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Measuring Osmotic Pressure of Sap within Live Cells by Means of a Visual Melting Point Apparatus[1]

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ABSTRACT

A freezing slide apparatus is described for visual observation of freezing water and melting ice within plant cells. The slide consists of an ordinary microscope slide glued into a Plexiglass jacket, through which cold 90% ethyl alcohol is pumped at varying rates for temperature control. Temperature is recorded by means of an iron-constantan thermocouple wire (25-micron diameter) connected to a recording potentiometer. Tissue strips were quick frozen (at a cooling rate of 33 C per ½ minute) and then warmed very slowly (at a rate of 2 C per minute) for observation of melting points. This apparatus has been used to determine osmotic pressures of cell sap of guard and adjoining epidermal cells of *Chrysanthemum morifolium* and *Pelargonium hortorum*. An accuracy of ± 1.2 atmospheres is possible. Wide variations among osmotic pressures of both guard and epidermal cells were found at any one stomatal aperture in both species.

During investigations of response of stomatal activity to increased salt concentrations in plant root media, many osmotic pressure determinations had to be made for guard and neighboring epidermal cells. Building and checking an apparatus for this purpose was a necessary prerequisite to actual study of stomatal response to salinity.

A number of devices have been used for microscopically observing ice formation and melting in plant and animal tissues. Smith, Polge, and Smiles (7) built a Perspex slide, fitted with copper conducting arms which were cooled by liquid air or warmed by hot water. The rate of cooling or heating was varied by adjusting the distance along the copper arms between slide and liquid air or hot water containers. A thermocouple connected with a reflecting galvanometer was used to record temperature.

Modlibowska and Rogers (5) used a similar apparatus for studying and photographing freezing of *Tradescantia* staminal hairs and other plant tissues. Solid carbon dioxide was placed on the copper plate conductor to freeze the tissue. Thermocouple output was recorded by a modified Kent recorder. In both of the above methods, illumination was from below and the slide was mounted on a standard microscope stage.

Idle (2) illuminated cut cross sectional surfaces of *Polyanthus* scapes with ultraviolet light, after treating the plant material with a fluorescent dye, and took photographs automatically at 1-min intervals of the material as it froze to detect the mode and direction of ice formation. The plant material was cooled within a chamber with double walls through which a water-methanol mixture from a refrigerated tank was circulated at varying rates.

About 1965, the Clifton Technical Physics Company of Wanamassa, New Jersey, developed a direct reading biological cryostat for determining melting point depressions of frozen plant sections or single cells on a digital readout. The melting point of the sample is observed through any conventional microscope with external or built-in illumination.

MATERIALS AND METHODS

The freezing slide which was constructed for determination of melting point depressions in guard and epidermal cells in this investigation consisted of an ordinary microscope slide glued into a Plexiglass jacket, through which cold 90% ethyl alcohol was pumped at varying rates for temperature control. A 2.5 cm-long section of 3-mm diameter copper tubing was glued into each end of the jacket to connect the Tygon tubing in which the alcohol flowed. The alcohol was cooled by pumping it through about 2 m of Tygon tubing coiled inside an ice bucket filled with Dry Ice. This resulted in an alcohol temperature of about −33 C minimum at the thermocouple junction on top of the glass slide. A vibrostaltic pump (Chemical Rubber Co., Cleveland) was used. Flow rate of the alcohol was regulated by adjusting a screw clamp on the nylon tubing at the inlet end of the freezing slide. Figure 1 is a picture of the apparatus minus the recording potentiometer. Figure 2 shows the dimensions of the freezing slide.

For microscopic observation of ice formation and melting within plant cells, a standard Zeiss microscope was used. A 5- × 5-cm Corning glass heat-absorbing filter, Color Spec. No. 1–69, was placed between the light source and substage condenser to prevent excessive heating of the plant tissue under the objective. A drop of glycerol was placed on the cover slip over the plant tissue to avoid loss of visibility from frost formation.

