THE UNIVERSITY OF MINNESOTA

GRADUATE SCHOOL

Report

of

Committee on Examination

This is to certify that we the undersigned, as a committee of the Graduate School, have given Robert Newton final oral examination for the degree of Master of Science . We recommend that the degree of Master of Science be conferred upon the candidate.

Minneapolis, Minnesota

May 25 1921

A. S. Gorton
Chairman

H. K. Hayes

L. I. Knight

THE UNIVERSITY OF MINNESOTA

GRADUATE SCHOOL

Report

of

Committee on Thesis

The undersigned, acting as a Committee of the Graduate School, have read the accompanying thesis submitted by Robert Newton for the degree of Master of Science. They approve it as a thesis meeting the requirements of the Graduate School of the University of Minnesota, and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science.

L. S. Ettor
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A COMPARATIVE STUDY OF

WINTER WHEAT VARIETIES WITH ESPECIAL REFERENCE

TO WINTER KILLING.

By Robert Newton.

A THESIS

Submitted to the Graduate School of the University of Minnesota in partial fulfilment of the requirements for the degree of

Master of Science.

St. Paul, Minnesota.

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A COMPARATIVE STUDY OF WINTER WHEAT VARIETIES WITH ESPECIAL REFERENCE TO WINTER KILLING.

By Robert Newton.

INTRODUCTORY.

Winter wheat, where it can be safely grown, usually outyields spring varieties quite markedly, but unfortunately it is much more restricted in distribution, due to its liability to winter-killing. Much progress has been made with this and other crops in breeding for cold resistance by empirical methods. It would appear, however, that greater and more certain progress should be made if the nature of cold resistance in plants were well understood. This subject has long been of interest to physiologists, and in its practical applications is of widest importance. The northern limit of profitable growth of our staple crops marks also the limit of profitable exploitation of our agricultural lands. The southern farmer has likewise to meet the problems of the frost killing of fruit buds and flowers and the more tender winter cereals.

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HISTORICAL.

The progress of our knowledge of the nature of cold resistance and frost effects has been reviewed quite fully at different times by Abbe (1895), Blackman (1909), Chandler (1913) and others. Of the earlier investigations it will be sufficient to note here in their order the most significant.

The theory of Duhamel and Buffon (1737) that death from cold was due to rupturing of the cell walls by expansion on ice formation is of historical interest. It was almost a century later that Goeppert (1830) found ice formation to occur in the intercellular spaces. Sachs (1860) showed this to be the usual occurrence, and developed the view, now generally considered erroneous, that disorganization took place on thawing, and might be prevented by warming very slowly, allowing time for reabsorption of the water by the cell.

Later Müller-Thurgau (1886) proved that this ice formation in the tissues was necessary for freezing to death, and concluded that death was due to the consequent desiccation of the protoplasm. This hypothesis received support from Matruchot and Molliard (1901) who demonstrated the identity of the modifications in cell structure produced by frost, plasmolysis and desiccation. The work of Greely (1901) supplied similar evidence. Mez (1905) opposed the theory of death by desiccation, since his investigations indicated that all solutes crystallize out at a temperature not lower than -6°C. He concluded that cold desiccation must therefore be complete at this temperature, and cannot explain injury to plants which resist much lower temperatures. He advanced instead the theory of a fatal minimum temperature for each plant.

Gorke (1906) showed another important effect of the witdrawal of water, namely, the precipitation of certain proteins by the increasing concentration of the cell sap, aided by its increasing acidity on cooling. He also showed that the precipitation occurred at varying temperatures for plants of varying degrees of hardiness. This was ascribed by Schaffnit (1910) to the splitting in varying degrees during the hardening process of complex proteins into simpler, less readily precipitated forms.

Lidforss (1907) found most hardy plants to have their starch reserves converted to sugar during the winter, and believed this an adaptation for cold resistance, the sugar having a protective action in preventing the precipitation of the proteins. Schaffnit (1912) tested the effect of adding sugars and various other substances to plant saps and to egg albumen solution, and was able to modify very greatly the precipitation by freezing. Equally striking results were secured by Maximov (1912) in increasing the hardiness of sections of red cabbage and Tradescantia by freezing them in solutions of either organic or inorganic substances, provided these were non-toxic and had a low eutectic point.

Recent Progress.

Recent investigations have dealt mainly with the hypotheses noted above, extending them in several important respects. Attempts have also been made to determine the correlation of various chemical, physical, physiological and morphological characters with apparent frost hardiness.

