UNFREEZABLE AND FREEZABLE WATER EQUILIBRIUM IN PLANT TISSUES AS INFLUENCED BY SUB-ZERO TEMPERATURES[1]

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Introduction

It is commonly accepted that with biocolloidal systems there is a definite temperature at which all of the freezable water will be frozen (**7, 11, 6,** and others). This temperature has been arbitrarily selected as about $-18°$ to $-20°$ C. In studying one phase of a problem on winter hardiness in red clover roots, it was desirable to determine the amount of unfreezable water in these tissues. Thus an opportunity was afforded to test the preceding hypothesis with regard to this tissue by determining the amount of water which does not freeze at temperatures of $-10°$ to $-50°$ C. Potato tubers were also included in the study.

Review of literature

ST.JOHN (**9**) made a study of the unfreezable and freezable water equilibrium of the thick portion of egg white, employing the method of RUBNER (**7**) as modified by THOENES (**11**) and ROBINSON (**6**). He found that at $-5°$ C., 80 per cent. of the water present was unfreezable, while at $-10°$ C. only 35 per cent. remained unfrozen. Determinations at $-12.5°$ C. showed 25.8 per cent. of the water unfreezable. From this temperature on through $-35°$ C. he found a flat straight-line curve indicating that no more of the water was frozen at a temperature of $-35°$ C. than at $-12.5°$ C.

JONES and GORTNER (**3**), using the dilatometer method on gelatin solutions, found that all of the freezable water was crystallized out at $-6°$ and no additional amount was removed at a temperature of $-50°$ C. With a 2 per cent. solution the percentage of total water unfreezable was 9.35. At a temperature of $-44.4°$ C. the unfreezable water percentage was exactly the same as at $-6°$ C. They state that animal and plant tissues and related systems would probably behave like the gelatin system. It does not necessarily follow, however, that such would be the case for plant tissues.

JONES and GORTNER'S (**3**) data on inorganic hydrogels indicate that there was no temperature within the range of 0.0° to $-50°$ C. at which a lower temperature did not cause the crystallization of additional quantities of ice.

[1] The writer expresses his appreciation to Dr. E. A. HOLLOWELL, of the Forage Crop Division, U. S. D. A., for furnishing the clover seed used in this investigation and for his valuable suggestions in culturing red clover plants.

Methods

A variety of methods have been used by investigators for determining the ratio of unfreezable to freezable water in organic materials. Of these the early method of MÜLLER-THURGAU (**5**), later modified by RUBNER (**7**), THOENES (**11**), ROBINSON (**6**), ST.JOHN (**9**), SAYRE (**8**), and MEYER (**4**), was chosen. This is the calorimetric or heat of fusion method. It has been modified slightly to meet the requirements of this particular problem. The symbols and equation used follow in general those developed by previous investigations.

The tissues used in the unfreezable water determinations were put through a Nixtamal mill. Portions were then taken for total and freezable water determinations, the total moisture being determined in a vacuum oven at 80° C. and 3 to 4 cm. pressure. The total moisture value was used in the unfreezable water calculations instead of the moisture determinations on the same following the calorimetric determinations, as has been done by a number of previous investigators.

For the freezable water determination, 8 to 10 gm. of tissue were placed in a tared tinfoil cup, similar in construction to that used by ROBINSON (**6**), which was then placed in a tared weighing bottle. The whole was weighed accurately and placed in the low temperature cabinet at the desired sub-zero temperature. For the temperatures of $-10°$ to $-23°$ C. a specially constructed cabinet was used. For temperatures of $-25°$ to $-50°$ C. an alcohol bath, to which dry-ice was added to give the desired low temperature, was employed. The inner container of tinfoil is essential in weighing the tissue and in making a quick transfer of the frozen tissue to the calorimeter. It likewise aids in preventing (1) floating of tissue on surface of water in calorimeter, (2) evaporation of water from tissue during weighing, etc., and (3) heat of solution of any soluble substances while in the calorimeter. The outer container aids in the identification and transfer of the tissue from the sub-zero temperature to the calorimeter with the minimum of time and exposure to room temperature.

