ment exactly one molecule of water is included by a monohydrate, $CuSeO_4 \cdot 4NH_3 \cdot H_2O$, and that a dihydrate is in this way simulated, as suggested by Hurd and Lenher; if inclusion took place, it would be of mother liquor and not pure water, and as a result the analyses would not show such good agreement.

Chemisches Institut der Universität Berlin
Willy Lange
Gerda v. Krueger

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THE TEMPERATURE AT WHICH UNBOUND WATER IS COMPLETELY FROZEN IN A BIOCOLLOID[1,2,3]

By J. L. St. John

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In a paper on the "Relation of Hydrophilic Colloids to Winter Hardiness of Insects" presented at the Colloid Symposium at the University of Michigan in June, 1927, Robinson stated,[4] in effect, that all of the free water in a hydrophilic colloid is frozen at a temperature of $-20°$, while none of the bound water is frozen. Foote and Saxton[5] using a dilatometer method on inorganic hydrogels found that all of the free and capillary water was not frozen at $-20°$. They found in some instances that a temperature of -28 to $-33°$ was required. They also found that repeated freezing appears to convert most of the capillary water into free water, while it has little effect on the bound water.

In studying one phase of a problem it was desirable to determine the amount of bound water in egg white. Preliminary to this it was necessary to determine the temperature which should be used for the measurement of bound water in such a material.

A variety of methods has been used for the determination of the nature of water in organic material. Of these the method described by Thoenes[6] and earlier by Rubner[7] was used.

[1] Published as Scientific Paper No. 189, College of Agriculture and Experiment Station, State College of Washington.

[2] Presented at the meeting of the Pacific Division of the American Association for the Advancement of Science on June 20, 1930, at Eugene, Oregon.

[3] The Assistance of Mr. Leo Clapsaddle in the preliminary part of this work is gratefully acknowledged. Valuable suggestions made by Prof. C. A. Isaacs regarding the development of the formula are also appreciated.

[4] Robinson, "Colloid Symposium Monograph," 5, 199 (1928).

[5] Foote and Saxton, This Journal, **38**, 588 (1916).

[6] Thoenes, *Biochem. Z.*, **157**, 174 (1925).

[7] Rubner, "Abhandlungen der Preussischen Akademie der Wissenschaften," Physikalisch-Mathematische Klasse, No. 1, 1–70.

Among a variety of other materials the latter author made a few measurements on egg white using a temperature of $-18°$ and found that only 5.61% of the water was firmly bound. Rubner's method was modified slightly by Robinson and again by the writer. The method is based on the principle that the amount of water frozen may be determined by measuring the amount of heat liberated when the ice melts, and the assumption that the water which is unfrozen is colloidally bound water.

The calorimeter consisted of a Dewar tube with an internal diameter of 3.6 cm. and a length of 19.5 cm. This was insulated by a thickness of 3 cm. of paper. The temperature was measured by the use of a thermocouple attached to a Leeds and Northrup Type K potentiometer. The three-junction thermopile was constructed of 28 gage copper and constantan wire. Each junction was placed in a glass tube, the three tubes bound together, and inserted through a cork which held the thermopile in place in the Dewar tube. The temperatures were read from a graph constructed through calibration. A low-temperature bath was constructed and the temperatures required were obtained by the use of solid carbon dioxide according to the general suggestions made by Dunn,[8] using 200 cc. of ether in the bath. The ether may be reused several times.

Fresh eggs, many of them still warm, were obtained from the poultry plant. All were used less than four hours after they were laid. The white and yolk were separated, after which the two portions of the white were separated and each placed in a special separatory funnel. This funnel was of 100-cc. capacity and was made with an 18-mm. opening at the top, while the stopcock opening was 4 mm. in diameter. The funnel had no stem. A definite quantity (10 to 12 g.) of the white was carefully weighed from this funnel, by difference, into a lead crucible (bottle cap similar in shape to a regular platinum Gooch crucible) of 20-cc. capacity which was in turn dropped into a test-tube, 3.5×14.5 centimeters, and stoppered.