Temperature at the slide surface was continuously recorded with a 38 British Standard Gauge iron-constantan thermocouple connected to a Speedomax type G recording potentiometer (Leeds and Northrup Co.). To prevent rusting, the reference end of the thermocouple wire was inserted in a glass tube filled with paraffin oil before placing it in an ice-distilled water mixture at 0 C. The thermocouple was calibrated with the recorder chart by immersing it in ice-water-NaCl mixtures of known temperatures. Output of the thermocouple was proportional to temperature.

Two factors made necessary the use of melting rather than freezing points for determining osmotic pressures in the cells.

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FIG. 1. The melting point apparatus.

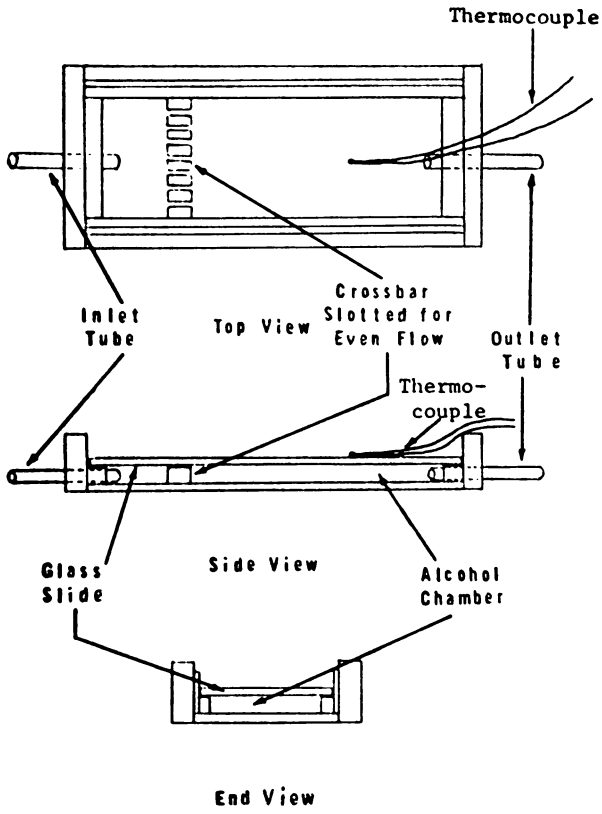

FIG. 2. Construction of the freezing slide.

The first was the tendency of the cells to supercool as much as 5 to 15 C below their freezing points before ice formation took place. The second factor was the formation, at slow cooling rates, of great masses of ice between the cells while simultaneous shrinking and collapse of the cells took place. Often, ice formation failed to take place in shrunken cells. When cooling rates were increased to about 16 C/min, the main location of ice formation became the epidermal cells, and these then swelled with ice while guard cells shrank. These observations bear out

Table I. *Melting Points of Calcium Chloride Solutions Observed on a Freezing Slide*

$CaCl_2$	Melting Points Observed	Freezing Points (3)
M	*C*	*C*
0.00	0.000	0.000
0.10	0.413	0.483
0.15	0.709	0.721
0.20	1.073	0.956
0.30	1.517	1.434
0.50	2.426	2.490
1.00	5.890	5.850

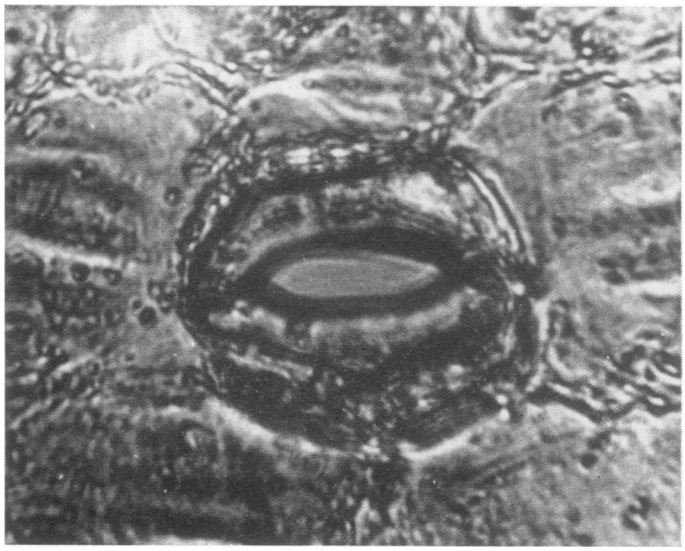

FIG. 3. Stomate and epidermal cells of *Pelargonium hortorum* leaf before freezing. × 1365.

those of Mazur (4), who concluded that cells cooled sufficiently slowly would dehydrate and would not freeze intracellularly.

