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Bacterial Spore Heat Resistance Correlated with Water Content, Wet Density, and Protoplast/Sporoplast Volume Ratio†

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Five types of dormant *Bacillus* spores, between and within species, were selected representing a 600-fold range in moist-heat resistance determined as a D_{100} value. The wet and dry density and the solids and water content of the entire spore and isolated integument of each type were determined directly from gram masses of material, with correction for interstitial water. The ratio between the volume occupied by the protoplast (the structures bounded by the inner pericytoplasm membrane) and the volume occupied by the sporoplast (the structures bounded by the outer pericortex membrane) was calculated from measurements made on electron micrographs of medially thin-sectioned spores. Among the various spore types, an exponential increase in the heat resistance correlated directly with the wet density and inversely with the water content and with the protoplast/sporoplast volume ratio. Altogether the results supported a hypothesis that the extent of heat resistance is based in whole or in part on the extent of dehydration and diminution of the protoplast in the dormant spore, without implications about physiological mechanisms for attaining this state.

The fundamental basis for the extraordinary resistance of bacterial spores remains unexplained despite important practical implications and a century of research. Pasteur in 1861 observed that drying increases the resistance of mold spores (20), and Lewith in 1890 predicted that bacterial spores should have an internal water content of about 10%, based on the stabilization of egg white by removal of water (16). From these historic roots, dehydration has come to be considered the most probable basis for wholly or partly explaining spore resistance, although recently challenged by Bradbury et al. (7b). A chronology of theories about spore resistance has been tabulated by Gerhardt and Murrell (11), and current ideas have been summarized by Murrell (18) and others (Spore Newslett. 7(5):1–83, 1981).

Accurate determination of the total water content of the fully hydrated dormant spore is accomplished gravimetrically by use of a large mass of wet spores with correction for interstitial water, or by use of a small mass of dried spores equilibrated to saturated water activity. The data available indicate that the water content in total, depending on species, varies from about 0.45 to 0.65 g of water per g of wet spore (7, 17, 23). Clearly, the total water contents are too great for dehydration of the entire spore to account for the observed heat resistances.

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Instead, the total water content would have to be distributed unequally within the spore, with more in an exterior compartment and less in an interior compartment. The problem was recognized and discussed thoroughly by Marshall and Murrell (17) and Gould (13). The historic microscopic image of a dormant spore with a refractile interior and a nonrefractile exterior qualitatively indicates, respectively, smaller and larger water contents. However, quantitation and structural identification of water distribution in situ within an intact spore has not been accomplished, although Watt (23) determined water contents of isolated fractions.

The problem of water distribution became complicated with recognition that the bacterial spore contains not only an inner membrane surrounding the cytoplasm but probably also an outer membrane surrounding the cortex (for literature review, see reference 8). The pericortex membrane apparently functions as a permeability barrier (10, 20a). In electron micrographs of thin-sectioned spores, the pericortex membrane is identified only as a line around the cortex and beneath the inner coat (see Fig. 2). The structures bounded by the pericytoplasm membrane are commonly called the protoplast, and we introduce the term sporoplast to describe the structures bounded by the pericortex membrane.

In this study we determined the wet and dry density and the solids and water content of the

entire spore and of isolated integument fractions, fully hydrated in aqueous suspension. Accuracy was obtained by using gram masses of material and by measuring weights and volumes directly. We also determined the volume ratio between the protoplast and sporoplast by measuring electron micrographs of medially thin-sectioned spores. The various biophysical parameters then were correlated with the moist heat resistance of five spore types, between and within species, which were selected to provide a wide range in heat resistance, determined as a D_{100} value. Correlations also were made with the average apparent refractive index of each spore type, as reported separately (10). The combined results enabled the correlation of heat resistance with reduced water content in the entire spore and sporoplast and the estimation of water distribution between the sporoplast and perisporoplast integument of the various spore types.

(A preliminary abstract of these findings appeared in Spore Newslett. 7(5):8–9, 1981.)

