Biochemical Studies of Bacterial Sporulation and Germination

XXII. ENERGY METABOLISM IN EARLY STAGES OF GERMINATION OF BACILLUS MEGATERIUM SPORES*

(Received for publication, February 10, 1970)

PETER SETLOW† AND ARTHUR KORNBERG

From the Department of Biochemistry, Stanford University School of Medicine, Stanford, California 94305

SUMMARY

Dormant spores of Bacillus megaterium contain DPN+, TPN+, and FAD at levels comparable to those in log phase vegetative cells, but little or no reduced pyridine nucleotides are present. The levels of free nucleotides, the most abundant of which are the adenine nucleotides, are also similar in spores and cells. In cells, however, 80% of the adenine nucleotide pool is ATP, whereas in spores it is less than 1%.

During germination, ATP levels develop in three stages. Within the first 5 min (Stage I), there is a sharp increase derived from the 3-phospho-D-glyceric acid depot and also from exogenous metabolites if available. The level is maintained for about 15 min (Stage II) by endogenous or exogenous metabolites. In Stage III, the ATP level increases as a result of biosynthesis of adenine nucleotides, and exogenous carbon and phosphate sources are required. The pathways of energy metabolism are aerobic and cyanide-sensitive in Stages II and III, and the enzymes required for energy metabolism in all three stages are previously formed and present in the dormant spore.

The breaking of dormancy in Bacillus spores appears to require neither energy nor macromolecular synthesis, since inhibitors of metabolism and of RNA and protein synthesis do not block the initiation of germination (2-4). However, RNA and protein synthesis do begin a few minutes after the initiation of germination (4, 5), and therefore ATP must be available at this time. Since the dormant spore contains almost no ATP, and has no detectable metabolic activity (6, 7), ATP must be produced shortly after the initiation of germination. Oxygen uptake and glucose oxidation are known to begin at this time (2).

There are several unanswered questions concerning this early ATP production. (a) Do dormant spores contain the coenzymes and nucleotides needed for ATP production, or do they appear early in germination? Although there is evidence that dormant spores contain DPN+ and CoA (8, 9), there are few quantitative data relating spore coenzyme levels to those in the vegetative cell.

(b) Do dormant spores contain an endogenous energy reserve which could provide an initial burst of ATP? Spores of Aspergillus niger are known to contain a depot of polyphosphate which is utilized for ATP production early in germination (10). Although the presence of polyphosphates has been considered in Bacillus spores (11), no firm support for this has been found (6). Another question which needs further resolution is the nature of the metabolic pathways in the germinating spore.

In this paper we attempt to answer these questions, giving special consideration to the source and means of production of high energy phosphate compounds. In the succeeding paper we consider the synthesis of RNA, one of the main uses of this high energy phosphate, and the relationship to nucleotide biosynthesis and metabolism (12).

The results presented in this paper show that dormant spores contain coenzymes and nucleotides at concentrations similar to those found in the vegetative cell, but that the spore contains almost no ATP and reduced pyridine nucleotides. ATP is generated early in germination, and its production can be divided into three stages as outlined in Scheme I.

In Stage I (0 to 5 min), ATP levels rise about 100-fold and the ATP is derived at least in part from a phosphate source stored in the dormant spore. This ATP production is prevented by fluoride but not by cyanide, and some of the energy is used for RNA synthesis and the phosphorylation of exogenous glucose. Although exogenous energy sources may contribute to the initial rise in ATP, endogenous energy reserves alone are sufficient to support this rise.

ATP levels remain approximately constant in Stage II (5 to 15 min), and endogenous energy supplies are sufficient to maintain this level. ATP production during this period is not prevented by fluoride but is dependent on oxygen and is blocked by cyanide. Metabolism in Stage III (15 to 50 min) is similar to that in Stage II, but exogenous energy sources are now required and there is an increase in the ATP level due to adenine nucleotide biosynthesis de novo.

EXPERIMENTAL PROCEDURE

Materials

Enzymes—Firefly lanterns, lactic dehydrogenase, glucose 6-phosphate dehydrogenase, and hexokinase were obtained from Sigma; pyruvate kinase and phosphoglycerate kinase were from

* This work was supported in part by grants from the National Institutes of Health (United States Public Health Service) and the National Science Foundation. The previous paper in this series is Reference 1.
† National Science Foundation Postdoctoral Fellow.
Calbiochem; and myokinase, glyceraldehyde 3-phosphate dehydrogenase, and yeast alcohol dehydrogenase were from Boehringer. \( \text{d-Amino acid oxidase and catalase were purchased from Worthington; d-amino acid oxidase was converted to the apoenzyme by the method of Massey and Curti} \) (14).

