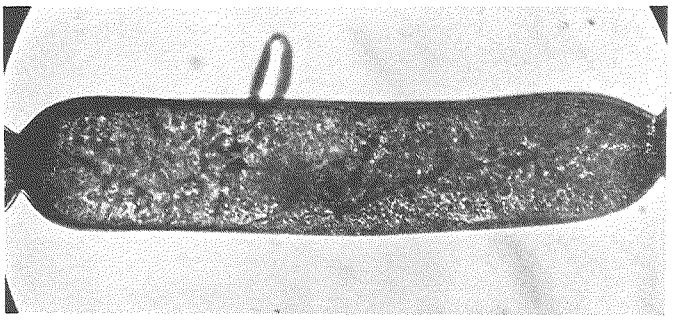


Fig. 30. Frozen cell 12 minutes after the "flashing" at —5.2°C, showing a net work of coagulated protoplasm at the surface layer of the cell. (× 230; exposure 1/10 sec.)
For symbols see Fig. 28.





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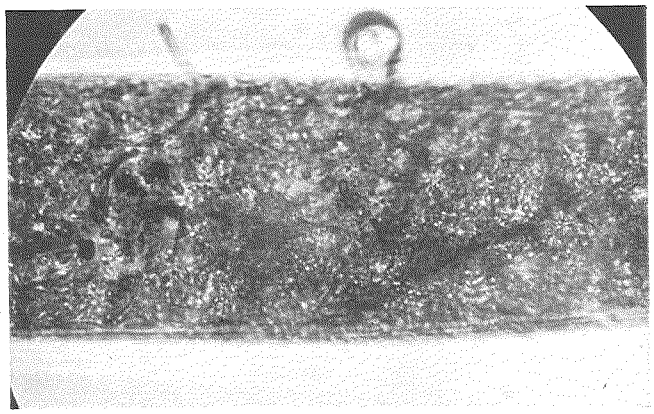

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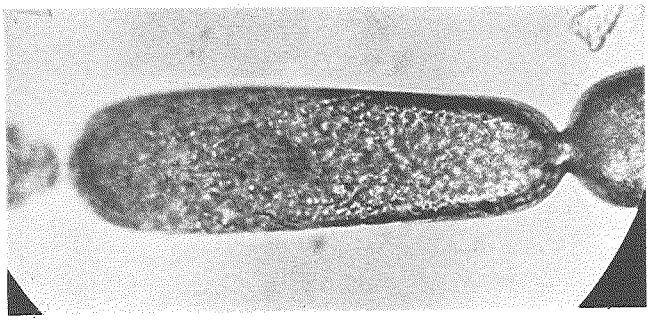

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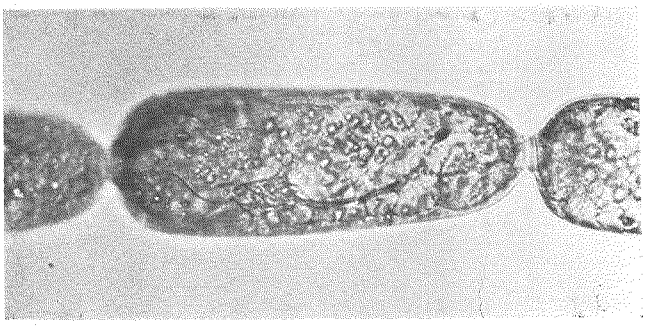

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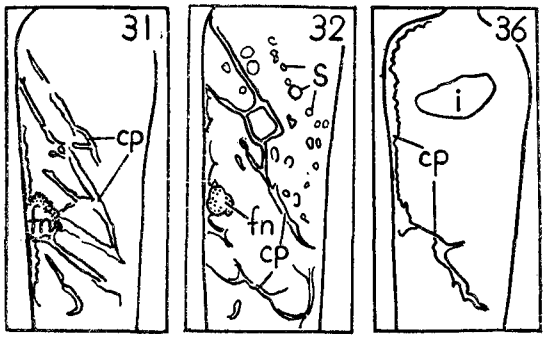
Plate VIII.—Flashed Cell of Staminal Hair of *Tradescantia.*

(× 440 : exposure 1/10 sec.)



cp, coagulated protoplasm;
fn, frozen nucleus;
i, ice;
s, droplet of condensed sap.

Fig. 31. At —5.2°C, 5 seconds after the "flashing".

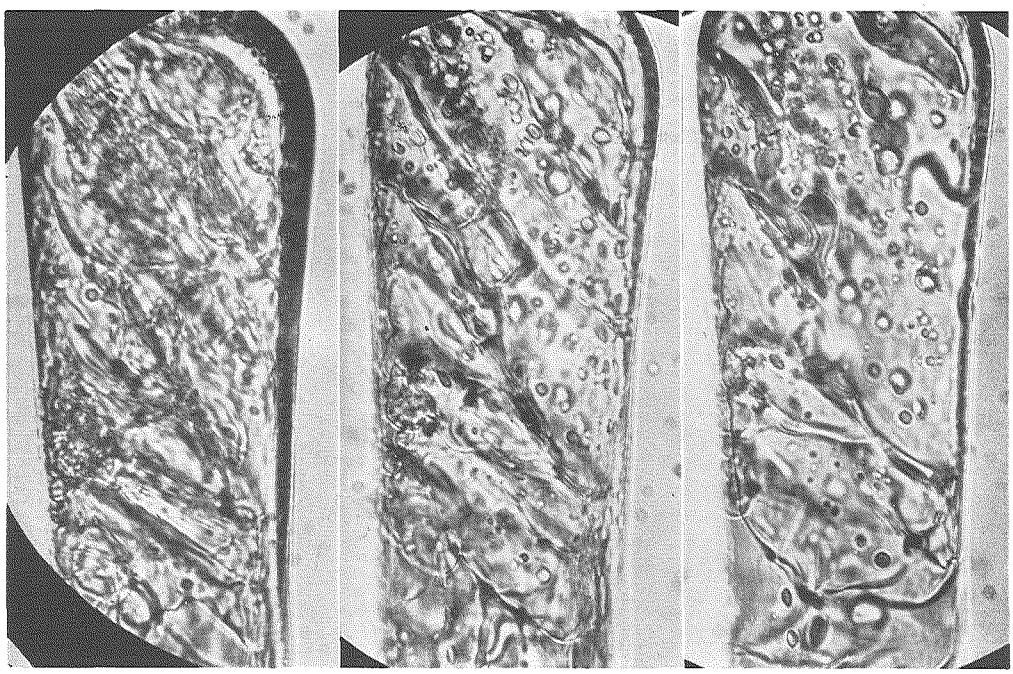
Fig. 32. At —5.5°C, 2 minutes after the "flashing", showing the deformed nucleus.

Fig. 33. At —5°C, 13 minutes after the "flashing", showing the lightened cell interior.

Fig. 34. Thawing at —3.6°C.

Fig. 35. Thawing at —1.7°C.

Fig. 36. Thawing, just before the disappearance of melting ice crystal.



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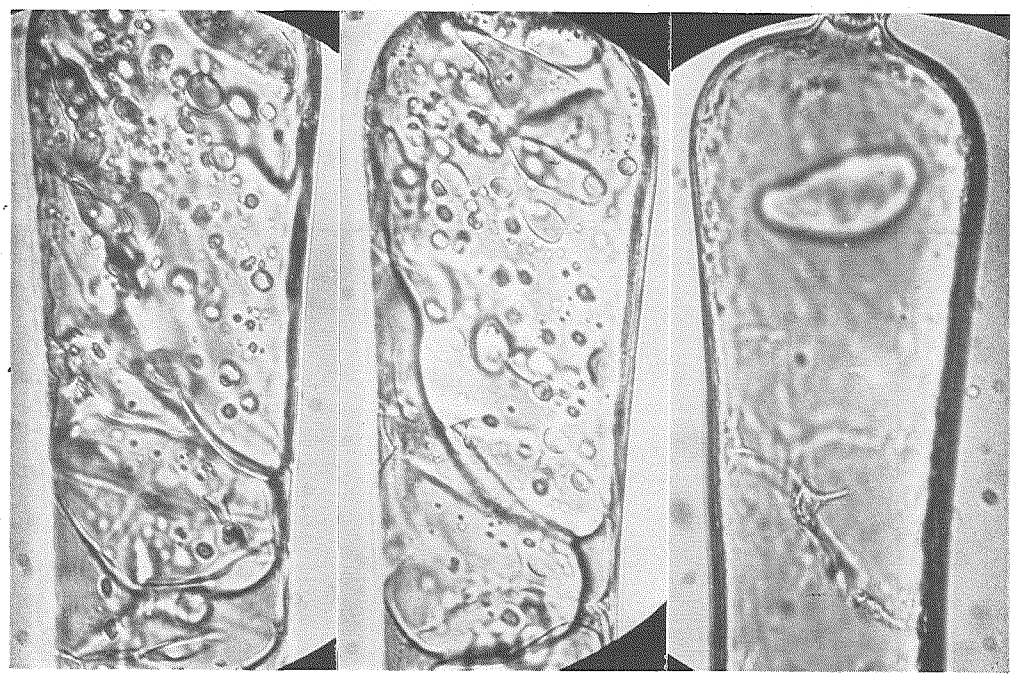

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Plate IX.— Intracellular Freezing in the Cells of Staminal Hair of *Tradescantia*.

Fig. 37. Supercooled cell at −2.1°C, showing the ice mass developing at the distal end of the neighbouring frozen cell. (× 315; exposure 1/10 sec.)
fc, frozen cell; i, ice mass in projected cell end; n, nucleus.

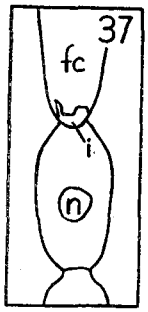


Fig. 38. Same field at −2.2°C, 5 seconds after the freezing of the cell interior. (× 315; exposure 1/2 sec.)

Fig. 39. Same field at −2°C, one minute later showing the rapid transformation in the figure in frozen cell. (× 315; exposure 1/10 sec.

Fig. 40. Freezing at −2.1°C; another cell, showing the form of growing ice crystal. (× 350; exposure 1/10 sec.)
fn, frozen nucleus; i, ice crystal; arrows indicating the direction of ice growing.

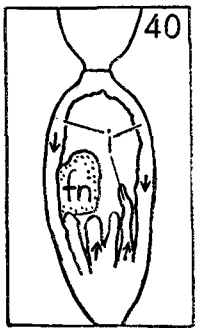
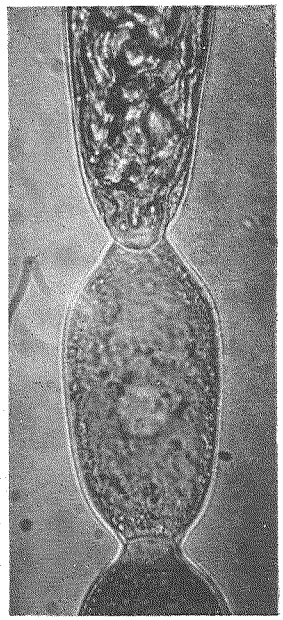


Fig. 41. Same cell 50 seconds later at −2.2°C. (× 350; exposure 1/5 sec.)



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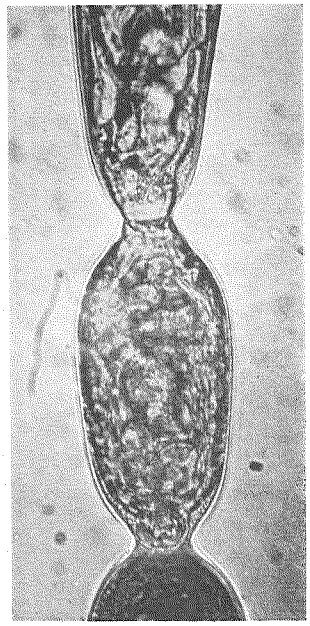

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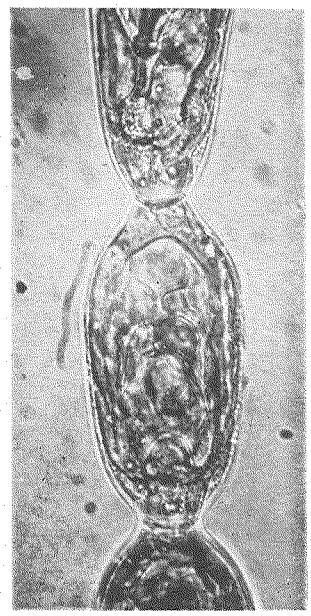

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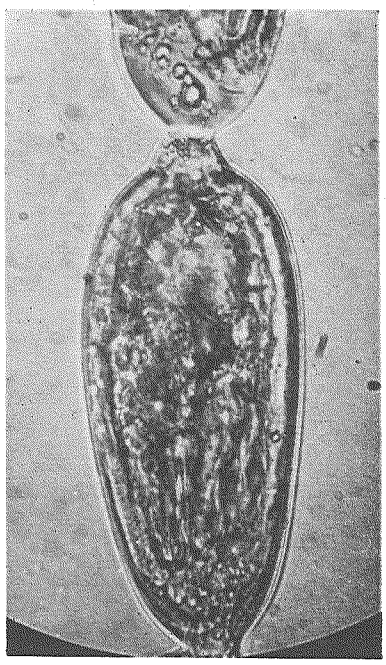

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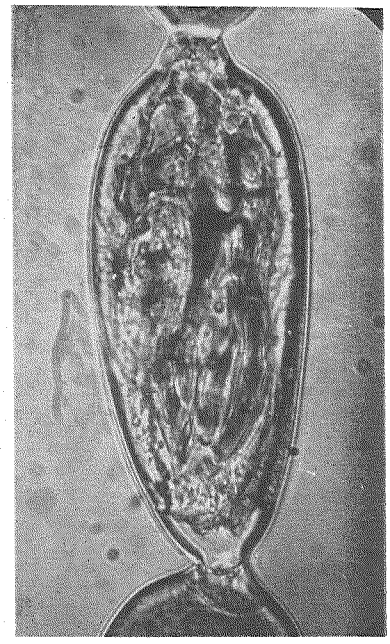

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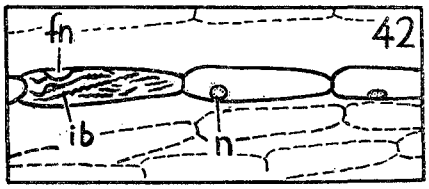
Plate X.—Propagation of Cell Freezing in Staminal Hair of *Tradescantia*.



fn, frozen nucleus;
ib, ice branch;
n, nucleus in unfrozen cell.

Figs. 42–44. At —2.2°C. (× 100; exposure 1/100 sec.)

Fig. 42. Freezing of the first cell, showing the broad ice branches growing in the surface layer.

Fig. 43. 2 secondes after the freezing of the second cell in which a clear ice mass is now forming at the cell end withdrawing water from the third cell.

Fig. 44. Just after the freezing of the third cell.

Fig. 45. Frozen cell maintained for 54 minutes at about —5°C, showing the concentrated unfrozen fraction around the ice mass. (× 230; exposure 1/10 sec.)

