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# Levels of $H^+$ and Other Monovalent Cations in Dormant and Germinating Spores of *Bacillus megaterium*

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Previous investigators using the extent of uptake of the weak base methylamine to measure internal pH have shown that the pH in the core region of dormant spores of *Bacillus megaterium* is 6.3 to 6.5. Elevation of the internal pH of spores by 1.6 U had no significant effect on their degree of dormancy or their heat or ultraviolet light resistance. Surprisingly, the rate of methylamine uptake into dormant spores was slow (time for half-maximal uptake, 2.5 h at 24°C). Most of the methylamine taken up by dormant spores was rapidly (time for half-maximal uptake, <3 min) released during spore germination as the internal pH of spores rose to ~7.5. This rise in internal spore pH took place before dipicolinic acid release, was not abolished by inhibition of energy metabolism, and during germination at pH 8.0 was accompanied by a decrease in the pH of the germination medium. Also accompanying the rise in internal spore pH during germination was the release of >80% of the spore $K^+$ and $Na^+$. The $K^+$ was subsequently reabsorbed in an energy-dependent process. These data indicate (i) that between pH 6.2 and 7.8 internal spore pH has little effect on dormant spore properties, (ii) that there is a strong permeability barrier in dormant spores to movement of charged molecules and small uncharged molecules, and (iii) that extremely early in spore germination this permeability barrier is breached, allowing rapid release of internal monovalent cations ($H^+$, $Na^+$, and $K^+$).

Despite extensive study, several key features of sporulation and germination in bacteria are not understood at the molecular level. In particular, the mechanisms whereby developing spores attain and maintain metabolic dormancy and heat and radiation resistance, yet lose these properties in the first minutes of spore germination, have not been clarified. Various factors, including decreased water content (9), binding of unique proteins to spore DNA (20), decreased levels of free divalent cations (26), and a high level of dipicolinic acid (DPA) (9), may be involved in the dormancy or resistance or both of spores. However, the significance and possible mechanism of action of these various factors are not understood.

Elevated heat and radiation resistance at one particular stage in the life cycle of an organism is seen in an extreme form in bacterial spores. However, many other types of organisms also have as part of their life cycle a stage of growth in which rates of metabolism and macromolecular synthesis are slowed. These dormant or metabolically quiescent stages of growth often show no significant increase in heat and radiation resistance. Two examples of this are unfertilized sea urchin eggs and dormant fungal spores (7, 10). Strikingly, in both of these systems the dormant or quiescent stage of growth may have a significantly lower internal pH than the actively growing form (2, 12). Furthermore, in unfertilized sea urchin eggs, artificial elevation of the internal pH results in large increases in the rates of endogenous metabolism and macromolecular synthesis (R. A. Steinhardt and M. M. Winkler, Fed. Proc. 38:465, 1979). This finding suggests that a decreased internal pH may be one cause of the metabolic quiescence of unfertilized eggs.

In common with the fungal (yeast) and sea urchin systems, dormant bacterial spores also have a pH within the spore core which is significantly lower (6.3 to 6.5) than that in germinated spores (~7.5) (21). This finding suggested that a low internal pH might be involved in the dormancy or resistance or both of bacterial spores. Consequently, we undertook further study of this system to determine (i) whether the low internal spore pH contributes to spore dormancy or resistance or both and (ii) how and when during spore germination the internal pH rises to the value of ~7.5.

## MATERIALS AND METHODS

**Chemicals, reagents, and bacteria.** Sources of labeled compounds were as described previously (21). HCl, double distilled from Vycor, was obtained from the G. Frederick Smith Chemical Co., Columbus, Ohio; $KNO_3$ and $NaNO_3$ standard solutions were purchased from Aldrich Chemical Co., Milwaukee, Wis.

Most of the work described was carried out using *Bacillus megaterium* QMB1551 (originally obtained from H. S. Levinson, U.S. Army Natick Laboratories, Natick, Mass.). Spores of this organism were grown, harvested, and washed as previously described (23). Cleaned spores were stored at 4°C in distilled water at a concentration of ~30 mg (dry weight) per ml. The spores were washed with distilled water every 24 to 48 h and were used only while free of germinated spores or bacterial contamination or both. Only with spores stored in this manner did we get rates of methylamine uptake which were reproducible from one spore crop to another. Lyophilization and dry storage of spores often resulted in spore crops which gave variable results. Spores of *Bacillus cereus* T (originally obtained from H. O. Halvorson, Brandeis University, Waltham, Mass.) were also used for a few experiments and were prepared as described above.