These test-tubes were placed in the low-temperature bath containing ether. Small pieces of solid carbon dioxide were dropped into the ether until the required temperature was obtained. The bath used was large enough to hold four of the test-tubes, with the thermometer placed in a smaller test-tube in the center. The bath was maintained at the required temperature for one hour, since no further freezing was obtained after this length of time.

In the meantime 50 cc. of water was pipetted into the calorimeter, and ice was placed in a second Dewar tube which contained the cold junctions of the thermopile. With the temperature of the calorimeter recorded, the crucible containing the frozen egg white was transferred quickly from the cold bath to the calorimeter, using rubber tipped tongs. The total time of transfer from bath to calorimeter was less than fifteen seconds. The calorimeter was stoppered by the cork through which the warm junction of the thermopile extended into the water. The contents of the calorimeter were gently stirred and the temperature recorded after equilibrium was attained. The water and dispersed egg white were transferred to a glass dish of 190-cc. capacity and the dry matter determined by evaporating overnight on a steam-bath and finally drying for an hour in a Freas oven at 100°.

In making a calculation of the free water in a sample it was necessary to have certain other data. The heat capacity of the calorimeter, including thermopile and stirrer, was determined by placing cold water or ice in it.

[8] **Dunn**, *Science*, **69**, 359 1787, (1929).

The ice was obtained by freezing weighed quantities of water by the same method that was used with the egg white. Knowing the required temperatures and the heat of fusion of ice, the heat capacity of the calorimeter was calculated. Using different quantities of ice a straight line curve was constructed from which a factor was obtained and used in the calculation of free water in each sample of egg white, the size of the factor, of course, depending on the weight of sample used. The value of the factor (F) varied from 1.08 to 1.16. Freezing point determinations were made and the average found to be $-0.68°$ for the thin portion and $-0.64°$ for the thick portion. Determinations of the specific heat of the white gave 0.85 as the average value.

With the above data available, the weight of free water in each sample was calculated by use of the formula

$$x = \frac{FN(T_3 - T_4) - (SW + S_1W_1)(T_4 - T_2)}{80 + 0.5(T_2 - T_1)}$$

The percentage of free water was calculated on the basis of the total water found. The bound water was that not frozen or the difference between the total and the free water.

Robinson[4] ignored the algebraic signs of the temperatures in using his formula. The formula presented above provides for the use of these algebraic signs. Such a formula is developed by considering that each temperature change is the result of the difference between two temperatures, and therefore by subtracting the lower temperature from the higher in each instance the required temperature range is obtained. The development of the formula correctly using the algebraic signs of the temperature is shown below.

		Illustrative Example
F	= Correction factor (heat capacity) of calorimeter	$F = 1.135$
N	= Cubic centimeters of water used in calorimeter	$N = 50$
W	= Weight of egg white used	$W = 11.0455$
W_1	= Weight of lead crucible	$W_1 = 5.5525$
S	= Specific heat of egg white	$S = 0.85$
S_1	= Specific heat of lead crucible	$S_1 = 0.03$
St	= Specific heat of total solids. The specific heat of water and of the bound water are taken as "one."	
T_1	= Average freezing point of egg white	$T_1 = -0.64$ °C.
T_2	= Temperature of egg white in bath just before being transferred to calorimeter	$T_2 = -20$ °C.
T_3	= Temperature of water in calorimeter before sample is added	$T_3 = 24.35$ °C.
T_4	= Final temperature of calorimeter	$T_4 = 10.35$ °C.
x	= Grams of free (frozen) water	$x = 7.1743$
z	= Total solids in sample	$z = 1.2250$
y	= Total water in sample	$y = 9.8205$

Then the calories of heat absorbed from the calorimeter and taken up by the material are represented as follows

$0.5x(T_1 - T_2)$ = warming ice to freezing point of material studied

$80x$ = calories absorbed by melting ice

$x(T_4 - T_1)$ = warming free water from freezing point to final temperature of calorimeter

