Dielectric Study of the Physical State of Electrolytes and Water Within *Bacillus cereus* Spores

EDWIN L. CARSTENSEN, ROBERT E. MARQUIS, AND PHILIPP GERHARDT

Department of Electrical Engineering and Department of Microbiology, University of Rochester, Rochester, New York 14627, and Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan 48823

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Dielectric measurements revealed that dormant spores of *Bacillus cereus* have extremely low conductivities at high frequencies (50 MHz) and so must contain remarkably low concentrations of mobile ions both within the core and in the surrounding integuments. Activation, germination, and outgrowth were all accompanied by increases in conductivity of the cells and their suspending medium, and this result indicated that intracellular electrolytes had become ionized and leaked from the spores. High-frequency dielectric constants of spores were consistent with normal states for cell water. These values increased during successive stages of development from dormant spore to vegetative bacillus, and they could be directly related to increases in cell water content. In all, the results refuted a model of the dormant spore involving freely mobile, ionized electrolytes and supported a model involving electrostatically bound electrolytes.

The extraordinary physiological properties of dormant bacterial spores seem to be associated with equally extraordinary intracellular accumulations of small organic molecules and inorganic ions. Dipicolinic acid (DPA) has long been recognized as a unique constituent of spores (31), but recently large amounts of L-glutamic acid (GA) and a few other amino acids (24), 3-phospho-D-glyceric acid (PGA; 25), and sulfo-lactic acid (4) also have been found. Similarly, calcium is present in much greater amounts in spores than in vegetative cells (9), and inorganic phosphate (P_i) too has recently been found to accumulate (25). Representative types of these pooled materials (GA, PGS, and P_i) are not released from representative spores by treatment with cold water or 5% trichloroacetic acid, but they are released completely by treatment with water at 100°C for 30 min, by physical disruption, or on germination. Moreover, the intracellular materials are almost completely inaccessible for exchange with their radioactive exogenous counterparts (24, 25, 27). Apparently some force or barrier holds small molecules within the spore.

Two fundamentally different hypotheses can account for these important findings. First, the electrolytes may be held in an electrostatically bound state within spores. Some of the anionic compounds in solution, DPA especially, form binary chelate complexes with divalent cations, and ternary complexes (e.g., DPA-glutamate-calcium complexes) may also form (34, 33). Nelson and Kornberg (24) have suggested that there may be a whole network of such complexes within spores. Black and Gerhardt (3) had proposed earlier that polymers in the spore core might be cross-linked to form a gel. Such a highpolymer matrix might be held together electrostatically by chelate links of divalent cations with DPA, glutamate, and other compounds. Regardless of the mechanisms by which they are bound in this model, the electrolytes are nonionized and relatively immobile in an electric field.

In the second hypothesis, it is suggested that electrolytes are retained in a free state within the spore by a membrane highly impermeable to them. Contraction (19) or rigidity of the cell might raise the core might compress the core membrane and effectively decrease its permeability. The spore core is accessible to water (3, 23) and some very small, lipid-soluble molecules such as ethylene glycol (15). However, most hydrophilic molecules permeate the dormant spore only to an extent corresponding to the volume outside the core and do not escape from the core once encased or synthesized there. In this second model, the electrolytes held within...
a permeability barrier are mobile and free to ionize.

A major distinction between these two models resides in the contrasting ionization states and consequently the abilities of the pooled electrolytes to conduct electric current. Previous studies (5-7, 13) have shown that dielectric measurements can be used to determine the distribution of mobile, conductive ions within intact vegetative cells of representative bacteria without disrupting or killing the cell. The measurements also yield dielectric constants, which indicate the physical state of internal water. Therefore, we applied the method to select between the bound-electrolyte and free-electrolyte models, by using spores of a widely studied bacterial species in various stages of development.

