APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1986, p. 1242–1246
0099-2240/86/121242-05$02.00/0

Vol. 52, No. 6

# Heat Resistance of Bacterial Spores Correlated with Protoplast Dehydration, Mineralization, and Thermal Adaptation†

TEOFILA C. BEAMAN AND PHILIPP GERHARDT*

*Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan 48824*

Received 24 June 1986/Accepted 12 September 1986

**Twenty-eight types of lysozyme-sensitive spores among seven *Bacillus* species representative of thermophiles, mesophiles, and psychrophiles were obtained spanning a 3,000-fold range in moist-heat resistance. The resistance within species was altered by demineralization of the native spores to protonated spores and remineralization of the protonated spores to calcified spores and by thermal adaptation at maximum, optimum, and minimum sporulation temperatures. Protoplast wet densities, and thereby protoplast water contents, were obtained by buoyant density sedimentation in Nycodenz gradients (Nyegaard and Co., Oslo, Norway). Increases in mineralization and thermal adaptation caused reductions in protoplast water content between limits of ca. 57 and 28% (wet weight basis), and thereby correlated with increases in sporal heat resistance. Above and below these limits, however, increases in mineralization and thermal adaptation correlated with increases in sporal resistance independently of unchanged protoplast water contents. All three factors evidently contributed to and were necessary for heat resistance of the spores, but dehydration predominated.**

The heat resistance of bacterial spores can be attributed to three main factors—protoplast dehydration, mineralization, and thermal adaptation—each of which can be quantified. Dehydration of the protoplast appears to be the primary component, possibly in itself accounting for the low resistance of *Bacillus megaterium* spores (4). In spores of more resistant species, including *B. stearothermophilus*, however, the protoplast water content is similar or higher, indicating that the resistance of these spores involves additional factors (10).

These findings have been extended by the observation of a correlation between the water content and the buoyant wet density of protoplasts of diverse spore types. Thus, a precise and easy method for measuring protoplast dehydration in other spores, even those in small impure samples, is now available (7). The key to these determinations is the use of spores in which the complex of coat and outer membrane is genetically defective or chemically disrupted, as evidenced by susceptibility of the cortex to digestion by lysozyme. These defects allow for the penetration of selected immersion media through the spore cortex to the protoplast membrane, so that the spore buoyant density reflects the protoplast water content.

Thermal adaptation has been assumed to be an inherent or intrinsic molecular component which is genetically determined; that is, the spores of thermophilic species generally are more resistant than those of mesophilic or psychrophilic species (12). However, the spores of a given species grown at maximum temperature are more resistant than those grown at optimum or minimum temperatures (14), and so there appears to be an extrinsic element imposed on the genetic element.

Mineralization changes sporal heat resistance extrinsically. This change can be accomplished to a limited extent by modification of the types and amounts of mineral cations in the sporulation medium (3) or, more completely, by demineralization through acid extraction of dormant spores followed by remineralization with specific salts (1, 5, 9).

The three main determinants of sporal heat resistance have been assumed to be independent variables. If they do indeed act independently to increase heat resistance, then one would expect additive effects; that is, the increase in heat resistance that occurs with decreased protoplast water content of spores (10) would be heightened by increased mineralization (5) and further augmented by increased thermal adaptation (12, 14).

In this paper, data are presented showing the effects of changes of protoplast mineralization and thermal adaptation separately on the heat resistance and the protoplast water content of water-suspended spores. The results are brought together in a graph correlating the three factors with the widely varying heat resistances of 28 spore types from seven *Bacillus* species.

## MATERIALS AND METHODS

The culture identities, sensitization methods, and experimental treatments for producing lysozyme-sensitive spore types from various species of *Bacillus* representative of thermophiles, mesophiles, and psychrophiles are shown in Table 1 and described further by Nakashio and Gerhardt (10) and Lindsay et al. (7). Each lot of clean spores was used within 1 week of refrigerated storage in distilled water. The spores of *B. stearothermophilus* were produced more efficiently (80 to 95% sporulation in 3 to 4 days) than described previously (3) by use of plates of medium containing 2% agar, 0.1% D-glucose, 0.1% L-glutamic acid, 0.05% yeast extract, 0.2% $MgSO_4 \cdot 7H_2O$, 0.001% NaCl, 0.01% $(NH_4)_2HPO_4$, and 0.05% $KH_2PO_4$. Mineral salts were added aseptically to final concentrations of 0.005% $CaCl_2$, 0.00078% $MnCl_2$, 0.0018% $ZnSO_4 \cdot 7H_2O$, and 0.0018% $FeSO_4 \cdot H_2O$. The pH was adjusted to 6.9 with NaOH. The plates were incubated at the desired temperature in humidified containers. Strain 0121 of *B. stearothermophilus*, more resistant ($D_{100}$ [minutes required for a decimal reduction in CFU on exposure to 100°C of $10^7$ to $10^8$ viable spores per ml] = 2,290 min with sporulation at 75°C) than the standard

* Corresponding author.

