

CELLULAR CHANGES
ASSOCIATED WITH
THE HARDENING OF PLANTS

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by

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INTRODUCTION.

Why do plants winterkill? How are some able to withstand the coldest temperatures while others are destroyed by the first touch of frost? As early as 1727, Stephen Hales attempted to answer these questions. "Plants of a less durable texture", he states, "as they abound with a greater proportion of Salt and Water, which is not so strongly attracting as sulphur and air, so are they less able to resist the cold; and as plants are observed to have a greater proportion of Salt and Water in them in the spring, than in the autumn, so are they more easily injured by cold in the spring, than in a more advanced age, when their quantity of oil is increased, with their greater maturity".

Two centuries later, most investigators are now willing to admit that they are unable to explain the phenomena of cold injury and frost resistance. And yet a tremendous amount of work has been done on the subject. Harvey (1935) in his bibliography lists 3400 references on the low temperature relations of plants. On account of this vast nature of the subject, no attempt will be made at a thorough review of its history, especially since excellent ones have in recent years appeared (Åkerman 1927, Maximov 1929, Wartenberg 1933).

In the past three decades, investigations have been especially numerous due to the practical significance of the subject. Workers, for the most part, have pursued the problem by means of physico-chemical studies on dead plant material. Much valuable and fundamental information has in this way been obtained. But progress along these lines now seems at a stand-

still. It is obvious that new methods of approach are desirable.

Consequently, the following research has been conducted on a totally different basis - the cellular physiology of the plant. Many of those who have attacked the problem of cold resistance by the usual methods (chemical and physical analysis) have been forced, eventually, to conclude that the seat of the solution is the living protoplasm. Of course, some have applied their results as a proof that protoplasmic changes do occur; but this is, unfortunately, a fallacy. If the protoplasm is altered in any way, this can only be discovered by means of an investigation on the living substance itself, and not on the products of its disorganisation. This fact has been kept constantly in mind during the following investigation, and all the experiments have been conducted on normal, living cells.

It is unfortunate that on account of the complex and but little understood nature of protoplasm, few methods for studying its changes are known. That, indeed, is why this aspect of the problem of cold resistance has been left so severely alone. One can, of course, determine the osmotic pressure of the vacuole fluid in the living cell, by means of the de Vries plasmolytic method. This is the simplest and most direct way to investigate the water relations of the living cell - a factor of paramount importance in cold resistance. Walter (1931) emphasizes that this not only gives information with

regard to the non-living sap, but is also a measure of the "hydrature" of protoplasm itself. The protoplasm and cell sap are constantly in contact, being separated only by the semi-permeable plasma membrane. The water systems of both are, therefore, in equilibrium. Clearly, then any change in the osmotic concentration or water-holding capacity of the cell sap will cause water to flow through the membrane in the direction that will reestablish the temporarily unbalanced equilibrium. Conversely, any change in the water-holding power of protoplasm itself will cause it either to give up water to the vacuole or to absorb more from it. The cell sap concentration is, in this way, a measurement of the water-holding power of the protoplasm.

Yet in the latter case, will any measurable change occur in the osmotic concentration of the cell sap? The quantity of protoplasm in a plant cell is so small that even if it were to give up half of its water content to the vacuole, it is very unlikely that any change in the concentration of the latter could be detected. But the protoplasm would be reduced to one half its bulk. Obviously, the simplest method of determining this is by measuring the thickness of the protoplasm layer. The water relations of the living protoplasm, then, were determined by measurements of the osmotic pressure of the cell sap, and of the thickness of the protoplasm layer. The osmotic pressure changes are, of course, due to fluctuations in soluble carbohydrate content, and as such have a two-fold significance in the problem of cold resistance.

A third cellular characteristic which one might consider worthy of investigation is permeability. That this plays a role in low temperature relations has sometimes been suggested, though little or no experimental work has ever been attempted. Some of the theories proposed to explain cold injury and frost resistance can be either refuted or substantiated by demonstrating a change in permeability. But even if permeability is not of direct importance to the subject, it is reasonable to expect that if other protoplasmal characteristics are altered, these may be reflected as a change in permeability, which would then act as a barometer.

The osmotic pressure and permeability were in most cases investigated simultaneously; however, they will be treated separately for the sake of clearness.

MATERIALS.

Since the principles here investigated are of very general significance, a large variety of plants was used to test them. The following is a complete list of the species employed:

(a) Herbaceous plants:-

Brassica oleracea capitata

Trifolium pratense

Helianthus annuus

Ricinus communis

Allium cepa

Phaseolus vulgaris

Lycopersicum esculentum

(b) Woody plants:-

Pyrus malus

Hydrangea paniculata

Catalpa sp.

Picea pungens

Liriodendron tulipifera

Caragana arborescens

Aesculus hippocastaneum

Pterocarya rhoifolia

OSMOTIC PRESSURE.

It is now a generally established principle that during the hardening period an increase in the sugar content and, consequently, in the osmotic pressure of the cell sap occurs. Russow already in 1882 asserted that starch disappears during November and reappears in March. Müller-Thurgau (1886) showed that exposure of plants (e.g. potatoes) to low temperatures causes a hydrolysis of starch to sugars. However, neither these investigators nor their contemporaries appeared to consider this phenomenon anything more than pathological. It remained for Lidforss (1896, 1907) to extend and expand on their observations. In an exhaustive survey of 130 phanerogams, he was able to show that the winter season causes a complete disappearance of starch from the leaves of evergreens and a corresponding increase in soluble sugars. Miyake (1902), however, observes that this depends more or less on the temperature. He subdivides Japan into three regions according to the severity of the winter. In the coldest of these, almost all the starch disappears, but in the other two, more or less amounts are still to be found in overwintering leaves. Further, this hydrolysis of starch does not apply to the storage cells in trees - the pith, medullary rays, and xylem parenchyma. These tissues are packed with starch throughout the winter in the case of "starch trees".

Lidforss (1907) further showed that the evergreens are resistant to cold only when their tissues possess these high quantities of sugars. Just as soon as starch appears in their tissues, frost resistance is lowered. It is but a short step

from here to attempt a distinction between the relative winter hardiness of plants by estimating the quantities of sugars occurring in them. Lidforss himself stopped short of this, but since his time numerous efforts have been made to distinguish in this way between hardy and tender varieties of a species. The one group of plants which has served most as experimental material is our winter cereals.

Gassner and Grimme (1913) were able to differentiate between frost resistant and non-resistant grain seedlings by means of their sugar content. Newton (1924) found that the freezing-point of the sap of wheat varieties is strictly proportional to their hardiness. Martin's (1927) results show the same trend. Åkerman (1927) exhaustively investigated the overwintering ability of wheat varieties in Sweden during a period of five years, and obtained a strict correlation between the values so obtained and the quantities of soluble carbohydrates. Osmotic pressure determinations by the plasmolytic method gave similar results. Tumanov (1931) proved that light is necessary for the hardening off of wheat seedlings, and that this is because the young wheat plant must photosynthesise in order to build up a sugar reserve. Dexter (1933) found the same relationship with regard to carbon dioxide supply. Govorov (1923) distinguishes between spring and winter wheats on the basis of sugars: the former lose glucose more quickly at warm temperatures, the latter gain it more rapidly at low temperatures. Newton and Brown (1931) further state that hardy varieties of wheat maintain their sugar reserves during winter better than the less

hardy. Mudra (1932) asserts that the relative hardiness of winter wheats can be determined refractrometrically, or better by sugar determinations.

This body of evidence from workers the world over (and it by no means exhausts the list) seems to prove beyond all doubt the fundamental importance of soluble carbohydrates to cold resistance. And yet some contradictory results have been recorded. Salmon and Fleming (1918) could discover no relation whatever between hardiness of rye, wheat, barley, and oats and their sap density during fall and early winter. Tumanov (1931) admits that winter vetch (which, however, is not a grain) is an exception among winter annuals for it is just as hardy as winter wheat and yet possesses only half the amount of soluble carbohydrates. According to Balde (1930), Swedish wheat varieties give sugar values which indicate their hardiness, and some German varieties show a similar trend; but it is not possible to obtain significant results by comparing Swedish and German varieties. Gassner and Goeze (1931) could discover no hard and fast series of varieties refractrometrically. Constantinescu (1934) found that varieties of winter barley of different hardiness showed the same increase in sugar content and in dry matter when hardened off. Also, less hardy varieties showed a greater total sugar content at low temperatures than more hardy ones.

Results with other groups of plants have, on the whole, not proved quite so satisfactory as with winter cereals.

Ohlweiler (1912) showed that twelve *Magnolia* species, when grouped according to the freezing points of their saps, showed a close relation to the injury suffered during an April freeze (25°F). The West Indian type of *Persea americana* was found by Harris and Popenoe (1916) to have a distinctly higher freezing point and to be less resistant to frost than the Guatemalan and Mexican types. Magistad and Truog (1925) were able to increase the osmotic pressure and the cold resistance of corn plants by fertilisation. Obviously, none of these cases involves true cold resistance. They are merely instances of tender plants escaping ice formation or, at best, reducing it to a minimum by the possession of a sufficiently high cell sap concentration. Thus Chandler (1913), after an exhaustive series of experiments with plants which kill at relatively high temperatures, concluded that their killing point is lowered when the sap density is increased.

Of greater importance are the results with more hardy plants. Gail (1926) describes a rapid increase in the osmotic pressure of five evergreens from mid-July to December and January. Meyer (1928), however, asserts that the increased osmotic pressure in the pitch pine is insufficient to account for the degree of cold resistance developed. Harvey (1918) comes to the same conclusion with regard to cabbage. Hildreth (1926) found a strikingly similar increase in sugars from fall to winter in the Duchess and Jonathan apple varieties, and though the former is much hardier than the latter, the difference

in sugar content during the winter is small. Non-electrolytes in spruce were shown by Lewis and Tuttle (1920) to increase from December to March. In *Pyrola*, on the other hand, there was a steady decrease from December to June. Weimer (1929) could find no correlation between hardness and the freezing point of alfalfa. Rein (1908) asserts that the cold-death point does not depend at all on osmotic pressure. But he worked with very dissimilar and unrelated species, many of which were water plants, and these Lidforss (1907) had shown do not undergo the same changes of starch into sugar as do land plants.

It is, of course, possible that some of the unfavorable results were due to faulty technique, especially in the method used to extract the juice. Yet the negative data are too numerous to be lightly waved aside in this manner. One is, therefore, forced to conclude that the relationship between osmotic pressure and cold resistance is not always demonstrable. Even Lidforss - the original and most vehement protagonist of the role of soluble carbohydrates in cold resistance - points out that some plants (e.g. the sugar cane and the sugar beet) are quickly killed by light frosts in spite of their high sugar concentrations. On the other hand, he admits that bacteria and mosses are examples of those individuals which are cold resistant without any considerable sugar content.

With the whole subject in this unsettled condition, more information is obviously needed. In the following investigation a considerable number of plants were tested in the hardened and

unhardened condition to determine whether they showed a correlation between cold resistance and the osmotic pressure of the cell sap, and further to ascertain whether this factor was of any importance in differentiating species or varieties.

Dixon and Atkins (1915), and Lewis and Tuttle (1920) have shown that there is no seasonal change in the concentration of electrolytes. Consequently, any increase in the cell sap concentration during the hardening period is solely due to an increase in soluble carbohydrates. Dexter (1934), it is true, has recently demonstrated a decrease in electrolytes during the hardening of wheat seedlings. This, however, is very slight and cannot appreciably affect the osmotic pressure. It is, therefore, assumed that changes in sugar content and in cell sap concentration run parallel during the hardening period, so that only the latter was measured.

Two methods are available for the determination of osmotic pressure in plants: one by means of the freezing-point lowering of the cell sap, the other by use of the de Vries plasmolytic method. The former has been employed in cold resistance investigations almost to the exclusion of the latter. Yet Lidforss (1907) and Åkerman (1927) obtained very good results with the plasmolytic method.

The advantage of the freezing-point method lies in the fact that it is amenable to a high degree of accuracy, provided that the correct technique is followed. Walter (1931) lists several methods of extracting the juice, some of which give widely diverging results. However, reliable procedures are now available, and by means of these an accuracy of .01 atmosphere can be obtained. Yet Walter admits that certain errors are involved, so that he expresses all his results only to the first decimal.

Fitting (1915), using the plasmolytic method, made his determinations to .0025M KNO_3 (or, approximately .1 atm.) and he states that Lepeschkin worked within even narrower limits - .001M. In their hands, then, the plasmolytic method was just as accurate as the cryoscopic. This, however, is possible only with very favorable material. In the following investigation determinations to .005M were sometimes possible, but since these were the exceptions, the solutions were made up so as to differ by .01M. Even this relatively coarse gradation entailed the use of some sixty solutions. In the case of small cells with high osmotic pressures it was impossible to obtain results closer than .02M. Under such conditions, however, the error was not more than 5%. All results are expressed only to .1 atm.

Other objections to the plasmolytic method are that the solute used may penetrate the cell and so raise its concentration, or it may alter the cellular permeability and allow exosmosis of solutes (Blagowestchenski 1926). These objections are undoubtedly well-founded in the case of potassium salts, which have frequently been used as the plasmolytic. In the following investigation CaCl_2 was always employed. As was shown by Fitting (1915) and many others, calcium salts do not penetrate. Scarth (1925), in fact, found that Ca prevented the penetration of other ions.

Walter (1931) further criticises the plasmolytic method on the ground that it deals only with one tissue instead of giving an average of all, as is afforded by the freezing-point method.

However, in an attempt to determine changes occurring in cell sap concentration, this would seem to be an advantage rather than a disadvantage. It is only natural to expect more exact information when dealing with one homogeneous tissue than with a mixture of several. A method of this sort is bound to prove more sensitive to changes. Translocation, for instance, from one tissue to another will not produce any difference in the freezing-point lowering of the whole mass unless condensation is also involved. Plasmolysis however, would readily detect the difference.

There is one fact which establishes the superiority of the plasmolytic method in determinations of the kind here involved. Since the object is to investigate changes only in the quantity of solutes, complications due to loss of water are undesirable. Pisek, Soehm, and Cartellieri (1935) have shown that transpiration continues, though diminished, throughout the winter. As long as the ground remains frozen, this loss is unreplaced. During thaws, water is again taken up. An excellent example of the wide fluctuations brought about in this way is given by Walter (1931). The cell sap of *Buxus sempervirens* attained the extraordinary osmotic pressure of 73 atms. at the end of February. A sudden thaw reduced this tremendously.

Thus it can be seen that freezing-point determinations are useless as a means of furnishing information with regard to change in quantity of solutes, so long as fluctuations in water content occur. The plasmolytic method is independent of passive changes in moisture, since the cells quickly come to equilibrium

with the solution in which they are immersed. Active changes in water content, such as occur during the ripening-off period of trees, cannot be compensated for by either method.

It is an obvious advantage to use the same individual in each species investigated, for all the seasonal determinations. This eliminates the variability that exists between different specimens. Further, in the case of trees, samples should always be taken from the same level to obviate differences due to height. Both these considerations are easily complied with when using the plasmolytic method, since such very small samples are sufficient.

Finally, a study of the literature reveals that seasonal investigations of osmotic pressure have been confined to evergreens. This, of course is due to the difficulty in obtaining a sufficient volume of juice from twigs. On the other hand, there is no obstacle in the way of a plasmolytic investigation of deciduous plants.

It seems obvious, then, that the plasmolytic procedure has advantages over the freezing point method, and merits a much wider application in the study of osmotic pressure and its relation to cold resistance.

PROCEDURE

Sections were first allowed to stain for half an hour in a hypotonic solution of $.15M$ $CaCl_2$ containing 5 ppm. of neutral red. In the few cases where this proved hypertonic a weaker solution was used. Neutral red is a distinct aid in determining incipient plasmolysis. Since only living cells are stained by this dye, it removes the danger of mistaking dead cells with coagulated protoplasm for plasmolysed living cells, or empty cell walls for unplasmolysed living cells. Furthermore, the slightest incipience shows up sharply.

