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Levels of Small Molecules and Enzymes in the Mother Cell Compartment and the Forespore of Sporulating *Bacillus megaterium*

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We have determined the amounts of a number of small molecules and enzymes in the mother cell compartment and the developing forespore during sporulation of *Bacillus megaterium*. Significant amounts of adenosine 5'-triphosphate and reduced nicotinamide adenine dinucleotide were present in the forespore compartment before accumulation of dipicolinic acid (DPA), but these compounds disappeared as DPA was accumulated. 3-Phosphoglyceric acid (3-PGA) accumulated only within the developing forespore, beginning 1 to 2 h before DPA accumulation. Throughout its development the forespore contained constant levels of enzymes of both 3-PGA synthesis (phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase) and 3-PGA utilization (phosphoglycerate mutase, enolase, and pyruvate kinase) at levels similar to those in the mother cell and the dormant spore. Despite the presence of enzymes for 3-PGA utilization, this compound was stable within isolated forespores. Two acid-soluble proteins (A and B proteins) also accumulated only in the forespore, beginning 1 to 2 h before DPA accumulation. At this time the specific protease involved in degradation of the A and B proteins during germination also appeared, but only in the forespore compartment. Nevertheless, the A and B proteins were stable within isolated forespores. Arginine and glutamic acid accumulated within the forespore in parallel with DPA accumulation. The forespore also contained the enzyme arginase at a level similar to that in the mother cell and a level of glutamic acid decarboxylase 2- to 25-fold higher than that in the mother cell, depending on when in sporulation the forespores were isolated. The specific activities of several other enzymes (protease active on hemoglobin, ornithine transcarbamylase, malate dehydrogenase, aconitase, and isocitrate dehydrogenase) in forespores were about 10% or less of the values in the mother cell. Aminopeptidase was present at similar levels in both compartments; threonine deaminase was not found in either compartment.

The system of sporulation and spore germination in the various *Bacillus* species has been considered a model system for the study of differentiation because of its relative simplicity and the ready application of biochemical and genetic analyses. An interesting period during this differentiation process occurs late in sporulation, as the spore is formed within the sporulating cell. This process creates two separate intracellular compartments, the mother cell and the forespore (11). Subsequently, the forespore is converted from a compartment carrying out protein and ribonucleic acid (RNA) synthesis and various metabolic reactions to a dormant spore, which is metabolically inactive (4, 15, 36). Understanding of the molecular events during this period in the sporulation process, as well as the attendant control mechanisms, requires distinction between the events occurring in the mother cell and those in the forespore. Until recently, isolation of intact forespores in amounts sufficient for biochemical analysis has been difficult, although techniques for forespore isolation have been described (3). However, Ellar and Postgate (10) have devised a simple and rapid technique for isolation of forespores from a lysozyme-sensitive strain of *Bacillus megaterium* KM. We have utilized this technique for determining the levels of a number of small molecules and enzymes in the forespore and in the mother cell compartment in another strain of *B. megaterium*. It is hoped that this knowledge will give insight into the metabolic and biosynthetic capacity of forespores and suggest possible control mechanisms that must operate within the mother cell com-

partment and the developing forespore during this period in sporulation.

MATERIALS AND METHODS

Reagents and enzymes. L-[1-^{14}C]glutamic acid was obtained from Schwarz Bio Research, Inc. (Orangeburg, N.Y.). All other enzymes and assay reagents were obtained from Sigma Chemical Co. (St. Louis, Mo.). Aminopeptidase, the dormant-spore protease, and a crude mixture of the A and B proteins were purified from *B. megaterium* spores as previously described (33, 34).

Growth of cells and isolation of the mother cell compartment and forespores. All work was carried out with *B. megaterium* QM B1551, originally obtained from Hillel Levinson (U.S. Army Development Center, Natick, Mass.). Spores of this organism were prepared by growth at 30°C in supplemented nutrient broth, harvested, lyophilized, and stored as previously described (35). All spore preparations were free of vegetative cells and cell debris and contained >95% refractile forms when viewed in a phase-contrast microscope. Growth of sporulating cells was also carried out at 30°C in supplemented nutrient broth (35).

