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Levels of Oxidized and Reduced Pyridine Nucleotides in Dormant Spores and During Growth, Sporulation, and Spore Germination of *Bacillus megaterium*

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Dormant spores of *Bacillus megaterium* contained no detectable reduced nicotinamide adenine dinucleotide (NADH) or reduced nicotinamide adenine dinucleotide phosphate (NADPH) despite significant levels of the oxidized forms of these nucleotides (NAD and NADP). During the first minutes of spore germination there was rapid accumulation of NADH and NADPH. However, this accumulation followed the fall in optical density that is characteristic of the initiation of spore germination. Accumulation of NADH and NADPH early in germination was not blocked by fluoride or cyanide, and it occurred even when germination was carried out in the absence of an exogenous source of reducing power. In addition to pyridine nucleotide reduction, de novo synthesis also began early in germination as the pyridine nucleotide levels increased to those found in growing cells. Midlog-phase cells grown in several different media had 20 to 35 times as much total pyridine nucleotide as did dormant spores. However, as growth and sporulation proceeded, the NADH plus NAD level fell four- to fivefold whereas the NADPH plus NADP level fell by a lesser amount. From min 10 of spore germination until midway through sporulation the value for the ratio of NADH/NAD is about 0.1 (0.03 to 0.18) while the ratio of NADPH/NADP is about 1.4 (0.3 to 2.4). Comparison of these ratios in log-phase versus stationary phase (sporulation) growth in all three growth media tested did not reveal any common pattern of changes.

Dormant spores of the various *Bacillus* species carry out no detectable macromolecular synthesis (7, 14) or oxidative metabolism (2) and have extremely low levels of adenosine 5'-triphosphate (ATP) (20). However, in the first minutes of spore germination, rapid oxidative metabolism (2), ATP production (20), and protein and ribonucleic acid synthesis (7, 14, 22) begin. Previous work indicated that dormant spores of *B. megaterium* also contain no detectable reduced pyridine nucleotides, although significant levels of oxidized pyridine nucleotides were found (20).

Since oxidative metabolism and ATP production begin in the first minutes of spore germination, it is likely that pyridine nucleotide reduction should also occur at this time. Indeed, from analysis of the germination of spores of various mutants of *B. subtilis*, Freese and his co-workers suggested that generation of reduced pyridine nucleotides may be an important event in the initiation of bacterial spore germination (12). Indeed, the first minutes of germination of spores of the fungus, *Neurospora crassa*, are accompanied by significant generation of reduced pyridine nucleotides (16). In this latter system, it was also suggested that production of reduced pyridine nucleotides may play an important role in the initiation of germination, possibly by allowing pyridine nucleotide-dependent reduction of some critical disulfide bonds (3, 15).

Since several workers (1, 5, 26) proposed that cleavage of critical disulfide bonds could facilitate the germination of bacterial spores, it is important to know if there is generation of reduced pyridine nucleotides early in spore germination. If one can show spore germination without pyridine nucleotide reduction, then models suggesting a crucial role for disulfide reduction in spore germination become less likely. However, the observation that pyridine nucleotide reduction always accompanied spore germination would be consistent with (although it would not prove) models that propose disulfide reduction as an important step early in spore germination. Furthermore, such an observation might prompt a search for disulfide bonds that are cleaved early in spore germination.

Despite the potential significance of measurements of levels of reduced pyridine nucleotides in the early minutes of bacterial spore germination, no such data are presently available. Consequently, we measured the levels of reduced and oxidized nicotinamide adenine dinucleotide (NADH and NAD) and of reduced and oxidized nicotinamide adenine dinucleotide phosphate (NADPH and NADP) in dormant spores of *Bacillus megaterium* as well as during germination, log-phase growth, and sporulation. Similar data were also obtained from dormant and germinated spores of *B. cereus*, *B. subtilis*, and *Clostridium bifermentans*.