In epidermal tissue, with stomates open wide, considerable differences in osmotic pressures were expected between guard and epidermal cells (6). The above observations, then, appear to indicate an increasing tendency for cells to supercool with increasing internal osmotic pressure.

For the above reasons, faster rates of cooling were attempted by shortening the inlet tube between ice bucket and slide and by increasing the alcohol flow rate. At a cooling rate of 33 C/half-min, guard and epidermal cells appeared to retain their original dimensions while freezing and so were assumed to undergo minimal change in osmotic pressure.

To determine the accuracy of the melting point determination, droplets of graded aqueous solutions of $CaCl_2$ were placed on the slide in the field of the tip of the thermocouple. The droplets were frozen by rapidly pumping the cold alcohol through the slide jacket and then allowed to warm at the rate of 2 C/min, by regulating alcohol flow to a slow drip from the outlet tube end into the reservoir flask. The melting point of the crystals at the droplet edge was recorded by snapping off the recorder chart so that the pen abruptly described a flat plateau instead of a sloping line as it moved across the chart in response to increasing temperature at the thermocouple junction. In Table I, the resulting melting point depressions are compared with those given by Levitt (3) for $CaCl_2$ solutions. Each value represents a single reading.

In view of the observed deviations from the values of Levitt, the melting points of several drops of distilled water were observed in the same fashion to correct for visual error. The 10 melting points had a mean deviation of 0.099 C, a value close to that of the largest deviation in Table I, where 1.073 − 0.956 = 0.117. This corresponded to an osmotic pressure mean deviation of ±1.2 atm.

Different warming rates were found to influence accuracy of melting point determinations of individual cells. A peridermal slice of epidermal tissue was taken from the underside of a *Chrysanthemum morifolium* leaf and placed cut side down on the slide. The tissue was frozen, and the melting points for a single epidermal cell were determined at three different warming rates. The first cell to melt was recorded and, to prevent loss of sap upon thawing, the cell was immediately refrozen. The melting point at a warming rate of 5.3 C/min deviated from the melting point at 1.76 C/min by 0.03 C. A warming rate of 10.6 C/min gave a melting point that deviated 0.16 C from that at 1.76 C/min. It was therefore decided to remain as close as possible to a warming rate of 2 C/min. The characteristics of the apparatus did not allow warming rates slower than this but did allow consecutive warming rates to agree within about 0.25 C/min of each other. The death of the cell after the first freezing could have caused possible changes in solute concentration of the cell sap by release of solutes from binding sites.

The above data were all obtained from melting of ice crystals at approximately the same distance from the tip of the thermocouple. Since melting points of distilled water droplets were influenced by the distance of the droplets from the thermocouple tip, a new zero point was recorded for each tissue slice. This was accomplished by breathing on the tissue immediately after

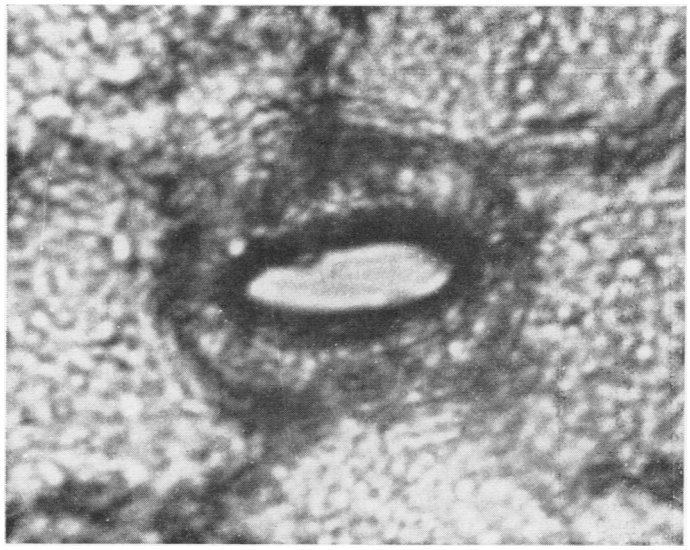

FIG. 4. Same cells as in Figure 3, frozen.

