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Studies on the Freezing of Living and Dead Tissues of Plants, with Special Reference to the Colloidally Bound Water in Living State

By

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It has been known that, when various plant tissues are cooled sufficiently, the temperature in the tissue goes up rapidly when the supercooling is broken, to fall soon and then to reach a value which lasts for a while (AOKI, 1–6; LUYET and GEHENIO, 22, 23; MAXIMOV, 25; and ZACHAROWA, 33). The first maximum is called the first freezing point and the temperature level following it the second freezing point. There have been many discussions about the cause of the double freezing.

MAXIMOV (1914) and ZACHAROWA (1926) demonstrated that the first freezing point was that of the liquid around the thermocouple, coming out of cells injured by the thermocouple. LUYET and GEHENIO (1937) pointed out that the freezing point of the intercellular liquid was exactly what was called the first freezing point. Their results, however, do not seem to be accurate enough since they used a mercury thermometer. AOKI (1946) stated that the freezing of the damaged surface layer of the tissue piece came out as the first freezing point, and the freezing of the inner living part as the second one. The second freezing is generally accepted to represent the freezing of living cells, either when the intracellular water diffused out of cells to be frozen or when the cell liquid is frozen inside cells.

The explanations so far proposed for the double freezing do not sufficiently explain the fact that it occurs only in living tissues but never in dead tissues.

The fact that the freezing point of the living tissue is lower than that of the dead tissue has also been widely recognized (AOKI, 4, 5; CURRIER, 10; LEWIS and TUTTLE, 21; LUYET and GEHENIO, 22, 23; review by CRAFT *et al.*, 9). WALTER (31) and WALTER and WEISMANN (32) insisted that the osmotic value of the living tissue must be the same as that of the dead tissue, and it was the lack of constant stirring that made the freezing point lower than the true value, in the case of living tissues.

The present paper reports some critical experiments which contribute to

these problems. The results were partly published in Japanese previously (16, 17 and 18).

Methods and Materials

A fine wedge-shaped thermojunction consisting of copper and constantan wires was used, each wire being 0.17 mm in diameter. This thermojunction was useful in measuring rapid and minute changes of temperature, since its heat capacity was calculated to be only 0.000115 g cal., assuming the part of the junction inserted in the tissue to be 3 mm long (HATAKEYAMA, 14). For more delicate measurements such as in cases of *Picea, Torreya* and *Nitella,* copper and constantan wires of 0.06 mm diameter were used. According to calculation, the heat capacity of the part inserted in the tissue is 0.000014 g cal., and the ice formation needed for raising its temperature by 1°C is only 0.00018 mg.

A small block of tissue was hung, by the thermojunction inserted in it, within a water-tight glass cylinder of 2 cm diameter, and this in turn was immersed in the freezing mixture of crushed ice and sodium chloride, at -10 ± 1 or -15 ± 1°C. Temperature of the sample was measured with a galvanometer every 15 seconds. Ice crystal was not applied to make freezing start, but spontaneous freezing was awaited. After about 15 minutes of freezing the glass cylinder was removed from the freezing mixture to let the sample packed hanging in it be thawed at room temperature. After about 5 minutes the glass cylinder was put back in the freezing mixture again and the temperature change was observed. Freezing and thawing were repeated several times in this way.

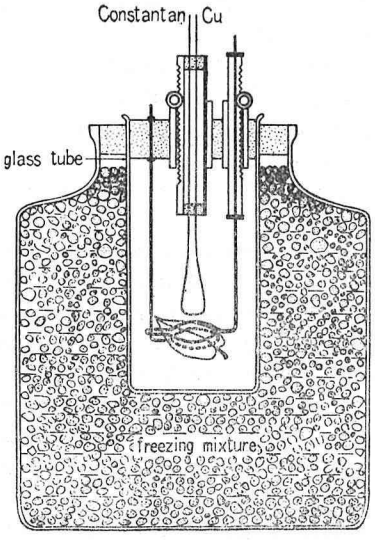


Fig. 1. Apparatus for measuring the freezing curve of a broad leaf. Cu: Copper wire, Constantan: Constantan wire.

The broad leaf used was too thin to insert a thermojunction in it. So a laminate thermojunction, 0.08 mm thick, 0.28 mm wide and 8 mm long, was used. It was lightly pressed on the surface of a leaf held as shown in Fig. 1. The part of the junction to be kept in contact with the leaf surface being 6 mm long, its heat capacity was calculated to be 0.000115 g cal.

Correction for the supercooling was made with a formula $\Delta=\Delta'\left(1-\frac{t}{80}\right)$ (where Δ=true freezing point, Δ'=observed freezing point, t=degree of supercooling).

A mercury thermometer for microcryoscopy was used to measure the freezing point of the sap expressed from heat-killed material (HATAKEYAMA, 15).

In the case of determining the freezing curves of pure water and expressed cell sap, the pith tissue of *Sambucus sieboldiana* or a small ball of absorbent cotton, purified by boiling in pure water for several hours, was infiltrated with them. As living tissues, about 5×5×10 mm cubic sections were cut from homogeneous part of tubers of potato (*Solanum tuberosum* (*danshaku*)), sweet potato (*Ipomoea batatas* (*okinawa* No. 100 and *gokoku*)) and taro (*Colocasia esculenta*). Fruit of banana (*Musa paradisiaca* subsp. *sapientum*) was also used. Tiny potato tuber that was about 5 mm in diameter was used as a sample of intact tissue material without any cut surface.

To obtain tissues killed by other means than freezing, material was heated by immersing an air-tight container in boiling water for 20 minutes, or it was exposed to chloroform gas being suspended in an air-tight container for 27 hours. The "unhealthy" material used was the whole tuber submerged in 1% acetic acid for 4 days. Tissue brei was prepared by rapid grinding in a glass-mortar and a thermojunction was inserted in a small ball of it wrapped in sulphate paper. Brei was also prepared in the same way from tissues treated with chloroform or acetic acid, though the cell wall grew tough owing to the treatment.

For leaf tissues, *Cucurbita moschata* var. *toonas*, *Diospyros kaki*, *Camellia sinensis*, *Buxus microphylla* var. *japonica*, *Picea excelsa* and *Torreya nucifera* were used. The internodal cell of *Nittella* sp. was used as a sample of single cell. As vacuolar saps, the juice expressed from the internodal cell of *Nitella* sp. and that from the vesicle of summer orange (*Citrus natsudaidai*) were used.

Results

The freezing curve of pure water held in a piece of *Sambucus* pith tissue, which was composed mostly of cells about 170×145μ in size and having numerous pits of 2–5μ diameter on the walls, or in a small mass of absorbent cotton was simple as to be expected (Fig. 2 A, B). And since the freezing point observed was just at 0°C, the method used is considered to be reliable.

When tissue cubes were prepared from potato tuber, a considerable amount of sap was seen squeezed by cutting pressure. Such a sample as this, which

contained much liquid in the intercellular space before cooling, often showed double freezing points (Fig. 2 C). The double freezing was found also with an intact tiny potato tuber which was considered to hold much intercellular liquid (Fig. 3 A), while it was not observed by AOKI (5).

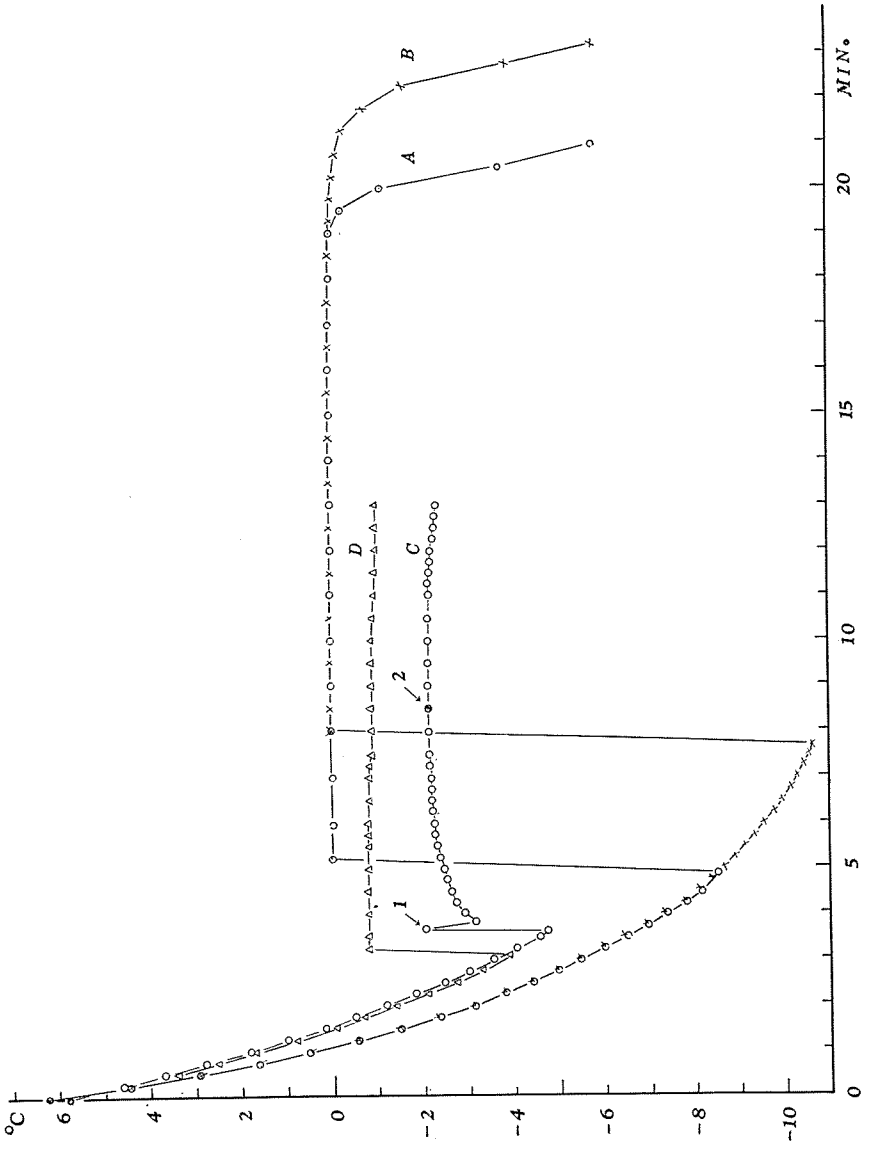
Many workers (MAXIMOV, 1914; WALTER and WEISMANN, 1936; LUYET and GEHENIO, 1937; and AOKI, 1946) observed the double freezing in excised potato tuber tissue, while KUME (1948) did not. The latter author ascribed the difference to the fact that, in his case, the surface of tissue piece was wiped and dried for a while. The present author, using excised potato tissue, observed the double freezing in seven cases out of nineteen, and irregular freezing curves showing local freezing in two cases (Fig. 3 C). The water content of the samples showing the double freezing varied from 375 to 397 per cent, while that of the samples which did not show it from 340 to 404 per cent. Thus with potato there seemed to be no correlation between the double freezing and the total water content on a dry weight basis. The double freezing phenomenon may be correlated rather with the amount of intercellular water than the total water content of whole tissue.

