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Photometric Immersion Refractometry of Bacterial Spores†

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Photometric immersion refractometry was used to determine the average apparent refractive index ($\bar{n}$) of five types of dormant *Bacillus* spores representing a 600-fold range in moist-heat resistance determined as a D_{100} value. The $\bar{n}$ of a spore type increased as the molecular size of various immersion solutes decreased. For comparison of the spore types, the $\bar{n}$ of the entire spore and of the isolated integument was determined by use of bovine serum albumin, which is excluded from permeating into them. The $\bar{n}$ of the sporoplast (the structures bounded by the outer pericortex membrane) was determined by use of glucose, which was shown to permeate into the spore only as deeply as the pericortex membrane. Among the various spore types, an exponential increase in the heat resistance correlated with the $\bar{n}$ of the entire spore and of the sporoplast, but not of the isolated perisporoplast integument. Correlation of the $\bar{n}$ with the solids content of the entire spore provided a method of experimentally obtaining the refractive index increment ($d\bar{n}/dc$), which was constant for the various spore types and enables the calculation of solids and water content from an $\bar{n}$. Altogether, the results showed that the total water content is distributed unequally within the dormant spore, with less water in the sporoplast than in the perisporoplast integument, and that the sporoplast becomes more refractile and therefore more dehydrated as the heat resistance becomes greater among the various spore types.

The water content of the entire dormant spore is inversely correlated with the heat resistance among various species and types over a wide resistance range (4). Although less than in vegetative cells, the total water content in spores is too great for dehydration to account for their heat resistance. A relatively less hydrated interior and more hydrated exterior is indicated by the historic microscopic image of a dormant spore with the two compartments appearing refractile and nonrefractile, respectively.

This apparently unequal distribution of water in the dormant spore seemed quantifiable by use of immersion refractometry. The use of light as an invasive but nondestructive probe is important because the property of heat resistance and the unequal distribution of water appear to depend upon unique in situ characteristics which are lost when the integrity of the spore is disrupted.

Immersion refractometry was first applied quantitatively in cell biology by Barer et al. (2). The technique involves matching the refractive index of immersion solutions with that of cells or cell components. The principles and applications were fully described by Barer (1a) and Ross (12). Generally, a phase or interference microscope is used to observe refractive index matching, e.g., as judged by the concentration of a bovine serum albumin solution required to cause half of the individual cells in a population to undergo phase reversal. The corresponding refractive index (n) of the immersion solution is considered to represent the average apparent refractive index ($\bar{n}$) of the cells.

Bacterial cells were first studied with immersion refractometry by Bateman et al. (3), who introduced the important use of photometry to measure refractility changes in the cell population as the n of the immersion solution changes. Marquis (11) adapted and refined the photometric technique using isolated bacterial cell walls, which provided a less complicated model for study.

In the present study, photometric immersion refractometry was used with differentially permeating solutes to determine the $\bar{n}$ of a membrane-bound compartment as well as of the entire spore. By use of an immersion solute such as bovine serum albumin which cannot permeate into the spore at all (8), the $\bar{n}$ represented the entire spore. By use of an immersion solute such as glucose, which was shown to permeate into the spore only as deeply as the outer pericortex membrane, the $\bar{n}$ represented the sporoplast (the structures bounded by the pericortex mem-

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brane). These differentiating $\bar{n}$ values were obtained for five types of dormant spores representing a wide range in heat resistance. Their refractive index increment ($d\bar{n}/dc$) was determined experimentally, thus enabling solids and water contents to be calculated.

(A preliminary abstract of these findings appeared in Spore Newslet. 7(5):12–13, 1981).

MATERIALS AND METHODS

Preparation of the various types of spores and of their isolated perisporoplast integument fraction and determination of their heat resistance were described previously (4).

