THE CRYOSCOPIC METHOD FOR THE DETERMINATION OF "BOUND WATER"*

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In 1922 Newton and Gortner (1) put forward the hypothesis that at least a portion of the water associated with the hydrophilic colloids in plant tissues was in a "bound" form, in which form it exhibited physicochemical properties which would serve to differentiate it from the "free" water of the vacuolar sap. It was suggested that bound water might be unavailable for the solution of sucrose and accordingly a method for the estimation of such bound water was proposed which consisted essentially in first determining the freezing point of the solution which contained the hydrophilic colloid and denoting this value as \( \Delta \). An amount of the sol was then taken which contained exactly 10 gm. of total water. To this portion there was added 0.01 mole of sucrose and a second depression of the freezing point, designated \( \Delta \alpha \), was determined. If all of the 10 gm. of water were free to dissolve sucrose \( \Delta \alpha \) should differ from \( \Delta \) by the freezing point depression of a molar solution of sucrose which according to Satchard (2) should be 2.085\(^\circ\), corresponding to a gram molecule of sucrose-hexahydrate dissolved in 892 gm. of water.

The formula which Newton and Gortner proposed

\[
\text{percentage of bound water} = \frac{\Delta \alpha - (\Delta + \Delta m)}{\Delta \alpha - \Delta} \times 89.2
\]  

(1)

was tested by them on aqueous solutions of gum acacia and on certain plant saps and appreciable amounts of bound water were apparently demonstrated to be present.

This method has been extensively used by numerous plant physiologists—especially Newton and his coworkers (3–6)—and has

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apparently given valuable information as to the physiological response of plants, especially in problems concerned with winter hardiness and drought resistance.

Bound water appeared to be more or less generally accepted as a physiological factor and the cryoscopic method as an approximate measure of its intensity. However, Grollman (7) has recently challenged the conception of bound water and the correctness of the formula proposed by Newton and Gortner (1).

Grollman suggests that Formula (1) does not provide for the concentration of electrolytes and other true solutes which are initially present in the biological fluids and that if sucrose forms a hexahydrate these solutes must be concentrated in the remaining water, thus forming a more concentrated solution which will freeze below the A as originally determined on the sap. He accordingly proposes the formula

\[
\text{percentage of bound water} = \frac{\Delta a - \frac{1000}{892} (\Delta + K_m)}{\Delta a - \frac{1000}{892} \Delta} \times 89.2
\]

as a measure of bound water, and adds:

"This modification of Newton and Gortner's method of calculation will markedly affect results quoted by these authors in which \( \Delta a \) is appreciable compared to \( \Delta a \) and \( K_m \) as is the case in practically all of the substances studied by these authors with the exception of gum acacia. In the latter case, the correction is comparatively slight.

"Newton and Gortner's results by the above method, as applied to the juices of the wheat plant (Triticum vulgare) led these authors to conclude that winter hardiness in such plants is related to the amount of bound water present in different varieties. If one corrects their results by the use of the modified formula described above, one finds very little evidence to substantiate this theory. Thus in the case of Triticum vulgare var. super, in which 4.4 per cent of the water was found to be bound, recalculation shows the results to indicate that all the water is actually free. Recalculation of the results for a second species of the same plant shows -3.6 per cent of the total water to be bound (an obviously impossible result) instead of -3.8 per cent as found by Newton and Gortner."

\footnote{"Approximate" only, since the method \textit{must} yield \textit{minimum} values as the assumptions are made (a) that bound water does not "dissolve" sucrose, \textit{i.e.} water is adsorbed but no sucrose is adsorbed, and (b) that a molar solution of sucrose in water does not shift the bound \( \leftrightarrow \) free water equilibrium."}
of 0.9 per cent as given by Newton and Gortner. The above two varieties of wheat were non-hardy, but in the case of *Triticum vulgare* var. *Minhardi*, a hardy species, recalculation also shows a 'negative' amount of 'bound' water to be present instead of 2.2 per cent as claimed.

"Obviously, then, the method employed by Newton and Gortner is unsuited for the determination of the amount of bound water in solutions and leads, in many cases, to the impossible conclusion that a negative amount of water is actually bound."