The method for isolation of forespores is a modification of the procedure described by Ellar and Postgate (10). Samples (50 ml) of sporulating cells were centrifuged (5 min, 10,000 × *g*) and washed with 25 ml of warm (37°C) buffer A (0.6 M sucrose, 0.1 M potassium phosphate [pH 7.0], and 16 mM $MgSO_4$). The cell pellet was suspended in 6 ml of warm buffer A, and, after addition of lysozyme (10 mg), cells from 50 ml of culture were incubated for 10 min at 37°C. This treatment does not result in formation of true protoplasts, since the cells do not separate and become spherical. However, they do round up to some degree and become very sensitive to sonic disruption. The lysozyme-treated cells were washed twice with cold buffer A by centrifugation (5 min, 10,000 × *g*), and a sample (20 to 40%) of the washed cells was saved for analysis of compounds in the mother cell compartment or the whole cell. The remaining cells were suspended in 6 ml of cold buffer A and sonically treated (30 s to 1.5 min, maximum output of a Sonifer-Cell Disruptor [Heat Systems-Ultrasonics, Inc., Plainview, N.Y.) to release forespores. The sample was centrifuged (3 min, 9,000 × *g*) and suspended in 6 ml of buffer A, and the sonic treatment was repeated twice, followed by centrifugation for 3 min at 7,000 × *g* and 3 min at 6,000 × *g*, respectively. The final forespore pellets had a very low level of contamination with mother cells and mother cell debris, as determined both by observation in a phase-contrast microscope and by the low level of a number of mother cell enzymes found in the forespores. Furthermore, the recovery of forespores from the sporulating cells was >75%, as determined both by cell count and by the recovery of several spore-specific compounds (dipicolinic acid [DPA] and 3-phosphoglyceric acid [3-PGA]) in the forespores.

In some experiments, KCN (10 mM) was included in all buffers to prevent both continued protein synthesis and energy-dependent protein degradation (32, 33). Some experiments also utilized a tris(hydroxymethyl)aminomethane buffer [0.6 M sucrose, 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.4), 0.15 M NaCl, 16 mM $MgCl_2$; buffer B] both for washing the lysozyme-treated cells and for the sonic treatment. The results of experiments done with or without these modifications were similar where tested.

Extraction of enzymes. Forespores from 30 to 40 ml of culture or lysozyme-treated, washed cells from 10 to 20 ml of culture were suspended in 2 to 3 ml of 50 mM tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.4) and 5 mM $CaCl_2$. Phenylmethylsulfonyl fluoride (0.1 mM) was also included, unless proteolytic enzymes were to be assayed. Enzymes were extracted from the mother cell compartment of lysozyme-treated cells (see above) by sonic treatment (30 s) in the presence of glass beads (100-μm diameter, 1.5 g). This brief treatment did not disrupt significant amounts of forespores within the mother cell, since forespore disruption required longer periods (4 to 10 min) of sonic treatment under these conditions. All sonically treated preparations were centrifuged (10 min; 10,000 × *g*) and dialyzed at 4°C against 50 mM tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.4) and 20% glycerol. Other additions to the dialysis buffer included: $CaCl_2$ (5 mM) for samples to be analyzed for protease (34); $MnCl_2$ (1 mM) for samples to be assayed for phosphoglycerate mutase or arginase (22, 25); dithiothreitol (1 mM) for samples to be assayed for enolase; dithiothreitol (1 mM) and KCl (50 mM) for samples to be assayed for pyruvate kinase (8); and dithiothreitol (1 mM) and $MgCl_2$ (2 mM) for all other enzymes.

Extraction of small molecules. Adenosine 5'-triphosphate (ATP) and total adenine nucleotides were extracted from lysozyme-treated cells and forespores by using boiling 80% 1-propanol, as previously described for extraction of adenine nucleotides from dormant spores (36). Reduced nicotinamide adenine dinucleotide (NADH) was extracted using 0.1 M KOH (27); NAD was extracted with HCl from lyophilized forespores or lysozyme-treated cells after dry rupture (24, 27). NADH was converted to NAD, and pyridine nucleotides were purified by passage through Sephadex G-10, as previously described (27). Amino acids, 3-PGA, and DPA were extracted with boiling water (10 min). After cooling in ice and addition of acetic acid to 3%, the sample was centrifuged (10 min; 10,000 × *g*), and the protein-free supernatant fluid was lyophilized. Amino acids were further purified by chromatography on Dowex-50 (37).

Assay procedures. ATP was analyzed using the luciferase reaction, and total adenine nucleotides were analyzed similarly after conversion to ATP (35). NAD was determined using the cycling procedures of Lowry and co-workers (14) and Setlow and Setlow (27). Amino acids were quantitated (37), and 3-PGA was quantitated fluorometrically (6). DPA was determined using the method of Rotman and Fields (23).

Deoxyribonucleic acid (DNA) and RNA were determined by the diphenylamine and orcinol procedures, respectively (26); protein was determined using the Lowry procedure (19). Treatment of cells or

spores to solubilize these macromolecules before analysis was as previously described (28, 37).