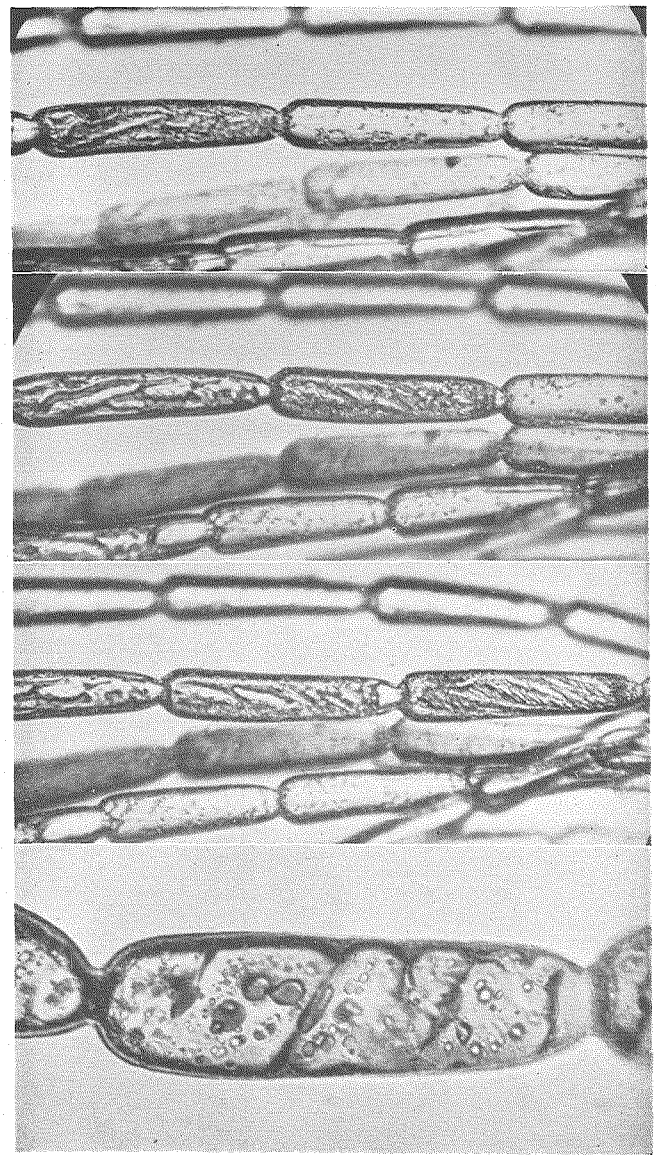


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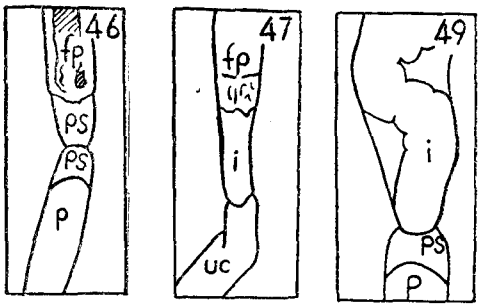
Plate XI.—Freezing in the Plasmolysed Cells of Staminal Hair of *Tradescantia.*



fp, frozen protoplost;
i, ice mass;
p, protoplast;
ps, plasmolysing solution;
uc, unfrozen cell.

Fig. 46. Ice formation confined only to contracted protoplast in the upper cell at −2.4°C. (× 100; exposure 1/50 sec.)

Fig. 47. Same field one minute later, showing the freezing of the space filled with plasmolysing solution in the upper cell. (× 100; exposure 1/100 sec.)

Fig. 48–52. Dehydration due to the ice formation in neighbouring frozen cell.

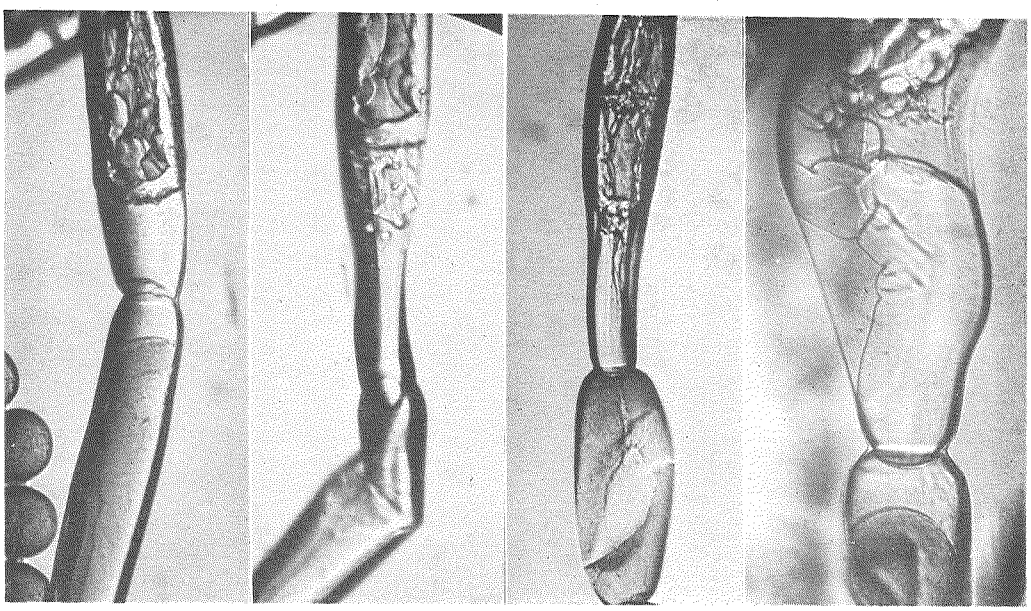
Fig. 48. A remarkable flattening of the unfrozen lower cell at −2.5°C. (× 100; exposure 1/50 sec.)

Fig. 49. Another field at −2.2°C, 3 minutes after the freezing of the upper cell. (× 230; exposure 1/25 sec.)

Fig. 50. Detail of Fig. 49, 2 minutes later at −2.4°C. (× 440; exposure 1/10 sec.)

Fig. 51. Same field 14 minutes later at −2.4°C. (× 440; exposure 1/1 sec.)

Fig. 52. Same cells in low magnification 3 minutes later at −2.4°C. (× 100; exposure 1/50 sec.)



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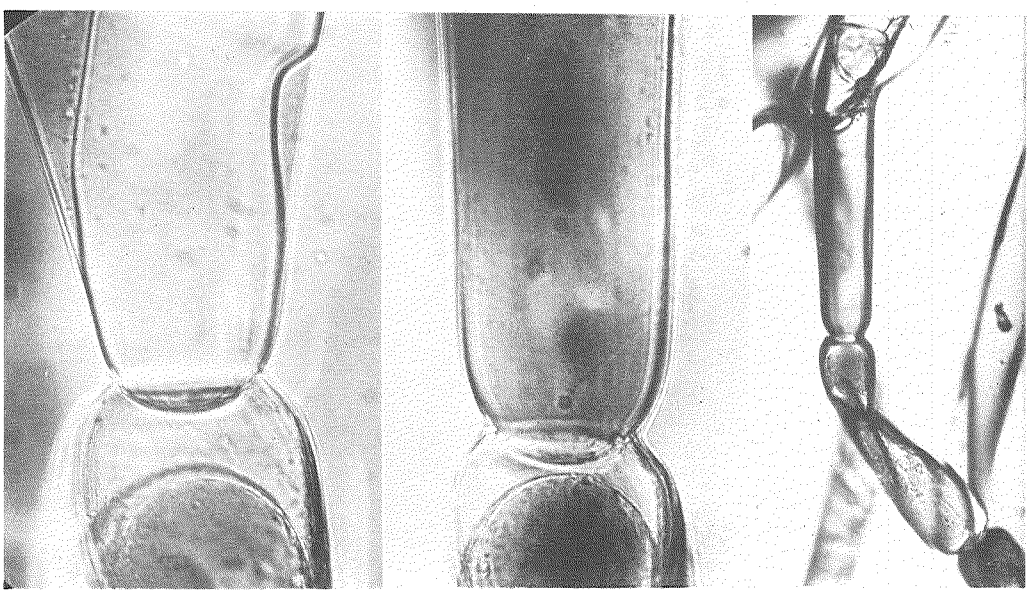

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Plate XII.—Intracellular Freezing in Young Cells of Staminal Hair of *Tradescantia*.

(× 440; exposure 1/5 sec.)

Fig. 53. Frozen cells at −6°C, 5 minutes after the "flashing".

Fig. 54. Same field at −5.2°C, 14 minutes after the "flashing".

Fig. 55. Thawing at −3°C, 29 minutes after the "flashing".

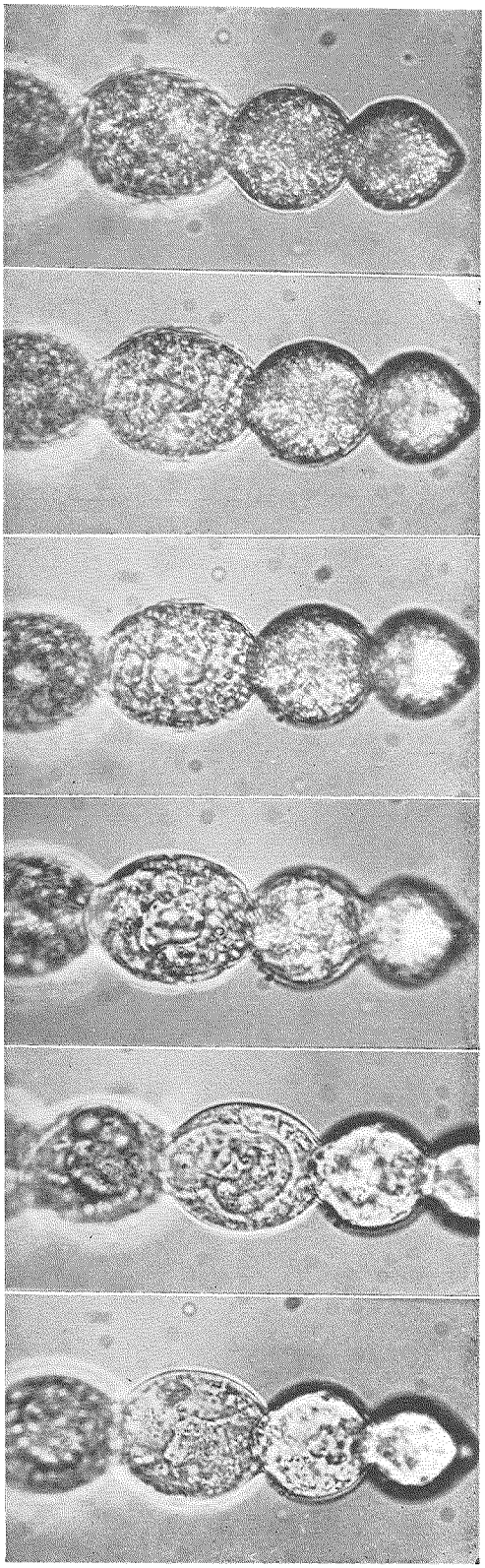
Fig. 56. Thawing at −1°C, 32 minutes after the "flashing".

Fig. 57. Just before thawing.

Fig. 58. Thawed cells, 36 minutes after the "flashing".

Fig. 59. Very young cells at −5°C, 15 minutes after the "flashing" at −6.5°C, showing the very granular appearance of frozen cytoplasm.

Fig. 60. Thawed cells, after have been frozen for 30 minutes at −6°C, showing the cytoplasm disorganized.



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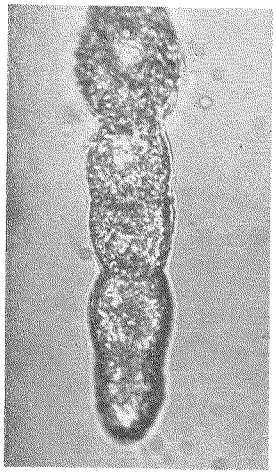

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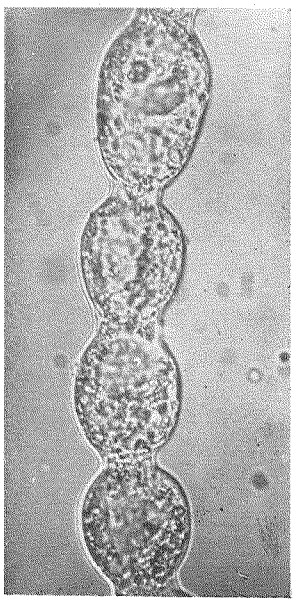

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Plate XIII.—Intracellular Freezing in the Epidermis of *Hardy* Welsh Onion.

Fig. 61. Frozen cells at −13.5°C. (× 100; exposure 1/100 sec.)

Fig. 62. Details of Fig. 61, 2 minutes after the "flashing". (× 350; exposure 1/10 sec.)
fn, frozen nucleus; s, droplet of condensed cell sap.

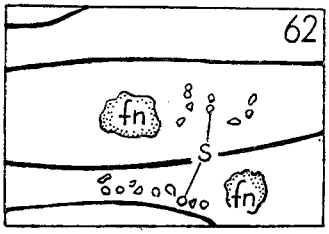
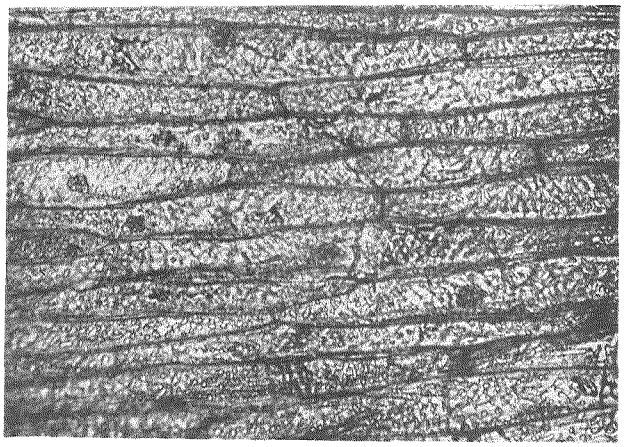


Fig. 63. Same field at −13°C, 12 minutes after the "flashing", showing the change in frozen cell content. (× 350; exposure 1/5 sec.)



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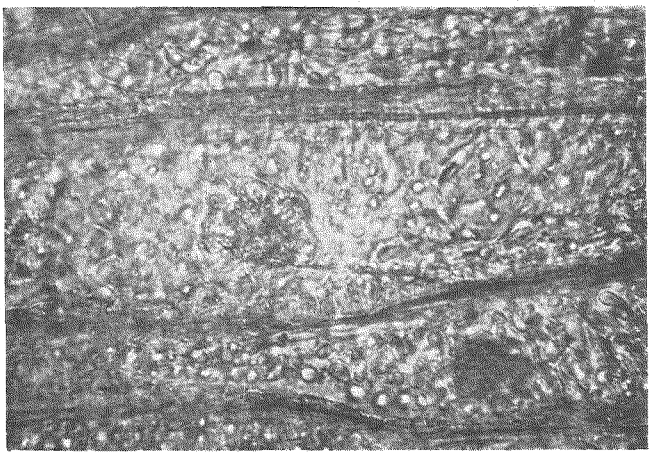

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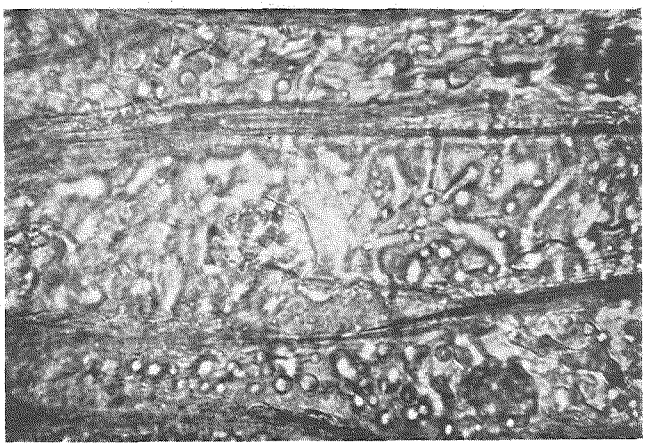
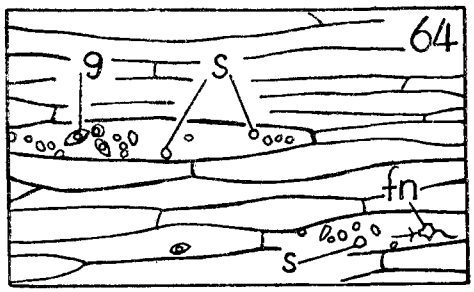


63

Plate XIV.—Intracellular and Extracellular Freezig in the Epidermis in *Hardy* Welch Onion.

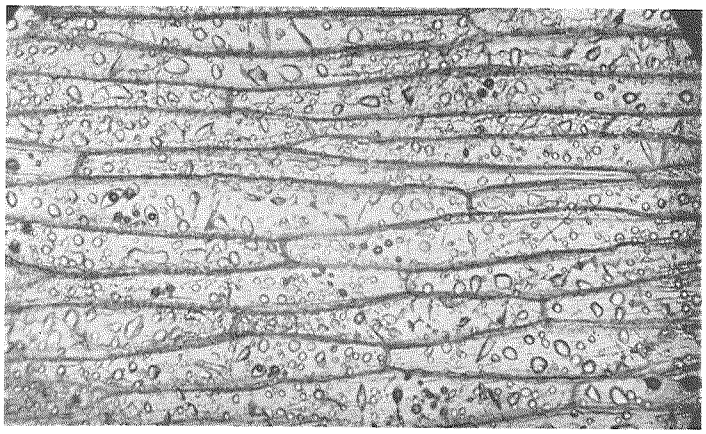


fn, frozen nucleus;
g, gas bubble;
s, condensed cell sap.