The $CaCl_2$ is a further aid to clear observation since it prevents the cell wall from becoming stained. A series of $CaCl_2$ solutions were used, varying in strength from $.10$ molar to molar, and all containing 5 ppm. of neutral red. The same solutions remained unchanged for several months. This is an obvious advantage over sucrose or other standard organic substances, both as a time-saver and for the sake of uniformity. Minute growths of fungal hyphae do eventually appear even in the strongest solutions, but since no nutrient is available the growth is exhausted as soon as the food reserves in the spore are used up. This minute growth cannot affect the concentration or utility of the solutions. From time to time, however, they were checked against other solutions and no alteration in the concentration could be detected. Their constancy was further shown by the fact that with the different series of solutions used throughout a period of three years, unhardened, healthy, cabbage seedlings always showed the same osmotic pressure - $.17$ to $.18M$ $CaCl_2$.

The osmotic pressure in atmospheres was read from tables of $CaCl_2$ equivalents worked out from the usual formula -

$P = iCRT$. Values for i were obtained from the dissociation constants: at $\frac{M}{2} = 61.2$, and at $\frac{M}{8} = 72.5$. The other values were interpolated.

From time to time results with CaCl_2 were compared with other solutions. Potassium nitrate and urea were used on plants previously found to be negligibly permeable to them (cabbage in the former case, Cordyline in the latter). Sucrose, a molar solution of which equals 26.6 atms. (Walter 1931) was, of course, applicable to all cells. The calculated pressures closely agreed in all cases.

RESULTS.

The plant material investigated is, for the sake of convenience, divided into two groups: (a) hardened under artificial conditions, and (b) hardened naturally.

(a) Artificial Hardening.

Seedlings of herbaceous plants were grown in the greenhouse until large enough for use (usually at the age of one to three months). In this state they were, of course, unhardened. These were subsequently subjected to a low temperature (5°C) in a cold chamber cooled with methyl chloride and having continuous artificial lighting. The latter was supplied by four 200 watt lamps, suspended outside the chamber about two feet above the plants.

The degree of cold resistance developed by a five-day hardening period of cabbage seedlings (variety Jersey Wakefield) was readily determined. Together with a control lot of unhardened plants, they were frozen for twelve hours at temperatures ranging between about -2° and -5°C . All the seedlings were then transferred to the greenhouse and left there for two weeks before estimating the injury. As shown in Table 1, the unhardened plants always suffered between 80 and 100% injury, whereas the hardened ones showed at most 20%. Each result in the table is an average of nine plants. The 20% injury in the case of the hardened plants was always confined to the lowest one or two leaves.

TABLE 1.

Relative frost resistance of hardened and unhardened cabbage plants.

Age	Temperature frozen at for 12 hours.	% Injury to unhardened	% Injury to hardened.
60 days	-1 to -4.5°C	80	10
49 "	-1 to -4	95	0
75 "	-1 to -4.5	100	30
57 "	-1 to -4	70	15
64 "	-2 to -6	100	25
73 "	-2 to -4.5	95	10

The time factor is of prime importance in the development of cold resistance. Day by day the plant becomes more hardy until the maximum is reached. This gradual increase offers an ideal opportunity for determining the relationship between hardiness and osmotic pressure.

For this purpose, some two dozen potted cabbage seedlings were placed in the cold chamber at 5°C. At daily intervals a leaf was removed from each of six plants, and the osmotic pressure of the petiole cells was determined. Longitudinal sections were used since the cells are markedly elongated in this direction. This enabled ready recognition of incipient plasmolysis. The cells examined were those of the chlorenchyma and pith. These two tissues never differed appreciably in cell sap concentration.

Fig. 1 shows that the osmotic pressure increased steadily during the hardening period. It is interesting to compare these results with those given by Harvey (1918). He determined the freezing-point lowering of the cell sap of hardened and non-hardened cabbage seedlings. Using four different methods to express the juice, he obtained results varying from $-.985$ to -1.822°C for the hardened, and $-.910$ to -1.668°C for the non-hardened, the values depending on the method employed. From his data it can easily be seen how far wrong freezing-point determinations can be. In all cases, the juice of hardened plants had a freezing-point which was about 10% lower than that of the unhardened. Fig. 1 shows that five-day hardened cabbage seedlings gain in osmotic pressure by about 25%.

Harvey's results are surprisingly high. When he prefroze the tissue in liquid air (as advised by Dixon and Atkins 1913) before extracting the juice, he obtained a freezing-point lowering of -1.668°C for unhardened cabbage. This is equal to an osmotic pressure of 20 atms., or double that shown in Fig. 1. And yet the plasmolytic method is usually accused of giving too high values!

Two clover varieties - a single cut and a double cut - were similarly hardened and tested for osmotic pressure with the same results. Though the former is supposedly more hardy, it did not exhibit any greater increase in osmotic pressure than the latter (Fig. 1).

Now, cabbage and clover are hardy plants. That is to say they are able to resist freezing temperatures when in the hardened condition. There are other plants, however, which under no conditions will become cold resistant. The question, then, is: Do these plants show any increase in osmotic pressure as a result of exposure to low temperatures? Sunflower and castor bean seedlings (which belong to this group) were subjected to a temperature of 0 - 5°C and tested for osmotic pressure. No change occurred after six days (Fig.1).

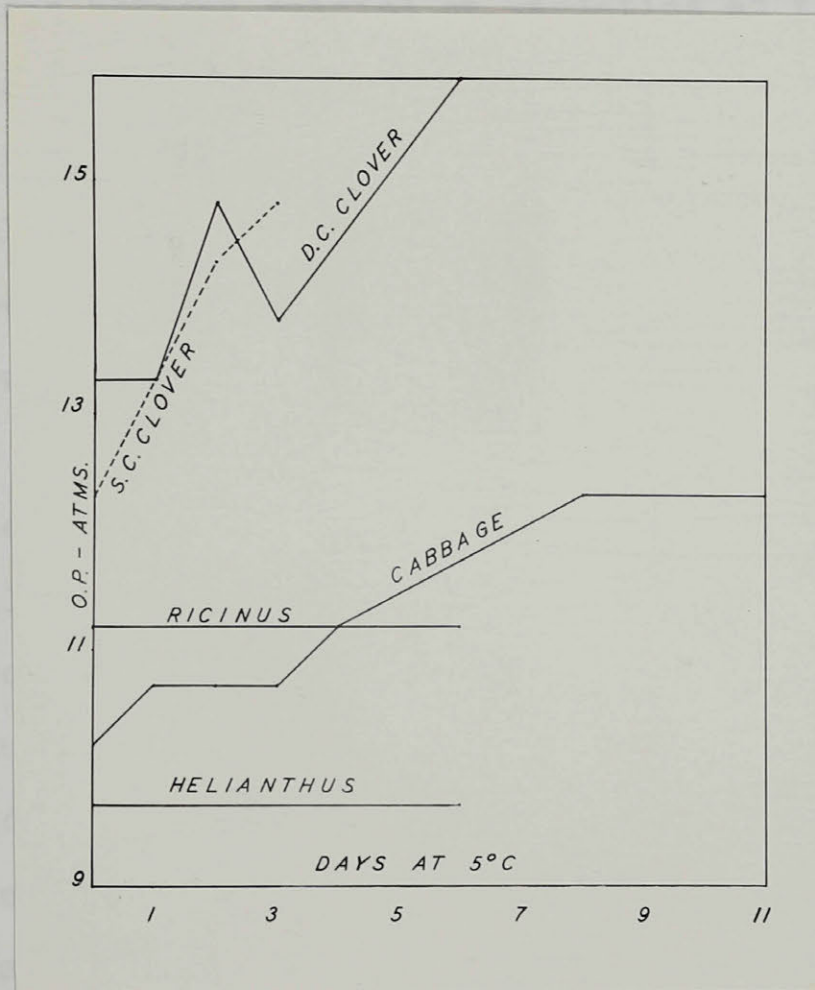


Fig.1. Change in osmotic pressure on hardening.

(see Tables 17,19,20,21).

(b) Natural Hardening.

Cabbage and clover, though relatively cold resistant, do not overwinter in this climate unless well protected. The attempt was, therefore, made to find out whether the very hardy woody plants show the same changes in osmotic pressure on hardening as do the above. Seasonal determinations were made on longitudinal sections of the cortex tissue. Terminal growth was always used. Since they had survived the severe test-winter of 1933-34, none of the plants investigated could be considered tender, and consequently, it was impossible to make any comparison between species or varieties at the two extremes of hardiness.

Four apple varieties were chosen. Hyslop (a crab) and Hibernial are the two hardiest, and Delicious and Milwaukee the two tenderest grown at Macdonald College. Since no really tender varieties can overwinter there, even the latter two possess a considerable degree of hardiness. Further, the individual specimens of these two varieties which furnished the material were necessarily the hardiest of their group, since all the more tender ones had been killed off by the winter of 1933-34. Thus, the Delicious used was the lone survivor in a row of its kind. Almost all the Milwaukee trees had also succumbed. Yet, though these trees were the hardiest in their respective varieties, they had suffered considerably in 1933-34. All the fruit buds and the terminal growth had been killed. They, therefore, did not flower during the spring of 1934, whereas

the Hyslop and Hibernial trees both flowered and fruited abundantly. Presumably, this again would tend to reduce the difference in hardness between the two groups during 1934-35. Finally, the tissue investigated - the cortex - is the hardest of all tissues. In spite of all these conditions which tended to reduce the differences in hardness between the two groups, there can be little doubt that marked differences still existed. Only current growth was used - the kind that killed on the Delicious and Milwaukee in 1933-34 but was unharmed on Hyslop and Hibernial. Determinations were made at intervals of three weeks throughout the fall and winter.

As can be seen in Fig. 2, an increase in osmotic pressure occurred in all varieties. From fall onward, Hibernial maintained the highest concentration while the other three showed little difference. But the tender varieties were later in reaching their maximum. Hyslop, for instance, had the highest cell sap concentration on Sept. 28th., a concentration not reached by Delicious till fully two months later.

It is highly probable that during the growing season, all varieties have more or less the same osmotic pressures. If this is so, then Hyslop had already considerably increased its pressure by the end of September at a time when the tender varieties had hardly, if at all, begun to do so. Hibernial, during the next three weeks quickly caught up and passed Hyslop. Milwaukee, too, was soon its equal. It would seem, however, that Hyslop ripens off the earliest. Whether this shows that it is prepared for the early cold snaps or that it is native to a region with earlier winters is hard to say.

During mid-winter six other varieties varying in hardness between the above two groups were tested for osmotic pressure. Tab.33 shows that the cell sap concentrations of the ten varieties during mid-winter is not correlated with their relative hardness. This agrees with Hildreth's (1926) results. He found very little difference between the mid-winter sugar contents of Duchess and Jonathan twigs, though the former is much the hardier.

Osmotic pressure, as such, is apparently not a determining factor in the cold resistance of apple varieties. Yet a seasonal study reveals that the autumn increase in osmotic pressure which occurs in all varieties is accomplished earlier in the more hardy than in the tender ones.

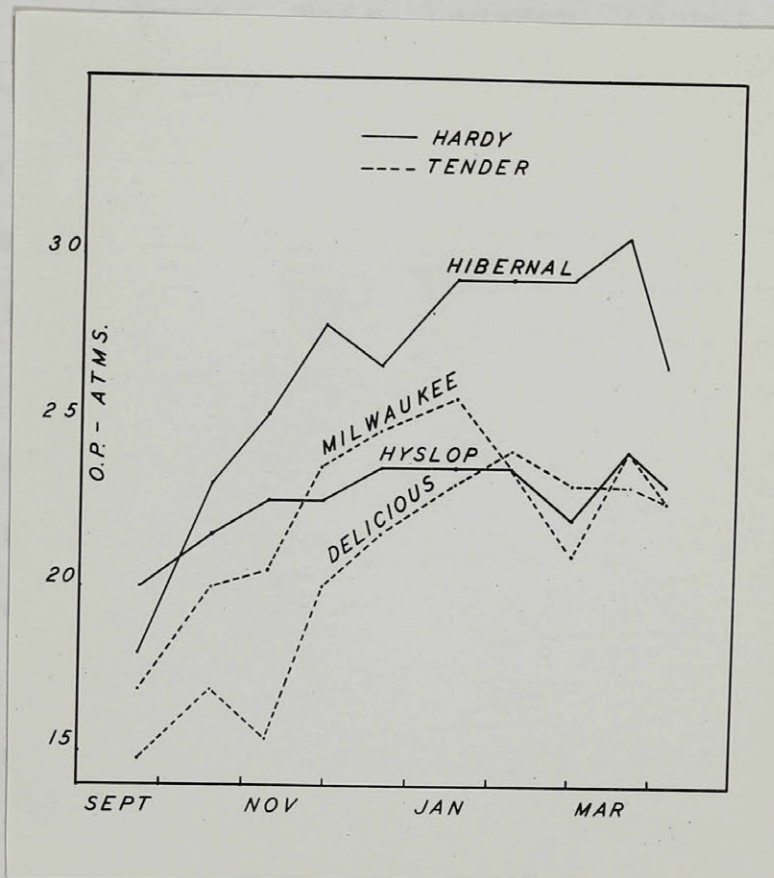


Fig.2. Seasonal change in osmotic pressure of apples.

(see Table 28).

Five woody, ornamental species were subjected to seasonal determinations of osmotic pressure during 1934-35. These were chosen on account of their relatively large cortical cells. Several native species, as well as some other introduced ones (e.g. *Acer*, *Fraxinus*, *Cornus*, *Syringa*, etc.) were tried but discarded on account of their extremely small cells. This, indeed, seems to be a characteristic of all very hardy plants.

The same individual plant was used throughout the year in all cases save that of *Caragana*. This shrub became inaccessible at the beginning of January, consequently, for the remainder of the season another plant was used.

All the species do not, apparently behave alike. (Fig. 3). *Liriodendron* and *Hydrangea* exhibit a strikingly steady increase in osmotic pressure. This, however, cannot be said for the other three. *Caragana* is characterised by the highest cell sap concentration. Yet little change occurred throughout the fall and winter. This high osmotic pressure which is already present in September is not characteristic of the plant in the unhardened condition. Thus when a twig taken in mid-winter was kept at room temperature for a week, its cell sap concentration dropped to 18.5 atms. Consequently, *Caragana* shows its unusually high degree of hardness by its extremely early preparation, just as in the case of the crab apple.

Picea and *Catalpa* are somewhat more difficult of interpretation. Neither shows any constant tendency. It is true that the lowest value occurred in both cases during September. Yet the winter increase was small and exhibited considerable fluctuation.

Nevertheless, the results with *Picea* are very similar to those obtained by Lewis and Tuttle (1920) by means of the freezing-point method. They, of course, worked with the leaves.

In all species, however, the relationship between cold resistance and osmotic pressure can be strikingly shown by comparing twigs before and after exposure to room temperature for several days (Fig.4). The rate of change in osmotic pressure also appears closely related to the emergence from the rest period. Thus, *Caragana* after seven days at room temperature was already bursting open its buds. As mentioned above, this was accompanied by a decrease in osmotic pressure from 28.3 to 18.5 atms. *Picea*, on the other hand, which never opened its buds in the laboratory, showed a relatively small change from 18.5 to 15.9 atms. in eleven days. In February, however, no decrease in the cell sap concentration of *Picea* occurred after a week in the laboratory.