Aminopeptidase and proteolytic activity either on ^{14}C-labeled carbamylated hemoglobin or on a crude mixture of the A and B proteins was determined as previously described (31). Aconitase and malate dehydrogenase were determined as described by Szulmajster and Hanson (38). NAD phosphate-specific isocitrate dehydrogenase was assayed using the method of Cleland et al. (5). Ornithine transcarbamylase was determined using the procedure of Hunninghake and Grisolia (13). Arginase was determined by measuring the release of urea from arginine (25). Glutamic acid decarboxylase was measured as described by Foerster and Foerster (12), but $^{14}CO_2$ was measured as previously described (29). Phosphoglycerate mutase was measured by coupling this reaction with the enzymes enolase, pyruvate kinase, and lactic dehydrogenase, as described by D'Alessio and Josse (7). Enolase was measured similarly by coupling the reaction with pyruvate kinase and lactic dehydrogenase. Threonine deaminase was assayed as described by Leitzman and Bernlohr (18); pyruvate kinase was assayed as described by Diesterhaft and Freese (8). Phosphoglycerate kinase was measured by coupling this reaction with glyceraldehyde-3-phosphate dehydrogenase, as described by D'Alessio and Josse (7); glyceraldehyde-3-phosphate dehydrogenase was measured as described by Amelunxen (1). The specific activities of proteolytic enzymes are given as nanograms of substrate degraded per minute per milligram of protein in extracts. All other specific activities are given as nanomoles of product formed per minute per milligram of protein.

The A and B proteins were obtained from lyophilized forespores or lysozyme-treated cells by using acetic acid extraction of dry-ruptured spores or cells (30). These proteins were quantitated after their separation by discontinuous gel electrophoresis at acid pH (30).

Chromatography of cell or forespore extracts on diethylaminoethyl-Sephadex was carried out as described for purification of the spore protease that degrades the A and B proteins during spore germination (34). However, the column volume was reduced to 35 ml, 75 ml of each gradient solution was used, and 3-ml fractions were collected.

RESULTS

Amounts of nucleic acid and protein in mother cells and forespores. At the earliest time in sporulation that forespores could be isolated (2.5 h before half-maximal accumulation of DPA and 3 h after the end of log-phase growth), they contained significant amounts of DNA, RNA, and protein (Fig. 1a). The amounts of DNA and RNA in the forespores remained relatively constant from this time on (Fig. 1a). However, the protein level in the forespores increased almost threefold (Fig. 1a), presumably due to accumulation of spore-specific proteins, such as coat proteins and the A and B proteins (30). The youngest forespores isolated contained 25 to 35% of the total cellular nucleic acid and ~10% of the total cellular protein (Fig. 1b). However, as sporulation proceeded, the synthesis of spore-specific proteins and the degradation of mother cell macromolecules resulted in an increase in the percentage of total cellular DNA, RNA, and protein in the forespores (Fig. 1b). Throughout sporulation, >85% of the DPA was found in the forespores (Fig. 1b), as has been observed by others (2, 17).

Amounts of ATP, total adenine nucleotides, NAD, and NADH. In addition to protein, RNA, and DNA, forespores also contained significant amounts of several key small molecules. At the earliest measurement, forespores contained ~10% of the cells' total free adenine nucleotides. The amount of adenine nucleotide in the whole cell fell ~2.5-fold as sporulation proceeded (Fig. 2b). In both the youngest forespores and the whole cells, 50 to 70% of the adenine nucleotide pool was ATP. However, while the ATP/adenine nucleotide ratio in the whole cells remained fairly constant, this ratio