The determination of unfreezable water by the calorimetric method requires the establishment of a stable phase equilibrium at the desired sub-zero temperature. The value for unfreezable water varies with temperature, within limits, and it is important to maintain the desired freezing temperature constant, preferably with $\pm 0.25°$ C., for a period of hours. The requirement of $-10°$ to $-23° \pm 0.25°$ C. was fulfilled in a special low temperature unit constructed by the Freas Electric Company.

The freezable water samples were allowed to remain in the cabinet for 10 to 12 hours. Triplicate samples with different time intervals indicated that the different phases had attained an equilibrium in this period.

The calorimeter consisted of a highly evacuated silvered Dewar flask, closed with a cork 3 cm. thick. Two holes were placed through the cork, one for the thermopile or Beckmann thermometer and the other for a spiral glass stirrer. Proper stirring of the liquid is essential to establish an equilibrium between the material and the liquid of the calorimeter.

Exactly 300 gm. of distilled water were placed in the calorimeter. As soon as the water had come to a temperature equilibrium with the calorimeter walls, glass stirrer, and thermo-elements, the frozen sample was introduced and the new equilibrium determined. The time required to reach equilibrium for the 8 to 10-gm. sample was 8 to 10 minutes, with a constant rate of stirring. The temperature was measured by a Beckmann thermometer or by the use of a 15-junction thermopile attached to a Leeds and Northrup type K potentiometer. The thermopile was constructed of 28 gauge copper and constantan wire. It was possible with this thermopile and accessory equipment to measure 0.001° C.

With the addition of the frozen sample to the water of the calorimeter there is a fall in temperature. In this investigation the heat lost by the water in the calorimeter is used in warming six different component parts of the system. Each of these quantities may be expressed as follows:

$$
\begin{aligned}
&(1)\ W_t\ S_t\ (T_e - T_s)\\
&(2)\ W_d\ S_d\ (T_e - T_s)\\
&(3)\ W_b\ S_b\ (T_\triangle - T_s)\\
&(4)\ W_i\ S_i\ (T_\triangle - T_s)\\
&(5)\ W_w\ S_w\ (T_e - T_\triangle)\\
&(6)\ H_f\ W_i
\end{aligned}
$$

W_t = weight of tinfoil cup;
W_d = weight of dry matter in sample;
W_b = weight of unfreezable water in sample;
W_i = weight of ice in sample;
W_w = total water in sample;
S_t = mean specific heat of tinfoil for temperature range used;
S_d = mean specific heat of dry matter for temperature range used;
S_b = mean specific heat of unfreezable water for temperature range used;
S_i = mean specific heat of ice for temperature range used;
S_w = mean specific heat of water for temperature range used;
H_f = heat of fusion of ice (79.75 calories) at $T_\triangle$;
T_e = equilibrium temperature;
T_s = sub-zero temperature of sample;
$T_\triangle$ = freezing-point of water in plant sap.

It is necessary to determine a correction factor for the calorimeter, since the walls of the calorimeter, stirrer, etc., absorb heat from the water the same as the components in the system. This correction factor was obtained by placing 8–10 gm. of water in the tinfoil cups, freezing at the desired temperature, and then transferring to the water of the calorimeter and determining how much heat was necessary to melt the ice and raise the temperature of the resulting water to the equilibrium temperature of the system. The values for the correction factor were plotted for the different sized samples. Thus the factor could easily be obtained for the sample that varied from 8 to 10 gm. For an 8-gm. sample the correction factor was found to be 1.085.

It is evident that the loss of heat energy in terms of calories by the water in the calorimeter may be expressed mathematically as follows: (7) $FNS_w(T_o - T_e)$

where F = calorimetric correction factor;

N = number of grams of water in calorimeter (300 gm.);

S_w = specific heat of water for temperature range used $(T_o - T_e)$;

T_o and T_e = original temperature of water and equilibrium temperature respectively.

Specific heat determinations were made on the dried tissue, following the procedure previously outlined, except that benzene was substituted for the water in the calorimeter. It was found that the use of a liquid of lower specific heat increased the accuracy of the determination. The values used for the specific heat of pure benzene were secured by plotting a curve for the values as given in the International Critical Tables (**12**).