MATERIALS AND METHODS

Spore preparation. Each of five spore types was produced in decagram quantity with >99% purity of brightly refractile spores, stored at 4°C in distilled water with a small amount of aminoglycoside antibiotic to prevent contamination, and analyzed within a few days.

Spores of *Bacillus cereus* T were produced in a 14-liter fermentor at 30°C, harvested by centrifugation at 5,000 × *g*, and repeatedly washed with distilled water as described previously (5). Calcium-sufficient dormant spores of maximal heat resistance were produced in G medium modified to contain 0.01% $CaCl_2$, and calcium-deficient spores of reduced heat resistance were produced in the original G medium containing 0.001% $CaCl_2$ (14). Germinated spores were prepared from the calcium-sufficient dormant spores as described previously (6).

The source of and the sporulation medium for *B. subtilis* subsp. *niger* were described by Warth (21). Culturing was managed in the fermentor at 41°C. After sporulation was complete, the spores were harvested by centrifugation at 5,000 × *g* for 5 min, suspended in 0.05 M potassium phosphate buffer (pH 7.0), and treated with lysozyme (EC 3.4.4.10; 200 μg/ml) for 2 h at 37°C. The spores were further centrifuged, washed several times in the buffer, resuspended, and treated with papain (EC 3.2.1.17; 1 μg/ml) for 2 h at 37°C. The spores were then washed repeatedly in distilled water by centrifugation at 3,000 × *g* for 5 min, with removal of the superficial sediment as well as the supernatant liquid until the desired standard of purity of the spores was attained.

Strain ATCC 7953 of *B. stearothermophilus* consists of a mixture of rough (opaque, larger) and smooth (translucent, smaller) colony variants, the spores of which differ in heat resistance (9, 15). A selected colony of the rough variant produced >99% spores of uniform character when subcultured, whereas the smooth variant did not. To produce >99% spores of the smooth variant successfully, a thick suspension of mostly smooth-variant spores was heated at 120°C for 5 min, and the survivors were used as inoculum. Both colony types were verified by routine determinative tests for *B. stearothermophilus*, which were consistent except for the reaction in litmus milk (R. L. Gherna, American Type Culture Collection, personal communication). Each variant was mass cultured at 65°C on plates of medium which contained 2.0% agar, 0.3% peptone, 0.25% tryptone, 0.25% beef extract, 0.4% yeast extract (all Difco), 0.2% K_2HPO_4, and 0.001% $MnSO_4$, and which was adjusted to pH 8.2 with NaOH. The sporulated growth was harvested from the agar surface with distilled water, centrifuged, treated with the lysozyme and papain (smooth variant) or allowed to autolyze (rough variant), and washed, all as described for the *B. subtilis* spores.

Heat resistance. Resistance of the spores to moist heat was determined as a D_{100} value, that is, the minutes required for a decimal reduction in colony-forming units on exposure to 100°C of about 10^7 viable spores per ml suspended in 0.05 M potassium phosphate buffer (pH 7.0). The spore suspensions (1.5 ml) were sealed in thin-walled glass ampoules (9 mm outer diameter by 9 cm), replicates of which were submerged on a rack in a stirred and thermoregulated bath containing heating-bath oil. Ampoules were removed at appropriate time intervals and cooled by immersing in an ice bath for at least 5 min. An ampoule was opened and a 1-ml sample was transferred into 9 ml of germination solution (0.6% L-alanine plus 0.4% adenosine in 1% peptone), which was incubated at an appropriate growth temperature for 30 to 60 min. (The heating alone did not cause germination.) Afterwards, appropriate decimal dilutions were made in 1% peptone (Difco) solution, and quintuplicate 0.1-ml samples at several appropriate dilutions were transferred and spread on the surface of predried agar medium (Trypticase-soy, BBL Microbiology Systems, Cockeysville, Md.). Colonies were counted after an appropriate incubation time. Plates at dilutions with 30 to 300 colonies were selected, and the counts were averaged and plotted. The D_{100} value was obtained from the thermal death curve by determining the slope of the linear, or near-linear, portion extending over at least one decade. The thermal death curves are exemplified in Fig. 1.

