

Regulation of Phosphoglycerate Phosphomutase in Developing Forespores and Dormant and Germinated Spores of *Bacillus megaterium* by the Level of Free Manganous Ions

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The large depot of phosphoglyceric acid (PGA) which is accumulated within spores of *Bacillus megaterium* is >99% 3-phosphoglyceric acid (3-PGA). The 3-PGA depot is stable in forespores and dormant spores, but is utilized rapidly during spore germination. When spores were germinated in KBr plus NaF, the PGA depot was not utilized, but 13% of the 3-PGA was converted to 2-PGA. These data suggest phosphoglycerate phosphomutase as the enzyme which is regulated to allow 3-PGA accumulation during sporulation. Young isolated forespores, in which 3-PGA was normally stable, utilized their 3-PGA rapidly when incubated with Mn^{2+} plus the divalent cation ionophore X-537A; Mn^{2+} or ionophore alone or Mg^{2+} or Ca^{2+} plus ionophore was without effect. Young forespores contained significant amounts of Mn^{2+} . However, forespore Mn^{2+} exchanged slowly with exogenous Mn^{2+} and was removed poorly by toluene treatment. This suggests that much of the forespore Mn^{2+} is tightly bound to some forespore component. Since phosphoglycerate phosphomutase from *B. megaterium* has an absolute and specific requirement for Mn^{2+} , these data suggest that the activity of this enzyme in vivo may be regulated to a large degree by the level of free Mn^{2+} . Indeed, the activity of this enzyme in forespore or dormant spore extracts was stimulated >25-fold by Mn^{2+} , whereas comparable extracts from cells or germinated spores were stimulated only two- to fourfold.

Dormant spores of various *Bacillus* species contain a large depot of 3-phosphoglyceric acid (3-PGA) (11, 19). In *Bacillus megaterium* the 3-PGA is accumulated late in sporulation only within the developing forespore (20). The 3-PGA depot is stable in the forespore and dormant spore despite the presence of enzymes of 3-PGA catabolism (phosphoglycerate phosphomutase, enolase, and pyruvate kinase) at levels similar to those in growing cells and germinated spores (11, 20). However, the spore 3-PGA is utilized rapidly in the first minutes of spore germination for generation of ATP; this occurs in the absence of new protein synthesis (19). When *B. megaterium* spores are germinated in KBr plus NaF, 3-PGA utilization is blocked, suggesting that this process goes via the fluoride-sensitive enzyme enolase (19, 21).

Since 3-PGA accumulates in forespores even though the enzymes for 3-PGA catabolism are detected in extracts, one of these enzymes must have very little activity within the forespore. This enzyme would then become activated upon spore germination. Previous work has implicated phosphoglycerate phosphomutase as a key en-

zyme in regulation of 3-PGA accumulation during sporulation (22). This enzyme has been purified from both *B. megaterium* and *B. subtilis* and has an absolute and specific requirement for Mn^{2+} for activity (12, 23, 25). In this communication we present further evidence that phosphoglycerate phosphomutase is the enzyme which is regulated to allow the accumulation of 3-PGA during sporulation and its utilization during germination. The regulation of this enzyme appears to be mediated, at least in part, by the level of free Mn^{2+} such that the enzyme has little or no activity in the forespore and dormant spore and becomes active only upon spore germination.

MATERIALS AND METHODS

Chemicals and enzymes. The ionophores X-537A (13) and A23187 (15) were the generous gifts of W. E. Scott (Hoffman-LaRoche) and Maurice Feinstein (University of Connecticut Health Center, Farmington, Conn.), respectively. Stock solutions (1 to 2 mg/ml) were prepared in ethanol. Phosphoglycerate phosphomutase and enolase were purified from log-phase cells of *B. megaterium* as previously described (21, 23). Other enzymes, nucleotides, and sugar phosphates

were obtained from the Sigma Chemical Co. ADP was freed of contaminating ATP by incubation with hexokinase and glucose and subsequent boiling.

Organism used and preparation of forespores, spores, and germinated spores. The organism used for these studies was *B. megaterium* QM B1551, originally obtained from Hillel Levinson (U.S. Army Research Laboratories, Natick, Mass.). Cells were grown and spores were formed in supplemented nutrient broth (SNB) and harvested, washed, and stored as previously described (18). SNB contains approximately 20 μM MnCl_2 , as well as 1 mM CaCl_2 , 1 mM MgSO_4 , 13 mM KCl , 55 mM glucose, and 8 g of nutrient broth (Difco) per liter.

Spores labeled with ^{32}P were prepared as previously described (19), and spores and forespores containing $^{54}\text{Mn}^{2+}$ were obtained by growth in SNB containing $^{54}\text{MnCl}_2$ (10 nCi/ml). Forespores were isolated from sporulating cells as previously described (20), but a Tris-protoplast buffer (0.6 M sucrose, 50 mM Tris-hydrochloride [pH 7.4], and 16 mM MgSO_4) was substituted for phosphate protoplast buffer (0.6 M sucrose, 50 mM KPO_4 [pH 7.4], and 16 mM MgSO_4) once sporulating cells were converted to protoplasts.

Spore germination was preceded by a heat shock (15 min; 60°C) of spores (20 mg/ml) in water, and germination was at 2.5 mg/ml and 30°C in 50 mM Tris-hydrochloride (pH 7.4) and 0.1 M glucose. In this medium >95% of the spores had initiated germination after 10 min. In some experiments spores were germinated in a similar fashion, but in 50 mM KPO_4 (pH 7.4) and 50 mM KBr .

