The Water and Solid Content of Living Bacterial Spores and Vegetative Cells as Indicated by Refractive Index Measurements

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SUMMARY: Refractive index measurements were made on the spores and vegetative cells of strains of *Bacillus cereus*, *B. cereus* var. *mycoides* and *B. megaterium* by phase contrast and interference microscopy with protein immersion. The refractive indices of the spores were found to be very high and comparable with that of dehydrated protein, suggesting that they contained very little water. Those of the vegetative cells were much lower, and indicated a solid content of about 30 %, w/v.

Because of their relatively high resistance to heat and other lethal agents, it has long been thought probable that bacterial spores contain appreciably less water than the vegetative forms. Only a few attempts to measure the water content of spores or vegetative cells have, however, been described (Virtanen & Pulkki, 1933; Henry & Friedman, 1937), and the methods used, all of which involved bulk weighing and varying degrees of desiccation, were necessarily rather crude and imprecise.

Refractive index measurements can now be made on microscopical objects with considerable accuracy (e.g. Barer, Ross & Tkaczyk, 1953; Mitchison & Swann, 1953; Davies, Wilkins, Chayen & La Cour, 1954; Ross, 1954; Barer & Joseph, 1954), and, as was first pointed out by Davies & Wilkins (1952) and by Barer (1952*a*), there is a close correlation between the refractive index of a cell and its water and solid content. It has been shown that the majority of substances found in living cells have approximately the same specific refraction increment (α) of *c.* 0.0018. This means that their combined refractive indices increase by about 0.0018 for every 1 % rise in their w/v concentration (Davies *et al.* 1954; Barer & Joseph, 1954).

In the present work, the refractive indices of the vegetative cells and spores of three selected organisms were measured with a view to obtaining a more accurate indication of their water and solid content than hitherto.

MATERIAL AND METHODS

Cultures. The vegetative cells and spores measured were of single typical strains of *Bacillus cereus*, *B. cereus* var. *mycoides*, both isolated from milk, and *B. megaterium* (National Collection of Type Cultures 2607). All were grown on a 1 % Lemco 1 % peptone agar medium and incubated at 30°.

Method of measuring the vegetative cells. The refractive indices of the vegetative cells from 18 hr. cultures were measured by the method of immersion refractometry first used by Barer & Ross in 1952. The cells were suspended in a range of concentrations of protein in aqueous solution (bovine plasma albumin, fraction V, supplied by the Armour Laboratories, Eastbourne), of measured refractive index, and examined with a phase-contrast microscope. They then all appeared darker than the background when mounted in solutions having a lower refractive index than their own, and brighter in solutions of higher refractive index. In media with refractive indices approximating closely to that of the cells, they became almost invisible. This method is a very sensitive one, capable of detecting refractive index differences of 0.001, which is approximately equivalent to 0.5 % of cell solids. It was found that the refractive indices of the individual vegetative cells varied by greater amounts than this, so that over a limited range of concentrations (in no case exceeding a refractive index range of 0.009, or 5 % of cell solids), dark and bright cells were both visible. The solution in which bright and dark cells were found to occur in approximately equal numbers was the one whose refractive index was taken as being equivalent to the mean refractive index of the cell population.

Method of measuring the spores. The method for the vegetative cells could not be used for the spores since it was found that their refractive indices were all higher than the most concentrated protein solutions obtainable, and non-toxic media of higher refractive index are difficult to find. Nevertheless, the fact that the middle of the spores all actually appeared bright under the phase-contrast microscope when they were mounted in water (Pl. 1, fig. 1), together with their small size, did indicate that the spores must have a considerably higher refractive index than the vegetative cells; because, with the particular phase plate used, this meant that the light passing through their maximum thickness was retarded by at least a quarter of a wavelength (Barer, 1952*b*). It is not possible, however, actually to measure such retardations with a phase-contrast microscope.

Instead, an interference microscope was used to measure the retardations of light through the spores when mounted in two media of different refractive indices, and from these measurements their mean refractive index was calculated. A Smith interference microscope (manufactured by Messrs Charles Baker of Holborn) was used, and measurements were made with a 2 mm. double focus objective in green light provided by a tungsten 'pointolite' lamp in conjunction with an Ilford 807 gelatin filter. The transmission of the filter with the light from this microscope lamp was investigated with a spectroscope, since these gelatin filters often show slight individual variations from the maker's specifications. It was found to give a narrow green band with a maximum intensity at 542 m μ ., which was taken as being the mean wavelength of the light used.