**Nucleotides and Coenzymes—**DPN\(^+\), DPNH, TPN\(^+\), FAD, and AMP were obtained from Sigma. All other nucleotides were obtained from P-L Biochemicals. Nucleotide and coenzyme concentrations were determined from extinction coefficients (15). FAD concentration was determined with a molar extinction coefficient of 1.14 \( \times \) 10\(^4\) at 448 nm.

**Miscellaneous—**Phosphoenolpyruvate, glucose 6-phosphate, chloramphenicol, Dicumarol, and rotenone were purchased from Sigma; 3-phospho-d-glyceric acid, imine and fluoroacetate were from Calbiochem; and 2,4-dinitrophenol was from Eastman Organic Chemicals. Actinomycin D was a gift from Merck, Sharpe, and Dohme, and amytal was from Eli Lilly. \( ^{32} \text{P-Orthophosphate} \) was obtained from Worthington; \( \text{n-amino acid oxidase was converted to the propanol, as described by Bieleski} \) (14). The propanol procedure avoids the problem of residual trichloroacetic acid which interferes with thin layer chromatography (20). The spore samples were diluted with 5 volumes of boiling propanol, boiled for 5 min, flash-evaporated, and resuspended in cold water prior to analysis. All adenine nucleotides were extracted from dormant spores in 5 min. These extractions were carried out in the presence of 0.01 M phosphate buffer, pH 7.5, to prevent the precipitation of low molecular weight phosphorylated compounds.

Germinating spores were extracted with the boiling propanol procedure described above, but without the added phosphate. All adenine nucleotides were extracted from the spores in 5 min, and there is little or no ATP breakdown (19). \( ^{32} \text{P-Labeled} \) germinating spores were extracted in a similar fashion. Extraction with 5% trichloroacetic acid was used in some early experiments, but proved to be ineffective for extracting small molecules from dormant spores and inefficient for carrying out kinetic studies.

To obtain DPN\(^+\) and TPN\(^+\) from vegetative cells, the latter were harvested from a chilled culture by centrifugation and then extracted with 5% trichloroacetic acid for 30 min at 4\(^\circ\). Extraction of reduced pyridine nucleotides was similar, except that 0.33 M NaOH was substituted for trichloroacetic acid. Removal of trichloroacetic acid and other operations were as described above for the spore extracts. To obtain adenine nucleotides the culture sample was mixed directly with an equal volume of cold 10% trichloroacetic acid and kept at 4\(^\circ\) for 30 min. The samples were centrifuged and the supernatant fluid was treated to remove trichloroacetic acid. The dry weight of vegetative cells was determined on samples which were centrifuged, washed once in distilled water, and evaporated from tared planchets under a heating lamp. Coenzyme and nucleotide levels were determined as nanomoles per g, dry weight. These values were converted into nanomoles per g of wet weight with ratios of grams, dry weight, to grams, wet weight in cells and spores of 0.20 and 0.39, respectively (21, 22).

**Germination Conditions—**Unless otherwise noted, the standard conditions for all spore germination experiments were as follows. Spores (25 \( \mu \text{g} \), dry weight, per ml) were heat-shocked for 10 min at 60\(^\circ\) and cooled prior to initiation. Three different media were used. (a) \( \text{KBr: 0.05 M KBr and 0.05 M potassium phosphate, pH 7.5} \); (b) \( \text{glucose: 0.1 M glucose and 0.05 M potassium phosphate, pH 7.5} \); and (c) \( \text{complete: Spizizen’s medium containing glucose, citrate, and salts} \) (23), and supplemented with 0.01% casam hydrolysate, 0.1 mM MnCl\(_2\), 0.1 mM CaCl\(_2\), and 7 \( \mu \text{M FeCl}_3\). Spores (2.5 \( \mu \text{g per ml} \)) were agitated in a gyratory water bath.

**Methods**

**Growth of Spores and Cells—**\( B. \text{megaterium QMB1551} \) spores and \( ^{32} \text{P-labeled} \) spores were obtained from cultures grown in supplemented nutrient broth, as previously described (6, 16), lyophilized, and stored at room temperature. Vegetative cells were also grown in supplemented nutrient broth and were harvested in midlog phase.