Most cultivated varieties of sweet potato so far observed did not show the double freezing except *Okinawa* No. 100 and *Taihaku*, which were somewhat juicy and contained a considerable amount of exuded liquid in the intercellular space. They showed the double freezing more prominently and more frequently than potato (Fig. 4 A). Taro tuber tissue also showed the double freezing (Fig. 4 C).

The tuber tissue of cultivated variety *Gokoku* of sweet potato did not usually show double freezing points even when the total water content was more than 290 per cent immediately after digging. But very clear double freezing was observed when water was penetrated in intercellular spaces by using a vacuum aspirator and the surface water was wiped off with filter paper (Fig. 5 C). As some water was absorbed by cells of this piece, the second freezing point was higher than control.

When the hole in which the thermojunction was inserted was filled with pure water, the double freezing occurred (Fig. 6 C). Double freezing occurred also when the surface of the sample was smeared slightly with water (Fig. 7 C). Especially in the latter case, because of both a very small amount of water being smeared and only the surface being treated, the freezing curve was almost the same as control except for the earlier start of freezing and the appearance of the first freezing point. The curve shows that the first freezing point was

Fig. 2. Freezing curves of pure water and of potato tuber tissue.—A: Freezing of pure water held in a piece, 4×4×10 mm in size, of pith tissue of *Sambucus sieboldiana*.—B: Freezing of pure water held in a mass of absorbent cotton.—C: Double freezing of a tissue piece, 5×5×14 mm in size, of potato tuber, the water content being 387 per cent of dry weight. The two arrows (1 and 2) indicate the first freezing point and the second one, respectively.—D: The second time freezing of the same piece under the same conditions as in C.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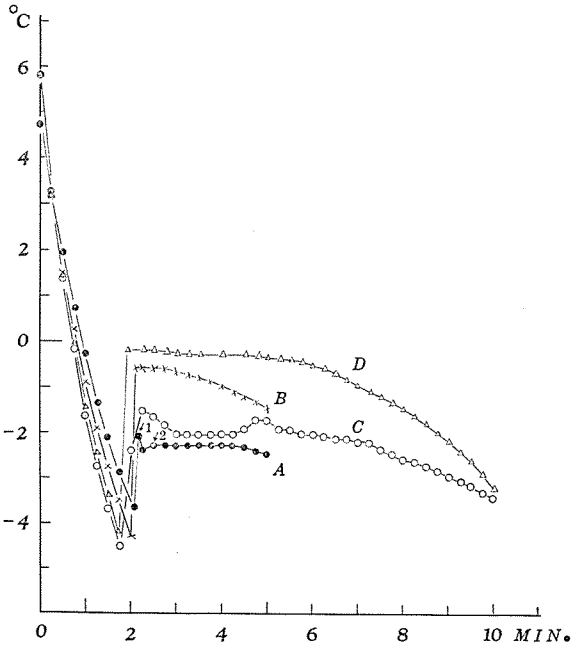

Fig. 3. Freezing curves of potato tuber.—A: The first time freezing of a whole (uncut) tuber of 5 mm diameter, the water content being 379 per cent of dry weight. 1: The first freezing point; 2: The second freezing point.—B: The second time freezing of the same tuber as in A.—C: An irregular freezing curve of a tissue piece, 4×4×6 mm in size, the water content being 392 per cent of dry weight.—D: The second time freezing of the same piece as in C.

reached rather slowly. This is considered to be due to the fact that the intercellular water which was responsible for the first freezing point existed only at the surface layer so that time was needed for the conduction of latent heat from there to the thermojunction.

The second freezing point was usually lower than the first freezing point. And the freezing curve was kept at that value for a considerable period, then to begin descending, and finally to assume the form of a simple cooling curve.

From the experimental results mentioned above, each of the three factors, viz. the liquid around the thermocouple (25, 33), the intercellular liquid (22) and the liquid at the damaged surface of tissue (1), seems to explain only one of the causes of the double freezing.

When the sample which was once frozen was thawed and cooled to freezing for the second time, the double freezing did not appear anymore and the freezing point was higher than in the first time freezing (Figs. 2-7). This conforms with the reports by AOKI (3, 4). The sample which gave an irregular curve

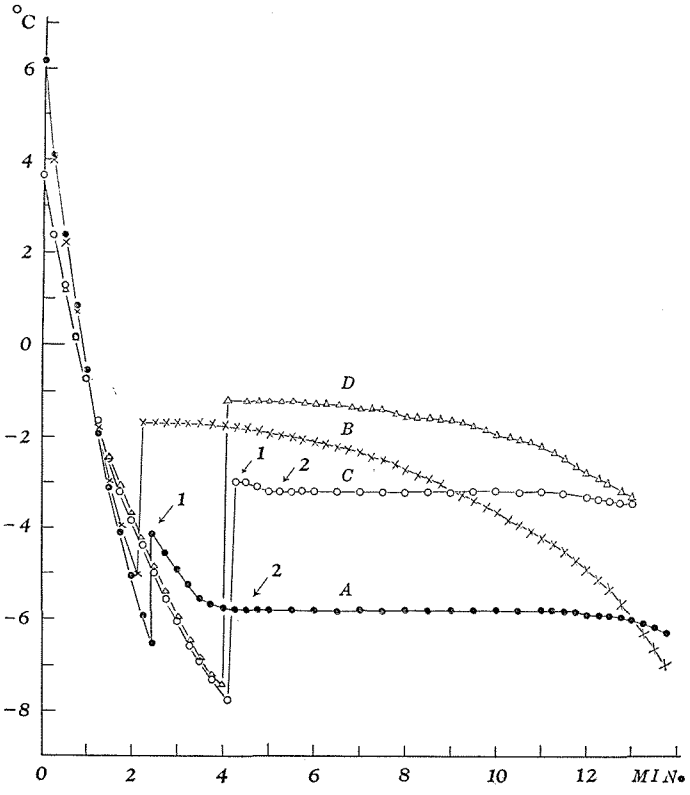

Fig. 4. Double freezing curves of tuber tissues of sweet potato and taro. 1: The first freezing point; 2: The second freezing point.—A: The first time freezing of a tissue piece, 5×5×10 mm in size, of *Ipomoea batatas* (*okinawa* No. 100), the water content being 239 per cent of dry weight.—B: The second time freezing of the same piece as in A.—C: The first time freezing of a tissue piece, 5×5×10 mm in size, of *Colocasia esculenta*, the water content being 305 per cent of dry weight.—D: The second time freezing of the same piece as in C.

in the first time freezing showed a smooth one in the second time (Fig. 3 C, D). However, the sample that was thawed immediately after the first freezing point, namely, before the second freezing stage, showed the double freezing in the second time freezing.