The n of immersion solutions was measured by use of a precision refractometer (model 33-45-01, Bausch and Lomb, Inc., Rochester, N.Y.) with a 33-45-01 prism, light of 589-nm wavelength from a sodium lamp, a sensitivity of 0.00002 units of n, and a temperature of 25°C. The immersion solutions were prepared in twofold serial dilutions from the maximal (saturation) concentrations of the solutes. Molecular radii, expressed as the Einstein-Stokes diffusion radius, were obtained from the tabulation of Scherrer and Gerhardt (14). For polydisperse polymers, the radius corresponded to the number average molecular weight ($\overline{M}_n$).

The $\bar{n}$ of an intact spore (or integument) was obtained by suspending and equilibrating an equal concentration of spores in distilled water and in each of the serial dilutions of a solute, and measuring the corresponding changes in optical density (OD). The OD was measured by use of a double-beam spectrophotometer (model DB-G, Beckman Instruments, Inc., Fullerton, Calif.) at a 700-mm wavelength with glass cuvettes of 10-mm light path. The changes were expressed as percentage of the initial OD, which represented the spores immersed in water at about OD = 0.6. A representative plot of data is shown in Fig. 1, and the procedure for obtaining $\bar{n}$ is described in Results.

Permeability of the spores was determined by the space (thick-suspension) technique (8, 14). A 3-g pellet of spores and 3 ml of a 3% solution were used to enhance accuracy. *Bacillus cereus* spores were centrifuged at 17,000 × g for 30 min. *B. subtilis* and *B. stearothermophilus* spores were centrifuged at only 10,000 × g for 30 min to prevent germination that occurred at higher force. Dextran of $\overline{M}_n$ 2,000,000 (T2000, Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) was used as a spore-nonpenetrating solute to determine the fraction of the pellet weight occupied by the interstitial volume (S_{in}^{W}). To determine the volume fraction of the spore permeated by glucose, the fraction of pellet weight occupied by the glucose-permeable volume less the dextran-permeable volume was determined on a weight basis ($R_{glucose}^{W}$). This value was converted to a volume basis ($R_{glucose}^{V}$) by use of the wet density and finally was expressed as the glucose-impermeable volume fraction ($1 - R_{glucose}^{V}$) of the spore.

RESULTS

Determination of $\bar{n}$. The procedure for determining an $\bar{n}$ value is exemplified in Fig. 1, which shows a plot of the OD of a spore suspension versus the n of an immersion solution. The plot originated at the measured n of the distilled water (1.3325). With increasing n of the immersion solution, the plot of OD decreased linearly at first but eventually became concave. The linear portion, plotted by means of the least-squares technique, was extrapolated to an abscissa intercept which represented the $\bar{n}$ of the spores. Observations with a phase-contrast microscope insured that, in the immersion solutions corresponding to the linear portion of the plot, all of the spores remained brightly refractile (that is, none underwent phase reversal) and unaggregated. With bacterial vegetative cells (3) or isolated cell walls (11), a parabolic curve is obtained because of their lesser refractility; consequently, complete phase reversal occurs with them in concentrated immersion solutions. With spores, however, only the beginnings of phase change occurred.

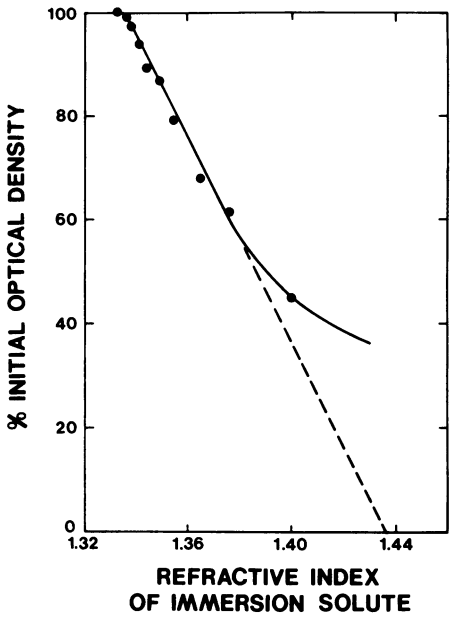

FIG. 1. Determination of the average apparent refractive index of spores ($\bar{n}$ = 1.436) by extrapolation from the least-squares line correlating OD of the spore suspension and refractive index of the immersion solution. The example shown is for the dormant spore of *B. stearothermophilus*, rough variant, with bovine serum albumin as the solute.