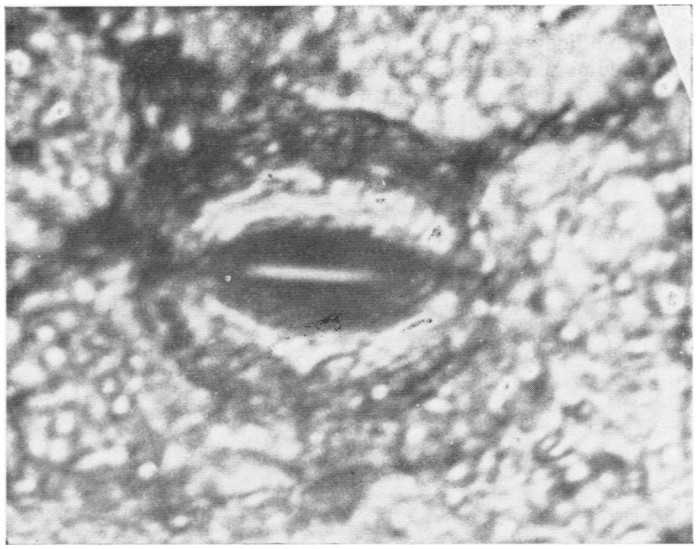

FIG. 5. Same cells as in Figure 3. Guard cells thawed. Stomate closed. Epidermal cells still frozen. Dark areas at periphery of guard cells represent overlapping frozen areas of epidermal cells. Ice crystals have coalesced into larger crystals.

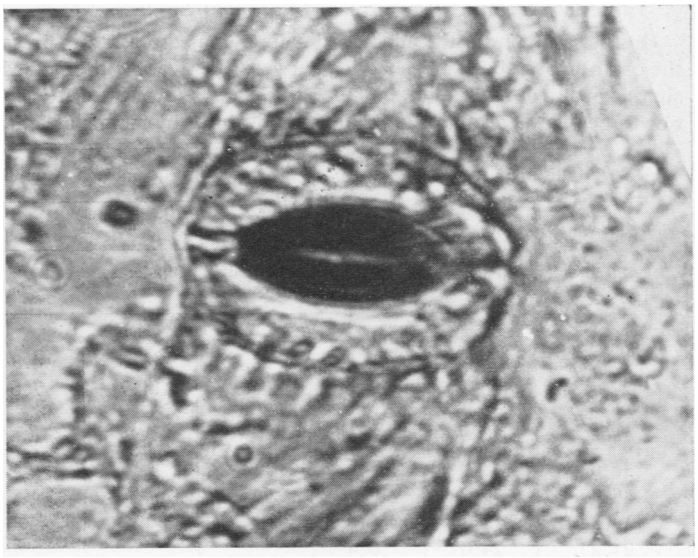

FIG. 6. Same cells as in Figure 3. All cells thawed.

Table II. *Osmotic Pressures of Epidermal Cells of P. hortorum Calculated from Melting Point Depressions of Cellular Ice*

Each value is from a different epidermal strip and represents an individual epidermal cell. Values were taken over a 5-day period from recently fully expanded leaves of four plants, all growing under the same conditions. Four separate determinations were made by plasmolysis. Melting point mean is the average for all melting point values listed here. Leaf sap osmotic pressure: 6.5 ± 0.3 atm. Epidermal cell osmotic pressure mean by melting point: 13.1 ± 0.9 atm; by plasmolysis: 15.0 atm ± 0.03.

Classes			
5–10	10–15	15–20	20–25
atm			
9.9	12.6		
9.9	12.7		
9.7	14.7		
8.7	14.5		
8.7	14.1		
8.0	12.6		
7.6	12.6	19.5	
6.5	12.6	19.3	24.7
6.5	12.3	17.6	21.2
6.5	12.1	16.4	20.8
6.1	10.3	15.1	20.4

Table III. *Range in Osmotic Pressure among Epidermal Cells of P. hortorum, as Determined by a Plasmolyzing Technique*
The method used is described in Reference 3.