The tissues, which would show the double freezing in the normal state or by water treatment, were kept at 100°C for 20 minutes in air tight containers and then made wet with water. These heat-killed tissues never showed the double freezing (Fig. 8 A). Tissues treated with chloroform also did not show any (Fig. 9 A). These dead tissues showed the same freezing point in the

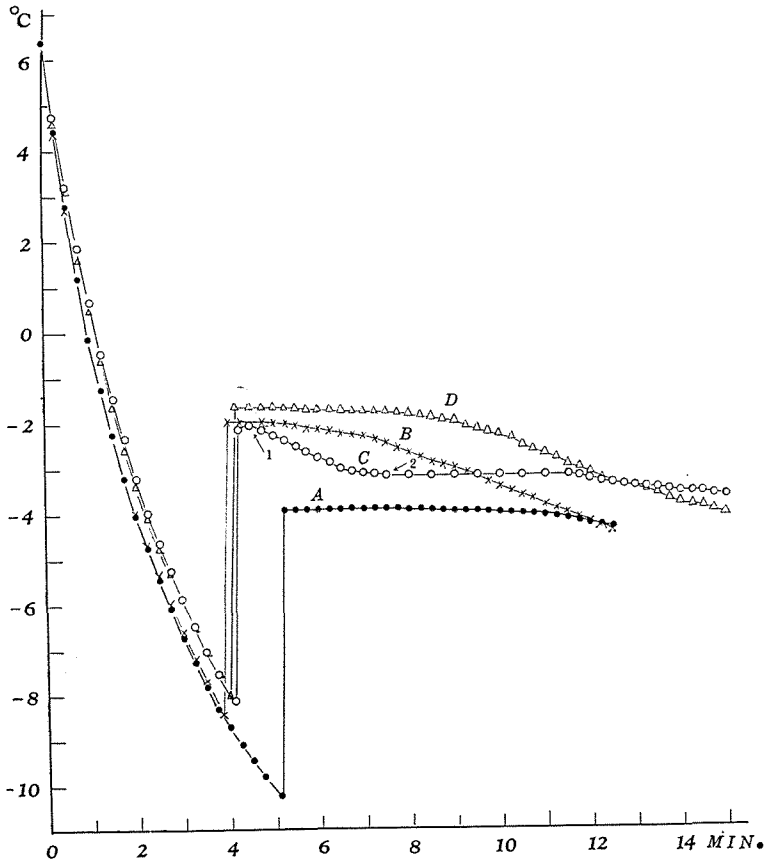

Fig. 5. Effect of water infiltration on the freezing curve of tuber piece of *Ipomoea batatas* (*gokoku*).—A: The first time freezing of control piece, 5×5×10 mm in size, the water content being 177 per cent of dry weight.—B: The second time freezing of the same piece as in A.—C: The first time freezing of a tissue piece, 5×5×10 mm in size, with intercellular spaces penetrated by water, the water content as a whole being 293 per cent of dry weight (1 and 2 indicating the first freezing point and the second one, respectively).—D: The second time freezing of the same piece as in C.

second time freezing as in the first time (Figs. 8, 9, A, B).

The freezing point in the second time freezing was always higher than in the first time (Figs. 2, 3, 4, 5, 6 and 7). The freezing point of tissues became higher when they were injured. When the tissue was frozen for the third time, the freezing point did not become higher than in the second time, provided that the tissue was dead before the second time freezing. The freezing point in the third time freezing was higher than in the second time in case the tissue

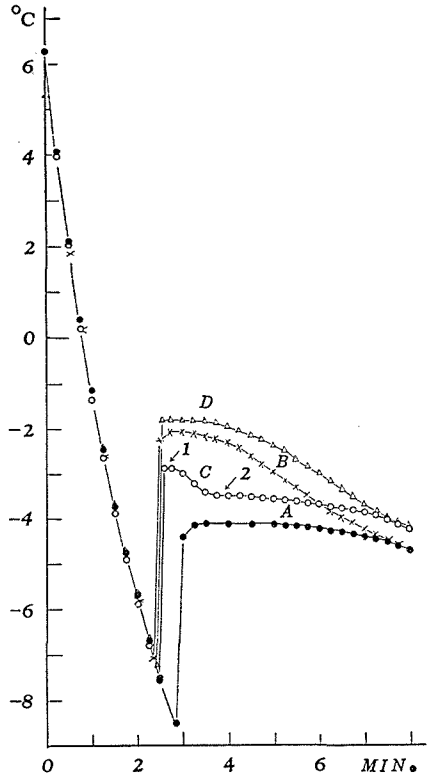

Fig. 6. Effect on the freezing curve of the water filling the thermojunction hole in *Gokoku* tuber piece.—A: The first time freezing of control piece, 5×5×10 mm in size, the water content being 165 per cent of dry weight.—B: The second time freezing of the same piece as in A.—C: The first time freezing of a piece, 5×5×10 mm in size, having the hole for the thermojunction filled with 0.03 ml of water, the water content as a whole being 169 per cent of dry weight (1 and 2 indicating the first freezing point and the second one, respectively).—D: The second time freezing of the same piece as in C.

was dead before the third time freezing. The same was true when freezing and thawing were repeated more than three times. The freezing point became constant at the second time freezing in the cases of potato, sweet potato, taro, summer orange and *Nitella* sp. (Figs. 2, 3, 4, 5, 6, 7 and 10; Table 3), while it became constant at the third time freezing in many other plant tissues (Figs. 11, 12, 13, 14, 15 and 16; Table 3), and occasionally at the fourth time (Table 3, *cf.* 19).

It may then be expected that the difference between the freezing point of the first time freezing and that of the second time reflects the healthiness of the tuber

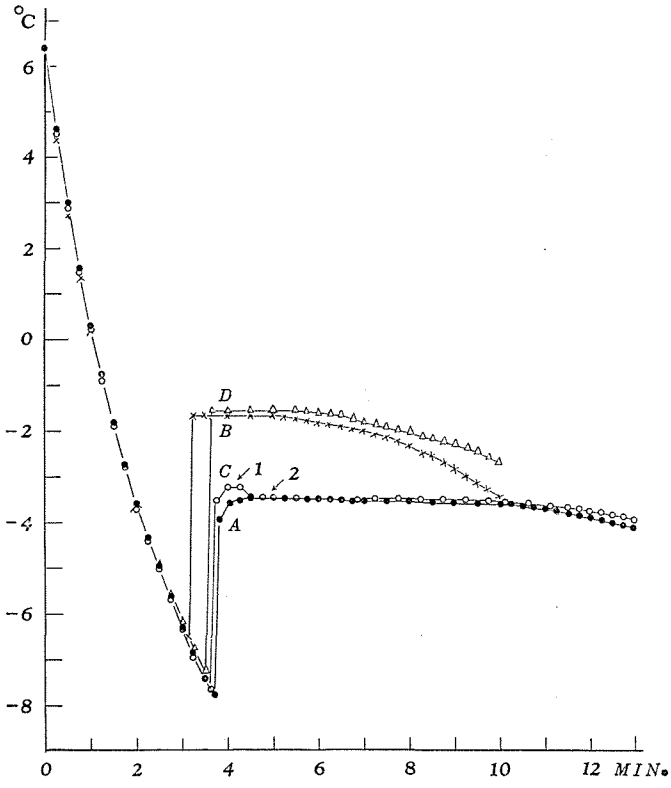

Fig. 7. Effect on the freezing curve of water moistening the surface of *Gokoku* tuber piece.—A (closed circles): The first time freezing of control piece, 5×5×10 mm in size, the water content being 195 per cent of dry weight.—B (crosses): The second time freezing of the same piece as in A.—C (open circles): The first time freezing of a piece, 5×5×10 mm in size, with the surface slightly wetted with water, the water content as a whole being 196 per cent of dry weight (1 and 2 indicating the first freezing point and the second one, respectively).—D (triangles): The second time freezing of the same piece as in C.

tissue. So an intact sweet potato tuber was submerged in 1% acetic acid for 96 hours and tissue samples were excised from various parts differing in the appearance of tissue. As seen in Table 1, the more injured the tissue, the higher the freezing point of the first time freezing, and the smaller the difference in the freezing point between the two successive freezings. The freezing point of the tissue brei, as well as that of the second time freezing, fell considerably at slight injury. This may be due to an increase in decomposed substances.

Freezing point of tissue brei of *Gokoku* tuber was the same whether it was

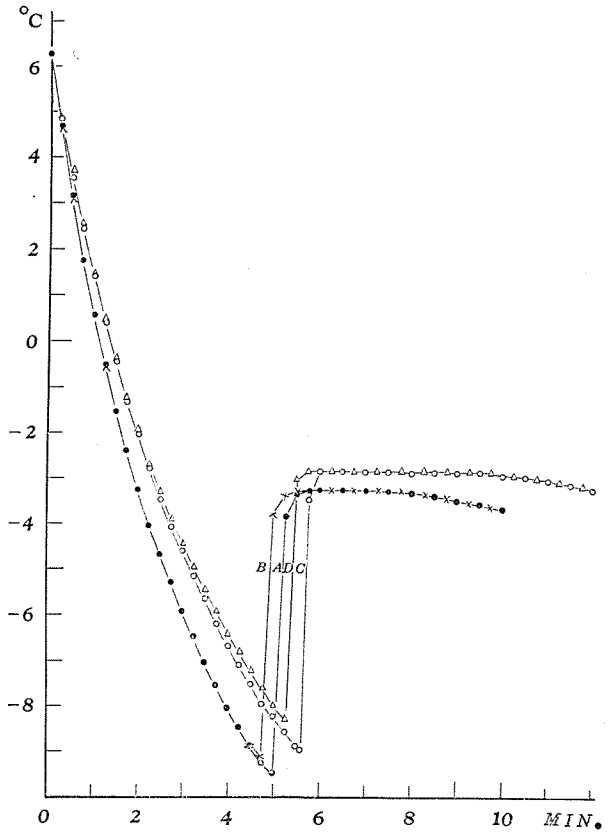

Fig. 8. Freezing curves of an excised piece and brei of heat-killed tissue of *Gokoku* tuber.—A (closed circles): The first time freezing of a piece, 5×5×10 mm in size, killed by heating at 100°C for 20 minutes and wetted with water, the water content as a whole being 185 per cent of dry weight.—B (crosses): The second time freezing of the same piece as in A.—C (open circles): The first time freezing of brei of the heat-killed tissue.—D (triangles): The second time freezing of the same brei as in C.