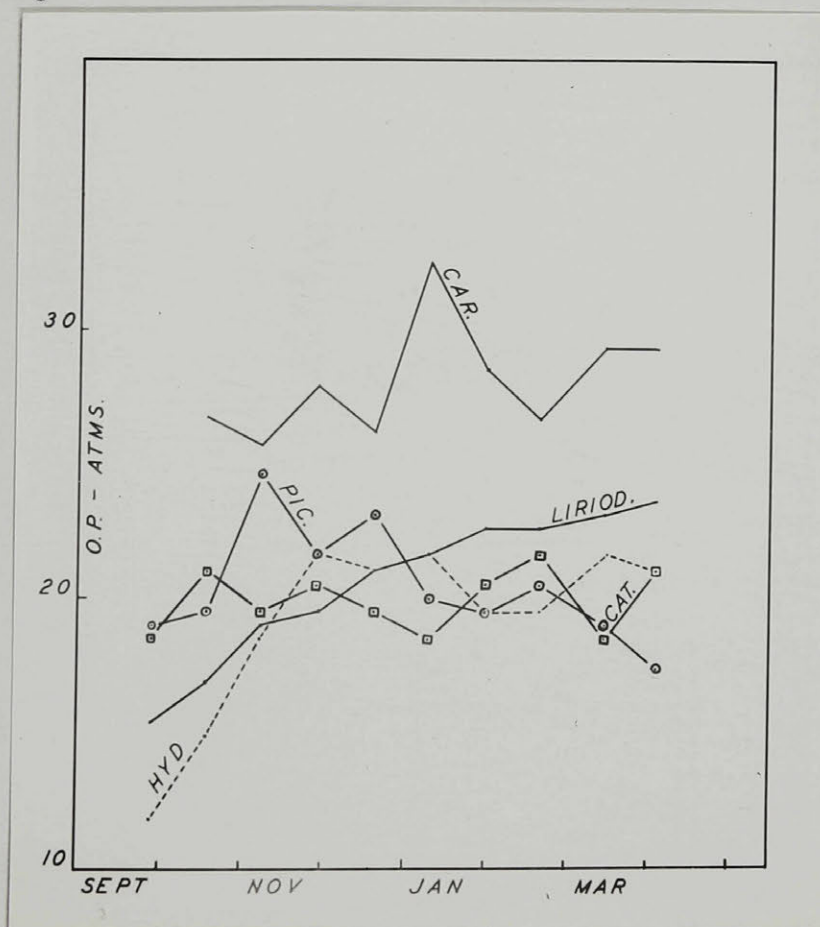


Fig.3. Seasonal change in osmotic pressure of ornamentals.
(see Table 27).

As a general rule, then, an increase in cold resistance, whether brought about artificially or naturally, is accompanied by an increase in osmotic pressure. Plants which are unable to harden off show no change in cell sap concentration on exposure to low temperatures. Some species do not exhibit any marked increase in osmotic pressure during the fall and winter when hardening occurs. Even in these cases, however, it was shown that their concentrations were unusually high. Thus keeping them at room temperature for several days always caused a decrease in cell sap concentration. Presumably, then, these plants had already reached their maximum concentrations by the end of September. This agrees with Gail's (1926) results. He found that evergreens begin to increase in cell sap concentration at the end of July.

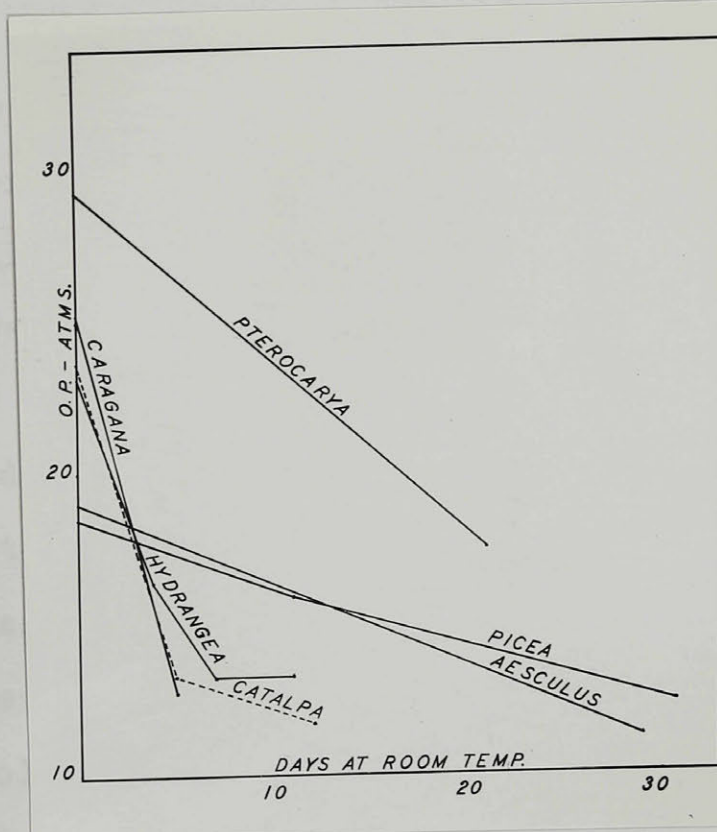


Fig.4. Change in osmotic pressure of ornamentals during loss of hardiness (see Table 26).

HYDROPHILY.

Of recent years, one of the commonest statements to be found in the literature on winter hardiness is that an increase in cold resistance is associated with an increased hydrophily, or in other words, a greater quantity of bound water. The conception of bound water, however, is as yet by no means clear. The extent of disagreement among experts as to the meaning of the term and the quantity to be found in specific cases, or even as to the actual existence of the entity is well illustrated in the symposium by Gortner (1930) and others. The term is often used to denote that water which remains unfrozen at some sub-zero temperature, usually -20°C . However, it is now coming to be accepted as defined by Hill in the above symposium. He describes it as that water which is held so strongly adsorbed that it is unable to act as a solvent.

It is from the point of view of the latter definition that most of the recent work has been done. There are, indeed some investigators who assert that on this basis bound water does not exist. Hill, himself strongly supports this view. He measured the decrease in vapor pressure caused by adding a definite quantity of solute to muscle containing a known amount of water. The observed depression of the vapor pressure was very near to the calculated amount, from which he concludes that all the water in muscle is free to act as a solvent.

Greenberg and Greenberg (1933) added definite quantities of solute to colloidal solutions (gelatine, blood serum, etc.) and then subjected these to ultrafiltration. The concentration of

solute in the filtrate was always very close to the amount added, so that, apparently, no water was bound. An objection to this has been raised (Gortner 1934) on the grounds that the solutes themselves must have been adsorbed and so left the concentration unchanged.

Yet the majority of workers now assume that the existence of bound water is an established fact. A number of methods have been used for determining its relationship to hardness. The dilatometer was one of the first instruments employed for this purpose. The tissue under investigation is submersed in oil and frozen at a definite temperature. From the expansion of the narrow oil column can be calculated the amount of ice which has formed, and if the water content of the tissue is known it is easy to determine the percent of unfrozen water. The latter is considered bound. Rosa (1921) found that there was a perfect correlation between hardness of cabbage and the amount of water unfrozen at -5°C . Lott (1926) asserts the same thing for brambles. Weimer (1929) working with alfalfa showed that the amount of unfrozen water was always greater in hardened than in unhardened plants. Yet there was no correlation among varieties to correspond with their differences in hardness.

Lebedincev (1930) pursued the question further than any of the above workers. She found that hardy varieties of wheat contain larger quantities of bound water than the less hardy. By determining the freezing point of the cell sap and combining these results with those obtained by the dilatometer, she

showed that the great majority of it was osmotically bound. In Minhardi, for instance, at -3.8° 48% of the water was bound, 39% osmotically and 9% colloiddally. Thus, any correlation obtained between cold resistance and bound water as determined by the dilatometer is far more likely to be due to an increase in that portion which is osmotically bound.

The calorimetric method has, until very recently, not found such wide use in hardiness investigations, though already employed by Müller-Thurgau (1880) in a simplified form. Meyer (1932) has recently applied it to determine the bound water in hardened and unhardened pine needles. He froze the tissue at -20°C . The same amount of water was bound per gram fresh weight in both the hardened and unhardened needles, and since the latter had a higher total moisture content, the unhardened contained a larger quantity of bound water per gram dry weight. Meyer points out investigators often fail to calculate their results on this basis with consequent erroneous conclusions. He considers that the ratio of bound to free water remains approximately constant, so that the characteristic low moisture content of hardened tissue applies to both the free and bound water.

The pressure method of Newton's (1922) has also been extensively used. He noticed that it is more difficult to express juice from hardy varieties of wheat than from the less cold resistant ones. He was in fact, able to place the varieties

investigated in their order of hardness according to the amount of juice expressed. Before expressing the sap, however, he froze the tissue. This treatment would obviously injure the less hardy plants more than the hardier ones, and the amount of juice obtained would, naturally, vary directly with the injury. Dexter (1932) has, in fact, made use of Newton's method from this point of view. Since the less hardy varieties will show greater injury, the semi-permeability of the membranes will be destroyed in more cells, and a larger amount of electrolytes will diffuse out. Thus by measuring the electrical conductivity of the expressed liquid after freezing the tissue, Dexter was able to determine the relative hardness of varieties.

Martin's (1927) work is further proof of this conception. He found the same correlation between hardness and the amount of sap expressed as did Newton, but this condition held true only after the samples were frozen. Unfrozen tissue, even though hardened, showed no relation between the amount of juice expressed and the hardness of the variety, and the actual quantity obtained was far smaller than from frozen samples.

Meyer (1928) came to the same conclusion as Newton. He found that no juice could be expressed from hardened pine needles, whereas unhardened ones gave a considerable amount. Later (1932), however, he realised that this was due to injury to the unhardened. As mentioned above, he demonstrated by means of the calorimeter that there was actually more bound water per gram dry weight in the unhardened than in the hardened needles.

The second method originated by Newton (Newton and Gortner 1922) involved the direct measurement, cryoscopically, of the colloiddally bound water. Sufficient sucrose was added to the plant juice to give a molar solution. The depression of the freezing point was then determined, and the difference between the observed and the calculated depression allowed an estimation of the amount of water which did not act as a solvent for the sugar. Several objections have been raised against this method. Hill (Gortner 1930) states that it is dangerous to add such a large quantity of sugar. In his own experiments only .1M was usually employed. It is necessary also to take into account the hydration of the crystalloids, and the 6% sucrose which Newton makes allowance for in the hardened wheat cannot cover this factor in the cell sap which has an osmotic pressure of 20 atms. and is further concentrated by freezing. As Grollman (1931) points out, Newton and Gortner's formula makes no provision for the concentration of solutes due to the separation of water as ice. Some of Newton's (1930) own findings are not in harmony with the theory of a great increase in hydrophilic colloids. Using dextrose instead of sucrose he obtains much lower amounts of bound water, sometimes nil.

The same objection of prefreezing applies to the results obtained with his cryoscopic as to those by his pressure method, and this alone may have been sufficient to cause the striking correlations that he obtained. Martin (1927) was also able to find a correlation after prefreezing, though there were irregularities in the amounts of bound water present.

Another means used to obtain the relative hydrophily of plant colloids is by determining the dye adsorption of the tissues. This does not measure bound water but its originators assume that the adsorptive powers of the colloids for water and dyes run parallel. Dunn and Bakke (1926) were able to establish a good correlation between the adsorption of basic dyes and winter hardiness in apple varieties. It is surprising that this does not apply to all basic dyes - e.g. safranin (Dunn 1933). As test material they used dried and ground apple twigs. Newton (1924), however, in his investigations on bound water found it impossible to dry plant material without destroying its colloidal properties. The ground twigs used by Dunn and Bakke, therefore, owed their adsorptive capacity to the cell wall material which, in fact, was by far the preponderating constituent of the test material. Since Beach and Allen (1915) were able to find that, on the average, hardier apple varieties have harder wood, it seems quite possible that some such factor was the cause of the correlation obtained by Dunn and Bakke. In some later studies Dunn (1930) found that seven out of twenty-two varieties showed no relation between dye adsorption and hardiness. Recently (1933), he has applied the method to the juices of herbaceous plants. The mean values seemed to show some correlation with hardiness, though overlapping always occurred.

It appears quite evident, then, that none of the methods so far used to determine the relationship between hydrophily and cold resistance is satisfactory. The calorimetric method is considered by Sayre (1932) to be the best, and as already

mentioned, it has given negative results (Meyer 1932). However, even if any one of the above procedures were without technical faults, there would still be one fundamental difficulty. In all cases dead material is used. The pressure method, it is true, is an exception, but as already shown, it is no longer tenable. In the use of the dilatometer and the calorimeter, the low temperature (which is necessary to remove the complication of crystalloid-bound water) kills the tissue. The oil, alone, accomplishes this in the case of the dilatometer. If the hydrophily of protoplasm is to be investigated, living tissue only can be used.

PROTOPLASM MEASUREMENTS.

It is obvious that if the hydrophily of protoplasm is increased, it will take up more water and so expand, provided that the cell sap remains unchanged in concentration, for as Walter (1931) has shown, the two are in equilibrium. Therefore, it should be possible to determine a change in the hydrophily of protoplasm by measuring its thickness.

The majority of vacuolate cells are surrounded by such a thin layer of protoplasm that it is almost or completely invisible, even under the highest power of magnification. Long cells, however, are usually characterised by the presence of a protoplasm "cap" at each end, which may be thick enough for measurements. It is necessary to choose cells which do not form starch, since this would be converted into sugars at low temperatures, and in that way affect the thickness of the protoplasm layer. Since they fulfill both these requirements, epidermal cells of onion (the yellow Spanish variety) were used.

The youngest cells were found most suitable on account of being richer in protoplasm. Each bulb was, therefore, cut in half, and strips of epidermis from an inner scale were stained for half an hour in the standard neutral red solution. This gave rise to a sharp contrast between the red cell vacuole and the unstained protoplasm. Camera lucida drawings were made of the caps at both ends of ten cells at incipient plasmolysis in a CaCl_2 solution. The cut bulb was then hardened off for six to seven days at 0°C . After this period, the other half of

the same epidermis layer served for measurements of hardened cells. These measurements were always made on cells about 5mm. from the base of the scale.

By means of a planimeter, the areas of the camera lucida drawings were determined. The average of the ten cells served as a measure of the total amount of protoplasm in the caps at both ends of the cells. The actual thickness of the caps would have been a satisfactory measure if the cells had all been regular. However, on account of the fact that some are much narrower than others, and that some have pointed apices while others are truncate, determinations of the area were more nearly proportional to the volume than mere measurements of the thickness of the layer.

The actual size of the cell did not seem to be in any way correlated with the quantity of protoplasm at the ends, so long as the cells occurred in the same region. The dimensions are, therefore, not given.

Injured cells were easily recognised. The dead ones, of course, remained unstained. Frequently, there occurred cells with stained vacuole and unstained protoplasm cap at one end, while at the other end the protoplasm was coagulated and stained. At other times the vacuole stained faintly, and the protoplasm caps were apparently normal but the cell did not plasmolyse. Finally, in some the nucleus was stained. None of these types served for measurements. If sufficient care was used in handling the tissue, abnormal cells were very few in number.

At times, difficulties arose due to the occurrence of strands of protoplasm which obliterated the sharp outline of the caps. In these cases, measurements were not attempted.

In Table 2 are presented the results with five onions. These were first kept in a warm room (25°C) for several days, and then hardened. Measurements were taken before and after hardening. The hardened cells showed a slight decrease in the quantity of protoplasm. Table 3 shows the variability that existed between ten-cell samples in a single epidermis strip. This variability is apparently smaller than the difference between hardened and unhardened samples. And since no perceptible change in osmotic pressure occurred, this would seem to indicate a slight decrease in hydrophily.

TABLE 2.

Total cross-sectional area ($\text{sq.}\mu$) of both protoplasm caps (average of 10 cells).

Onion no.	Days at 25°C (previous to hardening)	Unhardened	Hardened	Change
1.	7	300	306	+ 6
2.		303	281	-22
3.	14	376	338	-38
4.		477	370	-107
5.	28	376	314	-62
Average				-45

TABLE 3.

Comparison of 10-cell samples.

Onion	Sample a	Sample b	Divergence
1	309	319	10
2	370	389	19
3	284	296	12
Average			14

The cortical cells of twigs are in some species characterised by thick protoplasm layers. These are green, and therefore contain abundant chloroplasts. It is obvious that a change in the starch content will have a marked effect on the thickness of these layers. Therefore, if measurements are to denote changes in water content, they must be made in the absence of starch. In the fully hardened state this condition prevails. If a loss in hardness can occur without starch arising in the cells, then measurements taken from day to day on twigs kept at room temperature should tell something about the change, if any, in water content of the protoplasm.

Apple cortex is characterised by especially thick protoplasm layers - about $\frac{1}{2}$ the cross sectional area. Four varieties were chosen, and measurements were taken from time to time (Table 4.)

TABLE 4.

Twigs taken indoors Feb. 11/35. Each value is the average of 10 cells.

Days in lab	Percent of cell area occupied by protoplasm.				
	Alexander	Wealthy	Wolf River	Patten Greening	Average
0	47.7	45.2	53.1	47.7	48.4
1	46.3	42.2	53.0	57.4	49.7
3	50.5	46.3	49.5	51.7	49.5
7	52.6	51.1	54.4	49.6	51.6
14	46.7	41.3	50.8	57.6	49.1

The ten-cell samples were apparently too small to give representative values, for the fluctuations were large. The averages of the four varieties, however, seemed more constant. These show a slight rise until the last figure, which registers a drop. It is very doubtful whether these differences are significant.