Wet density. Weight and volume of a packed wet mass of the intact spores were measured essentially as described by Black and Gerhardt (7) and modified by Arnold and Lacy (1). Polycarbonate centrifuge tubes of 15 ml nominal volume were calibrated for exact volume as follows: a tared tube was filled with distilled water and closed with a tared microscopy glass cover slip; air bubbles were excluded from beneath the cover slip, and excess water was wiped from the external surfaces of the tube; the weight of water in the tube was determined and then was converted to volume by multiplying by the density of water at the ambient temperature. A calibrated tube was used to obtain a packed wet mass (1 to 3 g) of the spores, using centrifugation at 17,000 × *g* for the *B. cereus* spores and at 10,000 × *g* for the *B. subtilis* and *B. stearothermophilus* spores. (Especially with the latter spores, the lower gravitational force was necessary to prevent germination.) The supernatant solution was decanted, and the residue solution was carefully wiped from the inside of the tube with lint-free absorbent tissue

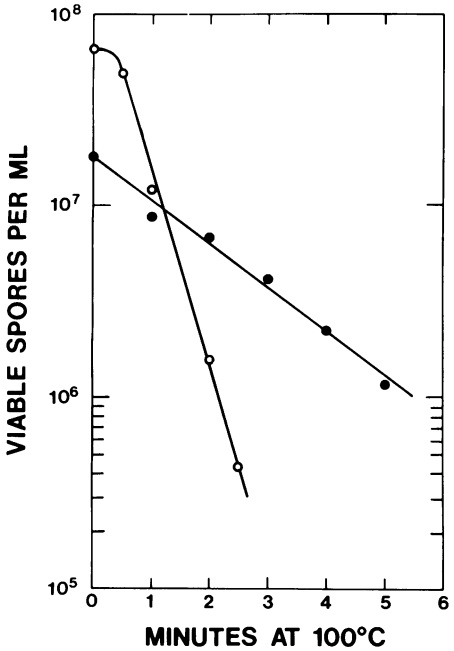

FIG. 1. Determination of heat resistance (D_{100}) from slope of thermal death curve for the calcium deficient (0) or calcium sufficient (●) dormant spore of *B. cereus* T. Respectively, the D_{100} values were 1.00 and 4.39 min, as calculated by least-squares analysis.

wrapped around a wood applicator stick. The weight of the wet pellet (W_p) was determined, and the tube was then refilled completely with distilled water without disturbing the pellet, covered with the companion cover slip, and weighed. The volume of the added water was determined and then subtracted from the volume of the calibrated tube to give the volume of the wet pellet (V_p). The fractions of the pellet weight and volume occupied by the interstitial volume (S_{in}^w and S_{in}^v, respectively) were determined as described by Gerhardt et al. (10). W_p and V_p then were corrected for the interstitial water of the pellet ($W_pS_{in}^w$ and $V_pS_{in}^v$, respectively). The corrected values represented the weight (W_c) and the volume (V_c) of the "cells" (that is, the spores) only. The wet density of spores (D^{wet}, grams of wet spores per milliliter of wet spores) thus was calculated as follows: $D^{wet} = [W_p - (W_pS_{in}^w)]/[V_p - (V_pS_{in}^v)] = W_c/V_c$.

Solids and water content. The centrifuged pellet of spores was resuspended and brought to 100 ml with distilled water in a volumetric flask. Replicate measured samples (1 ml) were transferred to tared weighing dishes, dried to constant weight over P_2O_5 under vacuum at 80°C, and cooled to and weighed at room temperature. The solids content so determined on a wet-weight basis (grams of solids per gram of wet spores) was converted to a wet-volume basis (grams of solids per milliliter of wet spores) by multiplication with the value for wet density of the spore type.