**Extraction Procedures—**Dormant spores were extracted by the procedure of Sacks (17). Dry spores, 100 mg, together with 100 mg of glass beads were shaken in a dental amalgamator (Wig-L-Bug) for 10 1-min intervals interspersed with cooling periods in ice. The powder was resuspended in 4 ml of cold 5% trichloroacetic acid, kept for 10 min at 4\(^\circ\), and then centrifuged. The pellet was re-extracted with 4 ml of trichloroacetic acid and the supernatant fluids were pooled. The trichloroacetic acid was removed by five extractions with ether, which brought the pH to 4.0. The solution was neutralized to pH 7.0 with 0.2 volume of 1 M potassium phosphate buffer (pH 6.8). Similar extractions designed to obtain the reduced pyridine nucleotides were performed, except that 0.33 M NaOH was used instead of trichloroacetic acid. The extract was neutralized with 1 M HCl, and was then adjusted with phosphate buffer to pH 7 and 0.15 M phosphate buffer. The spores were disrupted in the dry state in order to prevent enzymatic action on the spore contents. This precaution is especially important for determinations of pyridine nucleotides, since \( Bacillus \) species contain a heat-activated DPNase (18). ATP, DPN\(^+\), or DPNH was recovered almost completely by the extraction procedures described above.

\( ^{32} \text{P-Labeled} \) dormant spores were extracted in boiling propanol, as described by Bieleiski (19). The propanol procedure avoids the problem of residual trichloroacetic acid which interferes with thin layer chromatography (20). The spore samples were diluted with 5 volumes of boiling propanol, boiled for 5 min, flash-evaporated, and resuspended in cold water prior to analysis. All adenine nucleotides were extracted from dormant spores in 5 min. These extractions were carried out in the presence of 0.01 M phosphate buffer, pH 7.5, to prevent the precipitation of low molecular weight phosphorylated compounds.

Germinating spores were extracted with the boiling propanol procedure described above, but without the added phosphate. All adenine nucleotides were extracted from the spores in 5 min, and there is little or no ATP breakdown (19). \( ^{32} \text{P-Labeled} \) germinating spores were extracted in a similar fashion. Extraction with 5% trichloroacetic acid was used in some early experiments, but proved to be ineffective for extracting small molecules from dormant spores and inefficient for carrying out kinetic studies.

To obtain DPN\(^+\) and TPN\(^+\) from vegetative cells, the latter were harvested from a chilled culture by centrifugation and then extracted with 5% trichloroacetic acid for 30 min at 4\(^\circ\). Extraction of reduced pyridine nucleotides was similar, except that 0.33 M NaOH was substituted for trichloroacetic acid. Removal of trichloroacetic acid and other operations were as described above for the spore extracts. To obtain adenine nucleotides the culture sample was mixed directly with an equal volume of cold 10% trichloroacetic acid and kept at 4\(^\circ\) for 30 min. The samples were centrifuged and the supernatant fluid was treated to remove trichloroacetic acid. The dry weight of vegetative cells was determined on samples which were centrifuged, washed once in distilled water, and evaporated from tared planchets under a heating lamp. Coenzyme and nucleotide levels were determined as nanomoles per g, dry weight. These values were converted into nanomoles per g of wet weight with ratios of grams, dry weight, to grams, wet weight in cells and spores of 0.20 and 0.39, respectively (21, 22).

**Germination Conditions—**Unless otherwise noted, the standard conditions for all spore germination experiments were as follows. Spores (25 \( \mu \text{g} \), dry weight, per ml) were heat-shocked for 10 min at 60\(^\circ\) and cooled prior to initiation. Three different media were used. (a) \( \text{KBr: 0.05 M KBr and 0.05 M potassium phosphate, pH 7.5} \); (b) \( \text{glucose: 0.1 M glucose and 0.05 M potassium phosphate, pH 7.5} \); and (c) \( \text{complete: Spizizen’s medium containing glucose, citrate, and salts} \) (23), and supplemented with 0.01% casam hydrolysate, 0.1 mM MnCl\(_2\), 0.1 mM CaCl\(_2\), and 7 \( \mu \text{M FeCl}_3\). Spores (2.5 \( \mu \text{g per ml} \)) were agitated in a gyratory water bath.
Coenzyme and Nucleotide Levels in Dormant Spore

Coenzyme Levels—The levels of FAD, DPN+, and TPN+ found in the dormant spore were similar to those in cells at midlog phase (Table I). The latter values agree with those reported in other strains of B. megaterium (26, 31). Whereas the ratio of reduced to oxidized pyridine nucleotides in bacterial cells is in the range of 0.14 to 0.30 (32, 33), that in dormant spores was extremely low (0.02 or less). This ratio, as well as the levels of coenzymes, was not affected by storage for 5 months in the lyophilized state at room temperature.

