Osmotic Properties of some Plant Cells at Low Temperatures.

BY

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With five Figures in the Text.

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INTRODUCTION.

DURING the last few years the cryoscopic method of determining the osmotic pressure of plant saps has largely replaced the older plasmolytic methods. Investigations have been directed to seasonal changes in the constitution of the sap, as well as to changes due to differences in habitat, both in the same species and in different species characteristic of various plant formations.

The most recent work dealing with the first aspect of the subject is the work published by Dixon and Atkins in the Proc. Royal Dublin Society (5). These authors have carried out a fine series of researches on the seasonal changes in osmotic pressure and the relative proportion of electrolytes and non-electrolytes in the cell sap, both in leaves and in the conducting tissues of stems and roots of various plants. This work was directed particularly to the elucidation of the part played by osmotic pressure in the ascent of sap. Attention was paid to methods of extracting the plant sap to ensure that the extract was a fair sample of the sap within the living cell—a matter to which but little attention had been paid by previous workers.

The ecological side of the problem has been attacked by Harris and Lawrence (1), working on the osmotic pressures of Jamaican rain-forest vegetation. These authors found that vegetation of different ecological habitats possessed different osmotic pressures, but no information is given in their work as to the relative proportion of electrolytes and non-electrolytes in the several types they investigated.

It seems probable that ability to withstand low temperatures depends entirely upon the organization and biochemical characters of the cells. Although attempts have been made to recognize modifications of structure which would enable aerial or subaerial organs to withstand low temperature, none have been found whose presence would render the plant resistant.

An increase in the concentration of the cell sap will lower the freezing-point, but this seldom amounts to more than 2°C . Müller (2) has shown that living plants usually have a lower freezing-point than the expressed sap. The fact that the actual freezing-point of the cell is lower is due to surface tension forces, such as capillarity and imbibition. Mousson (3) found that in a capillary tube of not more than 0.4 mm. diameter the freezing-point of water was lowered 0.1° to 0.2°C . In order that sub-cooling may take place a localized formation of ice must be avoided. When contact with ice is avoided sub-cooling is observed in water. Thus Dufour (4) obtained the sub-cooling of -12°C . in small drops of water floating in a mixture of almond oil and chloroform.

In the experiments carried out by Müller-Thurgau, leaves were wrapped round the bulb of a sensitive thermometer and inserted in a freezing mixture with the thermometer scale projecting outside. With this method the temperature sinks until the maximum degree of sub-cooling is reached, and then rises to the true freezing-point, when ice formation takes place, after which it falls gradually. According to Pfeffer the sub-cooling in most plants is not more than -3° to -4°C . It is important to remember that most previous experiments have been performed with plants living in comparatively temperate climates, and results obtained in this investigation (in Western Canada) with plants subjected to long periods of -40° to -50°F . have some bearing on the question.

SCOPE OF WORK.

The scope of the present investigation may be indicated as follows:

1. To determine the osmotic pressure, relative amount of electrolytes and non-electrolytes, and sugars in a few typical plants living at low temperatures from the autumn throughout the winter.
2. To determine the temperature at which ice formation actually takes place in the living cells of the tissues.
3. To observe the condition of the cell contents at low temperature.

The materials chosen for investigation were *Picea canadensis* (Miller)

B.S.P., leaves; *Pyrola rotundifolia*, L., leaves; *Linnaea borealis*, L. var. *americana* (Forbes), leaves; *Populus tremuloides*, Michx., cortical tissues. During the winter some difficulty was experienced in collecting *Pyrola* and *Linnaea* from under the snow, thus limiting the number of observations that could be made with these plants.

METHODS.

According to the observation of Dixon and Atkins (5), sap expressed by simple pressure from living tissues does not give a fair sample of the sap in the vacuoles of the living cell. In the researches of these authors the protoplasm of the living cell was rendered permeable to all the solutes by the application of extreme cold, liquid air being used for this purpose. After freezing by this means the protoplasm is killed, rendered quite permeable, and the sap with all the solutes can be extracted by comparatively slight pressure.