Extraction and assay of small molecules. Small molecules were routinely extracted from spores or frozen forespore pellets by addition of 4 ml of boiling 80% 1-propanol (1, 19). After boiling for 5 min, the mixture was chilled and centrifuged, the supernatant fluid was flash evaporated, and the dry residue was dissolved in water. In a few cases extractions were carried out with 5 ml of 3% acetic acid for 20 min at 4°C. The mixture was centrifuged, and the supernatant fluid was lyophilized and redissolved in water.

ATP was assayed by the luciferase reaction, and total free adenine nucleotides were assayed similarly after their conversion to ATP (19). 3-PGA was also assayed by the luciferase reaction by measuring the 3-PGA-dependent conversion of ADP to ATP in a reaction mixture containing ADP, pyruvate kinase, enolase, and phosphoglycerate phosphomutase as previously described (22). Since this reaction measures the sum of 3-PGA, 2-PGA, phosphoenolpyruvate (PEP), and any endogenous ATP, separate reaction mixtures lacking phosphoglycerate phosphomutase or phosphoglycerate phosphomutase and enolase were used to determine either the sum of 2-PGA and PEP or PEP, respectively. Reaction mixtures lacking all three enzymes were used for ATP determinations. Controls were also run with added 3-PGA or 2-PGA to ensure that conversion reactions had proceeded to completion.

In one experiment the low-molecular-weight compounds were extracted from ^{32}P -labeled dormant spores (25 mg; 10^6 cpm/mg) with boiling 80% 1-propanol (19). After passage through charcoal in 1% acetic acid to remove nucleotides and dipicolinic acid (DPA),

PGAs (2-PGA plus 3-PGA) were purified by paper electrophoresis (11). 2-PGA and 3-PGA were resolved by paper chromatography (3) and sprayed for phosphate (8), and the respective spots were cut out and counted in a gas flow counter.

DPA was assayed by the method of Rotman and Fields (16), and protein was assayed by the method of Lowry et al. (10).

Enzyme extraction and assay. Samples of cells or forespores (30 ml) were centrifuged and cells were washed twice with 50 mM Tris-hydrochloride (pH 7.4), 50 mM KCl , and 1 mM phenylmethylsulfonyl fluoride (buffer A). Cell pellets were resuspended in 3 ml of cold buffer A, and cells and forespores were disrupted by sonication with glass beads (19) and then centrifuged (10 min; $15,000 \times g$). Supernatant fluids were either assayed immediately or frozen in dry ice.

Lyophilized dormant or 15-min-germinated spores (~100 mg) were disrupted in a dental amalgamator (Wig-L-Bug) as previously described with glucose as the abrasive (~100 mg) (17). The dry powder was extracted with 2 to 3 ml of cold buffer A, left for 15 min at 4°C, and centrifuged as described above.

Assays of enolase were carried out by measurement of conversion of 2-PGA to PEP as described previously, with or without MgCl_2 plus 2-mercaptoethanol (21). Assays of phosphoglycerate phosphomutase utilized the discontinuous assay described previously (4, 23) where the first incubation contained only enzyme plus 3-PGA. After boiling, a second incubation was used to determine the amount of 2-PGA formed in the first incubation. To assess the stimulation of the mutase by MnCl_2 , the first incubation was carried out with or without 1 mM MnCl_2 .

Forespore incubations. Forespores or ^{54}Mn -labeled forespores prepared as described above were suspended in Tris-protoplast buffer at a concentration twice that in the original culture. Additions were made as noted, and cultures were incubated at 30°C with slow shaking. In experiments in which an ionophore (in ethanol solution) was added to an incubation, ethanol was added to control cultures in the same amount. For assays of 3-PGA, DPA, or nucleotides, portions (5 ml) were taken at various times and centrifuged (5 min; $10,000 \times g$), and the pellets were frozen. For ^{54}Mn analysis, portions (1 ml) were passed through membrane filters (0.24- μm pore size) and washed once with 5 ml of prewarmed Tris-protoplast buffer, and the filter was dried and counted in a toluene-based scintillation fluid (18). For experiments in which toluene was added to remove Mn^{2+} as described with *B. subtilis* (5), incubations were carried out as described above with 2% toluene present.

^{54}Mn uptake in growth and sporulation and loss in germination. ^{54}Mn uptake during growth and sporulation in SNB was measured by collection of cells on filters as described above, washing twice with prewarmed SNB (3 ml) and then determining the radioactivity on the filter. At various times during sporulation, forespores were isolated from 30-ml portions of the culture, and ^{54}Mn levels were determined.

^{54}Mn release during spore germination was followed by germinating ^{54}Mn -labeled spores as described above, passing portions (500 μl) through a filter, washing with 3 ml of prewarmed germination medium, and

determining the radioactivity on the filter.

Ionophore-dependent metal ion transport. The ionophore-dependent transport of Ca^{2+} or Mn^{2+} through a bulk organic phase in vitro was measured as described by Pressman and deGuzman (13). The upper phase contained Tris-protoplast buffer without sucrose, but was 1 mM in the ion under study. The central organic phase contained 10^{-4} M ionophore, and the lower phase contained Tris-protoplast buffer which was 1 mM in the labeled ion under study. Incubations were carried out for a minimum of 2 h at 27°C, and over this period the rate of transport was linear.

Ionophore-dependent uptake of Ca^{2+} or Mn^{2+} by forespores was carried out at 30°C in Tris-protoplast buffer containing 1 mM Ca^{2+} or Mn^{2+} (10^5 cpm/ml) and with forespores at 20 times their concentration in the initial culture. Portions (150 μl) were passed through membrane filters, washed with 3 ml of Tris-protoplast buffer, dried, and counted. Control incubations without ionophore and without forespores were also analyzed.

RESULTS

3-PGA accumulation and levels of 2-PGA.