MATERIALS AND METHODS

The terminalis (T) strain of Bacillus cereus was cultured as described previously (1). Mature spores were isolated by differential centrifugation, and their homogeneity was assessed by several criteria (22). The spores were uniform in size with minor and major semiaxes of approximately 0.4 and 0.7 µm. The thick suspension techniques described by Black and Gerhardt (2) were used for activation and germination. Activation (heating for 2 hr at 65°C) was judged to be effective because almost complete germination occurred within 10 min when activated spores were incubated aerobically in a medium containing 6 mg of L-alanine per ml and 4 mg of adenosine per ml. When dormant spores were placed directly into the medium without activation, germination was delayed for as long as 30 min. Spores were kept in germination medium for a total of 30 min and then washed with deionized water to arrest further changes. Outgrowing cells were obtained by suspending germinated spores in Trypticase Soy Broth (BBL) and incubating them aerobically at 30°C for at least 90 min, at which time the cells were noticeably elongated. The cells were then washed in deionized water to prevent further changes (16). Longer periods of outgrowth produced vegetative, dividing cells. Cytological observations were made with a Reichert phase-contrast microscope at 1,500× magnification.

Dielectric measurements in the frequency range of 1 to 100 MHz (i.e., 1 to 100 Mcycles/sec) were performed at 29°C with an RX meter (Boonton Measurements Corp., model 250A) and, at 1600 Hz, with a Wayne Kerr model B221 Universal Bridge by using techniques which have been described previously (5, 30). With knowledge of the volume fraction (ρ) occupied by spores in a suspension, it is possible to calculate the complex, effective, homogeneous, spore conductivity (σₛ⁺) from values for the complex conductivity of the suspending medium (σᵣ⁺) and the complex conductivity of the suspension (σ⁺) by use of the following relationship (5, 14):

\[
\sigma⁺ - \sigmaᵣ⁺ / (\sigma⁺ + 2\sigmaᵣ⁺) = \rho (\sigmaₛ⁺ - \sigmaᵣ⁺) / (\sigmaₛ⁺ + 2\sigmaᵣ⁺)
\]

(1)

The complex conductivity is defined by the equation

\[
\sigma = \sigma + i\sigma'\omega \kappa
\]

(2)

where σ is the real conductivity, \(i = \sqrt{-1}\), \(\omega\) is the angular frequency, \(\kappa = 8.854 \times 10^{-12} \text{ F/m}\) is the permittivity of free space, and \(\kappa\) is the relative dielectric constant.

The fractional volume occupied by spores in a suspension was considered to be equal to the fractional volume that is impermeable to high-molecular-weight dextrans. Techniques for determining dextran-impermeable volumes for spore suspensions have been described in detail previously (1). For these measurements, the spores were centrifuged to a maximally packed pellet. Centrifugation compresses the exosporium so that the dextran-impermeable volume obtained includes the volume of this integument but not the volume of the suspending medium that seeps between the expanded exosporium and the outermost spore coat when the cells are resuspended.

Based on previous determinations (1, 2), a value of 0.90 was used in approximation of the partial volumes of dormant, activated, or germinated spores in pellets packed by centrifugation at 35,000 \(\times\) g for 60 min. Previous experience also indicated that the same value could be used for vegetative cells. The errors in calculating spore conductivity that result from errors in estimating \(\rho\) are minor, except in cases for which \(\sigmaₛ⁺\) is either much larger or much smaller than \(\sigmaᵣ⁺\) (12).

RESULTS

Theory. A detailed dielectric model for bacterial cells has been developed and described previously (5–7). For the present study, it was desirable to predict the dielectric characteristics of a cell that contains electrolytes both within a thin insulating membrane and in an outer, porous integument. The conductivities of such a cell characteristically undergo a major transition from the low-frequency values, which are dominated by effects of the integument, to the high-frequency values, to which the cytoplasm also contributes. Correspondingly, the cytoplasmic membrane dominates the effective dielectric constant at low frequencies but is unimportant at high frequencies. This transition has been called the \(\beta\)-relaxation (32). The effective, homogeneous conductivity (\(\sigma₂\)) above the \(\beta\)-relaxation frequency thus is a reflection of the total content of mobile ions in the cell. A complete quantitative analysis has been given by Fricke (14).