The maximum retardation in phase of the light through the middle of each spore was measured by extinction point measurement, first in preparations mounted in distilled water (refractive index 1.334), (Pl. 1, figs. 2, 3), and then in concentrated aqueous protein solutions (which had varying refractive indices

of about 1.40). The phase retardations of ten different spores were thus measured (as angles) in each of the two media, and the mean of each of the two sets of measurements taken.

The mean spore thickness was then calculated from the formula

$$t = \frac{\phi_1 - \phi_2}{(n_2 - n_1) 360} \times \lambda, \quad (1)$$

where t = the mean spore thickness (along the optical axis of the microscope) in μ ., ϕ_1 = the mean phase retardation of the spores in water, ϕ_2 = the mean retardation of the spores in the protein medium, n_1 = the refractive index of water (1.334), n_2 = the refractive index of the protein medium, and λ = the mean wavelength of the light used in μ . (0.542). The mean refractive index of the spores, n_s , was then calculated from the formula

$$n_s = \left(\frac{\phi_1}{360} \times \frac{\lambda}{t} \right) + n_1. \quad (2)$$

This method was similar to that employed by Barer in 1953, except that the phase-change measurements, instead of being made on the same individual cells suspended successively in two media, were made on a different sample of spores in each medium. This was because of the experimental difficulty of completely replacing one mounting medium with another without washing all the spores out of the field.

RESULTS

Vegetative cells. The refractive indices of the individual vegetative cells of *Bacillus cereus* were all found to lie between 1.3845 and 1.3880 and they had a mean refractive index of 1.3865. The other two organisms were rather more variable; those of *B. cereus* var. *mycoides* lay between 1.3960 and 1.4030 with a mean of 1.4000, and those of *B. megaterium* between 1.3830 and 1.3920 with a mean of 1.3880.

Spores. The results of two sets of measurements on different cultures of the spores of each organism are shown in Table 1. It will be seen that in each case the values for the mean thickness of the spores and their mean refractive index varied very little in the two successive experiments. The values for ϕ_1 are all comparable since the mounting medium was water in every case, but the values for ϕ_2 are not, because the refractive indices of the protein mounting media were different in the two experiments.

The accuracy of the values obtained. As has already been mentioned, the direct immersion and matching method used for measuring the refractive indices of the vegetative cells is very critical, and accurate to the nearest 0.001 which is less than the variation found in the refractive indices of the individual cells in each culture.

With the method used for the spores, it was not possible to obtain values for the refractive index of individual spores since, necessarily, different spores had to be measured in each medium. In deducing their mean refractive index, it

was assumed that the mean phase change and thickness of the spore population behaved in essentially the same way as that of an individual spore.

Such an assumption would seem to be a reasonable one to make in the absence of evidence to the contrary, but circumstances can be envisaged where it might give rise to a certain degree of error in the values obtained for the mean refractive index of the population. This might happen, for instance, if the refractive indices of individual spores varied appreciably according to their thickness, as might occur in a population containing germinating spores, which are known to swell and imbibe water; but the material used contained only resting spores.

Table 1. *The mean angular retardations of light, of wavelength 542 mμ., through spores mounted in water and in protein solutions measured by the interference microscope and their mean thicknesses and refractive indices calculated therefrom*

	<i>B. cereus</i>		<i>B. cereus</i> var. <i>mycoides</i>		<i>B. megaterium</i>	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 1	Expt. 2
Refractive index of protein medium (n_s)	1.3925	1.3960	1.3925	1.4000	1.3925	1.3880
Mean retardation in water (degrees) (from 10 measurements of different spores) (ϕ_1)	126.8	132.8	123.6	124.6	134.0	130.2
Mean retardation in protein (degrees) (from 10 measurements of different spores) (ϕ_2)	84.8	87.4	86.4	79.4	95.0	96.0
Mean thickness in μ . (t)	1.08	1.10	0.96	1.02	1.00	0.95
Mean refractive index (n_s)	1.521	1.513	1.528	1.519	1.537	1.540

It was thought advisable to get additional evidence to support this assumption, and so an alternative method was also used to obtain approximate values for the refractive indices of individual spores in the case of *Bacillus cereus*. Spores of this organism were mounted in distilled water and the phase retardations through ten spores were measured in the same way as before, but the thickness of each spore was also measured as accurately as possible with an eyepiece micrometer. The refractive index of each spore, n_s , was then calculated from the formula

$$n_s = \left(\frac{\phi_1}{360} \times \frac{\lambda}{t} \right) + n_m, \quad (3)$$

where ϕ_1 = the phase retardation through each spore, λ = the mean wavelength of the light used (0.542 μ .), n_m = the refractive index of mounting medium (1.3340) and t = the thickness of each spore estimated to the nearest 0.2 μ . These measurements, and the refractive indices calculated from them, are shown in Table 2.