**Measurements of methylamine incorporation and internal pH.** Measurement of methylamine incorporation was carried out as described previously (21). Unless otherwise noted, spores (12 to 15 mg [dry weight] per ml) were incubated in 200 mM Tris-hydrochloride buffer containing 20 μM [$^{14}$C]methylamine and, in some cases, a small amount of $^3H_2O$. At various times, samples (1.0 ml) were centrifuged (30 s) in an Eppendorf microcentrifuge, and the supernatant and pellet fractions were processed and counted as described previously (21). As noted previously (21), there was no detectable methylamine catabolism during any experiments reported in this work. The internal water volume of dormant or germinated spores was determined with [$^{14}$C]sorbitol as described previously except that 50 μM sorbitol was used (21). The internal pH of spores was calculated from the methylamine uptake and the values for the sorbitol-impermeable volume as described previously (21). Note that all values for internal spore pH are only calculated values.

**Measurements of methylamine or ion release during germination.** Spores (15 to 25 mg/ml) were heat shocked (15 min, 60°C) in water, cooled in ice, centrifuged (10 min, 15,000 × *g*), washed with an equal volume of cold water, and suspended in an equal volume of cold 200 mM Tris-hydrochloride (pH ~8.5 at 4°C) unless otherwise noted, and [$^{14}$C]methylamine was added. After incubation for 12 to 16 h at 4°C, the spores were added to a prewarmed (30°C) vessel containing germinants and any other compounds to be tested. The final spore concentration during germination was about 10 mg/ml, and the buffer concentration was 200 mM in Tris-hydrochloride unless otherwise noted. Samples were taken as described above, and the pellet fraction was analyzed for methylamine after processing as described above; the supernatant fraction was used for analysis of $K^+$, $Na^+$, and DPA. In cases in which only methylamine release was to be analyzed, a calibrated amount of NaOH was present in the prewarmed vessel to ensure that the pH during germination in Tris-hydrochloride at 30°C was the same as during methylamine uptake into dormant spores in Tris-hydrochloride at 4°C.

**Other methods.** $K^+$ and $Na^+$ were measured using a model 56 atomic absorption spectrometer (The Perkin-Elmer Corp., Norwalk, Conn.) with an HGA-2200 graphite furnace. All dilutions made for these determinations used milli-Q-water (Millipore Corp., Bedford, Mass.), and all pipettes and tubes used were plastic and were prewashed with HCl (double distilled from Vycor) and then milli-Q-water. Measurement of $K^+$ and $Na^+$ in dormant spores was carried out on samples which were washed with 200 mM Tris-hydrochloride (20 min, 4°C), washed with water, and then digested with HCl (30 min, 100°C).

All values for $Na^+$ and $K^+$ in germination supernatant fluid have been corrected for the $Na^+$ or $K^+$ contributed by the components of the germination medium alone. This correction was small (<4% of total spore level) for $K^+$, but was more significant (~30% of total spore level) for $Na^+$. Because Tris buffers were used, all pH values were measured at the temperature of the experiment. Conductivity was measured with a Radiometer conductivity meter. DPA was measured colorimetrically (17).

## RESULTS

**Extent and rate of uptake of methylamine by dormant spores.** Measurement of the extent of the uptake of the weak base methylamine is a simple way for measurement of the pH within a membrane which is permeable to unprotonated methylamine but not the protonated species (13, 14); this method has been used successfully for measurement of the pH within both cells and organelles (13, 14). With this technique, we calculated that the pH in an internal region of dormant spores of *B. megaterium* was 6.2 (Table 1), similar to the value obtained previously (21). The rate of uptake of methylamine by dormant spores was quite slow at pH 8.5 to 8.9; the time for half-maximal uptake was 2.5 and 7 h at 24 and 4°C, respectively (Fig. 1A). In contrast, the rate of uptake of methylamine by germinated spores was too fast to measure (time for half-maximal uptake, <2 min; data not shown) as has been the observation with other cells and organelles tested (13, 14).

Most of the methylamine taken up by spores at 4°C remained in the spores over a long period of time. In contrast, at 24°C much of the methylamine taken up was slowly lost (Fig. 1A). Because there was neither methylamine catabolism nor a change in the external pH under these conditions, there must have been some change in the spores themselves, presumably a slow increase in their internal pH. However, the latter phenomenon was reversible. If spores were preincubated at pH 8.7 and 24°C for 5 days, they

took up little methylamine. However, subsequent washing with water and incubation at 24°C in water for 4 days resulted in spores which took up an amount of methylamine similar to that of the original spores (Fig. 1A).

TABLE 1. *Alterations in internal pH of dormant spores of B. megaterium*

| Treatment | Calculated internal pH |
|---|---|
| Untreated[a] | 6.2 |
| Incubated with 20 mM $(NH_4)_2SO_4$ present[b] | 7.8 |
| Incubated with 20 mM $(NH_4)_2SO_4$ (12 h, 4°C) and then washed[c] | 6.4 |

[a] Spores were incubated at 12 mg/ml in 200 mM Tris-hydrochloride (pH 8.6), and the internal pH was calculated from measurements of the extent of incorporation of [$^{14}$C]methylamine at 4°C over an 18-h period as described in the text.