† Publication no. 11980 of the Michigan Agricultural Experiment Station.

TABLE 1. Identity, treatment, and analysis of lysozyme-sensitive spore types

| Identity no. | Species | Strain | Lysozyme sensitization | Treatment | | Protoplast wet density (g/ml) | $PWC^{WP}$ [a] | $PWC^{DP}$ [b] | $D_{100}$ (min) |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Sporulation temp (°C) | Mineralization | | | | |
| 1A | *B. stearothermophilus* | 7953 | TGC[c] | 75 | Calcium | 1.391 | 27.2 | 37.4 | 507 |
| 1A[1] | *B. stearothermophilus* | 0121 | TGC | 75 | Calcium | 1.385 | 29.5 | 41.8 | 933 |
| 1B | *B. stearothermophilus* | 7953 | TGC | 75 | Native | 1.392 | 26.8 | 36.6 | 311 |
| 1B[1] | *B. stearothermophilus* | 0121 | TGC | 75 | Native | 1.385 | 29.5 | 41.8 | 128 |
| 1C | *B. stearothermophilus* | 7953 | TGC | 75 | Hydrogen | 1.363 | 38.2 | 61.8 | 30.2 |
| 1D | *B. stearothermophilus* | 7953 | TGC | 60 | Calcium | 1.391 | 27.2 | 37.4 | 394 |
| 1E | *B. stearothermophilus* | 7953 | TGC | 60 | Native | 1.390 | 27.6 | 38.1 | 238 |
| 1F | *B. stearothermophilus* | 7953 | TGC | 60 | Hydrogen | 1.365 | 37.4 | 59.7 | 37.5 |
| 1G | *B. stearothermophilus* | 7953 | TGC | 45 | Calcium | 1.366 | 37.0 | 58.7 | 61.4 |
| 1H | *B. stearothermophilus* | 7953 | TGC | 45 | Native | 1.360 | 39.4 | 65.0 | 30.0 |
| 1I | *B. stearothermophilus* | 7953 | TGC | 45 | Hydrogen | 1.343 | 46.1 | 85.5 | 10.8 |
| 2 | "*B. caldolyticus*" | B697 | TGC | 60 | Native | 1.390 | 27.6 | 38.1 | 98.3 |
| 3 | *B. coagulans* | B666 | TGC | 50 | Native | 1.380 | 31.5 | 46.0 | 59.8 |
| 4A | *B. subtilis* | 4673 | Transductant | 50 | Native | 1.375 | 33.5 | 50.4 | 45.1 |
| 4B | *B. subtilis* | 4673 | Transductant | 37 | Calcium | 1.352 | 42.5 | 73.9 | 36.7 |
| 4C | *B. subtilis* | 4673 | Transductant | 37 | Native | 1.333 | 50.0 | 100 | 8.84 |
| 4D | *B. subtilis* | 4673 | Transductant | 37 | Hydrogen | 1.315 | 57.1 | 133 | 2.68 |
| 4E | *B. subtilis* | 4673 | Transductant | 20 | Native | 1.320 | 55.0 | 122 | 4.86 |
| 5 | *B. thuringiensis* | $S_1$ | Mutant | 30 | Native | 1.315 | 57.1 | 133 | 2.80 |
| 6A | *B. cereus* | 1OLD | Mutant | 42 | Calcium | 1.335 | 49.2 | 96.9 | 11.6 |
| 6B | *B. cereus* | 1OLD | Mutant | 42 | Native | 1.330 | 51.2 | 105 | 6.90 |
| 6C | *B. cereus* | 1OLD | Mutant | 42 | Hydrogen | 1.315 | 57.1 | 133 | 2.10 |
| 6D | *B. cereus* | 1OLD | Mutant | 30 | Calcium | 1.320 | 55.1 | 123 | 7.16 |
| 6E | *B. cereus* | 1OLD | Mutant | 30 | Native | 1.315 | 57.1 | 133 | 2.87 |
| 6F | *B. cereus* | 1OLD | Mutant | 30 | Hydrogen | 1.312 | 58.3 | 140 | 1.46 |
| 6G | *B. cereus* | 1OLD | Mutant | 17 | Native | 1.315 | 57.1 | 133 | 0.74 |
| 6H | *B. cereus* | 1OLD | Mutant | 17 | Hydrogen | 1.313 | 57.8 | 137 | 0.79 |
| 7 | *B. macquariensis* | 23466 | SDS-DTT[d] | 17 | Native | 1.320 | 55.1 | 123 | 0.33 |