Several workers have drawn attention to the possible importance

of the fact that plant sap is contained in cells of capillary dimensions. D'Arsonval (1901) estimated the osmotic pressure in very small cells at 1000 atmospheres, and notes that by the application of increasing pressure the solidification point of water can be lowered indefinitely. In buds with small cells, dense tissues and meager water content, Wiegand (1906c) found no ice at -18°C., though in most buds it was present in large quantity. He concluded that the degree of cold necessary to cause the separation of ice is proportional to the force which holds the water in the tissues. Lewis and Tuttle (1920) reported that living leaves of Pyrola wraped around the bulb of a mercury thermometer undercooled to -32.1°C. before ice formation took place. On the other hand, it has been a universal observation since the time of Goeppert (1830) that ice may form in the tissues without injury to hardy plants, so that undercooling is not of itself a sufficient explanation of hardiness.

The water content of tissues is related to structure, and it has been shown by several investigators (Schaffnit, 1910; Sinz, 1914; Beach and Allen, 1915; Akerman, 1917; Johnston, 1919; Rosa, 1919) that dry matter content is directly correlated with hardiness. Sinz and Beach and Allen noted also the importance of structures resisting desiccation, while Pantanelli (1918) found injury from frost to be always proporionate to loss of water from the tissues, even when freezing was done in a saturated atmosphere.

Between the concentration of the cell sap and winter hardiness, Ohlweiler (1912) and Chandler (1913) found a direct relationship; Salmon and Fleming (1918), working with winter cereals, found none.

Pantanelli (1918) partly reconciled the conflicting evidence by reporting a relationship in some crops and none in others, including wheat. Probably the sap of all plants increases in concentration during the hardening process, but not necessarily in proportion to the degree of hardiness attained. However, the earlier evidence as to the importance of the accumulation in the sap of substances of a protective nature, especially sugars, has received further support. Gassner and Grimme (1913) and Akerman (1917) reported that hardy varieties of winter wheat and other grains were richer in sugar, the differences between varieties corresponding to differences in degree of hardiness. Pantanelli (1918, 1919) found that sugar was rapidly used up during exposure to freezing temperatures, and that hardiness was related to the quantity of sugar retained by the plant. The association of sugar accumulation with hardening by cold has been pointed out again by Rosa (1919) and Coville (1920).

On the other hand, Harvey (1918) found that cabbages acquired hardiness on 5 days exposure to +3°C., before any great change occurred in the carbohydrate equilibrium. He believes the principal effect of the hardening process is a change in the constituents of the protoplasm, as indicated by an increase in the amino-acid content, and on freezing the sap by less precipitation of the proteins. He measured the increase in hydrogen-ion concentration of the sap on cooling, and was able to produce the same relative precipitation of proteins by adding equivalent quantities of acid. However, the ease of precipitation of proteins by freezing apparently cannot always be taken as an index of hardiness, since Chandler (1913) was unable to find any difference in this respect between the sap of

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tender and hardy twigs of fruit trees. Again, it must be remembered that only a small fraction of the total proteins is present in the expressed sap, and at most only 31.2 percent of this is reported by Harvey as precipitated by freezing the juice of unhardened cabbage. To take this as an index of the behaviour of the proteins within the cell may be too sweeping a conclusion.

There is some evidence that stability of the dormant condition may be an important protective adaptation. Lidforss (1907) noted that a succession of warm days caused regeneration of starch from sugar, with an increase in susceptibility to cold. Chandler (1913) found some varieties of peaches to have a longer rest period than others and to be started into growth more slowly by warm periods in the winter. Evidently varieties which can maintain continuous dormancy during the danger period must have a distinct advantage.

Our present concept of the causes of winter -killing may be briefly summarized. Without doubt, the ultimate cause of death by freezing must be the disorganization of the protoplasm. Irreversible coagulation or precipitation of the colloidal protein constituents may be caused by increase in concentration of electrolytes in the cell sap on withdrawal of water, or by increase in acidity, or by both factors acting together. The critical minimum temperature necessary to bring this about must be profoundly modified by rate of cooling, especially if this be slow enough to give time for the hardening process, and by the presence of substances which protect the proteins from precipitation. Splitting of the proteins during hardening may be a protective adaptation. Since the fundamental feature of the disturbance produced by freezing is withdrawal of

water from the cell, intracellular adaptations to resist desiccation must be of prime importance.

EXPERIMENTAL.

The Problem.

The present study seeks to establish a chemical or physico-chemical measurement of hardiness for winter wheat varieties. A number of varieties originated or selected by the Department of Plant Breeding of the University of Minnesota, and known to vary considerably in hardiness, were compared with reference to the physical constants of the cell sap, the content of amino nitrogen, water-soluble nitrogen and total nitrogen, and the content of sugars and starch. All material used was grown in field plots under normal conditions. Since it was desired to compare the varieties in the hardened condition rather than to study the hardening process, collections were not made until after the advent of freezing weather.