[b] Spores were treated as described in footnote *a*, but with 20 mM $(NH_4)_2SO_4$ present.

[c] Spores were incubated for 16 h as in footnote *b*, but without methylamine. They were then centrifuged, suspended in cold water for 3 h, and centrifuged; this procedure was repeated three times. The internal pH was then measured as described in footnote *a*.

Methylamine uptake also was slow, but less methylamine was taken up than in Tris alone, and it was then slowly lost (Fig. 1B). LiCl and NaCl gave similar results (data not shown). It seems likely that the effect of KCl (and presumably other alkali metal ions) was due to a $K^+$-$H^+$ exchange, resulting in elevation of internal spore pH, since the KCl effect was reversible (Fig. 1B). Also, addition of 0.25 M KCl to dormant spores incubating at 4°C in dilute (10 mM) Tris-hydrochloride (pH 8.7) led to a sevenfold increase in the rate at which the pH of the medium fell (from 0.01 to 0.07 per day) (data not shown).

**Effect of manipulation of the internal pH of spores on spore properties.** The finding that the internal pH (as measured by methylamine incorporation) of the spores could change slowly in response to their environment suggested that it might be possible to manipulate the internal pH in a simpler way. One method

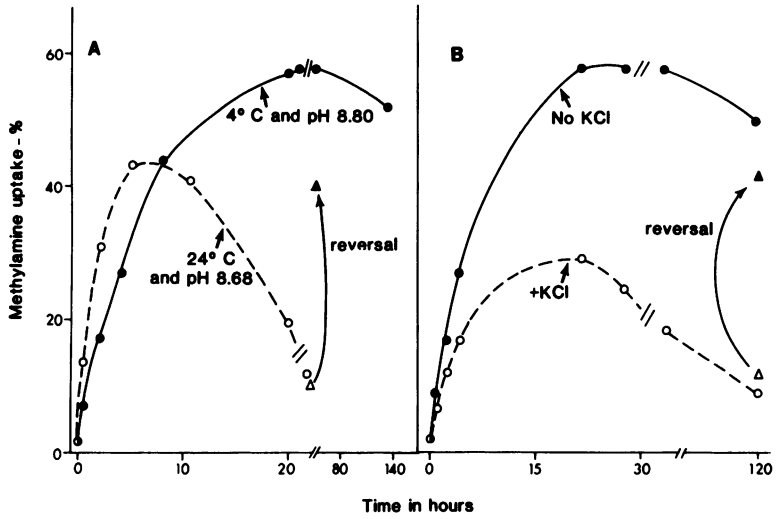


FIG. 1. *Uptake of [$^{14}$C]methylamine by dormant spores of B. megaterium at (A) different temperatures or (B) with and without KCl. (A) Spores (12 mg/ml) were incubated with [$^{14}$C]methylamine in 200 mM Tris-hydrochloride at the indicated pH and temperature and sampled at various times for methylamine uptake. The values for methylamine uptake represent the percentage of total methylamine in the culture which was taken up by the spores. The sample represented by Δ was incubated without methylamine at 24°C in Tris (pH 8.7) for 54 h. Methylamine was added to a portion of the culture, and its incorporation was measured after 5 h at 24°C. The remainder of the culture was centrifuged, incubated in water at 24°C, and washed by centrifugation (once every 24 h for 4 days). The spores were then returned to Tris-hydrochloride at pH 8.7 at 24°C, and at the point represented by ▲ methylamine incorporation was measured after 5 h. (B) Spores were incubated at 4°C in Tris-hydrochloride buffer (pH 8.8) with or without 250 mM KCl, and methylamine incorporation was measured. The point represented by Δ represents spores incubated 104 h in Tris-hydrochloride plus KCl alone, followed by methylamine addition and measurement of its incorporation after an additional 14 h. The sample represented by ▲ was treated as was the sample described above, but before the methylamine addition it was washed with water by centrifugation, resuspended in water, and rewashed once every 24 h for 170 h. The spores were then suspended in Tris-hydrochloride at 4°C, and methylamine incorporation was measured after 14 h.*

for manipulation of internal pH which has been successful in other systems (Steinhardt and Winkler, Fed. Proc. **38**:465, 1979) is the use of high levels of a weak base (such as $NH_3$) which can enter cells and act as an internal buffer. Indeed, soaking spores in $(NH_4)_2SO_4$ at pH 8.6 greatly reduced methylamine incorporation, raising the calculated internal pH by 1.6 U; again, this phenomenon was reversible (Table 1). A similar decrease in methylamine incorporation was seen when spores were soaked in $(NH_4)_2SO_4$ before methylamine addition (data not shown) or when $(NH_4)_2SO_4$ and methylamine were added simultaneously (Table 1). However, 10 μM $(NH_4)_2SO_4$, a concentration giving a level of weak base equal to that of the methylamine normally used for our measurements, had no effect on methylamine uptake (data not shown). Similarly, spores incubated with 20 mM methylamine exhibited a very low methylamine uptake and an internal pH of ~8.0 (data not shown).