The water content on a wet-volume basis (grams of water per milliliter of wet spores) was obtained from the solids content on a wet-volume basis by difference from the wet density of the spore type. The water content on a wet-weight basis (WC^{wet}, grams of water per gram of wet spores) was obtained from the corresponding solids content by difference and was converted to a dry-weight basis (WC^{dry}, grams of water per gram of dry spores) by use of the following equation: $WC^{dry} = (WC^{wet})/(1 - WC^{wet})$.

Dry density. The dry density (D^{dry}, grams of dry spores per milliliter of dry spore) was calculated by use of the following equation: $D^{dry} = 1/\{[(1 + WC^{dry})/D^{wet}] - [WC^{dry}/D_{HOH}]\}$, where D_{HOH} = grams of water per milliliter of water. An exact value for D_{HOH} within the spore was not available and so was taken to be 1.0, assuming that most of the water in a spore occurs in bulk liquid form.

Protoplast and sporoplast volume fractions. The procedures for electron microscopy were as described previously (3). Measurements were made of the average length and width of the entire spore, the sporoplast, and the protoplast of spores longitudinally sectioned through the center. Since the pericortex membrane usually was not discernable, the dimensions of the sporoplast were taken from the periphery of the cortex. The volume was obtained from the length and width measurements by assuming that the entire spore and both interior compartments geometrically approximated hemisphere-capped cylinders (volume = $2\pi b^2[a - b/3]$, where a = half-length and b = half-width). Essentially the same values for volume fractions were obtained by applying the formula for an ellipsoid of revolution (volume = $4\pi ab^2/3$).

In measuring the entire *B. cereus* T spore, the exosporium was considered to be collapsed around the spore proper, as occurs in the packed mass used for determining the density and water content. If the exosporium were considered to be expanded, as occurs in free suspension, the dimensions would become unrealistically large.

Integument isolation and analysis. Integument fractions were isolated essentially as described by Warth et al. (22). The spores (50 mg of dry spores per ml of phosphate buffer) were heat inactivated of autolytic enzymes and then disintegrated by shaking with glass beads (0.01 to 0.1 mm in diameter) in a Braun homogenizer (model MSK, Bronwill Scientific Inc., Rochester, N.Y.) for 60 to 180 s. Optical and electron microscopy revealed that this shaking regimen disintegrated virtually all of the spores, but did not markedly comminute their integument. The total integument fraction was sedimented, washed several times by differential centrifugation in distilled water, suspended in the phosphate buffer, treated with DNase (EC 3.1.4.5; 500 μg/ml) and RNase (EC 3.1.4.22; 150 μg/ml), incubated for 1 h at 37°C, washed repeatedly by differential centrifugation with 0.1 M NaCl, and treated with trypsin (EC 3.4.4.4; 500 μg/ml) for 2 h at 37°C. The residue was then washed twice with 0.1 M NaCl and repeatedly with distilled water. As monitored by electron micrographs of thin sections, the resulting fraction consisted of all of the periprotoplast integument, that is, the cortex, coats, sporoplast membrane (not visible), and exosporium (if present). This fraction was further treated with the trypsin and lysozyme for 6 h at 37°C and washed repeatedly by differential centrifugation, first with 0.1 M NaCl and then with distilled water. The resulting perisporoplast integument frac-

tion consisted only of coats and exosporium (if present).

The integument fractions were prepared in quantity, stored, and analyzed in essentially the same way as the spores.

RESULTS

Heat resistance and fine structure. The five types of dormant spores among three *Bacillus* species represented almost a 600-fold range in resistance to moist heat as measured by D_{100} values (Table 1).

The dormant spore of *B. cereus* T was relatively low in heat resistance compared to the other species and was made considerably less so by production in a calcium-deficient medium (Table 1). The germinated spore is considerably less resistant than the dormant spore, by several orders of magnitude (13). *B. cereus* T was selected because it has been widely used as a model in basic studies of spores. However, the spore contains a superficial, loose-fitting, bilayered exosporium (12). In the various biophysical determinations, the presence of exosporium introduces complicating considerations: the exosporium is expanded when the spores are freely suspended but collapsed when they are packed; it encloses liquid and sometimes crystals and other material between it and the spore coat; it occupies a substantial volume fraction of the spore; and it has an indeterminate surface because the outer layer is hirsute.