The procedure was also applied to spore-size polystyrene beads (0.79 μm, Dow Diagnostics, Indianapolis, Ind.). The value obtained by photometric immersion refractometry with bovine serum albumin ($\bar{n}$ = 1.522) was less than the known value (n = 1.5905; 5). Consequently the $\bar{n}$

of spores must be considered literally as an apparent value and was used only as an indicator of solids and water content until a correlation constant (that is, $d\bar{n}/dc$) was obtained in subsequent experiments.

Effect of immersion-solute size on $\bar{n}$. The procedure for determining the $\bar{n}$ of spores was applied using a series of immersion solutes that varied in molecular size. As the solute size increased, the spore $\bar{n}$ decreased (Fig. 2). The larger $\bar{n}$ of spores in solutions of smaller molecules can be related to the extent to which the molecules permeate into the spore structure (9). A similarly shaped curve at a lower range of $\bar{n}$ was observed by Marquis (11) with isolated vegetative cell walls.

The upper part of the curve in Fig. 2 was extrapolated by least-squares calculation to an intercept with an abscissa value of 0.15 nm, the radius of a water molecule. The resulting value of $\bar{n} = 1.511$ can be considered as an estimate of the $\bar{n}$ of the spore solids in the hydrated state, since water completely permeates the spore (9). This maximum $\bar{n}$ for spores was higher than that for isolated bacterial cell walls (11).

The lower part of the curve in Fig. 2 descends to a plateau representing the $\bar{n}$ of the entire spore as defined by solutes of extremely large molecular size, such as dextran of $\bar{M}_n$ 2,000,000, which are completely excluded from permeating the spore (9). Bovine serum albumin is almost but not completely excluded; however, it was selected for routine use here because of its previous use in measuring the $\bar{n}$ of spores (10, 13), its monodispersity, and its commercial availability in concentrated solution.

Structural identification of glucose-impermeable space. The volume fraction of the entire spore occupied by the glucose-impermeable space was determined for the various spore types and compared with the fraction occupied by the sporoplast and protoplast volumes, assuming that either the pericortex membrane or the pericytoplasm membrane functions as a permeability barrier to glucose (Table 1). Although the permeability and electron microscopy measurements were only roughly comparable, it was clear that the glucose-impermeable space corresponded to the physical volume occupied by the sporoplast rather than the protoplast. The comparisons were particularly significant for the two types of *B. stearothermophilus* spore lacking an