[a] Grams of water per 100 g of wet protoplast (see the text).
[b] Grams of water per 100 g of dry protoplast (see the text).
[c] TGC, Treatment with thioglycolic acid at the sporulation temperature.
[d] SDS-DTT, Treatment with sodium dodecyl sulfate plus dithiothreitol at 37°C.

strain 7953 ($D_{100}$ = 1,650 min with sporulation at 75°C), was isolated and presumptively identified at the Campbell Soup Company, Camden, N.J. The spores of "*B. caldolyticus*" and *B. coagulans* were produced on the sporulation medium of Warth (12) solidified with agar; the cultures were obtained from the Division of Food Research, Commonwealth Scientific and Industrial Research Organization, North Ryde, New South Wales, Australia. The spores of *B. subtilis* were produced in 200 ml of supplemented nutrient broth (11) without glucose in 2-liter shaken flasks. The spores of *B. thuringiensis* and *B. cereus* were produced as described by Aronson et al. (2). The spores of *B. macquariensis* were produced as described by Lindsay et al. (7).

Thermal adaptation was obtained by growth and sporulation at incubation temperatures corresponding to the maximum, optimum, and minimum for each species, consistent with a satisfactory percentage of sporulation.

Demineralization of the native lysozyme-sensitive spores to protonated or hydrogen-form spores, remineralization to calcified or calcium-form spores, and extraction for mineral analyses were accomplished by procedures essentially as described for lysozyme-resistant spores by Bender and Marquis (5). The mineral contents were analyzed by plasma emission spectroscopy (Spectra Span 3A; Beckman Instruments, Inc., Fullerton, Calif.). The results were expressed as micromoles per milligram of spore dry weight.

Wet density of the spore protoplasts was determined by buoyant density sedimentation at 5°C in incremental discontinuous gradients of Nycodenz (Nyegaard and Co., Oslo, Norway), as described by Lindsay et al. (7). The results were expressed as grams of wet protoplast per milliliter of wet protoplast.

Water content of the spore protoplasts was obtained from the experimental correlation $y = -0.00254x + 1.460$, where $y$ is the protoplast wet density and $\chi$ is the protoplast water content (7). The results were expressed as percentages on a wet weight basis, where $PWC^{WP}$ is expressed in grams of water per 100 g of wet protoplast, and were converted to a dry weight basis, where $PWC^{DP}$ is expressed in grams of water per 100 g of dry protoplast, by the following equation: $PWC^{DP} = [PWC^{WP}/(100 - PWC^{WP})] \times 100$.

Heat resistance was measured as described previously (3), except that the spores were suspended in distilled water instead of phosphate buffer so as not to affect mineralization. The results were expressed as $D_{100}$ values (see above). The $D_{100}$ values of less than 2 min (for *B. cereus* and *B. macquariensis* spores) were obtained by least-squares extrapolation from higher values obtained at 80, 85, and 90°C.