As liquid air was not obtainable for these experiments, liquid CO₂ was used. This gives a temperature of -72°C. , which results in the instant freezing of the tissues. The material was then placed in a glass vessel, rapidly thawed, and on treatment in a small screw-press about 25 c.c. of sap could be obtained from a comparatively small amount of material of all the plant tissues investigated. Care was taken to ensure that all the CO₂ was evaporated before the extraction of the sap. The increased ease with which the sap is extracted by this method is striking. One sample of untreated cortex of *Populus tremuloides* gave only a few drops of sap on pressing; an equivalent weight of cortex killed by freezing yielded 14 c.c. of sap with slight pressure.

The Δ of the expressed sap was determined at once—usually within the hour—by the ordinary Beckman method, and the measurement of the electrical conductivity was then measured by the method of Kohlrausch. The estimation of sugars was made by treating the extracted sap with a minimum amount of basic lead acetate to precipitate the tannins. The excess of lead was then removed by sodium carbonate. The extract freed from tannins was tested with Benedict's sodium citrate solution (6). All determinations were made in drops rather than in cubic centimetres, since the quantity of extract available would not permit of the latter method. The same burette was used throughout the investigation, thus ensuring a uniformity in the size of the drops. The results recorded in each case were the number of drops required to decolorize twenty-five drops of Benedict's solution; the relative reducing power of the extract before and after inversion being determined by comparison with that of a standard glucose solution. The relative amount of glucose, sucrose, and maltose were determined according to the methods described by Haas and Hill (7).

To minimize any marked variation of the sugar content due to variation in photosynthesis at different times of the day, all material was collected at 2 p.m.

METEOROLOGICAL CONDITIONS.

Records of the temperature were kept from January onwards by means of a Casella Recording Thermograph. The instrument was fixed on a wall facing north, about sixty feet above the ground. The temperatures recorded were probably slightly above those obtaining at the level of the ground. A reduced record of the maximum and minimum temperature curve is given in Graph I. As the instrument only recorded down to $+10^{\circ}\text{F}$. the breaks in the diagram indicate periods below this temperature. The minima are recorded in figures in these blanks.

The first part of the winter was unusually free from low temperatures, the lowest temperatures coming in February and March.

RECORDS OF OSMOTIC PRESSURES, &C.

All the material was obtained from the same habitat—a slope facing north. Trees of *Picea* of approximately the same age were used throughout—about fifteen years. To obviate any discrepancy due to individual differences of pressure in the trees, branches were cut from several trees, brought into the laboratory, and the leaves cut off. To eliminate any error due to differences in osmotic pressure of leaves of different ages only those from the previous year's shoot were taken. The leaves were then frozen and thawed in the manner already described and the cell sap expressed. The osmotic pressure was determined immediately, and then the conductivity, the whole process for one observation taking about three hours. The sugar estimation in some cases was made the following day, and in that case the sap remained frozen during the interval.

The depression of the freezing-point and the corresponding pressures in atmospheres are recorded in the tables and in the ordinates of the graphs. The figures in column C give the conductivity of the sap at 37°C . The figures under Δ_e are calculated from those under Δ and C, and represent the depression in freezing-point of solutions of potassium chloride having the same conductivity as those observed for the sap. They represent the total Δ due to electrolytes. The column $\Delta - \Delta_e$ represents the total Δ due to the presence of non-electrolytes in the sap. This method was used by Dixon and Atkins (8), and gives clear indication of the main changes going on in the cell.

The material for the observations on *Populus*, *Linnaea*, and *Pyrola* was obtained from the same habitat as *Picea*. The respective readings are given in Tables I to IV and recorded in Graphs II to V. The atmospheric

pressure P is the value corresponding to the observed Δ given in the Tables published by Harris and Gortner (9).

GRAPH I.

TABLE I. *Picea canadensis*.

Date.	Δ .	P .	Δ_e .	$\Delta - \Delta_e$.	$C \times 10^5$.	Sucrose %.	Maltose %.	Glucose %.
Oct. 23	1.424	17.13	—	—	—	—	—	—
Dec. 7	1.777	21.36	—	—	—	—	—	—
„ 15	1.655	19.91	—	—	—	—	—	—
„ 22	1.680	20.20	—	—	—	—	—	—
„ 28	1.635	19.66	0.179	1.456	771	—	—	—
Jan. 1	1.586	19.07	—	—	—	—	—	—
„ 8	1.705	20.50	0.187	1.518	793	—	—	—
„ 18	1.700	20.44	0.179	1.521	771	—	—	—
Mar. 7	1.909	22.94	0.214	1.695	921	0.20	0.97	0.24
„ 26	2.240	26.91	0.143	2.097	617	0.16	0.58	0.47
April 2	1.720	20.68	0.119	1.601	514	0.39	2.00	0.01
„ 7	1.625	19.53	0.142	1.483	612	0.62	0.00	1.00
May 26	1.750	21.04	0.191	1.559	822	0.00	0.77	0.03
June 4	1.635	19.66	0.261	1.374	1121	0.00	0.00	1.00

GRAPH II. *Picea canadensis* (leaves).