It must be admitted that Grollman is correct in his criticism of Formula (1) *in so far as it applies to plant saps and to solutions containing true solutes.* He was, however, unfortunate in concluding that winter-hardy wheats do not show the phenomenon of water binding, for the data which he selected for recalculation was taken from a table of data on greenhouse-grown plants and in all published work emanating from this laboratory or from the laboratories of Dr. Newton it has been expressly stated that there is no differentiation between winter-hardy and winter-tender plants grown in the greenhouse until after they have been subjected to a "hardening off" process by subjection to low temperatures for several days. Grollman's calculations were made on data taken from Table 31 (8) of tender plants. If he had recalculated the data in Table 32 (8) *(cf.* Newton (9), p. 31) his conclusions would not have been sustained. These data, together with recalculations according to Grollman's Formula (2) are shown in Table I.

In the collections of February 3 to 18 from the field we find that Super and Fulcaster wheats show no bound water. These wheats in Minnesota or Alberta are non-winter-hardy. Kanred, showing 3.65 per cent of bound water is moderately winter-hardy, Minhardi with 7.49 per cent of bound water is very winter-hardy. The order in the table from Minhardi to Fulcaster is exactly the order of winter hardiness shown by field tests, so that the corrected Formula (2) of Grollman does not change the essential conclusion that winter hardiness in wheat is accompanied by something that is measured by this cryoscopic method and which has been designated as bound water. The greenhouse-grown wheats were demonstrated to be in a winter-tender condition and again the figures confirm this fact.

Grollman does not discuss the data for gum acacia, merely noting
in the paragraph quoted "with the exception of gum acacia." It appears to us that this is a crucial exception. Here the corrected formula does not apply and the original Formula (1) of Newton and Gortner is essentially correct. In this colloidal sol appreciable

<table>
<thead>
<tr>
<th>Material used</th>
<th>Calculations by Formula (1) of Newton and Gortner</th>
<th>Calculated by Formula (2) of Grollman</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaves of</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Feb. 3-18, 1922, Collected from the open</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minhardi</td>
<td>$\theta$ 1.741 $\theta$ 4.226 $\theta$ 2.485 $\theta$ 0.400 $\theta$ 14.4 $\theta$ 7.49</td>
<td></td>
</tr>
<tr>
<td>Buffum</td>
<td>$\theta$ 1.719 $\theta$ 4.158 $\theta$ 2.439 $\theta$ 0.354 $\theta$ 13.0 $\theta$ 5.49</td>
<td></td>
</tr>
<tr>
<td>Turkey</td>
<td>$\theta$ 1.273 $\theta$ 3.612 $\theta$ 2.339 $\theta$ 0.254 $\theta$ 9.7 $\theta$ 4.10</td>
<td></td>
</tr>
<tr>
<td>Kanred</td>
<td>$\theta$ 1.461 $\theta$ 3.753 $\theta$ 2.292 $\theta$ 0.207 $\theta$ 8.1 $\theta$ 3.65</td>
<td></td>
</tr>
<tr>
<td>Super</td>
<td>$\theta$ 1.085 $\theta$ 3.279 $\theta$ 2.194 $\theta$ 0.109 $\theta$ 4.4 $\theta$ -0.89</td>
<td></td>
</tr>
<tr>
<td>Fulcaster</td>
<td>$\theta$ 1.202 $\theta$ 3.949 $\theta$ 2.192 $\theta$ 0.107 $\theta$ 4.3 $\theta$ -1.60</td>
<td></td>
</tr>
<tr>
<td>Feb. 10-16, 1922, From greenhouse.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minhardi</td>
<td>$\theta$ 1.147 $\theta$ 3.284 $\theta$ 2.137 $\theta$ 0.052 $\theta$ 2.2 $\theta$ -3.83</td>
<td></td>
</tr>
<tr>
<td>Super</td>
<td>$\theta$ 1.000 $\theta$ 3.106 $\theta$ 2.106 $\theta$ 0.021 $\theta$ 0.9 $\theta$ -4.37</td>
<td></td>
</tr>
<tr>
<td>Cactus (stems)</td>
<td>$\theta$ 0.505 $\theta$ 2.803 $\theta$ 2.298 $\theta$ 0.213 $\theta$ 8.3 $\theta$ 5.80</td>
<td></td>
</tr>
<tr>
<td>Gum acacia sols</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 per cent</td>
<td>$\theta$ 0.005 $\theta$ 2.147 $\theta$ 2.142 $\theta$ 0.057 $\theta$ 2.37 $\theta$ 2.32</td>
<td></td>
</tr>
<tr>
<td>3 per cent</td>
<td>$\theta$ 0.013 $\theta$ 2.186 $\theta$ 2.173 $\theta$ 0.088 $\theta$ 3.61 $\theta$ 3.48</td>
<td></td>
</tr>
<tr>
<td>5 per cent</td>
<td>$\theta$ 0.025 $\theta$ 2.221 $\theta$ 2.196 $\theta$ 0.111 $\theta$ 4.50 $\theta$ 4.37</td>
<td></td>
</tr>
<tr>
<td>7 per cent</td>
<td>$\theta$ 0.034 $\theta$ 2.254 $\theta$ 2.220 $\theta$ 0.135 $\theta$ 5.42 $\theta$ 5.26</td>
<td></td>
</tr>
<tr>
<td>10 per cent</td>
<td>$\theta$ 0.048 $\theta$ 2.294 $\theta$ 2.246 $\theta$ 0.161 $\theta$ 6.39 $\theta$ 6.15</td>
<td></td>
</tr>
</tbody>
</table>