This method, however, is not a very critical one, and its accuracy is limited by the accuracy with which one can measure the spore thickness by direct measurement. With the 2 mm. double focus objective used, it is very doubtful

if the thicknesses were measured accurately to more than the nearest 0.4μ ., and the final column of the table shows the limits of the refractive index of each spore assuming a maximum error of $\pm 0.2\mu$. in their measured thicknesses.

Table 2. *Individual measurements of the maximum width and angular retardation of light of wavelength 542 m μ . through spores of B. cereus mounted in water, and the refractive indices of the spores calculated therefrom*

Spore no.	Width (to the nearest 0.2μ .) measured by micrometer eyepiece (t) (μ .)	Retardation through spore in water (degrees) (ϕ_1)	Refractive index of spore (n_s)	Range of refractive index of spore, assuming a max. error in width (t) measurement of $\pm 0.2\mu$.
1	0.9	96	1.495	1.466–1.542
2	0.9	114	1.524	1.490–1.579
3	0.9	112	1.521	1.488–1.576
4	1.1	128	1.509	1.482–1.549
5	1.1	120	1.490	1.474–1.534
6	1.1	124	1.504	1.478–1.541
7	1.1	126	1.507	1.480–1.544
8	1.1	134	1.519	1.490–1.558
9	1.3	150	1.510	1.485–1.539
10	1.5	176	1.511	1.490–1.538

(Range of refractive index of vegetative cells of *B. cereus* 1.384–1.388.)

In spite of the relative inaccuracy of the method, however, two important conclusions can be drawn from these results.

(1) There appears to be no appreciable correlation between the refractive index and the thickness of the spores; and the fact that the phase change increases with the spore thickness suggests that the refractive indices of both the larger and smaller spores is similar. (The same kind of correlation between phase change and thickness was found in the cultures of *Bacillus cereus* var. *mycoides* and *B. megaterium*.)

(2) Even allowing for the relative inaccuracy of the method, it will be seen that the refractive indices of the spores are nevertheless all considerably greater than those of the vegetative cells.

It is also interesting to see that the average of these ten phase-change measurements is 128.0° as compared with the values of 126.8 and 132.8° obtained previously, and the average of their refractive indices is 1.509 compared to the previous values of 1.512 and 1.513 (see Table 1). While too much should not be made of this, it does serve to indicate that the values obtained by the previous method are probably, in general, correct.

A further indication of the accuracy of the double-immersion method is given by the results obtained by using exactly the same technique to measure the mean refractive index of the vegetative cells of *Lactobacillus bulgaricus* (Ross, 1957, to be published). Here, the values obtained could be compared with those obtained by direct immersion refractometry, and it was found that, in nine separate cultures, these differed from each other and from those obtained by direct immersion and matching by a maximum of 0.0085 , which is equivalent to less than 5% of cell solids.

CONCLUSIONS AND DISCUSSION

Water content. If one assumes that the mean refraction increment (α) of the dissolved substances in the vegetative cells is 0.0018 per 1 % rise in concentration, the mean w/v concentration of cell solids, C , can then be found from the formula (Barer, 1956):

$$C = \frac{n_c - 1.334}{0.0018}, \quad (4)$$

where n_c = the mean refractive index of the vegetative cells. Table 3 shows the percentage of cell solids thus calculated. It also shows the percentage of water, assuming, first that the solids in the vegetative cells are composed predominantly of protein, and secondly that 1 g. of protein occupies 0.75 ml. in aqueous solution, which is approximately true.

Table 3. *Solid and water content of vegetative cells, spores and other materials calculated from refractive index measurements*

Material	Mean refractive index	Assuming $\alpha = 0.0018$		Assuming $\alpha = 0.0015$
		Mean solid content (in g./100 ml.)	Mean water content (in g./100 ml.)	Mean water content (in g./100 ml.)
Vegetative cells				
<i>B. cereus</i>	1.386	29.0	78.0	—
<i>B. cereus</i> var. <i>mycoides</i>	1.400	36.5	72.5	—
<i>B. megaterium</i>	1.388	30.0	77.5	—
Spores				
<i>B. cereus</i> , Expt. 1	1.512	99.0	26.0	11.0
Expt. 2	1.513	99.5	25.5	10.5
<i>B. cereus</i> var. <i>mycoides</i>				
Expt. 1	1.528	108.0	18.0	3.0
Expt. 2	1.519	102.5	23.0	7.5
<i>B. megaterium</i> , Expt. 1	1.537	113.0	15.0	-1.5
Expt. 2	1.540	114.5	14.0	-2.5
Leather	1.530*	109.0	18.0	1.5
Wool	1.540*	114.5	14.0	-2.5
Dried casein	1.540*	114.5	14.0	-2.5

* Chamot & Mason (1938).

