BOUND WATER CONTENT OF VEGETATIVE AND SPORE FORMS OF BACTERIA

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In a previous paper (Henry and Friedman, 1937) we confirmed the works of Dyrmont (1886) and Virtanen and Pulkki (1933) which showed that little difference exists in the water content of vegetative cells and spores of a given species of bacteria. These results indicated that the commonly accepted idea that a low water content in the spore form is responsible for the observed heat resistance of this type of cell was not justified.

Virtanen and Pulkki advanced the theory that the enzymes present in the bacterial spore were in an inactive or resistant form. We suggested that the resistance, whether it concerned the enzymes or the bacterial protoplasm proper, might be due to differences in the percentage of bound water in the two types of cells. This suggestion was based on the report of Newton and Martin (1930) which shows that the resistance of certain plants to drought and freezing is, in part, due to their relatively high percentages of bound water.

The present paper is a report of the relative amount of bound water found in the vegetative cells and spores of *Bacillus mycoides*, *Bacillus megatherium* and *Bacillus subtilis*, as determined by the cryoscopic method (Newton and Gortner, 1922). This method was chosen because of its relative simplicity and because of the similarity of our problem to that of Skovholt and Bailey (1935) when they determined the bound water in flour. The procedure is based on the assumption that bound water does not alter the freezing point of a given solution of sucrose, and therefore if a weighed quantity of bacterial cells with a known water content, as determined by desiccation of a portion of a uniform sample, is added to a sugar solution, changes in the freezing point of the solution will be due to the unbound water; the difference between the amount of water which affects the freezing point of the solution and the total water content as determined by desiccation should represent water in a bound state. While the method employed is probably not sufficiently accurate to make the percentages of bound and free water found in a given cell suspension entirely reliable, it is possible to show that these errors will be similar in two determinations when conditions are controlled and that comparative data of significance may be obtained when suspensions of two types of cells are run under identical conditions.

METHODS

Suspensions of vegetative cells of *B. subtilis*, *B. mycoides* and *B. megatherium* were procured by growing the organisms on a medium of the following composition:

	grams
Glucose	2.5
Peptone	5.0
Sodium chloride	0.5
Dipotassium phosphate	5.0
Ammonium sulphate	2.5
Water	1000 ml.

Spores were obtained on a medium identical with that given above, except for the addition of 1.75 per cent agar. After harvesting, the cells were washed four times in distilled water and examined microscopically to determine the ratio of spore to vegetative cells. In all suspensions used the ratio of the desired type of cell to the other form was 200 to 1 or higher.

After thorough washing, the bacteria were blotted between silk and filter paper and prepared for sampling by being well mixed on a silk cloth. Representative samples were transferred to dry, weighed containers and one of these used to determine the water content while the others were used to measure the effect upon the freezing point of water or a standard sucrose solution. The total water content of the cells was determined by heating at 100–105°C. until a constant weight was obtained. This latter procedure was checked several times and shown to give consistent results.

EFFECT OF BACTERIA UPON THE FREEZING POINT OF DISTILLED WATER

By the addition of various quantities of a single mass of wet, vegetative cells of *B. mycoides*, the water content of which was determined, to known quantities of distilled water the effect of several concentrations of the cells themselves upon the lowering of the freezing point was obtained. After correcting for under-cooling by using the tables of Harris (1925) it was possible to calculate the true freezing points of these suspensions on the basis of grams of solids per 100 grams of water. The results obtained are as follows:

BACTERIA	WATER	FREEZING POINT
grams	*grams*	°*C.*
0.6627	100	−0.018
1.1803	100	−0.022
3.1705	100	−0.030

If these points are plotted, using lowering of the freezing point against grams of organisms per 100 grams of water, a straight line relationship can be demonstrated. Because of this linear relationship the effect of any quantity of bacterial material upon the freezing point of water can be obtained.

DETERMINATION OF BOUND WATER IN BACTERIAL CELLS

Accurately weighed quantities of moist bacteria with a known water content were placed in distilled water and in sucrose solution. This sugar solution was prepared by adding 16.000 grams of sucrose, which had been dried three days over fresh calcium chloride, to sufficient water to make 100 ml. of solution. Density measurements by the pycnometer method were made and the concentration of sucrose was found to be 0.1507(5) gram for each gram of solution used. The weights of total water, bacterial solids and sucrose were determined and the freezing point for each mixture was established. Masses of spores and vegetative cells of a given species were run in the same manner and all solutions and procedures were as nearly identical as possible for the two types of cells.

As an example of the results thus obtained the figures for *B. subtilis* are given in table 1.