amounts of bound water are shown to be present regardless of how the data are calculated.

Grollman adds that a "negative" bound water is an impossibility. This conclusion in itself is not correct for, as Bull (10) has recently pointed out, a negative bound water value is a necessary corollary
when adsorption of solutes is greater than adsorption of the solvent. If both solutes and water are adsorbed to the same extent then no bound water will be indicated.

Grollman suggests that sucrose, regarding the solutions of which we know relatively little, should be replaced by some electrolyte such as KCl, the solute behavior of which is relatively well known, and he finds in his studies of such systems little or no bound water. This finding may simply mean that the more mobile K⁺ and Cl⁻ ions are either preferentially adsorbed or equally adsorbed along with the water, whereas the non-ionic and highly polar sucrose molecule is negatively adsorbed.

It should be added that it appears to the authors that the available data (cf. especially Newton and Martin (5)), showing as they do an almost exact correlation between bound water values as determined by the cryoscopic method and the biological response of plants to their environment as demonstrated by field tests, is strongly suggestive of a parallel, if not a causative, phenomenon, and this biological correlation must be accounted for before the bound water theory is thrown into the discard on what appears to be more or less theoretical assumptions.

**EXPERIMENTAL**

While considering certain of the problems which have been noted, an alternative method of calculating cryoscopic data occurred to us. Grollman rightly suggests that the freezing point method may be subject to appreciable errors. There are the random errors of individual determinations and there are the errors of correcting for the ice which separates due to cooling below the true freezing point. The correction for undercooling

\[ \Delta = \Delta' - 0.125u \Delta' \]  

(3)

where \( \Delta \) = corrected freezing point  
\( \Delta' \) = observed freezing point  
\( u \) = degrees undercooling

was used in all earlier data published from this laboratory.

Formula (3) assumes a specific heat for the system which is identical with the specific heat of water, and assumes that all of the heat of
crystallization is utilized to raise the temperature of the solution. It also assumes that no heat exchange occurs between the system and its environment. Obviously these are ideal conditions which may not be realized experimentally.

It appeared, therefore, preferable to discard these assumptions and to assume only that there is a linear relationship between undercooling and the observed freezing point, which assumption appears to be well founded.

If then a series of data is secured in which are recorded the observed freezing point ($\Delta'$) and the degrees undercooling ($u$) such data can be treated by the method of least squares to find the constants ($a$) and ($b$) for the straight line which mathematically fits the data.

\[
a = \frac{\sum(x) \cdot \sum(xy) - \sum(x^2) \cdot \sum(y)}{[\sum(x)]^2 - n\sum(x^2)}
\]

\[
b = \frac{\sum(x) \cdot \sum(y) - \sum(xy)}{[\sum(x)]^2 - n\sum(x^2)}
\]

where $x = u$

$y = \Delta'$

$a = $ the true freezing point (i.e. $\Delta'$ where $u = 0$)

$b = $ tangent of the angle which the line makes with the $y$ axis

Such a study has been carried out on aqueous solutions of molar sucrose and $\frac{m}{2}$ KCl, KBr, and KI and on these solutions containing 3 or 5 per cent gum acacia. All solutions were weight molar. The gum acacia was selected from clear pieces and showed an almost negligible depression of the freezing point (0.024°) in a 5 per cent sol but the accepted true freezing points in Table II have been corrected for this slight depression. The data are shown in Table II and Figs. 1 to 4. The lines in the graphs are those calculated by the method of least squares.