With spores having an elevated internal pH, we examined several spore properties to determine whether there was some variation with internal pH. However, spores with calculated internal pH's of 6.2 or 7.8 had identical levels of DPA, showed no detectable utilization of their pool of 3-phosphoglyceric acid upon extended (6 days at 4°C) storage, germinated identically as measured by loss of DPA or optical density of the culture, and had essentially identical curves for killing by either a temperature of 90°C or by UV irradiation (data not shown).

**Changes in internal pH upon spore germination.** Although the exact value of the internal pH (at least between 6.2 and 7.8) of spores had no detectable effect on spore properties, it was still of interest to learn the time course and mechanism of how the internal pH of spores rises to 7.5 during spore germination (21). Most of the methylamine accumulated by dormant spores was released in the first minutes of germination in glucose (Fig. 2A). This rapid release of methylamine took place only in spores which were germinating; spores without germinants or spores which were not heat shocked showed little methylamine or DPA release (Fig. 2A and B). However, rapid methylamine release was seen with spores germinating in either a metabolizable (glucose) or non-metabolizable (KBr) germinant (Fig. 2A and B). The slower rate of release of methylamine during germination in KBr compared with glucose was almost certainly due to a slower rate of initiation of germination in KBr as measured either by DPA release (Fig.

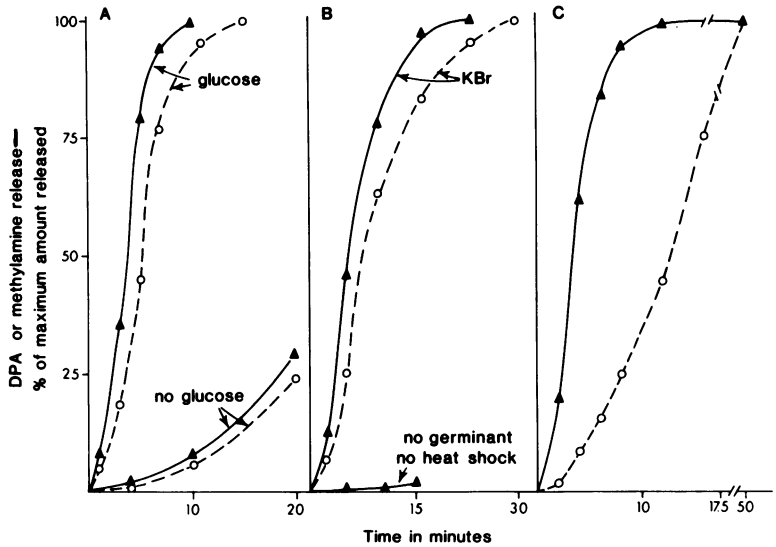


FIG. 2. *Release of methylamine and DPA during germination of B. megaterium spores (A) with or without glucose, (B) in KBr or with no germinants and no heat shock, or (C) with glucose and DCCD. After prior heat shock (unless otherwise noted), 13 mg of spores per ml was incubated in 200 mM Tris-hydrochloride (pH 8.8) plus methylamine for 16 h at 4°C, resulting in uptake of about 60% of the methylamine in the culture. The spores were then germinated at 30°C, and 10 mg of spores per ml with 100 mM glucose, 50 mM KBr, 100 mM glucose plus 1 mM DCCD, or no added germinant as described in the text. The maximum release of methylamine in these experiments amounted to more than 90% of the total originally present in the dormant spore. Symbols: ▲, methylamine; ○, DPA.*

2A and B) or the fall in optical density at 600 nm of the culture (data not shown). Rapid methylamine release was also seen during germination in glucose plus 3 mM Tris-hydrochloride (pH 8.5) or with *B. cereus* spores (data not shown). Surprisingly, it appeared that methylamine release preceded DPA release by 1 to 3 min depending on the experiment (Fig. 2A and B). This was shown even more dramatically by germinating spores with glucose plus the inhibitor dicyclohexylcarbodiimide (DCCD) (Fig. 2C). Under these conditions, methylamine release was relatively unaffected, whereas DPA release was significantly retarded. Similar results were obtained when 1 mM $HgCl_2$ was used instead of DCCD or when *B. cereus* spores were germinated with DCCD (data not shown).

One prediction from the experiments noted above is that before the excretion of DPA by spores germinating at pH values of >6.5 there should be a significant release of $H^+$. Indeed, when spores were germinated in dilute buffer (pH 8) with the non-metabolizable glucose analog α-methylglucoside (to eliminate generation of acid by catabolism of the germinant), there was a significant fall in the pH of the medium before a significant DPA release (Fig. 3A and B). This was most obvious when DCCD was also present during germination to block oxidative phosphorylation (Fig. 3B). Similar data were also obtained for spores germinated in KBr (data not shown). The rapid fall in the pH of the medium early in germination was not due to hydrolysis and release of spore cortex fragments, since the latter process follows DPA release (11).