TABLE II. *Populus tremuloides*.

Date.	Δ .	P.	Δ_e .	$\Delta - \Delta_e$.	$C \times 10^5$.	Sucrose %.	Maltose %.	Glucose %.
Oct. 22	1.247	14.99	—	—	—	—	—	—
Dec. 7	1.460	17.56	—	—	—	—	—	—
„ 16	1.202	14.48	—	—	—	—	—	—
„ 27	1.535	18.46	0.081	1.454	352	—	—	—
Jan. 18	1.344	16.17	0.083	1.261	354	—	—	—
Feb. 22	1.137	13.68	0.120	1.017	518	0.20	0.98	0.02
Mar. 23	1.410	16.96	0.078	1.332	335	0.20	0.98	0.02
May 28	0.877	10.56	0.114	0.763	493	0.08	0.28	0.45

GRAPH III. *Populus tremuloides* (bark and cortex).TABLE III. *Linnaea borealis*.

Date.	Δ .	P.	Δ_e .	$\Delta - \Delta_e$.	$C \times 10^5$.	Sucrose %.	Maltose %.	Glucose %.
Oct. 24	1.630	19.60	—	—	—	—	—	—
Dec. 26	2.080	24.99	0.342	1.738	1469	—	—	—
Mar. 21	2.127	25.55	0.343	1.784	1473	0.20	0.91	0.00
May 29	1.187	14.28	0.478	0.709	2056	0.00	0.00	0.31

GRAPH IV. *Linnaea borealis* (leaves).

TABLE IV. *Pyrola rotundifolia*.

Date.	Δ .	P.	Δ_e .	$\Delta - \Delta_e$.	$C \times 10^5$.	Sucrose %.	Maltose %.	Glucose %
Dec. 19	2.222	26.69	—	—	—	—	—	—
„ 29	1.863	22.42	0.223	1.640	986	—	—	—
Feb. 8	1.992	23.94	0.276	1.716	1186	0.12	1.21	0.00
April 11	1.430	17.20	0.181	1.249	780	0.00	0.11	0.37
June 4	1.045	12.58	0.239	0.806	1028	0.00	0.00	0.25

GRAPH V. *Pyrola rotundifolia* (leaves).AMOUNT OF UNDER-COOLING AND Δ OF LEAVES.

Experiments were made to ascertain the temperature at which ice first appears in the leaves of *Picea*, *Linnaea*, and *Pyrola*. *Picea* leaves were found unsatisfactory to use for this purpose, as they could not be packed tightly round the bulb of the thermometer. *Linnaea* leaves are too small for successful use, and most of the observations were carried out with the leaves of *Pyrola*.

Experiments have been carried out by Müller-Thurgau on the freezing-point of cell sap *in situ* by either placing the bulb of the thermometer in a succulent tissue such as potato, or wrapping the bulb with the leaves to be tested. This method, while not yielding results comparable in accuracy to those obtained by placing the Beckman thermometer in a solution, is still of considerable value, as it is capable of indicating the amount of under-cooling which the minute volume of sap in the cell vacuole is capable of undergoing without the formation of ice. That the amount of sub-cooling in minute volumes, when contact with ice is prevented, may be much greater

than in comparatively large volumes is indicated by the work of Dufour (4). Direct observations on plant tissues have confirmed this. Thus Müller (2) found the lowest sub-cooling point without freezing of the grape was -6.8° to -7.8° C., while the real freezing-point of the sap was -3.1° C. Dixon and Joly (10) found ice formation in the tracheides of *Taxus* began at -10° to -11° C. In our experiments we first carried out observations on greenhouse plants, and these are recorded in Table V.

TABLE V.