DISCUSSION

Several things are evident from an inspection of the figures and Table II.

In the first place there is a relatively large probable error for an individual freezing point determination. This error is probably larger in this series of determinations than would be the case if the
series were repeated. After all determinations had been completed it was observed that there was a distinct hysteresis effect observable in the acacia data. Those solutions which had stood for a time showed a slightly different freezing point scatter diagram than that shown a day or two previously by the same solution. Three series of runs (on different days) on the same solutions of sucrose + 3 per cent acacia and two series on sucrose + 5 per cent acacia are shown in

Table II

The True Freezing Point Depressions of Certain Solutions—with and without the Presence of Added Gum Acacia

<table>
<thead>
<tr>
<th>System</th>
<th>No. of freezing points to determine line</th>
<th>Calculated true freezing point at x = 0</th>
<th>Angle of regression line</th>
<th>Change in Δ due to colloid</th>
<th>Change in Δ due to colloid</th>
</tr>
</thead>
<tbody>
<tr>
<td>m/1 sucrose</td>
<td>10</td>
<td>2.058</td>
<td>0.0650</td>
<td>-0.028</td>
<td>2.104</td>
</tr>
<tr>
<td>m/1 sucrose + 3 per cent acacia</td>
<td>19</td>
<td>2.106</td>
<td>0.0511</td>
<td>+0.048</td>
<td>2.132</td>
</tr>
<tr>
<td>m/1 sucrose + 5 per cent acacia</td>
<td>21</td>
<td>2.133</td>
<td>0.0452</td>
<td>+0.075</td>
<td>2.152</td>
</tr>
<tr>
<td>m/2 KCl</td>
<td>14</td>
<td>1.664</td>
<td>0.0318</td>
<td>-0.016</td>
<td>1.679</td>
</tr>
<tr>
<td>m/2 KCl + 3 per cent acacia</td>
<td>16</td>
<td>1.640</td>
<td>0.0409</td>
<td>-0.024</td>
<td>1.663</td>
</tr>
<tr>
<td>m/2 KCl + 5 per cent acacia</td>
<td>24</td>
<td>1.658</td>
<td>0.0343</td>
<td>-0.006</td>
<td>1.674</td>
</tr>
<tr>
<td>m/2 KBr</td>
<td>10</td>
<td>1.699</td>
<td>0.0361</td>
<td>-0.024</td>
<td>1.715</td>
</tr>
<tr>
<td>m/2 KBr + 5 per cent acacia</td>
<td>11</td>
<td>1.650</td>
<td>0.0393</td>
<td>-0.049</td>
<td>1.679</td>
</tr>
<tr>
<td>m/2 KI</td>
<td>10</td>
<td>1.765</td>
<td>0.0399</td>
<td>-0.006</td>
<td>1.730</td>
</tr>
<tr>
<td>m/2 KI + 3 per cent acacia</td>
<td>10</td>
<td>1.743</td>
<td>0.0213</td>
<td>+0.068</td>
<td>1.741</td>
</tr>
</tbody>
</table>

Fig. 1, although all series are combined in the calculation of the constants for the line.

That this is a hysteresis (colloid aging) effect is made certain by unpublished data on gelatin-sucrose systems secured simultaneously with those presented in this paper. Here the trend was invariably from positive amounts of bound water to negative amounts with time. This would indicate a progressive removal of sucrose from the solution by the gelatin. Unfortunately we failed to keep exact enough
FIG. 1. Freezing point and undercooling data for \( \text{m/1} \) sucrose solutions with and without the addition of gum acacia.
Fig. 2. Freezing point and undercooling data for m/2 KCl solutions with and without the addition of gum acacia.
DETERMINATION OF BOUND WATER

records of the environmental conditions of storage of these solutions to justify publishing the data. We hope to repeat and extend the gelatin-sugar series in the near future.