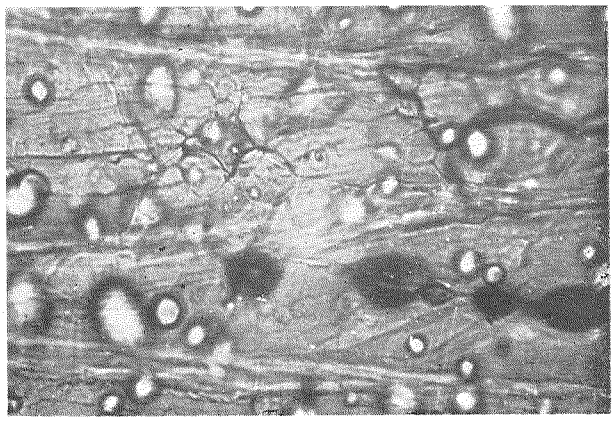
Fig. 64. Same strip as in Fig. 61 of Plate XIII kept at −15°C for 2 days; another field, showing the lightened cell interior. (× 100; exposure 1/100 sec.)

Fig. 65. Detail of the right below corner of Fig. 64, showing the deformed nucleus. (× 350; exposure 1/25 sec.)

Fig. 66. Extracellular freezing at −7°C, one minute after the inoculation, showing large ice crystals formed very rapidly as many dark masses on the cell surface. In this case most cells remained alive after thawing. (× 100; exposure 1/50 sec.)



64



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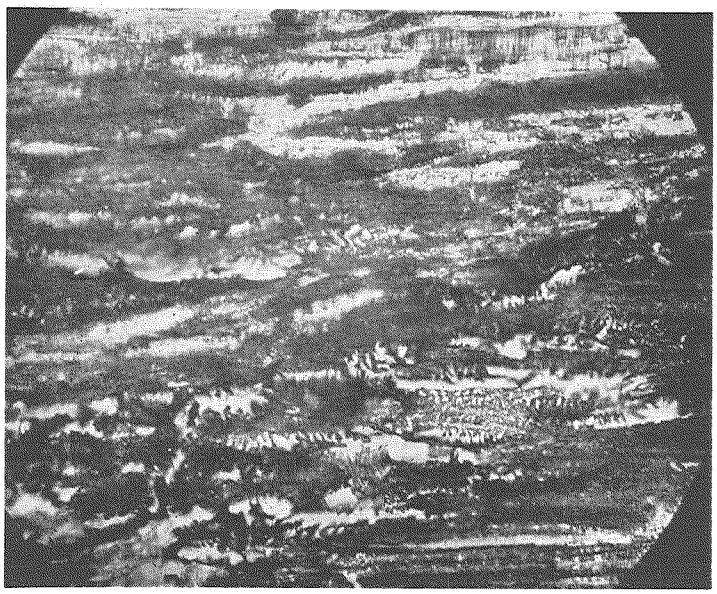
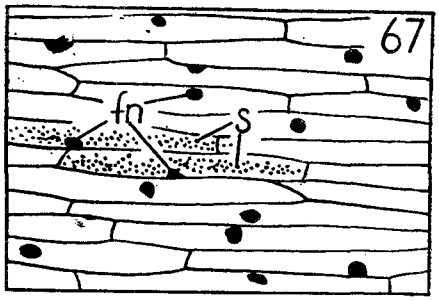


66

Plate XV.—*Unhardy* Welsh Onion (Figs. 67–69) and Onion (Figs. 70–71).



fn, frozen nucleus;
s, droplet of condensed cell sap.

Fig. 67. Intracellular freezing in epidermis at −11°C. (×100; exposure 1/25 sec.)

Fig. 68. Details of Fig. 67. 4 minutes after the "flashing", showing the finely granular appearance of the frozen nucleus. (× 440; exposure 1/2 sec.)

Fig. 69. Same strip as Fig. 67, slowly refrozen soon after thawing. Ice forms extracellularly. (× 230; exposure 1/25 sec.)
i, extracellular ice crystal;
cw, cell wall.

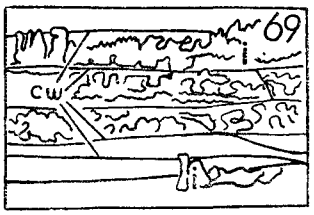
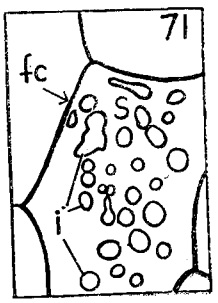
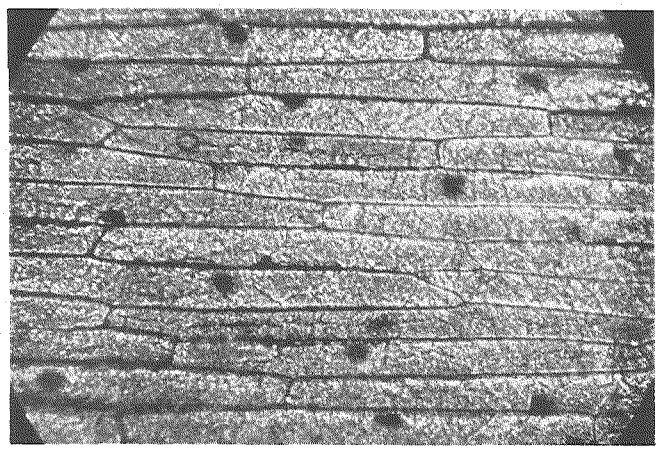


Fig. 70. Parenchymatous cell frozen at −3.2°C, showing the small ice crystals formed extracellularly before the freezing of cell interior. (× 440; exposure 1/2 sec.)

Fig. 71. Intracellular freezing in somewhat dehydrated epidermal cell at −6.6°C, showing a reticular conformation with many ice globules in frozen cell. (×440; exposure 1 sec.)
fc, frozen cell; i, ice globule;
s, condensed cell sap.





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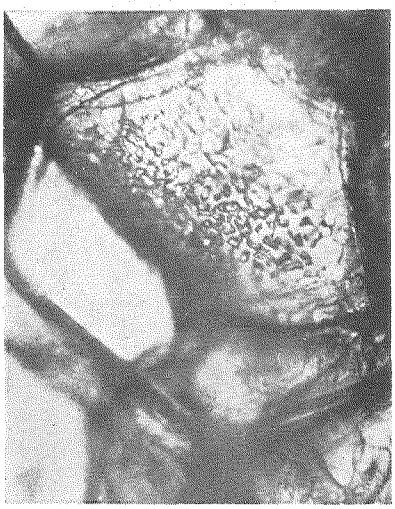

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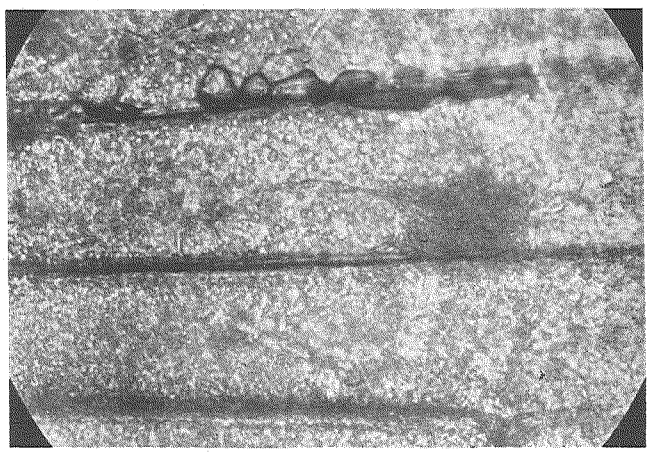

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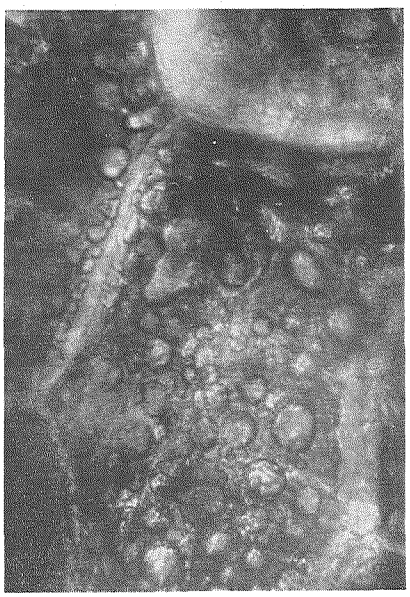

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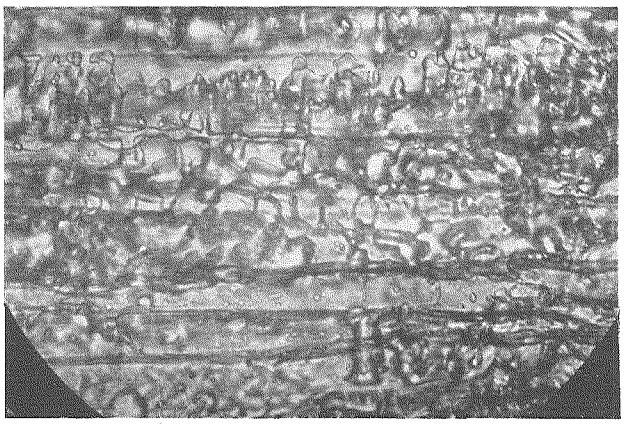


69

Plate XVI.—Intracellular Freezing in Epidermis of *Rhoeo*.

(× 440; exposure 1/2 sec.)

Fig. 72. Frozen cells at −8.7°C, showing the angular net work of coagulated cytoplasm in the surface layer of the cell.
cp, coagulated cytoplasm;
fn, frozen nucleus.

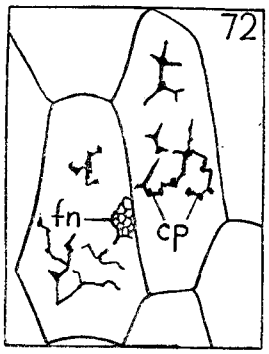


Fig. 73. Frozen cells at −8.8°C, 5 minutes after the "flashing".

Fig. 74. Same cells 8 minutes later, wormed to −4°C.
g, gass bubble; i, ice mass;
s, condensed cell sap.

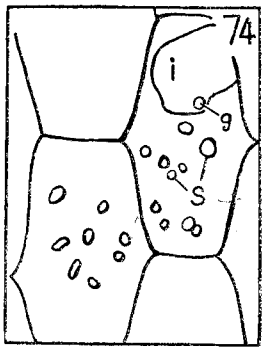
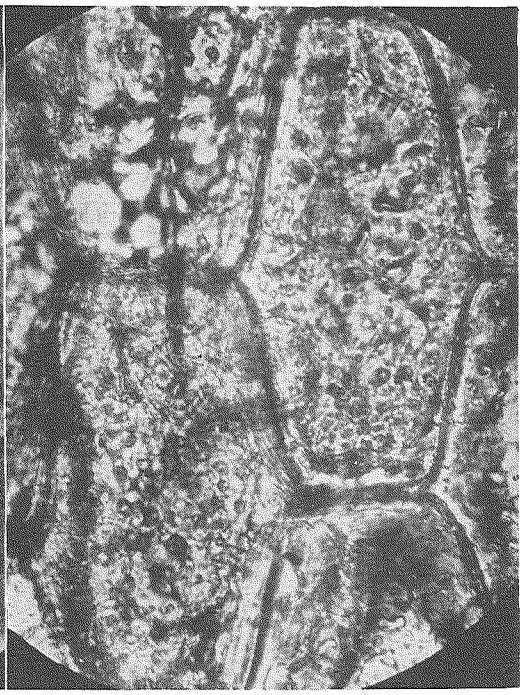


Fig. 75. Same field 4 minutes later, at −2.5°C.

72



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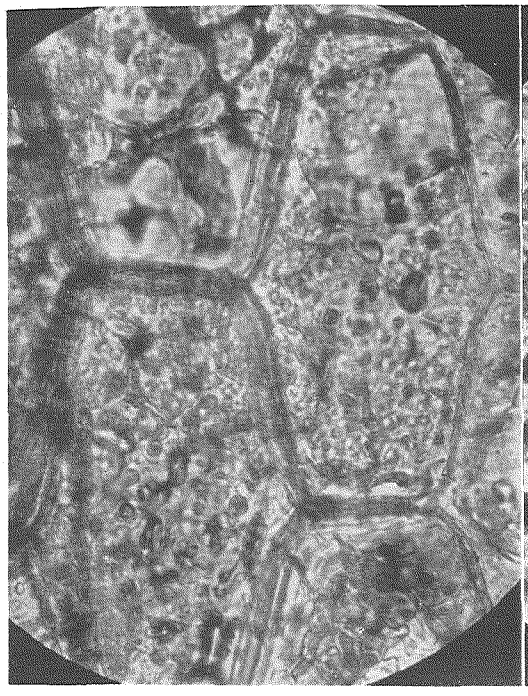

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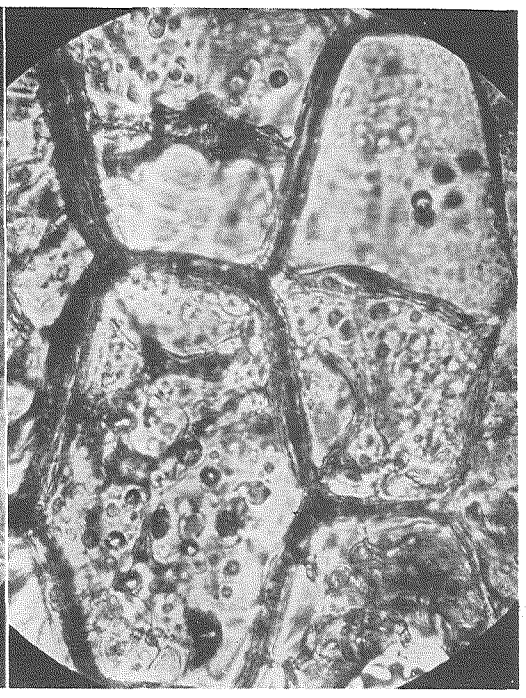


75

Plate XVII.—Thawing in Epidermal Cells of *Rhoeo*.

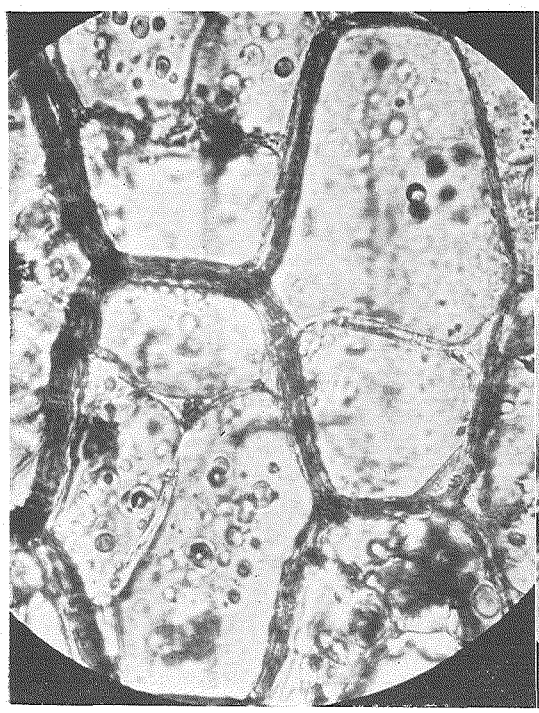
(× 440; exposure 1/5 sec.)

Fig. 76. Same cells as in Fig. 75 of Plate XVI, 3 minutes later, warmed to −1.3°C.

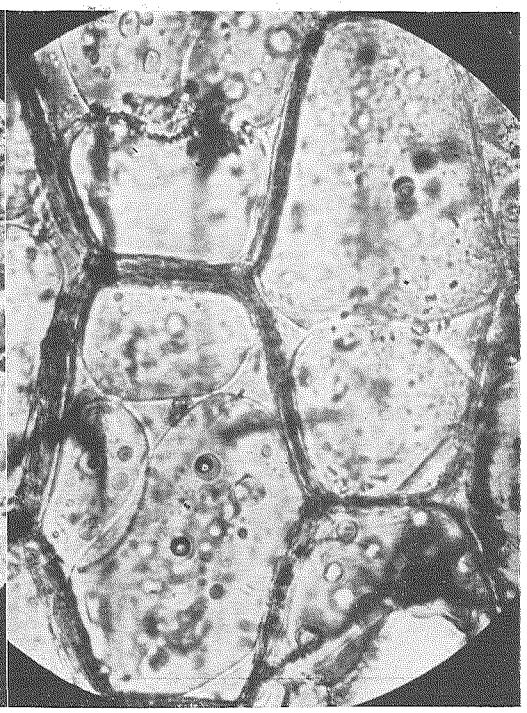
Fig. 77. Same field at −0.9°C, 2 minutes later.

Fig. 78. Same field at −0.3°C, one minute later.