A preliminary study of physical constants was carried out with eight varieties. Subsequent study was confined to four of these, two hardy and two tender. One variety, Minhardi, was collected from two plots some distance apart, and these are reported separately as the effect of location was quite marked. Table I indicates the hardiness of the varieties used as determined by survival under field conditions.

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Methods.

Collection of Samples.- All the samples of one series were collected from the field the same afternoon, though with the exception of the first series the leaves were frozen solid when collected, so that changes due to vital activities would be very slight. The plants were growing in rows, which were carefully gone over for the removal of dead leaves before taking the samples. For the collection of November 12,1920, it was necessary first to brush off a light covering of snow. As the leaves were cut, they were thrown on a wire screen for the removal of adhering bits of dirt and ice, then transferred at once to tight glass containers. Samples of approximately 100 grams were collected in duplicate, one lot for the study of physical constants, the other for analysis for nitrogen and carbohydrates. All samples were kept frozen until used.

Physical Constants.- The depression of the freezing point of the first collection was determined by the thermoelectric method, the accuracy of which has been shown by White (1910). The convenient arrangement of apparatus illustrated by Harvey (1919,fig.1) was used. The leaves were packed into a section of thin-walled glass tubing 2 cm. long, in which they were held in place by a small rubber band, the thermocouple then being inserted in the center. Undercooling seldom ammounted to more than 2°C. and was corrected for in the usual way. By this method duplicates often varied as much as 0.03°C., and in later collections it was abandoned in favor of the standard Beckmann method, by which it was always possible to obtain checks agreeing within 0.01°C.

The work of Dixon and Atkins (1913), extended by Gortner,

Lawrence and Harris (1916), has shown the necessity of rendering the cell membranes permeable by freezing the tissue previous to sap extraction in order that a representative sample may be obtained. However, having regard to the observation of Harvey (1918) that freezing permanently lowered the hydrogen-ion concentration of cabbage juice, and (1920) that on the other hand a certain amount of dilution did not affect this value, the samples of the first collection were not frozen before expressing the sap, as it was desired to study particularly the relationship of this constant to hardiness. The following comparisons of sap expressed from duplicate samples with and without previous freezing indicated that for wheat at least Harvey's observation does not hold true.

Variety	Not Frozen	Frozen
	pH	pH
Minhardi	6.580	6.376
Buffum	6.465	6.289
Padui	6.475	6.289

Therefore in later collections preliminary freezing of the tissues was carried out, and the expressed sap used for all constants studied.

The technique of Gortner and Harris (1914) was followed in the main. The rubber-stoppered bottles containing the samples were packed in a slushy mixture of pulverized ice and salt in an earthenware jar, which fitted snugly inside a well-insulated "fireless cooker". In this condition the contents remained frozen solid until required for use, but never in any case for less than twelve hours. For the freezing mixture, common salt was used at first,

and later calcium chloride. To thaw the samples, the bottles were placed under running water, then rinsed with distilled water and wiped dry before opening. The leaves were folded in pieces of strong cotton previously boiled in 3 changes of distilled water and dried free from dust, and the sap expressed either in a hydraulic press under 400 atmospheres pressure, or in a large hand screw press with a small steel cup which enabled heavy pressure to be brought to bear. The parts of the press with which the juice came in contact were kept coated with a thin layer of paraffin wax.

The depression of the freezing point was first determined with a Beckmann apparatus. Then the conductivity was measured with a Wheatstone bridge, using a Freas conductivity cell, and finally the hydrogen-ion concentration by means of standard Leeds and Northrup potentiometric equipment. Both of the latter determinations were carried out at 25°C. in a constant temperature room. Usually the work on any particular sample was completed within an hour of expressing the juice.

Preparation of Samples for Analysis.- As the samples for analysis were collected in the field they were placed directly in tared one-liter erlenmeyers with rubber stoppers. They were frozen when collected, and kept in that condition overnight. Immediately after thawing in the laboratory next day, samples for the determination of dry matter were weighed out; then in addition, sufficient material was removed to leave exactly 100 grams in each flask. To this was added 1.5 grams of pure precipitated calcium carbonate for the neutralization of plant acids, and sufficient 95 percent alcohol to make the final concentration 80 percent after allowing

for the dilution due to water in the leaves. The samples were then boiled half an hour under reflux condensers, and put away tightly stoppered until a convenient time for analysis. The procedure from thawing to boiling was carried out with the utmost expedition.