Osmotic Pressure of $CaCl_2$ Solution	No. of Cells Plasmolyzed per 100 Observed
atm	
7.4	3
12.3	17
17.3	69
22.6	86
26.7	96

placing it on the slide and recording the melting point in the water droplets nearest to the cells being observed.

During warming of frozen tissue, melting of ice usually took place first in the guard cells, next in epidermal cells, and finally in the ice masses formed from the condensed water droplets on top of the tissue. In this manner, three definite points were recorded by snapping the chart off and on, and the osmotic pressures of guard and epidermal cells were computed from the differences between their respective points and that of the water droplets (assumed to be 0 C) by the following equation:

$$\text{Chart divisions} \times 1.65 \times \frac{298}{273} \times 12.04 \text{ atm}$$

= osmotic pressure in atmospheres at 25°C

Table IV. *Osmotic Pressures of Guard and Epidermal Cells of Normal Chrysanthemum Leaves at Different Stomatal Apertures*
On the same line with each guard cell value is the osmotic pressure for an adjoining epidermal cell determined at the same time and the osmotic pressure difference (Δ) between the two. Neither guard cell nor epidermal cell osmotic pressure values were found to change significantly with stomatal aperture when a 5% level Student's *t* test was used. Any two osmotic pressure difference (Δ) means found not to be significantly different from each other by means of a Student's *t* test at the 5% level are marked by the same letter.

Osmotic Pressures												
0 μ			2 μ			4 μ			6 μ			
Guard cells	Epidermal cells	Δ	Guard cells	Epidermal cells	Δ	Guard cells	Epidermal cells	Δ	Guard cells	Epidermal cells	Δ	
atm			*atm*			*atm*			*atm*			
15.6	8.9	6.7	20.8	16.5	4.3	17.3	12.8	4.5	48.8	16.9	31.9	
27.3	16.2	11.1	19.3	11.7	7.6	16.2	9.8	6.4	31.6	18.9	12.7	
24.4	22.1	2.3				28.6	14.5	14.1	32.8	10.4	22.4	
38.6	19.3	19.3				39.4	15.4	24.0	38.6	10.8	27.8	
36.6	28.0	8.6				30.6	16.9	13.7				
45.9	32.2	13.7				45.5	13.0	32.5				
12.3	12.3	0.0				32.5	14.0	18.5				
24.4	24.0	0.4				28.7	8.9	19.8				
28.1 ± 4.1	20.4 ± 2.8	7.8[1] ± 2.3[a]	20.1 ± 0.8	14.1 ± 2.4	6.0 ± 1.7[a]	29.9 ± 3.5	13.2 ± 1.0	16.7[1] ± 3.2[b]	38.0 ± 4.0	14.3 ± 2.2	23.7[1] ± 4.1[b]	

[1] Denotes significant mean osmotic-pressure difference.

Table V. *Osmotic Pressures of Guard and Epidermal Cells of Normal Geranium Leaves at Different Stomatal Apertures*
On the same line with each guard cell value is the osmotic pressure for an adjoining epidermal cell determined at the same time and the osmotic pressure difference (Δ) between the two. Neither guard cell nor epidermal cell osmotic pressure values were found to change significantly with stomatal aperture when a 5% level Student's *t* test was used. Any two osmotic pressure difference (Δ) means found not to be significantly different from each other by means of a Student's *t* test at the 5% level are marked by the same letter.

Osmotic Pressure												
0 μ			2 μ			4 μ			6 μ			
Guard cells	Epidermal cells	Δ	Guard cells	Epidermal cells	Δ	Guard cells	Epidermal cells	Δ	Guard cells	Epidermal cells	Δ	
atm			*atm*			*atm*			*atm*			
8.0	8.0	0.0	33.3	20.4	12.9	36.8	17.3	19.5	21.7	6.5	15.2	
8.2	12.6	−4.4	25.4	12.6	12.8	38.6	15.2	23.4	51.1	10.0	41.1	
8.7	6.5	2.2	17.9	12.6	5.3	23.4	13.0	10.4	23.0	10.0	13.0	
8.7	8.7	0.0	14.1	9.7	4.4	27.8	10.0	17.8	30.3	14.7	15.6	
20.8	8.7	12.1				40.3	12.6	27.7	21.7	6.5	15.2	
15.2	19.5	−4.3				19.5	12.1	7.4				
14.1	14.1	0.0				23.0	17.6	5.4				
16.4	16.4	0.0				23.2	8.2	15.0				
12.5 ± 1.7	11.8 ± 1.6	0.7 ± 1.7[a]	22.7 ± 4.2	13.8 ± 2.3	8.9 ± 2.3[b]	29.1 ± 2.9	13.3 ± 1.2	15.8[1] ± 2.8[b]	29.6 ± 5.7	9.5 ± 1.5	20.0[1] ± 5.3[b]	