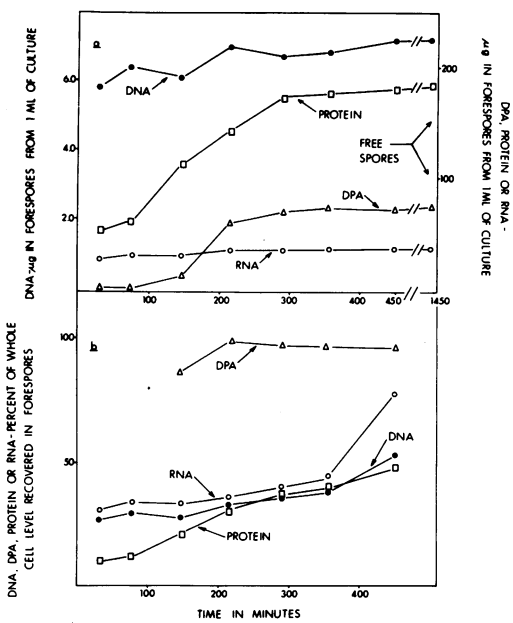

FIG. 1. *Amounts of DPA, DNA, RNA, and protein in the mother cell and forespore during sporulation. Forespores or lysozyme-treated sporulating cells were prepared, extracted, and analyzed for DPA, DNA, RNA, and protein. Dormant spores were treated similarly and were harvested ~20 h after accumulation of the maximum DPA level. (a) Levels of DPA, DNA, RNA, and protein in the forespore. (b) Percentage of total cellular DPA, DNA, RNA, and protein in the forespore.*

fell dramatically in the forespores at or slightly before the time at which DPA was accumulated (Fig. 2a and b). Indeed, when >95% of maximum DPA was accumulated within the forespores, less than 1% of the forespore adenine nucleotide pool was ATP, as has also been found in dormant spores (36).

Findings on pyridine nucleotides were similar to findings on adenine nucleotides. Throughout sporulation, the pyridine nucleotides in the forespores accounted for only a small part (6 to 16%) of the total cellular pool

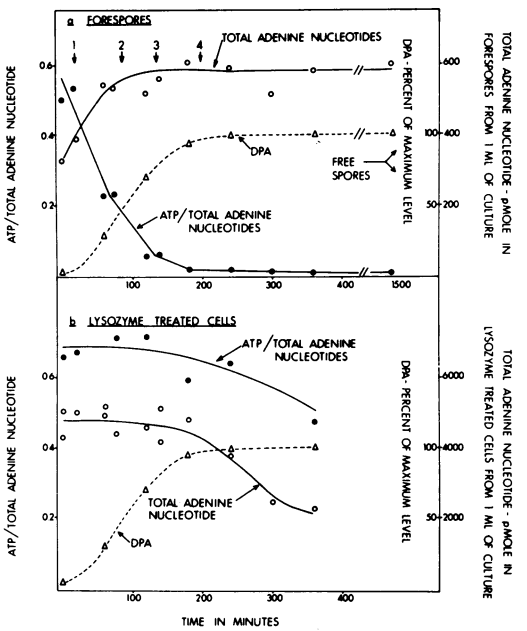

FIG. 2. *Amounts of ATP and total adenine nucleotides in either (a) the forespore or (b) the lysozyme-treated whole cell. Cells were isolated and extracted and analyzed for DPA, ATP, or total adenine nucleotides. The numbered arrows in (a) refer to the times samples were taken for analysis of pyridine nucleotides (see Table 1).*

(Table 1). Furthermore, forespore preparations with large amounts of DPA contained no detectable NADH, as was previously found for isolated dormant spores (Table 1) (37). In contrast, the whole cells, as well as forespores isolated before maximal DPA accumulation, contained significant amounts of NADH, but the maximum NADH/NAD ratios in forespores were much lower than those found in the whole cells (Table 1).

Levels of 3-PGA and enzymes of its synthesis and utilization. Although the mature spore contains low levels of the high-energy compounds ATP and NADH (Fig. 2, Table 1) (27, 36), it contains a large depot of 3-PGA, a compound utilized in the first minutes of germination to generate ATP and possibly NADH (27, 36). The 3-PGA accumulated during sporulation 1.5 h before the accumulation of DPA, as was previously noted (21), and >85% of the cellular 3-PGA was found in the forespores at all times tested (Fig. 3). The latter finding was true upon analysis of either lysozyme-treated cells and their forespores or untreated cells and their forespores (data not shown).

In addition to large amounts of 3-PGA, both the forespore and mother cell compartments of sporulating cells contained enzymes of 3-PGA metabolism (Table 2). These enzymes included those preceding 3-PGA (phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase) and those following 3-PGA (phosphoglycerate mutase, enolase, and pyruvate kinase) in the glycolytic pathway. These enzymes were present at similar levels in the mother cell compartment, forespore, and dormant spore and showed little variation in specific activity throughout sporulation (Table 2).