The advantage of using calcium carbonate as noted above has been discussed by Spoehr (1919). Davis, Daish and Sawyer (1916) have pointed out the necessity for rapid destruction of enzymes. In this connection the present study afforded opportunity for some observations of interest. An additional quantity of one variety was collected for experimentation in methods. Part of this was left 5 days in an ice chest, and was then put through the regular preparative and analytical procedure. In Table II the results are compared with those for the same material disposed of promptly after collection. When it is considered that the tissues were not crushed or injured to any appreciable degree, the effect of enzyme action even in such cold storage conditions is very striking.

Dry Matter.- Duplicate or triplicate samples of approximately 5 grams of green material were dried to constant weight in a vacuum oven at 98°C.

Total Nitrogen.- The total nitrogen was determined by the Kjeldahl-Gunning-Arnold method, using the residues from the dry matter determinations.

Extraction of Sugars and Soluble Nitrogen.- Large extractors of the Soxhlet type were made by drilling a small hole close to the bottom of a 750 c.c. wide-mouthed bottle, and fitting in a glass siphon tightly by making a ground glass joint or by wedging it with

a collar of rubber tubing. The bottle was closed with a large rubber stopper, in which were fitted two reflux condensers and a bent glass tube leading to the distilling flask below. The siphon also passed through the stopper of the distilling flask. The samples were transferred to one of these extractors and extracted with 80 percent alcohol on a steam bath for 30 hours, by which time the Molisch a-napthol test on the alcohol in the extractor always became entirely negative. In transferring material from one container to another, the emptied container was rinsed not less than 3 times with hot alcohol.

The extract was decanted off the small quantity of sediment (chiefly calcium salts) which collected, the last portion then being filtered and thoroughly washed with hot alcohol. The whole (extract and washings) was concentrated in the apparatus illustrated by Van Slyke (1911, fig. 1) under a pressure of less than 30 mm., with the distilling flask in a water bath at a temperature of 40° to 50°C. When reduced to a volume of 75 to 100 c.c., about 200 c.c. of distilled water was added and the solution reconcentrated to get rid of the last traces of alcohol. This precaution was found necessary since the presence of alcohol affected the subsequent determination of amino nitrogen. The reconcentrated extract was then transferred to a 250 c.c. volumetric flask by filtering through a pad of cheesecloth in a small funnel, making it nearly to volume by several successive washings of the distilling flask with small portions of boiling water. A pad of cheesecloth 4 layers thick was found the most satisfactory filter for removing the solid particles of chlorophyll which separated out. All other materials tried

clogged at once. The extract was then cooled to room temperature and made to volume.

All volumetric flasks and pipettes used throughout the analyses were standardized in true cubic centimeters at 20°C.

Amino Nitrogen.- The amino nitrogen was determined by the usual Van Slyke apparatus, using 10 c.c. portions of the extract. These were filtered for removal of fine particles which had escaped the cheesecloth filter. Since preliminary trials had given a somewhat higher yield when deamination was continued for 30 minutes instead of the usual 5 minutes, the former period was adopted. Of this time, shaking was done during the first minute and the last 2 minutes.

Water-Soluble Nitrogen.- The total water-soluble nitrogen was determined by the Kjeldahl-Gunning-Arnold method, using 10 c.c. portions of the extract, filtered as for amino nitrogen.

Clearing Extract for Sugar Analysis.- After the portions required for nitrogen determinations had been removed, the remainder of the extract was cleared with dry powdered lead acetate. The dry defecation method, first proposed by Horne (1904), has the advantage not only of largely eliminating the error due to the volume of the precipitate, but also of obviating the necessity of making to volume a second time. The lead acetate was added in small quantities at a time, successive small portions of the extract being filtered off and tested for completeness of precipitation. The solution was then filtered through a dry filter paper and deleaded with a minimum quantity of powdered sodium oxalate, following the same technique as for clarification. A second filtration through a

ary filter paper completed the preparation of the extract for sugar analysis.

Reducing Sugars.- The reducing sugars were determined by the very excellent method recently devised by Schaffer and Hartmann (1921), in which the cuprous oxide is determined directly by iodo-thiosulfate titration in the presence of an excess of potassium oxalate. The oxalate inhibits the reaction of cupric ions with soluble iodides. The reduction of Fehling's solution is carried out under the standard conditions prescribed for Munson and Walker's method (Official Methods, A.O.A.C.) and the sugar corresponding to the quantity of cuprous oxide read from the tables. By connecting the gas burners with water manometers, and carefully calibrating them in conjunction with the flasks used for the reductions, it was found possible to keep within 10 seconds of the 4 minutes prescribed for bringing the solution to boiling.