As shown previously, 3-PGA is accumulated late in sporulation of *B. megaterium* about 1 h before the accumulation of DPA (20) (Fig. 1). Previous work has shown that >90% of the 3-PGA accumulates within the developing forespore, but the site of 3-PGA synthesis (mother cell or forespore) is not known (20). The amount of 3-PGA accumulated within the dormant spore was not reduced by addition of fluoride late in sporulation (Fig. 1), nor was sporulation impaired (data not shown).

Analysis of the PGA pool from dormant spores or sporulating cells revealed that in dormant spores >99.5% of all PGA was 3-PGA, whereas in sporulating cells (forespores plus mother cell) >97.5% of all PGA was 3-PGA (Table 1). Similarly, when spores were germinated in KBr, the PGA pool was >96% 3-PGA even though the pool size of all PGA had been reduced by more than 90% (Table 1). However, during germination in KBr plus NaF, PGA catabolism was blocked as previously noted (19), yet 13% of the 3-PGA was converted to 2-PGA (Table 1). The decreased level of total PGA in spores germinated in NaF may be due in part to experimental error, but some PGA catabolism can take place under these conditions via a pathway not involving enolase (19).

Manganese ion stimulation of phosphoglycerate phosphomutase in various extracts. The data on 3-PGA and 2-PGA levels suggested that phosphoglycerate phosphomutase was the enzyme which was regulated to allow the accumulation of 3-PGA in sporulation and its utilization in germination. The specific activity of this enzyme has been shown to be

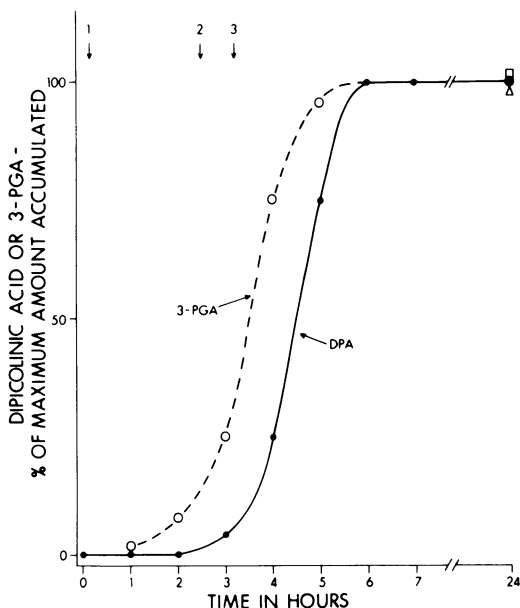


FIG. 1. Accumulation of 3-PGA and DPA during sporulation. Cells were grown in SNB; samples (15 ml) were taken and centrifuged (10 min; $15,000 \times g$); and the pellets were frozen, extracted, and analyzed for 3-PGA and DPA as described in the text. The amount of 3-PGA accumulated in the dormant spore was 20 nmol/mg (dry weight). At arrows 1 and 2 separate cultures were made 20 mM in NaF, and the spores produced in these cultures were analyzed for 3-PGA. The symbols (\square) and (\triangle) give the data for cultures to which fluoride was added at arrows 1 and 2, respectively. Arrow 3 gives the time of harvest of sporulating cells analyzed in Table 1. In this experiment, the optical density of the culture had reached a plateau at approximately 30 min before zero time. The 24-h points give the data for the dormant spore.

TABLE 1. Levels of 2-PGA plus PEP and 3-PGA at different stages of growth^a

Stage of growth	3-PGA (nmol/mg [dry wt])	2-PGA + PEP (nmol/mg [dry wt])
Sporulating cells ^b	4.2 ^c	<0.1 ^c
Dormant spores	21	<0.1 (<0.17) ^d
Spores germinated for 20 min without NaF	1.2	<0.05
Spores germinated for 20 min plus 10 mM NaF	14.6	2.2 ^c

^a Cells or spores were treated, harvested, extracted, and analyzed as described in the text. Sporulating cells were extracted with acetic acid, and the extract was passed through a Norit column to remove nucleotides.

^b Harvested at the point noted by arrow 3 in Fig. 1.

^c Nanomoles per milliliter of culture.

^d Value in parenthesis was determined chromatographically starting with ³²P-labeled spores.

^e <10% of this value is due to PEP.

similar in extracts of cells, forespores, and dormant and germinated spores when the assay was carried out with 1 mM Mn^{2+} present (20). However, it seemed worthwhile to test for enzyme activity under other conditions, because phosphoglycerate phosphomutase from *Bacillus* species (including *B. megaterium* cells and spores) has been shown to have an absolute and specific requirement for Mn^{2+} for activity (23, 25). Consequently, we prepared extracts from various stages of growth and assayed the enzyme with and without addition of $MnCl_2$. Although the phosphoglycerate phosphomutase in extracts of cells or germinated spores was stimulated ~3-fold by $MnCl_2$, the enzyme in extracts of forespores or dormant spores was stimulated >25-fold (Table 2). As found previously all extracts had specific activities which differed only by a factor of 2.5 under optimum assay conditions (20). In contrast to findings with phosphoglycerate phosphomutase, enolase was almost fully active in unsupplemented extracts of forespores and dormant spores (data not shown).

Although the findings noted above indicate that phosphoglycerate phosphomutase in forespore or dormant spore extracts is less active than that in germinated spore or cell extracts, it is difficult to extrapolate these data quantitatively to the *in vivo* situation. For example, both the total protein and thus the total Mn^{2+} concentration in our unsupplemented assays *in vitro* were well below those *in vivo* (Table 2). However, it is also possible that forespore or spore disruption, with the enzymatic action which can accompany this process, may have resulted in an increase in the amount of free Mn^{2+} available to the phosphoglycerate phosphomutase *in vitro* over that available *in vivo*. Despite these problems, the data in Table 2 suggest that phosphoglycerate phosphomutase might have little or no activity in forespores or dormant spores at least in part because of a low level of free Mn^{2+} *in vivo*.