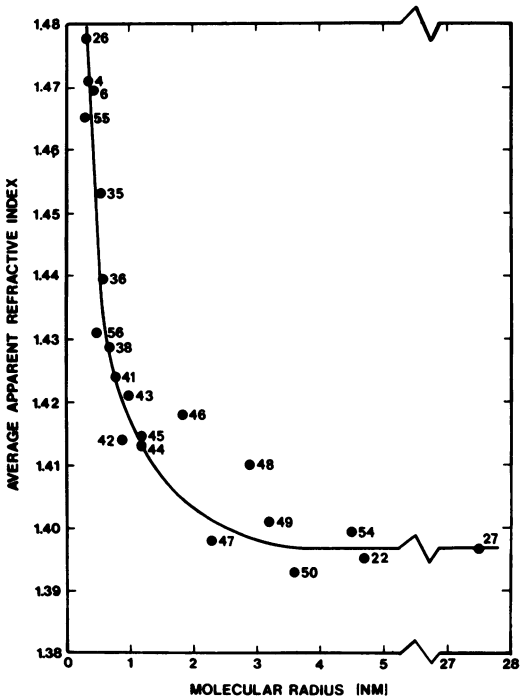

FIG. 2. Average apparent refractive index of the dormant spore of *B. stearothermophilus*, rough variant, as a function of the average molecular radius of immersion solutes. A similar curve was obtained using *B. cereus* T spores. The identification numbers correspond to the immersion solutes as follows and as tabulated by Scherrer and Gerhardt (14): 28, ethylene glycol; 4, glucose; 6, sucrose; 55, glycerol; 35, polyethylene glycol 200; 36, polyethylene glycol 300; 56, tetraethylene glycol; 38, polyethylene glycol 400; 41, polyethylene glycol 600; 42, methoxypolyethylene glycol 750; 43, polyethylene glycol 1000; 44, polyethylene E1450; 45, polyethylene glycol 1540; 46, polyethylene glycol 4000; 47, polyethylene glycol E4000; 48, polyethylene glycol E6000; 49, polyethylene glycol 6000; 50, polyethylene glycol E9000; 54, bovine serum albumin; 22, dextran 40; 27, dextran 2000.

TABLE 1. Comparison of the volume fraction of the entire spore occupied by structural compartments in various dormant spore types, determined from glucose permeability and electron microscopy measurements

Spore type (species, strain, variant)	Vol fraction of entire spore		
	Glucose-impermeable vol	Sporoplast vol[a]	Protoplast vol[a]
B. cereus T, calcium sufficient	0.55	0.71	0.32
B. subtilis subsp. *niger*	0.69	0.51	0.14
B. stearothermophilus, rough	0.63	0.61	0.22
B. stearothermophilus, smooth	0.65	0.75	0.20

[a] Data from Beaman et al. (4), determined from measurements on electron micrographs of medially thin-sectioned spores.

TABLE 2. Average apparent refractive index ($\bar{n}$) of the entire spore, the sporoplast, and the isolated perisporoplast integument of various spore types

Spore type (species, strain, variant)	D_{100} (min)	$\bar{n}$		
		Entire spore[a]	Sporoplast[b]	Perisporoplast integument[a]
B. cereus T, germinated	0.001	1.370		
B. cereus T, calcium deficient	1.00	1.404	1.450	1.372
B. cereus T, calcium sufficient	4.39	1.419	1.456	1.370
B. subtilis subsp. *niger*	40.0	1.429	1.468	1.380
B. stearothermophilus, rough	124	1.442	1.471	1.396
B. stearothermophilus, smooth	579	1.459	1.484	1.396

[a] Determined with bovine serum albumin.
[b] Determined with glucose.

exosporium, the presence of which complicates measurements in the other two species.

Values of $\bar{n}$ for entire spore and sporoplast of various spore types. Consequently, bovine serum albumin was used to determine the $\bar{n}$ of the entire spore and glucose was used to determine the $\bar{n}$ of the sporoplast in the five types of dormant spores and one type of germinated spore which represented a wide range in resistance to moist heat (Table 2). The $\bar{n}$ of the sporoplast was consistently higher than that of the intact spore in all of the dormant spore types. An $\bar{n}$ determined with glucose could not be obtained for the germinated spore because of active metabolism.

Among the various types of dormant spores, an exponential increase in the heat resistance correlated with an increase both in the $\bar{n}$ determined with bovine serum albumin for the entire spore and in the $\bar{n}$ determined with glucose for the sporoplast (Fig. 3).

Values of $\bar{n}$ for isolated integument fraction of various spore types. The $\bar{n}$ of the perisporoplast integument fraction isolated from the various spore types also was determined using bovine serum albumin (Table 2). For the *B. cereus* and *B. subtilis* spores, the fraction consisted of the coats and exosporium; for the *B. stearothermophilus* spores, it consisted only of the coats. Among the various spore types, the $\bar{n}$ of the perisporoplast integument was lower than that of the sporoplast or of the entire spore in all of the dormant spore types. Furthermore, the $\bar{n}$ of the perisporoplast integument varied irregularly and did not correlate with the heat resistance of the spores.