[1] Denotes significant mean osmotic pressure difference.

Figures 3 to 6 are photographs of cells as they appeared at certain stages of the freezing and thawing process. As frozen tissue warmed, cell ice became progressively coarser in appearance, and, shortly before melting, the ice crystals coalesced into large clear plates. If the observer were not watching carefully, ice plate formation might be taken for thawing. The melting point recorded was the point at which the large plates of ice disappeared after melting from the edges inward.

A further check for accuracy compared results from this method with those from incipient plasmolysis (3). Epidermal samples for melting point determination and plasmolysis measurements were taken from the centers of similar leaves. To determine whether epidermal cell sap was isotonic with sap expressed from entire leaves, sap samples were expressed from fresh, newly expanded leaves of *Pelargonium hortorum*. After freezing and thawing the leaves, sap osmotic pressure was measured with a Mechrolab-3000 vapor pressure osmometer.

Since osmotic pressures of guard and epidermal cells were expected to be important in determining ability of stomates to open, melting point depressions were determined for guard and epidermal cells of *P. hortorum* and *C. morifolium*.

RESULTS AND DISCUSSION

Average osmotic pressure obtained from plasmolytic determination approximated that from the melting point technique (Table II). No attempt was made to correct for volume change in plasmolytic determinations.

Concentration of sap in intact epidermal cells was consistently higher than that of sap expressed from the entire leaf. Possibly, juice expressed from frozen and thawed leaves may be more dilute than juice in intact leaves because of solute retention within pressed tissue by adsorption or filtering effects. Osmotic pressures for epidermal cells in Table II were confined to cells adjoining guard cells. In order to eliminate the possibility that these cells might possess higher osmotic pressures than cells farther removed from guard cells, osmotic pressures were obtained from 13 epidermal cells of *P. hortorum* bordered only by other epidermal cells. The average value obtained was 14.7 $\pm$ 1.4 atm. This was not significantly different from the 13.1 $\pm$ 0.9 atm average osmotic pressure shown in Table II for epidermal cells adjoining guard cells in geranium.

An additional finding in this investigation was the tremendous range of osmotic pressures among individual epidermal cells (Table II). This was also shown by the plasmolytic technique (Table III). The distribution of values is not the same for the two tables. This may be because samples were taken at different times of the year and from different geranium plants.

Osmotic pressures for guard and epidermal cells of chrysanthemum and geranium varied widely for all apertures (Tables IV and V). Because of these wide ranges of values, no significant correlation with stomatal opening could be found for any of the guard cell or epidermal cell osmotic pressures. Equally high values were obtained for some guard cells of closed stomates and some of stomates with apertures of 6 μ.

Wide variation also existed among osmotic pressure differences for any one stomatal aperture in both species. However, these values tended to be higher for open stomates than for closed. Actual numerical values of the averages appear to indicate a tendency for osmotic pressure difference to increase with extent of stomatal opening, but the wide range among individual values somewhat obscures any definite correlation.

In this connection, morphological variations among guard cells and differences in position of guard cells relative to epidermal cells have been noted (1). Differences such as these may exist to a lesser extent among stomates in the same species. Morphological variations may affect the ability of guard cells to expand. The extent to which guard cells are sunk in the epidermis affects the angle at which epidermal cell counterpressure is exerted against guard cells. These factors may combine to make opening of an individual stomate to a given aperture dependent on a guard cell-epidermal cell osmotic pressure difference necessary and specific for the particular structural environment of that stomate.

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