Figure 1 shows variations in \(\sigma₂\) and \(\kappa₂\) predicted by Fricke's theory (14) for particles with parameters appropriate for B. cereus spores. In all cases, the effective, homogeneous conductivity at 50 MHz is approximately equal to the mean conductivity of the entire spore, including the core. If one of the layers in the outer spore integument (e.g., exosporium) were an electrically insulating barrier, it would have a small effect at low frequencies but would have no effect at frequencies above 50 MHz.
**Effect of frequency.** Dielectric measurements of dormant, activated, germinated, and outgrown spores were made over a frequency range from 1 to 200 MHz. The results (Fig. 2) show the variation in dielectric properties of the different spore types suspended in media with essentially the same environmental conductivity and also of dormant spores with nearly a 100-fold change in environmental conductivity. In accord with the theoretical predictions described, β-relaxation frequencies are roughly proportional to internal cell conductivities.

The model used to treat the spore data indicates that, as long as the core conductivity remains very small, there should be no dispersion (i.e., frequency dependence) in the dielectric data above approximately 5 MHz, although ions from the environment invade the porous integument (curve 2, Fig. 1). In contrast, the data in Fig. 2 for dormant spores with $\sigma_1 = 0.4$ mho/m show almost as much dispersion as the model predicts for a spore with a core conductivity of 0.3 mho/m (curve 3, Fig. 1). The fact that dispersion is observed in the 1- to 20-MHz range suggests that a more complex model is needed which involves an outer, poorly conducting membrane, e.g., the exosporium. Alternatively, the low-frequency dispersion might be explained if environmental ions penetrate the core of dormant spores. In neither case would the conclusion drawn from the 50-MHz data be altered, namely that intrinsic core electrolytes become ionized during germination and outgrowth.

The conductivities of dormant spores in a low ionic strength medium, shown in the lowermost curve of Fig. 2, give a basis for assessing the con-
tributions made by water and cell polymers to the conductivities at frequencies of 50 MHz and above. The very high-frequency conductivity ($\sigma$) of a polymer solution in saline can be separated into three components such that (32)

$$\sigma = A_s + A_\ell f + A_w f^2$$  \hspace{1cm} (3)

The conductivity of small salt ions ($A_s$) is independent of the frequency ($f$) and usually predominates. The conductivity of proteins and other polymers ($A_\ell f$) contributes in direct proportion to the frequency, and the conductivity of water ($A_w f^2$) contributes in proportion to the square of the frequency. Calculations based on the data in Fig. 2 indicate that the last two terms in equation 3 contribute only about 0.005 mho/m to spore conductivity at 50 MHz. As discussed in the previous section, cell membranes significantly affect bacterial cell conductivity only at frequencies below about 50 MHz. Therefore, compromising between complications due to membrane effects and those due to polymer and water effects, we used effective homogeneous conductivities at 50-MHz frequency as measures of the total concentrations of mobile ions within the entire structure of intact spores.

**Effect of environmental conductivity.** Effective, homogeneous conductivities of spores arrested in several stages of development were measured with 50-MHz current over a range of environmental conductivities. The composite results are shown in Fig. 3. The different environmental conductivities were obtained by progressive additions of sodium chloride to dormant spores or by washes with deionized water of activated, germinated, or outgrown spores. Washing reduced the environmental conductivity of outgrown spore suspensions only to a limited extent because of the great release of ions from the cells. It is apparent that the effective conductivities for all of the cell types increased with increasing environmental ionic strength, as the predictable result of ions penetrating the porous integuments.

**Conductivities of dormant spores.** The high-frequency conductivity for dormant spores (Fig. 3) reached a plateau at low environmental conductivity, with a limiting spore conductivity of about 0.02 mho/m. This value gives a good index of the very low concentration of mobile ions that can be considered as intrinsic spore components, including counterions for spore polymers and any excess mobile ions that are not free to diffuse across the permeability barriers of the spore. The value of 0.02 mho/m is unusually low for cell conductivity at 50 MHz, especially considering that polymers and water may contribute as much as 0.005 mho/m. Comparable values for vegetative cells are an order of magnitude higher. For example, Micrococcus lysodeikticus cells have a conductivity ($\sigma_2$) of 0.2 mho/m in media with $\sigma_1 = 0.02$ mho/m (13), and Escherichia coli cells yield a $\sigma_2$ value of 0.3 mho/m in media with $\sigma_1 = 0.04$ mho/m (5). The 50-MHz conductivity of bacterial cells is a composite of protoplast and cell wall conductivity. Isolated protoplasts of M. lysodeikticus in 2 molal sucrose solution were found (13) to have a $\sigma_2$ value of 0.13 mho/m in a medium with $\sigma_1 = 0.02$ mho/m. Isolated cell walls of M. lysodeikticus were found (7) to have a $\sigma_2$ value of 0.35 mho/m in a medium with $\sigma_1 = 0.02$ mho/m.