Adenine Nucleotide Levels—The level of total adenine nucleotides, as with the coenzyme levels, was similar in spores and cells at 30°C. The rate of initiation of germination was measured by the decrease in optical density at 660 nm. Rates in the glucose and the complete media were similar, but the rate in KBr was slightly lower. Within 15 min, in all media, >90% of the spores appeared dark in the phase contrast microscope. Inhibitors of ATP production, including KF (10 mM), KCN (10 mM), and fluorocetate (10 mM), had no detectable effect on the initiation rate, nor did actinomycin D (100 µg per ml) or chloramphenicol (100 µg per ml).

Assays—FAD was determined by the method of De Luca, Weber, and Kaplan (24), and DPN+ and TPN+ were determined by the methyl ethyl ketone procedure described by Kaplan and Ciotti (25). DPNH and TPNH, extracted with alkali, were determined as DPN+ and TPN+ after prior conversion to the oxidized nucleotides by the method of London and Knight (26).

ATP was determined with firefly tail extracts as described by Streher and Totter (27). The rate of decay of light production was measured in a scintillation counter and extrapolated to zero time. Inhibition or facilitation of light production was corrected by the method of Atkinson et al. (28).

ADP and AMP were determined as ATP after conversion as follows: ADP by treatment of 1-ml samples with 5 mM MgCl₂, 1 mM phosphoenolpyruvate, and 20 µg of pyruvate kinase; AMP by similar treatment plus 50 µg of myokinase.

3-Phosphoglycerate was determined fluorometrically by the method of Czok and Eckert (29), and also by an isotopic method (6) in which ³H-labeled spores were analyzed.

Thin layer chromatography was carried out as described by Randerath and Randerath (30) with polyethyleneimine sheets obtained from Brinkman Instruments. Added markers were located by ultraviolet light and radioactive compounds by autoradiography.

RESULTS

Coenzyme and Nucleotide Levels in Dormant Spore

Adenine Nucleotide Levels—The level of total adenine nucleotides, as with the coenzyme levels, was similar in spores and cells (Table II). Strikingly different was the ATP charge (ratio of ATP plus 0.5 ADP to total adenine nucleotide (34)). The value was 0.1 in spores as compared to 0.8 to 0.9 in cells. The trace of nucleotide determined as ATP in dormant spores by the luciferase assay was further identified as ATP by its removal upon treatment of the spore extract with glucose and hexokinase. This trace of ATP was not extracted from spore preparations by treatment with trichloracetic acid (5%), suggesting that the ATP resides in the dormant spore rather than in vegetative cell debris or in germinated spores. Aging of spores (in the dry state for 5 months) did not affect the adenine nucleotide levels. Findings in dormant Bacillus subtilis and Bacillus cereus spores were similar to those in B. megaterium.

Other Nucleotide, PGA, and Pi Levels—As reported by Nelson and Kornberg (6), the principal low molecular weight phosphate compound in the dormant spore is 3-phospho-D-glyceric acid (Table III); Pi is the next most prominent component. (The importance of PGA as an early energy source is considered below.) The spore levels of uracil, cytosine, and guanine nucleotides are comparable to those in vegetative cells (6). As with the adenine nucleotides, the ratio of nucleotide in the triphosphate form to total nucleotide was very low.

1 The spore and vegetative cell levels may be even closer than indicated in Tables II and III. If the total adenine nucleotide levels were converted to intracellular or intraperosporular concentrations with the value for the ratio of grams of dry weight per gram of wet weight given under "Methods," the concentrations would be 1.1 µM in the cell and 0.9 µM in the spore.

*a The abbreviation used is: PGA, 3-phospho-D-glyceric acid.

---

TABLE I

<table>
<thead>
<tr>
<th>Coenzyme</th>
<th>Cells</th>
<th>Spores</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAD</td>
<td>22</td>
<td>20</td>
</tr>
<tr>
<td>DPN+</td>
<td>224</td>
<td>117</td>
</tr>
<tr>
<td>TPN+</td>
<td>31</td>
<td>25</td>
</tr>
</tbody>
</table>

*a Values for DPN and TPN in vegetative cells include both the reduced and oxidized nucleotides. In spores, less than 3 nmoles per g, wet weight, of DPNH plus TPNH were found.