Determination *of* $d\bar{n}/dc$. Figure 4 shows that, among the various types of dormant spores, an increase in the $\bar{n}$ correlated with an increase in the solids content determined separately by a direct mass method (4). The solids content is related to the $\bar{n}$ of cells according to the equation: grams of solids per milliliter of wet cells = $(\bar{n} - n_w)\,(d\bar{n}/dc)^{-1}$, where n_w is the refractive index of water and $d\bar{n}/dc$ is the refractive index increment of the cells (12). Consequently, from the line plotted in Fig. 4, the slope yielded the average $d\bar{n}/dc$ of the spores (0.168 ml/g). The close fit of points to the line indicated that the $d\bar{n}/dc$ was essentially the same for the various types of dormant spores. By use of this correlation constant and the foregoing equation, the solids content can be calculated from the $\bar{n}$ of these and presumably other spore types. The water content in turn can be calculated from the solids content by the difference from the wet density (4).

The method was verified by similarly measuring the solids content by the direct mass method and solving the equation to obtain the $d\bar{n}/dc$ for vegetative cells of *Serratia marcescens* (0.168 ml/g). This value agreed closely with the value of 0.170 ml/g obtained by interpolating the results from two other methods (3, 6) to the same wavelength (589 nm) used in the present study.

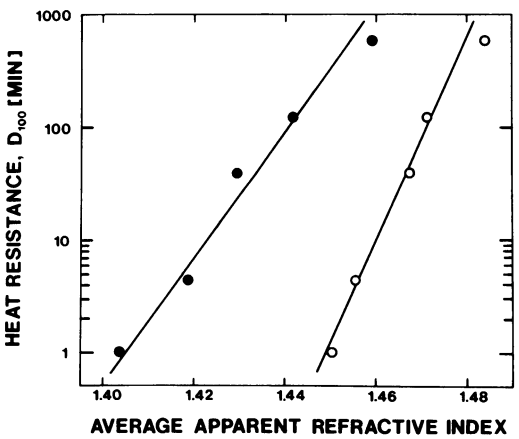

FIG. 3. Heat resistance determined as D_{100} correlated with average apparent refractive index determined with bovine serum albumin for the entire spore (●) and with glucose for the sporoplast (○) of various dormant spore types.