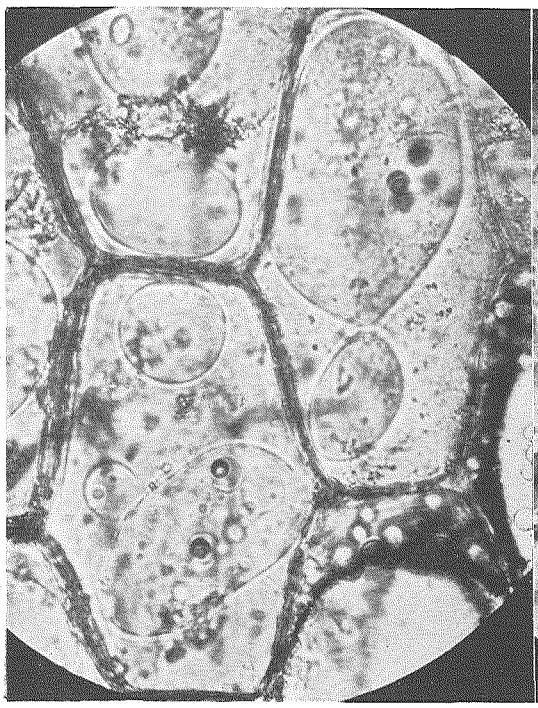
Fig. 79. Thawed cells at −0.1°C, 4minutes later.



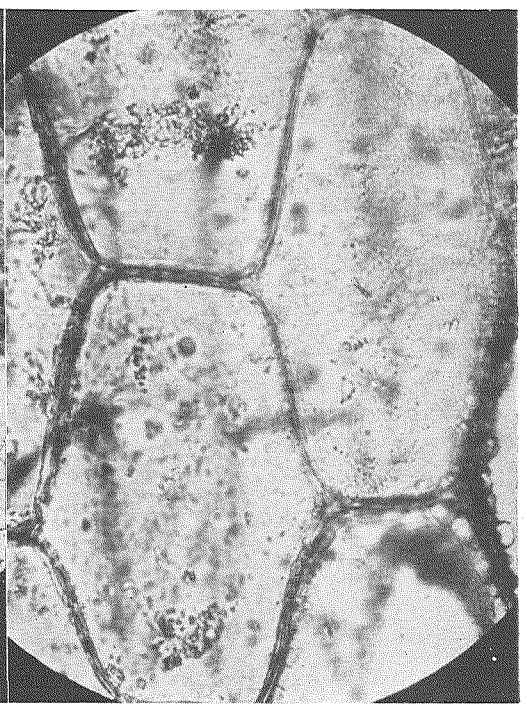
76



77



78



79

Plate XVIII.—Intracellular Freezing in Water Imbibed Cells of *Hardy* Table Beet.

(× 100; exposure 1/25 sec.)

Fig. 80. "Flashing" of the first cell (f_{C_1}), at −4.2°C.
fc, frozen cell; uc, cell yet unfrozen; i, ice.

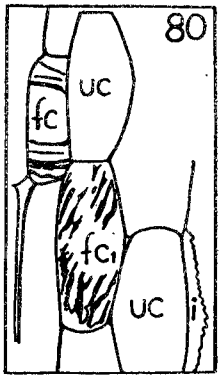


Fig. 81. At −4.6°C, 2.5 minutes later, when the second cell has just frozen.

Fig. 82. At −4.6°C, 6 minutes later. The third cell has already trozen.

Fig. 83. Same cells kept at −15°C for 3 days, showing the transformation of the cell interior.

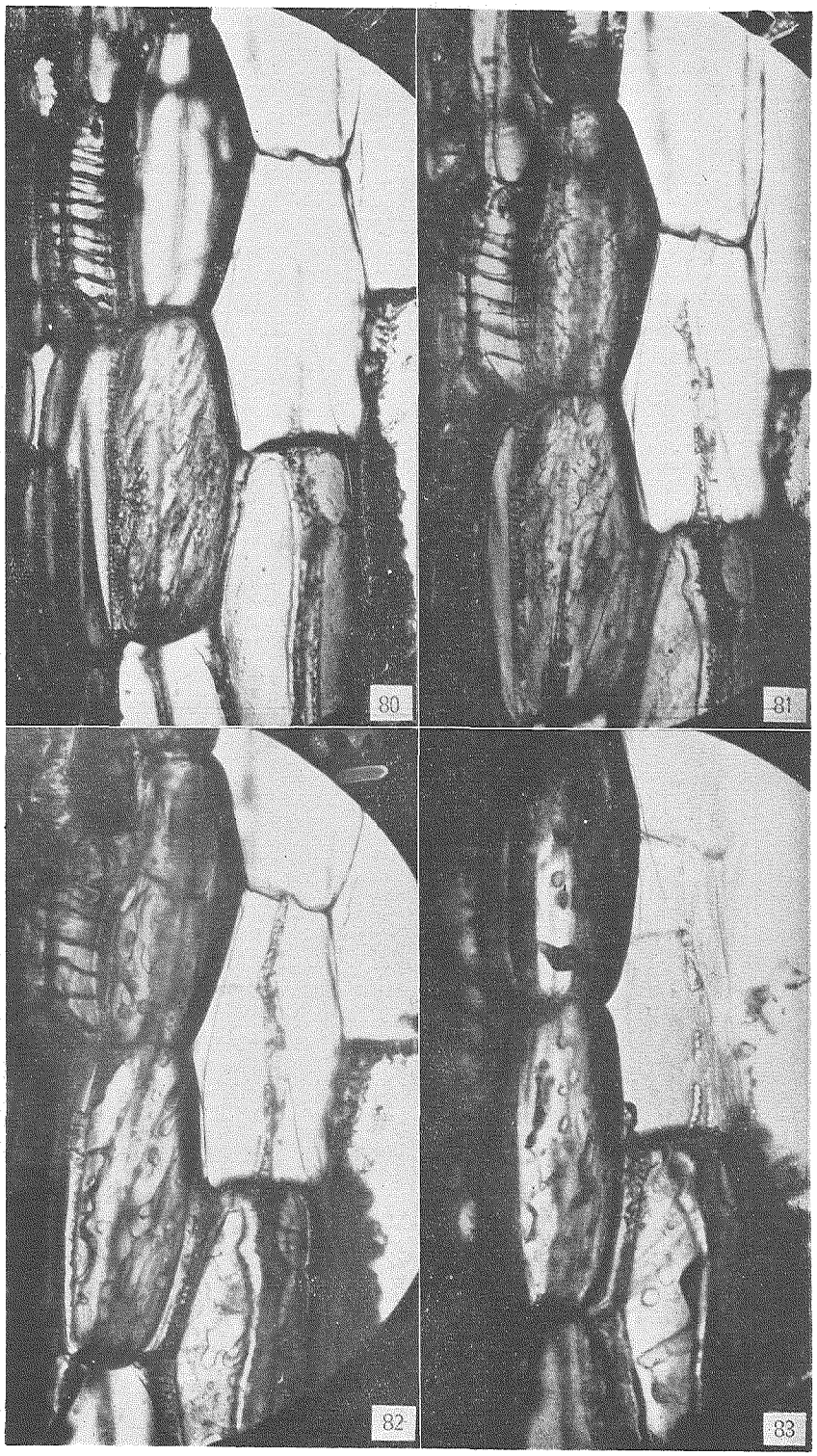

80
81
82
83

Plate XIX.—Thawing of the Frozen Cells of Table Beet.

(× 100; exposure 1/25 sec.)

Fig. 84. Same cells as in Fig. 83 of Plate XVIII kept at −15°C for 5 days and warmed to −7°C.
i, ice; p, condensed sap.

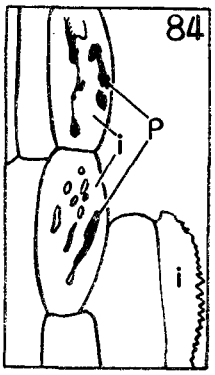


Fig. 85. Thawing at −4°C, 25 minutes later.

Fig. 86. Thawing at −1.4°C, 24 minutes later.
i, ice.

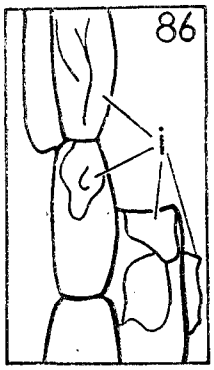
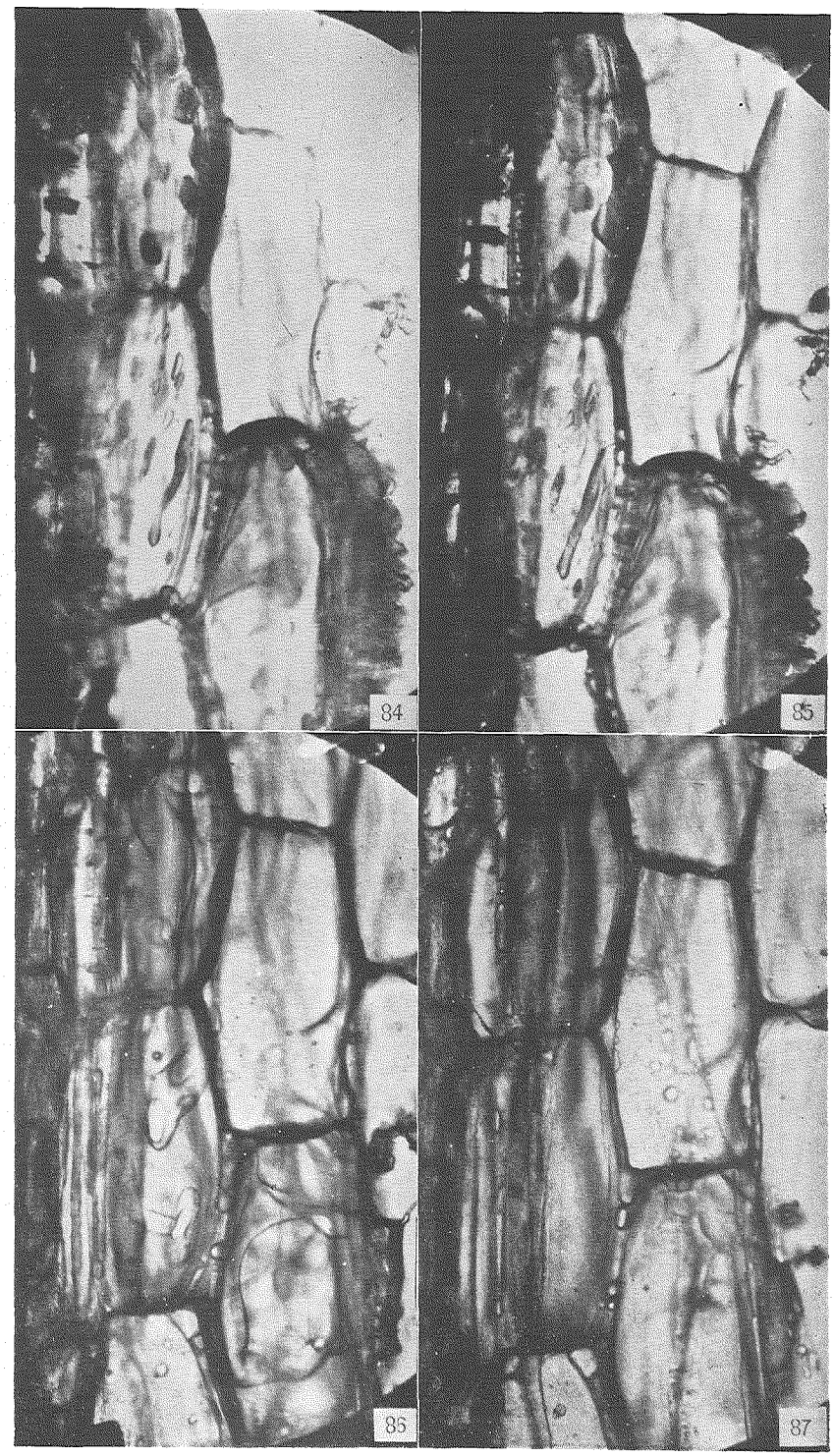


Fig. 87. Just-thawed cells, 9minutes later.


84
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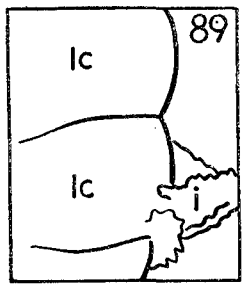
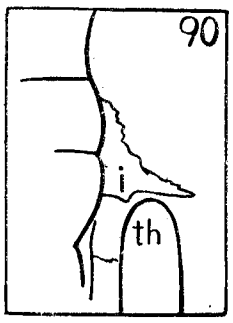
87

Plate XX.—Inoculation with Ice onto the Supercooled Cell of *Hardy* Table Beet.

Fig. 88. Supercooled cells at —4.2°C. (× 270; exposure 1/2 sec.)

Fig. 89. Same cells 15 seconds after inoculation at —4.2°C. Ice rapidly formed at the inoculated point on the cell surface and the tissue temperature was elevated to —3.2°C. (× 270; exposure 1/10 sec.)

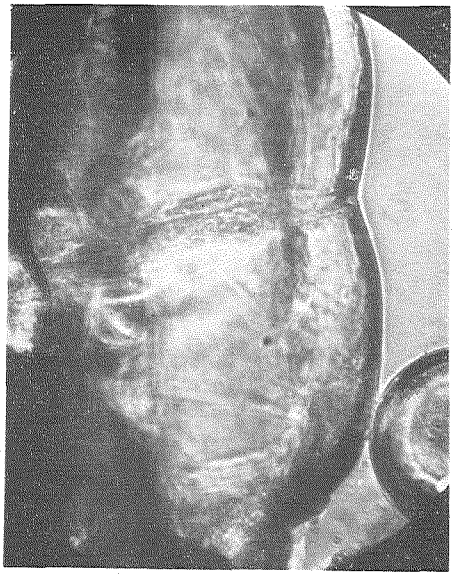




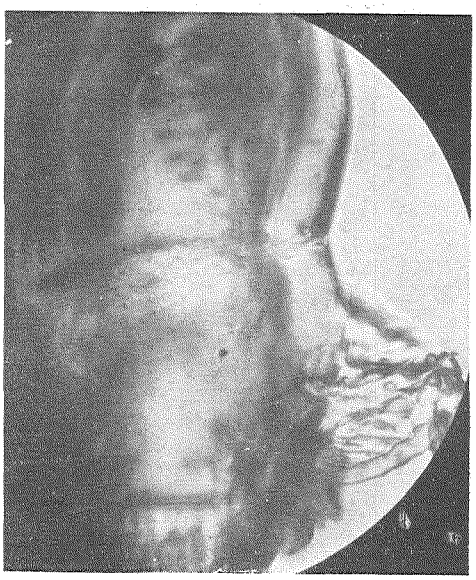
i, extracellular ice mass;
lc, living cell;
th, tip of thermocouple.

Fig. 90. Same cells in low magnification at —3°C, 4.5minutes after inoculation, showing remarkable ice formation on the cell surface. (×100; exposure 1/25 sec.)

Fig. 91. Same field just after thawing; thawed cells appear quite normal. (× 100; exposure 1/50 sec.)



88



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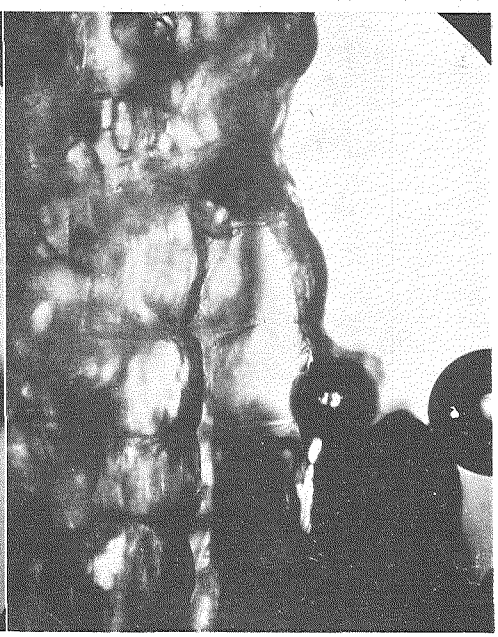


91

Plate XXI.—Intracellular Freezing in Isolated Cell of Tomato Fruit.