During the two weeks only traces of starch appeared. The osmotic pressure, however, showed a considerable drop, from about 25.5 atms. at the beginning, to about 19 atms. at the end of the period. One would expect this to cause an increase in the volume of protoplasm, if the hydrophily of the latter remained constant. And since no increase in the measurements occurred, a decrease in the hydrophily of protoplasm is indicated.

However, it must be remembered that the figures do not represent protoplasmic volume, but merely cross-sectional area. The change would, therefore, not be proportional to the increase in osmotic pressure but much smaller. It is doubtful whether a difference of this order could be readily detected, especially since the variability is so great. Further measurements using larger samples are necessary before conclusive evidence can be obtained.

The results so far obtained cannot be considered anything more than preliminary. Onions seemed to show a slight decrease in protoplasm on exposure to low temperatures, whereas apple protoplasm remained more or less unchanged during a two-week exposure to room temperature with a consequent loss of hardness.

In the former case, since osmotic pressure remained constant, a decrease in hydrophily during hardening is indicated. In the latter, on the other hand, due to a decrease in cell sap concentration, it would seem that loss of hardness is accompanied by a slight decrease in hydrophily. In both instances, however, the difference is very slight and probably within the experimental error. The only definite statement that can be made is that there is no evidence for a large change in the hydrophily of protoplasm, during an increase or decrease in hardness.

CELLULAR PERMEABILITY.

The possibility that cold injury and resistance to it may in some way be connected with permeability changes, has from time to time been suggested. Yet practically no experimental work on the subject has ever been attempted. Lidforss (1907) attached the foremost importance in cold resistance to the quantity of soluble carbohydrates formed during the hardening period. Yet he recognised that this could apply only to the higher plants. Tentatively accepting Gorke's (1906) hypothesis, he suggested that bacteria and mosses owe their high degree of resistance (in the absence of quantities of sugars) to a ready permeability for electrolytes. In this way, the high concentration of salts which Gorke emphasized must occur in the cell on ice formation, would be prevented by diffusion into the inter-cellular spaces. The experimental evidence which Lidforss offers is the fact that *Mnium cuspidatum* deplasmolyses within two hours in a 5.5% KNO_3 solution, and *Neckera* during 48 hours in 7% KNO_3 , both of which he considers unusually rapid.

Pantanelli (1919) found that cells of the mandarin orange (*Citrus nobilis*), when subjected to temperatures near freezing, suffer a progressive increase in permeability. Thus, there is a rapid emission of water from the tissue if it is kept dry, or an exosmosis of substances when immersed in water. Bennett (1934) recently showed that potato tissue, after being stored at low temperatures, was unable to retain its cellular contents when placed in tap water. The lower the temperature, the more rapid was the loss both of electrolytes and non-electrolytes.

Both these results have merely to do with changes caused by injury. Death by any method will cause cells to lose their power of semi-permeability, and the larger the number of cells killed, the greater will be the increased exosmosis of the affected tissue. As previously mentioned, Dexter et al. (1930) have, in fact, made use of this principle in determining the degree of hardness of plant material. They first subject the plant to freezing temperatures, and then determine the quantity of solutes that diffuse into distilled water by measuring its electrical conductivity.

Other suggestions with regard to permeability changes have been made, based mainly on theoretical considerations. Coville (1920) believes that the effect of low temperatures in breaking dormancy may be due to an increased permeability of the membrane surrounding the starch grains, thus enabling enzymes to come into contact with them and to convert them into sugars. It has been pointed out that this theory cannot explain the reverse process.

The problem undertaken in the following investigation is to determine just what changes in permeability, if any, occur on exposure to low temperature, and whether hardy and non-hardy plants show any differences. Only when a change has once been definitely established, is it in order to consider the theories suggested.

Cellular permeability may be divided into three types, according to the substances penetrating or going out: permeability to (a) water, (b) non-electrolytes, (c) electrolytes. There are several methods available for investigating it, none of which

is completely without objection. Scarth and Lloyd (1930) list five methods. The conductivity method can be used only for electrolytes, and there are several objections which have been raised against it. The chemical method, employing analysis of the medium or cell sap, is applicable only in the case of certain very large-celled Algae. The physiological method is qualitative and is of limited application. None of these three was attempted. The observation of a color change, as in the penetration of acid dyes, or of acids and alkalis in the presence of an indicator, was tried without success. Most cells are very difficult to penetrate by means of these substances.

This leaves only the plasmolytic method, which was adopted. It is suitable for investigating all three types of permeability, it is reasonably rapid, it can be used on almost any cells, and it is quantitative to a sufficient degree.

A. WATER PERMEABILITY.

Höfler (1931) conclusively proved that the rate of plasmolysis and deplasmolysis of a cell depends solely on the permeability of its protoplasm for water. The plasmolytic method can, therefore, be used as a measure of water permeability. Since among higher plants the cells do not plasmolyse at all regularly (*Majanthemum* is an exception according to Höfler) it is impossible to tell when the end point is reached. Consequently, measurements of the rate of deplasmolysis must be used instead. Höfler asserts that higher results are obtained in this way, but if the same method is constantly adhered to, this is of no importance in determining relative permeabilities.

De Haan (1931, 1933) produces evidence from which he concludes that permeability to water decreases with the degree of plasmolysis. That is, a plasmolysed cell allows less water to penetrate per unit surface area than an unplasmolysed one. He therefore, deduces that the higher the degree of hydration of protoplasm, the greater is its permeability to water. If this conclusion of de Haan's is correct, determinations of water permeability would serve as an invaluable weapon for attacking the problem of cold resistance. The question of bound water, or the hydrophily of living protoplasm could, in this way, be definitely settled.

De Haan's experiments were, therefore, repeated, using the same material - the inner epidermis of the yellow onion. The technique was somewhat modified. The cells were first slightly

plasmolysed to allow them to come away from the ends of the cells without remaining attached by means of protoplasmic threads. This also tended to prevent the breaking up of the cell into two or more parts which otherwise too frequently occurred. They were then plasmolysed for a shorter time in a stronger solution than de Haan used. Cells treated in this way seldom burst. If they are plasmolysed for a longer time in a weaker solution, they are able to come to equilibrium and this allows a certain amount of hardening of the plasma membrane, which causes them all to burst on deplasmolysis.

Table 5 shows that the experimental results were the same as those obtained by de Haan, i.e. the increase in length of the vacuole per unit time was constant during deplasmolysis. But his method of calculation was found unfeasible. He assumes that the plasmolysed cell remains square in cross-section. This was found to be untrue, at least for the outer epidermis. Cross-sections of a whole scale showed that the plasmolysed cell is oval and not square. Even the cell itself is not square but rectangular, being wider than deep. Both these facts introduce errors into his calculations. However, it must be admitted that it is difficult if not impossible to take these errors into account. Consequently, his assumptions were, in the main, retained.

There is, however, one assumption of de Haan's which introduces a large error into his results. He asserts that the cells on either side of the one being measured prevent the penetration of water where they overlap it. This, presumably, is based on his belief that the plasmolysed cell is square in cross-

section, a belief which we have seen is unfounded on fact. Even where the protoplasm is in contact with the wall there should be little if any hindrance to penetration, for as Höfler (1931) showed, the wall is so much more permeable than the protoplasm as to offer no barrier. This is easily proved by the fact that an isolated living cell gives the same result as one with living cells adjacent to it.

De Haan's belief that the side of the cell adjacent to the cutinised wall cannot be penetrated by water appears correct. This side is the longer one as seen in cross-section. The oval-shaped plasmolysed cell is, therefore, in contact with it for most of its width. Thus the water was assumed to penetrate freely through only three sides of the cell.

The formula ultimately used, then, was a modification of de Haan's:

$$f = \frac{\text{change in volume}}{\text{average surface} \times \text{average pressure difference}}$$

$$= \frac{(g_x - g_{x-1}) \text{ vol. cell}}{\{4\pi r^2 + (l_x + l_{x-1} - 4r) 3r\} \times \text{av. P.D.}}$$

and vol. cell = hb^2 where:

h = length of cell

b = width

g_x = degree of plasmolysis = $\frac{l_x - \frac{b}{3}}{h}$

r = half the width of the cell = $\frac{b}{2}$

l_x = length of the plasmolysed cell

av.P.D. = difference between the average cell sap concentration at any one interval and the concentration of the deplasmolytic.

Av. conc. at any one interval = conc. at incip. plas. $\div \frac{g_x + g_{x-1}}{2}$

Instead of the average pressure difference, de Haan used the average cell sap concentration. This, however, is only permissible when deplasmolysing in distilled water, which usually causes the cells to burst.

The formula for f then furnishes the actual rate of penetration of water in cubic μ per square μ per minute.

Using this formula, it can be seen (Table 5) that f shows a gradual and steady increase during deplasmolysis. This is in marked contrast to the results obtained by de Haan. With his formula he finds a slow increase at first and then an extremely rapid one in the later stages, so that the final value is often eight times as large as the initial one. When f is divided by g , the value obtained is constant. This suggests that the small increase in the rate of permeability is due to an alteration in the thickness of the protoplasm layer or in the pore size of the plasma membrane itself. This will be discussed later.

TABLE 5.

Deplasmolysis of onion epidermis. Plasmolysed first for 5 mins. in .24M CaCl_2 , then for 5 mins. in .75M CaCl_2 . Deplasmolysed in .13M CaCl_2 . O.P. = 11.24 atms., $h = 232.5$, $b = 82.5$.

Time (secs.)	l (μ)	g	av. P.D. (atms.)	f	$\frac{f}{g}$
60	120	.397	18.57	.34	.79
90	135	.462	15.12	.25	.51
135	150	.527	12.48	.27	.48
180	165	.591	10.41	.30	.48
225	180	.656	8.71	.33	.48
270	195	.720	7.32	.37	.49
315	210	.785	6.13	.40	.49
360	225	.849			

As already pointed out, epidermal cells of onion have certain disadvantages. For example, they are not absolutely regular and it is, therefore, impossible to calculate, accurately, either the volume or the surface area of the cell. Pulp cells of onion offer far more favorable material. They are isodiametric, or nearly so, in all directions. When plasmolysed (at least once they have begun to deplasmolyse) they form perfect spheres, which thus allow volume and area to be computed without any assumption being necessary. Further, the cells are very large, some being even visible to the naked eye. This is a twofold advantage for the larger the cell, the more accurate the measurement is likely to be; and, on account of the smaller relative surface, the rate of deplasmolysis is slower, this again facilitating accuracy in time measurements.

It is true that as soon as the vacuole has deplasmolysed to a sufficient extent to be in contact with the cell wall, the configuration is no longer perfectly symmetrical. On this account, measurements were not taken after this stage was reached, so that the later stages of deplasmolysis could not be obtained. Since it was not necessary completely to deplasmolyse the cell, a somewhat hypertonic solution was used for the deplasmolytic.

In this case the formula used was:

$$f = \frac{(r_x^3 - r_{x-1}^3) \frac{4}{3} \pi}{\frac{(r_x^2 + r_{x-1}^2)}{2} \times 4\pi \times \text{av. P.D.}} = \frac{\frac{2}{3}(r_x^3 - r_{x-1}^3)}{(r_x^2 + r_{x-1}^2) \times \text{av. P.D.}}$$

Table 6 shows that the permeability to water remained practically constant during deplasmolysis.

TABLE 6.

Deplasmolysis of onion pulp cell. Plasmolysed 10 mins. in M CaCl_2 ; deplasmolysed in .25M CaCl_2 .

Time (secs.)	r	g	av. P.D. (atms.)	f
90	120	.442	14.83	.41
165	127.5	.531	10.18	.45
270	135	.629	6.49	.38
450	142.5	.741	3.50	.37
795	150	.864	1.08	.49
1650	157.5	1.000		
3180	157.5			

There is, however, one objection to the use of these pulp cells. In the case of the epidermis the tissue is only one cell thick. In the case of the pulp tissue, on the other hand, it is necessary to make sections, and on account of the large cell size, these sections must be quite thick. This, undoubtedly, offers a considerable barrier to the ready diffusion of the water. When the cells are strongly plasmolysed, diffusion will not be a factor of much importance, on account of the large spaces between the plasmolysed cell and its wall. Toward the end, however, it will no longer be negligible. Consequently, an increase in permeability may actually exist in the later stages of deplasmolysis, in spite of the figures given in Table 6.

Further experiments were, therefore, conducted under conditions which did away with all complicating factors. These

ideal conditions involved the use of cells freed from their walls and liberated into the deplasmolysing solution.

Many a cellular physiologist has obtained free plant cells in a similar way. Höfler (1931) asserts that the technique was evolved by Küster. Höfler himself made use of the method to prove that the rate of plasmolysis is dependent on the protoplasmic permeability, and is unaffected by the cell wall. He did not actually free the cell but merely cut away one end wall. It is very doubtful, however, if isolated cells have ever before been used for quantitative work.

The technique finally adopted was as follows: A piece of onion scale (for the sake of uniformity it was always removed from the "equator" of the second scale from the outside) about a cm. square was placed in a strongly plasmolysing solution. The cutinised inner epidermis had previously been stripped off to allow ready access of the plasmolytic to the tissue. After a sufficient time had elapsed, sections were cut, dried for a moment on a piece of filter paper, and then placed on a chambered slide containing the deplasmolytic. Thus, some of the cell walls were cut without any damage to the strongly plasmolysed cell. To aid the liberation of these cells, the sections were gently teased by means of a blunt glass rod. The slide was greased to prevent evaporation from the edge of the cover glass. That this was accomplished was shown by the fact that even after $3\frac{1}{2}$ hrs. a measured cell remained unchanged in size.

The prepared slide was then examined for free cells, and one of these was photographed at definite time intervals (usually 30

secs.) until plasmolysis was completed. It was found necessary to use a water filter, for the strong light necessary for photography otherwise had a tendency to cause a bursting of the cells. In between photographs the light was kept off, except for the short time required for focusing. A mechanical time-interval device worked by means of a clock system was, at first, tried. But this proved insufficiently accurate for short periods, so that it was found necessary to operate the mechanism by hand, using a stop-watch to obtain accurate timing.

Lantern slides, made from the photographs were projected onto a screen. In this way, a final magnification of about 1500 times was obtained, thus enabling a degree of accuracy unattainable by micrometer measurements - the method previously used. Plate 1 shows the type of results obtained.

One of the main advantages in the use of free cells is that deplasmolysis can be conducted until the cell has expanded to past its original volume, since there is no cell wall to stop it. Thus, if there is any increase in permeability during the late stages, this should certainly show up when the membrane is still further stretched. It is, of course, impossible to determine the osmotic pressure of any individual free cell. It was, therefore, necessary merely to indicate the maximum and minimum pressures found among all the sections investigated.

In spite of the standardisation of procedure, results were not always obtainable. On some days it was necessary to examine dozens of preparations before a suitable cell could be located. On other occasions, several were found in quick succession.

Tables 7 and 8 show the results obtained with CaCl_2 as the solute. In the first, deplasmolysis was completed to the minimum osmotic pressure point and, therefore, most likely past the isotonic pressure of the cell. In the second case it most certainly went considerably past its original volume. The former, unfortunately, was only watched for twenty minutes afterwards to see if it remained alive. The latter, however, was still normal even after 24 hrs. A still weaker solution than the one used in Table 8 was tried, but the cells always ultimately burst.

TABLE 7.

Water permeability of free onion pulp cell at different stages of deplasmolysis. Plasmolysed 55 mins. in M CaCl_2 ; deplasmolysed in .15M CaCl_2 .

Time (secs.)	r μ	g	conc. (atms.)	av. P.D. (atms.)	f (μ per sq. μ per min.)
73	86.8	.362	23.92	12.61	.60
133	94.3	.465	18.62	7.76	1.15
193	103.2	.609	14.22	4.31	1.58
253	110.0	.738	11.73	2.44	1.73
313	114.2	.826	10.48	1.45	1.94
373	117.0	.888	9.75	.75	1.87
493	119.8	.954	9.08	.21	2.27
733	121.7	1.000	8.66		
1183	121.7				

TABLE 8.