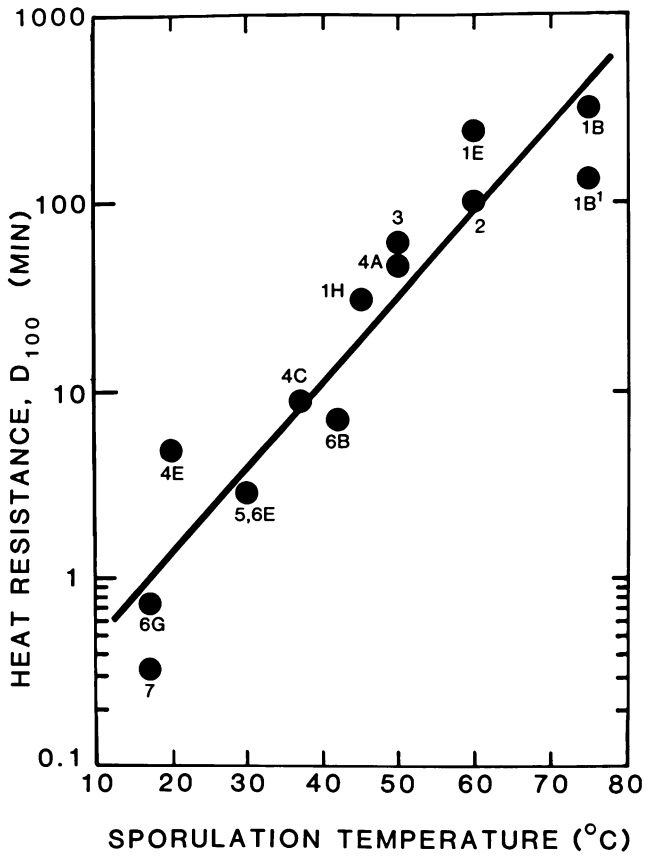


FIG. 1. Heat resistance correlated (by least-squares analysis) with sporulation temperature of lysozyme-sensitive native spores of seven species. The identity numbers correspond to those in Table 1.

## RESULTS

**Thermal adaptation.** A linear relationship over a wide range was found between the logarithm of heat resistance (log $D_{100}$) and the sporulation temperature of lysozyme-sensitive native spores of various *Bacillus* species (Fig. 1). The higher the optimum temperature for sporulation was, the greater the heat resistance was for the seven species. Within each species, sporulation at successively higher temperatures (from minimum to optimum to maximum) also caused successively greater heat resistances; e.g., lysozyme-sensitive native spores of *B. stearothermophilus* 7953 produced at 45, 60, and 75°C had $D_{100}$ values of 30, 238, and 311 min, respectively (Table 1). This general influence of higher sporulation temperature increasing sporal heat resistance was usually found in the protonated and the calcified spores, as well as in the native lysozyme-sensitive spores. The influence of sporulation temperature on sporal heat resistance was also evidenced in lysozyme-resistant spores; e.g., such native spores of *B. stearothermophilus* 7953 produced at 45, 60, and 75°C had $D_{100}$ values of 100, 580, and 1,650 min, respectively, confirming early findings (14).

Thermal adaptation during sporulation was found to affect protoplast dehydration over much, if not all, of the temperature range (Fig. 2). A complex, triphasic relationship occurred relating sporulation temperature to protoplast water content of the lysozyme-sensitive native spores. Increased temperature of sporulation was directly related to reduction in protoplast water content between limits of ca. 57 and 28%. Above and below these limits, however, increases in sporulation temperature did not affect protoplast water contents. Similar trends were observed with the protonated and calcified spores (Table 1).

**Mineralization.** Alteration of mineral contents brought about changes in heat resistance. The calcified spores were more resistant than the native spores, which were more resistant than the protonated spores, irrespective of sporulation temperature (Table 1). Quantitative analyses of the main mineral content in three representative species were unremarkable and minimally affected by thermal adaptation (Table 2). The changes in heat resistance occurring with changes in mineralization appeared not to involve changes in dipicolinic acid and generally were as expected from previous findings by Bender and Marquis (5).

However, changes in mineralization brought about changes in protoplast water content. Protoplasts of the protonated spores were more hydrated than those of the native or calcified spores, especially for the more resistant spore types (Table 1). This finding may seem contradictory to that of Marquis et al. (9), who reported that demineralization and remineralization do not change spore hydration. However, they used a measurement for water content of the entire spore rather than of only the protoplast, and they used *B. megaterium* spores, which are at the low limit of protoplast water content and have an anomalously low heat resistance (10).