$(y-x)(T_4-T_2)$ = warming bound water from low temperature used to final temperature
$zSt(T_4-T_2)$ = warming total solids as above
$S_1W_1(T_4-T_2)$ = warming crucible as above
$FN(T_3-T_4)$ = heat absorbed from calorimeter

Then

$$FN(T_3 - T_4) = 0.5x(T_1 - T_2) + 80x + x(T_4 - T_1) + (y - x)(T_4 - T_2) + zSt(T_4 - T_2) + S_1W_1(T_4 - T_2)$$

$$x = \frac{FN(T_3 - T_4) - y(T_4 - T_2) - zSt(T_4 - T_2) - S_1W_1(T_4 - T_2)}{80 + 0.5(T_1 - T_2) + (T_4 - T_1) - (T_4 - T_2)}$$

Since the total weight of material equals weight of water plus total solids

$$SW(T_4 - T_2) = y(T_4 - T_2) + zSt(T_4 - T_2)$$

Then

$$x = \frac{FN(T_3 - T_4) - SW(T_4 - T_2) - S_1W_1(T_4 - T_2)}{80 + 0.5(T_1 - T_2) + (T_4 - T_1) - (T_4 - T_2)}$$

Combining terms

$$x = \frac{FN(T_3 - T_4) - (SW + S_1W_1)(T_4 - T_2)}{80 + 0.5(T_2 - T_1)}$$

Data taken from one measurement are tabulated above and substituted in the formula to illustrate the calculation as follows

$$x = \frac{1.135 \times 50 \times (24.35 - 10.35) - (0.85 \times 11.0455 + 0.03 \times 5.5525)(10.35 + 20)}{80 + 0.5(-20 + 0.64)} = 7.1743$$

$$\text{Per cent. bound water} = 100\left(1 - \frac{7.1743}{9.8205}\right) = 26.94$$

The temperatures used were from -5 to $-35°$ inclusive by five-degree steps and in addition a series of measurements at $-12.5°$, to define better the curve between -10 and $-15°$; all measurements were made on whites from individual eggs, no attempt being made to mix a number of eggs to obtain a representative sample, since mixing would probably break down the gross colloidal structure postulated elsewhere by St. John and Green[9] and this might be expected to affect the water binding capacity. It is possible that this explains the low result obtained by Rubner.[7] Instead of mixing, determinations were made on from seven to thirty-five eggs for each temperature studied and the results averaged to give each of the points on the curve presented. Many of the measurements were made at two different temperatures on two different portions of the same egg, thus eliminating the possible effect of the variability between eggs.

Figure 1 is based on results obtained on the thick portion of egg white. The figure shows the percentage of bound water plotted against the temperature. At $-5°$ an average of about 80% of the water remained unfrozen, while at $-10°$ about 35% was not frozen. Determinations at $-12.5°$ showed about 25% of the water unfrozen. From this temperature on through $-35°$ we have a flat straight line curve showing that no more of

[9] St. John and Green, *J. Rheology*, **1**, 484 (1930).

the water is frozen at lower temperatures, down to at least −35°. This indicates that all of the freezable water is frozen at −12.5° and that the remainder cannot be frozen at considerably lower temperatures. This unfreezable water is considered to be bound water. Individual measurements at −20° gave the following results, 26.82, 25.01, 25.80, 26.23, 27.02, 26.94, 25.53, 26.08, 26.87, 27.02, 25.89, 26.24.