Water permeability of free onion pulp cell at different stages of deplasmolysis. Plasmolysed 40 mins. in M CaCl_2 ; deplasmolysed in .125M CaCl_2 .

Time (secs.)	r	g	conc. (atms.)	av. P.C. (atms.)	f (per sq. per min.)
68	63	.372	19.81		
128	70.3	.517	14.25	9.66	.75
188	76.7	.671	10.98	5.24	1.22
248	80.7	.782	9.42	2.83	1.41
308	83.0	.852	8.66	1.67	1.38
368	84.4	.894	8.24	1.08	1.29
518	85.7	.949	7.77	.63	1.33
668	87.0	.979	7.53	.28	1.32
788	87.6	1.000	7.37	.08	3.69
908	87.6				

When plotted against time, f shows the most rapid increase at first, followed by a gradual flattening out of the curve (fig.5). This is diametrically opposed to the results obtained by de Haan. When plotted against cell sap concentration, f tends to give a straight line relationship. It is interesting to note that the free cell shows a value for f two to three times as high as that for the cell still enclosed in its wall (Table 6). This, presumably, is due to the somewhat restricted diffusion in the latter case.

However, it may be considered unsafe to use CaCl_2 alone as

the plasmolytic. Consequently, further experiments were conducted employing a balanced solution consisting of approximately nine parts NaCl to one part CaCl_2 .

Tables 9 and 10 illustrate these results, in the former with a solution at most isotonic, in the latter with a hypotonic solution. In the first case the cell burst soon after reaching its end point. In the later stages it, therefore, shows abnormal permeability changes. The second cell, however, remained alive as long as watched. Results with these cells agree well with those obtained using CaCl_2 alone (fig.5). The slight dip followed by the sharp rise in the last stages is difficult to explain. It may be due to error, since the P.D. is very small toward the end, and a slight error in it would be large, proportionately.

TABLE 9.

Water permeability of free onion pulp cell at different states of deplasmolysis. Plasmolysed 28 mins. in 2.55M NaCl + .14M CaCl_2 (partial concentration). Deplasmolysed in .18M NaCl + .02M CaCl_2 .

Time (secs.)	r	g	conc. (atms.)	av. P.D. (atms.)	f (per sq. per min.)
74	64.4	.303	28.75	14.95	.67
134	75.5	.469	18.57	7.12	1.27
194	83.7	.665	13.09	3.37	1.43
254	88.5	.786	11.08	1.83	1.69
314	91.6	.871	10.00	1.07	1.41
374	93.1	.910	9.57	.71	.29
614	93.9	.939	9.28	.28	7.19
674	95.9	1.000	8.71		
854	95.9				
914	burst				

TABLE 10.

Water permeability of free onion pulp cell at different stages of deplasmolysis. Plasmolysed 28 mins. in 2.55M NaCl + .14M CaCl₂ (partial concentration). Deplasmolysed in .15M NaCl + .017M CaCl₂.

Time (secs.)	r	g	conc. (atms.)	av. P.D. (atms.)	f (per sq. per min.)
57	56	.406	18.13	8.41	.70
117	61.9	.549	13.41	4.70	1.02
177	66.7	.687	10.71	2.57	1.36
237	70.3	.804	9.15	1.42	1.41
297	72.3	.875	8.41	.83	1.56
357	73.6	.923	7.97	.42	1.43
477	74.8	.969	7.59	.11	2.42
657	75.6	1.000	7.36		
1147	75.6				

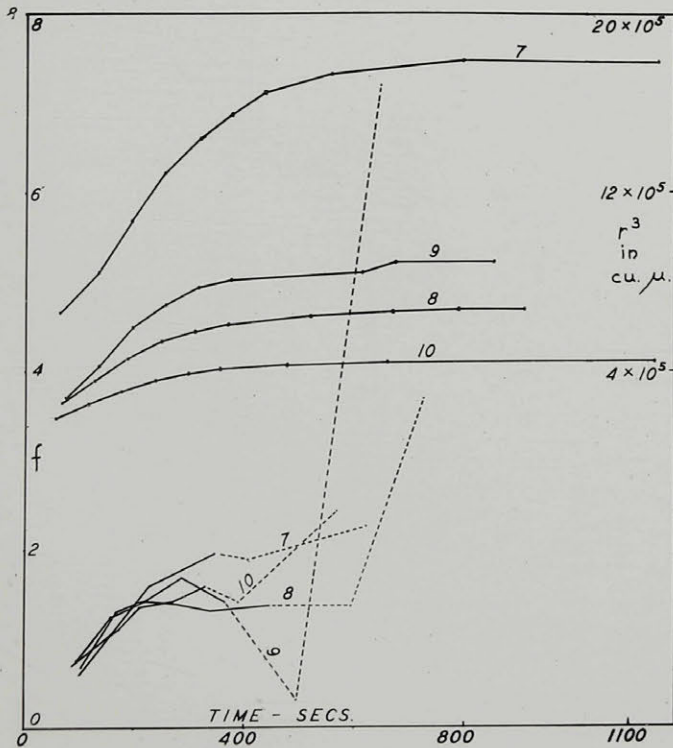


Fig.5. Deplasmolysis of free onion cell. Numbers correspond to tables. Cell 9 burst. Top curves volume to time; lower ones f to time. Uncertainty of last two values for f indicated by dotted lines.

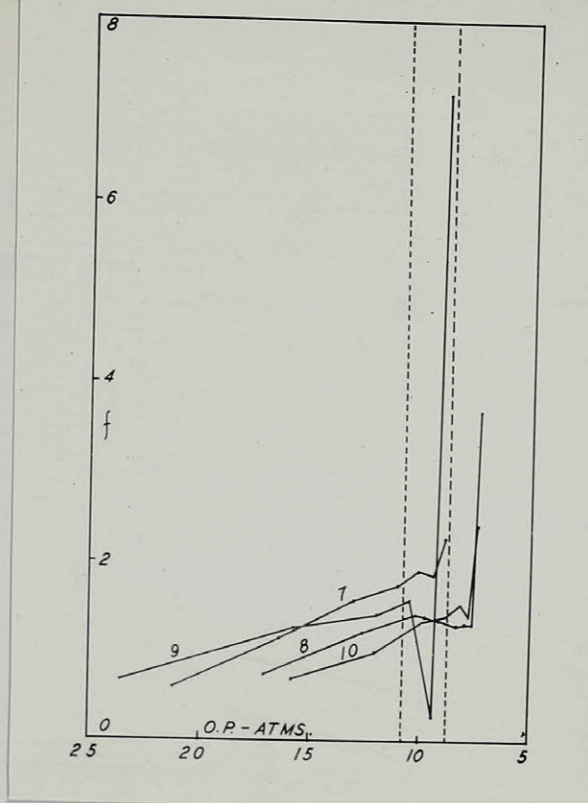


Fig.6. Relation of f to cell sap concentration in freed onion cell. Broken lines indicate the region in which the normal osmotic pressure of the cells lie.

The formula for f , however, does not take into account the thickness of the protoplasm layer. As deplasmolysis proceeds, the protoplasm becomes thinner, this change varying as r^2 . The symbol F has, therefore, been used to denote the true permeability rate - $F = \frac{f}{r^2}$ taking the final value for r^2 at the end of deplasmolysis as unity. Table 11, then shows the true change in permeability for the cells in Tables 7, 8, 9 and 10.

TABLE 11.

Values for F, or true permeability at the different stages of deplasmolysis in the previous four tables.

1.09	1.29	1.27	1.15
1.74	1.74	1.84	1.42
2.05	1.74	1.79	1.66
2.04	1.59	1.92	1.58
2.16	1.42	1.53	1.68
1.97	1.40	.31	1.49
2.31	1.35	7.43	2.44
	3.71		

It will be seen that the increase in the true permeability rate is relatively slight. Neglecting the last value, which is abnormally high due either to error or to an abnormal stretching of the membrane past its usual extent, the increase is never greater than 75%. This increase is presumably due to the greater hydration of the protoplasm.

In the face of these results, de Haan's contention can no longer be held. His method of calculation indicates a slow increase in permeability during the early stages of deplasmolysis, followed by an extremely rapid rise toward the end, so that the final value is often eight or more times the initial one. Our results, obtained under conditions which enable simple calculations, showed the reverse relation. That is, when plotted

against time, the permeability increase is greatest at first, and then slows up. The true permeability rate, obtained by taking into account the change in thickness of the protoplasm layer, shows a relatively slight increase, and it is only this which can be ascribed to the greater protoplasmic hydration.

This point being established, it is now more feasible to discuss any permeability changes associated with cold resistance.

After the above work was completed and written up it was discovered that Pfeiffer (1932) had previously conducted identically the same experiment, using a similar technique for the purpose of showing that quantitative work is possible with free protoplasts. He isolated cells of *Allium* epidermis and made measurements at different stages of deplasmolysis, or as he more correctly calls it, deplasmorrhysis. However, the only relationship he was interested in was the time to volume change, and his curves are similar to those shown in Fig.5. The measurements were taken with a micrometer eyepiece, instead of by means of actual microphotographs as in the technique here described, and consequently are less accurate.

As the only data he gives are the relative volumes, expressed in percent of the plasmolysed cell, it is impossible to calculate the true value for f . Relative values are shown in Table 11a. It can be seen that his results agree well with those in Tables 7-10. The maximum volume increase obtained by him is less than 70%, as compared with the 200% increase in Table 7. Furthermore, he deplasmolysed the cells only part way, so that the variation in cell sap concentration lies between 19 and 31 atms. Though Pfeiffer does not tell the normal osmotic pressure of the cell, it can safely be stated that even in the fully deplasmorrhysed condition, the isolated protoplast was deplasmolysed to, at most, only half its normal volume. Thus Pfeiffer's figures do not supply any information with regard to the final stages of deplasmolysis. However, as far as they go they do indicate the same thing as Tables 7-10 - that

there is no large increase in permeability with deplasmolysis.

The absence of the sudden increase for the last value seems to show that when these were obtained (Tables 7-10) it was due to an abnormal stretching of the membrane caused by the expansion of the cell to past its normal size.

TABLE 11a.

Change in permeability to water of isolated protoplasts on deplasmolysis. (adapted from Pfeiffer 1932).

Plasid in .8M NaCl Deplasid in .5" "			Plasid in .8M(CaCl ₂ - KCl) Deplasid in .5" " " "			Plasid in 1.2M sucrose Deplasid in .95M "		
Time (mins)	vol (%)	f (rela- tive)	Time (mins)	vol (%)	f (relative value)	Time (mins)	vol (%)	f (relative value)
1	108	.021	1	106.5	.008	.5	101.5	.008
2	124.5	.023	2	113	.012	2.5	107.5	.013
3	136.5	.022	3	122.5	.021	3.5	111	.015
5	150	.012	4	134.5	.017	4.5	114	.008
11	160	.020	5	141	.023	9.0	118.5	.022
13	162	.018	7	152	.018	11.0	121	.011
21	165		10	160	.015	20.0	122.5	
			20	167				

THE RELATION OF WATER PERMEABILITY TO COLD RESISTANCE.

Cabbage seedlings were hardened in the cold chamber in the usual way and determinations of water permeability made from time to time. The standard procedure was to plasmolyse for 15 - 20 mins. in twice isotonic dextrose, and then to deplasmolyse in an isotonic solution of the same substance. This, of course, gives only a relative measure, and not an absolute value as heretofore arrived at.

Table 12, shows that the water permeability increases, on hardening, until the end of a week, and then remains constant.

TABLE 12.

Water permeability of cabbage seedlings at different stages of hardening. Each value an average of 3 plants.

Hardening (days)	O.P. (atms.)	Deplasmolysis from 2i to i dextrose (mins.)
0	9.9	15
3	11.5	8
7	14.3	5
10	14.3	5

The experiments with woody plants under natural hardening conditions were, unfortunately, made before standardisation of the procedure. Consequently, in all cases, irrespective of

their osmotic pressures, the cells were plasmolysed for 15 mins. in a 2M dextrose solution and deplasmolysed in an $\frac{M}{2}$ dextrose solution (except Caragana, in which case on account of its high cell sap concentration double strength was used, i.e. M dextrose). But since the change in osmotic pressure was small during the period when the determinations were made, the difference in diffusion gradient was relatively slight. Table 13 indicates a definite increase in permeability to water during the late fall. From then on, the rate remained apparently constant, that is, deplasmolysis was so rapid as to be immeasurable.

TABLE 13.

Water permeability of woody plants during different stages of natural hardening. Plasmolysed 15 mins. in 2M dextrose; deplasmolysed in $\frac{M}{2}$ dextrose except Caragana which was deplasmolysed in M dextrose.

Date	Picea		Liriodendron		Caragana		Catalpa		Hydrangea	
	O.P. (atms.)	Depl. (secs.)	O.P.	Depl.	O.P.	Depl.	O.P.	Depl.	O.P.	Depl.
Oct.10	18.5	210	16.4	80	26.7	60	26.2	300	14.8	360
31	24.1	30	19.0	60	24.7	40	22.6	45	17.9	180
Nov.21	21.6	60	18.5	30	26.2	30	19.5	180	19.5	270
Dec.12	21.0	30	20.5	30	24.7		19.5	60	17.9	150

A further test was made on fully hardened twigs taken indoors during mid-winter, and then left in water at room temperature for a few weeks. Table 14 shows that during this time - that is, in the period when hardness was lost - a decrease in permeability to water occurred.

TABLE 14.

Water permeability before and after loss of hardness. Plasmolysed 15 mins. in 2i CaCl_2 ; deplasmolysed in $\frac{1}{2}$ CaCl_2 . Jan.16/35.

Days at room temp.	Hydrangea		Catalpa	
	O.P. (atms.)	Deplas. (secs.)	O.P. (atms.)	Deplas. (secs.)
0	20.5	20	19.5	35
37	10.2	120	14.3	85

However, it must be admitted that it was not always possible to demonstrate this change. At another time, for instance, even after several days at room temperature, the permeability was still immeasurably rapid. This, of course, does not prove that no true difference existed, for it may have been beyond the powers of measurement by the plasmolytic method.

It seems, then, that hardness is characteristically accompanied by an increase in the cellular permeability to water. This change is of the order of 300%, which is much

larger than the 75% increase obtained on deplasmolysis. It, therefore, appears unlikely that it can be due simply to an increase in hydrophily.

It can readily be seen that on account of the rapidity of cellular permeability to water, differences are not detectable unless very large. Solute permeability, being usually a far slower process, therefore offers better possibilities. It was for this reason that the relation of solute permeability to cold resistance was more thoroughly investigated.

B. PERMEABILITY TO NON-ELECTROLYTES.

Some objections have been raised to the use of the plasmolytic method for permeability determinations. Scarth (1926) found that plasmolysis caused an increased permeability to dyes. Lepeschkin (1932) reports the same thing. Weber (1933) showed that neutral salts in hypertonic concentration caused a greater cytolysis of plant cells by bile salts. Fitting (1915), however, proved that plasmolysis had no effect on the permeability of *Rhoeo discolor*. Huber and Schmidt (1933) attempted to settle the problem by working with four different species. They substantiated Fitting's results with *Rhoeo*. *Majanthemum* showed a slight but definite decrease in permeability to glycerine and urea - 10-15%. This was only detectable in careful experiments, and was well within the variability between several experiments. *Salvinia* exhibited abnormal behaviour, for deplasmolysis discontinued after a while in the solution, in spite of the sound appearance of the protoplasm. With *Gentiana*, however, extraordinary differences were obtained. This plant is very permeable to urea. After a 2 hr. plasmolysis in M cane sugar, urea deplasmolysis took hours, whereas unplasmolysed cells require only 10 - 20 mins. But it must be admitted that the two-hour plasmolysis was an extreme procedure, and may have caused a hardening of the membrane.

Weber (1931) concludes that the more easily cells plasmolyse, the less is their permeability disturbed, and the better do they withstand plasmolysis.