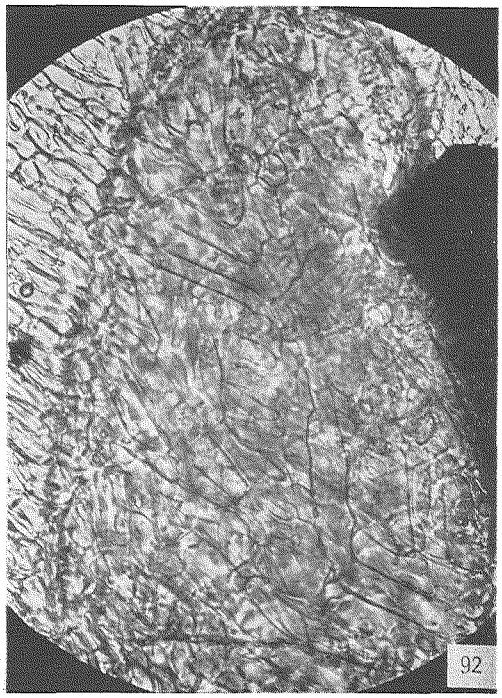
Fig. 91. Supercooled cell at —4.5°C with the tip of thermocouple. (× 100; exposure 1/100 sec.)

Fig. 92. Same cell 20 seconds after the "flashing" at —4°C. (×230; exposure 1/25 sec.)

Fig. 93. Same cell at —3.7°C one minute after the "flashing", showing the spherical particles formed in the frozen cell. (× 230; exposure 1/10 sec.)

Fig. 94. Thawing at —1°C. (× 100; exposure 1/25 sec.)

Fig. 95. Same field at —0.6°C, just before the disappearance of the ice in the cell. (× 100; exposure 1/25 sec.)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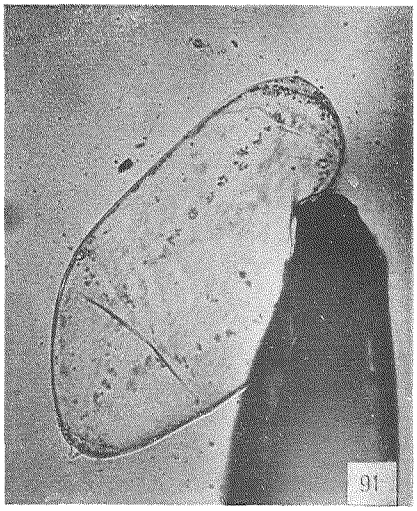
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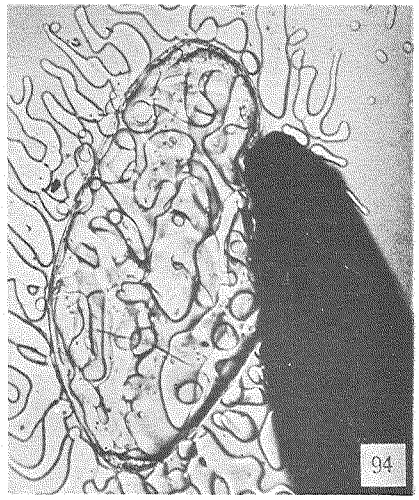
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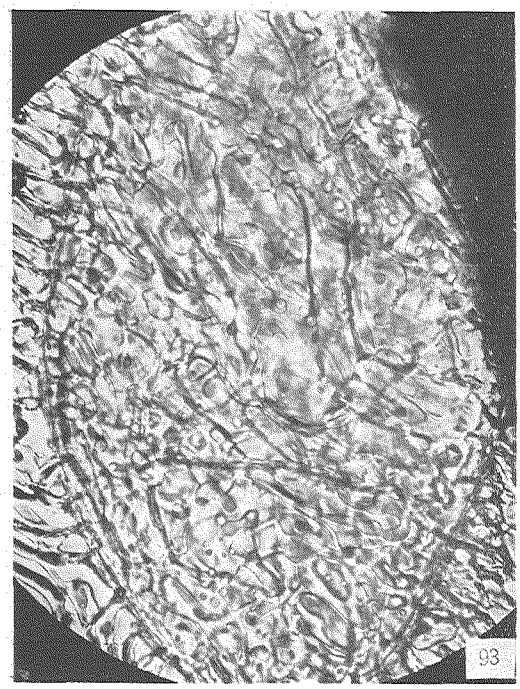
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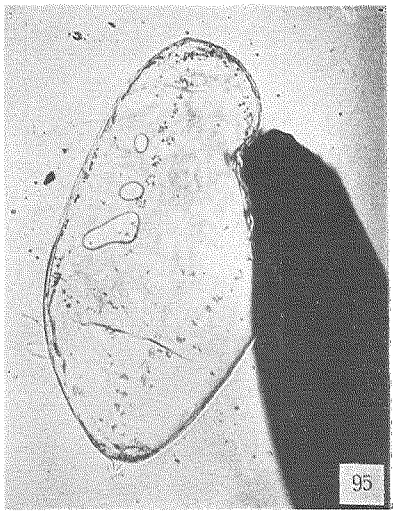

95

Plate XXII.—"Sherical Particle Type Flashing" in Isolated Cell of Tomato Fruit.

Fig. 96. Flashed cell at −3°C. (× 100; exposure 1/25 sec.)

Fig. 97. Details of Fig. 96 at −3.2°C, 12 minutes after the "flashing". (× 230; exposure 1/5 sec.)

Fig. 98. Same field at −2.7°C, 22 minutes after the "flashing". (× 230; exposure 1/5 sec.)
cp, coagulated protoplasm;
fn, frozen nucleus;
s, droplet of condensed cell sap.

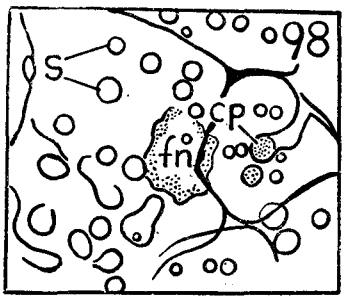
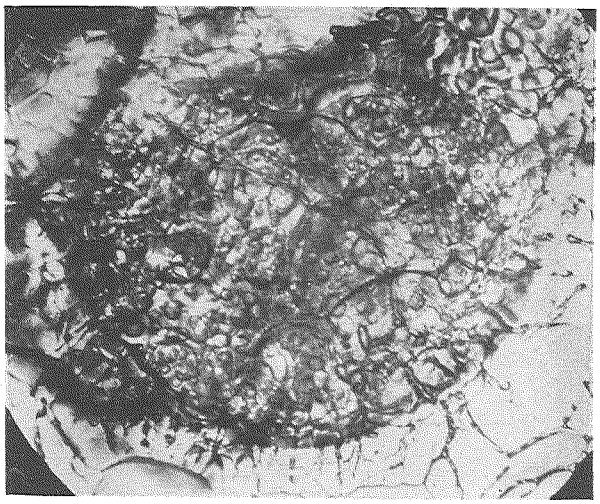


Fig. 99. At −2.5°C, 26 minutes after the "flashing". (× 230; exposure 1/5 sec.)

Fig. 100. At −1.6°C, 30 minutes after the "flashing". (× 230; exposure 1/10 sec.)



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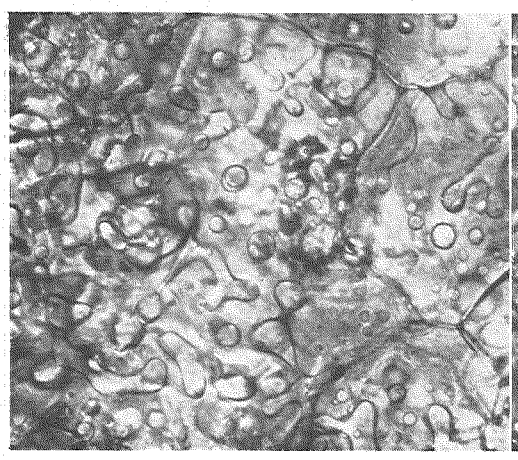
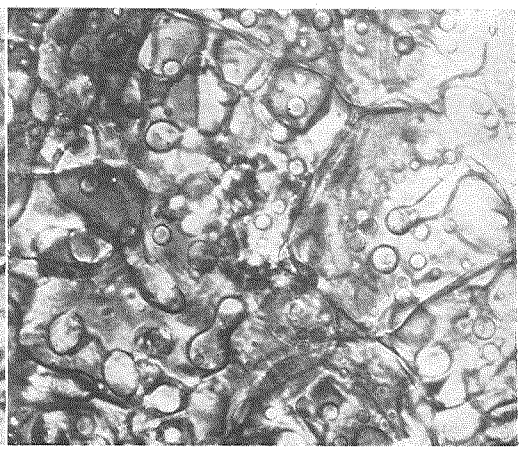
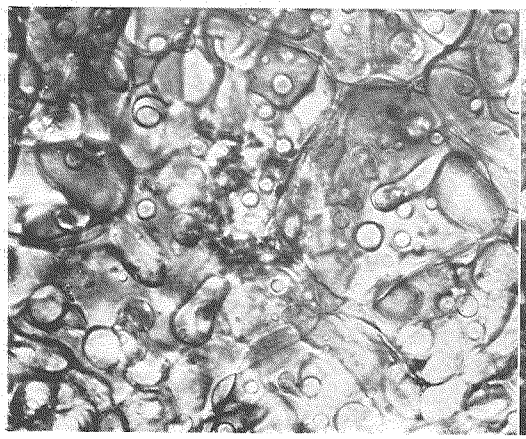


97



98



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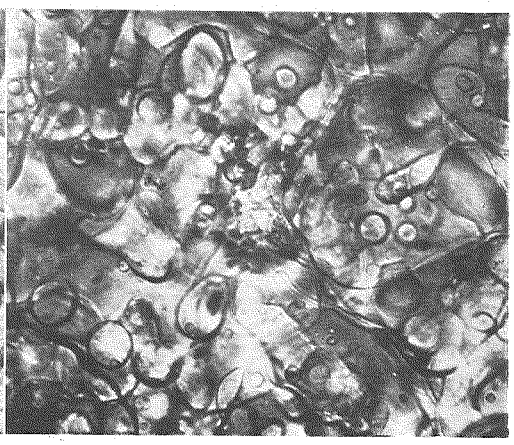


100

Plate XXIII.—Thawing of Isolated Tomato Cell.

Fig. 101. Same field as Fig. 100 in Plate XXII, at −1.2°C, 32 minutes after the "flashing". (× 230; exposure 1/10 sec.)

Fig. 102. Same field at −1°C, 34 minutes after the "flashing". (× 230; exposure 1/10 sec.) cp, coagulated protoplasm; i, ice mass; n, nucleus; s, condensed cell sap.

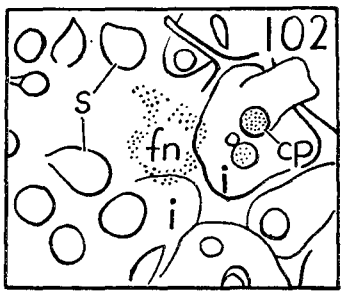
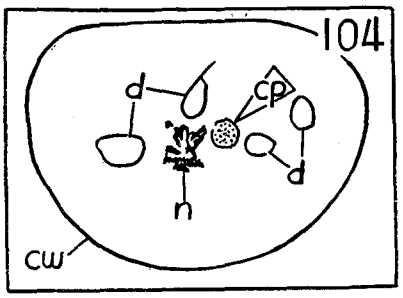
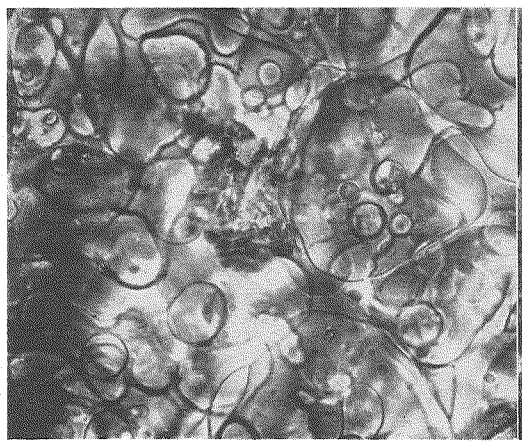


Fig. 103. Same cell in low magnification at −0.6°C, 37 minutes after the "flashing". (× 100; exposure 1/25 sec.)

Fig. 104. Same cell just thawed at −0.5°C, 41 minutes after the "flashing", showing the expansion of a mass of coagulated protoplasm indicated by white pointer. cf. Figs. 102 and 103. (× 100; exposure 1/50 sec.) cp, mass of coagulated protoplasm; cw, cell wall; d, sap drop attached on the outside surface of cell wall; n, deformed nucleus.





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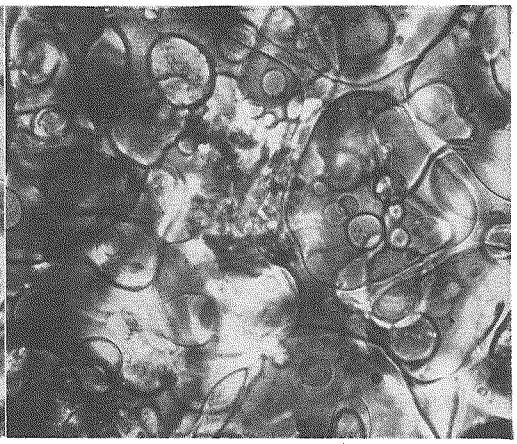

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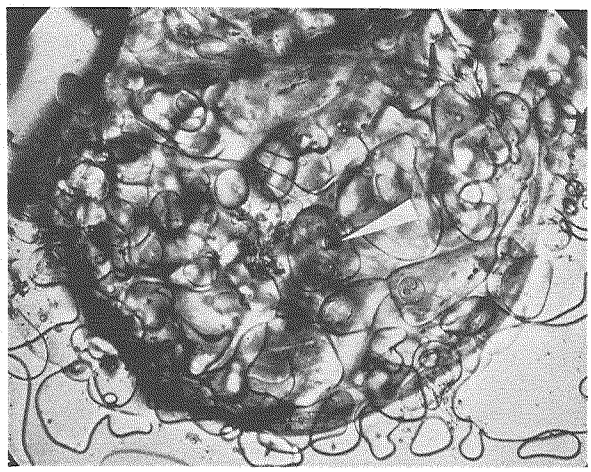

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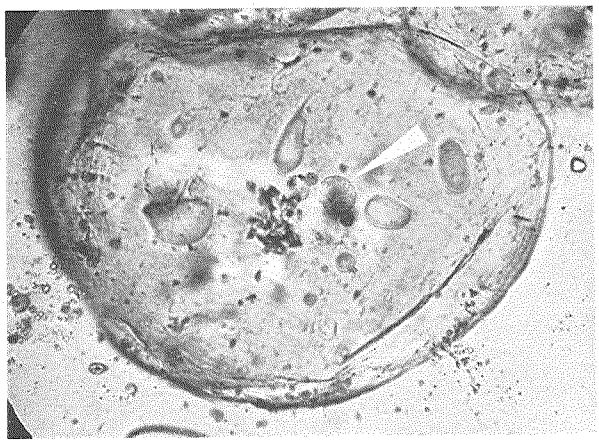


104

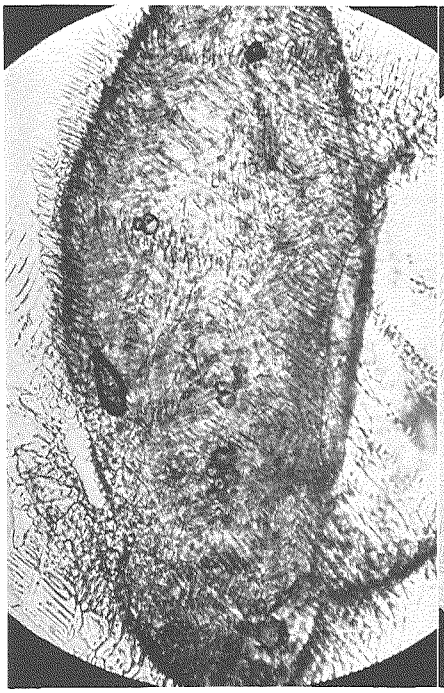
Plate XXIV.—Flashing Nearly Confined within the Vacuole in Isolated Tomato Cell.

Fig. 105. At the beginning of cell freezing at −2.9°C, showing the fine ice crystals forming in the cell. (× 100; exposure 1/25 sec.)

Fig. 106. At −3.1°C, 30 seconds later, when the vacuole was filled with ice crystals. (× 100; exposure 1/25 sec.)

Fig. 107. Details of Fig. 106, showing the unchanged appearance of the nucleus. Neither the net work nor the globule of coagulated cytoplasm is found on the frozen vacuole. cf. Plate XXII. (× 230; exposure 1/5 sec.)

Fig. 108. Same cell just thawed after having been frozen for 18 minutes. Note the appearance of cytoplasmic layer. (× 100: exposure 1/50 sec.)