Despite the presence of enzymes of 3-PGA catabolism in the forespore, the 3-PGA depot was rather stable, and >85% of the 3-PGA was retained when forespores containing 7% of the maximum amount of DPA (arrow A, Fig. 3) were incubated in buffer A for 30 min at 30°C

TABLE 1. *Levels of NAD and NADH in the whole cell and the forespore of B. megaterium*[a]

Sample no.[b]	DPA accumulated (% of maximum)	NAD plus NADH (pmol/ml of culture)		NADH/NAD	
		Whole cell	Forespore	Whole cell	Forespore
1	4	915	56	1.29	0.106
2	45	1,097	96	0.28	0.067
3	78	711	114	0.64	0.026
4	95	575	90	0.45	<0.02
Washed dormant spores[c]	100				<0.02

[a] Samples were harvested, prepared, extracted, and analyzed for DPA, NAD, and NADH.
[b] Samples were taken at times indicated by the arrow in Fig. 2a.
[c] Data taken from reference 27.

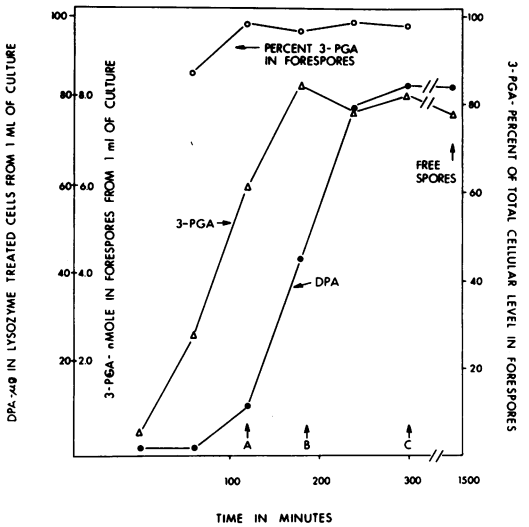

FIG. 3. *Accumulation of 3-PGA within the forespore during sporulation. Forespores or lysozyme-treated cells were isolated and extracted and analyzed for 3-PGA and DPA. Dormant spores were isolated 20 h after the accumulation of maximum DPA levels. The lettered arrows denote times of isolation of samples for enzyme analyses (see Table 2) or for subsequent incubation (see text).*

(data not shown). In older forespores containing more DPA (arrows B and C, Fig. 3), the 3-PGA was even more stable (data not shown). In contrast to 3-PGA, the DPA of young forespores was not retained (<10%) during an incubation for 30 min at 30°C; however, when forespore preparations contained >60% of maximum DPA, this compound was completely retained upon incubation (data not shown).

Levels of proteins A and B and proteolytic enzymes. Two other compounds destined for the mature spore that are accumulated late in sporulation are the unique low-molecular-weight proteins previously termed A and B proteins (30). As was seen above for 3-PGA, the A and B proteins were also accumulated only within the forespore beginning 70 to 100 min before the accumulation of DPA (Fig. 4). Furthermore, incubation of forespores containing 15% of the maximum DPA in buffer A for 30 min at 30°C resulted in the loss of <25% of the A and B proteins (data not shown).

In addition to the A and B proteins, forespores also contained a degradative enzyme(s) active on these proteins (Table 2). Diethylaminoethyl-Sephadex chromatography of an extract from forespores containing only 10% of their maximal DPA (arrow 1, Fig. 4) indicated that >80% of the degradative activity towards the A and B proteins in these extracts was due to a single enzyme (Fig. 5a). Furthermore, this degradative enzyme co-chromatographed with the dormant-spore protease previously shown to be responsible for degradation of the A and B proteins during spore germination (Fig. 5a) (34). This degradative enzyme in the forespores was separated from the proteolytic activity on the A and B proteins found in the mother cell, and indeed this latter enzyme appears to be present at extremely low levels in the forespore (Fig. 5a and b; and see below). Chromatographic analysis of extracts either of forespores containing 98% of their maximal DPA (arrow 2; Fig. 4) or of dormant spores (arrow 3; Fig. 4) also indicated the presence of a single degradative activity towards the A and B proteins, which co-chromatographed with the purified spore protease (data not shown). The specific activities of the diethylaminoethyl-Sephadex-purified enzymes from the older forespores or the dormant spores were similar, and were only 25% higher than that for the similarly purified enzyme from the younger forespores (data not shown). These data indicate that the specific spore protease previously implicated in degradation of the A and B proteins during germination (34) is accumulated in the forespore significantly before DPA is accumulated. Despite the presence of a protease active on the A and B proteins, forespores had very low levels of protease active on hemoglobin (Table 2). However, the mother cells had extremely high levels of protease active on hemoglobin (Table 2). This enzyme active on hemoglobin also degrades the