The conductivity curve for dormant spores (Fig. 3) appears to approach a constant slope at high environmental conductivities, where $\sigma_2/\sigma_1$ is about 0.40. The spore conductivity at high frequencies becomes (14)

$$\sigma_2 = \sigma_1 (1 - p_w) + \sigma_w p_w$$  \hspace{1cm} (4)

where $\sigma_1$ and $\sigma_w$ are conductivities of the core and integument, respectively, and $p_w$ is the volume fraction of the spore occupied by the integument. Equation 4 assumes that the dielectric constants of the core and integument materials are roughly equal. If environmental ions diffuse freely into the water space of the integument but do not enter the core, then at high $\sigma_2$ when $\sigma_1 \ll \sigma_w \approx \sigma_2$, equation 4 becomes

$$\sigma_2/\sigma_1 \approx \sigma_2/\sigma_1 \approx p_w$$  \hspace{1cm} (5)

This implies that about 40% of the volume of

![Fig. 3. Effective, homogeneous conductivities of B. cereus spores as a function of environmental conductivity. Data are given for 50 MHz conductivities with dormant, activated, germinated, and outgrown (1.5 and 5.0 hr) spores. Environmental conductivities were changed either by washing with deionized water or by adding NaCl solution.](image-url)
dormant spores can be penetrated by environmental ions, in agreement with previously reported values for the permeation of small solutes (3, 15).

Conductivities of developing spores. When dormancy was broken, the conductivities of developing spore suspensions increased many fold, with the increases partly due to leakage of ions into the suspending medium. The limiting high-frequency conductivity for activated spores at low environmental conductivity appeared only slightly higher than the value for dormant spores, whereas that for germinated spores was about three times higher. Outgrowing spores were even more highly conducting and, because of leakage, could not be maintained in the low conductivity media used for dormant spores. Because of the extensive loss of electrolytes from spores during development, there was uncertainty in the values of \( \sigma_1 \), which correspond to each value of \( \sigma \) in equation 1. Hence, values for environmental conductivity were obtained both before and after measurement of the suspension conductivities. Resulting uncertainties in the values for \( \sigma_1 \) and \( \sigma_2 \) for individual points in Fig. 3 are as much as \( \pm 30\% \) for developing spores, compared with about \( \pm 15\% \) for dormant spores.

It is apparent that the effective, homogeneous conductivities of spores increase during development. Furthermore, the evidence indicates that this increase results in large part from an increase in the concentration of mobile ions within the core. Meaningful comparisons of spore conductivities are complicated by the fact that environmental ions permeate the integument. However, at a given value of \( \sigma_1 \), the contributions of environmental ions to the spore conductivity (\( \sigma_2 \)) are roughly the same for all spores. Consequently, comparisons of ion content were made by calculating ratios for the conductivities of developing spores relative to the conductivity of dormant spores at the same \( \sigma_1 \). Average ratios, derived from the data of Fig. 3, are presented in Table 1. Of course, the ratios reflect the presence of all mobile ions which have been liberated during development, including ions in the core as well as fixed charge-counterion pairs in the integument. To estimate the contribution of the latter, the conductivities of dormant and germinated spores were compared at low frequency and low \( \sigma_1 \). Under these conditions, the intrinsic integument conductivities are dominant. At 1 MHz and with \( \sigma_1 = 0.01 \) mho/m, \( \sigma_2 \) values for dormant and germinated spores were about 0.015 and 0.025 mho/m, respectively. Thus, it appears that changes in intrinsic integument conductivity during development are only a small part of the total change. The data in Table 1 show that there are discernible increases in cellular conductivity during activation, germination, and outgrowth which cannot be attributed to increases in ion content of the porous integument. Instead, the increases probably result from changes in the degree of association of ions within the core itself.