<i>Material leaves.</i>	<i>Treatment.</i>	<i>Freezing mixture.</i>	<i>Maximum under-cooling.</i>	<i>Δ of sap within cells of leaf.</i>
Impatiens	Fresh material from greenhouse	NaCl + Ice -10°	— 1.3	— 1.1
Polypodium	" "	"	— 2.9	— 2.5
Begonia	" "	"	— 2.8	— 2.1
Syringa	" "	"	— 2.8	— 2.5
Pyrola	Killed by freezing in CO ₂ , thawed, and tested	Solid CO ₂	— 3.5	— 3.1
Pyrola	Fresh leaves not previously killed	"	— 32.1	— 31.65

All these plants give a sub-cooling from -1.3° C. in *Impatiens* to -2.9° C. in *Polypodium* and the Δ with formation of ice from -1.1° C. in *Impatiens* to -2.5° C. in *Polypodium*.

Pyrola leaves show very different values. The material was gathered outside during a spell of -20° F. weather. A thermometer graduated to 0.1° and registering to -40° C. was used in the Beckman apparatus, as the amount of under-cooling was too great to allow the use of the ordinary Beckman thermometer.

The behaviour of the living and dead leaves gave results of some interest. The leaves were killed by freezing in liquid CO₂, rapidly thawed, and the amount of under-cooling and Δ determined. The fresh leaves under-cooled to -32.1° C., rising to -31.65° C. on the formation of ice in the tissues. The leaves previously killed with liquid CO₂, thawed, and tested in the Beckman apparatus using a freezing mixture of solid CO₂, under-cooled to -3.5° C., rising to -3.1° C. on the formation of ice in the tissues. Further observations will be carried out next winter by us using the leaves of other northern evergreens. That the condition of the cell contents during periods of low temperature determines the resistance of the tissue to cold is suggested in a paper by one of us in 1919 (11). *Linnaea* leaves naturally de-starchified are able to withstand the cold of a northern winter, whilst plants in which the fat has been reconverted into starch are quickly killed on exposure to temperatures of about -19° F. (-28° C.).

CONDITION OF CELL CONTENTS AT LOW TEMPERATURES.

Pfeffer (12), in describing the work of Mohl, states that the brownish-green colour of evergreens during winter is due to a partial disorganization of the chloroplastids, accompanied by certain changes in the chlorophyll pigments. In some cases the browning only takes place when the cells are fatally affected, but with the restoration to normal conditions of light the chloroplastids in most conifers recover, turn green, and become functionally active.

During the progress of the present investigation we decided to examine the condition of the leaf cells of some of the material with a view to deter-

FIG. 1. *Picea canadensis*. Feb. 21. Mesophyll cells showing 'laking' of the chloroplast. Identity of individual chloroplasts completely lost, nuclei are prominent and very granular. *nu.* = nucleus, *ppl.* = protoplasm, *chl.* = chlorophyll.

mining whether similar features prevailed in this region. Microscopic examination of the mesophyll cells of *Picea* reveals a distinct localization of the cell contents during winter (Fig. 1). In this condition, which prevailed during the coldest weather, the identity of the individual chloroplast was completely lost. The diffuse chlorophyll mass, light yellowish green in colour, was segregated in a relatively small portion of the cell, closely associated with the very granular nucleus. The major portion of the cell was occupied by a large vacuole filled with fat (Fig. 2). All trace of starch disappears early in the autumn.

Leaves were examined frequently during the late winter and early spring, in order that the change to the summer condition might be observed. It was found that the change began quite early and took place within a short time. Cells examined on April 7 differed in several particulars from those of earlier preparations. The chlorophyll mass, though distinctly localized, gave evidence of the formation of distinct chloroplastids. Starch formation had commenced, but was localized in the region of the chloroplasts (Fig. 3). The central vacuole, though smaller, still gave a strong reaction when tested for fat with osmic acid. Three days later the change

had gone still farther; the chloroplasts, now definite in outline, were arranged at the periphery of the cell. Starch formation around the chloroplast was especially evident at this stage (Fig. 4). Material examined on April 14 revealed the cells in the normal summer condition, the chloroplast being entirely masked by the adherent starch granules (Fig. 5).

FIG. 2.

FIG. 4.

FIG. 3.

FIG. 5.