TABLE II

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Bacillus megaterium</th>
<th>Bacillus cereus T-spores</th>
<th>Bacillus subtilis SB-133 spores</th>
</tr>
</thead>
<tbody>
<tr>
<td>Count</td>
<td>Cells</td>
<td>Spores</td>
<td>Cells</td>
</tr>
<tr>
<td>ATP</td>
<td>725</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>ADP</td>
<td>94</td>
<td>82</td>
<td>61</td>
</tr>
<tr>
<td>AMP</td>
<td>450</td>
<td>485</td>
<td>35</td>
</tr>
<tr>
<td>ADP + AMP</td>
<td>195</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total adenine nucleotide</td>
<td>920</td>
<td>547</td>
<td>570</td>
</tr>
</tbody>
</table>

No adenine nucleotides were found in the growth medium.

TABLE III

<table>
<thead>
<tr>
<th>Compound</th>
<th>Level</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmoles/g, wet weight</td>
</tr>
<tr>
<td>PGA</td>
<td>6800</td>
</tr>
<tr>
<td>Pi</td>
<td>2000</td>
</tr>
<tr>
<td>AMP</td>
<td>405</td>
</tr>
<tr>
<td>UMP</td>
<td>216</td>
</tr>
<tr>
<td>CMP</td>
<td>97</td>
</tr>
<tr>
<td>GMP</td>
<td>123</td>
</tr>
<tr>
<td>ADP</td>
<td>74</td>
</tr>
<tr>
<td>UDP</td>
<td>&lt;10</td>
</tr>
<tr>
<td>ATP + UTP + CTP + GTP</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

Levels of PGA and Pi and nucleotides in dormant spores
Timo in minutes

FIG. 1. Kinetics of ATP production in several media; 0.5 ml samples were withdrawn and extracted, with the 80% propanol procedure. Inset, spores were germinated in the KBr medium. At various times, two samples were withdrawn. One was extracted for ATP by the propanol procedure and the other was diluted into 4 mM HgCl₂ for optical density measurement; HgCl₂ is known to stop changes in optical density of germinating spores (36).

Similar values for AMP were obtained with the luciferase assay, which measures only ribonucleotides (35), and by the isotopic method, which does not distinguish ribo- from deoxyribonucleotides. This finding suggests that there is little dAMP in spores. Furthermore, since UMP was present (Table III) and TMP could not be detected (<25 nmoles per g, wet weight), it seems likely that the cytosine and guanine nucleotides are also principally, if not entirely, ribonucleotides.

ATP Production in Stages I and II

Kinetics of ATP Production in Stages I and II—Although the dormant spore was found to contain significant levels of adenine nucleotides, there was essentially no ATP. Early in germination, however, there was a 100-fold increase in spore ATP (Stage I), after which the ATP level remained constant for about 10 min (Stage II, Fig. 1). ATP production in Stage I did not precede the loss in refractility, indicating that neither energy metabolism nor biosynthetic reactions are required before the loss in refractility and the other changes which are associated with initiation take place (Fig. 1, inset).

ATP generation in Stages I and II was the same in the germination medium which contained only KBr as in the more complex media (Fig. 1). Furthermore, in Stages I and II, spores germinating in the KBr medium without the phosphate generated ATP levels identical with those in spores germinating in the same medium with phosphate. It has been shown by other workers that endogenous phosphorous reserves will also maintain respiration early in spore germination (37).

PGA as Source of ATP in Stage I—The lack of a requirement for an exogenous energy source for ATP production in Stages I and II indicates that the dormant spore contains endogenous energy reserves. A potential energy source in the dormant spore is the depot of PGA. In the KBr medium, ATP production in Stage I was accompanied by the disappearance of the PGA in the dormant spore (Fig. 2) (38). Fluoride (10 mM) blocked both the PGA utilization and ATP production (Fig. 2). Similar PGA values were obtained by the isotopic method as compared to the fluorometric method for measuring PGA. In other organisms fluoride prevents the production of ATP from PGA via the normal glycolytic pathway by inhibiting enolase. Enolase extracted from dormant spores was inhibited by 10 mM fluoride by >97%. These results are consistent with PGA being the major source of ATP in Stage I when spores are germinated in the KBr medium.