MATERIALS AND METHODS

Reagents and enzymes. [^{3}H]NAD ([2,8-^{3}H]adenine) and [^{32}P]orthophosphate were obtained from the New England Nuclear Corp. NADP, glucose-6-phosphate, α-ketoglutarate, oxalacetate, yeast 6-phosphogluconate dehydrogenase type IV, beef liver glutamate dehydrogenase type II, pig heart malate dehydrogenase, and yeast alcohol dehydrogenase were obtained from Sigma Chemical Co., St. Louis, Mo. Adenosine 5'-diphosphate and NAD were obtained from P-L Biochemicals Inc., Milwaukee, Wis., and yeast glucose-6-phosphate dehydrogenase was obtained from the Boehringer-Mannheim Corp., New York. The malate dehydrogenase and alcohol dehydrogenase were treated with charcoal to remove pyridine nucleotides as described by Lowry and his co-workers (6, 9).

Production of spores and cells. Most of the work presented in this report 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 in supplemented nutrient broth (SNB) at 30°C and were harvested, washed, and stored as described previously (19). Cells of this organism were also grown at 30°C in either SNB, the minimal medium of Spizizen (25) supplemented with 0.1% Casamino Acids and containing only 12 mM phosphate, or the minimal medium of Slepecky and Foster (24). *B. cereus* T was originally obtained from Harlyn O. Halvorson (Rosenstiel Basic Research Center, Waltham, Mass.), and spores of this organism were prepared by growth of 30°C in SNB as described previously (18).

Spores of *C. bifermentans* were grown and washed as described previously (23) and were the gift of William M. Waites (A.R.C., Food Research Institute, Norwich, England). Spores of *B. subtilis* 168 were the gift of Donald Tipper (University of Massachusetts Medical School, Worcester, Mass.). All spore preparations used in this study were >90% refractile and were free of vegetative cells and cell debris.

Spore germination. Germination of spores of *B. megaterium*, *B. cereus*, and *B. subtilis* was preceded by a heat shock. Spores were suspended in water at 25 mg (dry weight) per ml and heated at 60°C for 10 min (*B. megaterium*), at 65°C for 30 min (*B. cereus*), or at 70°C for 30 min (*B. subtilis*). After the heated spores were cooled on ice, germination was carried out at 30°C at a spore concentration of 2.5 mg/ml. *B. megaterium* spores were germinated either in the minimal medium of Spizizen (25) containing no Casamino Acids and only 12 mM phosphate, or in a KBr medium (0.05 M KBr and 0.01 M KPO_4 [pH 7.4]). The initiation of germination of *B. megaterium* spores was followed by measuring the fall in optical density of a culture as described previously (20). The percent germination at any given time was calculated as the change in optical density at that time divided by the maximum fall in optical density during germination.

B. cereus spores were germinated in the modified medium of Spizizen described above supplemented with 10 mM adenosine and 10 mM L-alanine; *B. subtilis* spores were germinated in a solution of 0.1 M tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.4), 0.2 M KCl, 0.05 M glucose, and 20 mM L-alanine (4). Spores of *C. bifermentans* were germinated at 2.5 mg/ml at 37°C in a solution containing 83 mM $NaPO_4$ (pH 7.5), 100 mM NaCl, 25 mM L-lactate, 50 mM L-alanine, 5 mM L-arginine, and 5 mM L-phenylalanine (27). Germination of spores of *B. cereus*, *B. subtilis*, and *C. bifermentans* was monitored by observation in a phase-contrast microscope.

Extraction of pyridine nucleotides from cells or germinated spores. Extraction of pyridine nucleotides from germinated spores or growing cells was carried out without prior centrifugation of cultures, since this was shown to result in drastic alterations in the levels of reduced and oxidized pyridine nucleotides (28). The extraction procedure that was used is a slight modification of that described by Wimpenny and Firth (28). For extraction of oxidized nucleotides, samples (2 ml) of growing or germinating cultures were rapidly added to 1 ml of 0.2 N HCl preheated to 50°C. This suspension was incubated for 10 min at 50°C and then chilled and neutralized with 0.2 N NaOH. It was then centrifuged (10 min; 10,000 × *g*), and the supernatant fluid was retained. Reduced nucleotides were extracted similarly but 1 ml of 3 N KOH was substituted for the HCl. After neutralization of the latter extract with 3 N HCl, the suspension was centrifuged. The reduced nucleotides in the supernatant fluid were oxidized enzymatically, and the solution was acidified to inactivate the enzymes and then neutralized as described by Wimpenny and Firth (28). This latter procedure is necessary to destroy some compound that interferes with subsequent assay of the pyridine nucleotides as was noted previously (28). A small amount of [^{3}H]NAD (~0.2 pmol, 2.5 × 10^4 cpm/pmol) was then added to all neutralized extracts, and these were lyophilized. The dry residue was dissolved in 1.0 ml of 0.1 M acetic acid, desalted on a column of Sephadex G-10 (30 by 1.4 cm) equilibrated with 0.1 M acetic acid, lyophilized, and finally dissolved in 0.5 ml of water. The recovery of pyridine nucleotides from the column was determined from the recovery of [^{3}H]NAD and this value ranged between 65 and 80%. It has been reported that the extraction procedures described above give excellent (greater than