prepared from the living tissue or from the dead tissue. It was about 0.2°C higher than that of the second time and third time freezings of uncrushed tissue (Fig. 10). The freezing point of brei did not rise in the second time freezing. When the freezing point of the brei was −0.93°C by the thermoelectric method (Fig. 10), that of the expressed sap from the brei measured by the micro-cyoscopic method (15) was −0.912°C. Also in potato tuber the tissue brei showed the freezing point higher than that of dead tissue by about 0.1°C. Most tissue cells were from 80×70 to 60×50μ in size in *Gokoku* and from 130×110 to 90×70μ in potato. They were filled with starch grains and the

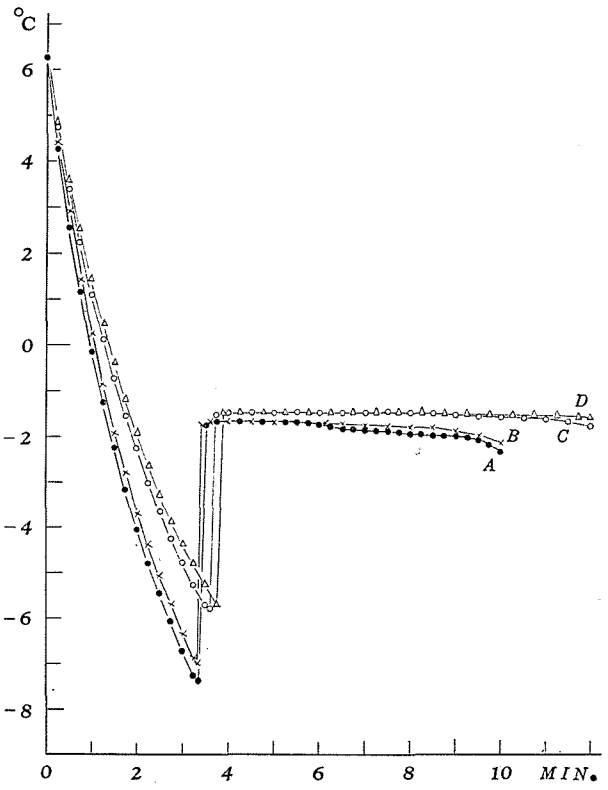

Fig. 9. Freezing curves of an excised piece and brei of *Gokoku* tuber tissue killed with chloroform.—A (closed circles): The first time freezing of a piece, 5×5×10 mm in size, treated with chloroform gas for 27 hours and wetted with water, the water content as a whole being 207 per cent of dry weight.—B (crosses): The second time freezing of the same piece as in A.—C (open circles): The first time freezing of brei of the chloroform-treated tissue.—D (triangles): The second time freezing of the same brei as in C.

intercellular spaces were very small. In *Gokoku* tuber the tissues killed with heat and with chloroform showed the freezing points lower than those of tissue breie by 0.41 and 0.24°C, respectively (Figs. 8, 9). Also the freezing point difference between the second time freezing and the tissue brei of *Gokoku* tuber treated with acetic acid was remarkably large (Table 1).

The difference between the freezing points of the living tissue and of its brei measured with some other cultivated varieties of sweet potato is presented in Table 2. The values correspond roughly to the so-called plasmolytic-cryoscopic discrepancy (e. g. CRAFTS *et al.*, 9).

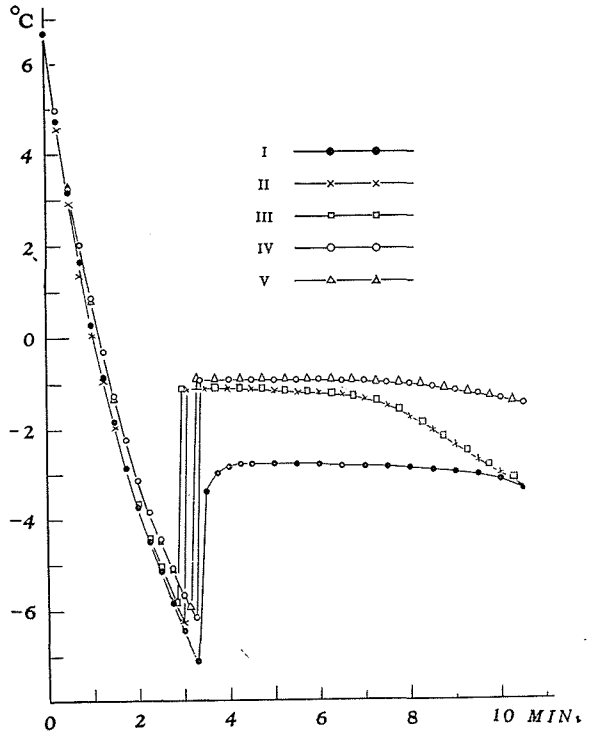

Fig. 10. Freezing curves of a tissue piece and brei of *Gokoku* tuber.—Closed circles: The first time freezing of a piece, 5×5×10 mm in size, the water content being 171 per cent of dry weight.—Crosses: The second time freezing of the same piece as in the former.—Squares: The third time freezing of the same piece.—Open circles: The first time freezing of brei of living tissue or of dead one.—Triangles: The second time freezing of the same brei as in the former.

Table 1. Relationship between freezing point and the state of tissue. *Gokoku* tuber was submerged in 1% acetic acid solution for 96 hours and tissue pieces were excised from various parts of it. No double freezing was observed with these tissue pieces.

State of tissue	Freezing point				Water content (% of dry wt.)
	Of the first time freezing (°C)	Of the second time freezing (°C)	Difference (°C)	Of tissue brei (°C)	
Control	−3.15	−1.42	1.73	−1.21	183
Color not yet changed	−2.95	−2.39	0.56	−1.83	251
Going to be colored	−2.71	−2.47	0.24	−1.97	252
Colored light brown	−1.45	−1.36	0.09	−1.05	259

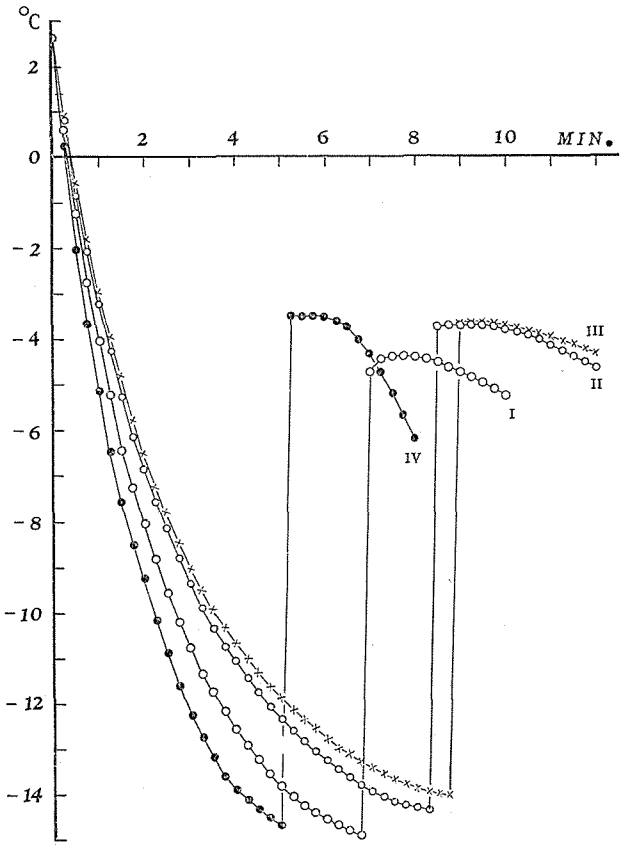

Fig. 11. Freezing curves of a piece, 3×3×5 mm in size, from the fruit of *Musa paradisiaca* subsp. *sapientum*, the water content being 329 per cent of dry weight. On June 25.

I, II and III: First, second and third time freezing, respectively. IV: Freezing of brei prepared from the piece killed by repeated freezing.

With other plant tissues, too, the same general tendency was observed in the freezing curves as shown in Figs. 11–17. The freezing point at each time of freezing in these and other experiments is shown in Table 3. The leaf of *Diospyros* measured was the eighth unfolded one from the shoot tip and that of *Camellia* the second one. In *Picea*, new leaves were not yet seen on May 1, so the last year's ones were used. And as its water content was low, the brei was not sufficiently fluid for the determination of the freezing point.

In *Torreya*, ice spots in intercellular spaces were observable through the lower surface of leaves under natural conditions on early mornings in winter.

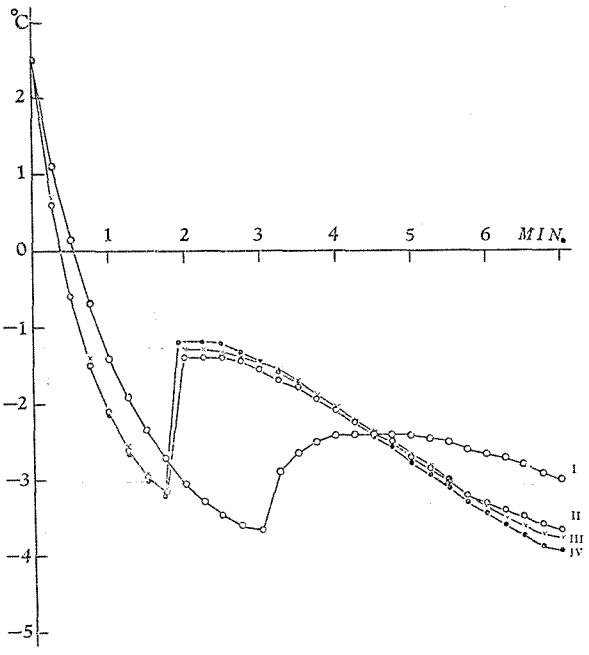

Fig. 12. Freezing curves of the second leaf, 15 mm wide and 40 mm long, from the shoot tip of *Camellia sinensis*, the water content being 306 per cent of dry weight. On May 6.