PGA Utilization in Glucose Medium—The utilization of PGA in the glucose medium appeared similar to that in KBr (Fig. 3). However, fluoride did not block PGA breakdown but caused only a brief lag (1 to 2 min) in both PGA utilization and ATP generation (Fig. 3). A possible explanation for this result is that 10 mM KF blocks PGA utilization through enolase, causing the lag
Effect of KCN-Germinating spores are known to have an aerobic metabolism (2). KCN blocks oxygen uptake during germination of the Bacillus strain under study although it does not inhibit the initiation of germination (2). KCN had little effect on PGA utilization in the glucose medium but had a striking effect on the ATP levels (Fig. 4). There was an initial increase in ATP as expected in Stage I, but the ATP level fell off and remained low during Stage II. Similar results were obtained in spores germinating under \( N_2 \). However, azide (10 mM), whose inhibitory effects on respiration are usually similar to those of KCN, had little effect (<10%) on the ATP level in Stages I or II. These data indicate that the conversion of PGA to ATP (Stage I) is insensitive to KCN and is anaerobic, whereas ATP production in Stage II is inhibited by KCN and is aerobic.

Effects of Other Inhibitors—Cytochromes, the usual sites of the inhibitory action of cyanide, are reported to be absent in the spore (39). Instead the spore is thought to contain a flavoprotein terminal electron transport system; a DPNH oxidase isolated from spores is inhibited by cyanide (39). However, amytal (2 mM) and rotenone (10 mM), which often inhibit flavin-mediated electron transport, were without effect on ATP production in Stages I and II (<5% inhibition), but a similar type of inhibitor, Dicumarol (0.1 mM), significantly inhibited ATP production in Stage II although it had little effect on the ATP level in Stage I (Fig. 4). 2,4-Dinitrophenol (50 \( \mu \)M), which uncouples oxidation from phosphorylation in electron transport, had no effect on ATP production in Stages I and II (<10% inhibition). Fluoroacetate, which inhibits aconitase after conversion to fluoro-citrate, also gave no inhibition (<5%), indicating that the tricarboxylic acid cycle is not functioning at this time.

Lack of Requirement for Protein and RNA Synthesis—Although many metabolic enzymes have been identified in the dormant spore (40, 41), it is not known whether all of the enzymes required for metabolism are present. Chloramphenicol and actinomycin D block protein and RNA synthesis during germination by >95% (3, 12). Even when both inhibitors were present, there was no effect on PGA utilization and the accumulation of ATP was actually enhanced (Fig. 5). When actinomycin D was omitted and ATP was being utilized for RNA synthesis (11), chloramphenicol had little effect on the ATP level in Stages I and II although it did inhibit ATP production profoundly in Stage III (Fig. 6). Furthermore, in the presence of chloramphenicol, exogenous inosine was converted to ATP, causing an increase in the ATP level of up to 6-fold (Fig. 6). This ATP production was
not inhibited by 10 mM KF. These data indicate that the enzymes needed for high energy phosphate production in Stages I and II are available in the dormant spore.

**ATP Production in Stage III**

**Kinetics of ATP Production**—After reaching a level of 400 to 500 nmoles per g of dry spores, the ATP level remains constant for the first 15 min of germination, but during the period from 15 to 50 min after the initiation of germination (Stage III) there is a 7- to 10-fold increase in the ATP level (Fig. 7). Exogenous carbon and phosphate sources are required for the ATP production in Stage III. The increase in ATP is the result of nucleotide synthesis *de novo* and is considered in the accompanying paper (12).

In addition to the change in ATP levels in Stage III, there is also a change in the ratio of ATP to total adenine nucleotide (Fig. 8). During Stage II this ratio was 0.6 to 0.65 in all media, but it rose or fell in Stage III depending on whether or not an energy source was supplied. The ratio of ATP to total adenine nucleotide is related to and is only slightly lower than the ATP charge which is known to control the rate of several bacterial enzymes (42), and the change in the ratio of ATP to total adenine nucleotide between Stages II and III may be of regulatory significance.

**Effects of Inhibitors**—The increased ATP production and the elevated ATP charge suggested that there might be a significant change in metabolism from Stage II to Stage III. Therefore, the inhibitors which were tested for their effects on the ATP production in Stage II were also tested in Stage III (Fig. 9). KF (10 mM), fluoroacetate (1 mM), and rotenone (10 μM) were without effect, while azide (10 mM), Dicumarol (0.1 mM), and 2,4-dinitrophenol (50 μM) caused significant inhibition. KCN (10 mM) and anaerobiosis caused almost complete blockage of ATP production. With the exception of 2,4-dinitrophenol and azide, the inhibitors had similar effects on ATP production in Stages II and III. Therefore, the pathways for energy metabolism are probably similar in both stages. The inhibition of ATP production in Stage III by 2,4-dinitrophenol and azide may reflect an increased rate of aerobic ATP production at this time.