**Dehydration.** Since increases in mineralization and sporulation temperature separately caused reduction in the protoplast water content of the spores, a generalized semilog plot was drawn correlating heat resistance (log $D_{100}$) with protoplast water content of the 28 lysozyme-sensitive spore

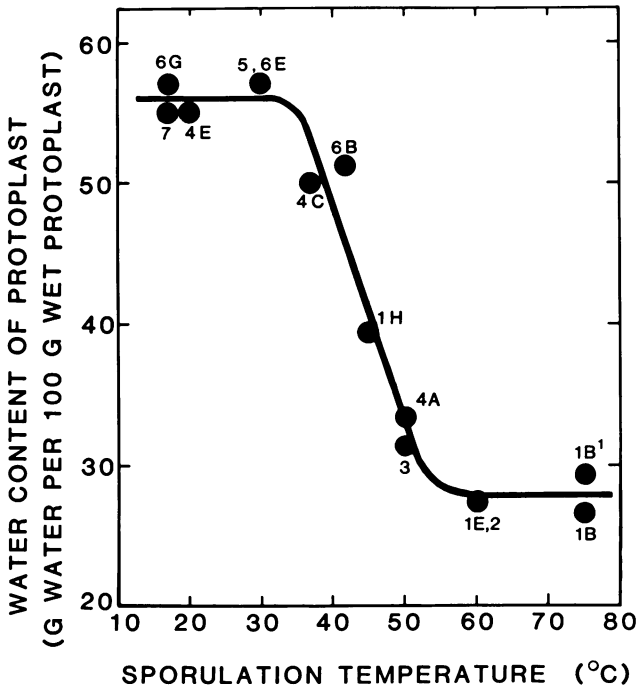


FIG. 2. Protoplast water content correlated (by least-squares analysis of linear regions) with sporulation temperature of lysozyme-sensitive native spores of seven species. The identity numbers correspond to those in Table 1.

TABLE 2. Mineral contents of representative lysozyme-sensitive spore types

| Identity no. | Species | Treatment | | Mineral content (μmol/mg of spores [dry wt]) | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Sporulation temp (°C) | Mineralization | Ca | Mn | Mg | K | Na |
| 1D | *B. stearothermophilus* | 60 | Calcium | 1.174 | 0.003 | 0.002 | 0.006 | 0.003 |
| 1E | *B. stearothermophilus* | 60 | Native | 0.777 | 0.056 | 0.037 | 0.040 | 0.019 |
| 1F | *B. stearothermophilus* | 60 | Hydrogen | 0.052 | 0.004 | 0.002 | 0.004 | 0.003 |
| 4B | *B. subtilis* | 37 | Calcium | 1.026 | 0.005 | 0.029 | 0.092 | 0.035 |
| 4C | *B. subtilis* | 37 | Native | 1.142 | 0.050 | 0.244 | 0.077 | 0.026 |
| 4D | *B. subtilis* | 37 | Hydrogen | 0.080 | 0.005 | 0.072 | 0.080 | 0.033 |
| 6D | *B. cereus* | 30 | Calcium | 0.886 | 0.042 | 0.036 | 0.053 | 0.047 |
| 6E | *B. cereus* | 30 | Native | 0.876 | 1.128 | 0.113 | 0.103 | 0.350 |
| 6F | *B. cereus* | 30 | Hydrogen | 0.021 | 0.018 | 0.015 | 0.016 | 0.023 |

[a] As in Table 1.

types from the seven species (Fig. 3). This resulted in a complex, triphasic relationship. Between limits of ca. 57 and 28% in protoplast water content, increases in mineralization or sporulation temperature caused decreases in protoplast water content and thereby corresponding increases in sporal heat resistance. Within these limits, the results resembled those previously reported for lysozyme-sensitive native spores of four species other than *B. megaterium* (10).