The dormant spore of *B. subtilis* subsp. *niger* was relatively intermediate in heat resistance (Table 1). The spore possessed a thick, close-fitting exosporium outside the outer coat (Fig. 2A). The exosporium consisted of a thin basal layer and a thick outer layer that was undifferentiated and uneven in thickness, and thus introduced complicating considerations in the various biophysical determinations.

The dormant spore of *B. stearothermophilus* was relatively high in heat resistance, and the smooth-colony variant was considerably more so than the rough one (Table 1). The two variants were alike in spore structure, and the spores did not possess an exosporium (Fig. 2B).

In all three species, the concentric internal structures were fairly typical of those in most bacterial spores: an outer electron-dense coat; an inner laminated coat; an outer pericortex membrane which usually is not discernable from the inner lamination of the inner coat, but which is functionally demonstrable (8, 10); a cortex with primordial cell wall; an inner pericytoplasm membrane; and cytoplasm containing ribosomes and nucleoplasm.

Wet and dry density of entire spore. Wet-density values are listed in Table 1. Among the various types of dormant spore, an exponential increase in the heat resistance correlated with an increase in the wet density (Fig. 3). However, the germinated spore type did not fit the correlation line.

The procedure for determining wet density was checked by use of polystyrene beads, which were about the same size as spores (0.868 μm; Dow Diagnostics, Indianapolis, Ind.). The re-

TABLE 1. Density, solids content, and water content (expressed in various ways) of the entire spore of various types

Spore type (species, strain, variant)	D_{100} (min)	Density		Solids content		Water content		
		(g of wet spore)/(ml of wet spore)	(g of dry spore)/(ml of dry spore)	(g of solids)/(g of wet spore)	(g of solids)/(ml of wet spore)	(g of water)/(g of wet spore)	(g of water)/(g of dry spore)	(g of water)/(ml of wet spore)
B. cereus, T, germinated	0.001[a]	1.075	1.369	0.259	0.278	0.741	2.86	0.797
B. cereus, T, calcium deficient	1.00	1.115	1.431	0.343	0.382	0.657	1.92	0.733
B. cereus, T, calcium sufficient	4.39	1.134	1.451	0.380	0.431	0.620	1.63	0.703
B. subtilis subsp. *niger*	40.0	1.196	1.445	0.533	0.637	0.467	0.88	0.559
B. stearothermophilus, rough	124.0	1.204	1.505	0.507	0.610	0.497	0.98	0.598
B. stearothermophilus, smooth	579.0	1.218	1.441	0.586	0.714	0.414	0.71	0.504

[a] Approximate value, from Gould (13).