**Requirement for Protein Synthesis**—Chloramphenicol blocked the increase in the ATP level which takes place in Stage III (Fig. 6). However, this was not due to inhibition of the production of high energy phosphate compounds, but, as shown in the accompanying paper (12), was due to inhibition of adenine nucleotide biosynthesis, *de novo*. Neither chloramphenicol nor actinomycin D inhibited the synthesis of high energy phosphate compounds during Stage III (Fig. 6), indicating that the enzymes required for energy metabolism throughout the 1st hour of germination are previously formed in the dormant spore.

**DISCUSSION**

**Coenzyme Levels in Dormant Spore**—The low levels of ATP and reduced pyridine nucleotides in the dormant spore are consistent with the absence of metabolic activity. However, the similarity of coenzyme and nucleotide levels in the spore to those in the vegetative cell indicates the potential of the spore to draw upon endogenous resources to return to metabolic and synthetic activ-
ity. In the first minutes of germination the AMP in the dormant spore is converted to ATP, and a part of the nucleotide pool is utilized for RNA synthesis (12).

**PGA as Source of ATP in Stage I**—The utilization of PGA during the ATP production in Stage I suggests that the dormant spore’s depot of PGA is utilized at this time to phosphorylate ADP (and AMP) via the glycolytic pathway. This suggestion is strengthened by two facts. (a) α-Phosphoglycerate mutase, enolase, and pyruvate kinase, the three enzymes required for the over-all reaction, are present in the dormant spore, as is adenylate kinase (43), and (b) KF, which inhibits spore enolase, blocks both the ATP production and the disappearance of PGA. This latter fact also indicates that PGA may be the major source of ATP during Stage I when spores are germinated in the KBr medium, since the dormant spore contains almost no other sugar phosphates (38). It is significant that, after 30 min of germination in the absence of exogenous carbon and phosphate sources (KBr medium) and in the presence of KF, 50% of the initial PGA depot is retained inside the spore.4

PGA, however, is probably not the sole source of the ATP produced in Stage I when spores are germinated in the presence of an exogenous energy source. Exogenous glucose enables the spore to produce an initial rise in ATP even in the presence of KF which blocks normal PGA utilization (Fig. 3). Furthermore, if PGA were solely responsible for the initial rise in spore ATP one would not expect this rise to be affected by KCN or anaerobiosis. However, as seen in Fig. 4, these conditions lower the extent of the initial rise in ATP. This indicates that metabolism of exogenous compounds can play a part in generating ATP in Stage I.

The eventual utilization of PGA during germination in the glucose medium in the presence of KF or with KF and KCN both present needs to be explained. Under these conditions high levels of reduced pyridine nucleotides may be generated from glucose oxidation, which, when coupled with a small amount of ATP, may cause utilization of PGA by an alternative pathway, possibly reversal of the glycolytic pathway. Exploratory experiments with 32P-labeled spores have shown that during germination in the glucose medium plus KF and KCN a 32P-labeled compound with properties similar to FDP accumulates.4 This compound accounts for about 40% of the 32P of PGA, the rest of the 32P appearing as P32.

**Uses for ATP Derived from PGA**—The PGA in the dormant spore represents a depot of γ ATP moles of high energy phosphate per g of wet spores. This depot is sufficient to convert the dormant spore’s pool of free nucleotides into the triphosphate form three times over, and is a potential source of 3.5 μmoles of triphosphate per g of wet spores.

Two of the main uses for the high energy phosphate depot are RNA synthesis and glucose phosphorylation during the first 10 min of spore germination. The contribution of the PGA depot to these processes can be determined quantitatively since during germination in KCl high energy phosphate is generated only from the PGA depot. In the presence of KCl 0.6 μmole of RNA nucleotide residues per g of wet spores is synthesized in Stage I (assuming that the RNA contains 25% uracil residues). It can be calculated that at least 17% of the dormant spore’s PGA depot is utilized for this synthesis (12). Similarly, in the glucose medium, one can calculate that more than 20% of the ATP produced from PGA is used to phosphorylate glucose producing FDP,4 assuming that 2 molecules of PGA are required to produce 1 molecule of FDP. The latter function of PGA, that of providing ATP for hexose phosphorylation, may be extremely important. If glucose metabolism proceeds via glucose 6-P and FDP as is indicated by the work of Nelson and Kornberg (38), it is essential to have a supply of ATP to phosphorylate the first hexose molecules.