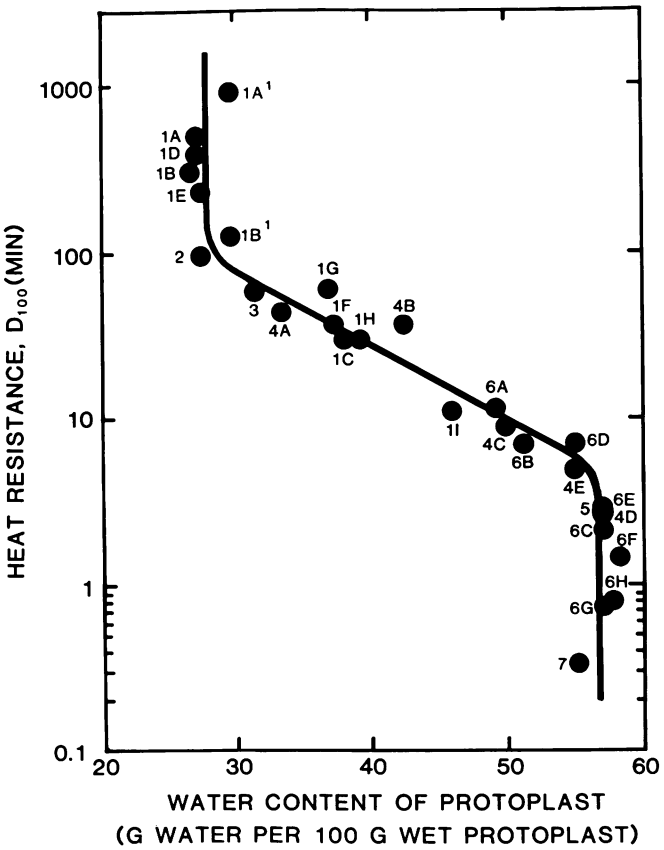


FIG. 3. Heat resistance correlated (by least-squares analysis of linear regions) with protoplast water content of 28 lysozyme-sensitive spore types from seven species varying in thermal adaptation and in mineralization. The identity numbers correspond to those in Table 1.

Outside these limits, however, increases in mineralization or sporulation temperature correlated with increases in sporal resistance independently of unchanged protoplast water content. Ten different types from four different species had essentially the same maximum in protoplast water content (ca. 57%), yet differed more than 10-fold in $D_{100}$ values. A similar difference in resistance occurred in seven spore types from two species having essentially the same minimum in protoplast water content (ca. 28%). All three factors evidently contributed to and were necessary for heat resistance of the spores, but dehydration predominated.

## DISCUSSION

The general correlation between the heat resistance of spores over a wide range with the sporulation temperature (Fig. 1) resembled the correlation previously shown with the maximum vegetative growth temperature (12). Thermal adaptation is generally attributed to intrinsic properties that stabilize tertiary and quaternary conformations of vital macromolecules. Our results (Fig. 2) show that, in addition to this intrinsic element, thermal adaptation during sporulation can affect spore heat resistance extrinsically by lowering the protoplast water content.

Mineralization is known to be a major extrinsic factor in sporal heat resistance (1, 5, 9) and was thought by Marquis et al. (9) to act independently of dehydration. Our results (Table 1), however, reveal that native or calcium-reminerализed protoplasts are more dehydrated, and thereby more resistant, than protonated protoplasts over much of the range. This suggests a possible molecular mechanism by which minerals might stabilize spores by replacement of water in biopolymers; evidence for this exists in the intercalation of DNA by calcium dipicolinate (8). At the upper and lower limits of sporal heat resistance, however, the protoplast water content is constant and heat resistance is changed independently by mineralization (Fig. 3), so that other mechanisms may occur.

The upper limit of protoplast water content in the spores (ca. 57%, wet weight basis) was remarkable in its abruptness and constancy. One might expect a gradual transition toward the protoplast water content of germinated spores and vegetative cells (for *B. cereus*, about 73 and 77%, respectively, assuming that the percent water content of the protoplasts is essentially the same as that for entire cells [6]). We are unable to explain why protoplast dehydration in spores should rise to no higher than ca. 57%.

The lower limit of protoplast water content in the spores (ca. 28%, wet weight basis) was yet more remarkable. This limit prevailed not only with the spores of two strains of *B. stearothermophilus* and a strain of "*B. caldolyticus*" (Fig. 2), but also with the spores of four strains of *B. megaterium*, albeit at much lower levels of heat resistance (4, 10). Why should protoplast dehydration fall to no lower than ca. 28%? Possibly, the physiological mechanisms (e.g., cortex pressure and molecular displacement) for removing water from the protoplast during spore formation are limited by the state of water occurring at this level. If so, the dehydration process would start with the early forespore, which probably contains about 75% water distributed at about the same percentage in the protoplast and in the integument as in the vegetative cell. As the spore matures, water mechanically trapped in the free liquid state would be progressively expressed and displaced from the protoplast. A lower and lower water content of the protoplasts with correspondingly higher and higher heat resistance would thus result for the various types of spores. The lowest protoplast water content (28%) would correspond to a threshold at which the remaining water is withheld in a bound state. Much higher pressure would be required to remove water in this bound state, and the spore would instead rely on mineralization and thermal adaptation (or some other mechanism) acting independently to attain yet greater heat resistance. This explanation is substantiated by the shape and explanation of water desorption isotherms, like those for intact *B. stearothermophilus* spores (13). In such graphs, there initially is a precipitous region representing readily removed free water, followed by a transition region representing loosely bound multilayered water, and finally a gradual plateau region representing tightly bound monolayered water.