FIGS. 2-5. *Picea canadensis*. Fig. 2. Feb. 28. Mesophyll cells after treatment with 1 % osmic acid showing vacuoles filled with fats. Fig. 3. Apr. 7. Mesophyll cells treated with iodine solution. Chloroplasts becoming distinct in outline although still localized in distribution; nuclei not visible; starch reaction localized. Fig. 4. Apr. 10. Mesophyll cells in iodine solution. Chloroplasts arranged at periphery of cell; nucleus visible and suspended in central vacuole; starch formation in region of chloroplasts. Fig. 5. Apr. 14. Mesophyll cells treated with iodine solution. Nucleus not visible at this stage. Chloroplasts closely packed round periphery of cell and masked by starch granules. *nu.* = nucleus, *v.* = vacuole, *chl.* = chlorophyll, *ppl.* = cytoplasm, *st.* = starch.

DISCUSSION.

The changes recorded in osmotic pressures cannot be due to peculiarities of individual plants. In the case of *Picea*, material for each reading was collected from several different trees of approximately the same age, and in *Populus* the cortical tissue for each pressing was stripped from two or three young saplings. To obtain sufficient sap for a reading of *Pyrola* leaves from at least 50 or 60 plants had to be used, and more than that number were taken for *Linnaea*.

In *Picea canadensis* the osmotic pressure rose during the late autumn months, fluctuated slightly at the end of the year, and then rose steadily to a maximum in late March of 26.91 atmospheres. By the beginning of June the pressure had fallen to 19.66 atmospheres, but was still higher than the October value of 17.13 atmospheres. The electrolytes show only slight changes throughout the period from December to June, although a decided rise began to be apparent at the end; approximately 7 weeks after the end of severe frost. The variation of osmotic pressure appears to be due to non-electrolytes. The sugars do not appear to play the chief part in the variation of the non-electrolyte curve $\Delta - \Delta_e$, but the rapid increase in the total sugars—especially maltose—at the beginning of starch formation is noteworthy. The possible part played by the metabolism of fats in the variation of the osmotic curve will be discussed in a future paper.

In *Populus tremuloides* two maxima are observed in the osmotic pressure, but, as in *Picea*, the pressure falls decidedly as the summer condition is reached. The difference between the maximum and minimum osmotic pressure is only about one-half what it is in the leaves of *Picea*. Here again, the variation in pressure is due chiefly to non-electrolytes, although the electrolytes show a slight increase from March onwards.

In *Linnaea borealis* the maximum osmotic pressure is attained about the third week in March and falls rapidly until June, and the electrolyte curve shows a decided rise at the end of the observations. It was impossible to avoid using a number of young leaves for the reading on May 29, and the small osmotic pressure and increase in the electrolytes may be due to this.

Pyrola rotundifolia, unlike the other examples, shows a fairly steady decrease in osmotic pressure from December to June, and the electrolytes remain fairly constant throughout the season. The same fall in the sugar content occurs as the spring is approached, but commences somewhat earlier than in the other plants.

SUMMARY.

1. Osmotic pressures, electrical conductivities, proportions of electrolytes and non-electrolytes, and the amounts of sucrose, maltose, and glucose have been determined in the leaf tissues of *Picea canadensis*, *Linnaea borealis*, *Pyrola rotundifolia*, and the cortical tissues of *Populus tremuloides* at intervals from the autumn until the summer.

2. No certain correlation between the above values and the daily or weekly fluctuations of air temperatures can be recognized without observations extending over more than one season.

3. The maximum osmotic pressure is reached in *Picea* and *Linnaea* towards the end of March; in December in the case of *Populus*, although a second maximum occurs in late March nearly of the same value as the December reading. *Pyrola* shows a fairly steady decrease from the middle of December till June.

4. The concentration of electrolytes shows very little variation in any of the plants during the whole period of observation.

5. The variations in osmotic pressure are due chiefly to the non-electrolytes.

6. The variation of the sugar content closely follows the variation of the osmotic pressure.

7. The sugars show a decided concentration during the winter months.

8. There is a progressive decrease of the sugars from the winter maximum towards the summer.

9. Leaves of *Pyrola* killed by freezing in liquid CO_2 show ice formation at -3.1°C .

10. In the living leaves of *Pyrola* ice formation does not begin until a temperature of -31.6°C . is attained.

11. During the winter months chlorophyll granules in *Picea* are completely laked, the chlorophyll being localized in the region of the nucleus.

12. During the early part of April the chloroplastids assume definite form and starch formation commences.

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