85%) recovery of pyridine nucleotides added to cultures (28), and we also found this to be true.

Extraction of pyridine nucleotides from dormant spores. Reduced pyridine nucleotides were routinely extracted from dormant spores with alkali as described above, but with 15 min of incubation at 50°C. This procedure extracts all small molecules from dormant spores (B. Setlow and P. Setlow, unpublished data). However, extraction of oxidized nucleotides from dormant spores required prior breakage of the spores. Dry spores (30 to 100 mg [dry weight]) were broken by 10 1-min periods of shaking in a dental amalgamator (Wig-L-Bug) with glass beads (100 mg) as the abrasive (13). The oxidized pyridine nucleotides were then extracted from the dry powder by using the HCl extraction method described above. In a few cases, the oxidized pyridine nucleotides were extracted from disrupted spores by using 5% trichloroacetic acid with subsequent removal of the trichloroacetic acid by extraction with ether as described previously (20). The results with both extraction procedures were similar. In all cases, the alkaline or acid extracts were neutralized, treated, and desalted as described above.

Assay of pyridine nucleotides. Pyridine nucleotides were assayed by using the enzymatic cycling procedures described by Lowry and his co-workers (6, 9). Using these assays, we found no significant inhibition of the cycling reaction by the extracts themselves at the concentrations used. However, if extracts were not desalted, massive inhibition was observed.

Incorporation of [^{32}P]phosphate into pyridine nucleotides. Spores were germinated in the medium of Spizizen (25) without Casamino Acids and containing 10 mM [^{32}P]phosphate (10^4 cpm/nmol). At various times, samples (100 μl) were passed through a membrane filter (Millipore Corp., Bedford, Mass.) presoaked in 10 mM phosphate (pH 7.5). The filter was then placed in 2 ml of 0.1 M HCl and, after 20 min at 4°C, the sample was centrifuged, and unlabeled NAD and NADP were added to the supernatant fluid which was then lyophilized. The dry residue was dissolved in 0.1 ml of water and a portion was run on high-voltage paper (Whatman 3MM) electrophoresis, using 0.025 M citrate (pH 3.5). The paper was dried, and NAD and NADP were located from the ultraviolet light absorbance of the pyridine nucleotides. The NAD and NADP spots were cut out, and the pyridine nucleotides were eluted with water. A portion of this eluate was then run on descending paper chromatography on Whatman no. 1 paper, using isopropanol-HCl-water (65:17:18) as the solvent. After the paper was dried, NAD and NADP were located both by their ultraviolet light absorbance and by autoradiography as described previously (20). On this final chromatogram there was excellent coincidence between the spots observed by both autoradiography and ultraviolet light absorbance. Recoveries of NAD and NADP through this procedure were about 70%, and all values were corrected using this number.