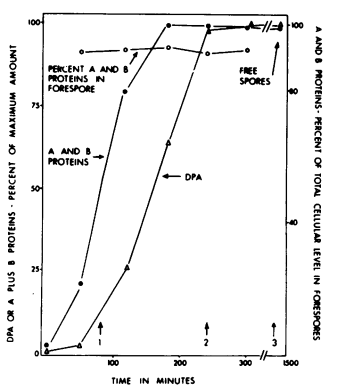

FIG. 4. *Accumulation of proteins A and B within the forespore during sporulation. Forespores or lysozyme-treated cells were prepared, extracted, and analyzed for DPA and proteins A and B. Dormant spores were isolated 20 h after the accumulation of maximum DPA levels. The numbered arrows denote times of isolation of samples for analysis of proteases (see text and Fig. 5).*

TABLE 2. *Enzyme levels in the mother cell compartment, forespore, or the dormant spore of B. megaterium*

Enzyme	Sp act (% of maximum)[a]						
	1[b]		2		3		Dormant spore
	Mother cell	Forespore	Mother cell	Forespore	Mother cell	Forespore	
3-Phosphoglycerate metabolism							
Glyceraldehyde-3-phosphate dehydrogenase	92	85	94	98	71	100	93
3-Phosphoglycerate kinase	89	99	88	70	53	93	96
Phosphoglycerate mutase	93	94	93	87	85	91	89
Enolase	100	95	91	88	73	85	85
Pyruvate kinase	84	87	67	94	58	98	89
Peptide bond hydrolysis							
Protease on A and B proteins	100	13	80	13	68	16	15
Protease on hemoglobin	100	8	85	5	60	4	4
Aminopeptidase	98	87	97	80	99	100	98
Amino acid biosynthesis or the tricarboxylic acid cycle							
Threonine deaminase[c]	<1	<1	<1	<1	<1	<1	<1
Ornithine transcarbamylase	100	10	54	5	17	4	<3
Aconitase	100	<1	65	<1	20	<1	<1
Isocitrate dehydrogenase	100	<1	54	<1	15	<1	<1
Malate dehydrogenase	90	12	100	11	40	10	10

[a] The maximum specific activity for a given enzyme is the maximum value in an extract (either a mother cell or a forespore extract) from sample 1, 2, or 3 or the dormant spore. The values for maximum specific activities obtained were: glyceraldehyde-3-phosphate dehydrogenase, 87; 3-phosphoglycerate kinase, 101; phosphoglycerate mutase, 58; enolase, 85; pyruvate kinase, 31; protease on A and B proteins, 37,200; protease on hemoglobin, 341; aminopeptidase, 78; ornithine transcarbamylase, 2.5; aconitase, 83; isocitrate dehydrogenase, 89; and malate dehydrogenase, 585.

[b] Samples were taken at times indicated by the lettered arrows in Fig. 3. Isolation of mother cell compartment and forespores and enzyme assays were as described in the text. Dormant spores were harvested 14 to 20 h after accumulation of maximum DPA levels.

[c] Since threonine deaminase could not be detected in either compartment late in sporulation, the maximum specific activity of this enzyme was determined on a late log-phase extract. This value was 57.

A and B proteins rapidly (Table 2) (31, 34). Consequently, the mother cell compartment has a higher level of proteolytic activity on the A and B proteins than does the forespore. However, the activity in the mother cell compartment is due to a different enzyme than the activity in the forespore (Fig. 5). Both compartments contained similar levels of aminopeptidase activity (Table 2).

Levels of amino acids and amino acid-catabolic enzymes. A third set of compounds found in large amounts in dormant spores of *B. megaterium* consists of the amino acids arginine and glutamic acid (20). Accumulation of these amino acids in the forespores occurred in parallel with the accumulation of DPA (Fig. 6). Whereas >90% of the cellular arginine was found in the forespores, the mother cell compartment contained the majority of the glutamic acid throughout sporulation (Fig. 6). In the experiments described above, comparison of the amounts of amino acid in lysozyme-treated cells with those in untreated cells extracted immediately after harvest revealed an only slightly higher (~10%) amount of amino acid in the untreated cells (data not shown).

In addition to arginine and glutamic acid, enzymes for catabolism of these amino acids were also present in both the mother cell compartment and forespores (Fig. 7). Whereas arginase was found at similar levels in both compartments at early times, the forespore level of this enzyme was three- to fourfold higher than the mother cell level later in sporulation. Similarly, the level of glutamic acid decarboxylase was 2- to 25-fold higher in forespores. The largest differences in the levels of the latter enzyme were seen at the latest times tested, since the enzyme level increased ~10-fold in the forespores as sporulation proceeded. Interestingly, the level of both arginase and glutamic acid decarboxylase in the mother cell compartment fell significantly during sporulation (Fig. 7).