Although dormant spores could be held in thick suspension for many days with the ion concentration in the suspending medium not rising above millimolar levels, developing spores lost large quantities of electrolytes. A quantitative estimate of this leakage was obtained by preparing dilute suspensions of spores in various stages of development with deionized water and measuring low frequency suspension conductivities for periods of approximately 1 hr. After an initial adjustment period of about 10 min, conductivities increased at constant rates for more than 30 min. Because of the low concentrations of spores, the suspending media served as almost infinite sinks for ions passing out of the cells, and increases in suspension conductivity were directly proportional to the rates of loss of ionic materials. The rates of change in conductivity, normalized in relation to spore volume, are presented in Table 2. Amounts of ionic material lost from the cells were estimated by converting conductivity values to NaCl equivalents. They are meant only to give an appreciation of the amounts of ions lost and do not imply that the major electrolyte released from spores was NaCl. Atomic absorption spectrophotometric measurements indicated that the ratios of Na\(^+\):K\(^+\):Ca\(^2+\):Mg\(^2+\) in the exudate from activated spores were 1.0:1.8:0.6:0.5, and the ratios from germinated spores were 1.0:0.4:330:30. In all, the loss of ions from germinated and outgrowing spores was at least 1,000 times greater than the loss from dormant spores.

High-frequency dielectric constants. Effective, homogeneous dielectric constants at 100 MHz for dormant, activated, and germinated spores are summarized in Table 3. The curves in Fig. 1 illustrated that the effects of the membrane (i.e., \( \beta \)-relaxation) on the dielectric constants were minimal for spores at 100 MHz. Therefore, the values presented in Table 3 reflect spore properties at a molecular level throughout the spore. There was some evidence of a \( \beta \)-relaxation near 100 MHz for outgrowing spores (Fig. 2), thus values for these cells are not included in Table 3. However, it appeared that the high-frequency limit in dielectric constant for outgrowing spores would be about 60. Thus, values for the dielectric constant increased progressively during the transition from dormant spores to fully outgrown cells. Parallel increases in water content have
been observed by Black and Gerhardt (3).

**DISCUSSION**

The above results indicate that the large amounts of electrolytes within dormant bacterial spores of *B. cereus* are electrostatically bound in nonionized, immobile forms, with resulting poor conductivity even at high frequency. The intracellular conductivity was roughly equivalent to that of only a millimolar NaCl solution. That the spores contained large amounts of potentially ionizable electrolytes was unequivocally demonstrated by the finding that the total conductivity of spore suspensions increased at least fivefold during germination.

The exact mechanisms by which the electrolytes are bound in the polymer-rich intracellular environment of dormant spores is still matters for speculation. The most apparent mechanism of binding involves the formation of chelate complexes (see introduction). Complex formation may also involve coprecipitation of small electrolytes and cell polymers, including cytoplasmic proteins and nucleic acids. The concentrations of many pooled electrolytes, especially calcium-dipicolinic acid (Ca-DPA), are supersaturated in relation to the amount of cell water (27), and thus amorphous precipitates may form. Moreover, the possibility exists that crystals could form, but they are not usually seen (except as parasporal bodies) in electron micrographs. Studies of the electrochemistry of Ca-DPA-polymer solutions in vitro may be fruitful in suggesting possible types of complexes that could form in vivo.

The finding that dormant spores have internal conductivities almost 10 times lower than those of vegetative cells indicates extensive neutralization of the ionizable groups of spore polymers so that they no longer require mobile counterions. This appears to be true not only for the core polymers but also for the integument polymers, including exosporium lipoproteins, coat proteins, and cortex peptidoglycans. Charge neutralization in flexible polymers, such as peptidoglycans, results in contraction (29). It appears, therefore, that the physical compactness and high density of dormant spores could be due in part to polymer contraction, especially of the cortical peptidoglycan, as suggested previously (10, 19, 21).