RESULTS

Pyridine nucleotide levels in dormant spores and during germination. As was observed previously (20), dormant spores of *B. megaterium* contained no detectable NADH or NADPH (Table 1). This was found to be the case both when spores were first disrupted in the dry state and then subjected to alkaline extraction and when they were extracted directly using the alkaline procedure. Similarly, no NADH or NADPH was detected in freshly prepared dormant spores, heat-activated dormant spores, or dormant spores incubated in 20 mM KCN (Table 1). Dormant spores did, however, contain significant amounts of both NAD

TABLE 1. *Pyridine nucleotide levels in dormant and germinated spores of several bacterial species*[a]

Spores	NAD + NADH (pmol/mg of dry spores)	NADH/NAD	NADP + NADPH (pmol/mg of dry spores)	NADPH/NADP
B. megaterium, dormant	108	<0.02[b]	18	<0.05
B. cereus, dormant	75	<0.02	4	<0.3
B. cereus, germinated 6 min[c]	198	0.26	104	2.2
B. cereus, germinated 15 min[c]	193	0.11	102	1.6
B. subtilis, dormant	72	<0.04	16	<0.05
B. subtilis, germinated 65 min[d]	26	0.36	60	1.1
B. subtilis, germinated 120 min[e]	43	0.26	73	1.2
C. bifermentans, dormant	31	<0.02	2.1	<0.3
C. bifermentans, germinated 19 min[c]	100	0.59		
C. bifermentans, germinated 35 min[c]	27	0.26	54	1.2

[a] Spores were germinated, extracted, and analyzed as described in the text.

[b] Less than 2 pmol of NADH per mg (dry weight) was also found in heat-activated spores and freshly harvested dormant spores as well as in dormant spores incubated in 20 mM KCN (pH 7.4) and at 4°C for 60 min.

[c] Germination greater than 90%.

[d] Germination about 25%.

[e] Germination about 60%.

and NADP, although the levels were two- to threefold lower than those determined in an earlier study (20). Although we have no complete explanation for the latter discrepancy, it should be noted that the assay used in the previous study would have detected not only NAD and NADP, but also other N-substituted nicotinamide derivatives that might have been derived from pyridine nucleotide breakdown.

When spores were germinated in a complete nutrient medium, both NADH and NADPH accumulated rapidly in the first minutes of germination (Fig. 1 and 2). However, there was no detectable accumulation during the lag period prior to initiation of germination (Fig. 1 and 2). Although the NADH/NAD ratio rose rapidly in the first minutes of germination, after reaching a maximum it began to fall until, after 90 min of germination, the ratio was only 10% of the maximum value (Fig. 1 and 3). In contrast, a NADPH/NADP ratio of 1.5 to 2 was maintained throughout 90 min of germination (Fig. 2 and 3).

In addition to pyridine nucleotide reduction early in germination, there appeared to be significant pyridine nucleotide synthesis at this time, since the level of NADP plus NADPH increased about fourfold during the first 10 min of germination (Fig. 2). However, the level of NAD plus NADH changed much less, exhibiting only a 60% increase through the first 30 min of germination (Fig. 1 and 3). After this time

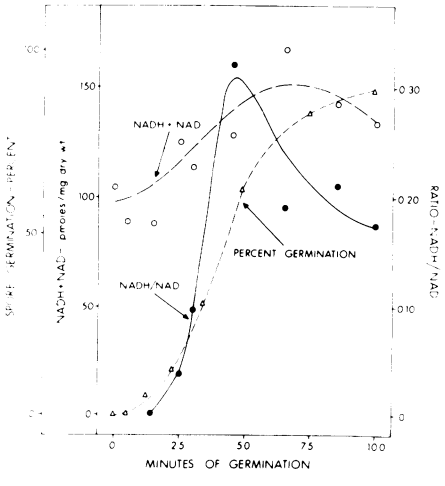

FIG. 1. *NAD and NADH levels in the first minutes of B. megaterium spore germination in the medium of Spizizen. NAD and NADH were extracted and assayed, and the percentage of spore germination was determined as described in the text. These data were obtained from three separate experiments. Symbols: ○, NAD + NADH; ●, NADH/NAD.*