As a test of the effect of plasmolysis on permeability, the rate of penetration both with and without plasmolysis was compared, adopting a modification of the method used by Huber and Schmidt. Two parallel determinations were made, one in the ordinary way, the other by use of a hypotonic solution which, of course, caused no plasmolysis. (The method of calculation is described in the next section.) In the latter case, a section was removed at intervals from the hypotonic "penetrating solution" and its osmotic pressure tested by means of a "plasmolysing solution" made up of a partial concentration of the penetrating substance equal to half that in the "penetrating solution". The purpose of the latter was to prevent exosmosis during the determination. The end point was taken as the time at which the cells were no longer plasmolysed by this composite "plasmolysing solution". At this end point, the concentration of the solute which had penetrated the cell was equal to half that in the "penetrating solution", or equal to that in the "plasmolysing solution". Since each section was tested only once and then discarded, the final one which established the end-point suffered no plasmolysis at all.

Table 15 shows that the rate of penetration with and without plasmolysis is identical.

TABLE 15.

Effect of plasmolysis on permeability.

Species	O.P. (atms.)	Time (mins.)		Penetration (mols per hr.)	
		plasmolysed	unplasmolysed	plasmolysed	unplasmolysed
		.8M thiourea	.4M thiourea		
Cabbage	11.2	60	100	.46	.40
		1.5M urea	.5M urea		
Hydrangea	19.5	52	56	.61	.71
Catalpa	19.5	75	90	.42	.44

METHOD FOR DETERMINING RATE OF PERMEABILITY:

Following the determination of osmotic pressure, the same sections were investigated for permeability to an organic penetrant. Thiourea was used where possible. When this penetrated too rapidly to cause plasmolysis, it was replaced by urea. Some others were tried but these two proved the most convenient. Since they tend to decompose after some time, fresh solutions were made up daily. Permeability was calculated from the time required for deplasmolysis to occur in solutions of standard concentrations. These concentrations necessarily depended on the osmotic pressure and the permeability of the cells under investigation. The resulting differences in concentration gradient were estimated and allowed for as follows:

The average pressure difference between the concentration of penetrating substance outside and inside the cells was taken as lying midway between the initial and final difference. The initial concentration of penetrating substance inside the cells is zero, and the final concentration (when deplasmolysis is completed) approximates that which is necessary to equalize the original internal pressure with the external. The latter remains virtually unchanged throughout the test.

Calculation of the formula involves the following symbols:

PD = average pressure difference (mols)

P = external concentration of penetrant (mols)

p = O.P. of cell (expressed as mols of penetrant)

t = time for deplasmolysis (hours)

Then the total amount penetrating per hour = $\frac{P - p}{t}$ mols

$$\text{also } PD = P - \frac{P - p}{2} = \frac{P + p}{2}$$

Therefore, the actual amount of substance entering a cell under a mean PD of 1 mol = $\frac{P - p}{t} \div \frac{P + p}{2} = \frac{2(P - p)}{t(P + p)}$

The validity of the formula was tested by observing the time for deplasmolysis to occur in solutions of different concentrations. The rate was found to be constant, within the experimental errors involved, irrespective of the osmotic gradient (Table 16). The results in Table 15 actually illustrate the same thing.

TABLE 16.

Comparison of the permeability rate in solutions of different concentrations.

Species	Conc. urea (mols)	conc. thiourea (mols)	O.P. (atms.)	deplas. (mins.)	perm. (mols per hr.)
Cabbage		.6	11.2	22	.49
		.7		36	.55
		.8		60	.46
Aesculus	.93		11.2	86	.42
	1.86			142	.49
	2.79			165	.50
Catalpa	.93		13.3	64	.42
	1.86			107	.58
	2.79			151	.51
Hydrangea	1.86		16.9	27	1.88
	2.79			35	1.93
Picea	1.86		19.5	110	.39
	2.79			127	.49

It is well to examine these results from the theoretical standpoint. Since the PD is not constant, the average degree of plasmolysis varies from one case to the next. But the greater the average PD, the smaller the surface area of the cell, and the less the total quantity of substance penetrating it, providing the permeability per unit area remains constant. Table 16 shows that the total quantity of substance penetrating is independent of the degree of plasmolysis. It would seem, then, that the permeability per unit area is increased by plasmolysis. But Table 15 proves this to be untrue. The only apparent explanation, then, is that the more strongly the cells are plasmolysed, the more ready is the diffusion of the solute through the tissue. This presumably counterbalances the decrease in surface area.

RESULTS.

(a) Artificial Hardening.

1. Cabbage: Though the osmotic pressure of the cells of different tissues was the same, the permeability differed. The epidermal cells and the chlorenchyma proved more permeable than the pith. Yet any variation on hardening occurred parallel in all cells. If the chlorenchyma was twice as permeable as the pith in the unhardened condition (as was often the case) the same ratio held in the hardened plants. Unless otherwise stated, the tables refer to the pith cells.

Plants were hardened in the cold chamber under continuous lighting at a temperature of 5°C , and determinations were made every few days. Each result is the average of six plants. Toward the end of the hardening period it was necessary to increase the concentration of thiourea used in order to obtain plasmolysis.

Table 17 shows that the osmotic pressure increases by about 20% while permeability is more than 300% greater. The period of increase of both coincides with the hardening period.

TABLE 17.

Effect of hardening on O.P. and permeability of cabbage cells.

Days hardened	O.P. (atms.)	conc. thiourea (mols)	Deplas. (mins.)	Perm. (mols per hr.)
0	10.2	.75	110	.27
1	10.7	"	70	.37
2	10.7	"	57	.46
3	10.7	"	54	.49
4	11.2	"	34	.70
8	12.3	.825	25	.96
11	12.3	.90	39	.74

To extend the series of substances investigated, glycol permeability was also determined. Table 18 shows that the same change occurs in the permeability of cabbage cells to thiourea and glycol.

TABLE 18.

Permeability of hardened and unhardened cabbage seedlings (chlorenchyma cells) to thiourea and glycol.

	O.P.(atms.)	Deplas.(mins.)		Perm.(mols per hr.)	
		thiourea .75M	glycol .75M	thiourea .75M	glycol .75M
unhardened	9.7	60	13	.56	2.60
hardened	12.3	15	3½	1.23	5.27

2. Clover: Here, as far as the tests extended, the increase of both osmotic pressure and permeability is somewhat less than in cabbage: viz. osmotic pressure 18%, permeability 200%. Each value is an average of four plants.

TABLE 19.

Effect of hardening on O.P. and permeability of double-cut clover.

Days hardened	O.P. (atms.)	conc. urea (mols)	Deplas. (mins.)	Perm. (mols per hr.)
0	13.3	.93	120	.22
1	13.3	"	72	.38
2	14.8	"	36	.56
3	13.8	"	38	.63
6	15.9	"	28	.57

TABLE 20.

Effect of hardening on O.P. and permeability of single-cut clover.

Days hardened	O.P. (atms.)	conc. urea (mols)	Deplas. (mins.)	Perm. (mols per hr.)
0	12.3	.93M	150	.20
1	13.3	"	110	.25
2	14.3	"	65	.34
3	14.8	"	48	.42

Single-cut clovers are usually considered more hardy than double-cut varieties. The change in permeability rate, however, is very similar in the two (Table 20). Whether a difference would show up after a longer hardening period was not determined. Also, since both varieties were obtained from Holland, it is perhaps doubtful whether any great difference in hardness really existed. Consequently, it is impossible to say whether or not varietal resistance in clover is related to permeability rate.

3. Sunflower: This is a non-hardy plant. It was subjected to the same conditions that caused the cabbage and clover to harden off but which do not confer any cold resistance on the sunflower. Table 21 shows that no change occurs either in osmotic pressure or permeability.

Table 21.

Effect of exposure of sunflower to low temperatures on osmotic pressure and permeability.

Days at 0 - 5°C	O.P. (atms.)	conc. thiourea (mols)	Depl. (mins.)	Perm. (mols per hr.)
0	9.7	.75	70	.48
7	9.7	.75	85	.39

Similarly, tomato, bean, and castor bean which are all tender plants, showed no increase either in osmotic pressure or permeability on exposure to hardening temperatures.

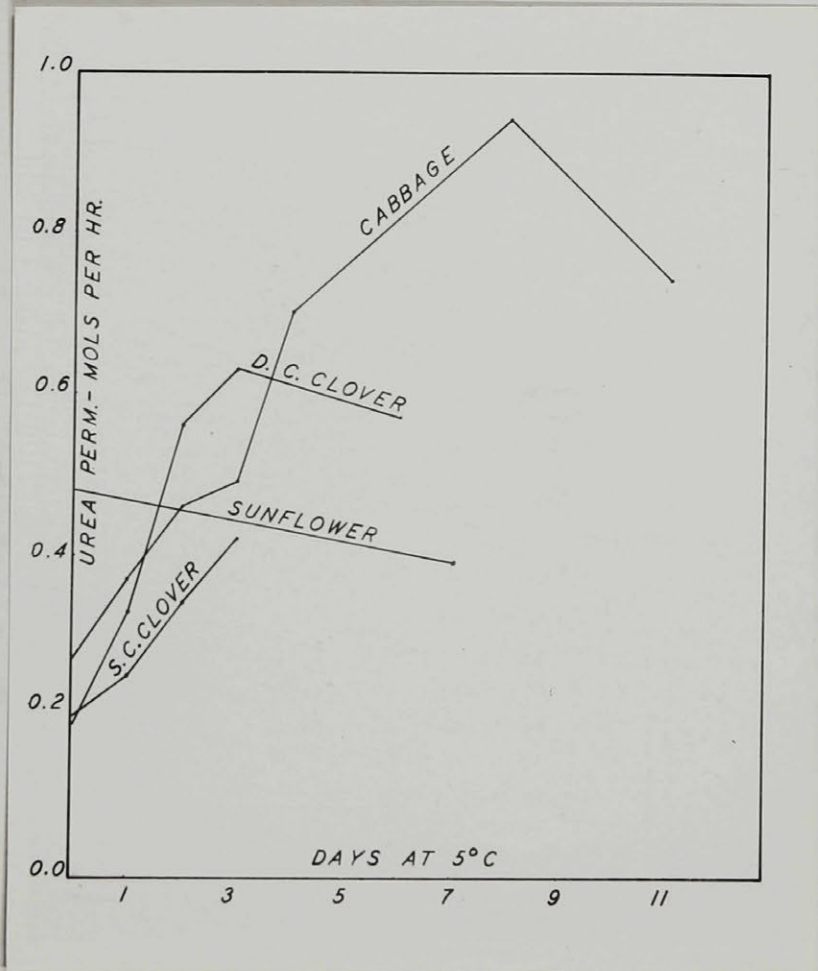


Fig.7. Change in urea permeability on hardening.

Other environmental conditions besides low temperatures affect hardness. It is important to determine whether these also change the permeability of the cell, or whether the latter is only the result of low temperatures.

It had been previously found that the hardness of cabbage plants varied inversely with the nitrogen supplied. The seedlings were grown in sand cultures under different nutrient treatments and were then hardened for five days at 5°C previous to freezing. Percentage injury is given in Table 22.

TABLE 22.

Effect of varying nitrogen supply on cold resistance of cabbage seedlings. Average of nine plants.

ppm. N	% injury
10	25
25	15
50	40
100	90
200	80
300	85

Seedlings similarly grown were, therefore, tested for osmotic pressure and permeability. Table 23 indicates that in this case, too, hardness is correlated with these factors even though the cause of the cold resistance is not low temperature.

TABLE 23.

Effect of quantity of available nitrogen on osmotic pressure and permeability of cabbage plants. Average of five plants.

ppm. N	O.P. (atms.)	Depl. (min.in .75M thiourea)	Perm. (mols per hr.)
10	10.7	46	.56
25	10.2	53	.55
50	9.7	55	.61
100	9.2	76	.48
200	8.7	113	.34
300	9.7	85	.39

It has frequently been shown that stunting, wilting, or in any way checking growth, will increase a plant's resistance to frost (Rosa 1921, Chandler 1913, Harvey 1918). In January 1935 a good example of this occurred. There were two distinct sets of cabbage seedlings growing side by side. One set had been sown at the end of September, the other at the end of November. The former group had been strongly checked during the first two months of growth by a combination of extremely dull weather and a heavy aphid infestation. From the time the second group was sown, they grew side by side under the same conditions. At the end of January both sets were approximately the same size, though differing markedly in appearance. The older checked

group was stemmier, the leaves were bluish due to a heavy bloom, and there was a slight development of red pigment on the stems and the under sides of the leaves. Further, the leaves were so turgid as to be almost upright, with the upper surfaces concave. The younger unchecked group, on the other hand, was a lush green, the leaves were larger and not so turgid and had a tendency to be convex on the upper surface. On hot, sunny days the younger group suffered wilting whereas the older set retained their full turgidity. Differences in drought resistance, therefore, obviously existed.

Here, then, were two groups of plants grown under the same temperature conditions and yet the one had all the appearances of at least partially hardened plants, whereas the other was obviously perfectly tender. Though their relative hardiness was not actually tested by freezing, there is little room for doubt that differences did exist, both on account of their characteristic appearance and because several investigators have proved that checking growth increases hardiness.

These plants were tested for osmotic pressure and permeability. Table 24 shows that marked differences existed between the two sets.

TABLE 24.

Osmotic pressure and permeability of checked and unchecked cabbage seedlings (chlorenchyma cells).

	O.P. (atms.)	Deplas. (mins.in .75M thiourea)	Perm. (mols per hr.)
unchecked	9.7	71	.48
checked	11.0	28	.89

As already mentioned, the checked plants were more drought resistant than the unchecked ones. Drought resistance has, in fact, frequently been considered as correlated with cold resistance (Maximov 1929). It is, therefore, interesting to ascertain whether a drought resistant plant exhibits any permeability change similar to that associated with hardiness.

Spartium junceum is a plant which is able to withstand considerable drought. A specimen of this species was kept in the greenhouse unwatered for two weeks. At the end of the first week the foliage showed little or no loss of turgidity. With the conclusion of the second week, however, the leaves were strongly wilted. Sections of the stem were examined at these time-intervals for osmotic pressure and permeability. Table 25 demonstrates that here, as with cold resistance, the plant undergoes an increase in both osmotic pressure and permeability during the "hardening" (to drought) period.

TABLE 25.

Osmotic pressure and permeability change during increase in drought resistance of *Spartium*.

Days unwatered	O.P. (atms.)	Deplas (mins.)	conc. urea (mols)	urea perm. (mols per hr.)
0	15.9	66	1	.44
7	15.4	55	1	.40
14	21.0	60	2	.72

(b) Natural Hardening.

The herbaceous plants so far investigated do not belong to the hardiest group of plants. Even clover, which overwinters in our climate, does so only when protected by a snow blanket, and it is doubtful whether the leaves (which were the parts tested) are as hardy as the overwintering stem. It is of prime importance to determine if the same law holds in the case of overwintering woody plants - i.e. do they also show higher solute permeabilities in the hardened than in the unhardened condition?

In the late winter of 1934 twigs of several woody plants were tested for permeability before and after being kept at room temperature for a number of days. Table 26 shows that in all cases a decrease in permeability occurred at room temperature.

A significant fact was noticed. Those species which burst their buds most rapidly, showed the quickest and most marked change in permeability (Caragana, Catalpa). Others which did not open their buds in the laboratory exhibited the slowest and smallest alteration (Picea). The question that suggests itself then is: Does this permeability change have anything to do with dormancy? In order to solve this problem, a seasonal study was made throughout the fall and winter of 1934 - 35.

TABLE 26.

Change in osmotic pressure and permeability of twigs kept at room temperature.