Effect of ionophores plus Mn^{2+} on forespore 3-PGA levels and Mn^{2+} transport. The data given above also suggested that elevation of endogenous Mn^{2+} levels might under some conditions activate phosphoglycerate phosphomutase *in vivo*. As we have shown previously (22), incubation of young forespores with the ionophore X-537A plus Mn^{2+} caused a rapid loss in the 3-PGA pool (Fig. 2A). In contrast to the behavior of young forespores, we have previously shown that mature forespores and dormant spores are resistant to the action of X-537A plus Mn^{2+} (22). In addition, neither Mn^{2+} alone, nor X-537A alone in protoplast buffer (which contains Mg^{2+}), nor X-537A plus Ca^{2+} ,

TABLE 2. Phosphoglycerate phosphomutase activity of various extracts with and without Mn^{2+} ^a

Extract prepared from:	Sp act plus Mn^{2+} (nmol/min per mg of protein)	Sp act minus Mn^{2+} (nmol/min per mg of protein)
Dormant spore	210	<8 ^b
Isolated forespores containing 5% of maximum DPA	330	<13 ^b
Isolated forespores containing 40% of maximum DPA	220	<8 ^b
Spores germinated for 15 min	230	55-120 ^c
Vegetative cells	430	100-220 ^c
Sporulating cells ^d	510	125-250 ^c

^a Cells or spores were grown, harvested, extracted, and assayed \pm $MnCl_2$ (1 mM) as described in the text.

^b This value is the maximum upper limit of activity observed in three separate experiments when assays used 100 to 350 μ g of protein per ml.

^c Range in three separate experiments. These assays had protein concentrations of 3 to 25 μ g/ml.

^d Containing ~5% of the maximum DPA, isolated ~6 h after the end of log-phase growth.

nor the ionophore A23187 plus Mn^{2+} had any significant effect on the 3-PGA pool of young forespores (Fig. 2A). Note that in the latter experiments the concentration of X-537A in the incubation mixtures with either Mg^{2+} or Ca^{2+} was three times that in the incubation mixture with Mn^{2+} . The effect of X-537A plus Mn^{2+} on young forespores was concentration dependent, and at high concentrations of the ionophore 3-PGA utilization was extremely rapid (Fig. 3).

As predicted by the data given above, the ionophore X-537A caused the rapid accumulation of Mn^{2+} by isolated forespores (Fig. 4). This ionophore also caused Ca^{2+} uptake by isolated forespores at one half the rate and to one half the extent of Mn^{2+} uptake (data not shown). In addition, for the two different ionophores tested there was good qualitative agreement between their rates of Mn^{2+} transport *in vitro* and their effect on the 3-PGA of isolated forespores (Fig. 2A, Table 3). Although A23187 did catalyze Mn^{2+} transport *in vitro*, it was only one eighth as effective as X-537A on a molar basis. Thus, the concentration of A23187 used in the experiment with forespores (Fig. 2) was equivalent only to ~1 μ g of X-537A per ml in its ability to transport Mn^{2+} . This concentration of X-537A would be expected to have almost no effect on 3-PGA utilization in isolated forespores (see Fig. 3). Despite the fact that X-537A generated significant transport of Ca^{2+} (*in vivo* and *in vitro*)

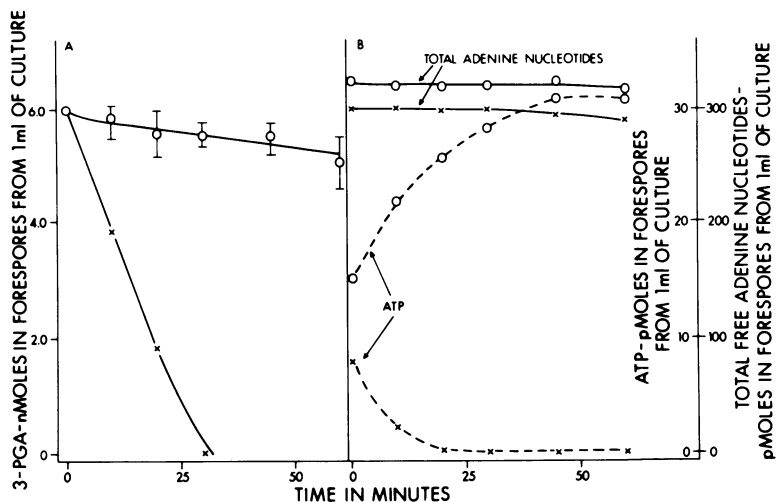


FIG. 2. Effect of ionophores plus metals on levels of (A) 3-PGA and (B) adenine nucleotides and ATP in isolated forespores. Forespores isolated when containing ~30% of the maximum amount of 3-PGA were incubated at 30°C in Tris-protoplast buffer with the following additions as noted: X-537A (24 $\mu\text{g}/\text{ml}$); X-537A (24 $\mu\text{g}/\text{ml}$) plus CaCl_2 (1 mM); MnCl_2 (1 mM); X-537A (8 $\mu\text{g}/\text{ml}$) plus MnCl_2 (1 mM); or A-23187 (7 $\mu\text{g}/\text{ml}$) plus MnCl_2 (1 mM). Note that Mg^{2+} is always present at 16 mM. Portions were taken at various times, harvested, extracted, and analyzed for ATP, total free adenine nucleotide, and 3-PGA as described in the text. Symbols: (A) O, no additions, or A-23187 + Mn^{2+} , or Mn^{2+} alone, or X-537A alone, or X-537A + Ca^{2+} ; X, X-537A + Mn^{2+} ; (B) O, no additions; X, +X-537A.

and Mg^{2+} (in vitro) (Table 3), these ions had no effect on 3-PGA utilization in vivo.