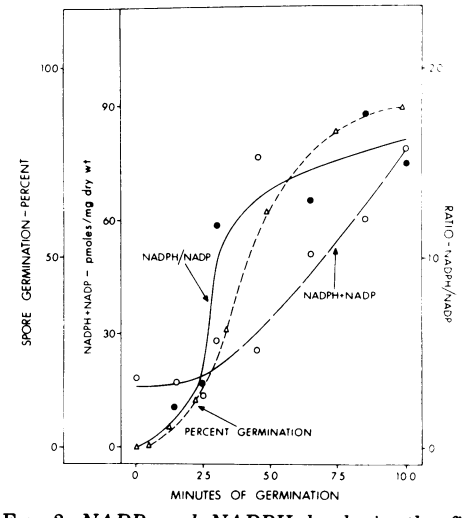

FIG. 2. *NADP and NADPH levels in the first minutes of spore germination in the medium of Spizizen. The cultures analyzed in Fig. 1 were also analyzed for NADP and NADPH as described in the text. Symbols: ○, NADP + NADPH; ●, NADPH/NADP.*

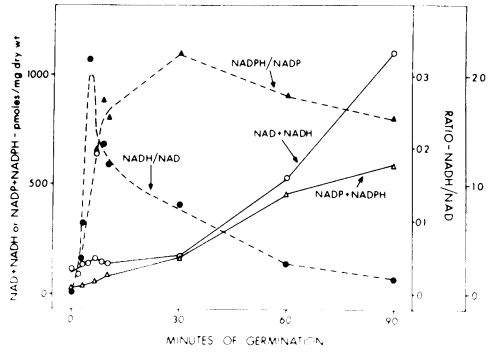

FIG. 3. *Pyridine nucleotide levels during germination and outgrowth of B. megaterium spores in the medium of Spizizen. NAD, NADP, NADH, and NADPH were extracted and analyzed as described in the text.*

the NAD plus NADH level increased rapidly while the NADP plus NADPH level also continued to increase. Since there was no net increase in spore dry weight through 90 min of germination (data not shown), the increases in pyridine nucleotide through this time represent 10- to 25-fold increases in the intracellular pyridine nucleotide content.

The changes in pyridine nucleotide levels during germination were paralleled by pyridine nucleotide biosynthesis as measured by incorporation of ^{32}P into NAD and NADP (Fig. 4). Significant NADP biosynthesis began by the third minute of germination and was rapid

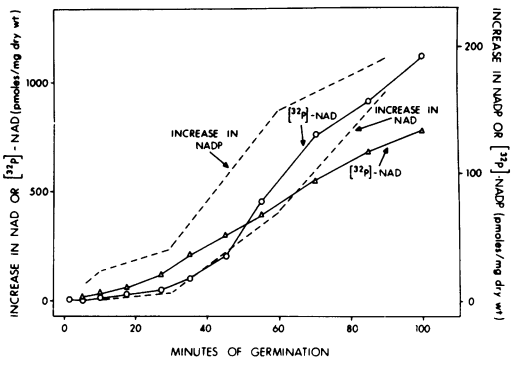

FIG. 4. *NAD and NADP biosynthesis during B. megaterium spore germination. Spores were germinated in the medium of Spizizen modified as described in the text to contain [32P]phosphate. Samples were isolated, extracted, and analyzed as described in the text, and the amount of [32P]NAD and NADP was calculated from the radioactivity in NAD and NADP and the specific radioactivity of the [32P]phosphate in the germination medium. The curve for the increase in NAD and NADP was obtained from the data in Fig. 3 by subtracting the dormant spore level of NAD or NADP.*

throughout germination. In contrast, NAD biosynthesis was slow early in germination and only became rapid later. Throughout germination, de novo synthesis accounted quite well for changes in the level of NAD and less well for changes in NADP (Fig. 4). However, since NADP is synthesized from NAD, the low specific radioactivity of NAD early in germination would be expected to lead to significant underestimation of NADP biosynthesis during this period.

Production of pyridine nucleotides during germination in KBr. Although it seems clear that generation of reduced pyridine nucleotides is an early event in spore germination, the experiments reported above used germination media containing exogenous compounds that could be readily metabolized to yield NADH or NADPH. Since it is known that many spores can be germinated by non-metabolizable compounds, it was of obvious interest to measure pyridine nucleotide reduction when germination was initiated by such non-metabolites. Significantly, when *B. megaterium* spores were germinated in a medium containing only KBr and KPO_4, production of both NADH and NADPH was an early event in spore germination (Fig. 5 and 6). Not surprisingly, the NADH/NAD and NADPH/NADP ratios in spores germinating in KBr were lower than those in spores germinating in a complete medium (cf. Fig. 5 and 6 and Fig. 1 through 3). However, this experiment indicates that dormant spores do contain significant levels of endogenous compounds that can be metabolized to generate reduced pyridine nucleotides.