Species	Date	Days at room temp.	O.P. (atms.)	conc. urea (mols)	Time to deplas. (mins.)	Molar penetration per hr.
<i>Hydrangea paniculata</i>	Feb. 23/34	0	23.1	1.86	30	1.15
		4	16.4	1.4	90	.42
		7	13.3	.93	60	.44
		11	13.3	.93	55	.48
<i>Caragana arborescens</i>	Mar. 14	0	25.2	2.79	40	1.28
		15	12.8	.93	145	.19
<i>Picea</i>	Mar. 16	0	18.5	2.79	120	.53
		11	15.9	.93	30	.53
		31	12.3	.93	125	.24
<i>Pterocarya rhoifolia</i>	Mar. 23	0	29.3	2.79	30	1.44
		21	17.4	1.86	40	1.23
<i>Catalpa</i> sp.	Apr. 11	0	23.6	2.79	16	3.39
		5	13.3	.93	64	.42
		12	11.7	.93	111	.30
<i>Aesculus hippocastaneum</i>	Apr. 19	0	19.0	2.79	21	3.04
		29	11.2	.93	86	.42

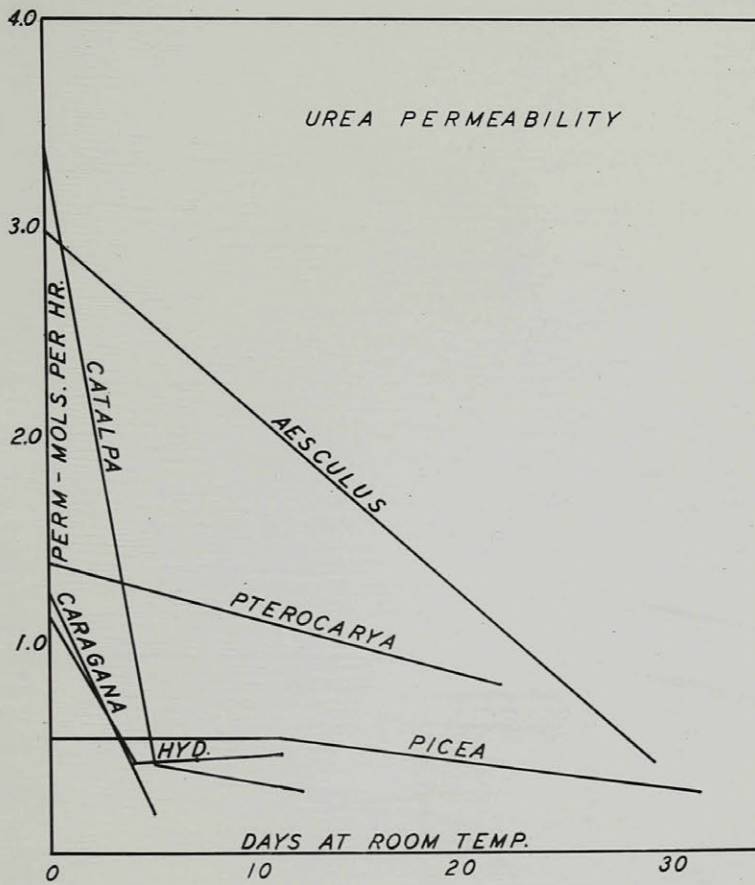


Fig.8. Change in urea permeability on loss of hardness.

Five species of ornamental woody plants, and four varieties of apples were tested at periods of three weeks. Results are given in Tables 27 and 28.

Table 27.

Seasonal determinations of osmotic pressure and permeability of ornamentals to urea 1934 - 35.

Date	Picea		Liriodendron		Caragana		Catalpa		Hydrangea	
Date	O.P.	Urea Perm	O.P.	Urea Perm.	O.P.	Urea Perm.	O.P.	Urea Perm	O.P.	Urea Perm.
Sept.27	19.0	.59	15.4	1.22			18.5	.62	11.7	.63
Oct.17	19.5	.29	16.9	.45	26.7	.69	21.0	.35	14.8	.63
Nov.7	24.7	.37	19.0	.63	25.7	.36	19.5	.33	18.5	.53
Nov.28	21.6	.61	19.5	8.25	27.9	.53	20.5	.89	21.6	.61
Dec.19	23.1	.43	21.0		26.2	.31	19.5	.62	21.0	.50
Jan.9	20.0	.54	21.6	6.48	32.5		18.5	.61	21.6	.63
Jan.30	19.5	.76	22.6		28.4	.61	20.5	3.74	19.5	.79
Feb.20	20.5	.67	22.6	14.89	26.7	.59	21.6	2.11	19.5	.78
Mar.14	19.0	.48	23.1	9.77	29.3	1.42	18.5	6.85	21.6	.70
Apr.3	17.4	.47	23.6	8.25	29.3	.58	21.0	.42	21.0	.72

TABLE 28.

Seasonal determinations of osmotic pressure and permeability in apple varieties 1934 - 35.

Date	Hyslop		Hibernal		Milwaukee		Delicious	
	O.P.	Perm.	O.P.	Perm.	O.P.	Perm.	O.P.	Perm.
Sept.28	20.0	1.07	17.9	.94	16.9	.46	14.8	.65
Oct.18	21.6	.76	23.1	.80	20.0	.65	16.9	.75
Nov.8	22.6	.63	25.2	.60	20.5	.47	15.4	.36
Nov.29	22.6	.67	27.9	.74	23.6	.69	20.0	.77
Dec.20	23.6	.80	26.7	.82	24.7	.48	21.6	.47
Jan.17	23.6	.98	29.3	.90	25.7	.54	23.1	.69
Feb.7	23.6	.81	29.3	.82	23.6	.50	24.1	.80
28	22.1	.91	29.3	.81	21.0	.43	23.1	.79
Mar.21	24.1	.83	30.6	.75	24.1	.43	23.1	1.04
Apr.4	23.1	.71	26.7	.76	22.6	.73	22.6	1.07

It is evident from these tables that, for the most part, no appreciable increase in permeability occurs. Liriodendron and Catalpa are obvious exceptions. But it is very doubtful whether the marked increase in these cases can be associated either with hardness or dormancy. Krassinsky (1929) demonstrated a seasonal change in the permeability of onion, and it is highly probable that the increase in Liriodendron and Catalpa is simply of this nature.

At first sight, it seems difficult to reconcile these results with the marked loss of permeability found on keeping

twigs at room temperature (Table 26). However, Gail (1926) showed that woody plants begin to ripen off at the end of July, as proved by the increasing osmotic pressure. It is, therefore, very likely that the increase in urea permeability had already occurred by the end of September when the first test was made. Unfortunately, this assumption has, so far, not been investigated.

With regard to varietal resistance, there appears to be some indication of correlation with urea permeability. From the beginning of November to the end of January, Hyslop and Hibernial were characterised by a higher permeability rate than the more tender varieties - Milwaukee and Delicious.

C. PERMEABILITY TO ELECTROLYTES.

Any permeability study would be incomplete without an investigation of the penetration of inorganic salts. It is these substances, in fact, which are most frequently used for work on cellular permeability.

The first plant tried was the cabbage. Petiole sections from hardened and unhardened seedlings were left in $\frac{M}{2}$ KNO_3 for 24 hours. After this time, their osmotic pressures were determined in the usual way, and the increase over the original cell sap concentration gave a measure of the amount of KNO_3 penetrated during the period. Table 29 shows that the same amount penetrated in both cases.

TABLE 29.

Permeability of cabbage cells to KNO_3 .

	original O.P. (atms.)	final O.P. (24 hrs. in $\frac{M}{2}$ KNO_3)	Increase
Unhardened	9.7	10.7	1.00
Hardened	11.3	12.3	1.00

Thus there is no apparent difference in the permeability to KNO_3 of hardened and unhardened cabbage. However, it must be admitted that the method is unsatisfactory. During such a long period of time the KNO_3 can be expected to injure the cells or

perhaps to alter their permeability. Injury did occur, for very many and sometimes even most of the cells were dead at the end of the 24-hour period. It seems hardly permissible, then, to trust the results with the few still alive. Further, the amount penetrating is so small that relatively large differences may pass unnoticed.

Consequently, if the permeability to KNO_3 is to be reliably determined, cells which are far more permeable to it than those of cabbage must be used. The same woody plants as heretofore employed were then tried and found satisfactory. Twigs collected in mid-winter were tested before and after remaining at room temperature (Table 30).

TABLE 30.

Permeability to KNO_3 of twigs before and after loss of hardiness. Twigs collected Feb. 6/35. (Picea in .75M KNO_3)

Species	Days at room temp.	O.P. (atms.)	Deplas. in 1.25M KNO_3 (mins.)	Perm. (mols per hr.)
Hydrangea	0	19.5	39	1.37
	7	16.9	73	.83
Catalpa	0	19.5	39	1.37
	7	19.0	215	.26
Caragana	0	28.3	16	2.11
	7	18.5	>720	< .15
Liriodendron	0	22.1	7	6.79
	7	20.5	106	.48
Picea	0	18.5	5	6.00
	7	20.0	58	.43

Extreme differences are evident, differences even larger than were obtained with urea. Penetration of KNO_3 was so rapid in the hardened cells that it is doubtful whether any comparably high degree of permeability to electrolytes is to be found in the literature.

Unfortunately, this marked difference in permeability to KNO_3 was not determined until the winter of 1935. Consequently, the only seasonal determinations were those made from mid-winter to spring. Figs. 9 & 10 and tables 31 and 33 show the changes occurring.

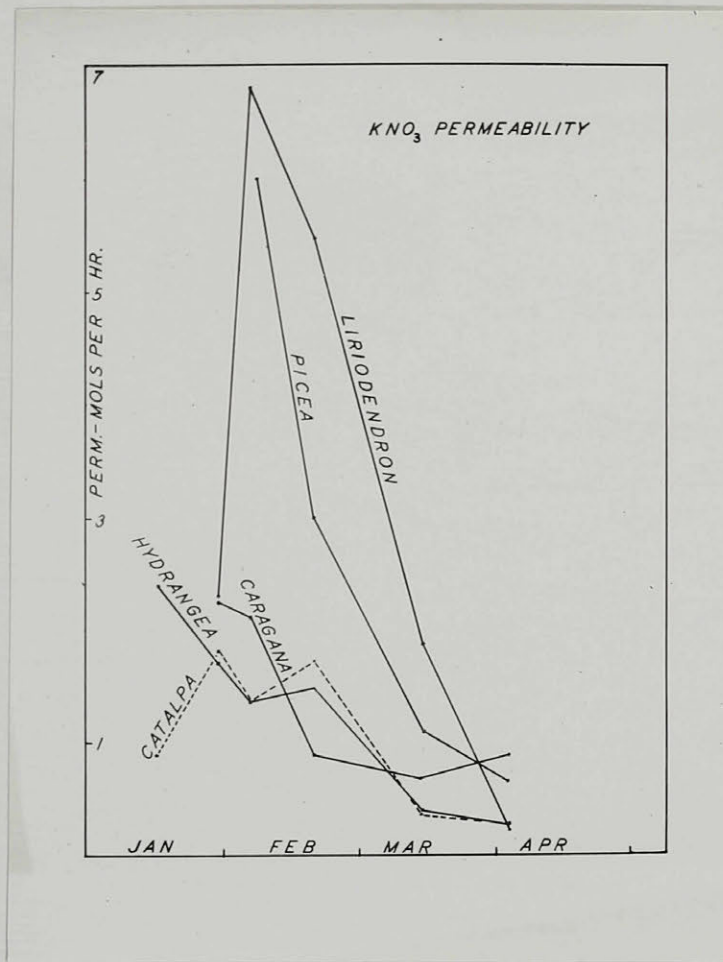


Fig. 9. Seasonal change in KNO_3 permeability of ornamentals.

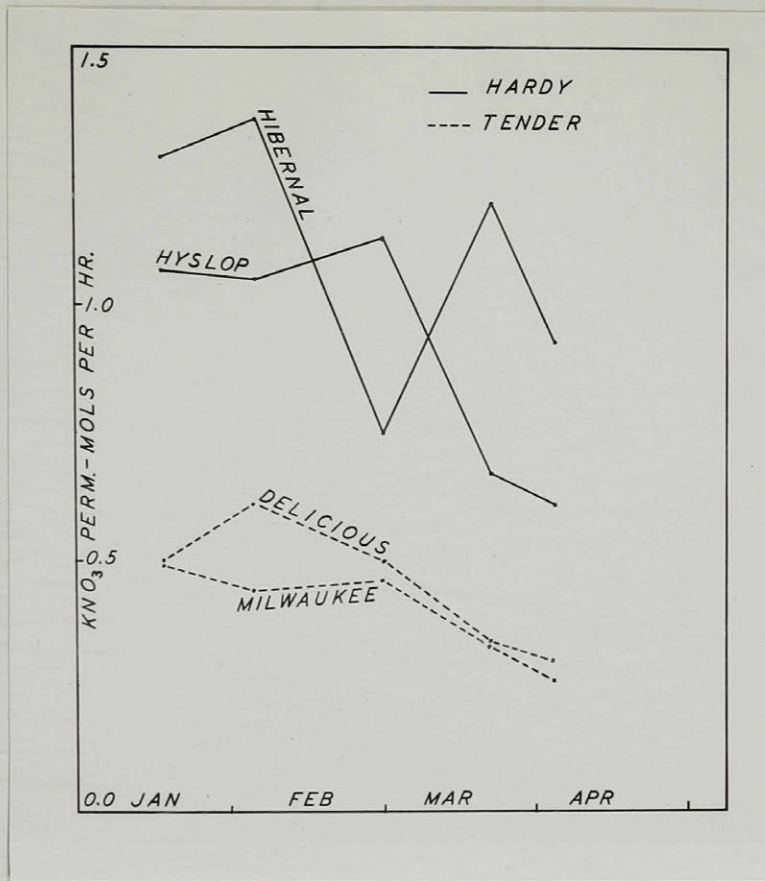


Fig.10. Seasonal change in KNO_3 permeability of apples.

TABLE 31.

Seasonal changes in permeability to KNO_3 of ornamental woody plants. Deplasmolysis in 1.25M KNO_3 (Picea in .75M).

Date	Hydrangea			Liriodendron			Caragana			Gatalpa			Picea		
	O.P. (atms)	Deplas (mins)	Perm. (mph.)	O.P. (atms)	Deplas (mins)	Perm. (mph.)	O.P. (atms)	Deplas (mins)	Perm. (mph.)	O.P. (atms)	Deplas (mins)	Perm. (mph.)	O.P. (atms)	Deplas (mins)	Perm. (mph.)
Jan. 16	20.5	10*	2.40							19.5	30*	.89			
29	19.5	31	1.72	22.6	20	2.31	28.3	15	2.25	20.5	31	1.82			
Feb. 6	19.5	39	1.37	22.0	7	6.79	28.3	16	2.12	19.5	39	1.37	18.5	5	6.00
20	19.5	36	1.49	22.6	8	5.47	26.7	41	.90	21.6	28	1.73	20.5	8	3.00
Mar. 14	21.6	125	.39	23.1	24	1.87	29.3	47	.69	18.5	150	.38	19.0	26	1.11
Apr. 3	21.0	180	.27	23.6	>180	<.24	29.3	36	.89	21.0	180	.27	17.4	50	.66

* = .75M KNO_3

TABLE 32.

Seasonal changes in permeability to KNO_3 of apple varieties. Deplasmolysis in 1.25M KNO_3 .

Date	Milwaukee			Delicious			Hibernal			Hyslop		
	O.P. (atms)	Deplas. (mins)	Perm. (m.p.h.)	O.P. (atms)	Deplas. (min.)	Perm. (m.p.h.)	O.P. (atms)	Deplas. (min.)	Perm. (m.p.h.)	O.P. (atms)	Deplas. (min.)	Perm. (m.p.h.)
Jan.17	25.7	56*	.49	23.1	65*	.50	29.3	25	1.29	23.6	41	1.07
Feb. 7	23.6	100	.44	24.1	70	.61	29.3	24	1.34	23.6	45	1.05
28	21.0	108	.46	23.1	92	.49	29.3	43	.75	22.1	42	1.13
Mar.21	24.1	131	.33	23.1	132	.34	30.6	25	1.20	24.1	64	.67
Apr. 4	22.6	180	.26	22.6	155	.30	26.7	40	.93	23.1	73	.61

* = M KNO_3

The apple varieties are especially interesting since there is a marked difference between the hardy and the tender group. The former are two to three times as permeable as the latter. In order further to investigate the relationship between varietal resistance and KNO_3 permeability, six more apple varieties varying in hardness between the above two groups were tested. Table 33 indicates a fairly good agreement among the ten varieties listed, there being only two exceptions. The varieties are placed in the order of hardness as found by Dr. R.F. Suit at Macdonald College, based on the injury suffered during the test winter of 1934 - 35.

TABLE 33.

Varietal frost resistance of apples as related to KNO_3 permeability. Average of 4 determinations.