**Nature of Metabolism in Stages II and III**—The mechanisms for ATP production in Stages II and III appear to be similar, as suggested by the effects of inhibitors in both stages, yet the nature of these metabolic pathways is unclear. The tricarboxylic acid cycle has been considered inoperative (44) and this is confirmed by the lack of inhibition of ATP production by fluorooacetate. The data of Blumenthal (44) and Goldman and Blumenthal (45) support the glycolytic pathway as the primary route of glucose utilization during germination. The demonstration that glucose 6-P, FDP, and α-glycero-P are the major phosphorylated sugars appearing during germination (38) also supports a major role for this pathway. Studies in vitro, however, have indicated that the pentose pathway is predominant (41), and this is further strengthened both by the fact that KF does not block ATP production during germination in glucose, and the demonstration by Hyatt and Levinson (2) that KF does not block O2 uptake during germination in glucose. As in our experiments with ATP production, KF did, however, cause a lag in O2 uptake (2).

Our experiments showing no inhibition of ATP production in Stages II and III are difficult to reconcile with glycolytic ATP production. Although KF does not inhibit ATP production during germination in glucose, it does inhibit enolase in vitro, and also presumably does so in vivo, since it blocks PGA utilization in the KBr medium. In the latter medium with KF present, 5 μmoles of PGA are utilized in 30 min. If this is taken as the rate of ATP production which might leak through a fluoride-inhibited enolase, this rate is still too low by at least a factor of 15 to account for the amount of ATP required for protein and RNA synthesis and for glucose phosphorylation in the glucose medium. It seems unlikely that new protein synthesis would diminish the fluoride inhibition since chloramphenicol does not reduce high energy phosphate production in the glucose medium when KF is present (Fig. 6). There also appears to be no shift in the metabolic pathways in Stages II and III in the presence of KF as judged by the virtual identity of electrophoretic and chromatographic patterns of the phosphorylated compounds produced by spores germinating in glucose plus or minus KF.4

A possible explanation to reconcile these observations is that the metabolic pathway does not go through enolase, but diverges earlier, perhaps at the triose phosphate level to connect with the pentose phosphate pathway. Clearly this question requires much further study.

**Inhibition of Stages II and III Metabolism by KCN and Azide**—Although metabolism in Stages II and III is aerobic (2), as indicated by the low ATP production in N2, the inhibition of ATP production in Stages II and III by KCN and in Stage III by azide is surprising in that spores have been reported to lack cytochromes, the usual targets of these inhibitors (30). Similar effects on oxygen uptake have been reported by Hyatt and Levinson (2). Although KCN and azide were effective inhibitors, other agents which inhibit terminal electron transport were,
except for Dicumarol, without effect, but the uncoupler 2,4-dinitrophenol was an effective inhibitor in Stage III. It is known that the flavoprotein, DPNH oxidase, isolated from B. cereus by Halvorson and Doi (39), is inhibited by both KCN and azide, and this may be the locus for the action of these compounds in vivo. Possibly cyanide acts by chelation of iron-heme iron residues in an electron transport protein. The mechanism of terminal electron transport in germinating spores is, however, still an open question.

Relationships between Dormancy, Stages I, II, and III—ATP production must be crucial to the sequence of stages in spore germination (Scheme I). Stage I provides ATP from endogenous and possibly exogenous sources to make the first mRNA molecule needed for protein synthesis and may also initiate metabolism by phosphorylations which prime the metabolic pump. Production of ATP is carried on in Stage II, maintained if necessary by endogenous nutrients. During this period there is both breakdown and resynthesis of RNA, and proteins needed for the initiation of Stage III are synthesized (12). The rate of ATP production increases in Stage III and nucleotide biosynthesis begins (12), initiating the large net synthesis of macromolecules essential for transformation of the spore into a vegetative cell.

Although many control mechanisms are undoubtedly involved during these three stages, one of the most dramatic transitions is from the dormant state into Stage I. Both PGA and the enzymes required to utilize it for ATP production are present in the dormant spore, but until germination is initiated the enzymes remain inert. How these enzymatic systems are masked and unmasked becomes a more specific statement of the general question about the maintenance of dormancy in quiescent systems such as that represented by the bacterial spore.

REFERENCES