Effect of inhibitors on pyridine nucleotide levels during germination. One endogenous reserve known to be present at high levels in dormant spores is 3-phosphoglyceric acid (3-PGA), and this compound is known to be metabolized early in germination to generate ATP (20). Although fluoride blocks 3-PGA utilization during germination of *B. megaterium* in KBr (20), fluoride did not block generation of NADH or NADPH during germination in this salt (Table 2). This suggests that metabolism of spore 3-PGA results in little pyridine nucleotide reduction and that spore reserves other than 3-PGA are used as sources of reducing

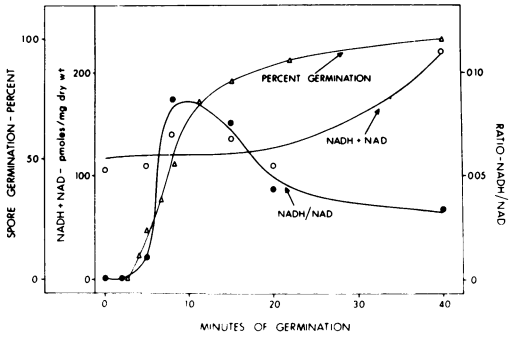

FIG. 5. *NAD and NADH levels in the first minutes of B. megaterium spore germination in the KBr medium. NAD and NADH were extracted and assayed, and the percentage of spore germination was determined as described in the text. The data points are averages from three separate experiments. Symbols: ○, NAD + NADH; ●, NADH/NAD.*

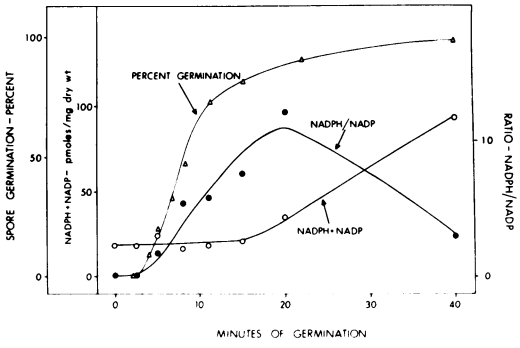

FIG. 6. *NADP and NADPH levels in the first minutes of spore germination in the KBr medium. The cultures analyzed in Fig. 5 were also analyzed for NADP and NADPH as described in the text. Symbols: ○, NADP + NADPH; ●, NADPH/NADP.*

TABLE 2. *Effect of inhibitors on pyridine nucleotide levels during germination of B. megaterium spores*[a]

Germination condition	NADH + NAD (pmol/mg [dry wt])	NADH/NAD	NADPH + NADP (pmol/mg [dry wt])	NADPH/NADP
Dormant spores	108	<0.02	18	<0.05
KBr medium + 10 mM NaF (20 min)[b]	84	0.09	29	0.4
KBr medium + 10 mM NaF (40 min)[c]	179	0.06	89	0.5
Spizizen medium, no aeration (30 min)[b]	186	1.9	83	2.1
Spizizen medium + 10 mM KCN (30 min)[b]	117	1.4	33	1.5

[a] Spores were germinated under standard conditions with changes as noted and were extracted and analyzed as described in the text.
[b] Germination about 70%.
[c] Germination greater than 90%.

power early in germination. However, it is also possible that 3-PGA metabolism is normally a primary source of reducing power, with other alternative sources used when 3-PGA metabolism is blocked.

Generation of NADH or NADPH was not blocked during germination in the presence of KCN or under mildly anaerobic conditions (Table 2). Indeed, under these conditions the NADH/NAD ratios were greatly increased, indicating that a primary route for NADH oxidation may be via a cyanide-sensitive pathway with eventual transfer of electrons to oxygen.