The specific heat values for ice were taken from DICKINSON and OSBORNE (**1**). The values for water above zero were taken from the Handbook of Chemistry and Physics (**2**). The specific heat values for water below zero were obtained by extending the curve of values from 0° to 30° C. as a straight line, assuming that the specific heat of water continues as a linear function, as has previously been done by SAYRE (**8**). It is assumed that the specific heat of unfreezable water is the same as that of freezable water at the same temperature.

Combining quantities 1, 2, 3, 4, 5, 6, and 7, equating and solving for W_i, the final complete formula becomes:

$$W_i = \frac{FNS_w(T_o - T_e) - W_tS_t(T_e - T_s) + W_dS_d(T_e - T_s) + W_wS_w(T_e - T_s)}{H_f - (S_b - S_i)(T_\triangle - T_s)}$$

The values for T_s and $T_\triangle$, being below zero, are indicated in the formula by a negative sign. The unfreezable water is determined by subtracting the freezable water from the total water, thus $W_b = W_w - W_i$.

Results

The temperatures used were from – 10° to – 50° C. by five degree steps. In addition measurements were made at – 22° C. The physical chemist (**10**) considers – 22° C. as the minimum freezing-point of pure water under any set of equilibrium conditions. The data presented in table I show the variation in results for typical triplicate samples and indicate the accuracy of the method.

TABLE I

PERCENTAGES OF UNFREEZABLE WATER IN UNHARDENED CLOVERS AT DIFFERENT SUB-ZERO TEMPERATURES (OHIO VARIETY)

SUB-ZERO TEMPERATURE °C. ± 0.25	UNFREEZABLE WATER	
	TOTAL WATER	TOTAL SOLIDS
	%	%
– 10	20.502	103.936
	20.548	103.988
	20.536	103.926
– 15	12.011	60.786
	13.940	70.543
	14.501	73.387
– 20	8.672	43.883
	9.555	48.353
	9.307	47.101
– 22	7.057	35.716
	7.925	40.106
	7.279	36.853

Table II presents representative data that have been calculated from measurements made upon clover and potato tissue when exposed to temperatures from – 10° to – 50°. It will be noted that as the temperature decreases from – 10° to – 50° C. (table II, A and C) in the case of the unhardened red clover root tissue, the unfreezable water value also decreases. A similar condition can be observed on the hardened-off clover root tissue between – 15° and – 20° C.; however, from – 20° to – 25° C. the unfreezable water value remains nearly constant. Part C of table II presents data to show the influence of very low temperatures upon the unfreezable water values. It will be noted that there is a lowering of the unfreezable water fraction with each five degree fall in temperature. This is especially marked between the temperatures of – 10° to – 30° C. The rate of decrease becomes slower with the lower sub-zero temperatures. Potato tissue (table II) behaves similarly to clover root tissue, with the exception that the unfreezable water ratio is shifted slightly farther to the right.

TABLE II

INFLUENCE OF SUB-ZERO TEMPERATURES ON PERCENTAGE OF UNFREEZABLE WATER IN CLOVER AND POTATO TISSUE

KIND OF TISSUE	SUB-ZERO TEMPERATURE °C. ± 0.25	UNFREEZABLE WATER		TOTAL MOISTURE
		TOTAL	TOTAL SOLIDS	
		%	%	%
RED CLOVER ROOTS— A: Ohio, unhardened, same age as B	−10	20.53	103.95	83.50
	−15	13.48	68.24	
	−20	9.18	46.45	
	−22	7.42	37.56	
B: Ohio, hardened, same age as A	−15	15.43	78.06	82.30
	−20	13.16	68.17	
	−22	14.03	71.04	
	−25	13.65	69.09	
C: Unknown variety grown in field, unhardened	−10	14.47	72.16	81.81
	−15	9.43	52.04	
	−20	7.36	38.66	
	−22	5.28	24.73	
	−25	2.66	14.23	
	−30	1.99	11.76	
	−35	2.11	12.84	
	−40	1.95	11.54	
	−45	1.50	10.63	
	−50	1.43	10.59	
D: Potato (Irish Cobbler) tubers	−10	11.05	47.31	81.07
	−15	9.16	38.18	
	−20	7.75	33.18	
	−23	5.59	23.96	

Discussion

A number of investigators have suggested that water binding in biological materials follows an adsorption reaction. The results of this investigation indicate more nearly an equilibrium between the external force (low temperature) and internal forces of the tissue.