TABLE 1

The effects of B. subtilis spores and vegetative cells upon the freezing points of distilled water and sucrose solution

	Vegetative cells 68.86 per cent water, 31.14 per cent solids		Spores 72.38 per cent water, 27.62 per cent solids	
	Distilled water	Sucrose solution	Distilled water	Sucrose solution
Moist weight of bacteria	0.3693	0.4609	0.5072	0.5581
Weight water added	13.0532		14.0895	
Weight sugar solution added		14.6864		15.1076
Freezing point	−0.019	−1.034	−0.020	−1.066
Undercooling, degrees C	1.0	0.8	0.6	1.1

From these data, again using *B. subtilis* as an example, it is possible to obtain or calculate:

1. The dry weight of the bacteria in sugar solution:
   Vegetative cells .... 0.1435 gram
   Spores .... 0.1541 gram
2. The weight of sucrose in the sugar solution:
   Vegetative cells .... 2.2139 grams
   Spores .... 2.2775 grams
3. The total weight of water in the sugar solution:
   Vegetative cells .... 12.7898 grams
   Spores .... 13.2340 grams
4. The weight of organisms per 100 grams of water:
   Vegetative cells .... 1.122 grams
   Spores .... 1.165 grams
5. The corrected freezing points in sugar solution:
   Vegetative cells .... −1.028°C.
   Spores .... −1.051°C.
6. The corrected freezing points in distilled water for quantities of bacteria equal to those used in sugar solutions:
   Vegetative cells .... −0.024°C.
   Spores .... −0.022°C.

7. The weight of sucrose per 100 grams water:

Vegetative cells	17.3098 grams
Spores	17.2094 grams

By applying the formula developed by Gortner and his coworkers (Newton and Gortner, 1922) to these figures, it is possible to calculate the bound water present in the system. This formula is:

$$\frac{T - (t + K)C}{T - t} = \text{per cent bound water.}$$

where T = observed lowering of freezing point in sugar solution containing bacteria.
t = lowering of freezing point in distilled water plus bacteria (corrected).
K = constant calculated for lowering of freezing point of sucrose solution.
C = constant dependent upon concentration, related to amount of available water.

For first calculations the value of K was that of Sayre's (1932). The value of C may be calculated:

$$100 - \frac{(\text{grams sucrose per 100 grams water})\ (18)\ (6)}{342.2}$$

The percentage of bound water multiplied by the weight of the total water in the system gives the weight of bound water. In the following calculation the change in C due to water added with the mass of organisms is disregarded as insignificant.

Results obtained by this treatment of the data gave, in some cases, a negative value and for this reason a constant K^1 was used. K^1 was obtained by assuming 0.014° less lowering, for all concentrations of sugar, than the values observed by Sayre. This procedure is justified in that our interest lies in comparative rather than absolute values for the amount of bound water present in the various cell suspensions. Also as Sayre says: "This difference may be due to some systematic error in all freezing point measurements, such as purity of sucrose used, the degree of undercooling, or the calibration of the thermometer."

By similar treatment of data obtained when the vegetative and spore forms of *B. megatherium* and *B. mycoides* were used the water binding capacity of bacterial cell materials of the three species was determined. Table 2 gives results expressed as grams of water bound per gram of solids and also the per cent of bound water in the moist bacterial masses. These latter figures were obtained in the following manner:

(Grams solids per 100 grams moist mass)
(grams water bound per gram solid) = per cent bound water.

If the amount of water which may be bound by the solids in a given cell mass is subtracted from the total water present, the percentage of free water in *B. subtilis* vegetative cells would be

TABLE 2

The bound water content of vegetative cells and spores of three bacterial species

	B. SUBTILIS		B. MEGATHERIUM		B. MYCOIDES	
	Vegetative	Spores	Vegetative	Spores	Vegetative	Spores
Grams bound water per gram solids	0	2.5	0.8	1.9	1.3	2.0
Per cent bound water in bacterial mass	0	69.0	17.7	62.6	28.2	58.7

68.9, in spores 3.4; for *B. megatherium*, 60.2 and 4.5, and for *B. mycoides* 50.0 and 11.9.

The thermal death time at 100°C. was determined for suspensions of spores of these three species which had been grown on the medium described above.

That a rather close correlation was found between the calculated free water and the relative heat resistance of the spores is shown below:

	FREE WATER	THERMAL DEATH TIME AT 100°C.
	per cent	*minutes*
B. subtilis spores	3.4	6
B. megatherium spores	4.6	4
B. mycoides spores	11.6	2

The free water, as determined by us, approximates the total water content which in the past has been assumed for spores in explaining their heat resistance on this basis.

Thermal death point determinations showed that the vegetative cells of all three species are destroyed at approximately the same temperature (50°C.). This would be expected if the work of Lewith (1890) on the effect of various concentrations of water on the coagulation temperature of albumin is accepted. Lewith showed that variations in high water concentrations did not materially affect the coagulation temperature of egg albumin, whereas low concentration differences markedly influenced the coagulation temperature.

SUMMARY

1. Bound water determinations, by the cryoscopic method, have been made on the vegetative and spore forms of *Bacillus subtilis*, *Bacillus megatherium* and *Bacillus mycoides*.

2. In all cases the spores were shown to have a far greater water binding capacity than did the vegetative cells.

3. The theory is advanced that the heat resistance of bacterial spores is due in part at least to the relatively high percentage of water in the bound state.

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