In the second place it is evident that the conventional undercooling correction Equation (3) does not yield entirely correct values. This formula gives a slightly curvilinear line with tangents 0.0246 at \( u = 1 \) and 0.0256 at \( u = 4 \). The tangents found in Table II are, with only one exception, greater than these, yielding a somewhat smaller true freezing point depression \( \Delta \) than the theoretical correction for undercooling.

![Fig. 3. Freezing point and undercooling data for m/2 KBr solutions with and without the addition of gum acacia.](image)

would indicate. However, when the difference in freezing point depressions due to the presence of colloid are considered (Columns 5 and 7, Table II) the values derived from the two methods of calculation are remarkably consistent. We believe, however, that the least squares method, involving as it does only experimental data, is the preferable one to use on reasonably extensive series of data.

In the third place the data indicate a positive amount of bound water in sucrose-gum acacia systems and a slightly negative amount in acacia-KCl and acacia-KBr systems. The acacia-KI system shows a positive value. While the values are not great, we believe that they
Fig. 4. Freezing point and undercooling data for 1/2 KI solutions with and without the addition of gum acacia.
are significant and actually represent positive and negative adsorption of the solvent by the colloid with concomitant negative and positive adsorption of the solute; i.e., the components of the solution are taken up differentially in hydrating the gum. A depression of the freezing point of 2.058°C indicates a 1.106 molar solution. Therefore the gram mole of sucrose is actually dissolved in 904.2 gm. of water. This corresponds almost exactly to a pentahydrate instead of the hexahydrate of Satchard (2). The freezing point of the 3 per cent acacia-sucrose solution corresponds to a 1.132 molar solution or 1 gm. mole of sucrose dissolved in 883.3 gm. of water. The 30 gm. of gum acacia have accordingly bound 20.9 gm. of water. In a like manner the 5 per cent acacia-sucrose system corresponds to a 1.147 molar solution in which 1 gm. molecule of sugar would be dissolved in 871.8 cc. of water, with the 50 gm. of acacia binding 32.4 gm. These values are small but consistent and indicate that each gram of the colloid has bound 0.6 to 0.7 gm. of water, assuming that no sugar molecules are adsorbed. If sugar molecules are simultaneously adsorbed then the binding of water is greater. In any event the 0.6 to 0.7 gm. water per gm. of this sample of gum acacia is a minimal value. Newton and Gortner (1) report 3.61 per cent of bound water in a 3 per cent acacia sol and 4.50 per cent in a 5 per cent sol. These figures, applying Grollman's correction (7) are 3.48 per cent and 4.37 per cent respectively. Our present series gives 2.09 per cent bound water for a 3 per cent acacia sol and 3.24 per cent for a 5 per cent sol. Considering that we are dealing with different lots of commercial gum acacia the disagreement is not surprising. Both sets of data indicate that the colloid gum acacia takes up water preferentially from a sucrose solution, resulting in a concentration of the sucrose in the body of the liquid. The water taken up is what Newton and Gortner designated bound water.

Theoretically, if the molecular orientation hypotheses of Hardy, Harkins, Langmuir, Adam, etc., have a basis of fact, and water molecules are oriented at solid-liquid interfaces, such water molecules must be more or less immobilized and have a reduced "activity." Such immobilized molecules constitute the bound water of Newton and Gortner (1).
SUMMARY

1. The criticisms by Grollman (7) of the cryoscopic method for the determination of bound water as proposed by Newton and Gortner (1) have been considered, and it is pointed out that even admitting the correctness of his contentions does not negative the conclusion that bound water values as determined by the cryoscopic method parallel in a remarkable manner the physiological responses of plants to environmental conditions.

2. A new method of calculating the true freezing point of a solution is proposed.

3. Gum acacia in aqueous sucrose solutions shows positive amounts of bound water to the extent of 0.6 to 0.7 gm. of bound water per gram of gum.

4. Gum acacia in aqueous solutions of KCl and KBr shows slightly negative amounts of bound water, indicating a preferential adsorption of the solute rather than the solvent.

LITERATURE CITED