The disappearance of forespore 3-PGA caused by X-537A plus Mn^{2+} was not due to 3-PGA leakage into the medium (22). We do not know whether the ionophore-dependent utilization of 3-PGA generated ATP, since the ionophore alone caused rapid loss of the ATP from the forespore (Fig. 2B). However, there was no effect on the level of free adenine nucleotides in the forespore (Fig. 2B). 3-PGA loss under the influence of X-537A plus Mn^{2+} was not accompanied by the loss of the small amount of DPA present in the forespore (data not shown).

Mn^{2+} levels in cells, forespores, and spores and its exchange. One obvious explanation for much of the preceding data is that Mn^{2+} is not present in young forespores of *B. megaterium*, but is only taken up later, possibly in conjunction with DPA. However, this is not the case. Spores eventually accumulated ~85% of the Mn^{2+} taken up by sporulating cells, as found previously with *B. subtilis* (5); at the earliest time tested, forespores contained significant amounts of Mn^{2+} (Fig. 5). Mn^{2+} accumulation into forespores took place well ahead of DPA accumulation (Fig. 5). In this experiment 5 to 13% of the cell's Mn^{2+} which was not found in the final forespore preparation was removed by or during the conversion of cells to proto-

plasts; 85 to 90% was removed by the first centrifugation after protoplast disruption, and only 5 to 8% was removed by the subsequent two centrifugations in forespore isolation (data not shown).

Although isolated forespores contained significant amounts of Mn^{2+} , it exchanged poorly with exogenous Mn^{2+} —even in the presence of X-537A (Fig. 6; note expanded scale). Similarly, even with the youngest forespores tested, toluene only released a small amount of Mn^{2+} (Fig. 6). Similar findings have been made with sporulating cells of *B. subtilis* (5). However, during spore germination ~70% of the endogenous Mn^{2+} was rapidly excreted, with the remainder exchanging rapidly with exogenous Mn^{2+} (Fig. 7). In the latter experiment >95% of the spore DPA was released (data not shown).

In contrast to the findings with forespores, 70 to 80% of the Mn^{2+} in vegetative cells was rapidly lost upon incubation with either toluene or exogenous MnCl_2 as shown previously with *B. subtilis* (5) (Fig. 8A). Mn^{2+} within stationary phase cells was more refractory to removal (Fig. 8A), but conversion of stationary phase cells to protoplasts labilized ~70% of their Mn^{2+} to rapid loss (Fig. 8B). That a significant amount of Mn^{2+} is refractory to removal from stationary phase cells by toluene has been found previously with *B. subtilis* (5).

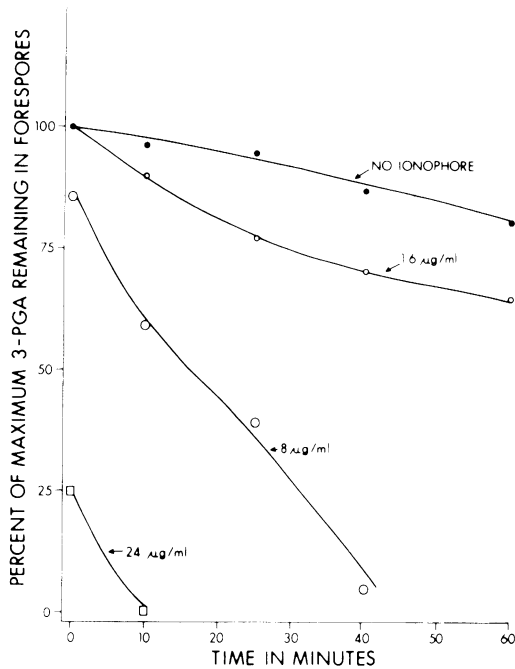


FIG. 3. Effect of increasing X-537A concentrations on levels of 3-PGA in isolated forespores. Forespores containing ~35% of the maximum amount of 3-PGA were incubated at 30°C in Tris-protoplast buffer plus 1 mM $MnCl_2$ and various concentrations of X-537A. At various times samples were taken, extracted, and analyzed for 3-PGA as described in the text. The ionophore alone had no significant effect on the 3-PGA depot at the highest concentration tested.

DISCUSSION

Enzymes of 3-PGA catabolism are present in extracts of forespores and dormant and germinated spores of *B. megaterium*. These enzymes act rapidly in vivo in the first minutes of spore germination. However, at least one of these enzymes must have little or no activity within the forespore and dormant spore in which a large stable pool of 3-PGA is accumulated. A reason which is often given for the absence of enzyme activity in dormant spores is their low water content (7). Although this may be true in the dormant spore, it seems unlikely to be the case in the forespore during 3-PGA accumulation, since 3-PGA accumulation is followed by other events (i.e., DPA accumulation) which should require a hydrated spore core. Similarly, spore heat resistance, also thought to be due to a very low spore water content, is not acquired until 1 to 2 h after 3-PGA accumulation (20). Consequently, a different mechanism seems necessary to explain regulation of the enzymes of 3-PGA catabolism at least during the period of 3-PGA accumulation during sporulation.