Pyridine nucleotide levels in dormant and germinated spores of other species. The extremely dramatic appearance of reduced pyridine nucleotides in the early minutes of germination of *B. megaterium* spores made it of obvious interest to see if this was a phenomenon general to bacterial spore germination. Indeed, spores of two other *Bacillus* species, *B. cereus* and *B. subtilis*, and one *Clostridium* species, *C. bifermentans*, contained no detectable NADH or NADPH although NAD and NADP were present (Table 1). Furthermore, there was extensive generation of both NADH and NADPH in the first minutes of germination of spores of all of these species, and there were significant increases in the total pyridine nucleotide pool (Table 1).

Pyridine nucleotide levels in log-phase and sporulating cells of *B. megaterium*. With extensive data on levels of pyridine nucleotides in dormant and germinating spores in hand, it was clearly of interest to compare these values to those in growing and sporulating cells of *B. megaterium*. Consequently, the levels were measured in three different media giving doubling times of 40 to 67 min (Fig. 7), all of which allow excellent sporulation (19) (P. Setlow, unpublished data). Cells growing in all three growth media had much higher pyridine nucleotide levels than those of dormant spores

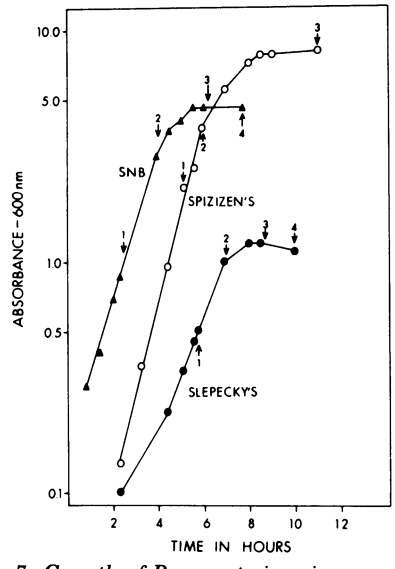

FIG. 7. *Growth of B. megaterium in several media. B. megaterium cells were grown in the three different media as described in the text. The numbers refer to the time at which the samples analyzed in Table 3 were taken.*

(Table 3). However, the levels fell during the shift from midlog phase to sporulation, with the NAD plus NADH level falling much more than the NADP plus NADPH level (Table 3; Fig. 7). Consequently, the NAD plus NADH/NADP plus NADPH ratio was three- to fourfold lower in sporulating cells than that in log-phase cells. The fall in the NAD level going from early log phase to sporulating cells was observed previously in cells of *B. cereus* (8).

In all media and periods of growth tested, the NADPH/NADP ratio was always higher (6- to 30-fold) than the NADH/NAD ratio (Table 3). There were significant changes in the values of these ratios with the stage of growth in a given growth medium, i.e., the fall in the NADH/

TABLE 3. *Pyridine nucleotide levels during growth and sporulation in several growth media*[a]

Growth medium	NADH + NAD (pmol/mg [dry wt])	NADH/NAD	NADPH + NADP (pmol/mg [dry wt])	NADPH/NADP	NADH + NAD/NADPH + NADP
Slepecky (1)	2,168	0.06	731	0.3	2.96
Slepecky (2)	1,216	0.11	581	1.1	2.09
Slepecky (3)	1,053	0.05	683	1.5	1.54
Slepecky (4)	500	0.10	670	2.4	0.75
SNB (1)	3,754	0.15	756	1.6	8.00
SNB (2)	2,098	0.08	764	1.0	2.75
SNB (3)	934	0.14	595	1.5	1.57
SNB (4)	735	0.15	454	1.2	1.62
Spizizen (1)	2,310	0.18	958	1.2	2.41
Spizizen (2)	1,322	0.06	1,668	1.3	0.79
Spizizen (3)	511	0.03	515	1.7	0.99

[a] Cells were grown in the three different growth media as described in the text. The numbers in parentheses refer to the arrows in Fig. 7 and give the times at which samples were taken and extracted.

NAD ratio going from log phase to sporulation in the medium of Spizizen (Table 3). However, when patterns for all three media were examined collectively, no general trends in the NADH/NAD and NADPH/NADP ratios were observed.