Earlier studies by Vary (29) and Rossignol and Vary (16) also have shown a release of protons early in the germination of the same strain of *B. megaterium* by constantly recording the pH of a germinating culture. The rate of acid release was significantly greater with the metabolizable germinant glucose than with the non-metabolizable analog α-methylglucoside. However, there was significant acid production during germination with α-methylglucoside (29). Addition of DCCD to spores germinating in glucose reduced the rate of acid production to ~25% of that of spores germinating in α-methylglucoside. However, the values reported for the rates of acid production during germination in α-methylglucoside or with DCCD must be considered approximate numbers, since a high background rate of acid release (almost equal to the value reported for acid release due to germination in α-methylglucoside) was subtracted from all of the data (29). Our conditions differ somewhat from those of Vary in that we used a three- to fourfold-higher spore concentration and a slightly lower buffer concentration. Consequently, the pH changes that we observed were much higher than those seen by Vary (29) and Rossignol and Vary (16). Comparison of the data in Fig. 3A and B show that DCCD addition did indeed decrease the rate of fall in the pH of

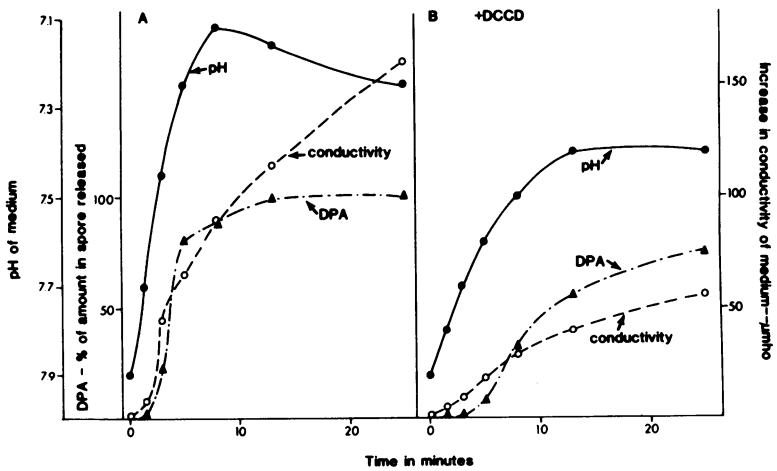


FIG. 3. *Release of DPA and change in medium pH and conductivity during germination of B. megaterium spores (A) without or (B) with DCCD. Spores were heat shocked, incubated in 200 mM Tris-hydrochloride (pH 8.5) for 14 h at 4°C, centrifuged, washed three times with cold water, and suspended at 13 mg/ml in cold 4 mM Tris-hydrochloride (pH 8.0 at 30°C). The spores were germinated at 10 mg/ml by addition to a prewarmed (30°C) flask containing α-methylglucoside (final concentration, 100 mM) (A) without or (B) with DCCD (final concentration, 1 mM). One-milliliter samples were taken at various times and centrifuged; the supernatant fluid was analyzed for DPA and conductivity, and pH was measured. The relative conductivity of the medium on our instrument at zero time was 20 μS (μmho).*

the medium slightly more than twofold. Although it is possible that this may reflect a direct effect of DCCD on acid release, it is more likely that this reflects only a significant slowing of the early steps in spore germination by DCCD as evidenced by the much slower DPA release with DCCD (compare Fig. 3A and B) in this experiment.

**Release of other monovalent ions during germination.** During germination of spores in α-methylglucoside with or without DCCD, we noticed that in addition to the early $H^+$ release there was an increase in the conductivity of the germination medium (Fig. 3A and B). Although the increase in conductivity of the medium during germination is due to several factors, including DPA and divalent cation release and excretion of metabolic end products (4, 21, 26), spores germinating with DCCD in particular showed a significant increase in the conductivity of the medium before DPA release (Fig. 3B). Because divalent cations are released only in parallel with DPA and because detectable metabolism during germination begins only after DPA release (8, 19, 24), there must then be a significant release of ions early in germination. However, it was impossible from this experiment to quantitate this early ion release.

To test the latter point directly, we measured levels of $K^+$ and $Na^+$ in dormant spores and the release of these ions during germination. Dormant spores of *B. megaterium* contained 880 μg/g (dry weight) of $K^+$ and 78 μg/g (dry weight) of $Na^+$, levels which are similar to those reported by others for the spores of *B. megaterium* and *B. cereus* (15, 28, 30). Strikingly, >80% of the $K^+$ of the spores was released during germination in glucose, but there was no $K^+$ release when the spores did not germinate (Fig. 4A). $K^+$ release was followed by rapid $K^+$ uptake (Fig. 4A); the $K^+$ uptake was presumably energy dependent, because it was abolished by DCCD (Fig. 4B). Under conditions in which $K^+$ reabsorption was blocked, $K^+$ release clearly preceded DPA release (Fig. 4B). Greater than 80% of the $K^+$ of the spores was also rapidly released when the spores were germinated with glucose in either 2 mM Tris-hydrochloride (pH 8.5) or 0.1 M Tris-hydrochloride (pH 7.2) (data not shown). The kinetics of $K^+$ release during spore germination were similar to those of methylamine release (Fig. 5A), and $Na^+$ was also released with kinetics similar to those for $K^+$ (Fig. 5B). Greater than 85% of the $Na^+$ of the spores was released in this process (data not shown). The release of $K^+$ before DPA release was also seen during