From the results presented in this paper, we wish to present a hypothesis for the regulation of 3-PGA accumulation in sporulation and its utilization during spore germination. The main features of our hypothesis are as follows. (i) Regulation is effected by control of the activity of phosphoglycerate phosphomutase such that this enzyme has little or no activity in forespores and dormant spores, and is activated early in spore germination. (ii) In forespores the low or lack of activity of phosphoglycerate phosphomutase is to a large degree the result of a low

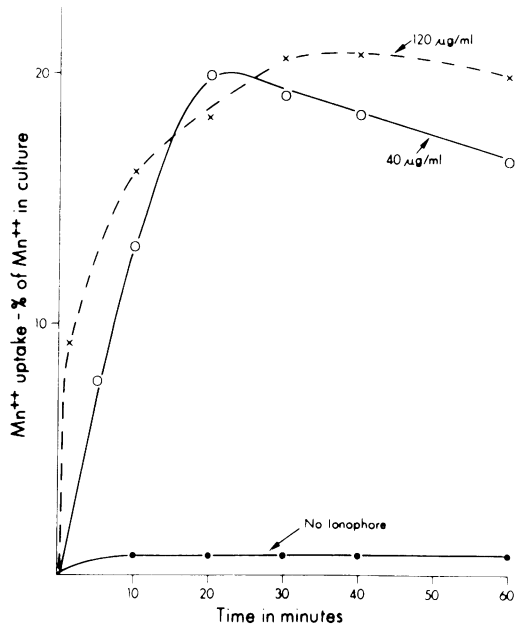


FIG. 4. Ionophore-dependent Mn^{2+} accumulation by isolated forespores. Incubations were carried out as described in the text and with a total ethanol concentration of 6%. All values have been corrected for Mn^{2+} bound in the zero-time value of the culture without ionophore. This amounted to 2 to 4% of the total Mn^{2+} in the culture in different experiments.

TABLE 3. Rates of ionophore-dependent metal ion transport through a bulk organic phase^a

Ionophore	Rate of transport		
	Mn^{2+}	Ca^{2+}	Mg^{2+} ^b
X-537A	1.25	0.67	4.3 ^b
A-23187	0.15	0.03	

^a Ionophore-dependent transport of cations through a bulk organic phase was measured as described in the text. All values given are: moles of ion transported/(hour × moles of ionophore).

^b Calculated from data on Ca^{2+} transport by using published data on the relative affinity of the ionophore for Ca^{2+} versus Mg^{2+} and the relative rates of transport of the two ions (13).

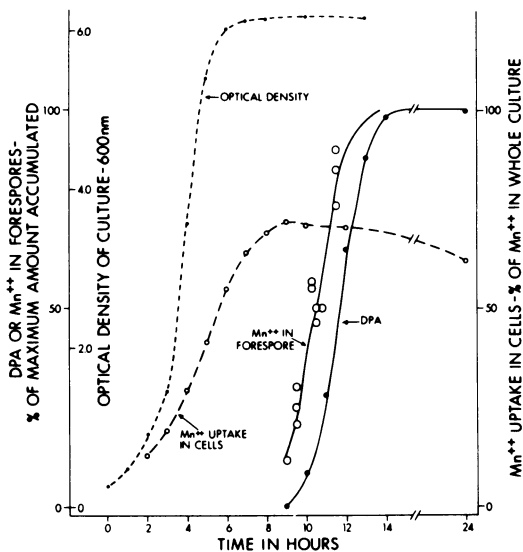


FIG. 5. Kinetics of uptake of Mn^{2+} into cells and forespores during growth and sporulation. Cells were grown in SNB containing $^{54}MnCl_2$ and at various times samples were analyzed for total Mn^{2+} uptake as described in the text. Portions were taken for analysis of DPA, and at various times aliquots (30 ml) were also taken and forespores were isolated for determination of the amount of Mn^{2+} in the forespore. The latter experiment was repeated a number of times, and the data from several experiments are plotted as the counts per minute in forespore samples/counts per minute in free spores $\times 100$. The 24-h point gives the data for the dormant spore.

concentration of free Mn^{2+} . Spores accumulate 170 nmol of 3-PGA per mg of soluble protein with the great majority of this accumulated in a 2-h period (see Fig. 1). If we assume that utilization or turnover of 25% of this 3-PGA could have been detected, then the maximum activity of phosphoglycerate phosphomutase within forespores is 42 nmol of 3-PGA utilized per mg of soluble protein in a 2-h period. The average maximum specific activity of the enzyme in forespore extracts *in vitro* is 33×10^3 nmol of 3-PGA utilized per mg of soluble protein per 2 h. Consequently, this latter value must be reduced at least 800-fold to reduce the enzyme activity *in vivo* sufficiently to account for our results. Although we suggest that a significant part of the decreased phosphoglycerate phosphomutase activity *in vivo* is due to a low free Mn^{2+} concentration, other factors may also be important. For instance, recent work has shown that the pH within dormant spores of *B. megaterium* is 1 to 1.5 pH units below the pH optimum for phosphoglycerate phosphomutase (B. Setlow and P. Setlow, unpublished data, 1979). Possibly the pH within forespores is also low. A low free Mn^{2+}

concentration may also be a significant factor in reducing phosphoglycerate phosphomutase activity in the dormant spore. Again, other factors, in particular the dormant spore's low water content, could be equally important, if not much more important, in preventing enzyme activity. However, in germinated spores the endogenous concentration of free Mn^{2+} rises and is now sufficient for rapid phosphoglycerate phosphomutase action. (iii) The low level of free Mn^{2+} in forespores (and possibly spores) is not due to the absence of Mn^{2+} from these stages of growth. Rather the forespore Mn^{2+} (and very probably the spore Mn^{2+}) is tightly bound to some forespore component(s) resulting in a very low level of free Mn^{2+} . During spore germination endogenous Mn^{2+} reserves are mobilized and become available to the phosphoglycerate phosphomutase.