DISCUSSION

Presently, we do not know the reasons for the changes in pyridine nucleotide levels that take place during growth and sporulation of *B. megaterium*. However, the large increases in both NAD plus NADH and NADP plus NADPH, which take place from 20 to 90 min of germination (Fig. 3), appear to convert the pyridine nucleotide levels from the low values in the dormant spore to the much higher values in the log-phase cell. We are also unclear as to the reason for the variations in pyridine nucleotide levels between the different growth media and, more significantly, for the variations in the NADH/NAD and NADPH/NADP ratios. Although it may be true that the pyridine nucleotide level or NADH/NAD or NADPH/NADP ratio is crucial to sporulation in a particular growth medium, the data suggest that these values, in particular the NADH/NAD ratios, are of themselves not crucial for sporulation. Cells sporulate well with either a low NADH/NAD ratio (medium of Spizizen) or a three- to five-fold higher ratio (SNB).

Although there are large changes in pyridine nucleotide levels during growth and sporulation, the most dramatic changes are those in the first minutes of spore germination. The absence of NADH and NADPH from dormant spores of several bacterial species is consistent with the known lack of metabolism in spores, and the extremely low levels of other common high energy compounds such as ATP, other ribonucleoside triphosphates, and aminoacyl-transfer ribonucleic acid (17, 20, 21). However, in the first minutes of germination, metabolism begins, and NAD and NADP are rapidly reduced. The accumulation of NADH and NADPH does not precede the initiation of spore germination, when the latter is measured by a fall in optical density of a germinating culture. Therefore, it is unlikely that spore germination is initiated by accumulation of a large amount of reduced pyridine nucleotides. However, since the values we measured are only steady-state levels, it is possible that NADH and/or NADPH is generated and immediately used to initiate germination in the short lag period preceding initiation, and that NADH and NADPH only accumulate after initiation has taken place.

If generation of NADH and/or NADPH is an important event in the initiation of spore germination, the reducing equivalents to generate these molecules need not come from exogenous sources. Spores germinating in KBr plus KPO_4 have endogenous reserves of reducing power that can generate much NADH and NADPH early in germination. This reserve of reducing power in spores is yet another example of the large number of compounds stored in the dormant spore for use in the first minutes of germination. These other reserves include those of energy, amino acids, and ribonucleotides (20–22). At present, we do not know the nature of the endogenous compound(s) that are oxidized to yield NADH and NADPH; however, they do not appear to be derived solely from 3-PGA, since NADH production is not blocked by fluoride. However, the spore contains high levels of several other compounds such as α-glycerophosphate (11) and glutamic acid (10) as well as

the large amount of amino acids generated by proteolysis in the first minutes of germination (22). These compounds could be oxidized to yield reduced pyridine nucleotides, and indeed the dormant spore is known to contain a number of amino acid dehydrogenases (22).

Whatever the endogenous reserve of reducing power in the dormant spore, it is important to ask the function of the NADH and NADPH generated in the first minutes of germination. It appears likely that much of the NADH is used to generate ATP via some type of oxidative phosphorylation. This is suggested by the facts that both cyanide and anaerobiosis inhibit both ATP production (20) and NADH oxidation in the first minutes of germination (Table 2).

A second possible function of NADH and/or NADPH early in spore germination may be the reduction of key disulfide bonds, which might result in activation of enzymes or enzyme systems. Pyridine nucleotide-linked reduction of the disulfide bond in oxidized glutathione was shown to be an early event in fungal spore germination (3). Previous work indicated that there is no change in the total sulfhydryl (SH) to disulfide (SS) ratio going from dormant to germinated spores of *B. megaterium* (19). However, this study measured both protein and nonprotein SH and SS and, in *B. megaterium*, the nonprotein SH plus SS is only 1 to 2% of the total SH plus SS (P. Setlow, unpublished data). It is possible that there may be large changes in the SH/SS ratio of the nonprotein fraction early in spore germination and that increased nonprotein SH may activate some key spore enzyme(s) via disulfide interchange. It may be fruitful to reinvestigate the possible involvement of reduced pyridine nucleotides in cleaving critical disulfide bonds in the first minutes of spore germination.

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