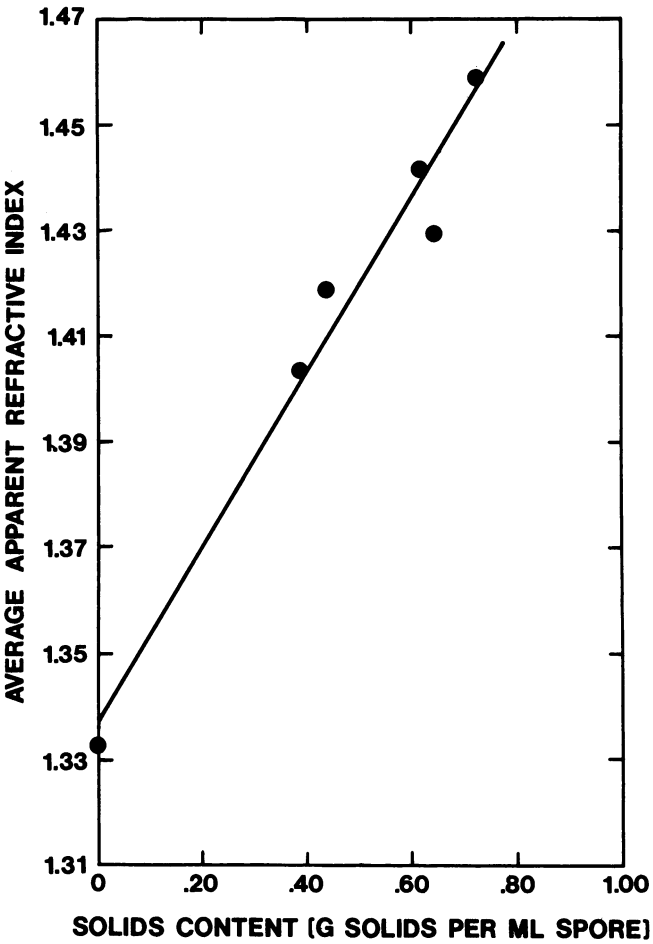

FIG. 4. Average apparent refractive index determined with bovine serum albumin correlated with the separately determined (4) solids content of the entire spore of various dormant spores types. The least-squares slope provided the average apparent refractive index increment ($d\bar{n}/dc$ = 0.168 ml/g) from the equation: grams of solids per milliliter of wet spores = $(\bar{n} - n_w)\ (d\bar{n}/dc)^{-1}$.

DISCUSSION

The main finding from the foregoing results was the quantitation and partial structural identification of unequal water distribution within the dormant bacterial spore. Not only was the sporoplast more refractile and therefore more dehydrated than the entire spore and the perisporoplast integument, but also the more resistant the spore type, the more refractile and dehydrated its sporoplast. That is, the $\bar{n}$ of the sporoplast > the $\bar{n}$ of the entire spore > the $\bar{n}$ of the perisporoplast integument; and the D_{100} of the spore correlated with the $\bar{n}$ of the sporoplast among the various spore types. Unfortunately, the actual water content of the sporoplast and the perisporoplast integument could not be calculated from their $\bar{n}$ values because their respective (and certainly different) $d\bar{n}/dc$ and wet density values were not known. Similarly, the relative water content of the protoplast importantly remains to be determined.

Identification of the glucose-impermeable space with the physical volume occupied by the sporoplast modified the conclusions previously drawn from permeability experiments by Gerhardt et al. (9). Their use of an exosporium-containing spore (*B. cereus* T), at a time when the existence of two spore membranes was not taken into account, led to the apparently erroneous conclusion that the inner pericytoplasm membrane functions as the primary permeability barrier of the intact dormant spore to glucose and similar molecules. The present results add further evidence that the outer pericortex membrane is intact and functional in the dormant spore (7, 11a). However, further work is needed to distinguish the role of each of the two mem-

branes in spore permeability and to determine whether the outer pericortex membrane corresponds to the pitted layer of the inner coat (1, 7a).

The determination of a constant $d\bar{n}/dc$ value for the entire spore of various types (0.168 ml/g) makes it possible in the future to calculate the solids and water contents from an $\bar{n}$ value. Experimentally determined values of $d\bar{n}/dc$ were obtained previously only for a few types of vegetative bacterial cells by means of specialized techniques (3, 6). In the previous studies of bacterial spores by Ross and Billing (13) and Leman (10) using immersion refractometry, a $d\bar{n}/dc$ of 0.180 ml/g had been assumed.

In the previous refractometric studies of spores, furthermore, a microscope had been used to observe phase changes in individual spores immersed in serial dilutions of bovin serum albumin. The refractive index matching apparently had been with the refractile interior, which dominates the microscopic image, rather than with the entire spore. This interpretation would explain the much higher values obtained by microscopic refractometry than by photometric refractometry. With spores of *B. cereus* T immersed in bovine serum albumin, for example, an $\bar{n}$ of 1.51 had been obtained in the previous studies (10, 13), whereas we obtained an $\bar{n}$ of 1.42. Even with these spores immersed in glucose to reflect the sporoplast, we obtained an $\bar{n}$ of 1.46. Only when we calculated a theoretical $\bar{n}$ for spores immersed in water to reflect the hydrated solids of the spore did we obtain a comparably high value, coincidentally $\bar{n} = 1.51$. The comparison suggests that the refractile interior of a spore observed in a microscope corresponds to the protoplast, and that it is even more refractile and therefore even more dehydrated than the sporoplast.

ACKNOWLEDGMENTS

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