The writer's experimental data with plant tissues exposed to sub-zero temperatures do not follow exactly the generalizations of JONES and GORTNER (3) on gelatin, or that of ST.JOHN (9) on egg white. The results of table II, parts A, C, and D, follow more closely the data that have been secured by different investigators on inorganic hydrogels; whereas the data in table II, B, more nearly coincide with the findings of JONES and GORTNER on gelatin and ST.JOHN on egg white. However, the constancy of unfreezable water values occurs at lower sub-zero temperature.

There are indications that the same tissue (table II, A and B), when grown under different conditions, as temperature, will produce entirely different values for the unfreezable water value when the determinations are made at −22° or −25° C.

Summary and conclusions

1. The hypothesis of RUBNER (7), THOENES (11), ROBINSON (6), and others that biocolloidal systems have a definite temperature (– 18° to – 20° C.) at which all of the unfreezable water will be frozen has been tested for plant tissues. This hypothesis is not supported by unhardened clover roots, but seemed to be by the hardened clover root tissue. The unfreezable water values decreased 1.40 per cent. for the cold hardened red clover roots with the lowering of the temperature from – 15° to – 22° C., whereas the unfreezable water values of the unhardened root tissue decreased 6.02 per cent. over the same temperature range.

2. With another lot of unhardened red clover root tissue there was a decrease in the amount of the unfreezable water expressed as the percentage of the total water, from 14.47 to 1.43 per cent. with the decrease of the temperature of – 10° to – 50° C.

3. Potato tissue gave similar results to those of unhardened clover root tissue, with the exception that the unfreezable water values were lower for similar sub-zero temperatures.

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LITERATURE CITED

1. DICKINSON, H. C., and OSBORNE, N. S. Specific heat and heat of fusion of ice. Bureau Standards Bull. 12. 49–81. 1915.
2. HODGMAN, C. D., and LANGE, N. A., Compilers. Handbook of chemistry and physics. 15th ed. pp. 1426. 1930.
3. JONES, I. D., and GORTNER, R. A. Free and bound water in elastic and non-elastic gels. Jour. Phys. Chem. **36**: 387–436. 1932.
4. MEYER, B. S. Further studies on cold resistance in evergreens, with special reference to the possible rôle of bound water. Bot. Gaz. **94**: 297–321. 1932.
5. MÜLLER-THURGAU, H. Ueber das Gefrieren and Erfrieren der Pflanzen. Landw. Jahrb. **9**: 133–189. 1880; **15**: 453–610. 1886.
6. ROBINSON, W. Free and bound water determinations by the heat of fusion of ice method. Jour. Biol. Chem. **92**: 699–709. 1931.
7. RUBNER, M. Über die Wasserbindung in Kolloiden mit besonderer Berücksichtigung des quergestreiften Muskels. Abhandl. Preuss. Akad. Physik-Math. Klasse, 3–70. 1922.
8. SAYRE, J. D. Methods of determining bound water in plant tissue. Jour. Agr. Res. **44**: 669–688. 1932.
9. ST.JOHN, J. L. The temperature at which unbound water is completely frozen in a biocolloid. Jour. Amer. Chem. Soc. **53**: 4014–4019. 1931.

10. TAYLOR, H. S. A treatise on physical chemistry. Vol. I. p. 381. D. Van Nostrand Co. 1925.
11. THOENES, F. Untersuchungen zur Frage der Wasserbindung in Kolloiden und tierischen Geweben. Biochem. Zeitschr. **157**: 174–186. 1925.
12. WASHBURN, E. W., Editor. International critical tables of numerical data, physics, chemistry, and technology. 1st ed. Vol. V. McGraw Hill Book Co., New York. 1929.