The exact interpretation, in terms of water and solid content, of the much higher values obtained for the refractive indices of the spores is more controversial, since it has been found that the results obtained from the extrapolation of formula (4) above do not accord exactly with the experimental evidence available (Davies *et al.* 1954; Barer & Joseph, 1954; Barer, 1956). This suggests that the refractive index of dried protein may be rather lower than might be expected, and Davies *et al.* (1954) have suggested that the refraction increment (α) of dried protein may be as low as 0.0015. It is certain, however, that these values indicate a very high content of solids, since they are comparable with the values obtained experimentally for the refractive indices of almost completely dehydrated protein products such as leather (1.53), wool and dried

casein (both 1.54), quoted by Chamot & Mason (1938), and they strongly suggest that the spores must contain very little water. Table 3 also shows the solid and water concentrations of the spores calculated on the same assumption used in the case of the vegetative cells. It is quite possible, however, that the values for the water content thus calculated are rather too high, and the final column of the table shows the even lower values obtained assuming the refraction increment of 0.0015 suggested by Davies *et al.* 1954. This method is, of course, only capable of indicating the total water content of the spores. It is not capable of showing whether, as was suggested by Friedman & Henry in 1938, a proportion of this occurs as 'bound water'.

Previous measurements of the water content of vegetative cells and spores. Henry & Friedman (1937) estimated the water content of the spores and vegetative cells of two of the three organisms used in the present investigation from bulk weighing and drying. A known weight of each organism, which had previously been blotted with filter paper, was air-dried, desiccated over CaCl_2 , and finally oven-dried; and it was reweighed at each stage. They found that, throughout the whole process, the spores of *Bacillus megaterium* lost 58 % by weight of water and those of *B. cereus* var. *mycoides* 71 %; while the vegetative cells of *B. megaterium* lost 80 %, and those of *B. cereus* var. *mycoides* 88 %. Although the method was almost certainly too crude for these figures to be regarded as being at all reliable absolute values, they do accord with the results of the present investigation in as far as they indicate that the spores contained appreciably less water than the vegetative cells.

Henry & Friedman did not, however, base their final estimation of water content on the overall loss of weight, but only on the percentage loss of weight between the stages of air drying and oven drying. The reason for their doing this is not altogether clear, for although, as they themselves point out, neither the blotted nor the air dried cells were very satisfactory starting materials, the former would seem to have been preferable in so far as the vegetative cells were probably mostly still intact, which they were unlikely to have been after air drying. The conclusion they consequently drew, to the effect that both the spores and the vegetative cells contained similar amounts of water (*c.* 16 %), is almost certainly incorrect.

Powell & Strange (1953) have also expressed the opinion that the water content of spores must be extremely low, and some preliminary weighing experiments of their own, with *Bacillus subtilis*, supported this view.

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K. F. A. ROSS AND E. BILLING—THE WATER CONTENT OF BACTERIA AND SPORES.
PLATE 1

(Facing p 425)

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EXPLANATION OF PLATE

Fig. 1 was taken with a Watson phase-contrast microscope with a 2 mm. fluorite objective and a 25 % absorbing 90° positive phase plate. Figs. 2 and 3 were taken with a Smith interference microscope with a 2 mm. 'double focus' objective and an Ilford 807 (mercury green) filter. The scale, indicated at the bottom of the page, is the same for all the photographs.

Fig. 1. Phase-contrast photograph of the spores of *B. cereus* mounted in water. The centres of the spores appear bright, indicating that they are appreciably more refractile than the vegetative cells.

Fig. 2. Photograph with the interference microscope of the spores of *B. cereus* mounted in water with the analyser goniometer set at 145°. At this setting the field appears maximally dark.

Fig. 3. The same preparation as in Fig. 2 with the goniometer analyser set at 80°. A number of the spores now appear maximally dark. The rotation of the analyser from the position in Fig. 2 represents a phase retardation of 130°.

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