Levels of enzymes of amino acid biosyn-

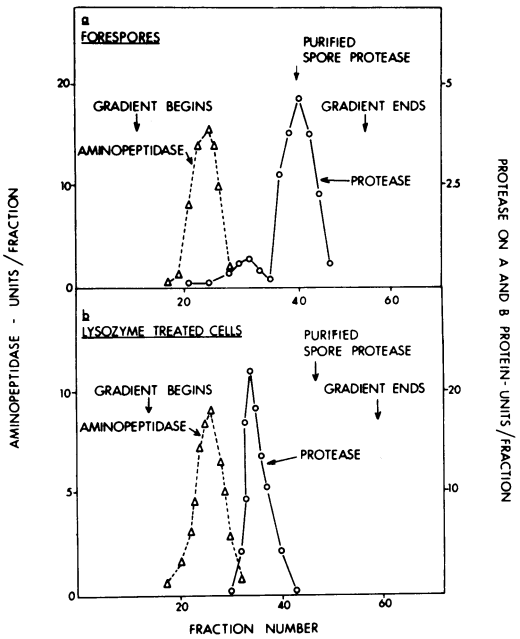

FIG. 5. *Diethylaminoethyl-Sephadex chromatography of the degradative activity toward the A and B proteins in (a) the forespore or (b) the mother cell compartment. Forespores from 700 ml of culture or lysozyme-treated cells from 100 ml of culture were isolated when only 10% of the maximum DPA level had been reached (arrow 1, Fig. 4). Buffer B was substituted for buffer A in steps following lysozyme treatment, and KCN (10 mM) was present. These preparations were then disrupted by sonic treatment. After centrifugation (10 min, 10,000 × g) the supernatant fluid was dialyzed for 20 h against one change of 20% glycerol, 50 mM tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.4), 100 mM NaCl, and 5 mM $CaCl_2$ (buffer C). The dialyzed material was then applied to a column of diethylaminoethyl-Sephadex A-50 (11 by 2 cm) equilibrated at 4°C in buffer C. Enzyme was eluted with a linear gradient of 100 to 400 mM NaCl in buffer C (75 ml of each), and 3-ml fractions were collected.*

thesis and the tricarboxylic acid cycle. In addition to the accumulation of high levels of a number of compounds, the dormant spore also lacks or has a low level of a number of compounds present in the log-phase or sporulating cell. Such compounds include enzymes of amino acid biosynthesis and the tricarboxylic acid cycle (16). Although high levels of three enzymes of the tricarboxylic acid cycle (malate dehydrogenase, isocitrate dehydrogenase, and aconitase) and one enzyme of amino acid biosynthesis (ornithine transcarbamylase) were found in the mother cell compartment at the earliest time tested, none of these enzymes was found in forespores (Table 2). The levels of all four enzymes in the mother cells fell rather rapidly, however, as sporulation proceeded. The amino acid-biosynthetic enzyme threonine deaminase could not be detected in either compartment at these times in sporulation. It has previously

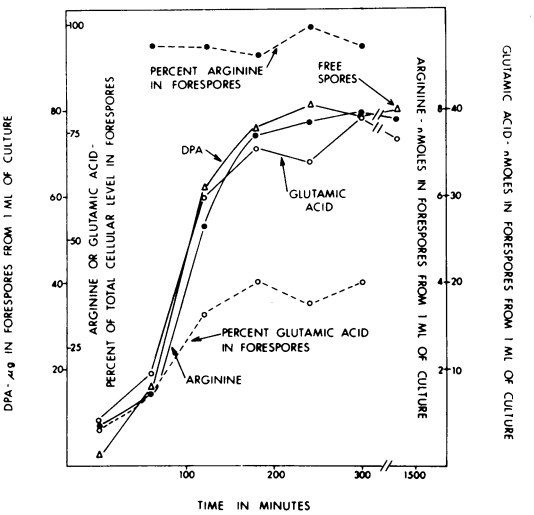

FIG. 6. *Levels of arginine and glutamic acid in forespores during sporulation. Forespores or lysozyme-treated cells were isolated and extracted and analyzed for DPA and free arginine or glutamic acid. Dormant spores were isolated 20 h after the accumulation of maximum DPA levels.*