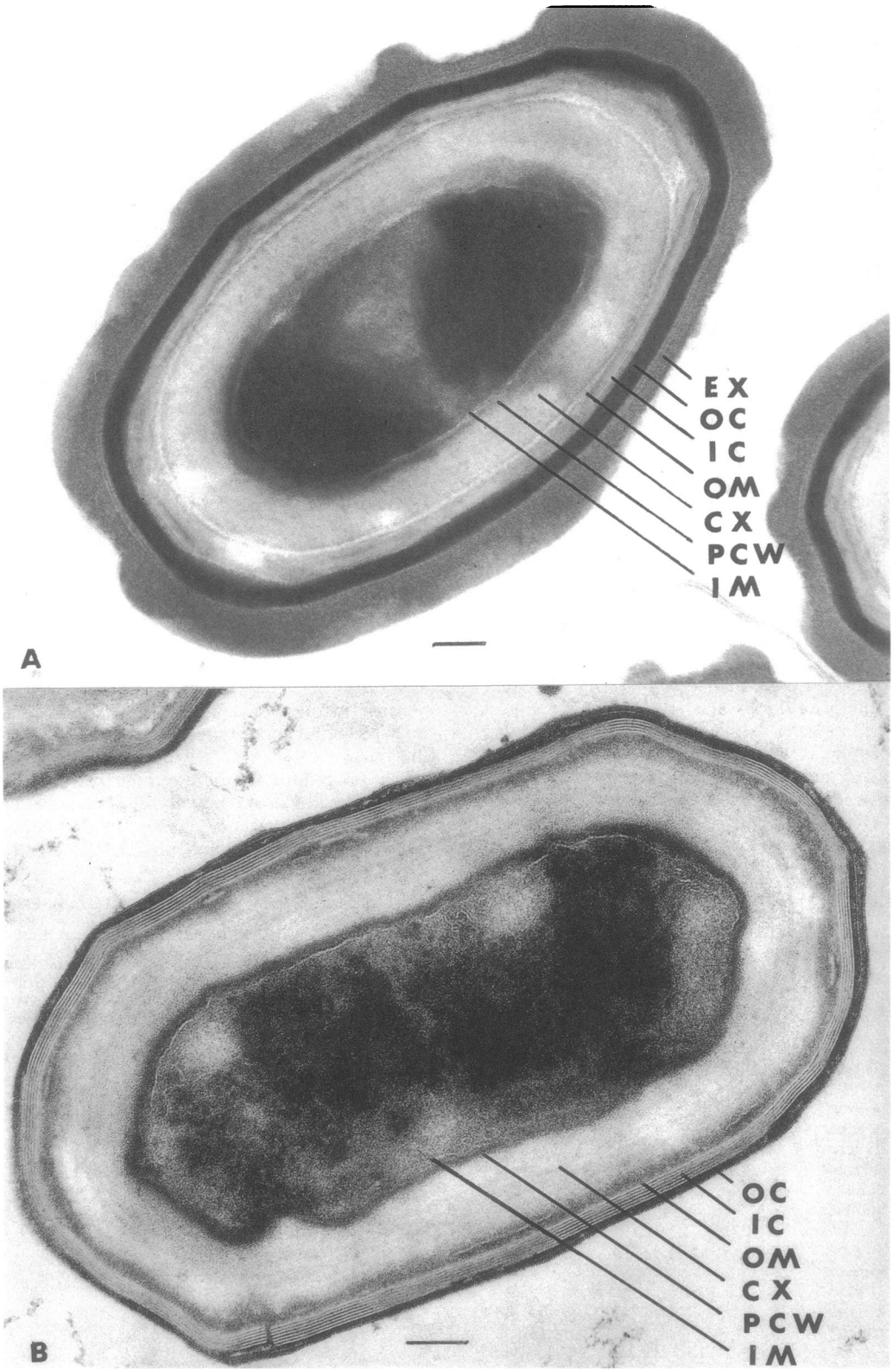

FIG. 2. Electron micrograph of a longitudinally medial section of a dormant spore of *B. subtilis* subsp. *niger* (A) and *B. stearothermophilus* smooth variant (B). Comparable micrographs of *B. cereus* T spores were published by Gerhardt and Ribi (12). The following structures are indicated in representative places: exosporium (Ex), outer coat (OC), inner coat (IC), location of outer membrane (OM), cortex (Cx), primordial cell wall (PCW), and inner membrane (IM). Bar = 100 nm.