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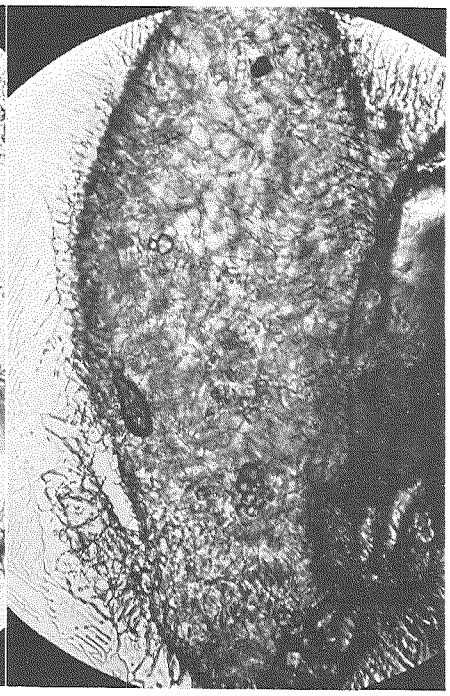

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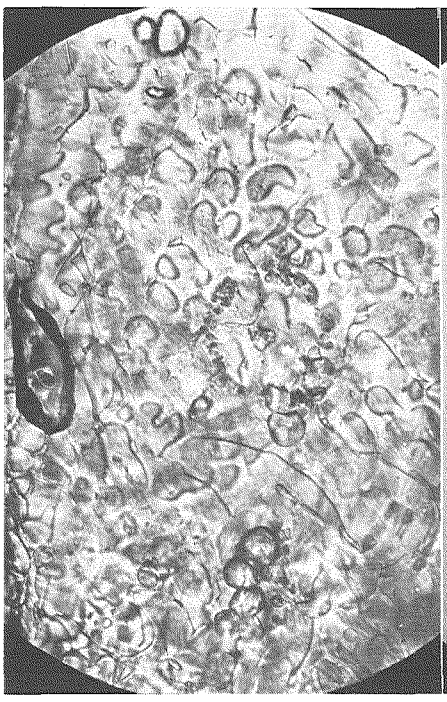

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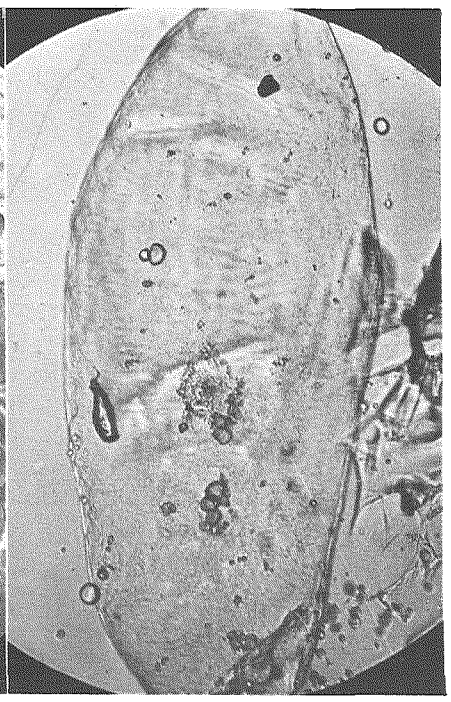


108

Plate XXV.—Flashing in Isolated Tomato Cell at a Low Temperature.

Fig. 109. A frozen cell at −10.2°C, 2 minutes after the "flashing". (× 230; exposure 1/2 sec.)
cp, coagulated protoplasm;
fn, frozen nucleus;
i, extracellular ice crystal;
s, droplet of condensed cell sap.

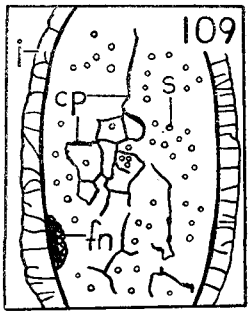
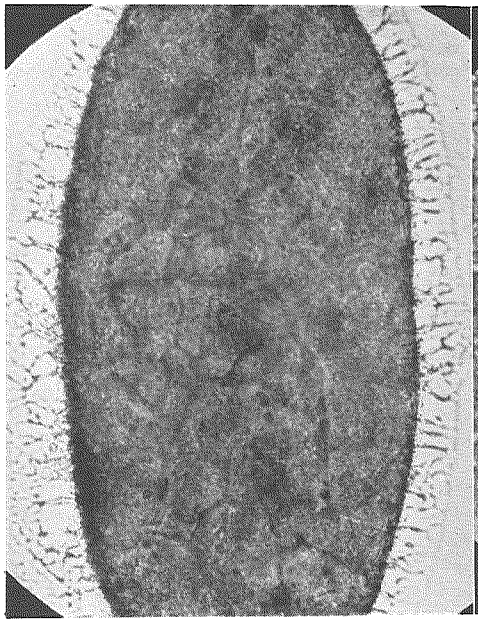


Fig. 110. Details of Fig. 109 at −10°C, 7 minutes after the "flashing". (× 440; exposure 1/2 sec.)

Fig. 111. Same cell at −9°C, 18 minutes after the "flashing", showing the "spherical particles" of condensed sap decreased in number and increased in size.

Fig. 112. Thawing at −1°C, showing the gas bubbles which have appeared in the sap drops in ice mass. (× 230; exposure 1/10 sec.)



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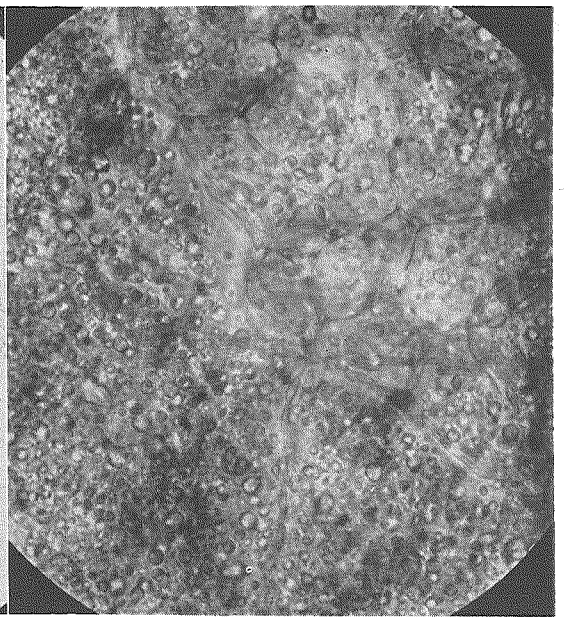

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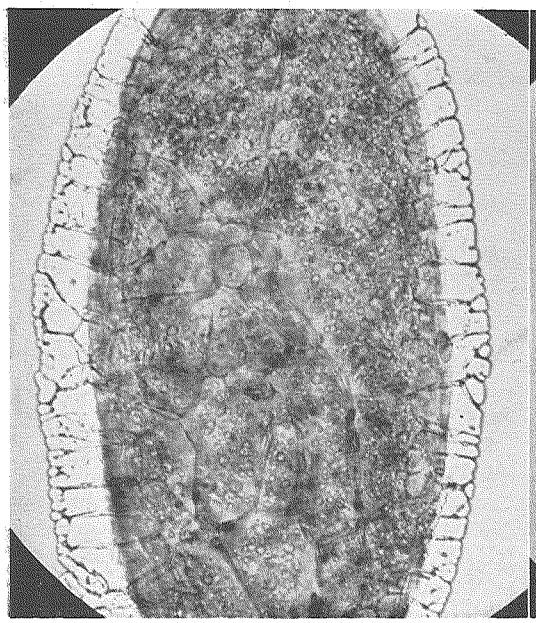

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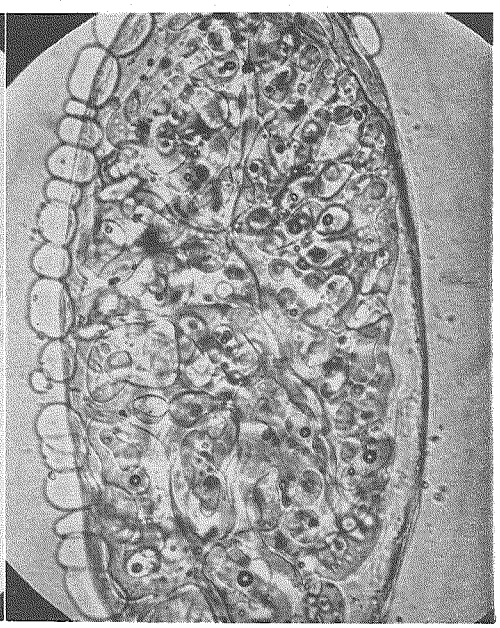


112

Plate XXVI.—Cells of Tomato Fruit.

Fig. 113. Extracellular freezing on a living cell at —3.6°C. (× 100; exposure 1/5 sec.)
i, ice formed on cell surface.

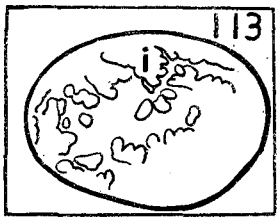


Fig. 114. Extracellular freezing of a cell injured by the freezing. A rapid ice formation occurred after a mild extracellular freezing for 15 minutes at about —2°C. (× 100; exposure 1/10 sec.)
c, injured cell; i, ice crystals; arrows indicating the direction of the jet of ice.

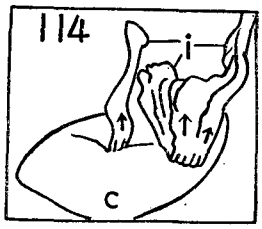


Fig. 115. Ice formation on the surface of cell wall just above the nucleus of the cell frozen intracellularly; photograph taken at —3.2°C, five seconds after the "flashing". (×440; exposure 1/5 sec.)

Fig. 116. Nucleus in a cell frozen extracellularly at —3.2°C, showing the abnormal appearance brought about by the extracellular ice formation. (× 230; exposure 1/25 sec.)

Fig. 117. Normal nucleus in a supercooled cell at —1°C. (× 230; exposure 1/10 sec.)
n, nucleus; nc, nucleolus;
m, grain of metaplasm.

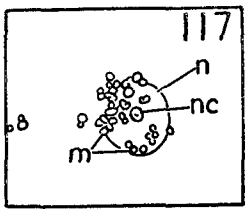
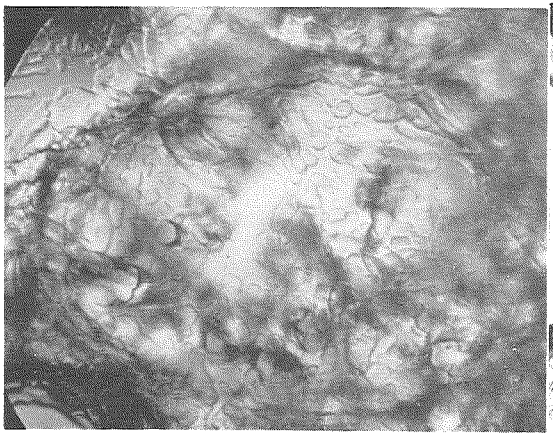


Fig. 118. Disorganized nucleus with the net work of coagulated cytoplasm in a cell thawed soon after the "flashing" at —15°C. (× 440; exposure 1/10 sec.)



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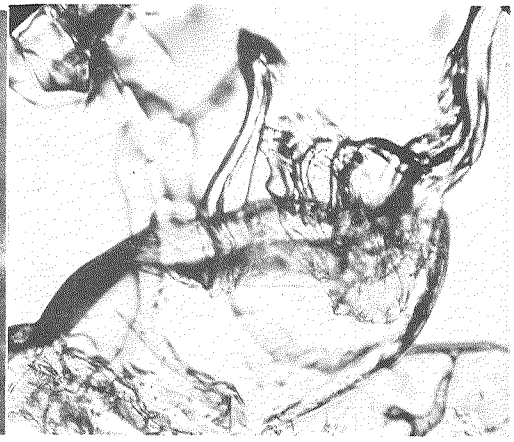

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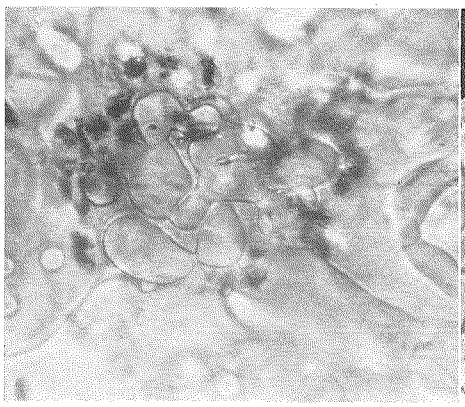

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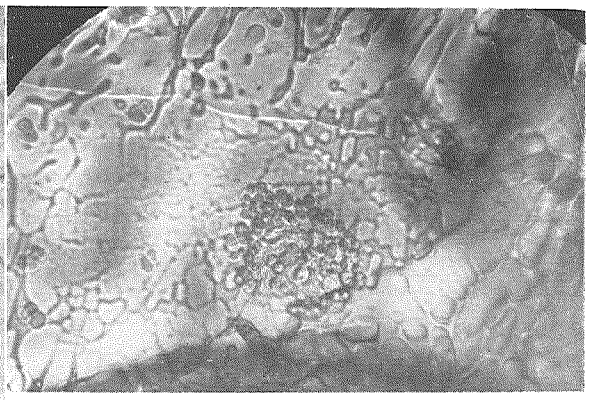

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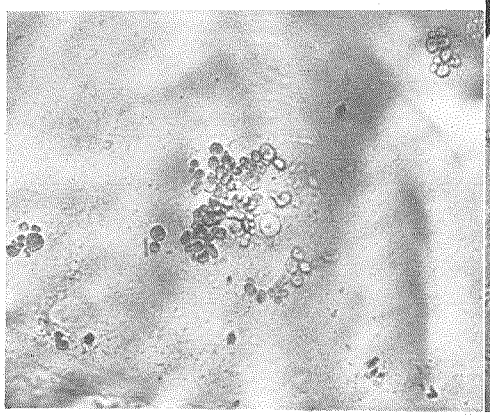

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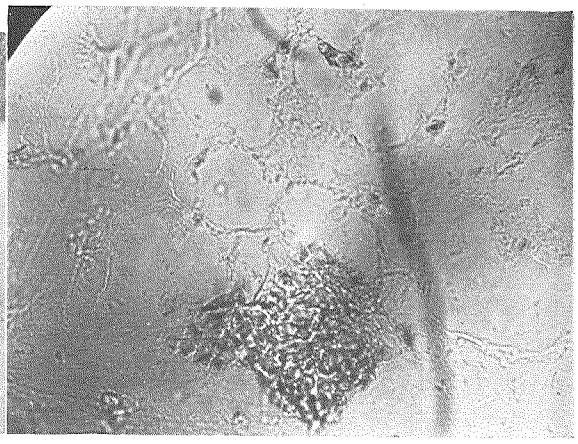


118

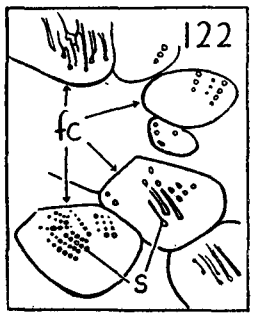
Plate XXVII.—Intracellular Freezing in Tomato Cells.

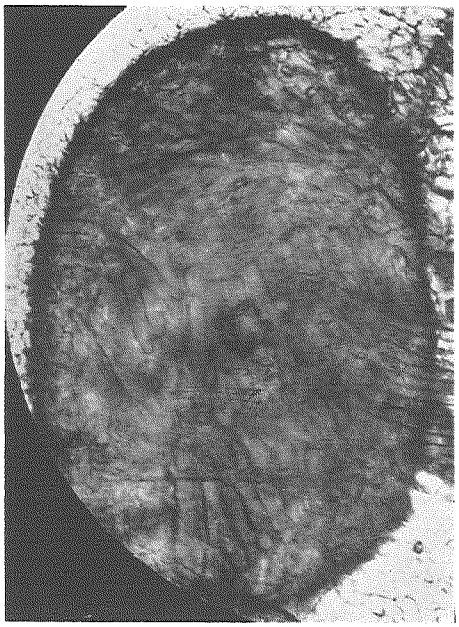
Fig. 119. Isolated cell frozen at —3.8°C, 10 seconds after the "flashing", showing the ice branches developed at first in the cytoplasmic layer. (× 100; exposure 1/25 sec.)

Fig. 120. Detail of Fig. 119; the unfrozen nucleus is deformed by the growing ice crystals. (× 440; exposure 1/5 sec.)

Fig. 121. Detail of Fig. 122 (right), showing the fine filamentous connection between the droplets embedded in ice mass. (× 350; exposure 1 sec.)

Fig. 122. Frozen tissue at —9°C, 17 minutes after the cell "flashing" at a temperature higher than —3°C. (× 100; exposure 1/25 sec.)
fc, frozen cell; s, condensed cell sap.