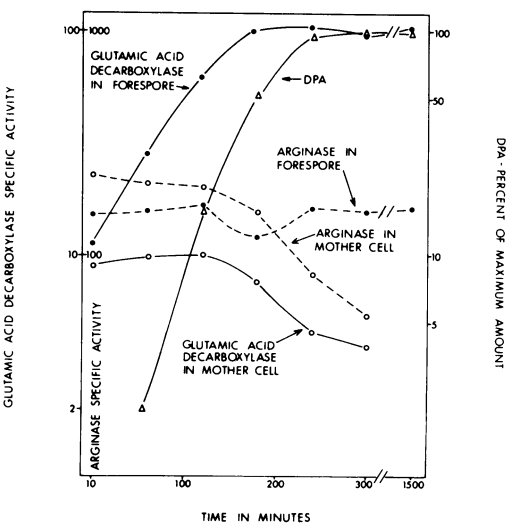

FIG. 7. *Levels of arginase and glutamic acid decarboxylase in the mother cell compartment and forespores during sporulation. Forespores or lysozyme-treated cells were isolated and extracted and analyzed for arginase, glutamic acid decarboxylase, and DPA. Dormant spores were harvested ~20 h after the accumulation of maximum DPA levels.*

been reported that in sporulating *Bacillus cereus*, alanine dehydrogenase is found only in the mother cell compartment, whereas several transaminases are associated primarily with the forespore (3).

DISCUSSION

We have found that five enzymes (protease active on hemoglobin, ornithine transcarbamylase, aconitase, isocitrate dehydrogenase, and malate dehydrogenase) known to be low or absent in dormant spores (16) were also absent or at low levels in forespores at the earliest time tested, despite significant levels in the mother cell compartment. Therefore, the loss or exclusion of these five enzymes from the forespore must occur before the earliest time at which we isolated forespores. This observation, coupled with the lack of high protease activity on hemoglobin in the forespore, is consistent with previous findings by Ellar et al. (9) that there is little protein turnover in the forespore compartment after the time when intact forespores can be isolated. However, it is possible that these particular enzymes are destroyed by proteolysis earlier in forespore development.

In addition to the loss of a number of mother cell proteins, the forespore also accumulates high levels of three other proteins (A and B proteins and glutamic acid decarboxylase). This indicates that either the proteins are synthesized in the mother cell and transported into the forespore or that the proteins are synthesized on forespore ribosomes using messenger RNA synthesized in either the mother cell or the forespore. At present we cannot decide between these alternatives, but forespores do have the capacity to generate high-energy phosphate for protein synthesis, since young forespores have a high ATP-total adenine nucleotide ratio. However, if forespores do carry out protein synthesis, it seems likely that amino acids for this synthesis would be obtained from the mother cell. Biosynthesis of amino acids, at least of methionine and arginine, should be low in forespores due to the absence of key biosynthetic enzymes (37). Indeed, dormant and young germinated spores carry out no biosynthesis of several amino acids, including arginine and methionine, until key amino acid-biosynthetic enzymes are synthesized during germination (37).

Although some spore proteins may be synthesized within the forespore, work in other laboratories has indicated that DPA is synthesized only in the mother cell and is transported into the forespore (2). We feel this may also be true for the arginine that accumulates in the forespore, since the forespore, but not the mother cell, lacks at least one enzyme of arginine biosynthesis (ornithine transcarbamylase). Since the forespore and mother cell both contain enzymes of 3-PGA biosynthesis, the site of synthesis of the 3-PGA that accumulates in the forespore is unclear.

One of the intriguing observations in this study is that forespores contain high levels of a number of compounds (3-PGA, A and B proteins, arginine, and glutamic acid) as well as the enzymes for catabolism or degradation of these compounds. In the case of 3-PGA and the A and B proteins, these compounds and the enzymes for their destruction are known to interact rapidly during spore germination in *B. megaterium*, resulting in >90% loss of both 3-PGA and the A and B proteins after 10 min (30, 36). In the case of 3-PGA, catabolism is known to take place via the enzymes phosphoglycerate mutase, enolase, and pyruvate kinase (36). Significant catabolism of glutamic acid by its decarboxylase and of arginine by arginase also takes place early in germination (12, 37). Despite this rapid breakdown during germination, there is evidence that several of these compounds (3-PGA and A and B proteins) do not turn over during sporulation, once accumulated in the forespore (21, 30); indeed, we have found these compounds to be stable within isolated forespores. These observations taken together indicate that there is some mechanism(s) operating within the forespore during its development that prevents these forespore enzymes from degrading their substrates. Furthermore, this mechanism(s) must be lost or bypassed in some fashion during spore germination. It is possible that elucidation of the mechanism(s) responsible for maintaining these enzymes in an inactive state in the developing forespore may give insight into the causes not only for the onset of spore dormancy, but also for its maintainence and its breaking during spore germination.

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