The thiosulfate was standardized by an adaptation of the simple method proposed by Peters (1912). About 100 mg. of copper foil, previously cleaned with dilute nitric acid, was weighed accurately, placed in a 200 c.c. erlenmeyer, and dissolved in 2.5 c.c. of a mixture of equal volumes concentrated nitric acid and water. After heating until brown fumes were no longer apparent, 50 c.c. of water and a knife-point of pure powdered talcum were added, and the mixture boiled vigorously for 10 minutes. It was then cooled to room temperature, 2.6 c.c. of concentrated sulfuric acid was added, the mixture recooled, 5.0 c.c. of a saturated solution of potassium iodide added, and the whole titrated with thiosulfate. Triplicate determinations by this method varied less than 0.01 mg. copper per

c.c. thiosulfate.

The accuracy of this sugar method was tested with pure dextrose obtained from the Bureau of Standards. Duplicate portions of 100 mg. and of 150 mg. were weighed out, dissolved in water and carried through the analytical procedure, with the following results:

Dextrose Present	Dextrose Recovered
100	99.8
100	99.6
150	149.4
150	149.2

The majority of the sugar determinations made fell within the limits of these quantities. Aliquots of 10.c.c. of the cleared extract were used for the reductions.

Sucrose.- The sucrose was determined by the increase in reduction on inversion of the cleared extract. For inversion the citric acid method of Davis and Daish (1913) was employed. Comparative tests with invertase and with the official hydrochloric acid method gave results varying by less than 0.5 percent. It appears, therefore, that sucrose was the only disaccharide present.

Starch.- A qualitative test for starch, with the usual iodine-potassium iodide solution, was made on the dried, ground residue from the alcohol extraction.

TABLE I.- PERCENTAGE SURVIVAL UNDER FIELD CONDITIONS OF VARIETIES STUDIED.(1)

Variety	1918	1919	1921		Classification.
	U.Farm, Minn. Waseca, " Gr.Rapids, "	Moccasin, Mon. Dickinson, N.D. Fargo, " Mandan, " Brookings, S.D. Highmore, " Archer, Wy. Akron, Col. Ashland, Wis. Saskatoon, Can.	University Farm. (Plots used in present study.) Ave. 4 Variety Plots	Ave. 4 Breeding Plots	
	Ave.%	Ave.%	%	%	
Turkey	27	-	-	80	Tender
Kanred	(2)	7	35	78	Tender
Minhardi	71	14	99	97	Very Hardy
Buffum	(3)	10	88	84	Very Hardy
Odessa	62	9	83	87	Very Hardy
Padui	74	7	80	90	Very Hardy
Minturki	58	8	95	91	Hardy
Red Rock	(4)	-	2	3	Tender

(1) Data for 1918 and 1919, and classification of varieties supplied by Dept. of Plant Breeding, University of Minnesota.

(2) Kanred killed as badly as Turkey in 1917 and 1918 at University Farm, when other wheats as Minhardi lived and produces good crops.

(3) Buffum is recognized as a very hardy wheat by the U.S. Dept. of Agriculture.

(4) Red Rock killed worse than other hardy wheats in 1919 variety tests.

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TABLE II.- CHANGES IN AMINO NITROGEN, TOTAL SOLUBLE NITROGEN AND SUGARS OF WINTER WHEAT LEAVES DURING FIVE DAYS STORAGE IN ICE CHEST.

	Fresh Leaves % Green Wt.	Stored Leaves % Green Wt.	Percentage Change
Amino Nitrogen	0.050	0.067	+34.0
Water-Soluble Nitrogen	0.157	0.237	+51.0
Reducing Sugar as Dextrose	3.286	4.174	+27.0
Invert Sugar as Sucrose	4.574	2.196	-52.0
Total Sugar as Dextrose	7.943	6.402	-19.4

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TABLE III.- PHYSICAL CONSTANTS OF SAP OF WINTER WHEAT LEAVES.

Collected October 29, 1920.*

Variety	Classification	△	P	pH
Turkey	Tender	2.08	24.99	6.360
Kanred	Tender	1.97	23.68	6.632
Minhardi,a	Very Hardy	2.11	25.35	6.528
Minhardi,b	Very Hardy	2.15	25.83	6.580
Buffum	Very Hardy	2.21	26.55	6.465
Odessa	Very Hardy	2.09	25.11	6.457
Padui	Very Hardy	2.05	24.63	6.475
Minturki	Hardy	2.07	24.87	6.536
Red Rock	Tender	2.02	24.28	6.632

* △ determined directly in tissue by thermoelectric method; pH determined on sap expressed after grinding tissue without previous freezing.