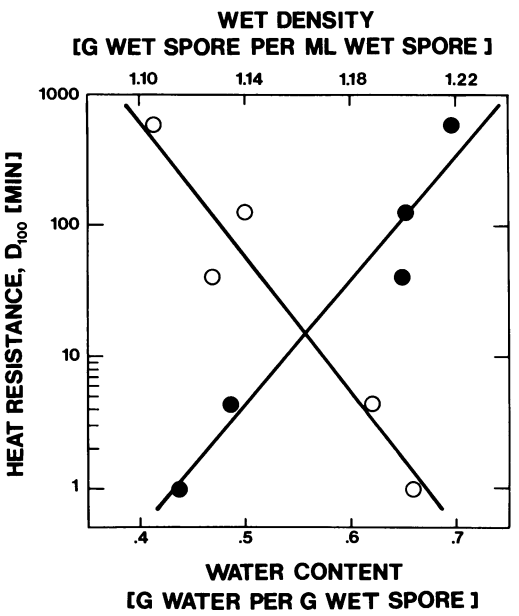

FIG. 3. Heat resistance determined as D_{100} correlated by least-squares analysis with the wet density (●) and the water content (○) of the entire spore of various dormant spore types.

sulting value of 1.040 g/ml agreed closely with the known value of 1.037 g/ml (7a).

In contrast to the wet density, the dry density varied inconsistently with the heat resistance among the various dormant spore types (Table 1). The average dry density was 1.455 g of dry spores per ml of dry spores.

Solids and water content of entire spore. By use of the wet density as a conversion factor, the solids and water contents were expressed on bases of wet weight, wet volume, and dry weight of the entire spore of the various types (Table 1). Among the various types of dormant spore (but not the germinated spore), an exponential increase in the heat resistance correlated with a decrease in the water content (Fig. 3).

Protoplast and sporoplast volume fractions. Neither the volume fraction of the entire spore occupied by the protoplast nor that occupied by the sporoplast changed consistently with the heat resistance among the various spore types (see Table 1, reference 10), probably because of differences in the volume of the total spore due to differences in the volume of the coats, and in the volume or absence of an exosporium.

Because of independence from these variables, however, the volume fraction of the sporoplast occupied by the protoplast provided useful information relative to heat resistance. Among the various types of dormant spore, an exponential increase in the heat resistance correlated roughly with a decrease in the protoplast/sporoplast volume ratio (Fig. 4).

Density, solids content, and water content of isolated integument. Results for the perisporoplast integument fraction and the condensed results for the periprotoplast integument fraction are shown in Table 2. In no case did the values change consistently with the heat resistance of the various spore types.

Water distribution. Attempts were made to determine distribution of the total spore water between the perisporoplast integument and the sporoplast (and between the periprotoplast integument and the protoplast), using the integument data in Table 2 and the volume fraction data in Fig. 4. However, the results were inconclusive.

DISCUSSION

The foregoing results inversely correlating heat resistance with water content of the entire spore prevailed not only between species but also within species (that is, calcium-deficient and -sufficient spores of *B. cereus* T, and rough- and smooth-variant spores of *B. stearothermophilus*). The distinction is important because of the correlation between spore heat resistance and maximum growth temperature among various species (21). However, water content alone may not account for the unique heat resistance

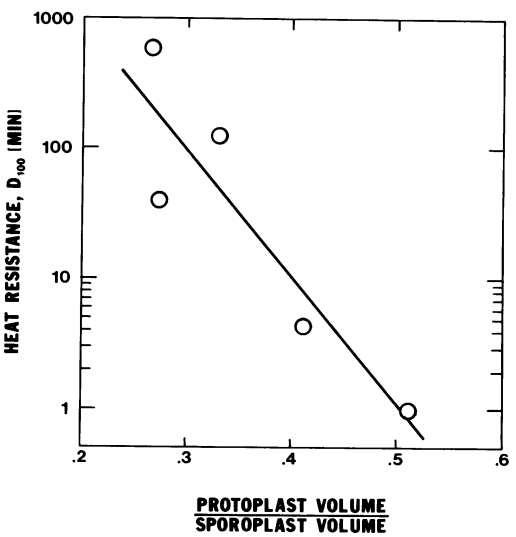

FIG. 4. Heat resistance determined as D_{100} correlated by least-squares analysis with the protoplast/sporoplast volume ratio of various dormant spore types. The size of the points indicates the largest among the range of standard deviations (±0.03 to 0.08) in values of the volume ratio.