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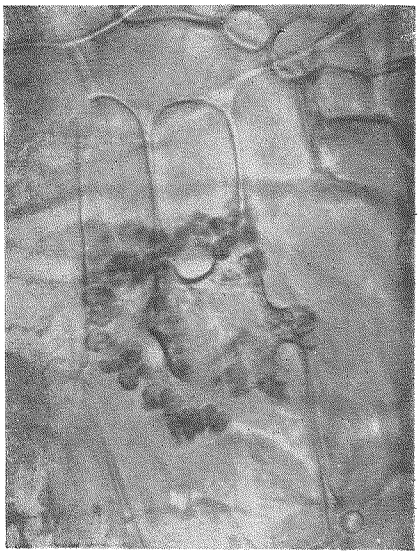

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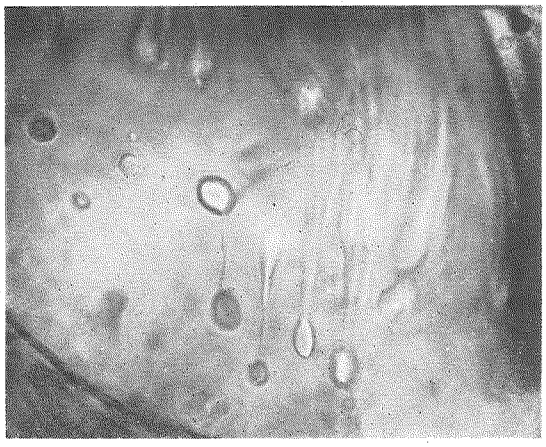

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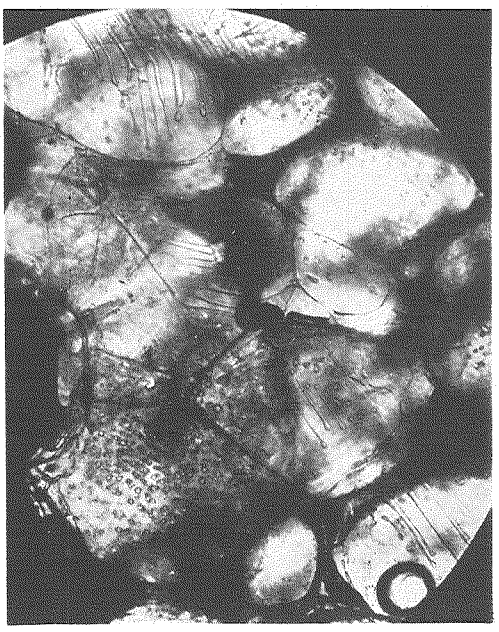


122

Plate XXVIII.—"Spherical-Particle Type Flashing" in Parenchymatous Tissue of Tomato Fruit.

Fig. 123. Detail of Fig. 124 (right) at —9.1°C, 17 minutes after the "flashing" at —10°C. (× 350; exposure 1/2 sec.)

Fig. 124. Frozen tissue at —9°C, about 50 minutes after the "flashing". (× 100; exposure 1/25 sec.)
a, air bubble; dc, cell has been dead before freezing; fc, frozen cell; s, condensed cell sap.

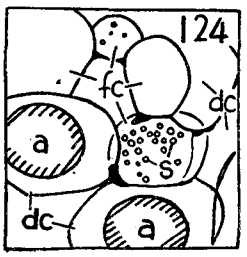
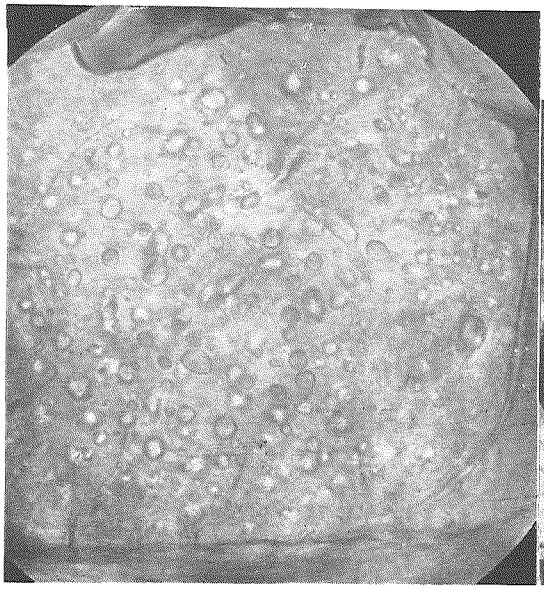


Fig. 125. Same field as Fig. 123 at —8.2°C, 55 minutes after the "flashing". (× 350; exposure 1/2 sec.)

Fig. 126. At —17°C, 17 hours after the "flashing". The cell interior becomes increasingly transparent as the embedded droplets in ice mass decrease in number. (× 350; exposure 1/2 sec).



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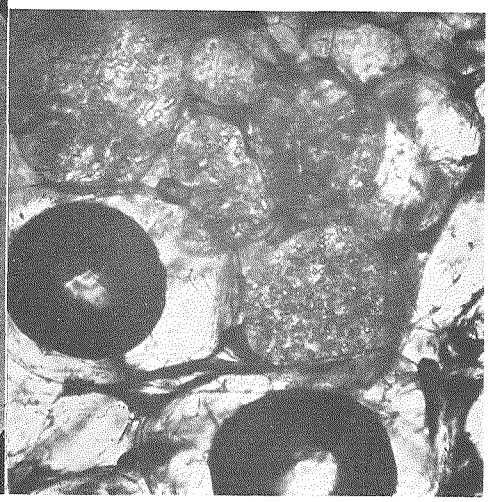

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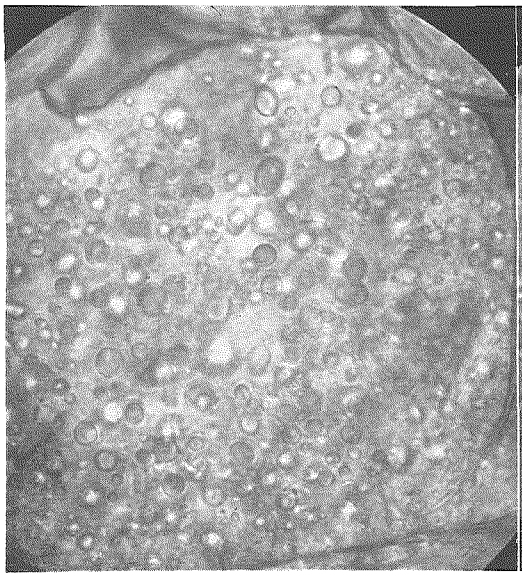


125

126

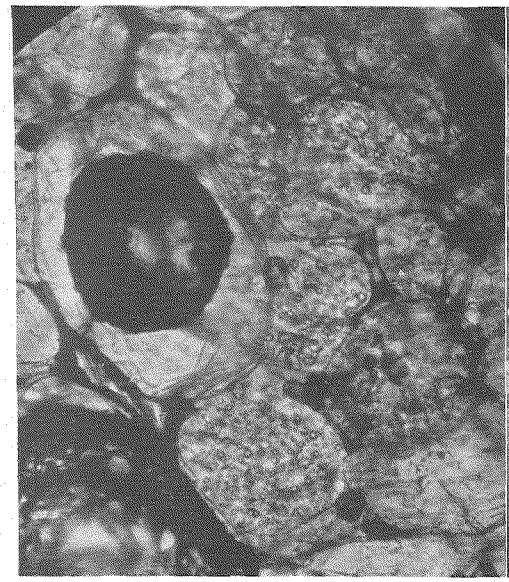
Plate XXIX.—Transformation in the Figure of Flashed Cells of Tomato Parenchyma.

Fig. 127. Same cells as in Fig. 124 of Plate XXVIII, kept at —20°C for 3 days, showing the lightened cell interior. (× 100; exposure 1/25 sec.)

Fig. 128. Details of Fig. 127. (× 350; exposure 1/2 sec.)

Fig. 129. Same cells as in Fig. 127, kept at —20°C for 4 days. (× 100; exposure 1/25 sec.)

Fig. 130. Details of Fig. 129. (× 350; exposure 1/2 sec.)



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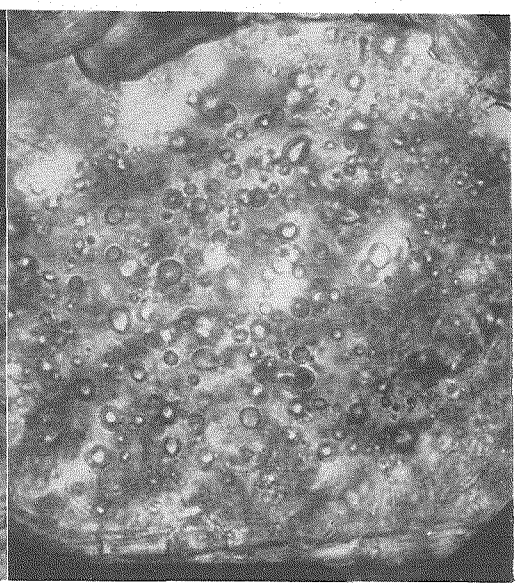

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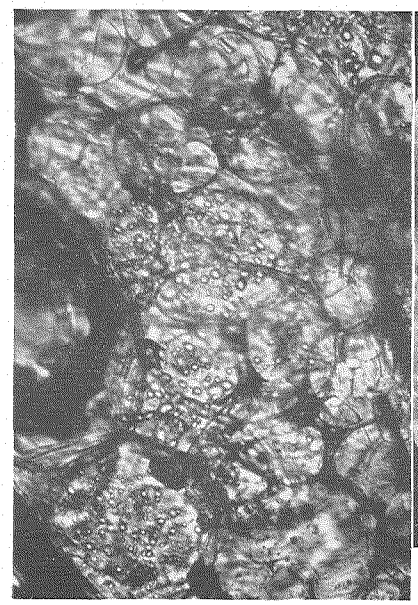

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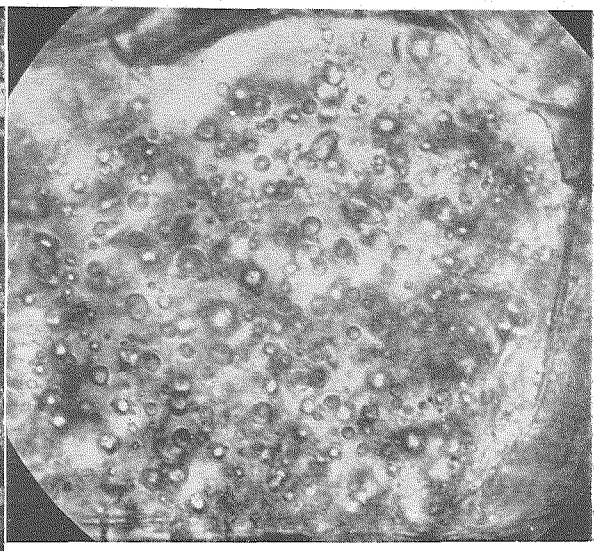


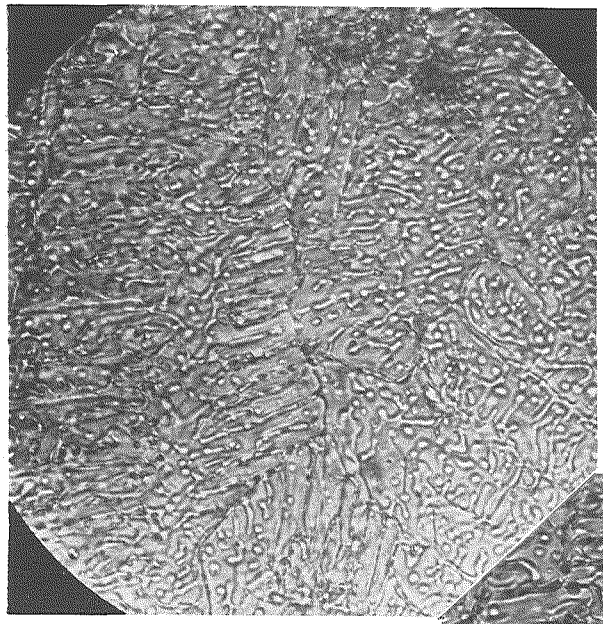
130

Plate XXX.—Process of Freezing of Squeezed Sap of Tomato Parenchyma.

Fig. 131. Frozen sap layer 5 seconds after the inoculation with ice at −7°C, showing veins of sap concentrated between the growing ice branches. (× 350; exposure 1/10 sec.)

Fig. 132. Same field at −7°C, one minute after the inoculation, showing the veins of condensed sap splitted into many droplets. (× 350; exposure 1/10 sec.)

Fig. 133. The phase-contrast photomicrograph of same field at −8°C, 6 minutes after the inoculation. (× 350; exposure 1 sec.)



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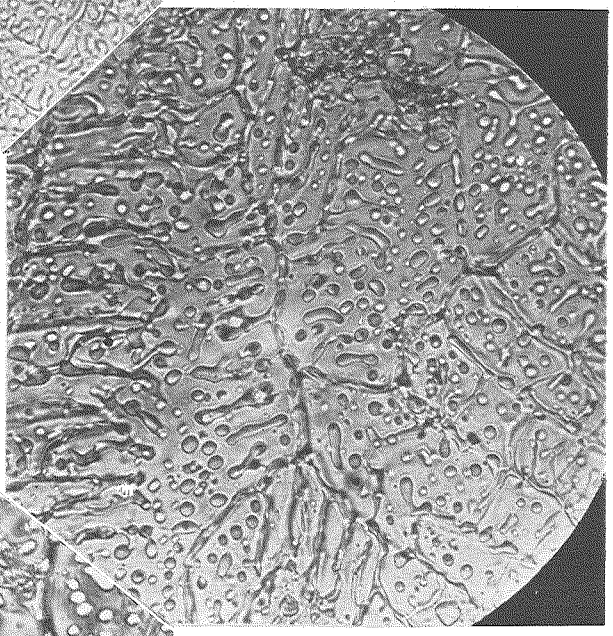

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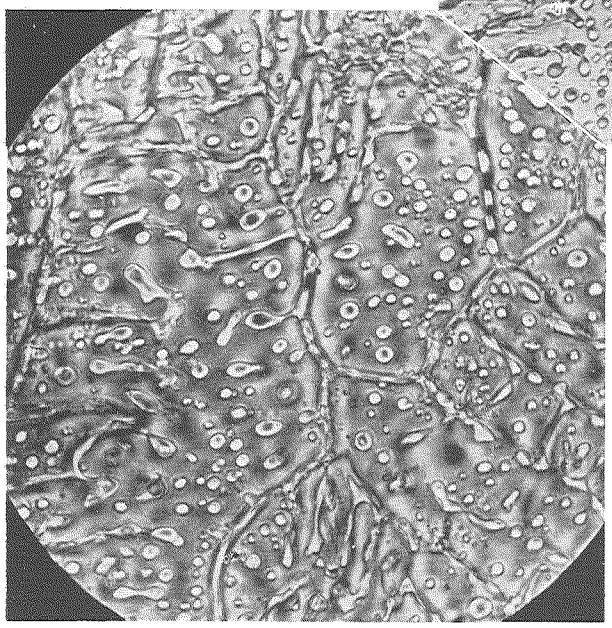


133

Plate XXXI.—Extracellular Freezing in Woody Plant.

Fig. 134. Cross section of leaf of white fir (*Abies*) supercooled at —2.5°C. (× 100; exposure 1/50 sec.)

Fig. 135. Extracellular freezing in the same tissue at —5.2°C, 10 minutes after the inoculation at —2.6°C, showing the ice formation in the intercellular space. (× 100; exposure 1/50 sec.)

Fig. 136. Paradermal section of a leaf of white fir at 1°C. (× 100; exposure 1/50 sec.)

Fig. 137. Same field at —4.6°C, 20 minutes after the inoculation, showing the ice formation in the intercellular spase. (× 100; exposure 1/25 sec.)

Fig. 138. Frozen tissue of cortical layer of red-berried elder at —2°C, showing the remarkably rapid growth of ice crystal curling up at the colder side (upper) of the tissue strip. (×100; exposure 1/25 sec.) i, ice crystal; t, tissue yet unfrozen.