TABLE IV.- PHYSICAL CONSTANTS OF SAP OF WINTER WHEAT LEAVES.

Variety	△	P	K x 10^3	pH
Collection of November 12,1920.*				
Turkey	2.26	27.15	11.86	5.840
Kanred	2.35	28.23	14.31	6.201
Minhardi,a	2.56	30.74	13.25	6.078
Minhardi,b	2.44	29.30	14.87	6.086
Buffum	2.68	32.17	14.83	5.980
Collection of December 9,1920.**				
Turkey	1.99	23.92	14.94	5.486
Kanred	1.42	17.08	14.04	5.557
Minhardi,a	1.83	22.00	13.93	5.548
Minhardi,b	1.69	20.32	15.02	5.733
Buffum	1.60	19.24	13.35	5.593

* Sap expressed under pressure of 400 atmospheres after freezing tissues.

** Sap expressed by large hand screw press after boiling tissues in pressure flasks.

TABLE V.- THE ROLE OF SUGARS AND ELECTROLYTES IN OSMOTIC PRESSURE.

Variety	P	$K \times 10^3$	P_s	$P - P_s$	$\frac{P - P_s}{K \times 10^3}$
Collection of November 12,1920.					
Turkey	27.15	11.86	11.50	15.65	1.32
Kanred	28.23	14.31	9.51	18.72	1.31
Minhardi,a	30.74	13.25	11.54	19.20	1.45
Minhardi,b	29.30	14.87	10.51	18.79	1.26
Buffum	32.17	14.83	12.32	19.85	1.34
Average	29.52	13.82	11.08	18.44	1.34
Collection of December 9,1920.					
Turkey	23.92	14.94	7.49	16.43	1.10
Kanred	17.08	14.04	5.40	11.68	0.83
Minhardi,a	22.00	13.93	7.91	14.09	1.01
Minhardi,b	20.32	15.02	6.59	13.73	0.91
Buffum	19.24	13.35	7.27	11.97	0.90
Average	20.51	14.26	6.93	13.58	0.95

TABLE VI.- NITROGEN OF WINTER WHEAT LEAVES.

Variety	Dry Matter Content	Amino Nitrogen		Water-Soluble Nitrogen		Total Nitrogen	
	%	Green %	Dry %	Green %	Dry %	Green %	Dry %
Collection of November 12,1920.							
Turkey	37.54	0.054	0.14	0.126	0.34	1.394	3.71
Kanred	32.89	0.045	0.14	0.129	0.39	1.242	3.78
Minhardi,a	37.99	0.051	0.13	0.161	0.42	1.416	3.73
Minhardi,b	33.51	0.048	0.14	0.153	0.46	1.228	3.66
Buffum	35.83	0.046	0.13	0.141	0.39	1.184	3.30
Collection of December 9,1920.							
Turkey	29.65	0.049	0.17	0.159	0.54	1.179	3.98
Kanred	25.48	0.050	0.20	0.134	0.53	0.936	3.67
Minhardi,a	31.74	0.058	0.18	0.192	0.60	1.276	4.02
Minhardi,b	28.20	0.065	0.23	0.175	0.62	1.049	3.72
Buffum	29.17	0.058	0.20	0.149	0.51	0.938	3.22

TABLE VII.- SUGAR CONTENT OF WINTER WHEAT LEAVES.

Variety	Dry Matter Content	Reducing Sugar as Dextrose		Invert Sugar as Sucrose		Total Sugar as Dextrose	
	%	Green %	Dry %	Green %	Dry %	Green %	Dry %
Collection of November 12,1920.							
Turkey	37.54	2.992	7.97	5.301	14.13	8.397	22.37
Kanred	32.89	3.090	9.39	3.900	11.86	7.055	21.45
Minhardi,a	37.99	3.237	8.52	4.797	12.63	8.127	21.39
Minhardi,b	33.51	3.335	9.95	4.351	12.98	7.760	23.16
Buffum	35.83	3.308	9.23	5.799	16.19	9.219	25.73
Collection of December 9,1920.							
Turkey	29.65	2.347	7.92	3.598	12.14	6.004	20.25
Kanred	25.48	1.840	7.22	2.660	10.44	4.540	17.82
Minhardi,a	31.74	2.579	8.13	3.356	10.57	5.991	18.88
Minhardi,b	28.20	2.027	7.19	3.391	12.03	5.472	19.40
Buffum	29.17	2.163	7.42	3.772	12.93	5.997	20.56

DISCUSSION.