The support for this hypothesis as well as its

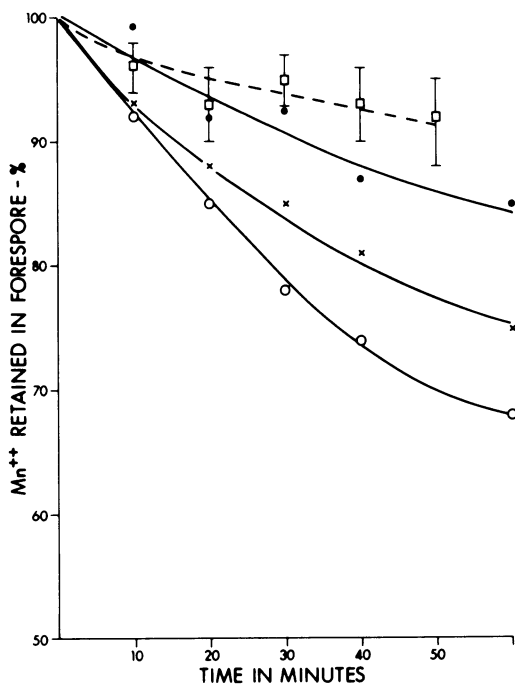


FIG. 6. Loss of $^{54}Mn^{2+}$ from prelabeled forespores. Cells were grown with $^{54}Mn^{2+}$, and forespores were isolated which contained 11% (solid lines) or 18% (dashed line) of the maximum DPA. These were then incubated in Tris-protoplast buffer containing: no additions or X-537A (8 $\mu g/ml$) plus $MnCl_2$ (1 mM) (\square); no additions (\bullet), 1 mM $MnCl_2$ (\times), or 2% toluene (\circ). $^{54}Mn^{2+}$ loss was quantitated as described in the text. The data are given as the percentage of Mn^{2+} remaining compared to that in the final isolated forespores. An amount less than 8% of the Mn^{2+} which was in the final forespore preparation was lost in the last two centrifugations in forespore isolation.

deficiencies are given below. (i) The data on 2-PGA and 3-PGA levels in cells and spores strongly suggest that the enzyme regulated is the phosphoglycerate phosphomutase. The 3-PGA/2-PGA ratio in spores germinated with fluoride is similar to ratios that others have

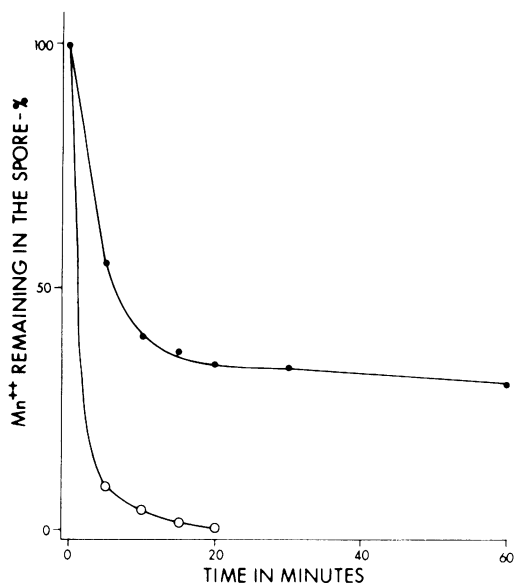


FIG. 7. Release of Mn^{2+} during spore germination. ^{54}Mn -labeled spores were germinated as described in the text with (○) or without (●) $MnCl_2$ (1 mM) in the medium.

measured for the equilibrium of phosphoglycerate phosphomutase (14). This suggests that this enzyme is active within germinated spores, and this is indeed the case (19). In contrast, the 3-PGA/2-PGA ratio in sporulating cells (forespores plus mother cell) and the dormant spore is far from equilibrium. It should be noted that variables such as pH and metal ion concentration have little effect on the phosphoglycerate phosphomutase equilibrium (14). Further evidence that phosphoglycerate phosphomutase is the regulated enzyme is the low activity of this enzyme (but not enolase) in forespore and dormant spore extracts. This is also suggested by the specific activation of 3-PGA catabolism in forespores by Mn^{2+} infusion. Only phosphoglycerate phosphomutase—not enolase or pyruvate kinase—has an absolute Mn^{2+} dependence (21, 23).

The observation that fluoride does not block 3-PGA accumulation during sporulation suggests that 3-PGA is made from a precursor above it in the glycolytic pathway. This would rule out enzymes which act before the mutase in glycolysis as being involved in regulating 3-PGA accumulation in the forespore. This conclusion depends, of course, on the ability of exogenous fluoride to inhibit enolase *in vivo* during sporulation. Although we do not know this to be true, exogenous fluoride enters and inhibits enolase within germinating spores (19).

(ii) The evidence that phosphoglycerate phosphomutase is inactive in forespores (and possibly

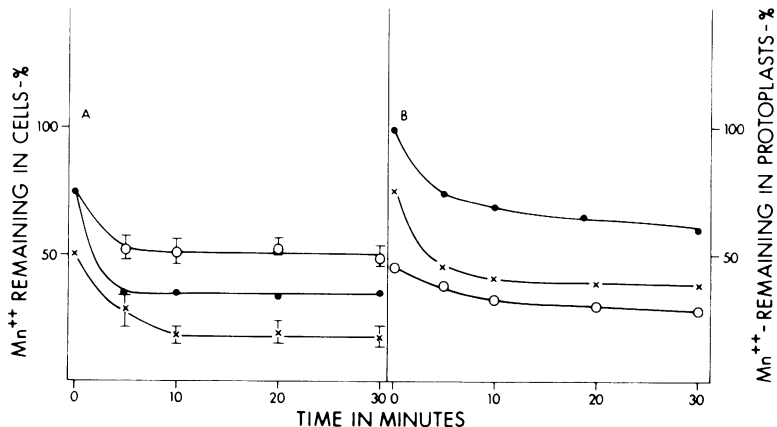


FIG. 8. Loss of $^{54}Mn^{2+}$ from prelabeled vegetative or sporulating cells. (A) Cells were grown in SNB plus $^{54}Mn^{2+}$, harvested, washed, and resuspended in fresh SNB, and the ^{54}Mn loss was quantitated as described in the text. Vegetative cells were in mid-log phase, whereas sporulating cells were 2 h into stationary phase. Values are calculated as the percent ^{54}Mn remaining of that present in cells at the time that they were harvested. Symbols: (A) ○, stationary phase cells with no additions or +2% toluene or +1 mM $MnCl_2$; ●, vegetative cells with no additions; ×, vegetative cells plus 2% toluene or 1 mM $MnCl_2$. (B) Cells were grown and harvested in stationary phase and converted to protoplasts which were incubated in Tris-protoplast buffer with (●) no additions; (×) 1 mM $MnCl_2$; (○) 2% toluene. Values are given as the percentage of Mn^{2+} remaining compared with that in the cells at the time that they were harvested.