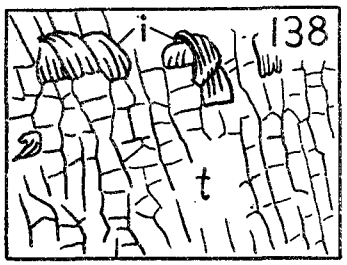
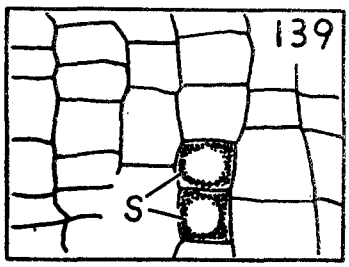
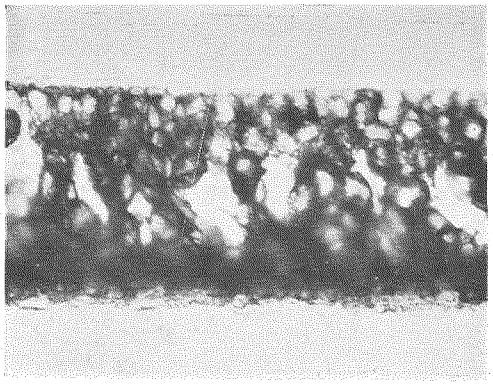


Fig. 139. Frozen cells of vitally stained cortical layer of mulberry tree at —7°C, showing the remarkable flattening of the centre area of each cell due to the dehydration by freezing. (× 350; exposure 1/2 sec.) s, condensed cells sap.





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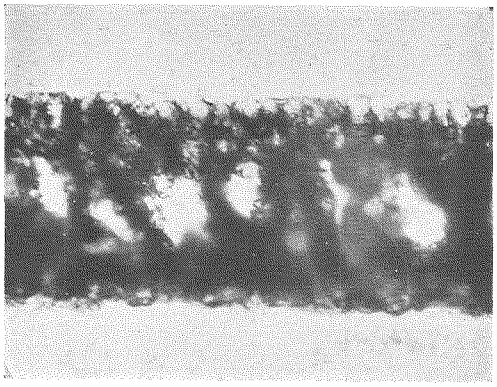

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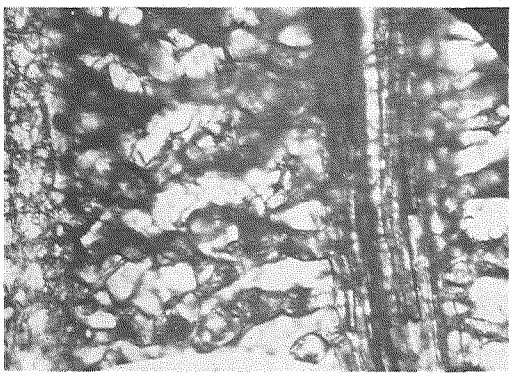

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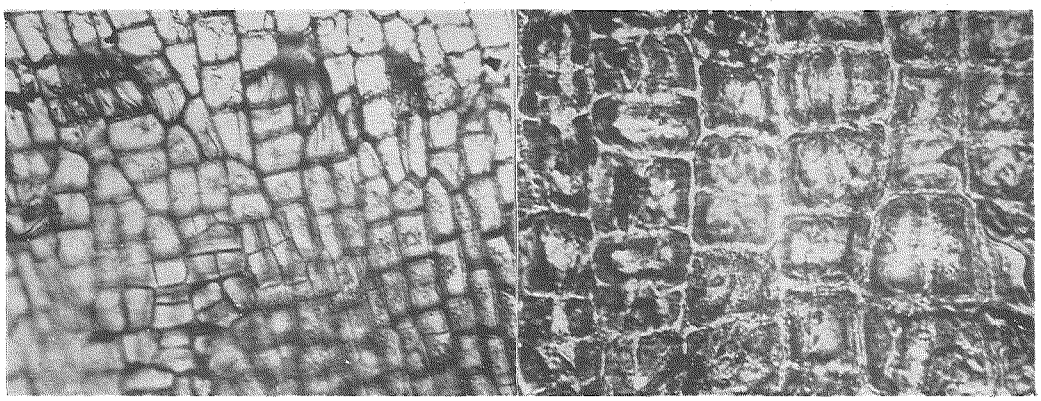

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Plate XXXII.—Freezing in Cortical Cells of Woody Plant.

Fig. 140. Vitally stained cells of *hardy* red-berried elder supercooled at —2°C (× 350; exposure 1/10 sec.)

Fig. 141. Extracellular freezing in same cells at —5.6°C, 9 minutes after the inoculation at —2°C. Note the ice mass formed just above each cell. (× 350; exposure 1/5 sec.)

Fig. 142. Intracellular freezing of young cells of *unhardy* red-berried elder 8 minutes after the "flashing" at —8.6°C. (× 350; exposure 1/10 sec.)
fn, frozen nucleus; s, droplet of condensed sap.

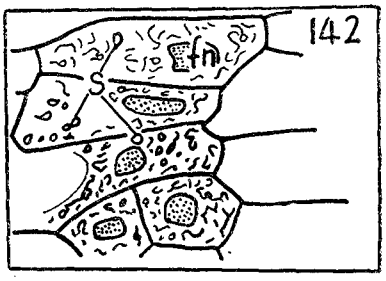
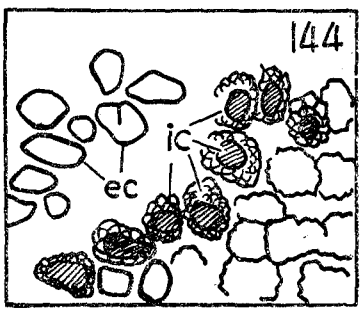
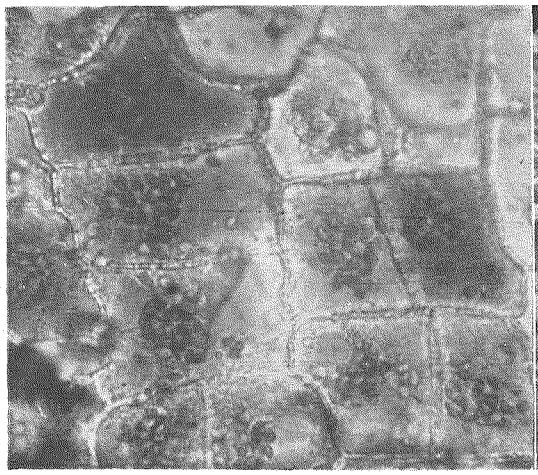


Fig. 143. Intracellular freezing of old cells of *unhardy* red-berried elder 9 minutes after the "flashing" at —6°C. (× 350; exposure 1/5 sec.)

Fig. 144. Intracellular freezing in very *hardy* cells of apple at —8.2°C, 6 minutes after the inoculation at —15°C, showing the foamy appearance of frozen cytoplasm around the unfrozen vacuole. (× 350; exposere 1/2 sec.)
ec, extracellularly frozen cell;
ic, intracellularly frozen cell.





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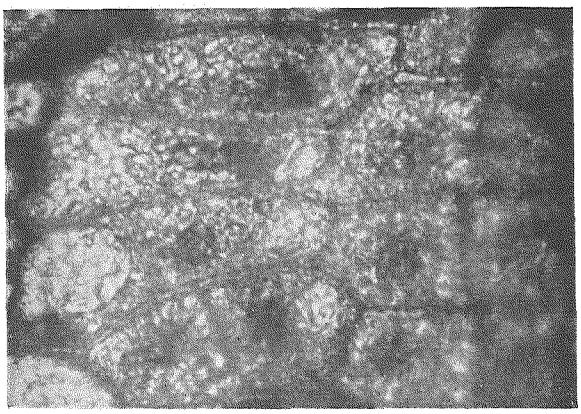

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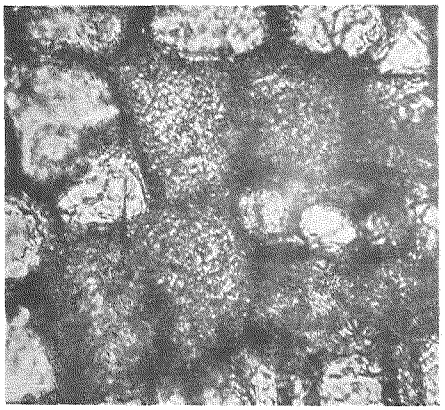

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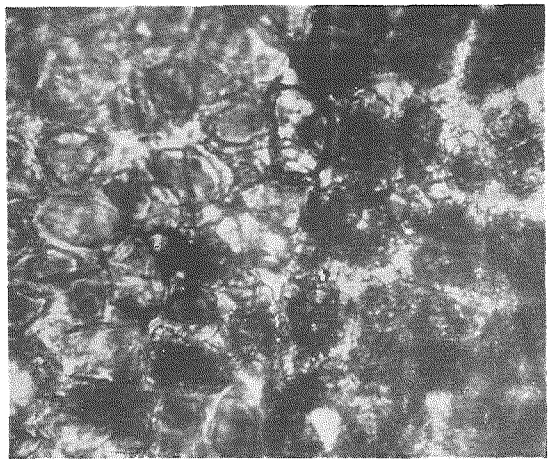

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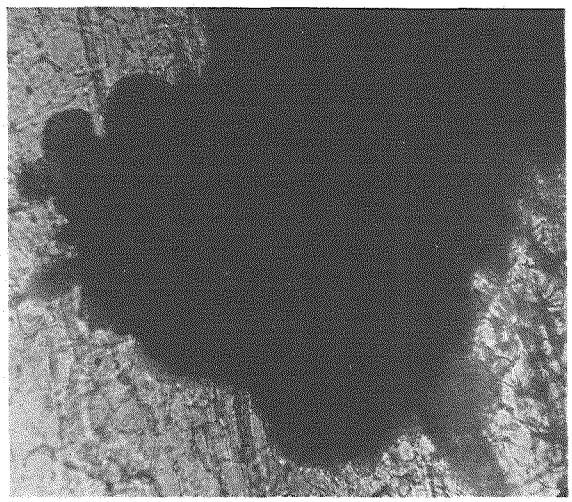
Plate XXXIII.—Freezing in Plasmodium of Slime Mould.

(× 300; exposure 1 sec.)

Fig. 145. Frozen plasmodium at −10°C, 11 minutes after the "flashing" at −7°C.

Fig. 146. Same plasmodium warmed to −1°C, 33 minutes after the "flashing".

Fig. 147. Plasmodium thawed after having been frozen at −10°C for 10 minutes.



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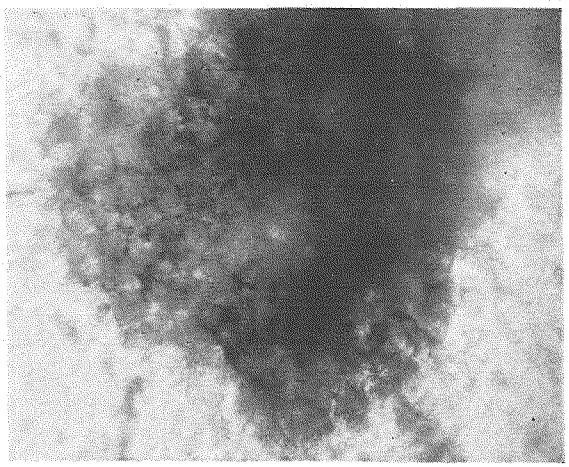

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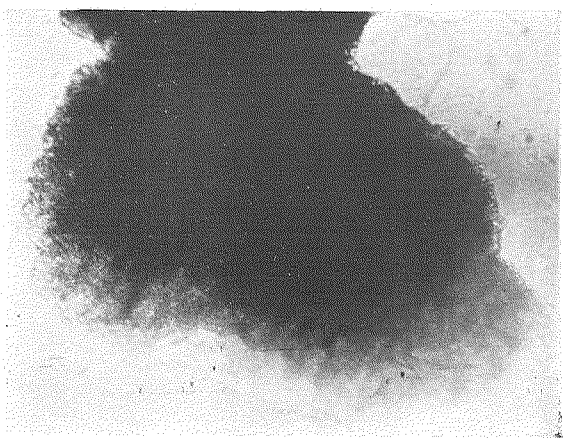

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Plate XXXIV.—Freezing in Plasmodium of Slime Mould.

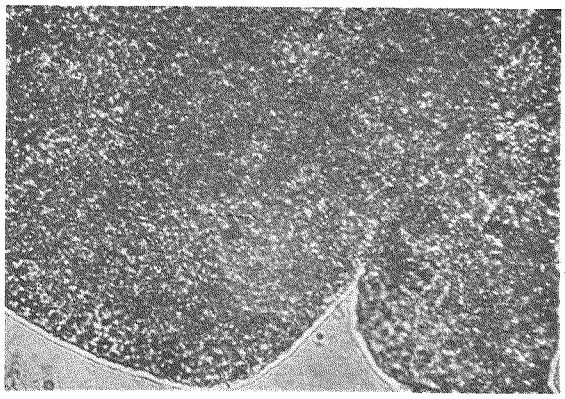
Fig. 148. Plasmodium before freezing. (× 300; exposure 1/2 sec.)

Fig. 149. Same field 4 minutes after the freezing of the plasmodium at —0.8°C. (× 300; exposure 1/2 sec.)

Fig. 150. Same field just after thawing, showing the disorganized plasmodium. (× 300; exposure 1/5 sec.)

Fig. 151. Frozen strand of the "reticulum" of plasmodium, 8 minutes after the freezing at —4°C. (× 100; exposure 1/25 sec.)

Fig. 152. Same strand thawed at 0°C after having been frozen at about —3°C for 42 minutes. (× 100; exposure 1/25 sec.)



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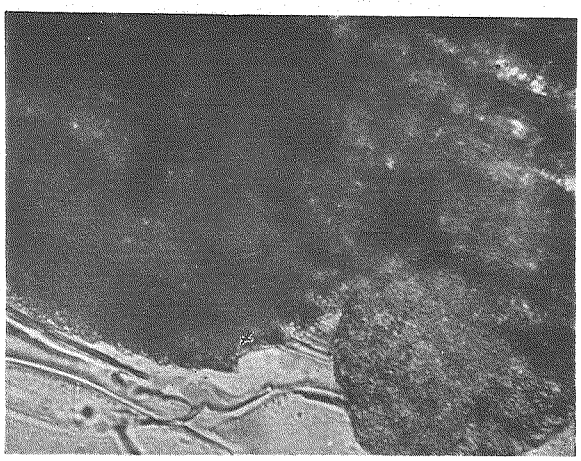

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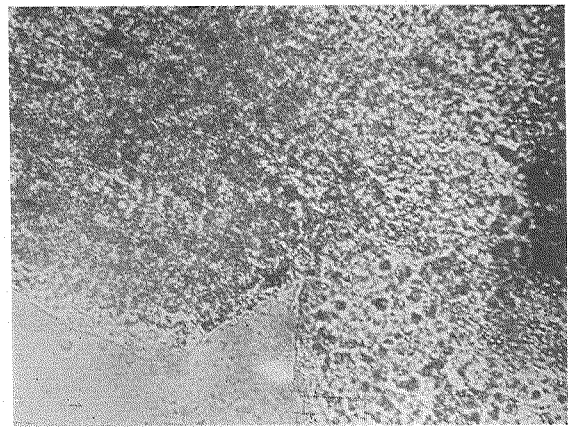

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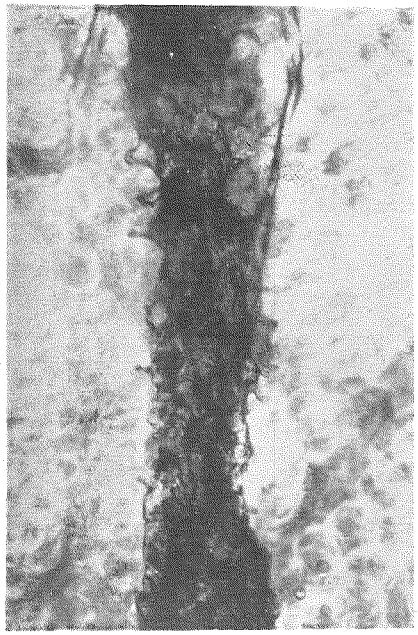

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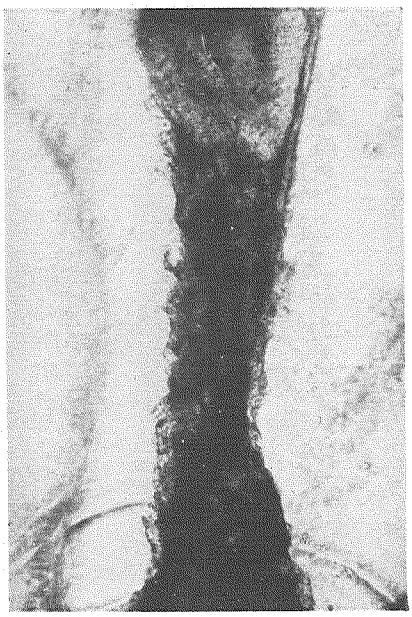

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