The depression of the freezing point, the corresponding osmotic pressure, and the hydrogen-ion concentration of the sap of the samples collected October 29 are given in Table III. The osmotic pressures recorded are based on the freezing point data, use being made of the published tables of Harris and Gortner (1914). The hydrogen-ion concentration is expressed in terms of pH value as read from the tables of Schmidt and Hoagland (1919). The classification of varieties given in Table I is repeated to facilitate comparison. It will be seen that in this collection at least there are no significant variations in these constants which could be correlated with the relative hardiness of varieties.

The same absence of correlation in physical constants holds true for the collection of November 12, reported in Table IV. It may be noted, however, that the concentration of the sap had increased somewhat in the varieties used. In the collection of December 9, included in the same table, all varieties exhibit a falling off in the depression of the freezing point and corresponding osmotic pressure, probably due in part to simple dilution of the sap, as the moisture content of the tissue was greater. One variety, Kanred, fell off in this respect appreciably more than the rest. It is perhaps noteworthy that this variety winter-killed considerably more than the others during the year of this test.

An unexpected difficulty was encountered in expressing the sap from the samples collected December 9. The samples were frozen by

the method already described, using a freezing mixture of pulverized calcium chloride and snow mixed in the proportions which should theoretically give a cryohydrate mixture with a corresponding temperature of -54.9°C. After 7 hours freezing, the samples were thawed under running water, and refrozen for a period of 11 hours. They were thawed again under the tap. Even after this treatment it was found impossible to express more than 2 to 3 c.c. of juice from 100 grams of material under 400 atmospheres pressure. The data of Table VI show that these samples contained a lower percentage of dry matter than did the earlier collection, consequently the failure to express the sap was not due to lack of moisture in the tissues but apparently to a failure to break down the colloidal complex of the protoplasm and to increase the permeability of the cell by freezing. Since the permeability of the tissues had not been affected by freezing, it was decided to attempt to destroy the colloidal complex and increase permeability by placing the material in a closed pressure flask and heating it in a boiling water bath. The juice was then expressed as readily by the hand press as by the hydraulic press, 30 to 40 c.c. (about the usual amount) being collected from each sample.

These observations give rise to some very important considerations. In the first place, the wheat plants were apparently not killed by exposure to temperatures lower than normally obtain in many places where they suffer severe winter=killing. The specific temperature must be only one of a number of factors involved. It is also apparent that the hardening process continued long after the advent of freezing weather, as this difficulty was not met with

in the collection of November 12. Further, and contrary to the findings of a number of workers, it was not in this case associated with an increase in the dry matter content, since as already noted the water content was greater in the collection of December 9. Nor was it associated with an increase in sugar content; this value had decreased, as will be seen later.

Most significant is the evident tenacity with which the hardened tissue grips its water content. Wiegand (1906a) noted that as the temperature falls the quantity of water separating in the form of ice becomes constantly less and less. In another paper (1906b), the same author develops the theory that the passage of water from the cell during freezing is due to an equalizing of the force of imbibition, acting from the outer cell membrane to the center of the system; this follows as a consequence of the disturbance of equilibrium set up by the force with which the formation of ice crystals takes water from the surface of the cell. Wiegand supports the view that death is due to drying of the protoplasm beyond its critical water content. Evidence of other workers as to the importance of resistance to desiccation has been presented in an earlier section of this paper. Whatever may be the precise mechanism by which withdrawal of water brings about disorganization of the protoplasm, it seems clear at least that hardiness must be intimately connected with forces which oppose this desiccation. In the light of our present knowledge of the properties of colloids, it seems most probable that the principal force is imbibition.

Spoehr (1919) found that in cacti the pentosans increase with decreasing water supply. MacDougal (1920) also points out that the

conversion of the diffusible sugars to the mucilaginous pentosans is one of the alterations which may result in the cell as a consequence of partial desiccation. The latter author pictures plant protoplasm as pentosan-protein colloid, and considers that the character and amount of the pentosans largely determines the hydration reactions of the protoplast. Winter conditions where the soil freezes solid are really xerophytic conditions, since the plant's usual water supply is cut off. Having this in mind, it may be reasoned from the evidence of MacDougal and Spoehr that under winter conditions pentosans would accumulate in the cell, contributing largely to the formation of a protoplasmic gel of high imbibitional powers. Investigation of this point will be reserved for a later paper.