spores) at least in part because of a level of low free Mn^{2+} is twofold. (a) The phosphoglycerate phosphomutase in forespore and dormant spore extracts is significantly less active than the enzyme in cell and germinated spore extracts in the absence of added Mn^{2+} . (b) 3-PGA catabolism can be initiated in forespores by incubation with an ionophore plus Mn^{2+} . In contrast, ionophore plus Ca^{2+} or Mg^{2+} is ineffective, even though Ca^{2+} is and Mg^{2+} should be transported into forespores. Although we have not proven that 3-PGA disappearance in forespores given Mn^{2+} plus ionophore is due to activation of phosphoglycerate phosphomutase, this seems likely. 3-PGA utilization via phosphoglycerate kinase and the hexose monophosphate shunt requires an energy input (19) which is not available in ionophore-treated forespores, since ATP is rapidly lost. Similarly, in spores germinated without an exogenous energy source, 3-PGA utilization is blocked when enolase is inhibited by fluoride (19).

That Mn^{2+} deficiency in a *Bacillus* species can result in a large decrease in the activity of phosphoglycerate phosphomutase in vivo has been shown directly by Oh and Freese (12). When *B. subtilis* is grown in a Mn^{2+} -deficient medium, a large pool of 3-PGA accumulates in stationary phase cells and sporulation does not take place (12). The phosphoglycerate phosphomutase in extracts from cells which accumulate 3-PGA has little or no activity unless Mn^{2+} is supplied (12).

(iii) If the first parts of our hypothesis are true, then a major reason for the little or no phosphoglycerate phosphomutase activity in forespores is not the absence of Mn^{2+} but rather a low free Mn^{2+} concentration in vivo. Our only data which support this latter contention directly are the exchange experiments with isolated forespores. Clearly, the Mn^{2+} which is in isolated forespores exchanges poorly with exogenous Mn^{2+} , more slowly than does Mn^{2+} in vegetative or stationary phase cells. It is, of course, possible that within the sporulating cell forespores contain significant exchangeable Mn^{2+} which it is lost immediately upon protoplast disruption before forespore isolation. However, protoplast disruption and forespore isolation were carried out at 4°C to reduce such loss, and only a small amount (<8% of that which remained in the forespores) of Mn^{2+} was lost during the last two centrifugation steps in forespore isolation; even this small loss may not be Mn^{2+} exchanging from forespores, but may be removal of residual mother cell cytoplasm, or physical loss of forespores, or both. In any case our data indicate that in isolated forespores Mn^{2+} is tightly bound to some forespore com-

ponents. In agreement with our data, an electron paramagnetic resonance study suggested that the great majority, if not all, of the Mn^{2+} in dormant spores was either chelated or tightly bound to some spore macromolecule(s) (26).

Clearly, our hypothesis requires the presence of Mn^{2+} binding moieties in the forespore and dormant spore. One candidate is DPA which is present in dormant spores in large excess over Mn^{2+} . Although DPA may bind much of the spore Mn^{2+} , it seems unlikely that DPA is the sole Mn^{2+} binder for a number of reasons. (i) Mn^{2+} uptake into forespores begins well before DPA accumulation. Furthermore, much of the DPA must be involved in Ca^{2+} uptake which begins slightly after Mn^{2+} uptake (Singh and Setlow, unpublished data, 1978). (ii) If all forespore and spore Mn^{2+} were bound to DPA, spore germination would not result in elevated free Mn^{2+} levels. However, binding of Mn^{2+} by some spore component that is degraded during spore germination (possibly the spore cortex) might result in an elevation in free Mn^{2+} . (iii) During germination of $^{54}Mn^{2+}$ -labeled spores, >95% of the DPA but only 70% of the ^{54}Mn is released. If all the Mn^{2+} in the spore was bound to DPA, than >95% of the Mn^{2+} should have been released upon germination. (iv) Spores of *B. cereus* which are genetically DPA negative take up 15 to 20% of the wild-type amount of Ca^{2+} and Mn^{2+} (9).

Although the data cited above indicate that much (70 to 80%) of the Mn^{2+} in the spore may be bound to DPA, there must also be other Mn^{2+} binding moieties in both the forespore and spore. Clearly, it will be of interest to identify such other Mn^{2+} binding moieties and to determine whether they might play a role in regulating free Mn^{2+} levels in vivo.

Although the hypothesis that we have proposed in this communication concerns the inactivity of a key enzyme in bacterial spores, similar types of proposals have been made, and in some cases substantiated, for other dormant systems. In particular, dormancy in unfertilized sea urchin eggs appears related to low endogenous levels of free Ca^{2+} (6). Upon fertilization and breaking of dormancy in sea urchin eggs, endogenous free Ca^{2+} levels rise with the Ca^{2+} being derived from endogenous reserves. Significantly, dormancy in sea urchin eggs can be broken artificially by administration of divalent cation ionophores (2, 24). Possibly regulation of dormancy by control of the concentrations of specific metal ions is a general process in biology.

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