I, II and III: First, second and third time freezing, respectively. IV: Brei prepared from the leaf killed by repeated freezing.

Table 2. Freezing points of living tissue and tissue brei of stored tubers of some cultivated varieties of sweet potato in February.

Name	Freezing point			Water content (% of dry wt.)
	Of living tissue (°C)	Of tissue brei (°C)	Difference (°C)	
Chiba No. 1	−2.97	−1.80	1.17	279
Akaiigo	−4.03	−2.83	1.20	249
Norin No. 1	−3.95	−1.85	2.10	160
Norin No. 2	−5.50	−3.63	1.87	156
Uchihara No. 1	−4.97	−2.75	2.22	162

Water seems to have come out from cells and jointed the freezing in the intercellular spaces. By having a high water permeability, cells may thus be saved from intracellular ice formation. Under the experimental conditions in

Table 3. Freezing points of various plant tissues, living, injured and killed by freezing, and ground.

	Remarks	Freezing point					Water content (% of dry wt.)
		Of living tissue (First time freezing) (°C)	Of transitional state (Second time freezing) (°C)	Of dead tissue (Third time freezing) (°C)	Of tissue brei (°C)	Difference between living tissue and brei (°C)	
Fruit of *Musa paradisiaca* subsp. *sapientum*	June 25	−4.40	−3.70	−3.62	−3.48	0.92	329
Bud of *Diospyros kaki*	April 7	−3.20	−1.45	−1.25	−1.15	2.05	294
Leaf of *Diospyros kaki*	May 9	−4.55	−1.85	−1.65	−1.60	2.95	310
Leaf of *Camellia sinensis*	May 6	−2.42	−1.40	−1.30	−1.20	1.22	306
Leaf of *Buxus microphylla* var. *japonica*	May 6 Normal leaf	1st fr. pt. −3.75 2nd fr. pt. −3.56	−2.20	−2.00	−1.87	1.69	204
	Young leaf (Water is seen in intercellular spaces)	1st fr. pt. −1.21 2nd fr. pt. −4.60	−2.13	−1.90	−1.75	2.85	212
Bud of *Picea excelsa*	May 1	−3.70	−2.35	−2.25	−2.07	1.63	547
Leaf of *Picea excelsa*	May 1	−5.55	−4.77	(IVth time freez.) −4.04	—	(Diff. betw. living and dead) 1.51	105
Leaf of *Torreya nucifera*	May 27	1st fr. pt. −5.80 2nd fr. pt. −6.60	−3.30	−2.50	−2.36	4.24	148
Summer orange (*Citrus natsudaidai*)	June 18 A small mass of 5 juice vesicles	−1.50		(IInd time freez.) −1.36	−1.29	0.21	1,024
	Sap only	−1.43		(IInd time freez.) −1.36		0.07	
Nitella sp.	Internodal cell	−0.68		(IInd time freez.) −0.41		(Diff. betw. living and dead) 0.27	
	Vacuolar sap	−0.64		(IInd time freez.) −0.60		0.04	
	Internodal cell, wiped thoroughly	−0.86		(IInd time freez.) −0.64		0.22	

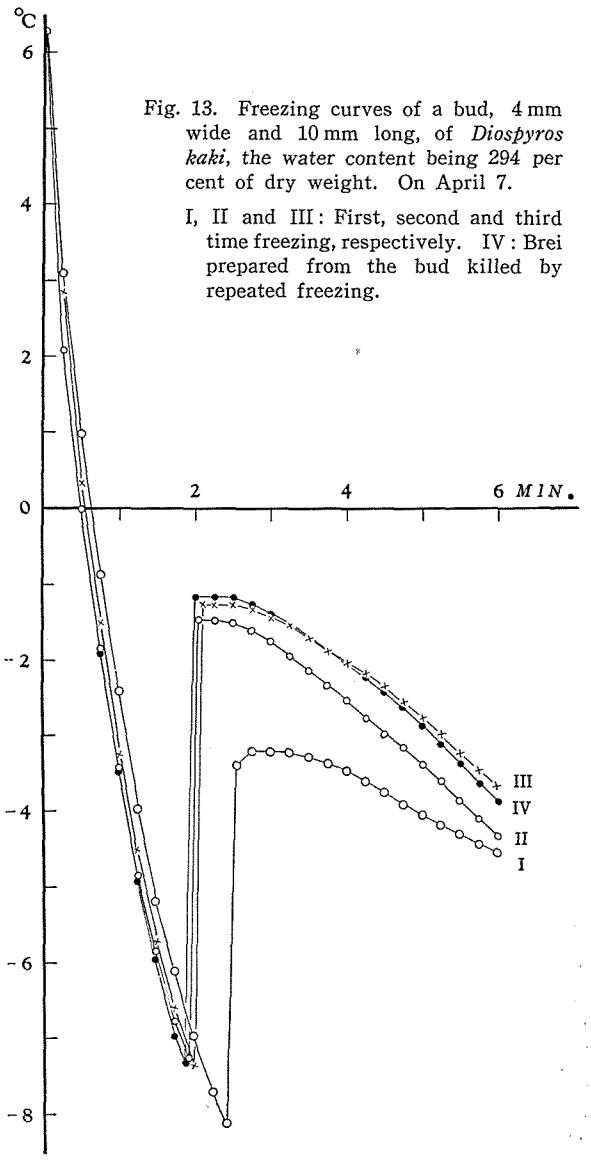

Fig. 13. Freezing curves of a bud, 4 mm wide and 10 mm long, of *Diospyros kaki*, the water content being 294 per cent of dry weight. On April 7.

I, II and III: First, second and third time freezing, respectively. IV: Brei prepared from the bud killed by repeated freezing.

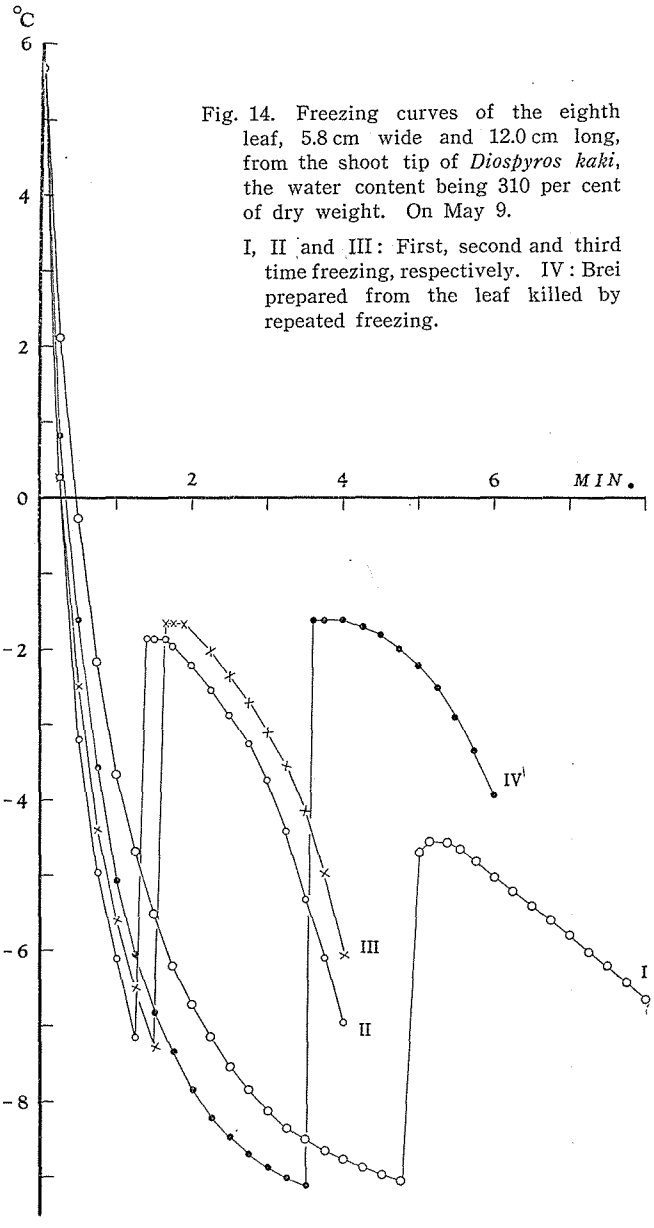

Fig. 14. Freezing curves of the eighth leaf, 5.8 cm wide and 12.0 cm long, from the shoot tip of *Diospyros kaki*, the water content being 310 per cent of dry weight. On May 9.

I, II and III: First, second and third time freezing, respectively. IV: Brei prepared from the leaf killed by repeated freezing.