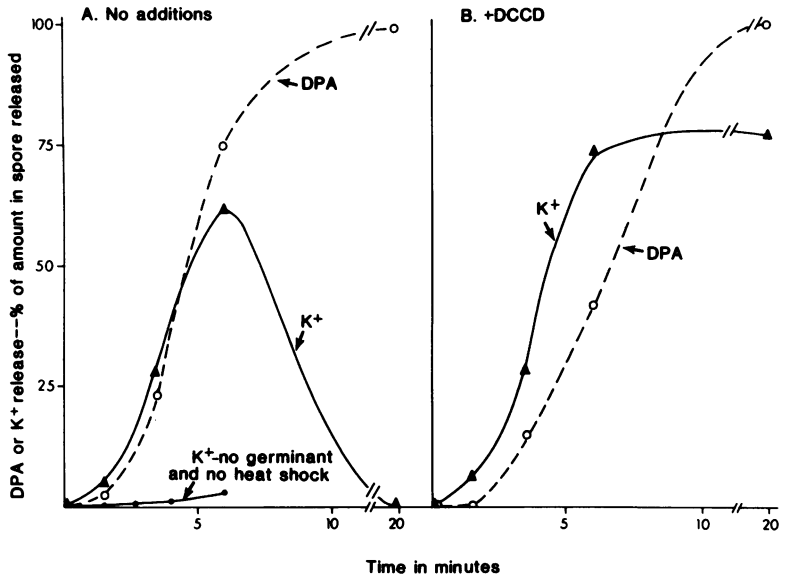


FIG. 4. *Release of DPA and $K^+$ by B. megaterium spores germinating in glucose (A) without or (B) with DCCD. Spores were incubated in 200 mM Tris-hydrochloride (pH 8.7) for 16 h at 4°C after a prior heat shock unless otherwise noted. The spores were centrifuged, resuspended in an equal volume of cold 200 mM Tris-hydrochloride (pH 8.6 at 4°C), and germinated in 100 mM glucose (A) without or (B) with 1 mM DCCD, samples were taken at various times and centrifuged, and the supernatant fluid was analyzed for DPA and $K^+$. The sample which was not heat shocked was added to a prewarmed (30°C) container without glucose and analyzed as described above.*

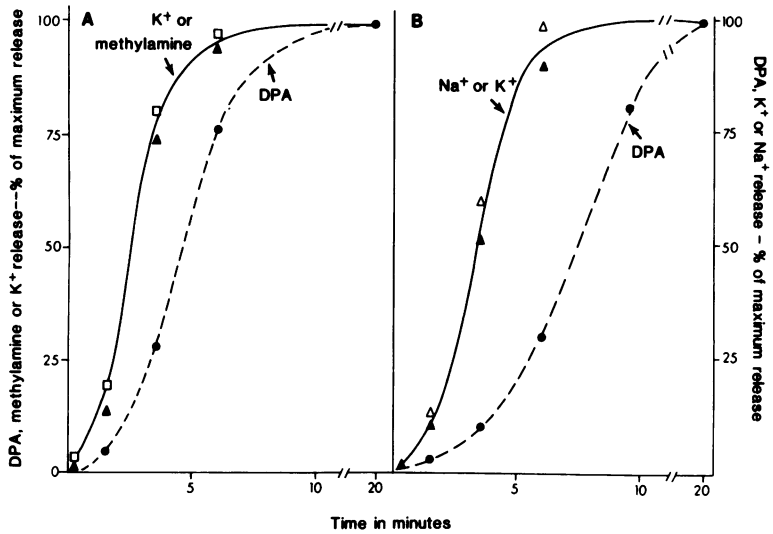


FIG. 5. *Excretion of $K^+$, $Na^+$, methylamine, and DPA during germination of B. megaterium spores. Spores (13 mg/ml) were heat shocked, washed with water, and incubated at 4°C in 200 mM Tris-hydrochloride (pH 8.8). After 1 h, the spores were centrifuged, resuspended in fresh 200 mM Tris-hydrochloride (A) with or (B) without [$^{14}$C]methylamine, and incubated for an additional 16 h at 4°C. The spores were added to glucose (final concentration, 100 mM) plus DCCD (final concentration, 1 mM) at 30°C as described in the text. Samples of the germinating culture were centrifuged, the supernatant fluid was analyzed for DPA, $K^+$, and $Na^+$, and the pellet fraction was analyzed for methylamine. For the spores containing methylamine, a calibrated amount of NaOH was added to the germination mix to ensure that the pH was the same at 30°C as it had been at 4°C. Symbols: ●, DPA; ▲, $K^+$; □, methylamine; △, $Na^+$.*

germination of *B. cereus* spores (data not shown); again, >70% of the total spore $K^+$ (780 μg/g [dry weight]) was released followed by $K^+$ reabsorption (data not shown).