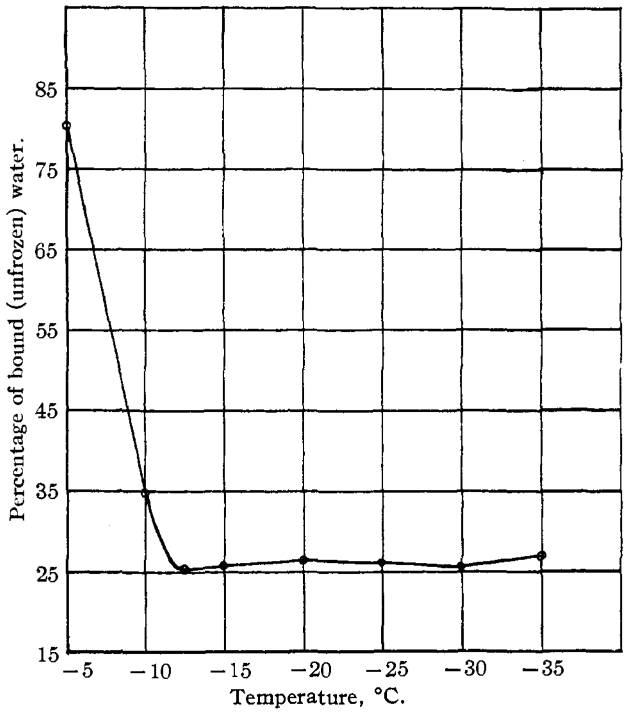

Fig. 1.—Relation of the temperature of freezing to the percentage of bound (unfrozen) water in egg white.

Working with certain inorganic hydrogels Foote and Saxton[10] found that lowering the temperature from −30 to −78° did not freeze an appreciable additional amount of the bound water in such hydrophilic colloids. A further somewhat indirect confirmation of the selection of −12.5° as the point at which all of the free water may be expected to freeze is the fact that the results on different eggs were somewhat more variable at −5 and −10° than at the other temperatures. On the basis of the data obtained it is calculated that there is about 1.97 g. of bound water for every gram of dry matter in the thick portion of egg white.

It has been suggested[11] that water found in biological material may exist

[10] Foote and Saxton, This Journal, **39**, 1103 (1917).

[11] Ostwald, quoted by Kuhn, *Kolloid-Z.*, **35**, 275 (1924).

in several different forms, namely: (1) as occlusion water, (2) capillary water, (3) osmotic water, (4) colloidal water, bound by physical forces, and (5) chemically bound water. The water which freezes at $-12.5°$ or below is probably either occlusion, capillary or osmotic water. It would not be expected that the osmotic pressure of the water in the white would be sufficient to prevent freezing at $-12.5°$. The ash of the white is about 0.60%. It is also probable that this bound water comes almost entirely under the class of colloidally bound water.

Summary

Using a freezing method for differentiating between bound and free water in a hydrophilic biocolloid, it is shown that a temperature of $-12.5°$ is sufficient to freeze all of the freezable (free) water and that the remaining water (bound water) is not frozen at temperatures ranging between -12.5 and $-35°$. The average amount of bound water in the thick portion of egg white is found to be about 26% as determined by this method. An improved formula for the calculation of free water is presented.

PULLMAN, WASHINGTON

[CONTRIBUTION FROM THE DIVISION OF LABORATORIES AND RESEARCH, NEW YORK STATE DEPARTMENT OF HEALTH]

A NOTE ON THE PREPARATION OF CEPHALIN

BY FRANK MALTANER

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The preparation of cephalin, of uniform composition and free from associated phosphatids, is of considerable importance not only for the study of the chemical and biological activity of this material but also for the study of its structure. While the results obtained by Levene and West,[1] with reduced cephalin, lend strong support to the generally accepted formula, $C_{41}H_{78}NPO_8$, the analytical figures found with the unreduced material, correspond more closely to the formula $C_{41}H_{78}NPO_{13}$.[2]

In a recent publication[3] a method was described for the preparation of cephalin, free from lecithin, and approximating in composition the latter formula. Different samples prepared by this method were of constant composition and contained all of their nitrogen in the amino form. The yield, however, was small and variable and the product was yellow in color, when dried, evidently due to slight decomposition. A better and more uniform yield (about one gram per pound of fresh brain) of a pure white prod-

[1] P. A. Levene and C. J. West, *J. Biol. Chem.*, **35**, 285 (1918).

[2] Hugh Maclean, "Lecithin and Allied Substances, the Lipins," Longmans, Green & Co., London, **1918**, p. 45.

[3] Augustus Wadsworth, Frank Maltaner and Elizabeth Maltaner, *Am. J. Physiol.*, **97**, 74 (1931).