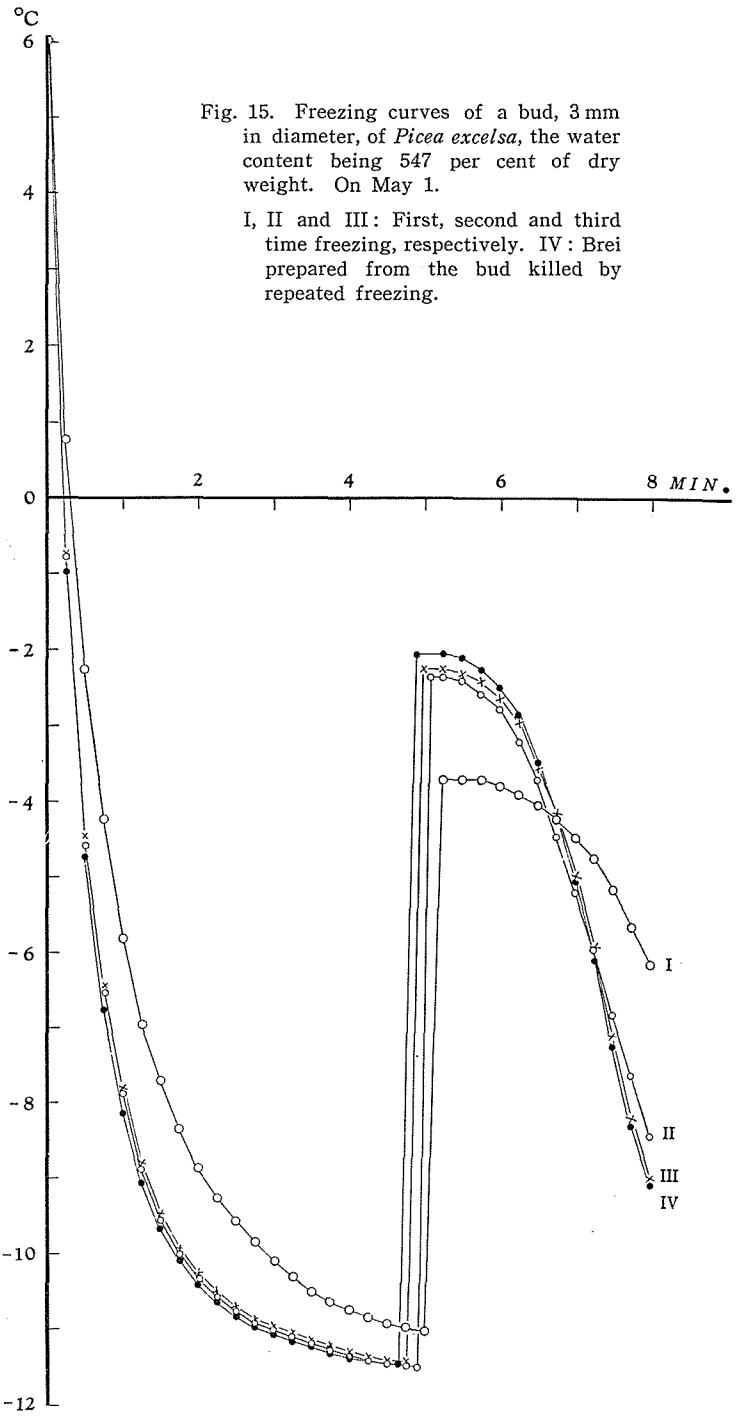

Fig. 15. Freezing curves of a bud, 3 mm in diameter, of *Picea excelsa*, the water content being 547 per cent of dry weight. On May 1.

I, II and III: First, second and third time freezing, respectively. IV: Brei prepared from the bud killed by repeated freezing.

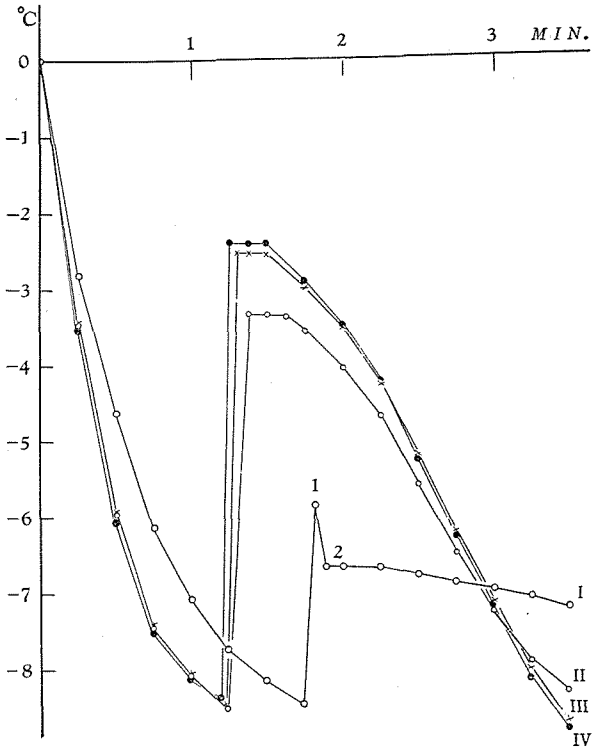

Fig. 16. Freezing curves of a previous season's leaf, 3 mm wide and 26 mm long, of *Torreya nucifera*, the water content being 148 per cent of dry weight. On May 27.

I, II and III: First, second and third time freezing, respectively. 1: The first freezing point; 2: The second freezing point. IV: Brei prepared from the leaf killed by repeated freezing.

May, *Torreya* leaves showed the double freezing. The first freezing point seemed to be due to the intercellular liquid, the freezing being propagated from the cut surface.

Normal leaves of *Buxus* scarcely showed the double freezing, the first freezing point being very small even under the best condition for its appearance, namely at −8°C. But the double freezing occurred distinctly when some water was remaining in the space between palisade tissue and spongy one a few days after the intercellular freezing (Fig. 17; Table 3).

A thermojunction was inserted in a small space surrounded by five juice vesicles of summer orange without injuring them. When frozen, they became turbid with ice crystals. The freezing point became constant already at the second time freezing. Drops squeezed out of vesicle with a slight pressure

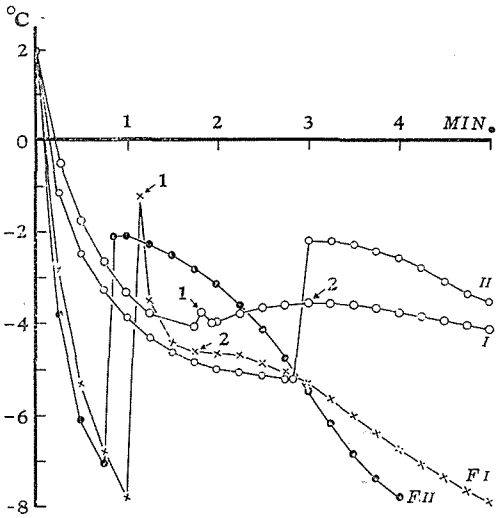

Fig. 17. Freezing curves of leaves of *Buxus microphylla* var. *japonica*. On May 6. I: The first time freezing of a normal leaf, 1.3 cm wide and 1.7 cm long, the water content being 204 per cent of dry weight. Temperature of freezing mixture, −8°C. 1: The first freezing point; 2: The second freezing point. II: The second time freezing of the same leaf as in I. FI: The first time freezing of a young leaf, 1.4 cm wide and 1.9 cm long, which retains some water in intercellular spaces, the water content as a whole being 212 per cent of dry weight. Temperature of freezing mixture, −13°C. FII: The second time freezing of the same leaf as in FI.

may be composed almost of the vacuolar sap. The internodal cell of *Nitella* was used as a cell, the surface being blotted lightly, or wiped thoroughly to make even the liquid held by the cell wall negligible. The finer thermojunction was inserted in the cell. For the measurement of the isolated vacuolar sap, the colorless, transparent liquid which dripped from a cut end of the cell was held in a small ball of absorbent cotton. Both in the summer orange and *Nitella* the isolated vacuolar sap showed a slight difference in the freezing point between the first time freezing and the second time (Table 3).

In *Cucurbita moschata* var. *toonas*, the tissue brei prepared from the living leaf, that prepared from the leaf killed by heating at 100°C for 20 minutes in an air-tight container, and the expressed sap from the latter showed the freezing points of −0.76, −0.75 and −0.743°C, respectively.

Discussion

The first freezing point.

The liquid in the intercellular space freezes first, as it is connected to the surface of the tissue. However, the quantity of the liquid is small and the

latent heat liberated is too small to raise the tissue temperature up to the freezing point of the intercellular liquid. The tissue temperature begins to descend before reaching the freezing point. In this case the first freezing point is to be accepted as an apparent one. This case occurs more easily when the heat capacity of the measuring apparatus is large and the cooling is rapid. TERUMOTO (30) observed that the first freezing point was affected by the grade of supercooling.

When a pressure is exerted on a living tissue, the expressed liquid at first must be less concentrated than the cell sap because of the semipermeability of the plasmamembrane. Liquid not much differing from the cell sap comes out after the plasmamembrane is injured. It, therefore, was observed in potato and sweet potato that the freezing point depression of the so-called expressed cell saps was the same as that of the juice from the dead tissue as well as of the tissue brei, only when it was squeezed very thoroughly from the living tissue. As shown in Fig. 2 A and B, water being held in a piece of pith tissue or in a mass of cotton, neither the heat capacity of the insoluble material nor the structure of it affects the freezing point depression. Also in microcryoscopic measurements of tuber tissues and *Cucurbita*-leaves, the freezing point of tissue brei was the same as that of the sap expressed from the brei. Hence pulp in tissue brei may not affect the freezing point provided that the brei is not juiceless.