The relative importance of sugars and electrolytes in the development of osmotic pressure is considered in TableV. Unfortunately there is no known method whereby the relative proportions of osmotic pressure contributed by electrolytes and non-electrolytes can be accurately calculated from conductivity and freezing point data. In the present instance the sugar percentages were known and these were assumed to represent the non-electrolyte materials. The theoretical osmotic pressure exerted by the sugars is calculated from the quantities of reducing sugars and sucrose determined by analysis to be present. The difference between this value P_S and the total osmotic pressure **P** (i.e., $P - P_S$) may be attributed chiefly, though probably not entirely, to electrolytes. In the collection of November 12, the sugars present were sufficient to account for an average of about 38 percent of the total osmotic pressure; in that of December 9, for about 34 percent. The ratio of the value

$P - P_s$ to specific conductivity ($x10^3$) is given in the last column of the table. This should be a constant in case the cell sap electrolytes in each variety were identical in composition. For the earlier collection this ratio varies from 1.26 to 1.45 and averages 1.34, but for the later collection it falls off somewhat, ranging from 0.83 to 1.10 with an average of 0.95. Possibly the heating of these later samples to 100°C. had released ions which would otherwise have remained adsorbed by cell colloids. This factor may account in part for the somewhat higher value of K, more striking in view of the dilution which had occurred, since the water content of the tissues was greater in the later collection. However, the diminution in the value of $P - P_s$ in this collection is so decided as to make it unlikely that the possible disturbing factors introduced by the heating could account entirely for the failure of K to diminish correspondingly. The evidence suggests that substances other than electrolytes, and variable in nature, must contribute somewhat to the quantity $P - P_s$, or in other words that sugars are probably not the only non-electrolytes which contribute to the osmotic values.

The amino nitrogen, water-soluble nitrogen and total nitrogen in percent of green and dry weights are given in Table VI. The percent of dry matter content is also included. It cannot be said that any of the figures exhibit a marked correspondence to differences in degree of hardiness. The increase in the water content of the collection of December 9 is accounted for by a mild rainy period of some days duration which occurred during the previous week. It has been remarked already that Kanred killed worse than the

other varieties during the season of this experiment, and this variety was lowest in dry matter content. Minhardi, the hardiest variety used, had a somewhat larger content of water-soluble nitrogen. All varieties show an increase in amino nitrogen and water-soluble nitrogen in the later collection. This is in harmony with the evidence of Harvey (1918) that splitting of the proteins is associated with the hardening process.

The high content of sugars, especially sucrose, reported in Table VII, is quite remarkable. It has been noted already that sucrose was apparently the only disaccharide present. But here again the varieties could not be classified according to hardiness on the basis of the values found. All suffered a loss between the collections of November 12 and December 9, but Kanred lost decidedly more than the others. The greater degree of killing in this variety thus lends support to the observation of Pantanelli (1918) that hardiness was proportional to the quantity of sugar retained during exposure to frost.

A qualitative test for starch on the dried, ground residue from the alcohol extraction gave entirely negative results in every case. This is as expected from the observations of Miyake (1902), Lidforss (1907) and others.

The above discussion indicates that we are still far from an exact analysis of the factors influencing winter hardiness, but certain of the observations, notably the failure of freezing the tissues to break down the protoplasmic structure in the hardened plants, are very suggestive. Further investigation of this phenomenon will be carried out in the near future.

SUMMARY.

1. A number of varieties of winter wheat, known to vary considerably in degree of winter hardiness, were compared in the hardened condition with reference to the physical constants of the cell sap, and the content of dry matter, nitrogen, sugars and starch.

2. No constant relation was found between depression of the freezing point, specific conductivity, or hydrogen-ion concentration of the cell sap and relative frost hardiness.

3. Sugars accounted for 34 to 38 percent of the total osmotic pressure of the sap.

4. The ratio of that part of the osmotic pressure not due to sugars (i.e., $P-P_s$) to the specific conductivity ($x10^3$) is not a constant. For the samples collected November 12, this ratio varied from 1.26 to 1.45 (average 1.34) and for those collected December 9, from 0.83 to 1.10 (average 0.95).

5. The relation between dry matter content and hardiness was not constant, though one of the two tender varieties had the lowest percentage.

6. All varieties increased in amino nitrogen and water-soluble nitrogen during the hardening process, but there was little relation between the amount of this increase and relative hardiness.

7. The sugar content did not correspond uniformly with the known hardiness. The percentage decreased between November 12 and December 9, falling lowest in one of the two tender varieties.

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8. Sucrose is an important storage material and is apparently the only disaccharide present.

9. All varieties were entirely free from starch.

10. The colloidal complex of the cell of the fully hardened tissue could not be broken down by exposure to the temperature of a calcium chloride - snow cryohydric mixture (theor.= -54.9°C.).

11. The hardened tissue retains its water content with great force. From tissue containing about 70 percent of moisture no appreciable amount of sap could be expressed by 400 atmospheres pressure, even after severe preliminary freezing.

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