## DISCUSSION

Of obvious importance to the interpretation of the data presented in this communication is that our measurements of the extent of methylamine uptake indeed give accurate estimates of the internal pH of dormant bacterial spores. Although the accuracy of this method has been verified in other systems, one can easily imagine potential artifacts in our system, especially in view of the long incubation times required. However, we believe that our measurements do give accurate estimates of the internal pH of the spores for the following reasons. (i) Fragments of spore coats, cortex, or membranes absorb little methylamine (21). (ii) Removal of spore coats from intact spores by detergent treatment at high pH alters neither the kinetics nor the extent of methylamine uptake (reference 21 and see below). These two findings argue strongly against nonspecific methylamine binding to spore layers external to the spore core. (iii) The percentage of total methylamine in a culture taken up by a constant amount of spores at an exogenous pH of 8.5 was constant with total methylamine concentrations in the culture from 5 to 300 μM (B. M. Swerdlow, B. Setlow, and P. Setlow, unpublished data). At 300 μM methylamine in the culture, this resulted in a methylamine concentration in spores of ~30 mM, again arguing against a nonspecific binding. (iv) The concentration of methylamine calculated to be inside spores varied inversely with the exogenous hydrogen ion concentration, at least between pH 7.1 and 8.4 (21). (v) In all cases in which spore methylamine levels fell (during germination or in dormant spores with KCl present), methylamine release was, as predicted, accompanied by a release of protons and presumably an increase in internal pH. Findings iii to v above are most consistent with the uptake of methylamine by dormant spores being driven by a pH gradient. In addition to the findings directly related to methylamine uptake by spores, there is also one independent measurement of internal spore pH which is in good agreement with our calculated values. Thus, the internal pH of dormant *B. subtilis* spores determined in a preliminary study with $^{31}$P nuclear magnetic resonance was 6.3 (2), essentially identical to the values that we determined for *B. cereus* and *B. megaterium* spores (21). A more difficult ques-

tion to answer is whether the long incubation used for the measurements for dormant spores alters them in some way. However, we do know that their germination properties are not affected by the incubation. Similarly, the stability of spore methylamine levels for 40 to 50 h at 4°C argues that any changes which do take place are very slow. It seems unlikely that large amounts of Tris enter spores during our routine 16- to 20-h incubation at 4°C in Tris, since this would have resulted in elevation of spore pH to values comparable to those generated by $(NH_4)_2SO_4$. Similarly, preliminary analysis of the incubation supernatants revealed that <10% of the DPA, $K^+$, $Na^+$, or $P_i$ of the spores was released during a 16- to 20-h incubation at 4°C (B. M. Swerdlow and P. Setlow, unpublished data). It is of course almost impossible to rule out subtle changes in spores during our measuring periods or that factors other than internal pH may play a partial role in spore methylamine uptake. However, we feel that the findings given above strongly suggest that the spores are not significantly altered during our measuring period and that the great majority of the methylamine uptake of spores at pH values from 7.5 to 8.8 is in response to a pH gradient.

Given the conclusions noted above, a third significant conclusion from work in this report is that the internal pH within dormant bacterial spores can vary significantly. Thus, soaking spores in $(NH_4)_2SO_4$ elevated the internal pH by 1.6 U. Similarly, we interpret the methylamine excretion during extended incubation of dormant spores with KCl as due to a rise in the internal pH, possibly caused by a $K^+$-$H^+$ exchange reaction. It is well documented that bacterial spores exhibit reversible $K^+$-$H^+$ exchange (as well as $H^+$ exchange with other cations) when more drastic procedures are used (1; R. E. Marquis, E. L. Carstensen, G. R. Bender, and S. Z. Child, 8th Int. Spores Conf., abstr. no. 80, 1980). Thus, soaking spores at low pH can result in exchange of spore cations for $H^+$ with little change in viability (1; Marquis et al., 8th Int. Spores Conf., abstr. no. 80, 1980). This treatment should lower the internal pH of spores, which it does in *B. megaterium* spores by at least 1.5 U (B. Swerdlow and P. Setlow, unpublished data). Both the latter $H^+$ loading of spores and the increase in pH of up to 1.6 U are reversible, suggesting that the spores behave in this regard as cation-exchange resins.