If the first freezing point of a tissue is that of the sap coming from the cells cut or damaged by insertion of the thermojunction, it must be so low as to correspond to that of the tissue brei (Fig. 18 A). In this case, if the sap is not diluted with the intercellular liquid, the freezing point must be nearly equal to that of the cell content, thus the separation of the first freezing point

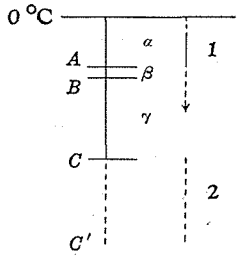

Fig. 18. Diagram representing relations among freezing points of a plant tissue.—A: Freezing point of tissue brei or of expressed tissue liquid.—B: Freezing point of dead tissue.—C: Freezing point of living tissue.—C′: Freezing point of living cells after dehydration of cell content by extracellular ice formation. α=A, β=B−A, γ=C−B. 1: Range of the first freezing point; 2: Range of the second freezing point.

from the second not being apparent. If, on the other hand, the first freezing point is owing to the intercellular liquid which is considered to be very dilute, it must not differ much from that of pure water (Fig. 18, 1). Many measurements showed, however, that the first freezing point was even lower than that of the dead tissue or of brei (Figs. 2–7, 16, 17 I and 18). The leaf of *Buxus* retaining a considerable amount of intercellular liquid after frost was exceptional

(Fig. 17 FI). The liquid obtained by collecting 10 ice pieces formed in leaves showed the freezing point of pure water, whereas the first freezing point of the leaf was −1.21°C and the peak disappeared in a moment. Hence the first freezing point must be so low because of the very small amount of the intercellular liquid which is responsible for it. In fact, the first freezing point was higher as much as 0.5°C than the freezing point of the dead tissue, when a large quantity of water was injected into the tissue of *Gokoku* tuber. Even in this experiment, however, the first freezing point did not reach 0°C, perhaps because some solutes had diffused out of cells.

The second freezing point.

When the tissue is cooled well below the freezing point of the cell content, intracellular water freezes, inside the cell or in intercellular spaces according to cases. When the cell content freezes a temperature level is kept for a considerable while since the amount of freezing liquid is large, and this freezing point is lower than the first freezing point as the concentration of the liquid is high. This is just the second freezing point. When the intracellular liquid comes out rapidly and much ice is formed in intercellular spaces, the second freezing point is manifest. When, on the other hand, the extracellular ice formation proceeds slowly and the quantity of the ice formed is small, the temperature of the tissue piece begins to fall before the second freezing point is clearly shown. Then a definite freezing point is observed when the freezing proceeds inside the cell (Fig. 2 C). In any of the author's experiments the freezing occurred not only outside the cells, but also inside them. When cells are dehydrated by ice formation outside the cell the freezing point of the cell content becomes lower than when the dehydration does not occur. Some hardy plants have a tendency to be dehydrated (Aoki *et al.*, 7; Scarth and Levitt, 28; and Siminovitch and Scarth, 29).

In Fig. 18, let C represent the freezing point of living cells when the dehydration does not occur, and C′ the value when they are most dehydrated by the growth of intercellular ice. Then the actual second freezing point, 2, due to the intracellular freezing appears somewhere between C and C′ depending upon the degree of dehydration of the cells. The freezing point of living tissue fluctuates considerably from measurement to measurement. This is probably because the degree of dehydration which has proceeded before the cell content freezes differs by chance. The views that the freezing point of living tissue appears to be low when the cooling temperature is very low (Luyet and Gehenio, 22; Maximov, 25; and Scarth and Levitt, 28) and that the freezing point measurement of living tissue depends largely on the cooling rate (Aoki, 5) are related to the dehydration of cell contents due to intercellular ice formation and the relation between the cooling rate and the latent heat liberation.

If there is no dehydration of the cell, the real freezing point (C) can be determined through a correction for supercooling. As shown in the freezing

curves of living tissues (Figs. 2–7, 10–17), it takes time to freeze throughout the whole tissue because of the resistance of cells against freezing. In some cases dealing with living tissues, the measured freezing points seem to be too low because of large heat capacity of thermometer bulb, especially when the cooling rate is large (e. g. LUYET and GEHENIO, 22). The reason may be that the cooling effect appears before the latent heat liberated makes the bulb assume the real freezing point. When a tissue is dead, on the other hand, the whole tissue freezes so rapidly that the cooling rate matters little. MAXIMOV (25) and WALTER and WEISMANN (32) also found that the freezing point measurement gave fairly constant values with dead tissues.

Conditions for the double freezing.

The double freezing takes place when relatively dilute intercellular liquid freezes at first (presenting the first freezing point) and the cell content freezes after a while (presenting the second freezing point). A typical example is shown by Fig. 2 C. Hence from physiological and ecological points of view the second freezing point has higher significance than the first. Even if the tissue is not injured when the first freezing point has appeared, it may be injured when it freezes as to show the second freezing point. When cells are injured in this way, the resistance against intracellular freezing may disappear and the inter- and intracellular liquids may be mixed together due to loss of semipermeability. Thus a distinct freezing point appears at the second time freezing.

The conditions which favour the double freezing are: 1) the relatively high water permeability of cells and the presence of dilute liquid outside the cell before cooling, and 2) resistance of cells against the intracellular freezing.

Some amount of juice may come out from cells which are cut or crushed at the preparation of the tissue block and at the insertion of the thermojunction. The juice may join the intercellular liquid. If only the liquid from injured cells contributes to the double freezing significantly, the double freezing should be observed with the samples which are low in the water permeability and those which are not resistant to intracellular freezing. But actually that was not the case. In living *Gokoku* tubers and banana fruits (Figs. 5 A, 6 A, 7 A, 10 A and 11) as well as in the dead tissues (Figs. 2–17), no double freezing was observed. Therefore, the idea (5, 6) that the cut surface is necessary for the first freezing point is concerned with only a part of necessary requirements. AOKI (6) did not find the double freezing phenomenon in intact small potato tubers, but the author found it when the tuber was supposed to contain intercellular liquid (Fig. 3 A).

Relations among freezing points of living and dead tissues and of expressed sap.

The freezing point of dead tissue is always higher than the first freezing point and the second one of living tissue, as seen in Figs. 2–7, 16 and 17. The

same is true even when, as in Figs. 5 A B, 6 A B, 7 A B and 10–15, the material contains only a small quantity of intercellular liquid which may dilute the cell sap as a result of loss of semipermeability by death. Moreover, with the internodal cell of *Nitella* wiped thoroughly, the difference between the freezing points of the first time and second time freezings was large, while the difference was small with the vacuolar sap (Table 3). Hence some change in the colloidal state of protoplasm seems to give the most probable explanation for the rise of freezing point due to death. LUYET and GEHENIO considered in their earlier work (22) that the protoplasmic water liberated at death was readily freezable. Later, however, they denied the idea and attributed the lower freezing point of living tissues to the resistance to the outward passage of water during freezing presented by the living cellular membranes (23). CURRIER (10) has also suggested that the plasmolytic-cryoscopic discrepancy is due to liberation of water which has been held by the protoplasm before killing.

Since the rise of freezing point due to repeated freezing was seen also in the vacuolar saps of summer orange and *Nitella* (Table 3), the rise of freezing point by death may be ascribed partly to the liberation of the water which has been bound to colloidal matters in the vacuolar sap. Then, naturally the water bound to the protoplasmic colloid may be liberated at the same time. Such liberation of colloidally bound water occurs not only by freezing but also by death from other causes as heating, chloroform treatment and grinding (Figs. 8, 9 and 10). The amount of colloidally bound water may differ according to the colloidal state of protoplasm and vacuolar sap (Tables 1 and 2).

The liquid in intercellular spaces and cell walls also may dilute the intracellular liquid after death. As seen in the young leaf of *Buxus* (Table 3), when a large amount of ice was formed in intercellular spaces the second freezing point became very low and the freezing point of the dead tissue became remarkably higher than this second freezing point, owing to the mixing of the cell content and the liquid which had frozen in intercellular spaces, in addition to the liberation of the colloidally bound water.

Using a mercury thermometer, WALTER and WEISMANN (32) observed that the freezing proceeded very slowly in the living tissue of potato, and the freezing area, which, being the area surrounded by the freezing curve and the cooling curve, corresponds to the quantity of ice formed in tissue, was the same in the living and the dead tissue when the tissue was cooled to the temperature −5 or −15°C. And they stated that the true freezing point specific to the living tissue was not observable and that, since the quantity of ice formed in the tissue was inferred from the freezing area to be the same as in the dead tissue, there might be no difference in the osmotic pressure between the living and the dead tissue.

They, however, did not think of the difference in the state of water in the living and the dead tissue. They seemed to consider that the quantity of water serving as solvent in the dead tissue was the same as in the living tissue.

According to their experiments, it took about 30 and 60 min. for the living tissue to be cooled to −5 and −15°C, respectively. Therefore, the tissue they used did not seem to be hardy against intracellular freezing. Since most cells die if the intracellular freezing occurs (8, 16, 24 and 26), a greater part of the tissue was probably dead already at the final stage of the long freezing, and accordingly, the freezing area even at the first time freezing was almost the same as that of the dead tissue. The quantity of the ice formed in living tissue is not considered to be the same as that in dead one, so far as the cooling temperature is not too low and the duration of cooling is not so long as to kill the tissue thoroughly.