The water content of intact spores decreases not only with increased temperature of sporulation (Fig. 2), but apparently also with increased temperature of exposure. Fully hydrated but compacted spores of *B. stearothermophilus* have a water content equivalent to 46% (wet weight basis) when exposed at 20°C, but only 35% at 100°C (13). If proportional and comparable, the protoplasts in situ within such water-suspended spores should change in water content from about 28% when exposed at 5°C (Fig. 2) to about 21% at 100°C. This important prediction needs to be tested experimentally.

Water content does not directly reflect the water activity within spores, since the two measurements are not equivalent (13). For molecular stabilization, it is the activity, rather than the content, of water which is important. What must be devised, if possible, is a method to measure protoplast water activity so that this parameter can be related to protoplast water content in sorption isotherms when both parameters are measured within intact water-suspended dormant spores. Furthermore, these measurements should be made not only at low temperatures, when spore preservation occurs, but also at high temperatures, when spore death occurs.

## ACKNOWLEDGMENTS

This work was supported by contract DAAG 29-83-K-0057 from the Biological Sciences Program of the U.S. Army Research Office.

We thank Jody McCormack, Laurie Jankowiak, and Rosita Cabrera for technical assistance.

## LITERATURE CITED

1. **Alderton, G., and N. Snell.** 1963 Base exchange and heat resistance in bacterial spores. Biochem. Biophys. Res. Commun. **10**:139–143.
2. **Aronson, A. I., N. Angelo, and S. C. Holt.** 1971. Regulation of extracellular protease production in *Bacillus cereus* T: characterization of mutants producing altered amounts of protease. J. Bacteriol. **106**:1016–1025.
3. **Beaman, T. C., J. T. Greenamyre, T. R. Corner, H. S. Pankratz, and P. Gerhardt.** 1982. Bacterial spore heat resistance correlated with water content, wet density, and protoplast/sporoplast volume ratio. J. Bacteriol. **150**:870–877.
4. **Beaman, T. C., T. Koshikawa, H. S. Pankratz, and P. Gerhardt.** 1984. Dehydration partitioned within core protoplast accounts for heat resistance of bacterial spores. FEMS Microbiol. Lett. **24**:47–51.
5. **Bender, G. R., and R. E. Marquis.** 1985. Spore heat resistance and specific mineralization. Appl. Environ. Microbiol. **50**: 1414–1421.
6. **Black, S. H., and P. Gerhardt.** 1962. Permeability of bacterial spores. IV. Water content, uptake, and distribution. J. Bacteriol. **83**:960–967.
7. **Lindsay, J. A., T. C. Beaman, and P. Gerhardt.** 1985. Protoplast water content of bacterial spores determined by buoyant density sedimentation. J. Bacteriol. **163**:735–737.
8. **Lindsay, J. A., and W. G. Murrell.** 1986. Solution spectroscopy of dipicolinic interaction with nucleic acids: role in spore heat resistance. Curr. Microbiol. **13**:255–259
9. **Marquis, R. E., E. L. Carstensen, S. Z. Child, and G. R. Bender.** 1981. Preparation and characterization of various salt forms of *Bacillus megaterium* spores, p. 266–268. *In* H. S. Levinson, A. L. Sonenshein, and D. J. Tipper (ed.), Sporulation and germination. American Society for Microbiology, Washington, D.C.
10. **Nakashio, S., and P. Gerhardt.** 1985. Protoplast dehydration correlated with heat resistance of bacterial spores. J. Bacteriol. **162**:571–578.
11. **Setlow, P., and A. Kornberg.** 1969. Biochemical studies of bacterial sporulation and germination. XVII. Sulfhydryl and disulfide levels in dormancy and germination. J. Bacteriol. **100**:1155–1160.
12. **Warth, A. D.** 1978. Relationship between the heat resistance of spores and the optimum and maximum growth temperatures of *Bacillus* species. J. Bacteriol. **134**:699–705.
13. **Watt, I. C.** 1981. Water vapor adsorption by *Bacillus stearothermophilus* endospores, p. 253–255. *In* H. S. Levinson, A. L. Soneshein, and D. J. Tipper (ed.), Sporulation and germination. American Society for Microbiology, Washington, D.C.
14. **Williams, O. B., and W. J. Robertson.** 1954. Studies on heat resistance. VI. Effect of temperature of incubation at which formed on heat resistance of aerobic thermophilic spores. J. Bacteriol. **67**:377–378.