TABLE 2. Density, solids content, and water content (expressed in various ways) of the isolated perisporoplast integument of various spore types[a]

Spore type (species, strain, variant)	D_{100} (min)	Density		Solids content		Water content		
		(g of wet integ.[b])/ml of wet integ.)	(g of dry integ.)/(ml of dry integ.)	(g of solids)/(g of wet integ.)	(g of solids)/(ml of wet integ.)	(g of water)/(g of wet integ.)	(g of water)/(g of dry integ.)	(g of water)/(ml of wet integ.)
B. cereus, T, calcium deficient	1.00	1.032	1.174	0.209	0.216	0.791	3.785	0.816
B. subtilis, subsp. niger	40.0	1.156	1.608	0.357	0.413	0.643	1.801	0.743
B. stearothermophilus, rough	124	1.122	1.792	0.246	0.276	0.754	3.065	0.846
B. stearothermophilus, smooth	579	1.159	1.360	0.571	0.662	0.429	0.751	0.497

[a] The perisporoplast integument of *B. cereus* and *B. subtilis* spores consisted of inner and outer coats and also exosporium. That of *B. stearothermophilus* spores consisted only of the coats. The periprotoplast integument of all the spore types additionally contained cortex. The density values (e.g., 1.043 to 1.099 g of wet integument per ml of wet integument) and the solids contents (e.g., 0.160 to 0.222 g of solids per g of wet integument) for this fraction were consistently lower than those for the perisporoplast integument fraction, apparently because of a higher water content of the cortex.

[b] Abbreviation: integ., integument.

of dormant spores. The vegetative sporogenous cell (T. Koshikawa, J. E. Algie, L. S. Tisa, and P. Gerhardt, Spore Newslet. 7(5):24–25, 1981) exhibits an entirely different relationship between heat resistance and water content.

Wet density (or solids content) of the entire spore mirrored water content in relation to heat resistance, as expected. However, the dry density appeared to be an independent variable without particular significance, as shown also by other investigators (4, 18).

The results correlating heat resistance with size of the protoplast relative to size of the sporoplast confirmed similar findings by other investigators (2; J. E. Algie, and L. S. Tisa, Spore Newslet. 7(5):20–21, 1981; A. D. Hitchins and R. A. Slepecky, Spore Newslet. 7(5):103–104, 1981). The results also were consistent with findings of a correlation between heat resistance and the amount of diaminopimelic acid as an index of the amount of cortex (19).

A difficult problem was posed by the use of measurements on electron micrographs of thin-sectioned spores to determine the protoplast/sporoplast volume ratio. However, no other method was available for this purpose, and precautions were taken to compensate for the intrinsic inaccuracies. Care was taken to select images in which a spore was longitudinally sectioned through the center, as indicated by elongate shape and good resolution of the integument around the entire periphery (Fig. 2). A reasonable number of images (6 to 11) were measured and averaged. The protoplast/sporoplast volume ratio, rather than absolute volumes, was used as a correlation parameter on the assumption that possible shrinkage during the dehydration and fixation steps would be proportional for the two internal compartments. The resulting statistical variation in the ratios seemed acceptable for determining correlations with comparably variable measurements of heat resistance (Fig. 4) and permeability (10).

The present data did not permit determination of the distribution of water into various structural compartments of the spore apparently because of the presence or absence of an exosporium, variable nature of the coat, change in water content of the cortex upon isolation, and other variables. However, the data provided a necessary basis for subsequently determining water distribution by means of immersion refractometry (10).

Altogether, the results supported the commonly held hypothesis that the extent of heat resistance is based in whole or in part on the extent of dehydration and diminution of the protoplast. However, the results shed no light on the mechanisms by which the protoplast is made drier and smaller as sporogenesis proceeds. It is important to distinguish between the physicochemical bases and the physiological mechanisms (11). The observed dehydration basis (and this not necessarily the only one) could be brought about in whole or part by any of several proposed mechanisms.

ACKNOWLEDGMENTS

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