Since the osmotic pressure of living tissue was estimated by WALTER and WEISMANN (32) from the final quantity of ice formed in a long run, it was natural that the value was almost the same as of the dead tissue. Their freezing curves of living tissues were simple in the form in the early process of freezing and the freezing point shown was lower than that of dead tissues. But they stated that there was no true freezing point in the living tissue because the ice formation was slow. If the ice formation proceeds slowly and is small in amount, the freezing curve will be irregular and the true freezing point will not be shown distinctly. The present author's experiments using the thermojunction did not show that sort of fact except two examples (Fig. 3 C). Ice formation seemed to be sufficient for making the temperature of both the living tissue and the thermojunction rise to the true freezing point.

It may be cared whether the temperature of the measuring instrument is lower than that of the sample, since the stirring for sufficient heat conduction is not performable. This is the case when the heat capacity of the instrument is large, especially as in a mercury thermometer. Provided that the freezing temperature is measured accurately by means of suitable thermojunction and the cooling temperature and the cooling rate are moderate, the plateau of the freezing curve may represent the freezing point of the tissue and this temperature also may be expected to be in proportion to the osmotic value of the tissue used. The present author does not agree with WALTER (31) who stated that the results indicating the lower freezing point of living tissue were unreliable, since the continuous stirring of the solution that was required was not possible.

The freezing point of the tissue brei, either prepared from living tissue or from dead tissue, is slightly higher than that of the dead tissue (Figs. 10, 8-16; Table 3). The destruction of micellar and capillary structures of the tissue may be chiefly responsible for this rise. The freezing point lowering of droplets of water is given by LAFARGUE's equation (27),

$$(Tm-T)/Tm = 2\sigma M/rL_f\rho .$$

Let Tm (normal melting point) = 273°C, T (solidification temperature of supercooled drop of radius r cm) = 272.9°C, σ (surface energy) = 119 erg/cm^2,

M (molecular weight) $=18$, Lf (molar heat of fusion) $=6034\times10^7$ erg, ρ (density of ice) $=0.916$, then $r=2\mu$. Cells of storage tissues used have much larger radii, but intercellular spaces, being 4–10μ in diameter, may deserve much consideration, if the above principle is applicable to plant tissues. In the tea leaf, epidermal cells are 18–27μ in diameter, parenchyma cells 14–23μ and intercellular spaces about 1.5μ. Hence, if the freezing point rises due to the destruction of the capillary structure, the droplets of liquid in the intercellular space may contribute to it rather than those inside the cell. And the liberation of the water imbibed or bound in the cell wall, protoplasm and organic matters in cell sap, due to the sudden change of the micellar structure as described by FREY-WYSSLING (11–13), may be another reason for the rise of freezing point of tissue brei.

The difference in freezing points between a dead tissue and its brei is considerably larger when the brei is prepared from the heat-killed tissue than when it is prepared from the tissue killed with chloroform or freezing (Figs. 8, 9 and 10). This fact may be due to that the capillarity is increased in the micro-structure when the tissue is macerated by heating. The conspicuous rise in freezing point of tissue brei caused by treating sweet potato tuber with acetic acid (Table 1) may be due to the volatilization of volatile substances as alcohol and the like during the process of tissue grinding.

In conclusion, the freezing point depression of the living tissue (Fig. 18 C) is the sum of the three, namely the freezing point depression corresponding to the expressed liquid of cells i. e. that of the tissue brei (α), the one due to micellar and capillary structures of the tissue (β) and the one due to the colloidal binding of water specific to the living state (γ). And when the intracellular liquid is dehydrated by the growth of ice crystals outside the cells, the freezing point of the tissue becomes still lower (C′).

Summary

Freezing curves of buds, leaves and tissue pieces of tubers and fruits were studied in their living and dead states, by using a fine thermojunction. Small intact potato tubers were used to avoid the effect of the cut surface of tissue. The internodal cell of *Nitella* and its vacuolar sap were also used. Tissues were killed or injured by freezing, heating and treatment with chloroform gas or 1% acetic acid. Tissue brei and the sap expressed from living as well as dead tissues were also used.

Principal results are as follows:

1. With living tissues, two freezing points appear successively. The first freezing point is higher than the second one. The first freezing point is ascribable to the intercellular liquid, and the second one to the cell content. The first freezing point appears distinctly when the intercellular liquid is not small in amount and the interval from its freezing to the intracellular freezing

is not short. The double freezing is not observed when a tissue is frozen for the second time after thawing, and when the dead tissue is used. In these cases the whole tissue freezes at a time.

2. By death the freezing point of a tissue becomes higher, even higher than the first freezing point. Therefore, the liberation of colloidally bound water by death seems to be chiefly responsible for the rise of freezing point accompanying the death. It is inferred that the micellar and capillary structures in the tissue are responsible for the difference in the freezing point between the dead tissue and the brei or the expressed liquid of dead tissue.

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References

1) AOKI, K., 1946. The first freezing point of plant tissues. Kagaku, Tokyo, **16**: 128. (In Japanese.)
2) ——— 1946. Irregular fluctuation on the freezing curve of plant tissues. Ibid., **16**: 129. (In Japanese.)
3) ——— 1946. Supercooling of living organisms. Ibid., **16**: 129. (In Japanese.)
4) ——— 1949. Freezing curve of potato tuber. Low Temperature Science (Hokkaido Univ.), **2**: 185. (In Japanese.)
5) ——— 1949. Freezing curve of plant tissues. Advances in Biology (Tokyo), **4**: 145. (In Japanese.)
6) ——— 1948. Analysis of the freezing process of living organisms. III. Two freezing points on the freezing curve of plant tissues. Low Temperature Science (Hokkaido Univ.), **4**: 65. (In Japanese, with English summary.)
7) ———, E. ASAHINA & I. TERUMOTO, 1953. Analysis of the freezing process of living organisms. IX. The relation between the shape of the freezing curve and the frost hardiness. Ibid., **10**: 69. (In Japanese, with English summary.)
8) ASAHINA, E., 1956. The freezing process of plant cell. Cont. Inst. Low Temperature Science (Hokkaido Univ.), **10**: 83.
9) CRAFTS, A. S., H. B. CURRIER & C. R. STOCKING, 1949. Water in the physiology of plants.
10) CURRIER, H. B., 1944. Water relations of root cells of *Beta vulgaris*. Amer. J. Bot., **31**: 378.
11) FREY-WYSSLING, A., 1937. Ueber die submikroskopische Morphologie der Zellwände. Ber. deut. botan. Ges., **55**: 119.
12) ——— 1938. Submikroskopische Morphologie des Protoplasmas und seiner Derivate.
13) ——— 1953. Submicroscopic morphology of protoplasm.
14) HATAKEYAMA, I., 1939. Ueber die Temperaturmessungen mit Thermoelementen im Freien. Botany & Zoology (Tokyo), **7**: 1359, 1539. (In Japanese.)
15) ——— 1947. Investigations on the water economy of plants. I. Especially as related to drought resistance. Physiology & Ecology (Kyoto Univ.), **1**: 15. (In Japanese, with English summary.)

16) HATAKEYAMA, I., 1951. The double freezing of living plant tissues. Kagaku, Tokyo, **21**: 95. (In Japanese.)
17) ——— 1951. The difference of freezing between the living and the dead tissue of a plant. Ibid., **21**: 138. (In Japanese.)
18) ——— 1952. An example of double freezing of a green leaf. Ibid., **22**: 652. (In Japanese.)
19) ——— 1957. Relation between the freezing point and the cold hardiness of plant tissue. Physiology & Ecology (Kyoto Univ.), **7**: 89. (In Japanese, with English summary.)
20) KUME, N., 1948. On the freezing of potato (*Solanum tuberosum* L.). Ecological Review (Tohoku Univ.), **11**: 129. (In Japanese.)
21) LEWIS, F. J., & G. M. TUTTLE, 1920. Osmotic properties of some plant cells at low temperatures. Ann. Bot., **34**: 405.
22) LUYET, B. J., & P. M. GEHENIO, 1937. The double freezing point of living tissues. Biodynamica, **30**: 1.
23) ——— & ——— 1939. The physical states of protoplasm at low temperatures. Ibid., **48**: 1.
24) ——— & M. C. GIBBS, 1937. On the mechanism of congelation and of death in the rapid freezing of epidermal plant cells. Ibid., **25**: 1.
25) MAXIMOV, N. A., 1914. Experimentelle und kritische Untersuchungen über das Gefrieren und Erfrieren der Pflanzen. Jb. wiss. Bot., **53**: 327.
26) MODLIBOWSKA, I., & W. S. ROGERS, 1955. Freezing of plant tissues under the microscope. J. Exp. Bot., **6**: 384.
27) PARTINGTON, J. R., 1952. An advanced treatise on physical chemistry. Vol. III. The properties of solids.
28) SCARTH. G. W., & J. LEVITT, 1937. The frost-hardening mechanism of plant cells. Plant Phys., **12**: 51.
29) SIMINOVITCH, D., & G. W. SCARTH, 1938. A study of the mechanism of frost injury to plants. Can. J. Research, (C), **16**: 467.
30) TERUMOTO, I., 1953. Analysis of the freezing process of living organisms. XI. On the significance of the first freezing point of potato tuber. Low Temperature Science (Hokkaido Univ.), **10**: 93. (In Japanese, with English summary.)
31) WALTER, H., 1955. The water economy and the hydrature of plants. Ann. Rev. Plant Phys., **6**: 239.
32) ——— & O. WEISMANN, 1936. Ueber die Gefrierpunkte und osmotischen Werte lebender und toter pflanzlicher Gewebe. Jb. wiss. Bot., **82**: 271.
33) ZACHAROWA, T. M., 1926. Ueber den Einfluss niedriger Temperaturen auf die Pflanzen. Ibid., **65**: 61.