The high-frequency dielectric constants also revealed important information about the physical state of spore constituents. The dielectric constants of most organic compounds are low, and therefore the effective dielectric constants of spores give at least rough indications of the amounts of intracellular water free to rotate in an electric field. Determinations of the water contents of *B. cereus* cells in aqueous suspensions have indicated that water accounts for about 65% of the weight of dormant spores, 73% of germinated spores, and 77% of vegetative cells (3). The high-frequency dielectric constants of 43, 57, and 60, respectively, observed for these cells are reasonably in accord with the water contents. A small part of the rise in the dielectric constant during spore development may be due

### Table 1. Ratios between the effective, homogeneous conductivity at 50 MHz for developing spores and the corresponding value for dormant spores

<table>
<thead>
<tr>
<th>Developmental stage of spores</th>
<th>No. of observations</th>
<th>Mean conductivity ratio</th>
<th>Standard error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activated</td>
<td>8</td>
<td>1.4</td>
<td>±0.2</td>
</tr>
<tr>
<td>Germinated</td>
<td>11</td>
<td>2.1</td>
<td>±0.2</td>
</tr>
<tr>
<td>Outgrowing 1.5 hr</td>
<td>3</td>
<td>2.7</td>
<td>±0.1</td>
</tr>
<tr>
<td>Outgrowing 5.0 hr</td>
<td>5</td>
<td>5.2</td>
<td>±0.9</td>
</tr>
</tbody>
</table>

* The ratios were calculated by dividing \( \sigma_c \) for the developing spores by the \( \sigma_c \) for dormant spores, with both in media of the same ionic strength (Fig. 3).

### Table 2. Leakage of ions from spores in successive stages of development

<table>
<thead>
<tr>
<th>Developmental stage of spores</th>
<th>Normalized rate of increase of suspension conductivity (mmho/m/min)</th>
<th>Leakage rate expressed in NaCl equivalents*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dormant</td>
<td>&lt;0.02</td>
<td>&lt;0.002</td>
</tr>
<tr>
<td>Activated</td>
<td>0.06</td>
<td>0.006</td>
</tr>
<tr>
<td>Germinated</td>
<td>14.0</td>
<td>1.40</td>
</tr>
<tr>
<td>Outgrowing (5 hr)</td>
<td>14.0</td>
<td>1.40</td>
</tr>
</tbody>
</table>

* Measured rates at 1.500 Hz were normalized by dividing by the fractional volume of cells in each suspension.

### Table 3. Effective, homogeneous dielectric constants at 100 MHz for spores in successive stages of development

<table>
<thead>
<tr>
<th>Developmental stage of spores</th>
<th>No. of observations</th>
<th>Mean dielectric constant</th>
<th>Standard deviation</th>
<th>Standard error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dormant</td>
<td>14</td>
<td>43</td>
<td>±5</td>
<td>±2</td>
</tr>
<tr>
<td>Activated</td>
<td>9</td>
<td>47</td>
<td>±5</td>
<td>±2</td>
</tr>
<tr>
<td>Germinated</td>
<td>12</td>
<td>57</td>
<td>±2</td>
<td>±1</td>
</tr>
</tbody>
</table>

**DISCUSSION**

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to conversion of compounds such as glutamic acid to ionized forms (dielectric increment of 26; reference 8), but most of the rise is probably due to water uptake. Some of the water may be bound to small molecules and polymers. Observations at microwave frequencies (10 GHz) on partially dehydrated cells have disclosed that the water in B. megaterium spores has a lower dielectric constant than the water in yeast cells (18, 20). This may imply a lower relaxation frequency for part of the spore water than for typical vegetative cells. Since there is probably a wide range in the degrees of binding of water in a single cell, however, the differences in microwave dielectric constants alone do not indicate a radically different state for spore water than that found in other cells. From our observations, it is clear that most of the water in fully hydrated B. cereus spores relaxes at frequencies well above 100 MHz, and thus that spore water is not as tightly bound as in clathrate hydrates (11, 17) or hexagonal ice (28). There is no dielectric evidence to suggest that water is in some unusual state in dormant spores.

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LITERATURE CITED