Group (increasing hardness)	Variety	O.P. (atms.)	Deplas. (mins in 1.25M KNO_3)	Perm. (mols per hr.)
1.	Milwaukee	23.4	98	.46
	Delicious	23.5	81	.55
2.	Alexander	26.7	45	.82
	Fameuse	21.7	67	.72
	McIntosh	24.1	64	.64
	Wealthy	22.2	70	.64
3.	Wolf River	30.3	44	.76
	Patten Gr.	22.8	59	.78
4.	Hibernal	29.3	31	1.04
	Hyslop	23.2	43	1.04

The four determinations of which Table 33 gives the average, were made between the end of January and the end of February. It may be mentioned that the first two (taken when hardness was at its height) gave an almost perfect agreement.

It is, therefore, possible that electrolyte permeability if determined at the right season may prove to be a good measure of varietal hardness.

D. RELATION BETWEEN OSMOTIC PRESSURE AND PERMEABILITY.

In most of the cases so far given, both osmotic pressure and permeability increased concurrently. It is of fundamental importance to know whether these changes necessarily go hand in hand. That osmotic pressure can increase without any concomitant change in permeability (or, incidentally in cold resistance) was strikingly shown by *Helianthus*. In the early stages of growth its osmotic pressure was low, partially on account of dull weather. Later an increase occurred, and finally as the flower bud formed, the highest osmotic pressure was recorded. This was, presumably, due to the increased C:N ratio which is correlated with reproduction. Yet as shown in Table 34, the permeability remained unchanged.

TABLE 34.

Osmotic pressure and permeability of *Helianthus* at different stages of growth.

Age (days)	O.P. (atms.)	Deplas. (min. in .75M thiourea)	Perm. (mols per hr.)
54	9.7	70	.48
80	11.2	50	.48
89	12.8	32	.51

But *Helianthus* is incapable of hardening off, and it may, therefore, be argued that this fact does not apply to cold

resistant plants. Consequently, experiments were conducted involving direct sugar feeding. To this end, cabbage leaves were stood in vials containing $\frac{M}{5}$ dextrose, and left in the laboratory for two days. From Table 35 it can be seen that a distinct increase in osmotic pressure did occur, but no permeability change accompanied it.

TABLE 35.

Effect of sugar feeding on osmotic pressure and permeability of cabbage leaves (chlorenchyma cells). Average of 3 plants.

Days in $\frac{M}{5}$ dextrose	O.P. (atms.)	Conc. thiourea (mols)	Deplas. (mins.)	Perm. (mols per hr.)
0	9.9	.75	42	.74
2	11.8	1.00	60	.63

This definitely establishes the fact that the increase in permeability which occurs during hardening is not caused by the sugar increase. Whether or not the reverse relation holds, it has as yet been impossible to prove.

With regard to electrolyte permeability, Tables 31 and 32 amply illustrate in all cases that the seasonal change takes place before any alteration in the osmotic pressure. Consequently, they seem to indicate that the permeability change is not the cause of the alteration in osmotic pressure.

E. PERMEABILITY TO APOLAR SUBSTANCES.

The three organic compounds used - glycol, thiourea, and urea - are characterised by small molecular weights (62, 76, and 60 respectively) and are not lipoid-soluble (Collander and Bärlund 1933). According to the modern conception of cellular permeability, the plasma membrane is a lipoid-sieve (Höber 1933; Höfler 1934). The above three substances, as well as water and electrolytes, penetrate through the pores, making use of the sieve mechanism. This is characteristic of all polar compounds. Non-polar substances, however, with larger molecules, cannot pass through the pores and must be lipoid-soluble in order to penetrate. Now does the increase in permeability which accompanies cold resistance apply also to these lipoid-soluble substances?

From the list of substances used by Collander and Bärlund (1933), two were chosen - urethane and succinimide. The former comes at the top of their list, penetrating about as rapidly as ethyl alcohol. The latter is further down in the series, but it, too, is rapidly permeable. On account of their quick penetration, a different technique was necessary in determining permeability. The solution causing incipient plasmolysis was found, this giving only a relative and not an absolute value. However, the substances could not be employed in pure solution since such high concentrations were necessary as to be toxic. Mixed solutions were found to serve the purpose.

These were made up of CaCl_2 having a partial osmotic pressure equal to that of the cell sap, together with varying concentrations of the penetrant. Table 36 shows the comparative permeabilities of hardened and unhardened cabbage seedlings to urethane, succinimide, and thiourea.

TABLE 36.

Permeability of hardened and unhardened cabbage seedlings (chlorenchyma cells) to polar and apolar substances.

	Unhardened	Hardened
O.P.	9.7 atms.	12.8
thiourea perm.	.82M per hr.	1.64
partial concentration causing incipient plasmolysis		
urethane	.50M	.50M
succinimide	.05M	.05M

Thus, the increased permeability caused by hardening, applies only to polar substances and not at all to the apolar, lipoid-soluble compounds. This is of fundamental importance for it indicates that the change is due to a widening of the pores in the plasma membrane. This may be considered a hydration of the plasma membrane. The protoplasm itself, however, appears not to be concerned in this "hydration", for otherwise one would expect an increase in the permeability to apolar substances as well as to polar.

DISCUSSION.

A completely new factor in the problem of the cold resistance of plants has been discovered, one which is apparently more closely related to hardiness than any of those to which cold resistance has been ascribed (e.g. osmotic pressure, sugars, hydrophily). This factor - cellular permeability - seems to involve a widening of the pores of the plasma membrane during the hardening-off period, for there is no change in permeability to apolar substances. Thus plants can be classified in the following way:

(1) Non-hardy. Cells slowly permeable to non-electrolytes (e.g. thiourea), and no increase occurs on exposure to low temperatures.

(2) Semi-hardy. Cells slowly permeable to non-electrolytes (thiourea or urea) but this, as well as water permeability, increases on hardening.

(3) Hardy. Slowly permeable to non-electrolytes (e.g. urea) and electrolytes in the unhardened state; very rapidly permeable to these and to water when in the hardened condition.

A similar relationship holds with regard to osmotic pressure. Thus the non hardy plants remain unchanged in cell sap concentration on exposure to low temperatures, whereas both the hardy and the semi-hardy show marked increases. The indications are that osmotic pressure and permeability are independent factors, i.e. an increase in the one does not cause an increase in the other.

It is interesting to note the high permeability found by Ruhland and Hoffman (1925) for *Beggiatoa mirabilis*. Thus, by our formula, urea penetrates at the rate of 98 mols per hr. This extraordinary rapidity is only partly explainable by the large surface. The fact seems significant when it is remembered that bacteria are, apparently unaffected by low temperatures.

It is important to see how this factor fits in with the prevalent theories. The accepted idea that cold injury can be prevented only by reducing the amount of ice formation (Maximov 1929) can find no use for an increased permeability rate. As previously mentioned, however, Lidforss applied Gorke's salting-out hypothesis and suggested that if a cell is highly permeable the salts might diffuse out and so prevent the precipitation of proteins. Attempts to prove this have produced only negative results. Sections of hardy plants taken in mid-winter were found to be absolutely unchanged in osmotic pressure after several hours in distilled water. More extensive tests were made by Howat (1935). He placed the living bark of apple branches (collected during winter) in distilled water for 2 - 3 days and analysed the extract. After this time he was unable to detect any substance in the water, either organic or inorganic, except for a faint unidentifiable precipitate with lead acetate. This lack of exosmosis is rather surprising in view of the very high permeability. However, there are many

similar unexplainable problems in the field of permeability, including even the simplest everyday occurrences, such as the transportation of sugars to which the cell is completely impermeable under experimental conditions.

It must be admitted, then, that the evidence is all opposed to Lidforss' hypothesis. Yet there are some interesting findings recorded by other investigators which may perhaps be correlated with our results. Maximov (1912, 1914) showed that the cells of tender plants which are normally killed by the first touch of frost can resist very low temperatures if frozen in solutions. The solutes used need only one property - that their eutectic points be below the temperature at which the sections are frozen. He ascribed this partly to the decrease in ice formation, and partly to a protective action of the solutes on the plasma membrane preventing mechanical injury by the ice.

Åkerman (1927) repeated and confirmed Maximov's results but proved his second explanation to be wrong. He showed that the protective action occurs only on plasmolysis. Iljin (1927) independently found that the same technique conferred drought resistance on plant cells.

Following these and other results, Iljin (1933) has built up his own theory of cold injury. He has revived an idea long thought disproved by most investigators. Iljin (1934) admits that Sachs' hypothesis of the occurrence of death during thawing instead of freezing, has been opposed by the negative

results of many workers. It has long ago been definitely and repeatedly proved (Wartenberg 1933) that death can occur while the plant is still frozen, and the vast majority of investigators have failed to produce any effect on the amount of frost injury by varying the rate of thawing. The few who were able to find any differences under laboratory conditions (Åkerman 1927, Hildreth 1926, Janssen 1929, etc.) considered it of little if any practical importance.

It is interesting to note Åkerman's viewpoint. He believes that between certain narrow limits slow thawing can protect a plant. Below these limits no injury occurs, no matter how rapid the thawing, and above them the plant is killed by the cold and so cannot be revived no matter how slow the thawing. Janssen's results indicate the same thing. Wheat seedlings which he was able to kill during mid-winter by means of sufficiently low temperatures followed by rapid thawing, were removed from a plot which subsequently proved to be uninjured by the winter.

Now, although Iljin admits that death can be caused by cold, he believes that the rate of thawing is the more important factor. It must be admitted that he produces some remarkable experimental results to substantiate this belief. He was able to freeze the cells of tender plants at even -80° C without injuring them, provided that the rate of thawing was sufficiently slow. Previously to his experiments,

it is doubtful whether any of the investigators on cold resistance would have considered this possible.

His method of slow thawing includes the use of a strong sugar solution to prevent the cell from taking up water too rapidly. This, then, is essentially the same method as used originally by Maximov, except that Iljin has shown it to be necessary only during the period when the cell is thawing out. If it thaws in the normal way, its wall immediately expands to full size. But the protoplasm is unable to keep pace with it, and a pathological "pseudoplasmolysis" occurs, this eventually resulting in death, either before or after it is able to reach its expanded wall. Now, if the protoplast were sufficiently permeable to water, would not this injurious "pseudoplasmolysis" be prevented or at least reduced, since the cell would be able to come rapidly to equilibrium? Similarly, in the case of rapid freezing, which Iljin (and others before him) believes causes death by intracellular ice formation, this can occur only because the sudden drop in temperature does not allow enough time for the water to move out of the cell into the intercellular spaces, and consequently, permits intracellular ice formation.

In this connection, it is interesting to note that the temperature coefficient of cellular permeability to water is 2 - 3 (de Haan 1933). At sub-zero temperatures, then, the passage of water through the protoplasm will be greatly slackened, and the need for an increased permeability rate will be greater.

But it must be admitted that it is hardly plausible to generalise from Iljin's results. Because cells can be made to resist temperatures of -80°C for a few hours is no reason to suppose that the same method will be effective over longer periods. It has long been known that cold injury involves a time-factor. In nature it is hardly possible that the rate of thawing can be the determining factor in frost injury. Thus, during the month of February 1934, more injury occurred than in any previous month since 1917. Yet every day was so cold as to register below 0°F for at least part of the 24 hours. Obviously, there was no thawing during this period, and the injury was due solely to the freezing.

In cases where injury occurs relatively rapidly, then, it appears caused by the slow permeability of the cell to water, either during freezing or thawing. Under these conditions, an increased permeability rate can be readily understood as a protection against injury. In nature, however, severe injury is often if not usually caused directly by the cold and involves a time-factor. In this case it is difficult to see how an increased permeability rate can have any effect. Similarly, in what way solute permeability can be of any significance in the resistance of a plant to frost is, for the present, unanswerable.

The existence of other possible explanations cannot be denied. But until the cause of cold injury is definitely established, it will, of course, be impossible to state with any degree of assurance how a plant can be protected from it. That

an increased permeability to water or solutes or both does increase the plant's resistance to cold appears beyond doubt in the light of the results here reported, and this is all that can be said with any certainty.

SUMMARY.

Plants hardened both artificially and naturally were examined in the resistant and unresistant condition. The cellular changes accompanying cold resistance were found to be as follows:-

(1) An increase in the osmotic pressure of the cell sap (determined plasmolytically) amounting to 20 - 25% in those artificially hardened, and to as much as 100% in the naturally hardened.

(2) The question of change in hydrophily was approached by a new method - measurement of the protoplasmic layer. In the onion a slight decrease in the protoplasm layer and, therefore, in its hydrophily, occurred on hardening. In apples there was no change but in the presence of the decrease in osmotic pressure, a slight drop in hydrophily on loss of hardness is indicated. These opposing results are presumably within the experimental error so that if any change in hydrophily does exist, it must be small.

(3) (a) Preliminary investigations were made on the water permeability of cells at different stages of deplasmolysis. This was with the object of testing the results found by de Haan, i.e. that the permeability rate greatly increases on deplasmolysis due to the hydration of the protoplasm. Cells freed from their walls were used to eliminate complications caused by a hampered diffusion, and to enable a simple computation of volume and area. With these improved methods

it was found possible to disprove de Haan's statement and to show that the change in true permeability on deplasmolysis is, at best, very slight. This appears to indicate that a change in the hydration of protoplasm has little if any effect on the permeability of water.

(b) The most conspicuous cellular change found on hardening was an increase in the permeability - to water and non-electrolytes in the case of those artificially hardened, and to these as well as to electrolytes in the case of the more resistant, naturally hardened plants. The changes were extraordinarily large, the hardened cells being in some cases twenty times as permeable as when unhardened.

(c) Though the increase in osmotic pressure and permeability usually occurred simultaneously, they are not cause and effect. Thus, sugar feeding allowed an increase in osmotic pressure without any change in permeability. On the other hand, there was a sharp drop in the permeability to electrolytes during spring without any change in osmotic pressure.

(d) The permeability rate of apolar substances was unaffected by hardening, so that the process appears to involve a widening of the pores of the plasma membrane.

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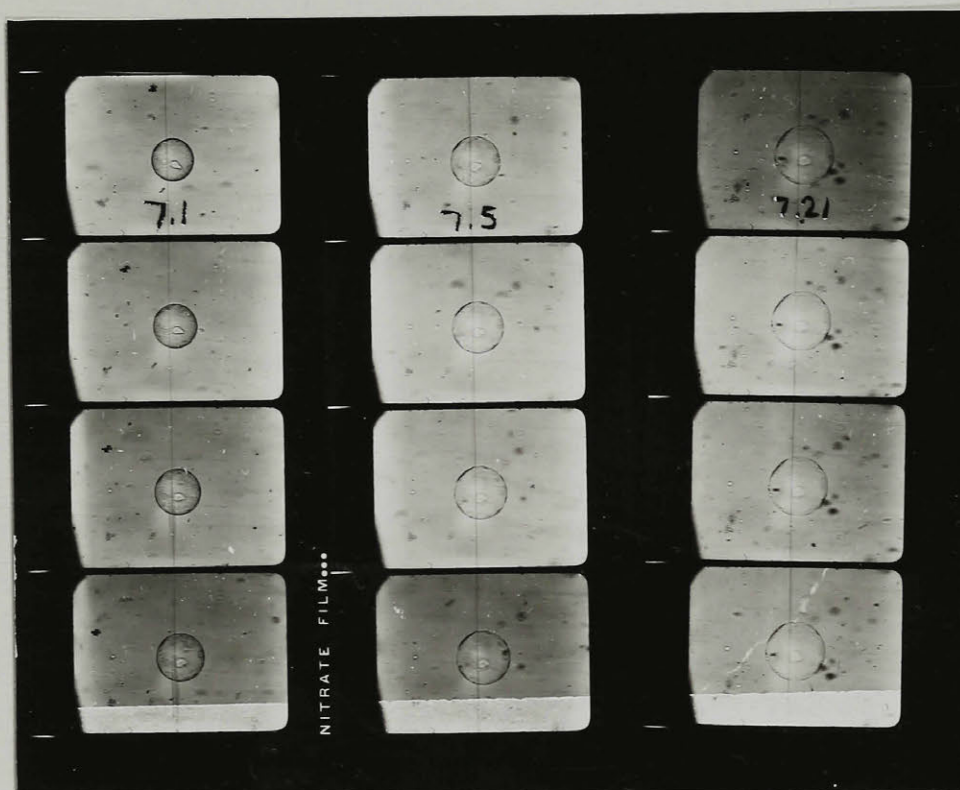


PLATE 1. Deplassmolysis of free onion cell (see Table 7).