Although we could vary the internal pH of the spores between 6.2 and 7.8, within these limits the internal pH of the spores had no detectable effect on their ability to germinate or their dormancy or resistance or both. Although spores which have been $H^+$ loaded often have greatly decreased resistance properties (1; Marquis et al., 8th Int. Spores Conf., abstr. no. 80, 1980), it is not clear whether this is due to the lowered internal pH or to the loss of spore cations or both. It is, of course, possible that a fall in pH within developing spores during sporulation (from 7.5 to 6.2) plays a role in the acquisition of some spore property such as dormancy, but that subsequently some other change in spores (e.g., dehydration) is the primary mechanism for maintaining dormancy. However, it is also possible that a fall in the intrasporular pH may be only an effect of the exhaustion of the ATP pool of the developing spores during sporulation (25) rather than a cause of such a phenomenon.

One striking result in the present work was that the rate of methylamine uptake by dormant spores was slow, in contrast to results from other systems. This suggests that there is a permeability barrier in dormant spores which not only blocks passage of charged species ($CH_3NH_3^+$) (8) but also retards passage of uncharged lipid-soluble species ($CH_3NH_2$). Because previous work has suggested that most of the methylamine enters the spore core (21), a likely candidate for the permeability barrier is the inner spore membrane. More external spore layers (coats, cortex, and outer spore membrane) are thought not to be permeability barriers to small hydrophilic compounds, whereas the inner membrane is (8). Indeed, spores whose coats (and presumably much outer membrane) were removed by detergent treatment at high pH and then soaked in water also took up methylamine slowly (B. Setlow and P. Setlow, unpublished data). The inner dormant spore membrane may well have properties resulting in a low permeation rate for small lipophilic molecules, since the phospholipids in this membrane may be tightly packed and in a crystalline structure. However, this structure is disrupted early in spore germination (6, 27).

Whatever the permeability barrier resulting in slow uptake of methylamine by dormant spores, this barrier is rapidly destroyed upon spore germination as most of the methylamine taken up was released along with $H^+$, $Na^+$, and $K^+$. In contrast, dormant spores released these ions slowly. This permeability change accompanying germination took place before DPA release and is an extremely early event. Although no other biochemical events have been characterized which precede DPA release during spore germination, loss in spore heat resistance precedes DPA release (4).

In the case of $K^+$, the ions released early in germination were reabsorbed in a DCCD-sensi-

tive process, and an energy-requiring $K^+$ transport system has been identified in dormant spores of *B. subtilis* (5). Although the $K^+$ reabsorption required energy, we have no evidence that the release of $H^+$, $K^+$, or $Na^+$ early in germination required energy. Indeed, the ion release took place during germination in DCCD and during a period before the accumulation of detectable ATP or NADH or both (18, 19, 24).

Although the release of monovalent ions early in germination was studied most extensively with *B. megaterium* spores, methylamine and $K^+$ release also preceded DPA release during germination of *B. cereus* spores. *B. subtilis* spores containing high $K^+$ levels also lose much $K^+$ early in germination (3). However, it was unclear in the latter report whether $K^+$ was lost by release or by exchange with exogenous $K^+$. The present work indicates that monovalent ions are simply released, presumably in conjunction with some anion(s). The finding of this comparable phenomenon with spores of three different species strongly suggests that monovalent ion release and the permeability change that allows the ion release are common features of the first minutes of bacterial spore germination. An obvious question then is whether the $K^+$ or $Na^+$ release or both is a process essential for early steps in spore germination or only the result of other essential processes. At present this question cannot be answered definitively, because ion release has been observed under all germination conditions and with all species tested. However, it seems unlikely that $Na^+$ and $K^+$ release generates energy by dissipation of a concentration gradient. Indeed, it seems more likely that the net release of $Na^+$ and $K^+$ early in our germination experiments is only the result of an increased permeability at this time plus the low level of $Na^+$ or $K^+$ in the germination medium. This net $Na^+$ and $K^+$ release would be driven solely by the concentration gradient between the inside and the outside of the spore. However, it appears unlikely that this is essential for spore germination, because spores of our strain of *B. megaterium* germinate well in 0.4 M $K^+$ or $Na^+$ (P. Setlow, unpublished data), concentrations well above that of free $K^+$ of $Na^+$ within spores.

The location of most of the methylamine within dormant spores (and thus the region of low pH) is thought to be the spore core (21). However, this assignment is based on indirect measurements, and direct measurement seems impossible. However, there is direct evidence that the $K^+$ excreted during germination comes from the spore core. Thus, in spores of *B. cereus* containing $K^+$ levels similar to those in our experiments, >85% of the spore $K^+$ was shown by electron probe microanalysis to be in the spore core, with at most 15% in the cortex plus coat regions (28). Given the high percentage of total spore $K^+$ released in the first minutes of germination, most of the $K^+$ must come from the core. This finding further supports the concept presented previously by Vary (29) that a large change, possibly a transient change, in permeability in the inner spore membrane is one of the first events in bacterial spore germination.

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